The Role of the Tumour Suppressor p53 in Cooperation with Ras and Raf Oncogenes

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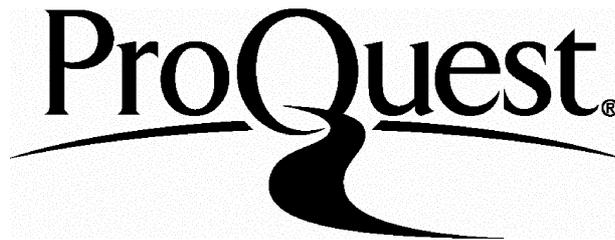Lincoln's Inn Fields,
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Abstract

To study the molecular mechanisms of oncogene cooperation, rat Schwann cells were used in which oncogenic Ras or Raf alone cause a cell cycle arrest, whereas cooperating oncogenes lead to transformation (Ridley et al., 1988; Lloyd et al., 1997). This model system was used to search for cooperating oncogenes and to investigate the underlying biochemical mechanisms of oncogene cooperation.

Interestingly, mutants of the tumour suppressor p53 efficiently cooperate with oncogenic Ras or Raf to transform Schwann cells. This result prompted me to further investigate the role of p53 in Schwann cells expressing an inducible activated Raf (ΔRafER). Activation of ΔRafER led to the induction of the cell cycle inhibitor $p21^{WAF1/Cip1}$. Induction of $p21^{WAF1/Cip1}$ is p53-dependent, since dominant negative mutants of p53 abolish this induction and subsequently the G1 cell cycle arrest.

The pathway leading to the p53-dependent induction of $p21^{WAF1/Cip1}$ by activated Raf was investigated by addressing the possible role of the MAP-kinase pathway. The contribution of MAP-kinase kinase and MAP-kinase in the signalling pathway triggering the G1 cell cycle arrest were examined using dominant negative and constitutively active mutants as well as a specific chemical inhibitor. My results indicate that MAP-kinase kinase is necessary for $p21^{WAF1/Cip1}$ induction in Schwann cells. The contribution of the JNK-kinase pathway in the Raf-initiated cell cycle arrest was addressed.

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Abbreviations

aa	amino acids
APS	ammonium persulfate
ATP	adenosine triphosphate
AC	anchor cell
BSA	bovine serum albumin
bp	base pairs
CIP	Calf intestine alkaline phosphatase
CDK	Cyclin-dependent kinase
DTT	dithiothreitol
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
EDTA	sodium-ethylenediaminetetraacetate
ECL	enhanced chemiluminescence
EGF	epidermal growth factor
FACS	fluorescence assisted cell sorting
FCS	foetal calf serum
FGF	fibroblasts growth factor
HEPES	(N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid)
h	hours
IPTG	isopropylthio-β-D-galactoside
IGF-BP3	insulin-like growth factor binding protein 3
IGF-1	insulin growth factor-1
l	litre
min	minutes
mM	millimolar
M	Molar
ml	millilitre

MBP	myelin basic protein
MOPS	(3-(*N*-morpholino) propanesulfonic acid
mA	milliampere
mt	mutant
nt	nucleotide
dNTP	desoxyribonucleotides (all four)
NGF	nerve growth factor
PLL	poly-*L*-lysine
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
PBS A	phosphate buffered saline A
PIPES	piperazine-*N,N''*-bis (2 ethane-sulfonic acid)
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
Py	pyrimidine
Pu	purine
RT	room temperature
rpm	rounds per minute
Rb	retinoblastoma protein
sec	seconds
SDS	sodium dodecyl sulfate
SRE	serum response element
Tris	2-amino-2 (hydroxymethyl)-1,3 propandiol
TCA	trichloroacetic acid
TEMED	*N,N,N',N'*-tetramethylethylenediamine
UTR	untranslated region
V	volt
VPC	vulval precursor cell

CHAPTER 1.0

INTRODUCTION

Molecular Mechanisms of Oncogene Cooperation

1.0   Molecular mechanisms of oncogene cooperation

Tumorigenesis is a multistep process in which multiple genetic defects are acquired over time. The requirement for multiple genetic changes in tumorigenesis has been indicated by many studies *in vitro* and *in vivo* (Hunter, 1991). Furthermore, in humans the correlation between the increase of cancer incidences and age has supported this notion (Peto et al., 1975). In multistep tumorigenesis, oncogenes cooperate towards cellular transformation (Land et al., 1983; Ruley, 1983).

1.0.1   Oncogene cooperation *in vitro*

First evidence that more than one oncogene is necessary for cellular transformation was provided by experiments with DNA tumour viruses. The viral functions of DNA tumour viruses which are required for transforming primary non-established cells differed from the viral functions required to transform already established cells. For instance, the adenovirus E1A gene immortalises primary cells. The action of additional viral oncogenes such as E1B leads to full transformation in these immortalised cells (Houweling et al., 1980). Similarly, large T antigen on its own is able to immortalise non-established fibroblasts (Rassoulzadegan et al., 1983b). Likewise, the polyoma virus middle T antigen is only capable of transforming established fibroblasts (Treisman et al., 1981), while the joined action of small-, middle- and large T antigen is required to transform non-established fibroblasts (Rassoulzadegan et al., 1983a).

The necessity for oncogene cooperation in cellular transformation has also been shown for cellular oncogenes. The introduction of *ras* oncogenes into non-established primary fibroblasts did not result in transformation (Newbold & Overell, 1983; Land et al., 1983; Ruley 1983; Sager et la., 1983, Franza et al., 1986), whereas activated Ras clearly leads

to a transformed phenotype in established NIH3T3 fibroblasts (Stacey & Kung, 1984; Pulciani et al., 1985). However, the transformation of non-established primary cells can be achieved when an activated *ras* gene is introduced together with a cellular oncogene such as *c-myc* (Land et al., 1983) or the adenovirus *E1A* gene (Ruley, 1983). These results clearly indicate that at least two or more oncogenes are required for cellular transformation of non-established cells.

1.0.2   Oncogene cooperation *in vivo*

The cooperative effect of oncogenes *in vivo* has been widely observed in transgenic mice. Co-expression of *v-Ha ras* and *c-myc* in the mammary gland of trangenic mice led to a dramatic acceleration in the formation of carcinomas compared to transgenic mice with only a single oncogene (Sinn et al., 1987). A similar cooperative effect in tumorigenesis of leukemias was observed in transgenic mice expressing the *Eμ-myc* gene, under control of the immunoglobulin heavy chain enhancer, infected with a retrovirus harbouring *v-Ha ras* (Langdon et al., 1989). Cooperation of oncogenes *in vivo* is not only characterised by an enhanced initiation of tumorigenesis. Furthermore, increased tumour invasiveness and the appearance of tumours in tissue originally not affected in parental mice have been reported in many transgenic mice studies (Donehower et al., 1995).

Transgenic mice have also been employed in the search of cooperating onocgenes. For instance, transgenic *Eμ-myc* mice were infected with the Moloney murine leukaemia virus (MuLV) acting as an insertional mutagen to activate protooncogenes (van Lohuizen et al., 1991; Haupt et al., 1991). Given that the retroviral insertion of MuLV is random, any repetitive integration in a specific locus ("proviral tagging") found within

the early stages of an outgrowing tumour would suggest that the affected gene cooperates with the *Eμ-myc* transgene. The analysis of loci with repetitive integrations, therefore, should reveal genes capable of cooperating with the *Eμ-myc* transgene (Berns 1991; Allen & Berns, 1996; Jonkers & Berns, 1996). Using this approach, it was discovered that the serine/threonine kinase *pim-1* is activated with high frequency in *Eμ-myc* transgenic mice (van Lohuizen et al., 1991; Haupt et al., 1991). This result was confirmed in double transgenic mice carrying oncogenic *myc* and *pim-1* genes which develop tumours in a very short time compared to single transgenic parental mice (Verbeek et al., 1991; Möröy et al., 1991). Reversibly, *L-myc* and *N-myc* genes have been identified which cooperate with *pim-1* transgenic mice (van Lohuizen et al., 1989). Similarly, proviral tagging with MuLV in mice carrying an *Eμ-myc* transgene in a *pim-1* nullizygous genetic background results in the activation of a related *pim-2* gene in 80% of observed tumours (van der Lugt et al., 1995).

Despite the value of transgenic mice in studying tumorigenesis and oncogene cooperation, homogenous expression of oncogenes in targeted tissues does not appropiately mimic an *in vivo* situation of early onset of tumorigenesis. Oncogenic mutations will occur most likely in single cells surrounded by normal cells. Such a scenario was modelled by introducting a few retrovirally infected cells into a reconstituted murine prostate (Thompson et al., 1989). Interestingly, incorporation of prostate gland cells containing oncogenic *ras* causes only dysplasia, whereas cells expressing an activated *myc* gene alone cause a hyperplastic phenotype (Thompson et al., 1989). This finding suggests that single oncogenes can induce different phenotypes in premalignant stages of tumour development. In contrast, both oncogenes jointly expressed, gave raise to prostate

carcinomas, although additional genetic events are likely to participate in the formation of these tumours (Thompson et al., 1989).

1.0.3 Loss of tumour suppressor function cooperates in tumorigenesis

Synergistic oncogenic effects towards cellular transformation have also been observed between a growth promoting gene and the loss of tumour suppressor gene function. In contrast to oncogenes, mutations in tumour suppressor genes are mainly recessive. Therefore, the loss of function is often associated with the loss of both alleles as in case of the tumour suppressors Rb (Levine, 1990; Cowell, 1990) and p53 (Levine, 1992). However, the function of the tumour suppressor p53 can be strongly impaired by mutations in only one allele, since p53 mutants exhibit a dominant negative effect on the function of the remaining wild type p53 (Milner et al., 1991a; 1991b; Martinez et al., 1991). Mutants of the tumour suppressor gene *p53* cooperate with an activated *ras* gene in transforming primary rat embryo fibroblasts as measured by foci formation and tumorigenicity in nude mice (Parada et al., 1984; Eliayahu et al., 1984; Hinds et al., 1990). Similarly, p53 mutants can release the *ras*-induced cell cycle arrest in REF52 fibroblasts and cooperate in transformation (Hicks et al., 1991). In p53 nullizygous mice, the introduction of oncogenes such as Ras (Kemp et al., 1994; Hundley et al., 1997), myc (Elson et al., 1995), E7 (Howes et al., 1994) and SV40 large T (Symonds et al., 1994) can significantly enhance tumorigenesis confirming that the functional loss of p53 cooperates with various oncogenes *in vivo*.

Apart from a cooperative effect between a growth promoting gene and the loss of tumour suppressor function, loss of the two tumour suppressor genes p53 and the retinoblastoma (Rb) can also have a

synergistic effect on tumorigenesis (Williams et al., 1994; Harvey et al., 1995). Mice deficient for p53$^{-/-}$ and heterozygous for Rb$^{-/+}$ exhibit a reduced viability due to extensive tumour growth (Williams et al., 1994). Furthermore, p53$^{-/-}$/Rb$^{-/+}$ mice have a wider tumour spectrum compared to the parental p53 nullizygous or Rb heterozygous mice. In addition, some tumours in p53$^{-/-}$/Rb$^{-/+}$ mice did loose the remaining Rb-allele, arguing for a selective pressure towards the complete loss of Rb function (Williams et al., 1994).

The inactivation of tumour suppressor genes also appears to be the underlying principle for the cooperation of many viral oncogenes. A number of viral genes abrogate the function of p53 and Rb. For instance, SV40 LT inactivates p53 by physical interaction (Gannon & Lane, 1987) and the adenovirus E1A protein can inactivate Rb or family members p300 and p107 (Barbean et al., 1992; Ludlow & Skuse, 1995). Similarly, the human papilloma virus 16/18 (HPV) E6 and E7 proteins inactivate p53 (Scheffner et al., 1990) and Rb function (Scheffner et al., 1992), respectively. The transgenic expression of both viral proteins (Pan & Griep, 1994) or the Rb inactivating HPV E7 protein in a p53 nullizygous genetic background (Howes et al., 1994) lead to tumorigenesis in murine ocular lens. The functional loss of both p53 and Rb has also been frequently observed in human tumours (Lin et al., 1996; Paggi et al., 1996), arguing that this form of cooperation may play an important role in human carcinogenesis.

1.0.4   Cooperation of oncogenes affect G1-S phase transition

Cooperating oncogenes enable non-proliferative cells to proceed through the cell cycle in the absence of external growth signals. At the restriction point, the regulation of the Rb-protein is instrumental for the

progression into S-phase (Lin et al., 1996; Paggi et al., 1996). The regulation of the Rb-protein can be influenced by cooperating oncogenes. For instance, cyclin D forms active cyclin-dependent kinase complex with cyclin-dependent kinases 4 and 6 (CDKs) which are responsible for the phosphorylation-dependent regulation of Rb (Lin et al., 1996; Paggi et al., 1996). Overexpressed cyclin D can cooperate with oncogenic Ras in cellular transformation of rat embryo fibroblasts indicated by immortalisation, anchorage-independent growth and tumour formation after injection of activated Ha-Ras/cyclin D expressing fibroblasts into nude mice (Lovec et al., 1994a; Hinds et al., 1994). In transgenic mice, the *cyclin D1* gene can cooperate with *L-myc* and *N-myc* genes in forming B-cell lymphomas (Lovec et al., 1994b; Bodrug et al., 1994). In support of these findings, overexpression or amplification of cyclin D has been widely observed in various tumours (Lammie et al., 1992; Motokura et al., 1991; Schuuring et al., 1992; Leach et al., 1993; Hall & Peters, 1996).

Oncogene cooperation can also occur between activated Ha-Ras and Cdc25A and B phosphatases (Galaktionov et al., 1995a). The Cdc25A and B phosphatases activate cyclin-dependent kinases through dephosphorylation of inhibitory threonine and tyrosine phosphorylation sites (Dunphy & Kumagai, 1991; Galaktinonov & Beach, 1991; Gautier et al., 1991; Strausfeld et al., 1991) and thereby regulate cell cycle progression positively. Moreover, the loss of the retinoblastoma gene (Rb) enables Cdc25A to transform primary mouse embryo fibroblasts (Galaktionov et al., 1995), indicating a cooperative effect between the loss of tumour suppressor function and a phosphatase which activates cyclin-dependent kinase complexes.

1.0.5   Oncogene cooperation and apoptosis

Genes which regulate apoptosis have been found to cooperate with genes regulating cell proliferation. The overexpression or deregulation of *c-myc* is often associated with neoplastic growth (Spencer & Groudine, 1991; Marcu et al., 1992). However, *c-myc* overexpression in the absence of survival factors can also cause apoptosis (Askew et al., 1991; Evan et al., 1992; Shi et al., 1992). The anti-apoptotic gene *bcl-2*, on the other hand, either prolongs cell survival (McDonnell et al., 1989; Nunez et al., 1991) or inhibits apoptosis depending on the cell type (Sentman et al., 1991; Henderson et al., 1991). In the absence of survival factors, the coexpression of c-Myc and Bcl-2 abrogates c-Myc-induced apoptosis (Fanidi et al., 1992; Wagner et al., 1993). The expression of Bcl-2 does, however, not influence c-Myc´s mitogenic properties (Fanidi et al., 1992). Therefore, c-Myc and Bcl-2 can cooperate in cellular transformation. Furthermore, cell proliferation in c-Myc and Bcl-2 expressing fibroblasts occurs in the absence of mitogens (Fanidi et al., 1992). Other features of transformation, however, such as the formation of foci or morphological transformation are absent in c-Myc and Bcl-2 expressing fibroblasts (Reed et al., 1990; Wagner et al., 1993). This cooperative effect *in vitro* has also be shown in *in vivo*-studies. Bcl-2 transgenic mice exhibit a small number of malignant lymphomas. Coexpression of the *c-myc* gene dramatically increases the number of tumours compared with the number of tumours in either transgenic parental mice (Vaux et al., 1988; Strasser et al., 1990).

Oncogenes can also cooperate with survival factors such as Insulin-like growth factor I and II (IGF-I/II) in order to abolish an apoptotic response and enable tumour progression. A subset of hyperproliferative islets expressing SV40 large T in a transgenic mouse model (Hanahan 1985), show secretion of IGF-II, whereas normal non-proliferative islets did not

secret this factor. The secretion of IGF-II appears to be responsible for an abolition of SV40-large T-induced apoptosis in this system (Christofori et al., 1995), since expression of SV40 LT in IGF-II nullizygous mice exhibit a greatly reduced number of smaller and more structured tumours compared with tumours which develop in SV40 large T/IGF-II$^{+/+}$ mice. Furthermore, tumours arising in IGF-II-deficient mice exhibit a five-fold higher rate of apoptosis, suggesting that the absence of the survival factor IGF-II reduces the tumour growth due to a high apoptosis rate (Christofori et al., 1994; Ueda & Ganem 1996). In agreement with these findings, the IGF-I receptor has been shown to promote survival in tissue culture and animals (Resnicoff et al., 1995a, b; Harrington et al., 1994).

Cooperating oncogenes can also influence apoptosis through the functional inactivation of apoptosis-inducing regulators such as p53. Apoptosis which occurs in the development of ocular lenses in Rb-heterozygous mice is lost in a p53-deficient background (Morgenbesser et al., 1994). Retinal tumours develop in Rb heterozygous/ p53$^{-/-}$ mice but do not develop in a p53$^{+/+}$ background (Howes et al., 1994; Pan & Griep, 1994). Similarly, the slow tumour growth due to apoptosis in transgenic mice expressing a SV40 large T antigen mutant inactivating Rb (Symonds et al., 1994) was dramatically accelerated in a p53 nullizygous background and tumour growth was indistinguishable from cells expressing wild type SV40 large T-antigen capable of inactivating both Rb and p53. In agreement with this finding, a strong decrease of apoptosis accompanies the development of malignant tumours (Symonds et al., 1994). In summary, the cooperation of oncogenes can occur between a proliferative oncogene and the loss of cellular apoptotic function through either overexpression of an anti-apoptotic protein, secretion of a survival factor or inactivation of an apoptosis inducing protein.

1.0.6 Oncogene cooperation and human tumorigenesis

In human tumorigenesis sequential mutations in proto-oncogenes are required before cells become fully transformed. Intensive studies into the initiation of colorectal tumourigenesis as a model system have established that seven to ten mutations have to occur before epithelial cells become transformed (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996).

In colon carcinogenesis, the effect of cooperating oncogenes on tumour initiation and progression can be analysed in morphological well-defined stages (Vogelstein & Kinzler, 1993). Although the genes affected and the overall number of genetic lesions appear to be crucial for the onset of tumorigenesis (Fearon & Vogelstein, 1990), defects in specific genes can be correlated with specific stages of tumorigenesis. For instance, loss of the *APC* gene are correlated with early hyperproliferation of normal epithelium (Levy et al., 1994; Luongo et al., 1994; Jen et al., 1994). Mutations in the *ras*-gene are often associated with further progression of early premalignant adenomas (Vogelstein et al., 1988; Shibata et al., 1993) and the loss of the tumour suppressor genes *DCC* and *p53* accompanies the development towards malignant carcinomas (Fearon & Vogelstein, 1990).

The progression from hyperproliferation towards malignant tumours is understood to occur via clonal expansion. Clonal expansion describes a process by which the first mutation in a single cell results in limited cell proliferation within a tissue. These hyperproliferative cells may acquire a second mutation resulting in a small benign tumour. Cells within the benign tumour which acquire further mutations clonally expand and may outgrow the original benign tumour towards malignant stages (Fearon & Vogelstein,

1990; Kinzler & Vogelstein, 1996). This hypothesis has been supported by the finding that malignant cells within a tumour exhibit the same set of mutations as found in benign sections, but with additional mutations (Fearon & Vogelstein, 1990; Kinzler & Vogelstein, 1996).

CHAPTER 1.1

INTRODUCTION

The Ras-pathway in signal transduction and transformation

1.1 The Ras pathway in signal transduction and transformation

The Ras signalling pathway is of central importance in mediating cellular growth and differentiation signals from the cell surface to the nucleus. The cellular *ras* genes *H-ras*, *K-ras* and *N-ras* belong to the family of small GTPases which include other members such as Rap, Rho and Rac (Boguski & McCormick, 1993). Ras has also been widely implicated in tumorigenesis, and mutations of the *ras* gene have been found in 10-15% of human tumours (Bos, 1988).

1.1.1 The Ras signalling pathway

The Ras signalling pathway mediates growth and differentiation signals, initiated by receptor and non-receptor tyrosine kinases, from the cell surface to the nucleus. The importance of the Ras signalling pathway was initially shown by the requirement of Ras-activity for cell proliferation (Mulcahy et al., 1985; Feig & Cooper, 1988). Blocking the Ras-MAP-kinase pathway via a dominant negative mutant of Ras (Feig & Cooper, 1988) or an interfering antibody to Ras (Mulcahy et al., 1985; Stacey et al., 1991; Moodie et al., 1993) is sufficient to abrogate S-phase entry after mitogenic stimulation.

The stimulation of various growth-factor receptors, such as the Insulin receptor (Skolnik et al., 1993a, 1993b; Baltensperger et al., 1993), the Nerve Growth Factor (NGF) receptor (Li et al., 1992) and the Epidermal Growth Factor (EGF) receptor (Buday et al., 1993a, 1993b) triggers p21^{Ras} activation. Activation of these tyrosine kinase receptors leads to the autophosphorylation of tyrosine residues within their cytoplasmic domains. These specific phosphorylations provide an anchor for adapter molecules

such as Grb2 (Rozakis-Adcock et al., 1993). Grb2 consists of protein-interaction domains, src-homology domains 2 and 3 (SH2/SH3) (Clark et al., 1992) and has no enzymatic activity (Suen et al., 1993; Bar-Sagi et al., 1993). Grb2 bound to the receptor via an SH2 domain (Lowenstein et al., 1992) attracts the Ras guanine nucleotide exchange protein, mSOS-1, to the multi-protein complex via its SH3 domains (Batzer et al., 1993; Egan et al., 1993; Gale et al., 1993). This physical interaction between the phosphorylated receptor, Grb2 and mSOS-1 increases the guanine nucleotide exchange activity of mSOS-1 on Ras (Aronheim et al., 1994). G-protein coupled receptors signal also towards Ras. This involves the $G\beta\gamma$-mediated tyrosine phosphorylation of Shc, which leads to an increase in Shc-Grb2-Sos association and subsequent Ras-dependent activation of downstream targets (Crespo et al., 1994; van Biesen et al., 1995).

1.1.1.1   Mechanism of Ras activation

Ras is a small signal transducer protein whose activation is regulated by the binding to guanine nucleotide phosphate (Lowy & Willumsen, 1993). In unstimulated mammalian cells the majority of Ras molecules are found in the inactive conformation bound to guanine nucleotide diphosphate (GDP). Upon activation of p21[RAS], GDP is exchanged for guanine nucleotide triphosphate (GTP). Activated GTP-bound Ras is slowly hydrolysed by an intrinsic GTPase activity to GDP and free phosphate (Lowy & Willumsen, 1993). Both steps, the activation of Ras through the exchange of GDP to GTP and its inactivation through hydrolysis of GTP to GDP and phosphate, are regulated by a number of accessory proteins. Ras is positively regulated by guanine nucleotide-exchange factors (GEF) (Boguski & McCormick, 1993) such as mSos (Chardin et al., 1993; Simon et al., 1993), GRF/CDC25

(Crechet et al., 1990), Vav (Margolis et al., 1992; Gulbins et al., 1994), Dbl (Shou et al., 1992), which facilitate GTP binding to Ras (Egan et al., 1993). Contrary to GEFs, GTPase-activating proteins (e.g. GAP and NF1) act as negative regulators by increasing the hydrolysis of GTP bound to Ras (Boguski & McCormick, 1993), thereby shifting the equilibrium towards inactive Ras. Oncogenic Ras, however, remains in a constitutively active GTP-bound conformation through mutations that either inhibit the hydrolysis of bound GTP or increase the rate of GDP to GTP exchange, thus resulting in higher levels of GTP-bound Ras (Lowy & Willumsen, 1993).

In addition to their role as negative regulators of Ras, some GTPase-activating proteins may also be effectors of Ras. For instance, the Ras GTPase-activating protein p120^{GAP} contains SH2 and SH3 domains in the amino-terminus which, when expressed separately, can induce transcription from the promoter of the c-fos-gene in a Ras-dependent manner (Medema et al., 1992). The amino-terminus of p120^{GAP} can also increase transformation when co-transfected with v-src into NIH3T3 cells (De Clue et al., 1993). Further evidence that p120^{GAP} may be a Ras effector has come from the observation that the amino-terminus of p120^{GAP} binds to two proteins, p62 (Carpino et al., 1997; Yamanashi & Baltimore, 1997) and p190 (Bryant et al., 1995). This binding is facilitated via SH2/SH3 domains of p120^{GAP} in cells stimulated by mitogens (Boguski & McCormick, 1993). The cytoplasmic protein p62 can also bind to other proteins such as phospholipase Cγ, Grb2 and Src (Richard et al., 1995). The second GAP-binding protein p190 (Settleman et al., 1992a), exhibits GAP-activity towards Rho (Foster et al., 1994) and can inhibit Rho-mediated formation of stress fibers (Ridley et al., 1993). These findings suggest that p190 is a RhoGAP and that Ras activation may influence Rho via the RasGAP-p190RhoGAP interaction (Ridley et al., 1993). However, the formation of a

complex with p120^{GAP}/p62/p190 and its association with the plasma membrane does not require activated Ras, thus raising doubts whether p120^{GAP} is indeed a Ras effector (Pronk et al., 1993).

Neurofibromin (NF-1), another mammalian GTPase activating protein, binds to Ras via its GAP-homology domain and negatively regulates Ras activity (Martin et al., 1990; Xu et al., 1990a). Oncogenic Ras, however, is insensitive to negative regulation by neurofibromin (Martin et al., 1990; Xu et al., 1990a). Neurofibromin shows sequence homology with the *S.cerevisiae* Ras-regulatory proteins, IRA1 and IRA2 (Ballester et al., 1990). The catalytic domain of neurofibromin can functionally replace IRA proteins in regulating Ras in yeast (Xu et al., 1990b; Ballester et al., 1990). IRA1 and IRA2 bind to Ras but exhibit only negative regulatory functions (Tanaka et al., 1990; 1991). Similarly, there is no evidence that neurofibromin is an effector of Ras. In support of this notion, mammalian neurofibromin appears to have no additional known motifs besides its GAP-related domain (Boguski & McCormick, 1993). Furthermore, apart from Ras, the only other protein known to bind to neurofibromin is tubulin (Bollag et al., 1993). Nevertheless, neurofibromin can inhibit Ras-dependent growth in NIH3T3 cells by a mechanism apparently independent of its ability to accelerate the intrinsic GTPase activity of Ras (Johnson et al., 1994).

1.1.1.2   Downstream effectors of Ras

Active Ras can bind to several effector proteins which mediate signals into distinct pathways. The interaction of Ras with its different effectors can occur through two distinct regions of the Ras protein: The first region comprises amino acids 32-38, designated Switch I and a second, the Switch II region of residues 60-76. Both regions dramatically change their conformation in the active GTP-bound state (Krengel et al., 1990; Pai et al.,

1990; Schlichting et al., 1990). Furthermore, these two regions may determine the specificity of Ras signalling towards different cellular target proteins (Moodie et al., 1995).

The best characterised Ras effector so far is the Raf serine/threonine kinase (Kyriakis et al., 1992, 1993; Rapp et al., 1983, 1991) which is part of the Ras-MAP-kinase signalling pathway. Raf-1 kinase is associated through its amino-terminus with GTP-bound Ras as well as oncogenic Ras (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang XF. et al., 1993; Vojtek et al., 1993).

Another Ras effector, phosphatidylinositol-3-OH kinase (PI3K) (Rodriguez-Viciana et al., 1994), consists of two subunits, a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. Ras can bind to the catalytic subunit *in vitro* (Rodriguez-Viciana et al., 1994), and the expression of both PI3K subunits together with Ras rapidly increase the generation of the second messenger phosphatidylinositol-3,4-bisphosphate. Furthermore, the increase in phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate after the addition of mitogens can be inhibited by coexpression of a dominant negative Ras(N17) mutant, arguing for the Ras-dependency of this process (Rodriguez-Viciana et al., 1994). The activation of phosphatidylinositol-3-OH kinase, and subsequential generation of phosphatidylinositol-3,4-bisphosphate triggers the specific activation of the PKB/Akt kinase (Burgering & Coffer, 1995; Franke et al., 1995; 1997a) and has also been shown to activate the serine/threonine kinase p70^{S6} (Chung et al., 1994).

MEK-kinase, a homologue of the yeast protein kinases Bry2 and Ste 11 (Lange-Carter et al., 1993), binds directly via its catalytic domain to GTP-bound Ras and was therefore suggested as a possible Ras effector (Russell et al., 1995). Initially identified as a kinase able to activate MAP-kinase

kinase in a Raf-independent manner (Lange-Carter et al., 1993), MEK-kinase, however, has been shown to be a member of the JNK-1 kinase pathway rather than the Ras-MAP-kinase pathway (Sanchez et al., 1994; Yan et al., 1994). Thus, MEK-kinase links Ras with the JNK-kinase pathway (Minden et al., 1994a).

Other proteins, initially identified as Ras effector candidates by their binding capacity to Ras *in vitro* or by their interaction in yeast two-hybrid system screens, have not yet been clearly characterised as Ras effectors. For instance, the association of Ras with the Ral guanine nucleotide dissociation stimulator (Ral-GDS), an exchange factor for the Ras-related protein Ral has been observed in a yeast two-hybrid system screen (Hofer et al., 1994; Spaargaren et al., 1994). To date, it is not clear whether Ral-GDS is a direct Ras effector, although it does appear that Ral-GDS is involved in the Ras-dependent activation of phospholipase D (Carnero et al., 1994; Jiang et al., 1995). Activated phospholipase D generates various second messengers such as phosphatidic acid, lysophosphatidic acid and diacylglycerol (Jiang et al., 1995). Other Ral-GDS, such as RGL (Kikuchi et al., 1994; Peterson et al., 1996) have been found to bind to GTP-bound Ras *in vitro* (Van Aelst et al., 1994).

1.1.2   The Ras-MAP-kinase signalling pathway

Ras can activate several different signalling pathways (Denhard, 1996; Marshall, 1996). One of these pathways branching from Ras, the Ras-MAP-kinase signalling pathway has been intensively studied since the activation of this pathway is required for cell proliferation in many cell types. As an initial step in this kinase cascade, Ras in its active conformation as well as oncogenic Ras bind to the amino-terminus of the serine/threonine

kinase c-Raf 1 (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang XF. et al., 1993; Vojtek et al., 1993; Koide et al., 1993). However, the physical interaction between activated Ras and the regulatory domain of Raf (amino acids 51-131) is not sufficient to stimulate Raf-kinase activity. The activation of Raf appears to require multiple activation steps within a multi-protein complex, localised at the cell membrane (Wartmann & Davis, 1994; Morrison & Cutler, 1997), with the binding of GTP-bound Ras to Raf only being involved in a first activation step of localising Raf to the plasma membrane (Traverse et al., 1993; Marais et al., 1995). This localisation appears to be the only requirement of activated Ras in the activation of Raf, since Raf targeted to the membrane does not require Ras for activation (Leevers et al., 1994; Stokoe et al., 1994).

Several additional steps have been proposed, although the exact mechanism of Raf activation is not clear. Further activation steps have been suggested involving the possible binding of phosphatidylserine and ceramide directly to Raf (Ghosh et al., 1996; Huwiler et al, 1996), phosphorylation events, especially on tyrosine residues for the increase of kinase activity (Fabian et al., 1993, Jelinek et al., 1996), triggered by a ceramide-activated protein kinase (CAP-kinase) (Yao B. et al., 1995) or the involvement of 14-3-3 proteins (Li et al., 1995) as part of the multi-protein complex. The CAP-kinase was recently identified as the kinase suppressor of Ras (KSR) protein (Zhang et al., 1997), having been initially discovered in genetic screens in *Drosophila* (Therrien et al., 1995) and *C.elegans* (Kornfeld et al., 1995a; Sundaram & Han, 1995). In *Drosophila*, loss-of-function *ksr* alleles were recovered as a dominant suppressor of an activate Ras1-induced rough-eye phenotype (Therrien et al., 1995). The KSR-protein appears to function in parallel or upstream of Raf in *Drosophila* and *C.elegans* (Downward, 1995). Biochemical studies have revealed that

KSR/CAP-kinase binds to Raf in a ceramide-dependent manner at the plasma membrane, and can activate Raf-kinase activity through phosphorylation on two adjacent threonine residues (Zhang et al., 1997). CAP-kinase also increases the effects of activated Ras in *Xenopus laevis* oocytes and cell transformation (Therrien et al., 1996).

Dimerisation of c-Raf 1, as facilitated by chemicals, activates Raf-kinase activity and the MAP-kinase cascade in the absence of membrane compounds (Farrar et al., 1996; Luo et al., 1996). The Ras-dependency of this process is, however, unclear since activation of dimerised Raf has been observed in a Ras-dependent (Luo et al., 1996) and Ras-independent manner (Farrar et al., 1996). It has been speculated that *in vivo* 14-3-3 proteins may facilitate dimerisation of Raf (Farrar et al., 1996; Luo et al., 1996), although the evidence for a role of 14-3-3 proteins in Raf activation is controversial.

Various 14-3-3 proteins appear to be involved in Raf activation, since the expression of 14-3-3 proteins in *Xenopus* oocytes enhances Raf-1 activity and oocyte maturation (Irie et al., 1994; Fantl et al., 1994) and in *Drosophila* 14-3-3 proteins are positively modulating Ras/Raf signalling (Chang & Rubin, 1997; Kockel et al., 1997). In contrast, some groups have reported a constitutive association between Raf-1 and 14-3-3 proteins independent of Raf-1 activation (Freed et al., 1994a, 1994b; Suen et al., 1995), while others have indicated that Raf-1 kinase activity is suppressed by 14-3-3 proteins (Michaud et al., 1995).

The main target of Raf appears to be the dual specific tyrosine/threonine kinase, MAP-kinase kinase (MKK) also designated MAP-kinase/ERK-kinase kinase (MEK) (Crews et al., 1992; Huang et al., 1993). Activated Raf-1 binds and activates MAP-kinase kinase (Dent et al., 1992; Kyriakis et al., 1992, 1993; Howe et al., 1992) through phosphorylation of

two adjacent serine residues (Alessi et al., 1994; Zheng CF. & Guan, 1994; Papin et al., 1995). These two phosphorylation steps are necessary and sufficient to activate MAP-kinase kinase (Alessi et al., 1994), and point mutations of these phosphorylation sites have led to the generation of a constitutively active MAP-kinase kinase (Cowley et al., 1994; Mansour et al., 1994).

Activated MAP-kinase kinase phosphorylates various isoforms of Mitogen-activated-protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (Boulton et al., 1991; Crewes et al., 1992a; Wu J. et al., 1993; Zheng CF. & Guan, 1993a, 1993b). Upon phosphorylation of both threonine 183 and tyrosine 185 residues MAP-kinase activity increases dramatically (Payne et al., 1991; Haystead et al., 1992). The phosphorylation of threonine 183 facilitates the second phosphorylation step on tyrosine 185 (Payne et al., 1991; Haystead et al., 1992), but both phosphorylation events are required for the increase of kinase activity (Anderson et al., 1990). Removal of either of these phosphorylations through the action of specific phosphatases (Anderson et al., 1990) or site-specific mutagenesis (Cowley et al., 1994) leads to inactivation. The position of MAP-kinases downstream of Ras and Raf was confirmed by the observation that blocking of upstream members such as Ras through coexpression of a dominant negative mutant, abolishes MAP-kinase activity (de Vries-Smits et al., 1992; Wood et al., 1992; Schaap et al., 1993). Furthermore, MAP-kinases are found constitutively activated in v-ras (Leevers et al., 1992) and v-raf transformed cells (Howe et al., 1992).

1.1.2.1   MAP-kinase target proteins

In response to various stimuli, activated MAP-kinase translocates to the nucleus (Chen et al., 1992; Lenormand et al., 1993; Gonzalez et al., 1993), where the MAP-kinase-dependent phosphorylation of transcription factors is understood to mediate immediate early gene expression (Treisman, 1996). A clear effect of MAP-kinase phosphorylation on its ability to transactivate genes has been observed for some of these transcription factors. For instance, proteins in the ternary complex bound to the serum response element (SRE) such as ELK1/p62^{TCF} are regulated in part through the phosphorylation of MAP-kinase (Gille et al., 1992; Janknecht et al., 1993; Marais et al., 1993). The MAP-kinase-dependent phosphorylation of ELK1 increases the ELK1-mediated formation of the ternary complex (Gille et al., 1995a, 1995b) and subsequent transactivation from the serum response element (Gille et al., 1995a, 1995b; Janknecht et al., 1993). As another example, the phoshorylation of the c-Fos transcription factor by MAP-kinase (Chen RH. et al., 1993) has been associated with enhanced stability of the protein and an increase in transactivation activity (Chen RH. et al., 1996; Okazaki & Sagata, 1995). The phosphorylation of other Fos family members, in particular Fra-1 and Fra-2, also increased the DNA-binding affinity of these transcription factors towards target genes (Gruda et al., 1994). The MAP-kinase-dependent phosphorylation of the amino-terminus of c-Myc at serine 62, as observed *in vitro* has been proposed to increase the transactivation activity of c-Myc *in vivo* (Seth et al., 1991; Seth et al., 1992). The transcription factors Ets-1 and Ets-2 are direct targets of MAP-kinase *in vitro* and *in vivo* and positively influence transactivation activity (Rabault et al., 1996; Yang et al., 1996). The c-Jun transcription factor is also phosphorylated by MAP-kinase (Pulverer et al., 1991, Alvarez et al., 1991). The major phosphorylation site *in vitro* is serine 243 (Chou et

al., 1992), a carboxy-terminal site which inhibits the DNA-binding capacity of c-Jun (Alvarez et al., 1991; Baker et al., 1992; Chou et al., 1992). MAP-kinase-dependent phosphorylation therefore appears to have an inhibitory effect (Minden et al., 1994a, 1994b), whereas the related JNK-kinases can phosphorylate c-Jun N-terminal phosphorylation sites and enhance c-Jun's transcriptional activity (Minden et al., 1994a, 1994b), suggesting a coordinated control of transactivation activity converging from at least two signalling pathways.

The phosphorylation of a number of additional transcription factors *in vitro* has been reported for p53 (Milne et al., 1994), c-Myb (Aziz et al., 1993) and oncoprotein 18 (Marklund et al., 1993), but a clear physiological effect *in vivo* has not yet been demonstrated.

Activated MAP-kinases also phosphorylate a great number of other proteins at serine or threonine residues in the consensus motif P/LXT/SP (X representing any amino acid) (Alvarez et al., 1991; Gonzalez et al., 1991; Haycock et al., 1992). Apart from cytoskeletal proteins (Drewes et al., 1992; Drechsel et al., 1992), other downstream kinases such as S6 kinases p90[rsk1/2] (Chung et al., 1991; Erikson et al., 1991; Sutherland et al., 1993) and MAPKAP kinase-2 (Stokoe et al., 1992) have been found up-regulated in their activity upon phosphorylation by MAP-kinase. Furthermore, the cytoplasmic phospholipase A_2 (cPLA_2) has been convincingly shown to be activated through phosphorylation by MAP-kinase (Lin et al., 1993). The activation of cytoplasmic phospholipase A_2 results in the release of fatty acids from phospholipids which generates various second messengers (Lin et al., 1993).

1.1.3 Negative regulation of the Ras-MAP-kinase signalling pathway

The Ras-MAP-kinase pathway is negatively regulated at various points and through a number of mechanisms. For instance, the phosphorylation of upstream members such as Sos by MAP-kinase, which inhibits the binding of the Sos/Grb2 complex to phosphorylated tyrosines of the EGF-receptor and thereby provide a negative feedback loop (Cherniack et al., 1994; Waters et al., 1996; Buday et al., 1995; Porfiri & McCormick, 1996; Corbalan-Gracia et al., 1996). The phosphorylation of the EGF-receptor itself by MAP-kinase (Alvarez et al., 1991; Northwood et al., 1991; Takishima et al., 1991) may be required for internalisation since mutants had an impaired uptake (Heisermann et al., 1990). This mechanism could reduce the number of EGF-receptors available at the cell membrane after prolonged stimulation. Furthermore, MAP-kinase-dependent phosphorylation of MAP-kinase kinase (Matsuda et al., 1993; Brunet et al., 1994b; Gardner et al., 1994) and c-Raf-1 (Lee et al., 1992; Anderson et al., 1991; Ueki et al., 1994) has been observed, although it is currently not clear whether these phosphorylation events were sufficient to reduce kinase activity in all cases.

Further negative regulation of the Ras-MAP-kinase signalling pathway can be provided by the regulated dephosphorylation of activated protein kinases by either cytoplasmic protein phosphatases or MAP-kinase-specific phosphatases. The protein phosphatase 1 (PP1) and the protein phosphatase 2A (PP2A) can dephosphorylate Raf-kinase (Dent et al., 1995) and MAP-kinase kinase 1/2 *in vitro* (Nakielny et al., 1992). PP2A also dephosphorylates the activating phosphorylation at threonine 183 in MAP-kinase 1 and 2 *in vitro* (Anderson et al., 1990), while the other phosphorylation site at tyrosine 185 can be dephosphorylated by several

protein phosphatases (Anderson et al., 1990). The importance of these *in vitro* results was strengthened by the fact that SV40 small T expression, which inhibits PP2A protein phosphatase 2A, led to the up-regulation of MAP-kinase kinase and MAP-kinase activity followed by stimulation of cell proliferation (Sontag et al., 1993).

MAP-kinase is specifically inactivated by a family of phosphatases consisting of PAC-1 (Rohan et al., 1993; Ward et al., 1994), HVH1 (Zheng & Guan, 1993c), HVH2 (Guan & Butch, 1995), MKP-1/3CH134/ERP (Charles et al., 1993; Sun et al., 1993; Noguchi et al., 1993) and a human homologue of MKP-1/3CH134, CL-100 phosphatase (Keyse & Emsilie, 1992; Alessi et al., 1993; Lewis et al., 1995). Most of the MAP-kinase specific phosphatases are localised in the nucleus (Rohan et al., 1993, Brondello et al., 1995) where they are understood to act on translocated and activated MAP-kinase. These dual-specific tyrosine/threonine phosphatases are mainly regulated at transcriptional level (Charles et al., 1993; Noguchi et al., 1993). Mitogens as well as oxidative stress and heat shock, which activate MAP-kinase, also rapidly induce the transcription of these phosphatases (Sun et al., 1993; Charles et al., 1992; Keyes & Emslie, 1992), thereby providing a negative feedback loop. However, individual growth factors have a different potential to induce these phosphatases (Noguchi et al., 1993) and the protein phosphatase CL-100 dephosphorylates activated MAP-kinase in response to EGF in a cell-type specific manner (Alessi et al., 1995b).

The action of MAP-kinase-dependent phosphatases have clear physiological effects in a number of cell types. The expression of the MAP-kinase-specific phosphatase MKP-1 inhibits Ras induced DNA-synthesis (Sun et al., 1994), S-phase entry after serum stimulation (Brondello et al., 1995) as well as the serum stimulated transcription from the *c-fos* promoter in fibroblasts (Brondello et al., 1995). The constitutive expression of the

MAP-kinase specific phosphatase PAC-1 inhibits phorbol ester-induced transcriptional activity from the *c-fos* serum response element (Ward et al., 1994). Moreover, the CL-100 phosphatase inhibits the activated Ras-induced MAP-kinase activation in a cell-free system derived from *Xenopus laevis* oocytes (Alessi et al., 1993).

1.1.4 The Ras-MAP-kinase pathway in *Drosophila melanogaster*

In *Drosophila melanogaster* homologues of mammalian proteins of the Ras-MAP-kinase signalling pathways regulate development and differentiation, for instance, the cell fate of the R7 photoreceptor cell and the termini via the torso-pathway.

The adult *Drosophila* eye consists of approximately 800 ommatidia which are each made up of twenty cells. Each ommatidium contains eight photoreceptor cells, designated R1-R8, four lens-secreting cone cells and eight accessory cells. The fate of each cell appears to be decided by locally restricted cell-cell interactions (Perrimon, 1994; Zipursky & Rubin, 1994). The ligand Boss protein exclusively expressed on the differentiating R8 photoreceptor cell (Kramer et al., 1991) induces the R7 cell to become a photoreceptor cell via activation of the sevenless receptor (Van Vactor et al., 1991). The sevenless (sev) receptor tyrosine kinase is part of a signalling pathway consisting of homologous proteins to the mammalian Ras-MAP-kinase pathway which are both necessary and sufficient to determine the cellular fate of R7 (Zipursky & Rubin, 1994). In agreement with this model, mutations in various genes such as *sevenless* (Ready, 1989), *boss* (Reinke & Zipursky, 1988) and the transcription factor *sina* (Carthew & Rubin, 1990) disrupt the recruitment of the R7 cell to the ommatidium. The signal for

determining the cell fate in R7 cells is further mediated by the Drk protein, a homologue of the mammalian Grb2 (Olivier et al., 1993). Drk binds to the sevenless receptor as well as to Sos and is required for Ras activation (Simon et al., 1993). Furthermore, D-raf (Dickson et al., 1992) and a MAP-kinase homologue encoded by the *rolled*-gene (Biggs et al., 1994) have been genetically identified to mediate the differentiation of precursor cells into R7 photoreceptor cells. Target proteins for the MAP-kinase Rolled, such as the transcription factors Sina/Yan and Phyllopod are involved in translating the differentiation signal into specific gene expression patterns (Brunner et al., 1994; O`Neill et al., 1994; Rebay & Rubin, 1995; Chang et al., 1994; Dickson et al., 1995).

The torso receptor pathway in *Drosophila* determines cell fate at the posterior termini of the Drosophila. Ras (Lu X. et al., 1993) and a MAP-kinase kinase homologue D-MEK (Tsuda et al., 1993) are required for this differentiation process. The generation of gain-of-function mutations in the MAP-kinase Rolled and MAP-kinase kinase homologue D-MEK have indicated that both kinases are members of three pathways in Drosophila: the sevenless pathway, the torso pathway and the EGF receptor (DER) tyrosine kinase pathway (Brunner et al., 1994; Hsu & Perrimon, 1994).

1.1.5   The Ras pathway in *Caenorhabditis elegans*

The nematode *Caenorhabditis elegans* requires the activity of various proteins homologous to the mammalian Ras-MAP-kinase pathway proteins for vulva development (Kornfeld, 1997; Sundaram & Han, 1996). Vulval induction is initiated by the anchor cell (AC) of the gonad which induces three of six vulval precursor cells (VPC) to form vulval tissue, while the remaining three VPCs develop into epidermal cells (Kornfeld, 1997;

Sundaram & Han, 1996). Several genes involved in vulval induction are homologous to members of the mammalian Ras-MAP-kinase pathway such as the *lin-3* gene which encodes an EGF-like molecule, secreted from the anchor cell (Hill & Sternberg, 1992), the *let-23* gene which encodes a tyrosine kinase receptor of vulval precursor cells (Aroian et al., 1990), the *sem-5* gene encoding a Grb-2 homologue (Clark et al., 1992) and the *let-60* and *lin-45* genes which encode a *ras* and *raf* homologues, respectively (Han & Sternberg, 1990; Beitel et al., 1990). Deregulation of this pathway by reducing activity causes a vulvaless phenotype, whereas an increased activity of the Ras-homologue *let-60* results in a multivulva phenotype (Han et al., 1990a, 1990b).

1.1.6 MAP-kinase pathways in *Saccharomycetes sp.*

In the yeast *S. cerivisiae* a number of pathways with mammalian MAP-kinase pathway member homologues have been described (Ammerer, 1994) which regulate various aspects such as the pheromone response, invasiveness in haploid strains, pseudohyphal development and spore formation in diploid strains (Ammerer, 1994; Herskowitz, 1995).

The pheromone response signalling pathway consists of homologous members of the mammalian MAP-kinase pathway and is important for the preparation of a- and α-cells prior mating. Haploid cells of *S. cerivisiae* occur in two distinct phenotypes, a or α-cells. Both haploid cell types grow via mitotic cell division but can also mate to form a diploid a/α cell. In order to prepare cells for mating, a-cells secrete an a-factor which binds to a specific receptor on α-cells, whereas α-cells secrete α-factors, a peptide which binds to a specific receptor on a-cells (Herskowitz, 1995). The pheromone signalling pathway activated by these peptide factors

consist of various yeast homologues of the mammalian MAP-kinase pathway which appear to be identical with the exception of the respective receptors, the a-factor receptor (*STE2*) or the α-factor receptor (*STE3*). Both receptors are coupled to G-proteins and it is understood that the Gβγ-proteins mediate the activation of the MEK-kinase STE11 possibly via protein kinase STE20, although the exact mechanism as to how Gβγ-proteins mediate STE11 activation is not yet clear. Activated STE11-kinase phosphorylates and activates STE7, a MAP-kinase kinase homologue (Neiman & Herskowitz, 1994). Activated STE7 phosphorylates and thereby activates the MAP-kinase homologues FUS3 or KSS1 (Errede et al., 1993). It has been suggested that these three kinases are bound to a "scaffolding" protein, STE5 which thereby creates a kinase module (Whiteway et al., 1995). The activated MAP-kinases FUS3 and KSS1 appear to phosphorylate the transcription factor STE12 which leads to its activation (Errede & Ammerer, 1989). The transcription factor STE12 induces transcription of genes required for the fusion between a- and α-cells as well as genes encoding members of the pheromone response signalling pathway itself (Fields & Herskowitz, 1985).

The activation of this pathway also leads to a G1 cell cycle arrest mediated by the FAR-1 protein, which binds and inhibits the cyclin-dependent kinase complexes CDC28-CLN1 and CDC28-CLN2 (Peter & Herskowitz, 1994). The protein level of FAR-1 is elevated by pheromones (Peter et al., 1993; Chang & Herskowitz, 1992). It seems that the activity of FAR-1 is modulated through phosphorylation by FUS3 MAP-kinase, thereby establishing a connection between the activation of the pheromone signalling pathway and the cell cycle machinery (Peter et al., 1993).

1.1.7 The Ras-MAP-kinase pathway in apoptosis

Serum and a set of growth factors can inhibit programmed cell death (apoptosis) and increase the survival rate of cells, whereas the removal of these factors can result in apoptosis (Askew et al., 1993; Raff et al., 1993; Harrington et al., 1994). For example, IGF-1, insulin and PDGF increase survival and inhibit cell death, whereas EGF or FGF are poor survival factors in fibroblasts (Harrington et al., 1994). Both classes of factors can stimulate the Ras-MAP-kinase pathway to various degrees in different cell types, but it is unclear how a Ras-dependent pathway mediates the inhibition of apoptosis. Suppression of apoptosis has been correlated with the activation of the Ras effector PI3-kinase (Yao R. & Cooper, 1995) which is mediated via the Ras and PI3 kinase-dependent activation of PKB/Akt (Franke et al., 1995, 1997a, 1997b) but not the activation of $p70^{S6}$ kinase (Kauffmann-Zeh et al., 1997; Dudek et al., 1997). Corresponding with these results, the inhibition of PI3-kinase accelerated apoptosis and an activated form of PKB/Akt blocked apoptosis (Franke et al., 1997b). Activated PKB/Akt did not modulate the expression of negative apoptosis regulators Bcl-2 or Bcl-X_L, but blocked Ced3/ICE-like protease activity (Kennedy et al., 1997), required for the execution of programmed cell death (Ellis et al., 1991; Nicholson et al., 1995; Korsmeyer, 1995).

1.1.8 The Ras-MAP-kinase pathway and tumorigenesis

The significance of Ras in tumorigenesis was initially elucidated by the discovery that retroviruses with an acquired activated ras-gene were able to transform mammalian cells (Simons et al., 1964; Kirsten et al., 1968; Harvey & East, 1969). In support of this finding, mutations of cellular Ras,

constitutively activating the protein, were found in 10-15% of human tumours examined. In particular, in up to 95% of pancreatic carcinomas mutations of the *ras* gene occurred (Bos, 1989; Almoguera et al., 1988).

Ras-dependent transformation has mainly been attributed to the continuous activation of members of the Ras-MAP-kinase pathways and the resulting prolonged activation of downstream transcription factors. This notion is supported by the fact that dominant negative mutants of Raf-1 (Kolch et al., 1991), MAP-kinase kinase (Cowley et al., 1994) and MAP-kinase (Khosravi-Far et al., 1995) are all inhibitory to Ras-dependent cell transformation. Moreover, constitutively active Raf (Rapp et al., 1988), Raf targeted to the membrane (Leevers et al., 1994) and constitutively active MAP-kinase kinase (Cowley et al., 1994; Mansour et al., 1994) can transform fibroblasts indistinguishable from cellular transformation caused by oncogenic Ras.

Despite the evidence of the important role of the Ras-MAP-kinase pathway in transformation, other Ras-dependent but MAP-kinase-independent signalling pathways appear to contribute or are even sufficient for cellular transformation (White et al., 1995; Qiu et al., 1995; Khosravi-Far et al., 1995; 1996; Rodriguez-Viciana et al., 1997; Olson et al., 1995). Experimental evidence which implies that multiple pathways were required for Ras transformation was provided by cooperation assays with Ras effector domain mutants. The effector domain mutant Ha-Ras (V12,T35S), although capable of binding Raf and stimulating MAP-kinase activity, is reduced in its transforming activity, suggesting that additional Ha-Ras effectors may contribute towards cellular transformation (White et al., 1995). However, mutant Ha-Ras (V12,T35S) is able to cooperate with another mutant Ha-Ras (G12V, E37G) to effectively transform fibroblasts as measured in a focus assay (White et al., 1995). The mutant Ha-Ras (G12V,

E37G) is unable to transform on its own, because it has lost the capability of binding to Raf but can still bind other Ras effectors. The requirement for both Ha-Ras mutants to efficiently cooperate towards cellular transformation suggests that multiple Ras functions may be involved in the transformation of mammalian cells (White et al., 1995). Similarly, two Ras mutants, Ha-Ras (V12, 37G) and Ha-Ras (V12, 40C), both defective in activating the Raf/MAP-kinase pathway, cause oncogenic transformation when expressed together, indicated by growth in low serum, anchorage-independent growth and formation of tumours after injection of transformed cells into nude mice (Khosravi-Far et al., 1996).

Investigations into additional Ras-dependent signalling pathways required for Ras transformation have focused on the role of Rac1, RhoA and PI3-kinase (Qiu et al., 1995; Khosravi-Far et al., 1995, 1996; Rodriguez-Viciana et al., 1997). Activated Rac1, Rac1(Val12) transforms Rat-1 fibroblasts cells as indicated by the partial loss of contact inhibition, anchorage-independent growth and in vivo tumour growth of Rac1(V12) expressing cells in athymic nude mice (Qiu et al., 1995). Furthermore, dominant negative Rac1(N17) reduces foci formation by oncogenic Ras in NIH3T3 fibroblasts (Qiu et al., 1995; Khosravi-Far et al., 1995) but not by a constitutively active form of Raf, RafCAAX (Leevers et al., 1994) which activates the MAP-kinase signalling pathway independently of Ras, suggesting that Rac1 activation is an essential step in Ras transformation (Qiu et al., 1995). It is interesting to note that RafCAAX or a weakly transforming Raf mutant, Raf(340D) cooperates with activated Rac1 in a focus formation assay (Qiu et al., 1995; Khosravi-Far et al., 1995), suggesting that the Rac1-dependent pathway is independent of the Ras-MAP-kinase signalling pathway and that both pathways are required for strong cellular transformation.

The Ras effector phosphoinositide 3-OH kinase (PI3K) (Sjolander et al., 1991; Rodriguez-Viciana et al., 1994) also appears to be involved in Ras transformation. The coexpression of a dominant negative PI3-kinase mutant (Dhand et al., 1994) strongly inhibits the cellular transformation by oncogenic Ras as measured in a focus formation assay (Rodriguez-Viciana et al., 1997). Constitutively active mutants of PI3-kinase cooperate with Raf and Rho but not Rac1, suggesting that PI3-kinase and Rac1 are members of the same signalling pathway (Rodriguez-Viciana et al., 1997).

Experiments with Ras mutants which selectively bind to various Ras effectors have confirmed these results. For instance, the Ha-Ras mutant (V12, T35S) can still activate Raf cooperates in a focus formation assay with activated mutants of PI3-kinase, Rac1 and Rho, but not with Raf itself. Similarly, Ha-Ras (V12, C40) activates only the PI3-kinase/Rac pathway cooperates with activated Raf and Rho, but not with activated PI3-kinase or Rac1, respectively (Rodriguez-Viciana et al., 1997). Taken together, these findings suggest the Raf/MAP-kinase pathway and the PI3-kinase/Rac pathway downstream of Ras can cooperate in cellular transformation.

The involvement of Rho in Ras transformation has been suggested since the expression of dominant-negative versions of Rho(N19) mitigate transformation by oncogenic Ras (Qiu et al., 1995; Khosravi-Far et al., 1995). Furthermore, activated forms of Rho and Rac, although not transforming on their own, can cooperate in a focus formation assay suggesting that they are acting on different signalling pathways (Khosravi-Far et al., 1995; Rodriguez-Viciana et al., 1997). Thus, despite the fact that many of these oncogene cooperation studies were carried out in established cells, they, nevertheless, have provided first evidence that apart from the Ras-Raf-MAP-kinase pathway, a number of Ras-dependent

downstream pathways, explicitly Rho-and PI3-kinase/Rac1-dependent pathways, may contribute towards cellular transformation by activated Ras.

CHAPTER 1.2

INTRODUCTION

The p53 tumour suppressor gene

1.2   The p53 tumour suppressor gene

The tumour suppressor p53 was first identified as a 53 kiloDalton protein associated with a transforming protein of the SV40 virus, large T (SV40 LT) (Lane & Crawford, 1979; De Leo et al., 1979; Linzer & Levine, 1979a, 1979b). The genomic p53 clones and p53 cDNA clones were subsequently isolated by various groups (Oren & Levine, 1983; Bienz et al., 1984; Pennica et al., 1984; Harlow et al., 1985). p53 is a transcription factor which mediates various cellular activities such as G1 cell cycle arrest (Michalovitz et al., 1990; Martinez et al., 1991), apoptosis (Lowe et al., 1993a, 1993b), the maintenance of genetic stability (Cross et al., 1995; Fukasawa et al., 1996) and the suppression of tumorigenesis (Baker et al., 1990). The p53 protein is mutated in many human tumours and mutant p53 can transform eukaryotic cells in cooperation with oncogenic *ras* (Eliayahu et al., 1984; Parada et al., 1984). Wild type p53, however, is unable to transform eukaryotic cells (Eliyahu et al., 1989; Hinds et al., 1989) and suppresses cellular transformation by other oncogenes *in vitro* (Finlay et al., 1989) as well as the formation of tumours in nude mice (Chen et al., 1990). Due to these features, p53 was classified as a tumour suppressor gene (Finlay et al., 1989; Eliyahu et al., 1989; Lane & Benchimol, 1990).

1.2.1   The tumour suppressor p53 in human cancer

Somatic mutations of the tumour suppressor gene p53 are the single most common genetic alteration in human tumours (Levine, 1992). The p53 gene is lost or functionally inactivated by mutations in more than half of all human tumours (Hollstein et al., 1994, 1996; Hainaut et al., 1997). 75-80% of colon tumours and 50% of lung and breast cancers in particular and many other tumours arising from various tissues exhibit somatic mutations

in the p53 gene (Greenblatt et al., 1994). Most somatic mutations of the p53 gene are missense mutations (Greenblatt et al., 1994) which are not randomly distributed throughout the p53 gene, but occur in specific clusters located in the region encoding amino-acid residues 130-290 (based on 393 amino acids in human p53). Four regions of p53, residues amino acids 117-142, amino acids 171-181, amino acids 234-258 and amino acids 270-286, which are highly conserved across p53 from different species are the target for most of these mutations (Levine et al., 1990). The DNA-binding domain of p53 (Pavletich et al., 1993) carries most mutations, "hot-spots" in particular involving amino acid residues 175, 248 and 273 (Levine et al., 1991; Hollstein et al., 1994). Mutations of p53 in this region dramatically alter the overall structure of the p53 protein (Cho et al., 1994; Gannon et al., 1990). Interestingly, the distribution of "hot-spot" mutations in human tumours appear to be tissue-specific which has been speculated to be either due to the type of mutagen a particular cell-type was exposed to or a selective growth advantage of certain p53 mutations in a tissue-specific context (Levine, 1992).

Besides somatic p53 mutations, a genetic disorder has been reported in which p53 mutations are inherited. The Li-Fraumeni syndrome (Li & Fraumeni, 1969) is an autosomal dominant genetic disorder which is characterised by the high risk of an early onset of tumours and the development of many independent tumours. Half of individuals affected by Li-Fraumeni syndrome develop tumours by the age of 30 compared with 1% of the normal population (Malkin et al., 1990). A number of patients have presented heterozygously inherited p53 missense mutations (Malkin et al., 1990; Srivastava et al., 1990). This heterozygosity for p53 increases the risk for loss of both p53 alleles.

The homozygous loss of p53 has a dramatic effect in mice which become highly prone to acquiring a wide variety of malignant tumours at an early stage of their life (Donehower et al., 1992; Jacks et al., 1994). Within six months, 74% of p53$^{-/-}$ mice develop various tumours (Donehower et al., 1992). By comparison, mice heterozygous for p53 (p53$^{+/-}$) exhibit a delayed onset of an altered spectrum of tumours (Jacks et al., 1994) which can be accelerated by the application of the carcinogen dimethylnitrosamine (Harvey et al., 1993). Mice nullizygous for p53 do not reveal substantial developmental alterations, except for a small percentage of female embryos exhibiting exencephaly (Sah et al., 1995).

1.2.2   Cellular functions of p53

1.2.2.1   Wild type p53 in tumour suppression

Wild type p53's function as a potent tumour suppressor was first discovered when wild type p53 was introduced into colorectal carcinoma cell lines resulting in a cell cycle arrest (Baker et al., 1990). Additionally, the overexpression of wild type p53 suppresses transformation by cooperating oncogenes in rat embryo fibroblasts (Chen et al., 1990). Furthermore, the introduction of p53 re-establish the G1 cell cycle arrest point in response to DNA damage and reduces the rate of gene amplifications of transformed cells (Yin et al., 1992). Therefore, wild type p53 can suppress oncogenic transformation which is surprisingly independent from any p53-dependent transcriptional transactivation activity (Crook et al., 1994) which is required for p53-dependent growth suppression (Pietenpool et al., 1994). One mechanism of transformation suppression may be p53-dependent apoptosis (Lowe et al., 1994), especially since p53-dependent apoptosis has been observed which is independent of transcriptional transactivation (Haupt et al., 1995).

1.2.2.2   Wild type p53 and cell cycle arrest

Following DNA damage by a wide range of agents including UV-radiation, γ-irradiation and exposure to DNA damaging agents, p53 protein levels increase and cells arrest in G1 (Fritsche et al., 1993; Hall et al., 1993; Kastan et al., 1992; Kuberbitz et al., 1992). It has been proposed that this G1 arrest allows cells to repair damaged DNA before they proceed to replicate their DNA (Kastan et al., 1991). On the other hand, DNA damage in normal human fibroblasts induces an irreversible cell cycle arrest in G1 (DiLeonardo et al., 1994), questioning the generality of this hypothesis. In addition to p53 playing a role in the G1 cell cycle arrest, recent evidence suggests an involvement of p53 in a cell cycle arrest at the G2/M boundary (Agrawal et al., 1995; Stewart et al., 1995; Guillouf et al., 1995).

The ability of p53 to arrest cells after DNA damage has been implicated in maintaining stability and integrity of the genome. For instance, gene amplification is below detectable levels in normal diploid fibroblasts ($<10^{-9}$), but has been observed at much higher frequencies in transformed cells (10^{-3}-10^{-5}) (Livingstone et al., 1992), resulting in chromosomal abnormalities in tumour cells. This increase of gene amplification has been associated with a parallel loss of the ability of p53 to arrest cells in response to the uridine biosynthesis inhibitor PALA (Livingstone et al., 1992; Yin et al., 1992). Interestingly, expression of exogenous wild type p53 re-established the G1 arrest and reduces the frequency of gene amplification to the same low levels as observed in normal diploid fibroblasts (Yin et al., 1992).

The p53 molecule has also been implicated in the control of a mitotic cell cycle checkpoint (Cross et al., 1995; Fukasawa et al., 1996). Application of spindle inhibitors or the loss of wild type p53 function *in vitro* and *in vivo*

lead to a increased population of tetraploid cells, suggesting that p53 has an important function in maintaining the diploid status of cells (Cross et al., 1995). In addition, cells deficient in functional p53 or the p21$^{WAF1/Cip1}$-inhibitor exhibit a substantial lack of coordination between S-phase and mitosis after DNA damage (Waldman et al., 1995). This lack of coordination causes additional rounds of S-phase without a following mitosis. A high proportion of these cells exhibit aneuploidy and are eliminated by apoptosis (Waldman et al., 1995). Further studies suggest that p53 is involved in the regulation of centrosome duplication (Fukasawa et al., 1996). In the absence of p53, the number of centrosomes already increases dramatically with the second passage. This abnormal number of centrosomes results in an unequal segregation of chromosomes which suggests one possible mechanism by which the loss of p53 may cause genomic instability (Fukasawa et al., 1996). These reports support the idea that p53 has the ability to prevent DNA-damaged cells proceeding through the cell cycle, thereby controlling the integrity of the genome.

1.2.3.3   p53-dependent apoptosis

Apoptosis is a mechanism for eliminating proliferating cells damaged beyond DNA repair or affected by oncogenes which are susceptible to malignant progression. Programmed cell death is characterised by cell shrinkage, genomic DNA fragmentation and fast removal of the cell debris from the surrounding tissue (Hale et al., 1996). In response to various stimuli such as DNA damage (Clarke et al., 1993; Lowe et al., 1993a), UV-irradiation (Ziegler et al., 1994), γ-irradiation (Lowe et al., 1993b), the expression of c-myc (Hermeking & Eick, 1994; Wagner et al., 1994) and adenovirus E1A (Debbas & White, 1993; Sabbatini et al., 1995a, 1995b), p53 can induce apoptosis.

The decision, whether a cell undergoes G1 growth arrest or apoptosis depends on various factors which are only partly understood. In response to γ-irradiation the cellular decision between induction of growth arrest or p53-dependent apoptosis appears to be cell-type specific with fibroblasts arresting in G1 phase of the cell cycle and apoptosis occurring in hematopoietic and lymphoid cells (Midgley et al., 1995; Haupt et al., 1996; Canman et al., 1995). Apart from cell specificity, other factors such as the presence of growth factors (Canman et al., 1995), the expression of viral proteins (Howes et al., 1994; Sabbatini et al., 1995) and the protein levels of the retinoblastoma protein (Rb) (Morgenbesser et al., 1994; Pan & Griep, 1994) and the transcription factor E2F (Qin et al., 1994) appear to determine the decision between p53-dependent growth arrest or p53-dependent apoptosis as response .

The presence of survival factors, for instance, leads to a p53-dependent G1 cell cycle arrest after γ-irradiation, whereas their absence can trigger programmed cell death (Canman et al., 1995). Interestingly, expression of v-src could mimic the shift towards G1 cell cycle arrest. It has been speculated, that changes in p21$^{WAF1/Cip1}$ and GADD45 protein levels are involved in this decision (Canman et al., 1995).

The co-expression of viral proteins is another factor which can influence the decision between growth arrest and apoptosis. The expression of adenovirus protein E1A shifts the cellular response after γ-irradiation from a p53-dependent growth arrest towards induction of apoptosis (Debbas & White, 1993; Lowe & Ruley, 1993; Lowe et al., 1994). Inactivation of retinoblastoma protein (Rb) by adenovirus E1A and E7 protein results in unregulated E2F-DP1 activity. Similarly, overexpression of E2F causes S-phase entry and induction of apoptosis (Qin et al., 1994; Wu & Levine 1994), whereas the overexpression of Rb blocks p53-dependent

apoptosis (Haupt et al., 1995). Thus, a model emerges in which the decision for p53-dependent growth arrest or apoptosis depends on the availability of survival factors and whether activated oncogenes enable cells with DNA damage to enter the cell cycle, leading to apoptosis in the presence of wild type p53 (Levine, 1997).

Both p53-dependent G1 cell cycle arrest and apoptosis, are mainly regulated via p53's transactivation activity. The inhibition of cell proliferation by p53 is mediated through up-regulation of transcription of target genes such as p21$^{WAF1/Cip1}$ (El-Deiry et al., 1993). Similarly, the regulation of p53-dependent apoptosis is understood to be mediated via the transactivation of genes responsible for the induction of apoptosis (Miyashita & Reed, 1995; McCurrach et al., 1997; Sabbatini et al., 1995). However, a p53 molecule with two mutations in amino acids critical for transcriptional activity (Lin et al., 1995) was nevertheless capable of triggering apoptosis without inducing any sequence-specific transactivation (Haupt et al., 1995). This unexpected result was confirmed by the observation of p53-dependent apoptosis in the presence of RNA or protein synthesis inhibitors (Caelles et al., 1994). These results indicate that p53-dependent induction of apoptosis may occur in a cell type-specific manner (Haupt et al., 1996) by either an unidentified transactivation-independent mechanism or via transactivation of p53 target genes which are involved in the apoptotic process (Sabbatini et al., 1995; Levine, 1997).

1.2.4   Functional domains of wild type p53

The human p53 protein can be separated into four functional domains: the N-terminal transcriptional activation domain (amino acids 1-42) (Unger et al., 1992), the sequence-specific DNA-binding domain (amino

acids 102-292), the oligomerisation domain (amino acids 324-355) and the very C-terminal activation domain (amino acids 363-393) (Levine, 1997; Ko & Prives, 1996).

The N-terminal transactivation domain interacts with basal transcription factors in regulating transcription from p53 target genes (Lin et al., 1994). Particularly, the physical interaction with TATA-associated factors of the TFIID-complex are understood to mediate the p53-dependent increase in transcription (Thut et al., 1995; Lu H. & Levine, 1995). Amino acid residues 19, 22 and 23 have been shown to be crucial for p53's transcriptional activation ability (Lin et al., 1995). The interaction of the TATA-binding protein with p53 has also been mapped to this region (Liu X. et al., 1993; Truant et al., 1993, Liu X. & Berk, 1995). In addition, the cellular protein Mdm-2 and the viral protein E1B-55 kDa abrogate p53's transactivation activity by binding to this domain (Chen J. et al., 1993; Braithwaite et al., 1991).

The central domain of p53 confers sequence-specific DNA binding (Wang et al., 1993a, 1994a; Pavletich et al., 1993; Bargonetti et al., 1993). The importance of the specific DNA-binding domain has been illustrated by the fact that most p53 mutations in tumours negatively affect this domain through changes to the structural integrity rendering the molecule unable to bind DNA specifically (Cho et al., 1994; Bargonetti et al., 1993). The DNA-binding domain alone exhibits sequence-specific DNA-binding affinity *in vitro* (Bargonetti et al., 1993; Pavletich et al., 1993; Wang et al., 1993). This specific DNA-binding affinity can be abrogated by SV40 LT whose binding to p53 has been mapped to the DNA-binding domain (Jenkins et al., 1989).

The C-terminal oligomerisation domain mediates dimerisation and tetramerisation of p53 (Stürzbecher et al., 1992; Pavletich et al., 1993; Reed et al., 1993; Lee et al., 1994; Cho et al., 1995; Jeffrey et al., 1995). The

minimal domain required for transformation lies within the oligomerisation domain (Shaulian et al., 1992), although the significance of oligomerisation for the various biological functions of p53 is unclear. It has been suggested that oligomerisation of p53 is required for DNA binding (Halazonetis & Kandil, 1993; Shaulian et al., 1993; Pietenpol et al., 1994) and that the C-terminal oligomerisation domain is required for p53 transactivation activity (Halazonetis & Kandil, 1993; Pietenpol et al., 1994; Zhang W. et al., 1994). However, transfected monomeric p53 molecules resulting from a deletion in the oligomerisation domain and lacking sequence-specific DNA-binding capacity, still exhibit transactivation activity in transient transfection assays (Shaulian et al., 1993; Tarunina & Jenkins, 1993) and the ability to suppress oncogene-induced transformation (Shaulian et al, 1993).

The p53 activation domain induces the latent p53 sequence-specific DNA-binding activity in response to a number of modification at the C-terminus. The deletion of the last 30 amino acid of p53 as well as binding of the monoclonal antibody 421 to residues amino acids 370-378 activate p53's sequence-specific DNA binding activity (Hupp & Lane, 1994). More physiological stimuli, such as phosphorylation at serine 378 by protein kinase C and serine 392 by casein kinase II, or short single DNA strands can also activate the sequence-specific DNA binding of p53 (Hupp & Lane, 1994; Jayaraman & Prives, 1995). The C-terminal domain contains residues that enable p53 to oligomerise and to bind both single-stranded DNA and RNA (Pavletich et al., 1993; Clore et al., 1994; Wu et al., 1995).

1.2.5   Regulation of transcription by p53

Wild type p53 regulates transcription in two ways. Firstly, activated p53 positively regulates transcription from promoters or introns of target genes that contain p53-specific DNA-binding sites (Farmer et al., 1992; Zambetti et al., 1992). Although the exact sequence of p53 DNA-binding sites varies, a palindromic consensus site consisting of two copies of a 10 base pair sequence 5′- PuPuPuC(A/T)(T/A)GPyPyPy-3′ has been defined (El-Deiry et al., 1992; Funk et al., 1992).

Secondly, wild type p53 can repress transcription from promoters with a TATA-binding site but without a p53 consensus site, probably by interacting with the basal transcription machinery (Subler et al., 1992, 1994; Jackson et al., 1993; Seto et al., 1992).

1.2.5.1   Transcriptional activation of p53 target genes
mediating growth inhibition

Wild type p53 mediates growth inhibition mainly through the activation of target genes. Activated p53 strongly induces the expression of the wild type p53-activated fragment 1 gene (WAF1) (El-Deiry et al., 1993). The same protein was independently isolated as a Cdk-interacting protein 1 (Cip1) which was found to bind to Cdk2/cyclin complexes and to inhibit their kinase activity (Harper et al., 1993). This parallel discovery immediately led to the suggestion that p21[WAF1/Cip1] could mediate the G1 growth inhibitory function of p53 since, in particular cyclin A-Cdk2, cyclin E-Cdk2 and cyclin D-Cdk4 complexes are essential for the G1-S phase transition of the cell cycle (El-Deiry et al., 1993; Harper et al., 1993). In fact, it was shown that p21[WAF1/Cip1] can inhibit a wide range of cyclin/Cdk complexes (Xiong et al., 1993b; Gu et al., 1993) and the overexpression of p21[WAF1/Cip1] leads to

growth inhibition in human tumour cell lines (El-Deiry et al., 1993) as well as in normal diploid human fibroblasts (Harper et al.,1993). Conversely, a decrease of p21$^{WAF1/Cip1}$ protein levels by antisense RNA allows arrested fibroblasts to progress into the cell cycle (Nakanishi et al., 1995). Moreover, the p53-dependent inhibition of cyclin/Cdk kinase activity after γ-irradiation is due to induction of p21$^{WAF1/Cip1}$ (Dulic et al., 1994) and the ability to arrest in G1 is impaired in p21$^{WAF1/Cip1}$ nullizygous mice (Brugarolas et al., 1995; Deng et al., 1995). However, further investigations revealed that p21$^{WAF1/Cip1}$ is only partly responsible for mediating the growth inhibitory functions of p53, since p21 nullizygous mouse fibroblasts exhibited only a partial loss of G1 arrest in response to γ-irradiation (Deng et al., 1995). In contrast, nullizygous p53 fibroblasts exhibit a complete loss of the G1 arrest after γ-irradiation (Deng et al., 1995), indicating the existence of p53-dependent, but p21$^{WAF1/Cip1}$-independent ways of cell cycle arrest. However, the G1 arrest in response to a specific inhibitor of the *de novo* uridine synthesis, PALA, was absent in both p21$^{-/-}$ and in p53$^{-/-}$ cells (Deng et al., 1995), arguing for an important role of p21$^{WAF1/Cip1}$ in p53-dependent cell cycle arrest.

Many well-characterised cellular functions of p53 were not affected in p21$^{-/-}$ mouse fibroblasts. Wild type p53 has been implicated in tumour suppression (Baker et al., 1990), emphasised by the fact that p53-nullizygous mice displayed a wide variety of tumours at an early stage (Donehower et al., 1992; Jacks et al., 1994), whereas p21$^{-/-}$ mice develop normally without any malignancies for the first 7 months (Deng et al., 1995). Two other functions of wild type p53 in controlling a mitotic spindle checkpoint and thereby cell ploidy (Cross et al., 1995) as well as apoptosis in response to DNA damage in thymocytes (Lowe et al., 1993b; Clarke et al., 1993) were not negatively affected in p21$^{-/-}$ mice (Deng et al., 1995).

Thus, these results indicate that p21$^{WAF1/Cip1}$ mediates only certain functions of p53.

The function of p21$^{WAF1/Cip1}$ depends on protein concentration within the cell. In growing diploid human fibroblasts, p21$^{WAF1/Cip1}$ is associated in a complex with the proliferating cell nuclear antigen (PCNA), cyclin-dependent kinases (Cdks) and their respective cyclin partners of type A, E and D (Zhang H. et al., 1994a). Nevertheless, p21$^{WAF1/Cip1}$ did not appear to inhibit the kinase activity of these complexes (Xiong et al., 1993a). As an explanation for this observation, it was proposed that p21$^{WAF1/Cip1}$ containing cyclin-Cdk-PCNA complexes can exist in active and inactive states, depending on the stoichometry of p21$^{WAF1/Cip1}$ molecules in the complex (Zhang H. et al., 1994b). Indeed, a low protein concentration of p21$^{WAF1/Cip1}$ facilitates the assembly of cyclin D-Cdk4 complexes by stabilising the formed complexes. At higher concentrations, p21$^{WAF1/Cip1}$ inhibits the kinase activity of these complexes (LaBaer et al., 1997). In addition, p21$^{WAF1/Cip1}$ targets cyclin D1 and Cdk4 protein to the nucleus. This function is independent of its function to promote the assembly of cyclin D1-Cdk4 complexes (LaBaer et al., 1997).

The elevation of p21$^{WAF1/Cip1}$ protein level during development, in senescent and differentiated cells is mostly regulated in a p53-independent manner (Huppi et al., 1994; Macleod et al.,1995; Parker et al., 1995; Jiang et al., 1994). The cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ has been implicated in cell senescence and differentiation (Noda et al., 1994; Parker et al., 1995; Macleod et al., 1995; Halevy et al., 1995; Steinman et al., 1994). In fact, p21$^{WAF1/Cip1}$ was cloned as a protein induced in senescent cells (Noda et al., 1994). A p21$^{WAF1/Cip1}$ related p24 protein has also been implicated in senescence (Mazars & Jat, 1997). In addition to its role in senescence and differentiation, p21$^{WAF1/Cip1}$ blocks proliferating cell

nuclear antigen (PCNA) activity to activate DNA polymerase δ *in vitro*, which is required for DNA replication (Flores-Rozas et al., 1994), through direct interaction (Waga et al., 1994).

Apart from the p21$^{WAF1/CIP1}$ gene, the Insulin-like growth factor binding protein 3 (IGF-BP3) gene has been described as a p53 target gene involved in growth inhibition (Buckbinder et al., 1995). In response to DNA damage, IGF-BP3 expression and secretion are induced. The secreted IGF-BP3 inhibits signalling initiated by insulin growth factor (IGF-1), presumably through complex formation (Buckbinder et al., 1995). Since IGF-1 can act as a mitogen or as a survival factor (Harrington et al., 1994), it may be a possibility that p53 can activate an autocrine/paracrine loop to negatively regulate mitogenic or survival signals.

Another transcriptional target of p53 is cyclin G (Okamoto & Beach, 1994; Zauberman et al., 1995). Its role in mediating p53 function, however, has remained unclear, since overexpression of cyclin G did not lead to growth arrest making it unlikely that cyclin G mediates p53´s growth-inhibitory function directly (Okamoto & Beach, 1994). However, the regulatory subunit of the protein phosphatase 2A has been found to bind in a p53-dependent way to cyclin G, although the biological implications of this interaction remain unclear as well (Okamoto et al., 1996).

1.2.5.2 Genes with other functions

Wild type p53 can regulate its own transcriptional transactivation activity through the transcriptional induction of the murine double minute 2 gene (*mdm-2*) (Barak et al., 1993; 1994; Wu X. et al., 1993). The *mdm-2* gene has been identified as a gene amplified in a spontaneously transformed mouse BALB/c 3T3 cell line (Fakharzadeh et al.,1991) and shown to be amplified in a number of sarcomas (Oliner et al., 1992). Wild

type p53 induces transcription from the *mdm-2* gene (Barak et al., 1993) through a p53 DNA-binding site of an internal promoter (Juven et al., 1993). The relationship between Mdm-2 and p53 represents an autoregulatory negative feedback loop to regulate p53 activity (Wu X. et al., 1993; Barak et al., 1993, 1994). The Mdm-2 protein binds to the amino terminal activation domain of p53 (Momand et al., 1992; Picksley et al., 1995) and thereby inhibits its function (Momand et al., 1992; Oliner et al., 1993; Chen J. et al., 1995). Furthermore, Mdm-2 binding to p53 enhances the degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997) via a ubiquitin-dependent pathway (Haupt et al., 1997).

The critical importance of the Mdm-2 protein in regulating p53 activity was confirmed by experiments with transgenic mice. *Mdm-2* nullizygous mice are not viable due to early embryonic lethality (Montes de Oca Luna et al., 1995; Jones et al., 1995). However, in a p53-deficient genetic background *mdm-2* nullizygous mice become viable and develop normally (Montes de Oca Luna et al., 1995; Jones et al., 1995). This surprising result shows the important role of the Mdm-2 protein in negatively regulating p53´s function at different stages of development. These experiments confirmed that overexpression of wild type p53 is not compatible with normal development (Godley et al., 1996; Nakamura et al., 1995; Hoever et al.,1994).

Wild type p53 activates the thrombospondin-1 (TSP-1) gene (Dameron et al., 1994), which is a strong inhibitor of angiogenesis. The absence of functional p53 causes a rapid decline of thrombospondin-1 and formation of an angiogenic phenotype in fibroblasts (Dameron et al., 1994).

A number of other genes have been described as possible p53 targets and these include the muscle creatine kinase (Weintraub et al., 1991; Zambetti et al., 1992; Zhao et al., 1994, 1996), the p53 gene itself

(Deffie et al., 1993), the transforming growth factor α (TGF-α) (Shin et al., 1995), the hsp70 gene (Agoff et al., 1993), the retinoblasoma gene (Osifchin et al., 1994), the epiderminal growth factor (EGF) receptor (Deb et al., 1994; Ludes-Meyer et al., 1996) and proliferating cell nuclear antigen (PCNA) (Shivakumar et al., 1995; Morris et al., 1996). However, the biological significance of p53-dependent induction of these genes are currently not understood.

1.2.6   General suppression of transcription by p53

Elevated p53 protein levels have been shown to generally repress transcription from viral and cellular promoters (Ginsberg et al., 1991; Subler et al., 1992). Those promoters which contain a TATA-box but lack a specific p53 DNA-binding site, in particular, can be suppressed by wild type p53 (Mack et al., 1993). This suppression of transcription is mediated through the interaction of p53 with basal transcription factors, required for the initiation from TATA-box containing promoters (Mack et al., 1993). Although p53 physically interacts with two TBP-associated factors (TAFs) TAF$_{II}$40 and TAF$_{II}$60 in the basal transcriptional machinery, this interaction is required for the transcriptional activation by p53 but not for repression of transcription (Thut et al., 1995). However, p53 also binds to the TATA-binding protein (TBP) another important component of the transcription machinery (Seto et al., 1992; Liu et al., 1993; Truant et al., 1993; Martin et al., 1993; Chen et al., 1993). It has been suggested that p53 binding to TBP is responsible for the observed general suppression of transcription (Seto et al., 1992; Horikoshi et al., 1995). In contrast to this hypothesis, however, a p53 mutant which fails to interact with TAF$_{II}$40 and TAF$_{II}$60 but binds to TBP is incapable of repressing transcription (Sabbatini B. et al., 1995). The exact   molecular

mechanism and the proteins through which p53 mediates its general suppression of transcription therefore remain to be identified.

1.2.7 Genes regulating p53-dependent apoptosis

The special significance of p53's apoptosis-inducing function has been clearly established by the effect of its inactivation in human tumours (Bardeesy et al., 1995) as well as by its role in growth and progression of tumours in an animal model (Symonds et al., 1994). The p53-dependent expression of the *bax* gene is an important step in the induction of apoptosis (Selvakumaran et al., 1994; Miyashita & Reed, 1995; McCurrach et al., 1997). Bax encodes a protein with homology to the survival factor Bcl-2. Both proteins Bcl-2 and Bax can heterodimerize (Oltvai et al., 1993) and the equilibrium between these two proteins influences apoptosis (Reed, 1994), although other p53 effectors may modulate this decision (McCurrach et al., 1997). The induction of Bax in response to γ-irradiation correlates with the p53 status in human cells (Zhan et al., 1994), and in p53$^{-/-}$ fibroblasts expression of the Bax protein can not be detected (McCurrach et al., 1997). Alternatively, the proposed p53-dependent induction of the fas/APO-1 gene (Owen-Schaub et al., 1995) may be another way for p53 to mediate apoptosis, since the fas/APO-1 factor triggers apoptosis.

1.2.8 p53 function in DNA replication and DNA repair

Wild type p53 may also regulate DNA replication and DNA repair via its own enzymatic activity (Mummenbrauer et al., 1996), by protein-protein interactions (Dutta et al., 1993; Waga et al., 1994; Flores-Rozas et al., 1994) or by regulating transcription from target genes (El-Deiry et al., 1994; Harper et al., 1994; Kastan et al., 1992).

The regulation of DNA replication by p53 appears to occur in multiple ways. The p53-dependent transcriptional activation of the cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ arrests cells before entering S-phase (El-Deiry et al., 1993; Harper et al., 1993; Dulic et al., 1994). Cells in S-phase could be prevented from replicating DNA due to p21$^{WAF1/Cip1}$ binding to proliferating-cell nuclear antigen (PCNA) and blocking its ability to activate DNA polymerase δ (Flores-Rozas et al., 1994; Waga et al., 1994; Warbrick et al., 1995). Interestingly, the PCNA-gene can also be transcriptionally activated by low p53 protein levels (Shivakumar et al., 1995; Morris et al., 1996). In addition to transcriptional regulation, wild type p53 can directly inhibit viral SV40 DNA replication in vitro (Braithwaite et al., 1987; Wang E. et al., 1989; Friedman et al., 1990), and p53 constitutively active in DNA binding is able to inhibit eukaryotic DNA replication in the absence of any transcription, suggesting that there is a direct involvement of p53 (Cox et al., 1995). Moreover, wild type p53 binds to the replication factor RPA and inhibits its binding to single-stranded DNA, an essential step for the initiation of DNA replication, although certain p53 mutants also bind and inhibit RPA, so that the biological significance of this interaction is not yet fully understood (Dutta et al., 1993; He et al., 1993).

The possible role of wild type p53 in DNA repair has been strengthened by the recent findings that the p53 protein itself exhibits 3′-′5 exonuclease activity which is absent in mutant p53 (Mummenbrauer et al., 1996). Furthermore, wild type p53 can recognise DNA structures resulting from damage-induced lesions such as insertion/deletion mismatches. Wild type p53 binds as tetramers to these lesions (Lee et al., 1995). This specific binding capacity is mediated via the C-terminus (Lee et al., 1995) which regulates the DNA-binding activity of p53 (Hupp et al., 1992, 1995; Bayle et al., 1995; Jayamaraman & Prives, 1995). In addition to these findings, wild

type p53 interacts with a number of proteins involved in DNA repair. Wild type p53 binds to ERCC3, a transcription factor involved in nucleotide excision repair (Wang X. et al., 1994), and binds the DNA repair factors XPD (RAD3) and XPB, as well as inhibiting DNA helicase activity (Wang X. et al., 1995). Although many reports suggest that the p53-dependent modulation of DNA repair occurs via protein-protein interaction, the p53-dependent induction of a Growth arrest and DNA damage gene, GADD45 (Zhan et al., 1994; Kastan et al., 1992), appears to contribute to DNA repair. The GADD45 protein has been shown to interact with the proliferating cell nuclear antigen (PCNA) and to stimulate nucleotide excision repair activity *in vitro* and inhibited S-phase entry (Smith et al., 1994; Hall et al., 1995).

1.2.9   Mechanisms of p53 activation and regulation

1.2.9.1   Translational regulation and protein degradation of p53

Wild type p53 activity is highly regulated by various mechanisms. In normal cells wild type p53 is in an inactive state and only detectable at very low concentrations. Following DNA damage, the activation of p53 has been correlated with an increase of p53 protein levels (Maltzman & Czyzyk, 1984; Kastan et al., 1991; Hall et al., 1993; Midgley et al., 1995), although no increase in p53 mRNA can usually be detected (Kastan et al., 1991). Moreover, the addition of the RNA polymerase II and III inhibitor α-amanitin does not inhibit the accumulation of wild type p53 (Maltzman & Czyzyk, 1984). The enhanced translation of existing mRNA molecules may be partially responsible for the increase of p53 protein levels since the translation inhibitor cyclohexamide blocks an increase in p53 protein, and at the same time partially abrogates the G1 arrest in response to DNA

damage (Kastan et al., 1991). It has been suggested that p53 3'UTR may play a role in the translational control of p53 protein expression (Fu et al., 1996). Interestingly, elevated p53 protein levels show an increase both in DNA-binding activity and sequence-specific transactivation activity (Hupp et al., 1992; Lu X. & Lane, 1993; Zhan Q. et al., 1993; Hupp & Lane, 1994; Hupp et al., 1995).

Altering the half-life of p53 from approximately 35 minutes to around 150 minutes after γ-irradiation (Maltzman & Czyzyk, 1984) also contributes to the increase in p53 protein. Moreover, since p53 is specifically degraded via the ubiquitin-dependent degradation pathway (Scheffner et al., 1990; 1993; Maki et al., 1996), changes in this pathway appear to regulate p53's steady-state levels. As an illustration of this regulation, the expression of the HPV E6-protein together with the cellular ubiquitin-ligase E6AP-protein lowers p53 protein levels (Scheffner et al., 1990, 1993; Huibregtse et al., 1991; Hubbert et al., 1992), while an inactivating mutation in the ubiquitin-dependent degradation pathway leads to an increase in p53 protein levels (Chowdary et al., 1994). Recently, the proteolytic cleavage of p53 at the amino terminus by calpain has been correlated with an increase in protein stability (Kubbutat & Vousden, 1997), although the significance for p53 regulation requires further investigations.

The regulation of p53 protein degradation also contributes to the down-regulation of activated p53. The down-regulation of p53 steady-state levels as well as p53's activity can be regulated by the Mdm-2 protein. The *mdm-2* gene itself is induced by wild type p53 (Barak et al., 1993). The binding of Mdm-2 to p53 enhances the ubiquitin-dependent protein degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). In addition to Mdm-2 enhanced degradation of p53, Mdm-2 binds to the amino-terminus of p53 (Momand et al., 1992; Picksley et al., 1994) thereby abolishing its

transactivation activity (Barak et al., 1993; 1994; Wu X. et al., 1993). Thus, the Mdm-2 protein provides a negative feedback and modulates the duration of p53 activity in response to DNA damage.

1.2.9.2   Phosphorylation of p53

Wild type p53 is extensively phosphorylated at amino- and carboxy-terminal sites *in vivo* and can be phosphorylated at these sites by several protein kinases *in vitro* (Meek, 1994). For example, the p53 protein is phosphorylated by the double-stranded DNA activated protein kinase (DNA-PK) (Lees-Miller et al., 1992; Wang & Eckhart, 1992), Casein kinase I (CKI) (Milne et al., 1992b), MAP-kinase (Milne et al., 1994) and c-Jun kinase (JNK) (Milne et al., 1995) at the amino-terminus. Cyclin A and cyclin B-associated forms of p34cdc2 (Bischoff et al., 1990; Sturzbecher et al., 1990), protein kinase C (PKC) (Baudier et al., 1992; Milne et al., 1996) and cyclic nucleotide-independent serine/threonine protein kinase, casein kinase II (CKII) (Meek et al., 1990b; Hupp et al., 1992) and PAK1 (Thiagalingam et al., 1995) have been reported to phosphorylate the p53 C-terminus.

Despite the number of kinases capable to phosphorylate p53 *in vitro*, a clear effect of individual phosphorylations in modulating p53 function has only been shown in a few cases. The phosphorylation of the PKC site has been suggested to enhance p53´s DNA-binding affinity (Hupp et al., 1993) and to increase its transactivation and suppression ability (Takenaka et al., 1995). Similarly, phosphorylation of p53 by casein kinase II *in vitro* potentially activates p53´s specific DNA-binding activity (Hupp et al., 1992). However, the mutational analysis of the CKII site has led to contradictory results since an inactivating mutation resulted in either a reduction of p53-dependent growth inhibition (Milne et al., 1992) or in no measurable physiological effect whatsoever (Fiscella et al., 1994). P53 has also been

reported to be specifically phosphorylated by S and G2/M-Cyclin-dependent kinases. The phosphorylation of p53 results in increased sequence-specific DNA-binding activity and confers a preference for specific p53 binding sites (Wang & Prives, 1995). The effect of phosphorylation on p53's activity is apparently complex because individual phosphorylation sites have different effects on p53's sequence-specific DNA-binding activity which depends on the specific nature of p53 binding sites in target genes (Fuchs et al., 1995; Wang & Prives, 1995; Lohrum & Scheidtmann, 1996; Hecker et al., 1996).

1.2.9.3   Posttranslational modifications of the p53 C-terminus

The p53 C-terminal region (amino acid residues 363-393) has been shown to be essential in activating the sequence-specific DNA-binding activity of p53. The carboxy-terminal domain contains residues that confer on p53 the ability to oligomerise and to bind both single-stranded DNA and RNA (Pavletich et al., 1993; Clore et al., 1994; Wu et al., 1995). The very C-terminus of wild type p53 recognises products of DNA damage such as short single DNA strands, DNA insertion/deletion mismatches (Jayaraman & Prives, 1995; Lee et al., 1995). In contrast, low levels of UV radiation can induce the sequence-specific activation of p53 without an accompanied increase in protein levels (Lu X. & Lane, 1993). The finding that lesion binding is mediated via the C-terminal domain (Hupp et al., 1992; Bayle et al., 1995) shows that the C-terminal domain communicates with the core DNA-binding domain of p53.

This activation of p53's sequence-specific DNA-binding activity can be achieved through phosphorylation at the carboxy-terminus. Examples which activate DNA-binding are phosphorylations at serine 378 by protein

kinase C and at serine 392 by casein kinase II (Hupp & Lane, 1994). Other modifications, such as O-glycosylation, occur at the carboxy-terminus although it is unclear whether this modification can activate p53 (Shaw et al., 1996). Deleting the last 30 amino acid residues of the p53 molecule (Hupp & Lane, 1994) and binding of the monoclonal antibody 421 to amino acid 370-378 activates sequence-specific DNA-binding activity of p53 (Hupp & Lane, 1994) as well as short single DNA strands which can activate the sequence-specific DNA binding of p53 (Jayaraman & Prives, 1995; Hupp et al., 1995). Recent findings have convincingly shown that the p53 sequence-specific DNA binding activity can also be activated by acetylation of the p53 C-terminal domain (Gu & Roeder, 1997).

1.2.9.4   Redox regulation

The conformation of *in vitro* translated p53 and its sequence-specific DNA binding can be modulated by metal chelators and oxidising agents. Oxidation disrupts the protein conformation and inhibits p53´s sequence-specific DNA-binding activity, whereas reduction restores this activity as well as protein conformation (Hainaut & Milner, 1993; Hupp et al., 1993). This redox regulation can be partly attributed to the central Zn^{2+} atom in the p53 molecule and to cysteine residues implicated in sequence-specific DNA binding (Rainwater et al., 1995). However, the biological relevance of the redox regulation of p53 *in vivo* was questioned, since a metal chelator which inactivates p53 *in vitro* unexpectedly activates p53´s transactivation activity *in vivo* (Sun et al., 1997).

1.2.9.5   Alternative splicing of p53

Alternative splicing involving the p53 carboxy-terminus has been reported for murine and human p53 leading to the shorter p53as protein

(Han & Kulesz-Martin, 1992; Kulez-Martin et al., 1994; Will et al., 1995; Flaman et al., 1996). The murine p53as mRNA, which contains an additional 96 bp derived from intron 10, represents 25-30% of regularly spliced p53 mRNA (Han & Kulesz-Martin, 1992) and is predominantly found in G2 of the cell cycle (Wu Y. et al., 1994). The predicted protein from p53as mRNA is 9 amino acids shorter and exhibits 17 different amino acid between residues 364-390 at the carboxy-terminus (Kulesz-Martin et al., 1994; Wu L. et al., 1995). The human p53as protein which is truncated at amino acids 341 includes 10 new amino acids and is mainly detected in quiescent cells (Flaman et al., 1996).

In contrast to p53, the p53as protein lacks non-specific DNA-binding activity (Bayle et al., 1995) but exhibits strong constitutive sequence-specific DNA-binding ability compared to p53. The DNA-binding activity of p53as does not require activation and can be inactivated through the formation of heterodimers with p53 *in vitro* (Wu Y. et al., 1994; Bayle et al., 1995; Wolkowicz et al., 1995). This strong constitutive sequence-specific DNA-binding ability of p53as has been attributed to the changes in the C-terminus, which in full-length p53 has a negative effect on sequence-specific DNA-binding (Bayle et al., 1995; Wolkowicz et al., 1995).

The alternatively spliced p53 protein has been implicated in the delayed induction of apoptosis compared with p53 (Almog et al., 1997). Furthermore, p53 promotes the association of single-stranded RNA or DNA into duplex hybrids (Pavletich et al., 1993; Clore et al., 1994). The p53as protein lacks this function of normal p53 (Wu L. et al., 1995). These first examples point to alternative splicing as part of the regulation of p53.

1.2.10   Properties of wild type and mutant p53 protein

The vast majority of p53 mutants can be characterised by functional and biochemical features which clearly distinguish them from wild type p53. Firstly, most mutants have an extended protein half-life compared to the wild type p53 . Whereas wild type p53 has a protein half-life of approximately 30 minutes, p53 mutants exhibit an extended half-life of up to several hours (Finlay et al., 1988; Hinds et al., 1990). P53 mutant protein therefore accumulates at higher concentrations than wild type p53 within the cell (Iggo et al., 1990). Wild type p53 inhibits the growth of tumour cells *in vitro* (Michalovitz et al., 1990; Baker et al., 1990; Diller et al., 1990; Chen et al., 1990; Mercer et al., 1990a; 1990b) and can inhibit the transformation of primary fibroblasts by cooperating oncogenes (Finlay et al., 1989; Eliyahu et al., 1989). Conversely, mutant p53 has lost the ability to suppress growth of tumour cells and lacks the ability to inhibit transformation by cooperating oncogenes (Finlay et al., 1989; Eliyahu et al., 1989). These biological functions of p53 are mediated through the specific DNA-binding activity of wild type p53 (Kern et al., 1991a; Bargonetti et al., 1991) which is strongly impaired in p53 mutants. As a result, the induction of essential cellular p53 target genes is abrogated (Kern et al., 1992). Many p53 mutants exhibit an altered protein conformation which can be detected through altered affinity towards certain monoclonal antibodies (Gannon et al., 1990) and by crystallographical studies (Cho et al., 1994).

1.2.10.1   Mutated p53 transdominantly Inactivates wild type p53

The functional inactivation of wild type p53 through co-expression of mutant p53 is a common mechanism of p53 inactivation. In general, missense mutations in one p53 allele not only result in high cellular

concentrations of mutant p53 protein; mutant p53 also binds to DNA with a much lower affinity than wild type p53 both *in vitro* and *in vivo* (Kern et al., 1991; Bargonetti et al., 1993). Moreover, complexes between wild type and co-expressed mutant p53 can be formed (Milner et al., 1991a; 1991b; Martinez et al., 1991). This stable interaction abrogates wild type p53-mediated transcriptional activation of target genes (Kern et al., 1992). Co-expression of p53 mutants from all evolutionary conserved regions of "hot spot" mutations are capable of abolishing wild type p53-dependent transactivation from p53 DNA-binding sites (Kern et al., 1992). Therefore, mutant p53 is believed to act in a negative trans-dominant fashion by sequestering wild type p53 into inactive complexes (Finlay et al., 1988; Martinez et al., 1990; Milner & Metcalf, 1991; Kern et al., 1992; Barnonetti et al., 1992). This idea is supported by recent findings in transgenic mice where the expression of a mutant p53 transgene accelerate tumorigenesis in a heterozygous p53 genetic background but not in a homozygous p53 deficient background (Harvey et al., 1995).

1.2.10.2   Inactivation of p53 by viral proteins

The transactivation function of p53 can also be inactivated by a number of DNA tumour virus proteins. For instance, the SV40 large T antigen blocks p53-dependent transactivation by binding to the p53 DNA-binding domain (Mietz et al., 1992). In addition to SV40 LT protein, the E1B 55 kDa proteins encoded by adenovirus 2 and 5 inactivate p53-dependent transcriptional activation by binding to the N-terminal transcriptional activation domain within the p53 protein (Braithwaite et al., 1991).

The inactivation of wild type p53 function by the human papillomavirus 16 & 18 (HPV16 & 18) E6 protein appears to be an important contributing factor in the development of cervical carcinomas. The

papillomavirus E6 protein binds to p53 protein (Werness et al., 1990) and accelerates the degradation of the p53 protein through the ubiquination degradation pathway (Scheffner et al., 1990; Mietz et al., 1992). As a result of increased p53 degradation, the expression of the E6 protein abrogates the G1 arrest in response to DNA damage (Kessis et al., 1993).

Proteins of two other viruses, the Hepatitis B virus protein X (HBX) and the Epstein-Barr virus proteins BZLF1 have been implicated in the inactivation of p53 (Wang et al., 1994; Zhang et al., 1994). The Hepatitis B virus protein X (HBX) is capable of inactivating the transactivation activity of wild type p53 (Wang et al., 1994) as well as possibly preventing the translocation of p53 into the nucleus by direct binding (Ueda et al., 1995).

1.2.10.3 Gain-of-function of p53 mutants

The current understanding of p53 inactivation by most p53 mutants is that wild type p53 and mutant p53 form stable complexes which are non-functional in transactivating target genes (Milner & Metcalf, 1991; Kern et al., 1992; Barnonetti et al., 1992). However, since there seems to be a positive selection for the presence of a mutated p53 gene in human tumours even at later stages of tumorigenesis, it has been proposed that a gain-of-function is associated with certain p53 mutations (Levine, 1992). Indeed, some p53 mutants exhibit a gain-of-function in p53-null cell lines reportedly capable of transactivating a specific target gene (Dittmer et al., 1993, Chin et al., 1992; Crook et al., 1994). However, experimental evidence suggests that a gain-of-function of p53 mutants strongly depends on the nature of the p53 mutation (Crook et al., 1994), the p53 binding site of the reporter construct (Chen et al., 1993) and the cell line used for the investigation (Dittmer et al., 1993; Sehgal & Margulies, 1993). The relevance of these gain-of-function mutations in tumorigenesis *in vivo* is therefore difficult to assess, although

positive modulation of the proliferating-cell nuclear antigen (PCNA) promoter (Deb et al., 1992) and the multi-drug resistance gene expression (Chin et al., 1992; Dittmer et al., 1993) by p53 gain-of function mutations may confer some growth advantage.

1.2.10.4   Exclusion of p53 from the nucleus

Wild type p53 is a nuclear protein and requires localisation within the nucleus in order to function (Shaulsky et al., 1991). Translocation has been attributed to three nuclear localisation signals (NLS I, II, III) in the C-terminus of p53 (Addison et al., 1990; Shaulsky et al., 1990b). A pattern of cell cycle specific regulation of the localisation of wild type p53 has been observed with p53 accumulating in the cytoplasm during G1 and entering the nucleus for a short period at early S-phase (Shaulsky et al., 1990a). Wild type p53, as well as mutant p53, require intact nuclear localisation signals to suppress transformation or enhance transformation in cooperation with activated *ras*, respectively (Shaulsky et al., 1991). Thus, the exclusion of wild type p53 from the nucleus may be another mechanism of inactivating the function of p53 and thereby increasing the risk of genetic instability of cells affected (Knippschild et al., 1996). In this context, it is interesting to note that in some tumours wild type p53 is only found in the cytoplasm (Moll et al., 1995) and that functional inactivation by binding to a viral protein, such as HBX, in the absence of any p53 mutations can exclude p53 from the nucleus and increase the risk of tumour formation (Ueda et al., 1995).

1.2.11   The role of p53 in tumorigenesis

The important role of p53 in protecting eukaryotic cells from tumorigenesis has been indirectly emphasised through the high incidences of loss or p53 mutation in tumours (Hollstein et al., 1991, 1996, 1997). By

investigating the functions of wild type p53, it has become clear why loss of functional p53 dramatically increases the risk of tumorigenesis. Although p53 appears not to be directly involved in cell cycle progression, p53 regulates the G1 cell cycle arrest in response to DNA damage. The p53-dependent G1 arrest provides the time for DNA to be repaired. Experimental evidence suggests that p53 is also involved in the coordination of DNA-repair mechanisms in damaged and arrested cells and recently p53 has been shown to have an intrinsic 3´-5´ exonuclease activity (Mummenbrauer et al., 1996). The loss of functional p53 would not only abolish the G1 cell cycle arrest but also impair essential DNA repair mechanisms. Severely DNA damaged cells are usually eliminated by p53-dependent apoptosis. P53-dependent control of apoptosis is an important part of p53 function in normal cell growth. The abrogation of p53's apoptotic function would also allow these cells to proliferate.

Finally, a very interesting p53 function in spindle checkpoint control has been reported which monitors the ploidy of cells (Cross et al., 1995). Tumours and tumour-derived cell lines are very often characterised by a degree of aneupody and, coincidentally, a lack of functional p53. The inactivation or abolition of any of these described p53 functions can lead to severe damage of the integrity of the genome, with the risk of mutations and aneuplody being established after further cell division.

Another important factor for progression of tumours is angiogenesis. Wild type p53 expression results in secretion of inhibitors of angiogenesis (Dameron et al., 1994). The mutation of wild type p53 would not only reduce apoptosis within the tumour dramatically but also reduces the secretion of angiogenesis inhibitors.

1.3   Aim of my project

The aim of my project was to further explore the mechanisms of oncogene cooperation in Schwann cells. Based on previous results with SV40 larger T (Ridley et al., 1988), I sought to find a cellular oncogene which would be able to cooperate with Ras. Furthermore, my aim was it to delineate the pathway for the growth inhibitory signal initiated by Ras in Schwann cells and to understand the mechanism by which a cooperating oncogenes could abrogate this response leading to cell proliferation.

CHAPTER 2.0

MATERIALS

2.0 Materials

2.1 Stock solutions

<u>PBSA</u>

100 mM NaCl

4.5 mM KCl

7 mM Na_2HPO_4

3 mM KH_2PO_4

<u>TE-buffer</u>

10 mM Tris pH 8.0;

1 mM EDTA pH 8.0

<u>TAE-buffer</u>

40 mM TrisOAc,

2 mM EDTA

<u>TBE-buffer</u>

89 mM Tris base,

89 mM boric acid,

2 mM EDTA

<u>LB-medium</u>

10 g/l Bacto-tryptone

5 g/l yeast extract

10 g/l sodium chloride

Adjusted to pH to 7.5 with 5 N sodium hydroxide

<u>LB-agar:</u>

LB-medium, supplemented with 1.5% (w/v) bacto agar

<u>BHI-medium</u>

Brain heart infusion broth powder

37g/l, dissolved in destilled H_2O, autoclaved

<u>2x Ca^{2+}-Transfection Buffer</u>

50 mM HEPES, pH 7.1

280 mM NaCl

1.5 mM Na_2HPO_4

KCl

Dextrose

<u>DNA loading buffer</u>

0.25% bromphenol blue

0.25% xylene cyanol FF

30% glycerol in water

<u>Protein loading buffer (4x)</u>

200 mM Tris/HCl pH 6.8

8% (w/v) SDS

0.4% (w/v) bromophenol blue

40% (v/v) glycerol

400 mM DTT (added shortly before use)

Luciferase buffer

25 mM glycylglycine pH 7.8

5 mM ATP pH 8.0

15 mM $MgSO_4$

SDS-running buffer

25 mM Tris

192 mM glycine

0.1% (w/v) SDS

Tfb I-Buffer

30 mM KOAc

50 mM $MnCl_2$

100 mM KCl pH 5.8

10 mM $CaCl_2$

15% glycerol

adjusted to pH 5.8 with 0.2 M acetic acid,

filtered and stored at 4°C

TfIIb-buffer

10 mM Na-MOPS pH 7.0

75 mM $CaCl_2$

10 mM KCl

15% glycerol

filtered and stored at 4°C

2.2   Chemicals

Chemical compound	Supplier
Acetyl CoA	Sigma
Acetic acids (glacial)	BDH
Acrylamide solution	Scotlab
N,N'-methylene bisacrylamide solution	Scotlab
Agarose	SeaKemFMCBioProducts
Agarose, low gelling temperature	SeaPlaque FMC BioProducts
Ammonium persulfate	Bio-Rad
Ampicillin (Na salt):	Sigma
Aquasol	Du Pont
BactoAgar	Difco
Boric acid	FSA
BSA Fraction V	Boehringer Manheim
Charcoal	Sigma
HEPES	Sigma
PIPES	Sigma
Tris acid	Sigma
Tris base	Sigma
Trichloroacetic acid	BDH
Calcium chloride	BDH
Chloramphenicol	Sigma
Chloroform	FSA
Dextran T-70	Sigma
DMSO	Sigma
DTT	Sigma
EDTA	FSA
EGTA	Sigma

Ethanol	Hayman Ltd.
Film	Fuji RX,Kodak XAR-5
Filters 0.2 and 0.45 μm	Millipore
Formaldehyde	BDH
Gentamycin	Life Technologies
Guanidine isothiocyanate	Fluka
Hygromycin B	Calbiochem
Intensifying screens	DuPont Lighting Plus
Isopropanol	FSA
β-mercaptoethanol	Sigma
Potassium chloride	FSA
Potassium acetate	Sigma
Kanamycin	Sigma
Leupeptin	Sigma
Methanol	BDH
Mitomycin C	Sigma
Magnesium chloride	BDH
Molecular weight marker 1 kb ladder	Life Technologies
Puromycin	Sigma
Sodium chloride	BDH
Sodium fluoride	BDH
Sodium hydroxide	BDH
Sodium vanadate	BDH
Triton x-100	Sigma
Hybond-N filter	Amersham
Needles and syringes	Sabre
Nonidet NP-40	Sigma
Nucleotides	Pharmacia

Oligonucleotides	Oligo-synthesis Unit, ICRF
O-nitrophenyl-β-D-Galactopyranoside	Sigma
Parafilm	American National Can
Petri dishes	Sterilin
PMSF	Sigma
Poly-L-lysine	Sigma
Aprotinin	Sigma
Protein Assay Mix	BioRad
SDS	Sigma
TEMED	BioRad
Tissue culture dishes	Falcon
Triton X-100	Sigma

2.3 Radiochemicals

All radiochemicals were purchased from Amersham Life Science and the most recently dated radiochemicals were employed for experiments. In particular, the following radiochemicals; Redivue (γ^{32} P) dATP, aqueous solution >5000 Ci/mmol and ^{3}H-thymidine 25Ci/mmol, 1mCi/ml were employed.

2.4 Enzymes

Calf Intestinal Alkaline Phosphatase	Boehringer Mannheim
Taq DNA polymerase	Boehringer Mannheim
DNA polymerase I, Klenow Fragment	Pharmacia
Proteinase K	Boehringer Mannheim
Restriction endonucleases	New England Biolabs
RNase A	Sigma
DNA T4-ligase	Boehringer Mannheim

2.5 Media and sera

Low glucose Dulbecco's modified Eagle medium (DMEM) minus phenol red containing 1 mg/ml glucose and 0.11 mg/ml sodium pyruvate was supplemented with 4 mM glutamine and used for Schwann cells. High glucose DMEM containing 4.5 mg/ml glucose without sodium pyruvate was used for all other cell types, provided by Media Production, ICRF.

Foetal calf serum (FCS), batch 2321 (Globepharm) was used for Schwann cells, whereas foetal calf serum batch 940278 (Autogen Bioclear) was used for all other cells.

2.6 Medium supplements

Low glucose DMEM for Schwann cells was supplemented with forskolin, Glial Growth Factor (GGF), gentamycin and kanamycin. Forskolin (Calbiochem) was solubilised at 25 mM in ethanol and stored at -20°C. A final concentration of 2 mM was added to the media. GGF was produced using a baculovirus-system and an empirically measured dilution of 1:5000 of the supernatant was added for growth promotion. Gentamycin and kanamycin were soubilised in water, sterile filtered and kept at 4°C. A final concentration of 2 mg/ml gentamycin and 100 mg/ml kanamycin were added to low glucose DMEM. High glucose DMEM for all other cells was supplemented with gentamycin and kanamycin at the same final concentration.

2.7   Cell lines

All Schwann cell had to be infected via co-cultivation with GPE-virus producer cells. The following table shows the generated GPE-virus producer cell lines, introduced retroviral vector and their resistance.

Tab. II.1: Generated GPE-virus producing cell lines

GPE virus-producing cell line	retroviral vector	resistance
GPE Puro	pBabe Puro	puromycin
GPE P175	pBabe Puro p53^{R175H}	puromycin
GPE p53CT	pBabe Puro p53CT$^{302-90}$	puromycin
GPE P248	pBabe Puro p53^{R248W}	puromycin
GPE ΔMEK1:ER^{TM}	pBabe PuroΔMEK1:ER^{TM}	puromycin
GPE MKK	pBabe Puro MKK	puromycin
GPE MANA	pBabe Puro MKK (MANA)	puromycin
GPE LIDA	pBabe Puro MKK (LIDA)	puromycin
GPE ERK2	pBabe Puro ERK2	puromycin
GPE ERK2 (AF)	pBabe Puro ERK2 (AF)	puromycin
GPE ERK2 (A52)	pBabe Puro ERK2 (A52)	puromycin
GPE Bleo	pBabe Bleo	phleomycin
GPE B/SEK1	pBabe Bleo SEK1	phleomycin
GPE B/SEK1/AL	pBabe Bleo B/SEK1-AL	phleomycin
GPE ΔMEK1: ER^{TM}/H	pBabeHygro ΔMEK:ER^{TM}	hygromycin
GPE LXSN	LXSN	neomycin
GPE LXSN cHa-Ras Val12	LXSN cHa-Ras Val12	neomycin
GPE LXSN-ΔRaf	LXSN-ΔRaf	neomycin
GPE LXSN-Raf-CAAX	LXSN-Raf-CAAX	neomycin

GPE WT-tk-luc2F   pBPSTR1 WT53 tk luc 2F   puromycin

Generated cell lines as listed in Tab. II.2 are sorted into groups according to their parental cell line. Cell lines which harbour more than one retroviral vector are separated by a dash showing both introduced genes. Additionally all resistances of a cell line are indicated. Cell lines with an asteriks were generated by Alison Lloyd.

Tab.II.2 : Generated cell lines and introduced genes

Cell line	Retroviral vectors	Resistance markers
11ras2 Puro	pBabePuro	neomycin/puromycin
11ras2 p53^{175}	pBabePuro p53^{R175H}	neomycin/puromycin
11ras2 p53^{248}	pBabePuro p53^{R248W}	neomycin/puromycin
10ras3 Puro	pBabePuro	neomycin/puromycin
10ras3 p53^{175}	pBabePuro p53^{R175H}	neomycin/puromycin
10ras3 p53^{248}	pBabePuro p53^{R248W}	neomycin/puromycin
10ras3 p53CT	pBabePurop53CT$^{302-90}$	neomycin/puromycin
NSPuro	pBabePuro	puromycin
NSP175	pBabePuro p53^{R175H}	puromycin
NSp53 CT	pBabePurop53CT$^{302-90}$	puromycin
NSPuro/	pBabePuro/	puromycin/
cHa Ras Val12	LXSN-cHa Ras Val12	neomycin
NSP175/	pBabePuro p53^{R175H}/	puromycin/
cHa Ras Val12	LXSN-cHa Ras Val12	neomycin
NSp53CT/	pBabePurop53CT$^{302-90}$	puromycin/
cHaRasVal12	/LXSN-cHa Ras Val12	neomycin

NSPuro/	pBabePuro/	puromycin/
	LXSN-cHa Ras Val12	neomycin
NSP175/ Ras	pBabePuro p53^{R175H}/	puromycin/
	LXSN-cHa Ras Val12	neomycin
NSp53CT/ Ras	pBabePurop53CT$^{302-90}$	puromycin/
	/LXSN-cHa Ras Val12	neomycin
NSΔRafER *	LXSN ΔRafER	neomycin
NSempty*	LXSN	neomycin
NSΔRafER/Puro	LXSN ΔRafER/	neomycin,
	pBabe Puro	puromycin
NSΔRafER/SV40 LT	LXSN ΔRafER,	neomycin,
	pBabe Puro SV40LT	puromycin
NSΔRafER/p53^{R175H}	LXSN ΔRafER,	neomycin,
	pBabe Puro p53^{R175H}	puromycin
NSΔRafER/p53^{R175H}	LXSN ΔRafER,	neomycin,
	pBabe Bleo p53^{R175H}	phleomycin
NSΔRafER/p53 CT90	LXSN ΔRafER,	neomycin,
	pBabe Puro p53CT^{90}	puromycin
NSΔRafER/ERK2	LXSN ΔRafER,	neomycin,
	pBabe Puro ERK2	puromycin
NSΔRafER/A52	LXSN ΔRafER,	neomycin,
	pBabe Puro A52	puromycin
NSΔRafER/AF	LXSN ΔRafER,	neomycin,
	pBabe Puro AF	puromycin
NSΔRafER/MKK	LXSN ΔRafER,	neomycin,
	pBabe Puro MKK	puromycin

NSΔRafER/MANA	LXSN ΔRafER,	neomycin,
	pBabe Puro MANA	puromycin
NSΔRafER/LIDA	LXSN ΔRafER	neomycin,
	pBabe Puro	puromycin
NSΔRafER/ΔMKK	LXSN ΔRafER,	neomycin,
	pBabe Puro ΔMKK	puromycin
NSΔRafER/Bleo	LXSN ΔRafER	neomycin,
	pBabe Bleo	phleomycin
NSΔRafER/B/SK	LXSN ΔRafER	neomycin,
	pBabe Bleo SEK1	phleomycin
NSΔRafER/B/AL	LXSN ΔRafER	neomycin,
	pBabe Bleo SEK1-AL	phleomycin

2.8   DNA constructs

pBabe Puro CT[90] was constructed by Trevor Littlewood (ICRF) by PCR and contains the murine carboxyterminus of murine p53 from aa 302-390, cloned BamH I- EcoR I into pBabe Puro (Morgenstern & Land, 1990).

pBabe Puro MKK, MANA (221S to A), LIDA (217S to A) were kind gifts from Sally Cowely in Chris Marshalls Laboratory (Cowley et al., 1994). The inserts, rabbit MAP-kinase kinase, were cloned into BamH I -EcoR I of pBabe Puro (Morgenstern & Land, 1990).

pBabe Puro ERK2, AF, A52 were constructed by inserting the murine ERK2, the mutation of murine ERK2 designated AF (183T to A, 185Y to F) and A52 (52K to A) into the BamH I- restriction site of pBabe Puro.

pBabe Puro SV40 LT was constructed by excising the cDNA of SV40 LT from the plasmid pZipSVLT (a kind gift by Parmjit Jat) with BamH I. The plasmid pZIPSV40 LT contains the cDNA of SV40 LT from Bgl I position nt 5235 to Hpa I at position nt 2666 cloned into the BamH I site with BamH I - linkers. The BamH I-fragment was cloned into the polylinker BamH I site of pBabe Puro.

pBabe Puro p53^{R175H} argenine to histidine at position aa175 was constructed by excision of mt p53^{R175H} BamH I from pBluescript and cloned into BamH I site of pBabe Puro.

pBabe Bleo p53^{R175H} argenine to histidine at position aa175 was constructed by excision of mt p53^{R175H} BamH I from pBluescript and cloned into BamH I site of pBabe Bleo .

pBPSTR wt p53 tk luc 2F

pBPSTR1 was cut Sal I-BamH I, thereby removing a total of 1.5 kb containing the TET- system elements. The luciferase gene from pGL3 basic vector (Promega) was excised through Bgl II-Sal I and ligated into pBPSTR1 with Sal I -BamH I, leading to pBPSTR1 luc 2 F. pBPSTR1 luc 2F was cut Bgl II - Not I and the p53 DNA binding site, previous ligated into BamH I-Not I into pcDNA 3, was cut and ligated into Bgl II-Not I. The HSV-tk minimal promoter was cut Hind III - Not I and ligated into pBPSTR1 p53 luc 2F.

pGEX2T c-Jun is a GST-expression vector with the amino acids 1-257 of c-Jun (a kind gift by Jim Woodgett).

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pBabe Puro ΔMEK1:ER™ and pBabe Puro ΔMEK1:ER were generated by Martin McMahon (DNAX) and contain a constitutivley active form of MEK1 (Masour et al., 1994) fused to the hormone binding region of the human oestrogen receptor cloned BamH I-Sal I. pBabe Puro ΔMEK1: ER™ is not responsive to oestrogen concentrations up to more than 250 nM (M. McMahon, pers. comm.).

pBabe Hygro ΔMEK1: ER™ was constructed by subcloning the BamH I-Sal I fragment from pBabe Puro ΔMEK1: ER™ into pBabe Hygro.

pBabe Bleo SEK1 and pBabe Bleo SEK1-AL were constructed by excising SEK1 and SEK1-AL from pcDNA3 with Kpn I and Xba I, blunted (kind gifts by Jim Woodgett) .These inserts were cloned into the SnaBI site of pBabe Puro.

2.8.1   Oligomers

p53 RGC oligomer flanked by BamHI and Not I sites

5`AGG CCA GAC CTG CCC GGG CAA GCC TTG GCA 3´

2.9.   Antibodies

2.9.1   Primary antibodies

Antibody 122 against p42$^{ERK 2}$ (Leevers et al., 1992)

Ab 122 is a rabbit polyclonal antibody and recognises both the phosphorylated and unphosphorylated form of p42^{ERK2} MAP-kinase.

Antibody erk2 against p42$^{ERK 2}$ (UBI Ltd.)

Ab erk2 is a monoclonal antibody, specific for MAP-kinase ERK2.

Antibody 240 against mutant p53 protein (Oncogene Science Ltd.)

Ab 240 is a monoclonal mouse antibody, raised against mouse epitope aa 206-211 of p53 (Gannon et al, 1990). This antibody specifically recognises mutant human and rat p53 in immunoprecipitation.

Antibody 423 against SV40 large T

Ab 423 is a monoclonal mouse antibody, raised against SV40 large T (Harlow et al., 1981). This antibody recognises SV40 large T on western blots. Ab 423 was provided by the Monoclonal Antibodies Unit (ICRF, Clare Hall).

Antibody 9E10 against c-myc tag

Ab 9E10 is a monoclonal mouse antibody raised against c-myc (Evan et al., 1985) and is used for the detection of various tagged proteins. Ab 9E10 was a kind gift by Gerad Evan (ICRF, London).

Antibody JNK-1 (C-17) (Santa Cruz Ltd.)

Antibody JNK-1 (C-17) is a rabbit polyclonal antibody and can be used for western blot analysis and immunopreciptiation with subsequent *in vitro* kinase assay of Jun- N-terminal kinase.

Antibody MKK-1 and MKK-2 (Transduction Laboratories Ltd.)

Antibodies MKK-1 and MKK-2 are monoclonal antibodies which recognise the amino-terminus of MAP-kinase kinase 1 or 2 in western blot analysis and immunoprecipitation prior to *in-vitro* kinase assays.

2.9.2   Secondary Antibody

Secondary antibody against rabbit immunoglobulins coupled to horseraddish peroxidase (Amersham Life Science).

Secondary antibody against mouse immunoglobulins coupled to horseraddish peroxidase (Amersham Life Science).

Secondary antibody against mouse immunoglobulin (Amersham Ltd.)

CHAPTER 3.0

METHODS

3.0. Methods

3.1 Tissue culture techniques

3.1.1 Growth conditions for Schwann cells

All eukaryotic cells were grown in humidified incubators in 10% CO_2 : 90% air at 37°C. Normal Schwann cells and all cell lines derived from Schwann cells were maintained in low-glucose Dulbeccos Modified Eagle Medium (DMEM, Gibco Ltd.) minus phenol-red, containing 3% stripped foetal calf serum (FCS) (batch no. 2321, BRL-Gibco Ltd.), 4 mM glutamine, 100 mg/ml kanamycin, 2 mg/ml gentamycin, 2 mM forskolin (Calbiochem Ltd.) and 100 μl recombinant glial growth factor (GGF) solution. Medium was changed every 2-3 days.

Schwann cells containing proteins fused to the hormone-binding domain of the oestrogen receptor were grown in medium without phenol-red and stripped foetal calf serum, since it had been reported that phenol-red as well as components of foetal calf serum can activate fusion proteins with the oestrogen receptor leading to an undesired background activity (Littlewood et al., 1995). Stripping of foetal calf serum was carried out by stirring serum with 0.1% (w/v) charcoal and 0.01% (w/v) dextran T-70 for three hours at 37°C. After this time serum was filtered (0.45 μm), aliquoted and stored at -20°C until further use.

All Schwann cells were grown on poly-L-lysine coated dishes. Poly-L-lysine (PLL) was solubilised in destilled water to give a 0.4 mg/ml stock solution. Dishes were coated with 2.5 ml of PLL-solution (13.3 mg/ml in destilled water) for 10 minutes at room temperature, drained for 5 minutes, washed twice with sterile destilled water and allowed to dry for 5-10 minutes.

Cells were passaged by first washing them in PBSA and then briefly incubating them with 1:5 (v/v) mix of trypsine/versene at 37 °C. Once the cells detached from the dish surface, they were diluted with fresh medium and replated. Schwann cells were routinely split 1:3 and cells with low passage numbers (below passage number 10) were used in all experiments.

3.1.2   General growth conditions for eukaryotic cells

Unless otherwise stated in individual experiments, all other cells were maintained in Dulbecco's modified eagle medium (DMEM) containing 10% foetal calf serum (FCS) (Autogen, batch no. 940278) plus 100 mg/ml kanamycin, 2 mg/ml gentamycin. Medium was changed every 2-3 days and cells were routinely split between 1:3 to 1:5.

3.1.3   Freezing and storage of eukaryotic cells

In order to maintain stocks of eukaryotic cells, the following freezing procedure was employed. Cells were trypsinized as previously described (see section 3.1.1) and resuspended in a pre-cooled freezing mix containing the specific medium required for these cells, mixed 1:1 with foetal calf serum. To this freezing mix 10% (v/v) dimethyl sulfoxide (DMSO) was slowly added. Each freezing vial (NUNC) was filled with 1 ml of cell suspension and immediately placed on ice. The vials were transferred into styrofoam freezing boxes into the -80 °C freezer for two days and then stored under liquid nitrogen. Frozen cells were recovered by rapidly thawing them in a 37°C water bath. The cell suspension was slowly mixed on a plate with 10 ml of prewarmed medium. After 2 hours, the medium was changed again to remove remaining traces of DMSO.

3.1.4   Retroviral infection of eukaryotic cells

3.1.4.1   Transfection and selection of GPE-virus producer cells

Transfection procedures were based on the protocol by Wigler et al, 1979. Prior to transfection, medium was changed to fresh 10 ml medium supplemented with 10% foetal calf serum. For every 90 mm-dish of GPE-cells (Markowitz et al., 1990) to be transfected, up to 10 µg plasmid DNA was mixed in 500 µl 1x transfection buffer followed by the addition of 23 µl of 2.5 M sterile $CaCl_2$ solution. A white translucent precipitate formed after approximately 10 minutes, and this tranfection mixture was added dropwise to the cells. Cells were incubated with the $DNA-Ca^{2+}$-precipitate for 16-20 hours and medium was change afterwards. Two days after transfection, GPE-cells were split 1:5, 1:10 and 1:20 into the appropriate selective medium. Cells were maintained under selection pressure with medium changed every 2-3 days until single colonies appeared. Single colonies usually appeared after 1-3 weeks depending on the drug selection used. For the following co-cultivation experiments, these selected GPE-virus producing cells were pooled.

All cell lines with retrovirally introduced genes were grown under constant drug selection. The table below gives the parental cell line and various drug concentrations routinely used for this purpose.

Tab.III.1: Drug concentrations for selection

Cell line	selective drug	final concentration
Schwann cells (NS)	neomycin, gentamycin	400 µg/ml
	puromycin	0.4 µg/ml
	hygromycin	75 µg/ml

	phleomycin	0.5 µg/ml
NSΔRafER cells	neomycin	0.4 µg/ml
	puromycin	0.4 µg/ml
GPE cells	puromycin	5 µg/ ml
	hygromycin	150 µg/ml
	neomycin	400 µg/ml
	phleomycin	25 µg/ml

Neomycin (G418) was prepared as a 100 mg/ml stock solutions in 10 mM HEPES pH 7.5. A puromycin stock solution of 25 mg/ml in sterile destilled water was used. Hygromycin was directly used as provided by the supplier. Bleomycin (=phleomycin) was prepared as a 25 mg/ml solution. All stock solution were sterile filtered and stored at 4°C.

3.1.4.2 Co-cultivation of Schwann cells with GPE-producer cells

Selected pools of GPE-producer cells were trypsinised and cells were collected in DMEM medium containing 10% FCS, washed once with PBSA and placed on ice. An aliquot of cells was counted with the coulter counter model ZF (Coulter Electronics Ltd.). Subsequently 2×10^6 GPE-cells were seeded per 90 mm-dish. Following an overnight incubation, 125 µl mitomycin C (final concentration 25 µg/ml) was added to the medium and cells were incubated for a further 2 hours. After this time cells were trypsinised and spun for 5 minutes at 1500 rpm in a bench-top centrifuge (Centaur 2/MSE) and washed once with PBSA. Cells were re-suspended

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in medium used for the co-cultivation and an aliquot of the cell suspension was counted as previously described. For the co-cultivation, 7.5 x 10⁵ cells of both cell types (i.e. GPE Puro + Schwann cells) were seeded onto a PLL-coated 90 mm-dish and incubated for 2-3 days. After this period cells were usually split 1:5, 1:10, 1:20 into the appropriate selection medium until resistant colonies appeared.

3.2 Proliferation assays

3.2.1 Growth assay without growth-promoting factors

Normal Schwann cells or those cell lines derived from Schwann cells were seeded at 2×10^4 cell per well in a 6-well dish in 2% low glucose DMEM without any growth promoting supplements. Schwann cells were trypsinised daily with 500 µl of 1:5 (v/v) trypsine/versine for 5-10 minutes at 37°C. Subsequently 500 µl medium was added. 100 µl of this cell suspension was diluted in 10 ml of isotone and counted with the Coulter Counter. The number of cells of two wells were counted in triplicates.

3.2.2 ³H-thymidine DNA-synthesis assay

In order to measure DNA-synthesis as an indication of S-phase entry of cells, to 2×10^4 cells in a 1 ml well 0.5 µCi/ml ³H-thymidine (used 2.5 µl/ml medium) was added for the indicated times. Cells were washed once with PBSA and 0.5 ml of 1% SDS solution was added. After 10 minutes at room temperature the lysate was transfered and the well washed once with 500 µl PBSA. 1 ml 15% (w/v) trichloroacetic acid was then added. After 10 minutes on ice the solution was filtered through a 2.5 µm glass fibre filter (Whatman). These filters were washed twice with 10 ml 5% TCA solution and once with 5 ml 100% ice-cold ethanol, air-dried and scintillation counted.

3.2.3   FACS-analysis

FACS-analysis was performed to establish the distribution of cells in the various phases of the cell-cycle. Bromodeoxyuridine (BrdU) with a final concentration of 10 μM was added to cells for times as indicated in the individual experiment. Thereafter cells were trypsinised and transferred into complete medium to inactivate trypsine. Cells were spun for 5 minutes at 1500 rpm in centrifuge Centaur 2, washed once with cold PBSA and re-suspended in 200 μl PBSA. Whilst vortexing, 2 ml 80% (v/v) cold ethanol was slowly added to fix cells. Samples were stored for 30 minutes on ice and afterwards at 4 °C in the dark. The fixed cells were then incubated with fluorescein isothiocyanate-conjugated anti-BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 μg/ml). Replicative DNA synthesis and DNA content were analysed using bivariate flow cytometry. FACS-analysis was carried out by Derek Davies and Clare Hughes in the ICRF FACS-laboratory.

3.3   DNA-techniques

3.3.1   Isolation of plasmid DNA through alkaline lysis

BHI-medium (2-3 ml) containing 100 μg/ml ampicillin was inoculated with a single bacterial colony from a BHI-agar plate. After an incubation period of between 5 hours to overnight, 1.5 ml of the bacterial suspension was spun for 5 minutes at 13000 rpm in a microfuge. The pellet was re-suspended in 200 μl resuspension buffer (25 mM Tris/HCl pH 8.0, 10 mM EDTA pH 8.0). 100 μl lysis buffer (0.2 M NaOH, 1% SDS) was added. After 5 minutes at room temperature, 150 μl of neutralisation buffer (3 M potassium acetate, pH 5.5) was added and the tubes were incubated on ice for 5 minutes. After a centrifugation step for 10 minutes at 13000 rpm, the

supernatant was mixed with 2.5x volumes of 100% (v/v) ice-cold ethanol and precipitated by spinning at 13000 rpm for 2 minutes at room temperature. The DNA-pellet was washed once with 70% (v/v) ethanol and then solubilised in 20 µl TE-buffer plus 100 µg/ml RNase A.

3.3.2 Plasmid DNA-preparation with Qiagen resin column

Bacteria were grown in 100 ml LB-medium containing 100 µg/ml ampicilin at 37°C overnight. The bacterial cells were harvested by centrifugation at 5500 rpm for 7 minutes. Isolation of plasmid DNA was carried out according to the manufacture's protocol. Bacterial pellets were completely re-suspended in 10 ml resuspension buffer P1 (100 µg/ml RNase A, 50 mM Tris/HCl, 10 mM EDTA pH 8.0) and 10 ml of lysis buffer P2 (200 mM NaOH, 1% SDS) were added. The suspension was neutralised with buffer P3 (3 M potassium acetate, pH 5.5). Samples were centrifuged at 5800 rpm for 30 minutes. In the meantime a Quiagen-tip 500 column was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, 15% (v/v) ethanol, pH 7.0, 0.15% Triton X-100). After the centrifugation the supernatant was immediately added onto the equilibrated column. The column was washed twice with 30 ml of wash buffer (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0) and the DNA was eluted by applying 15 ml buffer QF (1.25 M NaCl, 50 mM Tris/HCl, 15% ethanol, pH 8.5) onto the column. The eluted DNA was precipitated after addition of 0.7x volumes of isopropanol (10.5 ml). After a centrifugation step at 5800 rpm for 30 minutes at 4°C, the precipitated DNA was solubilised in 200 µl TE-buffer. This protocol routinely led to 0.5-3.0 mg/ml DNA depending on the plasmid used.

3.3.2.1 Quantification of DNA

The concentration and purity of DNA solutions was assessed on the basis of absorption at wavelength of 260 nm and 280 nm. One unit of optical density A_{260} corresponds to the concentration of 50 µg/ml for dsDNA, 40 µg/ml for RNA or 33 µg/ml for ssDNA including oligonucleotides. With proteins generating peak absorbance at 280 nm of the spectrum, the ratio A_{260}/A_{280} is indicative of the extent of protein contamination in nucleic acids solution. A ratio of 1.7 or bigger was considered acceptable.

3.3.3 Restriction analysis, modification and ligation of DNA

Restriction digests were performed using 0.5 to 1 µg of DNA and a 3-5x units excess of restriction enzyme in 20 µl total volume. Multiple restriction digests with incompatible buffer conditions were subsequently carried out with a phenol/chloroform extraction and ethanol precipitation between reactions. DNA molecules of 0.1-20 kb length were resolved on agarose gel matrixes. 0.5%-2% (w/v) agarose powder (SeaKem) was dissolved in 1x TAE-buffer and then melted in a microwave oven. Ethidium bromide (0.5 µg/ml) was added to the pre-cooled gel mix and poured into prepared electrophoresis trays. DNA samples and 0.5 µg 1 kb marker were mixed 1:5 with loading dye (50 mM EDTA pH 8.0, 100 mM Tris pH 8.0, 50% (v/v) glycerol, 0.4% (w/v) bromphenol blue) and loaded into the wells. DNA molecules were separated with a constant voltage electric field of 5 V/cm gel. Photographs of gels were taken under UV-illumination using Polaroid type 57 film.

DNA fragments used in ligations were prepared by digesting plasmid DNA with the appropriate restriction endonucleases. Modification of 5´ or 3´

DNA ends were carried out with T4-DNA polymerase I, Klenow fragment, and T4-DNA polymerase according to the supplier's standard protocols. To obtain blunt ends, 1 μl (10 units) of T4-DNA polymerase was added at the end of a restriction digestion. After 15 minutes of incubation at room temperature, the reaction tube was heated at 65°C for 20 minutes. To fill recessed 3' ends, DNA polymerase Klenow fragment was employed along with 33 nM dNTP. Self-ligation of DNA-vectors with compatible ends was prevented by dephosphorylation with calf intestinal alkaline phosphatase (CIP). 3 units per μg restricted DNA were added to the restriction reaction and incubated at 37°C for 30 minutes.

Analytical separation of DNA molecules for ligations were carried out by resolving DNA in up to 1% low-melting-point agarose gels (LMP-agarose) in 1x TAE running buffer. DNA bands were visualised under long wave UV-light (340 nm) and excised with a clean razor blade. Standard preparations provided with about 1 μg of an isolated fragment in 50 μl LMP-agarose. The LMP-agarose slices were transferred to sterile tubes. The LMP-agarose was melted for 10 minutes at 68°C and cooled down in a 37°C water bath. In a ligation of compatible and overhanging ends, 1 μl (20 ng) vector DNA was combined with 4 μl (100 ng) insert DNA in 50 mM Tris pH 7.8, 10 mM $MgCl_2$, 1 mM ATP, 20 mM DTT, 1 μl T4-DNA ligase (6 Weiss units) in a total volume of 20 μl. Ligations were incubated for between 4 hours to overnight at 14-16°C depending on the generation of cohesive or blunt ends. To stop the ligation, tubes were heated for 5 minutes to 68°C, cooled down to 37°C and a 10 μl aliquot was used to transform 100 μl freshly thawed competent DH5α bacteria.

3.3.4 Preparation and transformation of competent bacteria

A single colony of DH5α bacteria from a BHI-agar plate was inoculated into 3 ml BHI-medium and grown overnight at 37°C. 1 ml of overnight culture was further diluted in BHI-medium and left to grow at 37°C for approximately 2-3 hours until the density $OD_{550 \, nm} = 0.5$. Bacteria were pelleted at 3.5 k for 10 minutes at 4°C. The supernatant was discarded and the remaining bacteria pellet were gently re-suspended in 20 ml ice-cold Tfb I -buffer for each 50 ml volume of bacterial suspension. After 15 minutes incubation on ice, bacteria were pelleted again at 3.5 k for 10 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in 4 ml sterile ice-cold Tfb II -buffer by gentle swirling on ice. It was important to re-suspend the bacteria slowly to obtain a high degree of competence. 100 µl aliquots of competent DH5α bacteria were frozen at -80°C.

To transform ligated DNA into bacteria, a frozen aliquot of DH5α was thawed on ice. Purified DNA or melted LMP-agarose containing DNA was added and gently mixed with bacteria suspension. Bacteria were incubated for 20 minutes on ice. After this incubation period bacteria were incubated for 120 sec in a 37°C water bath and then placed on ice again for a further 3 minutes. After a further 3 minute-incubation at room temperature, 1 ml of BHI-medium was added. Bacteria were incubated for 1 hour at 37°C with occasional inversion of the tube. Bacteria were then spun down at 6500 rpm in minifuge, re-suspended in 200 µl BHI-medium and plated onto BHI-agar plates containing ampicilin (100 µg/ml). Incubations were carried out at 37°C overnight until colonies were clearly visible.

3.4 Protein techniques

3.4.1 Preparation of protein extracts for Western blotting

Prior to cells lysis, cells were rinsed twice with PBSA to remove last traces of tissue-culture medium. Cells were then scraped with a rubber policeman and transfered into an 1.5 ml-tube. Cells were spun at 1000 rpm for 5 to 10 minutes. The pellet was then re-suspended in 1x EDTA sample buffer, boiled for 2 minutes, vortexed and boiled again for 8 minutes. After 5 minutes on ice samples were spun for 5 minutes at 13000 rpm at 4°C and the supernatant was transfered into a new tube and immediately frozen at -70°C.

Prior to separation on an SDS-PAGE gel, protein samples were thawed on ice. The protein concentration was determined as described below. Aliquots were mixed with 4x protein sample buffer (0.4 M Tris pH 6.8, 10% (w/v) SDS, 4 M DTT, 50% glycerol, 10 mg bromophenol blue), boiled at 95°C for 5 minutes and loaded onto an SDS-PAGE gel.

3.4.1.1 Quantification of protein concentration

The protein concentration of a total cell lysate was measured with the BioRad Protein Assay kit following the manufacturer's recommendations. The stock solution was diluted 1:5. Aliquots of 1 ml were filled into disposable plastic cuvettes. The cell extract (1.0 µl) was added and after 5 minutes the samples were measured at $OD_{595\ nm}$. A standard curve for 1 to 10 µg BSA (BioRad) was used to calibrate the sample readings.

3.4.2   Protein analysis by SDS-PAGE

Different polyacrylamide gel concentrations were used to analyse various sizes of proteins. The following table shows the standard mixtures used in all experiments unless not otherwise stated:

Table III.2: SDS polyamide/acrylamide gels

	7.5%	**10%**	**12.5%**	**15%**
acrylamide/ bisacrylamide 30% : 0.8%	3.8 ml	5.0 ml	6.3 ml	7.5 ml
1 M Tris pH 8.8	5.6 ml	5.6 ml	5.6 ml	5.6 ml
water	5.5 ml	4.3 ml	3.0 ml	1.8 ml
20% SDS	0.075 ml	0.075 ml	0.075 ml	0.075 ml
20% APS	0.05 ml	0.05 ml	0.05 ml	0.05 ml
TEMED	0.005 ml	0.005 ml	0.005 ml	0.005 ml

For the analysis of the MAP-kinase phosphorylation shift, a special ratio of acrylamide to bisacrylamide was used in order to separate the phosphorylated and non-phosphorylated form of these protein.

Tab.III.3: Running gel for MAPK-phosphorylation shift (15% acryiamide/0.075% bisacrylamide)

compound	**volume**
30% acrylamide	15 ml
2% bisacrylamide	1.126 ml
1 M Tris pH 8.8	11.2 ml

water	2.2 ml
20% SDS	0.150 ml
20% APS	0.150 ml
TEMED	15 μl

A 5% acrylamide/bisacrylamide stacking gel was universally used for all gels. The exact specifications are listed in the table below.

Tab. III.4: Concentrations of stacking gel

compound	volume
Acrylamide/Bis 30%: 0.8%	0.835 ml
1 M Tris pH 6.8	0.625 ml
water	3.49 ml
20% SDS	0.025 ml
20% APS	0.025 ml
TEMED	0.005 ml

Gels were run with SDS-runing buffer (50 mM Tris base, 380 mM glycine, 0.1% SDS) at a constant 0.5 mA.

3.4.3   Western blotting

After separating the protein of interest on an SDS-PAGE gel, the gel was electroblotted onto PVDF-membrane using the BioRad semi-dry transfer system (15 V for 30 minutes to 1 hour, depending on the size of protein and thickness of gel) using transfer buffer (50 mM Tris base, 380 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol). All the following steps were

carried out at room temperature on a shaker. The PVDF-membrane was blocked with 5% non-fat dried milk in TBST (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween 20) for one hour up to overnight at 4°C. The primary antibody was diluted in blocking buffer with 5% non-fat dried milk (for dilutions of individual antibodies, see Chapter 2.0) and incubated for one hour in a sealed plastic bag. To remove unspecifically bound primary antibody, the blot was rinsed twice and then washed with TBST. The secondary antibody (sheep anti-rabbit antibody, coupled to horse raddish peroxidase) was diluted 1:2000 in TBST + 5% (w/v) milk powder and incubated for one hour. The blot was washed again for 3 x 20 minutes in TBST plus 25 mM EDTA. Subsequently proteins were detected using the enhanced chemiluminescent system (ECL, Amersham Ltd.), according to the manufacture's protocol.

3.4.3.1   Stripping of Western blots

Western blot membranes were stripped of bound antibodies to allow detection of other proteins on the same filter. To strip filters, these filters were washed for 3 x 10 minutes in 0.1 M glycine pH 2.5. Thereafter, the blot was rinsed with 1 M Tris pH 8.0 and washed 3 x 10 minutes with a large volume of PBSA. The blot was then blocked in 5% milk in PBSA for 1 hour or overnight at 4°C and incubated with a different antibody as described. Prior the storage at -20°C western blots were washed with PBSA once and then wrapped in foil.

3.4.4   Immunoprecipitation

3.4.4.1   *In vivo* labelling with ^{35}S-methionine

Various Schwann cells were grown in 3% FCS in low glucose DMEM plus glial growth factor (GGF) and forskolin up to 80% confluency on 90 mm

dishes. Schwann cells were washed once in prewarmed DMEM-medium without methionine. 2.5 ml of DMEM-medium without methionine containing 2% dialysed foetal calf serum and ^{35}S-L-methionine (300 µCi per 90 mm dish) were added for times as indicated in the individual experiment.

3.4.4.2 Harvest of radioactive labelled cells

After this time cells were washed twice with cold PBSA and scraped from the dish. All cells were lysed for 15 minutes on ice in reaction tubes with 0.5 ml of lysis buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mM EDTA, 1% NP-40, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin). After a spin for 5 minutes at 13000 rpm at 4°C the supernatant was transferred into new reaction tubes. To the radioactive cell lysates 20 µl of beads (50% (v/v)) were added to preclear non-specific interactions prior to immunoprecipitation. After an incubation period of 1 hour on a rotating wheel at 4°C, reaction tubes were spun for 30 seconds at 10000 rpm at 4°C and the supernatant was transferred into new tubes.

As normalisation for the following immunoprecipitation, cell lysate with the same number of TCA-precipitable counts was used. 1µg of mAb 240 was added to the cell lysates and after incubation on a rotating wheel for 1 hour at 4°C, 20 µl of protein G -beads (50% (v/v) lysis buffer) were added. After a further incubation for 1 hour at 4°C, the immunoprecipitates were pelleted by centrifugation for 30 seconds at 10000 rpm at 4°C and subsequently washed 4x with lysis buffer at 4°C. After the final wash the remaining liquid was removed completely and equal volumes of 2x protein sample buffer were added to the pellet. Tubes were heated to 95°C for 10 minutes, spun and the supernatant was loaded onto an SDS-PAGE gel.

SDS-PAGE gels were fixed by soaking them twice for 5-10 minutes in methanol/acetic acid (40:10). Thereafter twice for 5-10 minutes in

methanol/acetic acid (10:5) followed by washing with aqua dest. for 15 minutes.

To further enhance the detection of radioactive labelled protein, fixed gels were soaked in a 1 M salicylic acid solution and then dried for an hour before being subjected to autoradiography.

Protein G sepharose (Sigma) was supplied as a suspension. After 3 washes with the buffer used in the specific experiment, protein G sepharose beads were used for immunoprecipitation. Protein A sepharose (Sigma), supplied as a powder, was re-suspended in PBSA according to the manufacturer's instructions. Before usage an aliquot of this suspension was washed several times with the specific buffer used for the immunoprecipitation.

3.5 *In vitro* kinase assays

3.5.1 *In vitro* kinase assay for MAP-kinase p42^{ERK2}

All the following steps were carried out at 4°C. Nearly confluent cells were washed once in cold PBSA. Cells were scraped off the dish with a rubber policeman and spun for 1 minute at 6500 rpm in a microfuge. Cells from a 150-mm dish were re-suspended in 150 µl buffer (10 mM Tris pH 8.0, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 µM Na$_3$VO$_4$, 1% Triton X-100 containing 1mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.6). After an incubation of 10 minutes on ice, lysates were spun for 5 minutes at 13000 rpm. The supernatant was aliquoted into new tubes and snap frozen. Due to the lack of SDS in the lysis buffer which strongly inhibits phosphatase activity, these cell lysates could be stored for approximately one month at -20°C without loss of kinase activity. Prior to immunoprecipitation the protein concentration was estimated using the Biorad protein assay. For each

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immunoprecipitation 30 μl protein A sepharose beads were incubated with 5 μl of antibody 122 (Leevers & Marshall, 1992) in 200 μl lysis buffer for 1 hour at 4°C. After a centrifugation step and aspiration of the supernatant, 100 μg total protein in 200 μl lysis buffer were added and rotated for 1 hour at 4°C. Beads were washed twice in lysis buffer and twice in ice-cold kinase buffer (15 mM Tris/HCl pH 7.5, 15 mM Mg Cl_2) prior to the *in vitro* kinase assay.

Following immunoprecipitation, beads were incubated in 25 μl of kinase assay mix containing kinase buffer with 0.5 mM EGTA, bovine myelin basic protein (MBP) at 7.5 μg per reaction as substrate, 50 μM ATP, 50 μCi/ml of (γ^{32}P) ATP, 2 μM cAMP-dependent protein kinase inhibitor peptide and 100 nM microcysteine LR at 30°C for 30 minutes by shaking on the Eppendorf Thermomixer. The kinase reaction was terminated by adding 1/4 volume 4x protein gel sample loading buffer (SB) and reactions were loaded onto a 15% SDS-PAGE gel. The gel was fixed, dried and exposed to X-OMAT-film (Kodak) overnight.

3.5.2 Production and purification of GST-fusion proteins

Bacteria transformed with a GST-fusion protein construct were grown overnight and diluted 1:10 into a 100 ml BHI-medium culture plus 100 μg/ml ampicillin. At an optical density of OD_{550} of 0.8 protein production was induced with 1 mM IPTG for 4 hours. After this incubation time the suspension was spun down and re-suspended in 1/10 vol. PBSA + 1% (v/v) triton x-100 and 10 μg/ml aprotinin and 10 μg/ml leupeptin. The re-suspended bacteria were transferred into 1.5 ml tubes and sonicated for 3 x 15 seconds on half maximal power. Tubes were kept on ice in between the sonication steps. After a spin of 10 minutes at 14000 rpm the supernatant

was pooled and filtered through a 0.45 μm filter. Supernatant was incubated overnight with GST-sepharose beads on a rotating wheel at 4°C and washed 5 x with PBSA + Triton X-100 (1%). The bound GST-fusion protein was eluted with freshly prepared 10 mM glutathione (Sigma) in 50 mM Tris pH 8.0. At least 5 washes were performed and eluted fractions were kept separately, run on a gel and stained.

Overnight cultures of DH5α were diluted 1:10 and grown for 1 hour. GST-ERK2 was induced with 1 mM IPTG for 4 hours at 37°C and GST-MEK induced with 30 μM IPTG at 27°C overnight. The bacteria were then pelleted, frozen and thawed. Bacteria were sonicated on ice 3 x for 15 seconds with Sonicator model Soniprep 150 (MSE) at 14 microm. Cell debris was removed by centrifugation and GST-fusion proteins were purified by adding 0.5 ml glutathione-Sepharose beads and rotating for 30 minutes at 4°C. The GST-ERK2 was cleaved from the GST in thrombin buffer while the GST-MEK was eluted from the glutathionine-sepharose beads with 5 mM glutathione in 50 mM HEPES.

3.5.3   *In vitro* kinase assay for MAP-kinase kinase

MAP-kinase kinase activity was measured in a coupled assay with recombinant MAP-kinase and myelin basic protein as final substrate. Initially MAP-kinase kinase was precipitated with a 1:1 mixture of monoclonal anti-MEK-1 and anti-MEK-2 antibodies for 2 hours with 40 μl of protein A-agarose (1:1 slurry) added for the another hour. Immune complexes were collected by centrifugation and washed three times in lysis buffer without PMSF and once with buffer A (50 mM Tris-HCL, pH 7.5, 0,1 mM EGTA, 0.5 mM sodium vanadate and 0.1% 2-mercaptoethanol). Pellets were resuspended in 30 μl of MEK/MAPK buffer (30 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% 3-mercaptoethanol, 100μg/ml GST-MAPK (a kind gift of

Thomas Seufferlein, ICRF), 0.03% Brij-35, 10 mM Mg-ATP and 20 mM N-octyl-β-D-glucopyranoside) and incubated at 30 °C for 30 minutes. The reaction was stopped by addition of 4X protein loading buffer.

3.5.4   *In vitro* kinase assay for JNK-1 kinase

Cells were washed twice with cold PBSA and left to drain. 600 µl lysis buffer was added to a large tissue-culture dish. The large tissue-culture dish was left on ice for 5 minutes. Cells were scraped with a rubber policeman, resuspended in JNK-lysis buffer (20 mM HEPES pH 7.5, 2 mM EGTA, 50mM β-glycerolphosphate, 1 mM DTT, 1 mM Na$_3$ VO$_4$, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 400 µM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 0.1 ng/µl ocadaic acid) and after another 5 minutes on ice, spun for 10 minutes at 4°C. The supernatant was transfered to new tubes. The protein concentration was immediately measured using the BioRad protein system. The indicated concentration of total cell protein lysate was subjected to immunoprecipitation. After incubation with 1 µg of antibody JNK1 (C-17, Santa Cruz Ltd.) for 2 hours, immunoprecipitates were washed 3x in lysis buffer, 3x in LiCl-buffer (500 mM LiCl, 100 mM Tris pH 7.5, 0.1% (v/v) Triton x-100, 1 mM DTT) and 3x in JNK-kinase buffer (20 mM MOPS pH 7.2, 2 mM EGTA, 10 mM MgCl$_2$, 1 mM DTT, 0.1% (v/v)Triton x-100), before a kinase reaction was carried out. For the JNK-1 kinase reaction, an ATP-MIX was prepared consisting of 2 µl of 0.1 mM ATP, 5 µl of 50 mM MgCl$_2$, 2.66 µl of $^{32}\gamma$-ATP (= 26,6 µCi) and 90.34 µl water. 7.5 µl of the ATP-MIX was added to the protein A sepharose beads together with 20 µl JNK-1 kinase buffer and 3 µl GST-Jun aa 1-257 (1 mg/ml). The kinase reaction was incubated at 30°C for 20 minutes and stopped with 4x protein loading dye.

3.6   Luciferase activity assay

Prior to harvest cells were washed twice with ice-cold PBSA and drained for 5 minutes. 100 µl 0.25 M Tris pH 7.5 was added and cells were scraped with a rubber cell scraper. After transfer of the cell suspension into reaction tubes, the cells were lysed by snap-freeze and thaw three times. Tubes were subsequently spun for 5 minutes. The supernatant was removed and used for the following luciferase assay. 30 µl of supernatant were added to luminator cuvettes containing 350 µl luciferase reaction buffer (25 mM glycylglycine pH 7.8, 5 mM ATP (pH corrected to 8.0), 15 mM $MgSO_4$). Samples were loaded into an LKB 1251-luminator and luciferase activity was measured after injection of 30 µl of 3 mM luciferin into the individual sample. Luciferase activities were normalised against protein concentration measured by the BioRad protein assay as previously described (see section 3.4.1.1).

CHAPTER 4.0

Mutants of the tumour suppressor gene p53 cooperate with oncogenic Ras and oncogenic Raf in Schwann cells

4.0 Mutants of the tumour suppressor gene p53 cooperate with oncogenic Ras and oncogenic Raf in Schwann cells

4.1 Introduction

Oncogenic Ras mutants introduced into Schwann cells cause a cell-cycle arrest, which can be rescued by a second cooperating oncogene such as the Simian virus 40 large T antigen (SV40 LT) (Ridley et al.,1988). To facilitate the investigation of oncogene cooperation, Schwann cell clones contain v-Ha Ras and a temperature-sensitive mutant of SV40 large T antigen (tsA58 LT) (Tegtmeyer et al., 1975) were generated (Ridley et al., 1988). Cells shifted to the non-permissive temperature (39°C) arrested in the G1-phase of the cell-cycle, revealing the growth inhibitory effect of oncogenic Ras in Schwann cells. In contrast, at the permissive temperature (33°C) Schwann cell clones 10ras3 and 11ras2 proliferated rapidly, exhibit a transformed morphology and a reduced requirement for growth factors due to v-Ha Ras and SV40 LT cooperativity (Ridley et al., 1988). Since a viral protein had been convincingly shown to cooperate with activated Ras in transformation of Schwann cells, I was interested to find a cellular oncogene which could be utilised for my investigations into the mechanism of oncogene cooperation.

SV40 LT is a multifunctional protein which interacts with a number of cellular target proteins. One important cellular target of SV40 LT is the tumour suppressor protein p53 which is inactivated in its function through the interaction with SV40 LT (Lane & Crawford, 1979). Therefore, I investigated whether mutants of p53 itself were able to cooperate with activated Ras in our Schwann cells model.

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4.2 Mutant p53 cooperates with v-Ha Ras in 10ras3 and 11ras2 Schwann cells

To address the question whether mutants of the tumour suppressor p53 were capable of cooperating with oncogenic Ras in the transformation of Schwann cells, I introduced two mutants of the human p53 gene into the described Schwann cell clones 10ras3 and 11ras2 (Ridley et al., 1988). Based on previous results with Ras and SV40LT (Ridley et al., 1988), I reasoned that Schwann cells at the non-permissive temperature should be enabled to overcome the *ras*-induced growth arrest, if mutants of p53 cooperate with oncogenic Ras. To test this hypothesis, two missense mutants of the human p53 gene (R175H and R248W) in the DNA binding domain (Baker et al., 1990) which naturally occurred with high frequency in various human tumours (Hollstein et al., 1996) were used. These two p53 mutants were cloned into the retroviral vectors pBabe Puro (Figure IV.1) or, alternatively into pBabe Bleo (Morgenstern & Land, 1990).

To derive Schwann cells expressing these mutant p53 proteins, Schwann cells were infected by co-cultivation with GPE-virus producer cells for pBabe Puro p53^{R175H} and pBabe Puro p53^{R248W} including pBabe Puro vector as control. After infection of 10ras3 and 11ras2 cells with these viruses, Schwann cells were split and selected for two days at the permissive temperature. Then, half of the dishes were shifted to the non-permissive temperature (39°C), whereas the other half were kept at the permissive temperature (33°C). Three weeks after co-cultivation, outgrowing colonies at both temperatures were stained and counted. Interestingly, at the non-permissive temperature both introduced mutant p53 cDNAs could rescue the *ras*-induced cell-cycle arrest in 10ras3 and 11ras2 cells (Figure IV.2A and IV.3A). Between 60-100% of colonies found at the permissive temperature were also able to grow at the non-permissive

Figure IV.1: Mutant p53^{R175H} and mutant p53^{R248W}

in pBabe Puro vector

Two missense mutations of the human p53 gene, mutant p53^{R175H} and mutant p53^{R248W} were cloned into the BamH I-restriction site of the retroviral vector pBabe Puro (Morgenstein & Land, 1990).

Cap Site —

CAAT TATA
R U5

S— (point mutant)
D AGGT → AGGC
ATG⁻

gag

amp^R

pBS Ori

SV40 early promoter

puro^R

U3

BstX I
EcoR I
SnaB I
BstX I
Sal I

Bam HI

0

p53

CGC → CAC
R 175 H

CGG → TGG
R 248 W

393

BamH I

poly A site
CAA TAT A
R U5
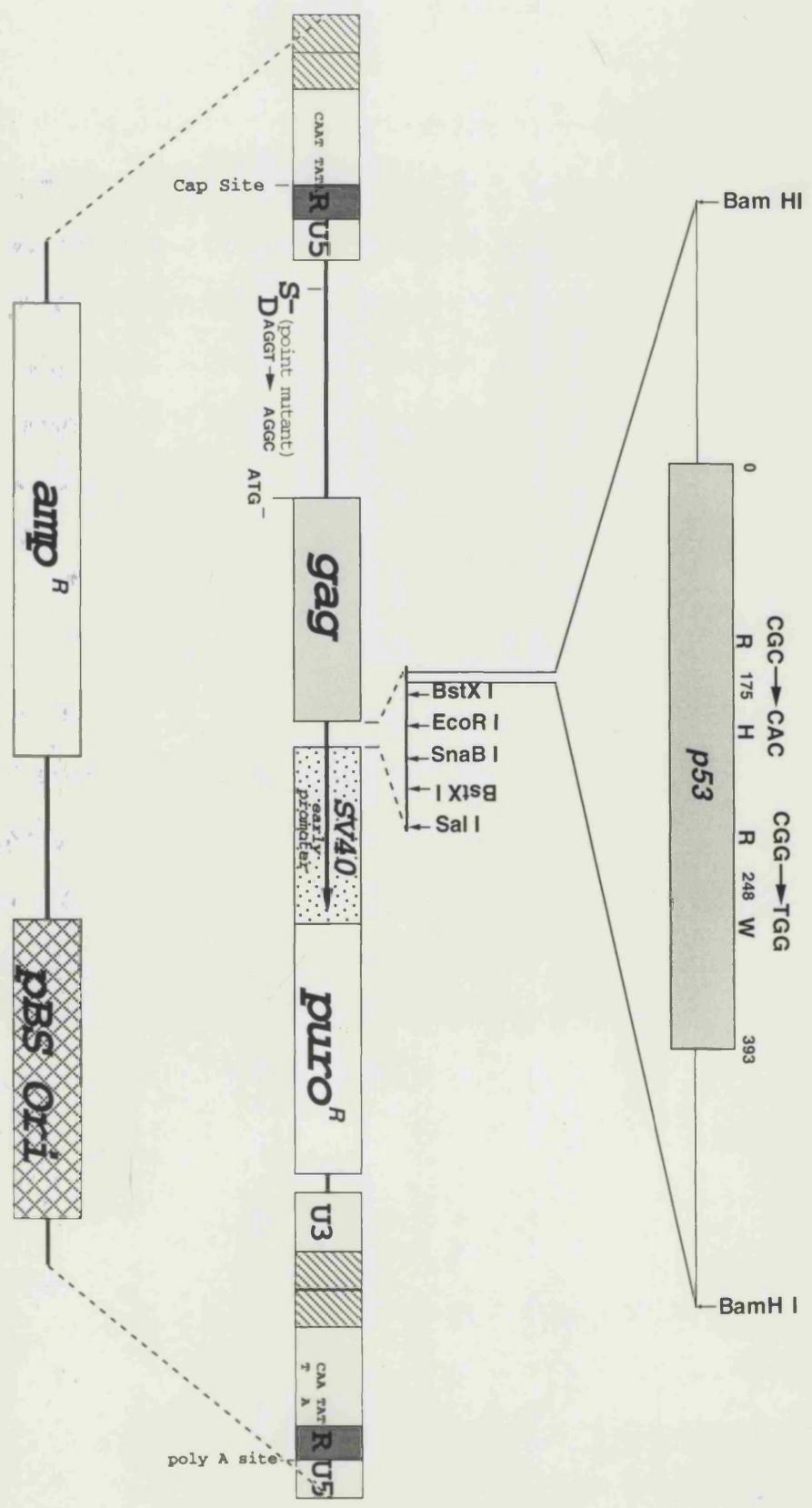
Figure IV. 2A: Mutant p53 can rescue *ras*-induced growth arrest in 11ras2 cells

Schwann cells 11ras2 were co-cultivated with GPE-virus producer cells shedding pBabe Puro, pBabe Puro p53^{R175H} and pBabe Puro p53^{R248W} viruses over a period of two days as described (see Methods). Cells were split 1:10 and kept for two days under puromycin selection. Half of the dishes were shifted to the non-permissive temperature (39°C), whereas the other half was kept at the permissive temperature (33°C). Outgrowing colonies were stained and counted three weeks after infection. The standard deviation of the number of 11ras2 Puro colonies at 39 °C was too small to be seen in the presented graph.

Figure IV.2B: Mutant p53 is expressed in 11ras2 cells

11ras2 Puro, 11ras2 p53^{175} and 11ras2 p53^{248} cells were grown at indicated temperatures. After addition of ^{35}S-methionine for four hours, cells were lysed and equal numbers of TCA-precipitable counts were immunoprecipitated with the mutant p53 specific antibody 240 or a control antibody as described (see Methods). Immunoprecipitates were separated on a 10% SDS-PAGE gel and exposed to film.

1: 11ras2 Puro, 33°C, Ab 9E10

2: 11ras2 Puro 33°C, Ab 240

3: 11ras2 p53^{175}, 39°C, Ab 9E10

4: 11ras2 p53^{175}, 39°C, Ab 240

5: 11ras2 p53^{248}, 39°C, Ab 9E10

6: 11ras2 p53^{248}, 39°C, Ab 240

7: radioactive size-marker

A.
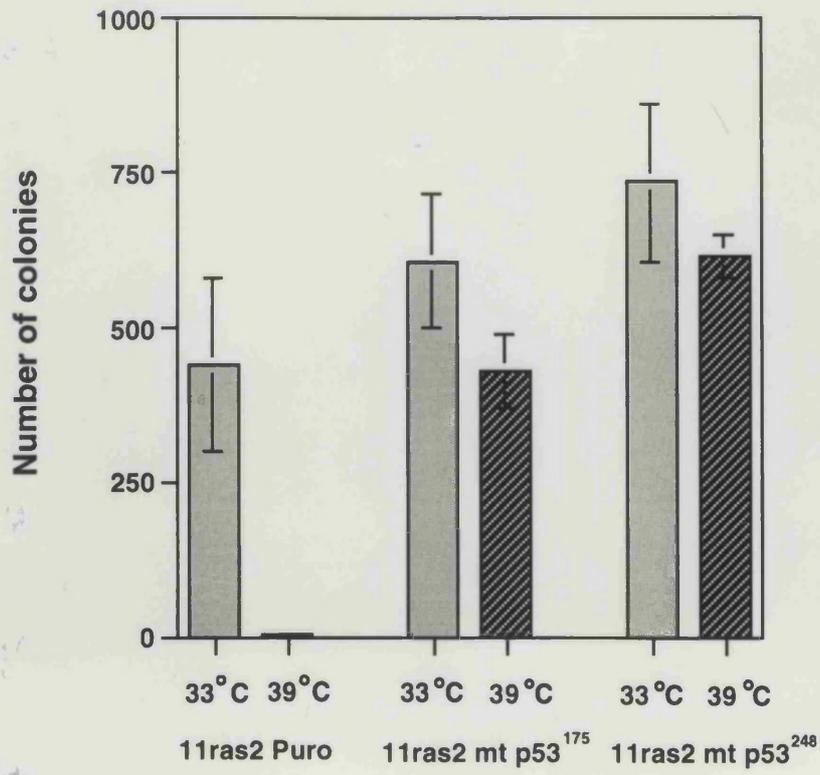
B.
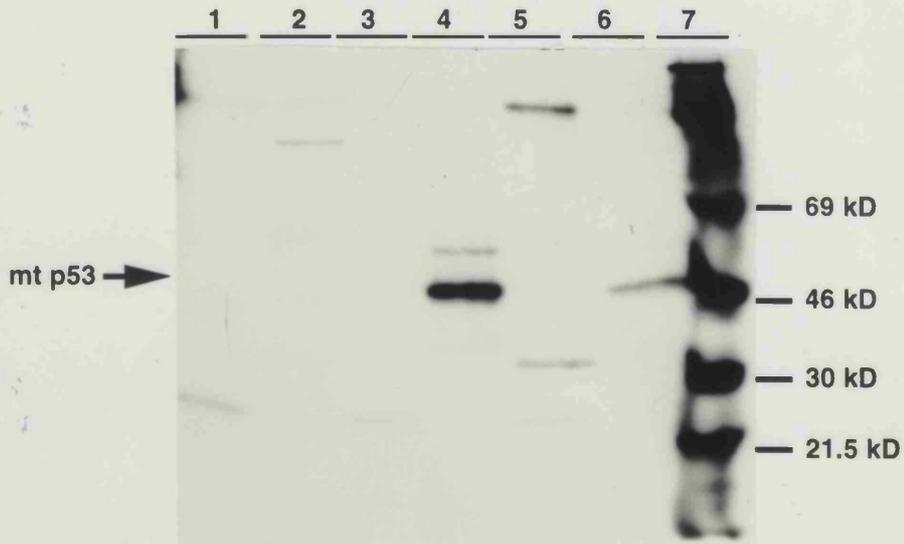
Figure IV.3A: Mutant p53 can rescues *ras*-induced growth arrest In 10ras3 cells

10ras3 cells were co-cultivated with GPE-virus producer cells shedding pBabe Puro, pBabe Puro p53^{R175H} and pBabe Puro^{R248W} viruses over a period of two days (see Methods). Cells were split 1:10 and kept for two days under puromycin selection. Half of all dishes were shifted to the non-permissive temperature (39°C), whereas the other half remained at the permissive temperature (33°C). Colonies were stained and counted three weeks after co-cultivation. The standard deviation of the number of 10ras3 Puro colonies at 39 °C was too small to be seen in the presented graph.

Figure IV.3B: Mutant p53 is expressed in 10ras3 cells

10ras3 Puro, 10ras3 p53^{R175H} and 10ras3 p53^{R248W} cells were grown at indicated temperatures. After labelling with ^{35}S-methionine for four hours, cells were lysed and equal numbers of TCA-precipitable counts were immunoprecipitated with the mutant p53 specific antibody 240 or a control antibody as described (see Methods). Immunoprecipitates were separated on a 10% SDS-PAGE and exposed to film.

1: 10ras3 Puro, 33°C, Ab 9E10

2: 10ras3 Puro, 33°C, Ab 240

3: 10ras3 Puro, 39°C, Ab 9E10

4: 10ras3 Puro, 39°C, Ab 240

5: 10ras3 p53^{175}, 39°C, Ab 9E10

6: 10ras3 p53^{175}, 39°C, Ab 240

7: 10ras3 p53^{248}, 39°C, Ab 9E10

8: 10ras3 p53^{248}, 39°C, Ab 240

9: radioactive size-marker

A.

Number of colonies

B.

mt p53

69 kD
46 kD
30 kD
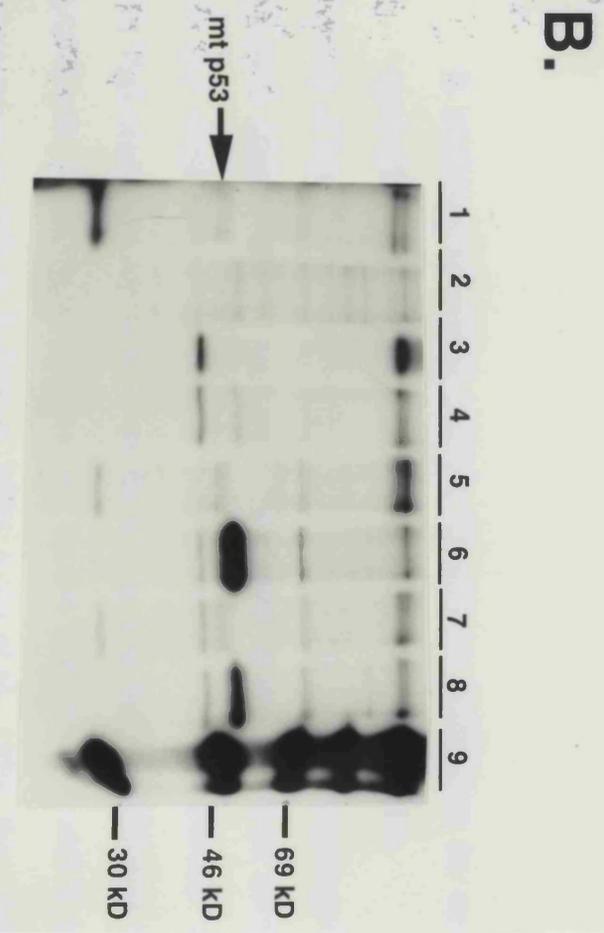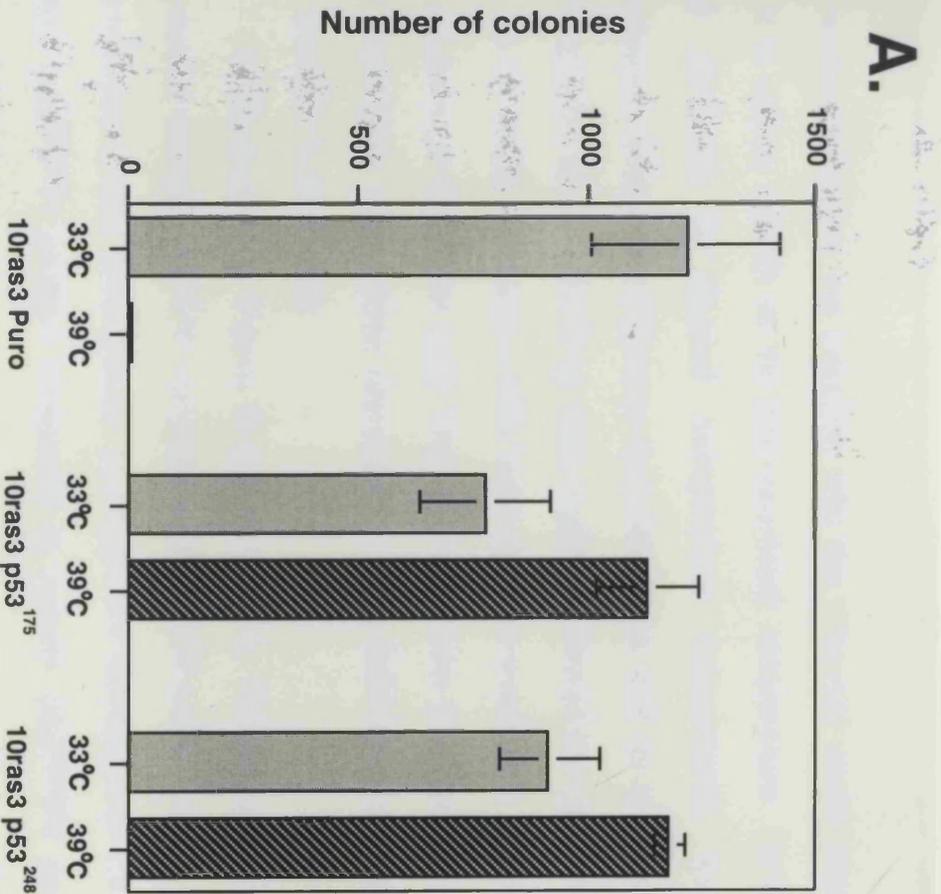
temperature whereas the infection with the retroviral vector alone did not result in colony growth at the non-permisive temperature. Similar results were obtained in several independent experiments. In addition, experiments with other Schwann cell clones as well as with the retroviral vector pBabe Bleo, containing mutant p53 confirmed the presented results that up to 100% of infected cells were able to overcome the *ras*-induced growth arrest in Schwann cells. These results show that the effect of mutant p53 expression was neither dependent on the Schwann cell clone nor on the retroviral vector used.

As illustrated in Figure IV.2B and IV.3B, 10ras3 and 11ras2 cells expressed mutant $p53^{R175H}$ or mutant $p53^{R248W}$ protein at the permissive and non-permissive temperature. The apparent difference in the amount of mutant $p53^{R175H}$ protein compared to mutant $p53^{R248W}$ protein after immunoprecipitation was very likely due to a reduced affinity of the antibody Ab240 used as reported by Gannon et al., 1990 and did not necessarily reflect differences in expression levels.

4.3   A carboxy-terminal region of p53 is sufficient to co-operate with oncogenic Ras in Schwann cells

Mutants of the p53 tumour suppressor can overcome the *ras*-induced growth arrest in 10ras3 and 11ras2 cells as shown in the previous section. However, the mechanism by which p53 mutants can cooperate with oncogenic Ras remained unclear. Despite ample supportive evidence that mutant p53 binds to wild-type p53 leading to the formation of stable non-functional complexes (Milner & Metcalf, 1991; Kern et al., 1992; Bargnonetti et al., 1992), this explanation may not be fully satisfactory, since certain p53 mutants have been reported to transactivate a specific target gene when

introduced into p53-null cell lines (Dittmer et al., 1993, Chin et al., 1992; Crook et al., 1994). This indicated the possibility that p53 mutations could also lead to the gain of certain functions. The two p53 mutants (R175H, R248W) employed in my experiments have been reported to show a gain-of-function in p53-null (10)3 cells in terms of transactivation of a reporter construct containing the promoter region of the multi-drug resistant gene and tumourigenicity after injection into nude mice. However, in p53 negative SAOS-2 cells the same p53 mutants displayed no gain-of-function (Dittmer et al., 1993). In contrast to this report, mutant p53^{R175H} has been shown to be transcriptionally inactive towards a different p53 binding site, p53CON (Funk et al., 1992) in p53-null K562 chronic myelogenous leukemia (CML) cells (Zhang et al., 1993) and in a p53-null ovarian carcinoma cell line (SKOV-3) (Chumakov et al., 1993). Nevertheless, a missense mutation at the same position substituting arginine to a proline, p53^{R175P}, resulted in a clearly transcriptional active p53 mutant (Crook et al., 1994). Some experimental evidence suggested that the reported observation of a gain-of-function of p53 mutants depended strongly on the specific mutant (Crook et al., 1994), the nature of the p53 binding site of the reporter construct (Chen et al, 1993) and the cell line used for investigation (Dittmer et al., 1993, Sehgal & Margulies, 1994).

Intensive studies into the functions of the p53 protein have established a minimal carboxy-terminal domain which is required for transformation in primary cells (Shaulian et al., 1992). The transforming activity of this C-terminal region is not due to any mutation within but has been correlated with the ability to form stable oligomers with wild-type p53 (Shaulian et al., 1992; Bowman et al., 1996). This minimal transformation domain of p53 (Figure IV.4A) widely overlaps with the oligomerisation domain of amino acids 330-390 in murine p53 (Shaulian et al., 1992;

Stürzbecher et al., 1992; Wang et al., 1994) which is required for the formation of functional p53 complexes and, in particular, tetramers (Stürzbecher et al., 1992). Similarly to stable complexes formed between full-length mutants of p53 with wild-type p53, oligomers between wild-type p53 and the carboxy-terminal domain abrogate any sequence-specific DNA-binding of wild-type p53 (Shaulian et al., 1992) and the transactivation from p53 reporter genes (Reed et al., 1993; Shaulian et al., 1995). Consistent with this notion, a full-length mutant of p53 with a truncation of this C-terminal domain did not inhibit the ability of wild-type p53 to activate transcription (Unger et al., 1993). Conversely, the carboxy-terminal region of p53 cooperated with activated Ras in the transformation of rat embryo fibroblasts (Shaulian et al., 1992) and enhanced the transformation by oncogenic *ras* and *E1A* (Reed et al., 1993). Moreover, overexpression of the p53 carboxy-terminus interfered with p53-mediated apoptosis in response to cytokine withdrawal in haematopoietic cells (Gottlieb et al., 1994). The effectiveness of the p53 carboxy-terminus in inactivating wild-type p53 was confirmed in a transgenic mouse model for brain tumour development in which tissue-specific expression of the p53 carboxy-terminus resulted in a reduction of apoptosis and accelerated tumourigenesis (Bowman et al., 1996). Thus, the carboxy-terminal domain provides a way to investigate the cooperation of oncogenic Ras and a functional defective p53 protein in the absence of any possible gain-of function activity.

To test whether the rescue of the *ras*-induced growth arrest by p53 mutants required a gain-of-function of these mutants, I repeated the experiments described above using the carboxy-terminal region of wild-type p53 rather than the full-length mutant protein. The murine p53 carboxy-terminus from amino acid 302 to 390 (Figure IV.4A) expressed in pBabe Puro designated pBabe Puro p53CT was introduced into Schwann cell

Figure IV.4A: Schematic representation of p53 carboxy-terminus in comparison to full-length murine p53 protein

The murine p53 carboxy-terminus amino acids 302-390 is aligned to the major functional regions of p53 after Osbun & Butel, 1995, indicating the overlapping region in particular with the oligomerisation domain of p53.

Figure IV.4B: p53CT$^{aa302-390}$ rescues *ras*-induced growth arrest in 10ras3 cells

10ras3 cells were co-cultivated with GPE Puro and GPE p53 CT$^{aa302-90}$ cells for two days as described (see Methods). Cells were split and kept under puromycin selection for three days. Half of all dishes were then shifted to the non-permissive temperature of 39°C, whereas the other half remained at the permissive temperature of 33°C. Colonies were stained and counted three weeks after co-cultivation. The standard deviation of the number of 10ras3 Puro colonies at 39 °C was too small to be seen in the presented graph.

A.

wild-type p53

1   42   99   289   337   352 393

transactivation   DNA-binding region   oligomerisation
domain   domain

302   390

p53 carboxy-terminus

B.

Number of colonies

1500

1250

1000

750

500

250

0

33°C   39°C   33°C   39°C

10ras3 Puro   10ras3 p53 CT aa 302-390
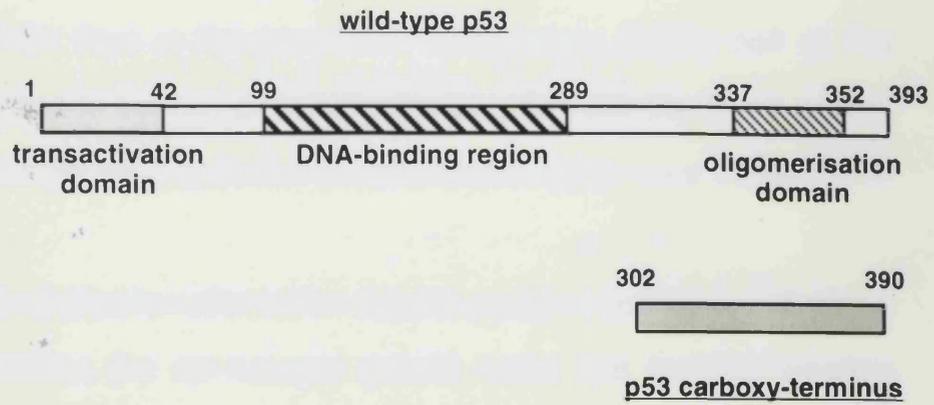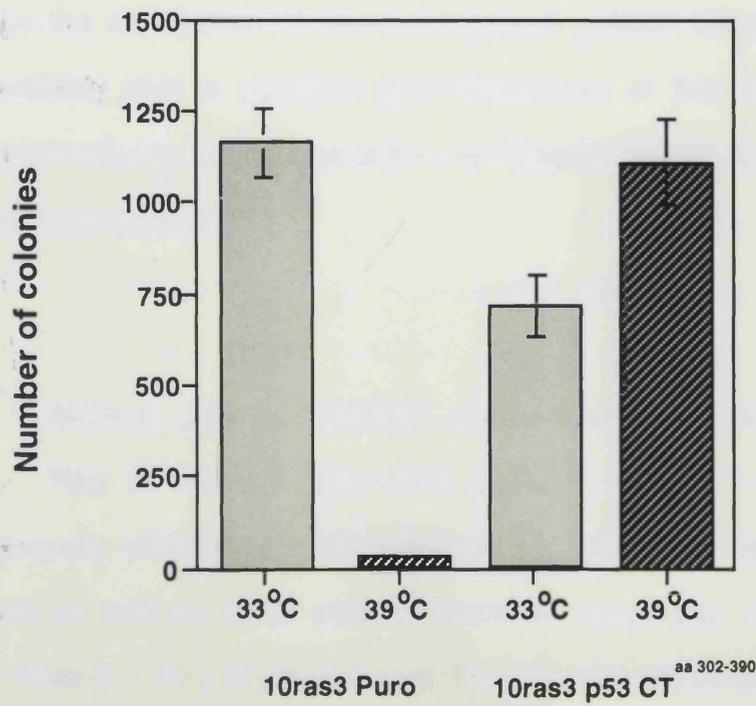
clone 10ras3. This carboxy-terminal region of the p53 mouse and rat sequence differ in only a very few amino acid positions (Stürzebecher et al., 1992). Following infection, Schwann cells were split into drug-selective medium. After two days at the permissive temperature (33°C) half of the dishes were shifted to the non-permissive temperature (39°C). Three weeks after infection, outgrowing colonies at both temperatures were stained and counted.

The p53 minimal transformation domain enables Schwann cell clone 10ras3 to overcome the ras-induced growth arrest and to form growing colonies at the non-permissive temperature. The number of colonies obtained at the permissive (33°C) and non-permissive temperature (39°C) indicated that up to 100% of infected cells were enabled to overcome the ras-induced growth arrest by expression of the p53 carboxy-terminus (Figure IV.4B). These results showed that the inactivation of wild-type p53 is required for the abrogation of the ras-induced growth arrest. It therefore seemed unlikely that a possible gain-of-function of the full-length p53 mutants contributed to the rescue of the ras-induced growth arrest described in previous experiments.

4.4 Mutant p53 and p53CT cooperate with oncogenic Ras in normal Schwann cells

The results presented in the previous sections have established that p53 mutants as well as a p53 carboxy-terminal region can cooperate with oncogenic Ras in 10ras3/10ras3-1 and 11ras2 cells. However, it has been reported, that various cells expressing the temperature-sensitive large T (tsLT) arrest or enter senescence after withdrawal of the LT antigen which indicates that proliferation had become dependent on the presence of LT

(Ikram et al., 1994; Gonos et al., 1996). If this phenomenon also occurs in Schwann cells used in previous experiments, it would make it impossible to distinguish between growth arrest initiated by activated Ras or due to the withdrawal of LT at the non-permissive temperature. As a consequence, the abrogation of the growth arrest by mutant p53 may not necessarily have directly contributed to the cooperation with activated Ras.

To investigate whether mutant p53 and activated Ras could cooperate in the absence of any LT protein, I introduced the retroviral vectors pBabe Puro p53^{R175H} or pBabe Puro p53CT into normal Schwann cells. After selection, Schwann cells with empty vector (NS Puro), p53^{R175H} (NS mt p53^{175}) and the p53 carboxy-terminus (NSp53CT) were pooled and subsequently infected with oncogenic c-Ha Ras Val12-LXSN or the empty vector LXSN virus (Miller & Rosman, 1989).

In line with previous findings (Ridley et al., 1988), c-Ha Ras Val12 introduced alone did not result in any sustainable Schwann cells colonies. Therefore, Schwann cells expressing c-Ha Ras Val12 were not available for further experimentation. Similar numbers of resistant control and mutant p53 or p53CT expressing colonies were recovered, although colonies expressing mutant p53 or the p53 carboxy-terminus were larger compared with controls. Colonies resistant to both selective markers were pooled.

Cooperation of c-Ha Ras Val12 and p53^{R175H} or p53CT was measured as growth factor-independent proliferation. The cell proliferation rate was determined to establish whether p53 mutants and oncogenic Ras were able to alter the growth factor requirements of Schwann cells since growth factor-independent cell proliferation is a hallmark of Schwann cell transformation (Ridley et al., 1988). Generally, proliferation of normal Schwann cell is weakly stimulated by foetal calf serum and required

Figure IV.5: Growth curve of NS p53^{175}/c-Ha Ras Val12 cells

Schwann cells were infected with either pBabePuro or pBabePuro p53^{R175H} as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10^4 cells were seeded into 2% FCS containing DMEM in the absence of glial growth factor and forskolin. Cells from two wells of a 6-well dish were trypsinised daily and counted three times for six days. The average cell number was plotted against time. The standard deviation of some points was too small to be seen in the presented graph. Growth curves presented in Figure IV.5 & 6 were obtained in the same experiment. Therefore, the control growth curves are identical in both figures.

Figure IV.6: Growth curve of NS p53 CT$^{aa302-390}$/c-Ha Ras
Val12 cells

Schwann cells were infected with either pBabe Puro or pBabe Puro p53CT$^{aa302-290}$ as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10^4 cells were seeded into 2% FCS containing DMEM in the absence of glial growth factor and forskolin. Cells from two wells of a 6-well dish were trypsinised daily and counted three times for six days. The average cell number was plotted against time. The standard deviation of some points was too small to be seen in the presented graph.

133
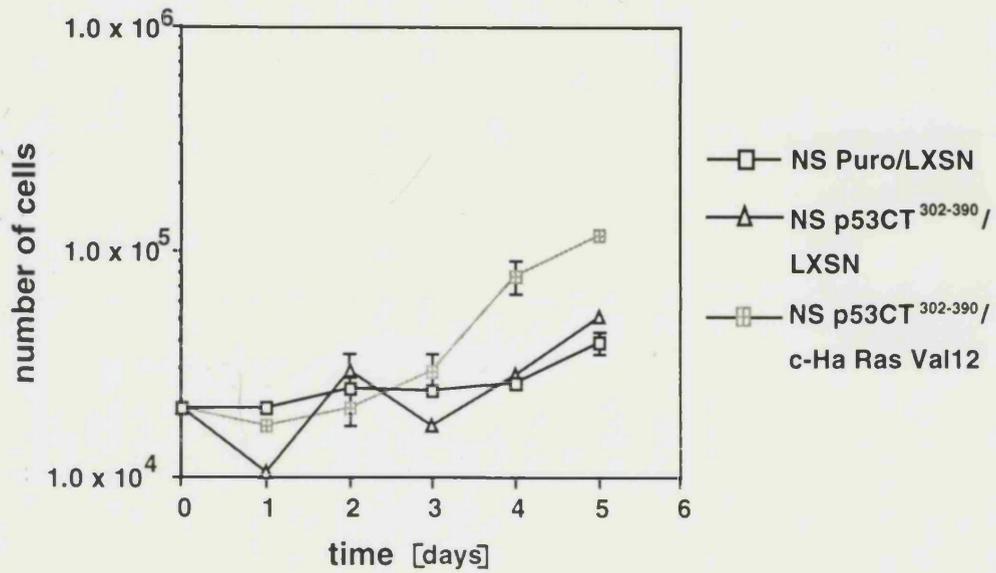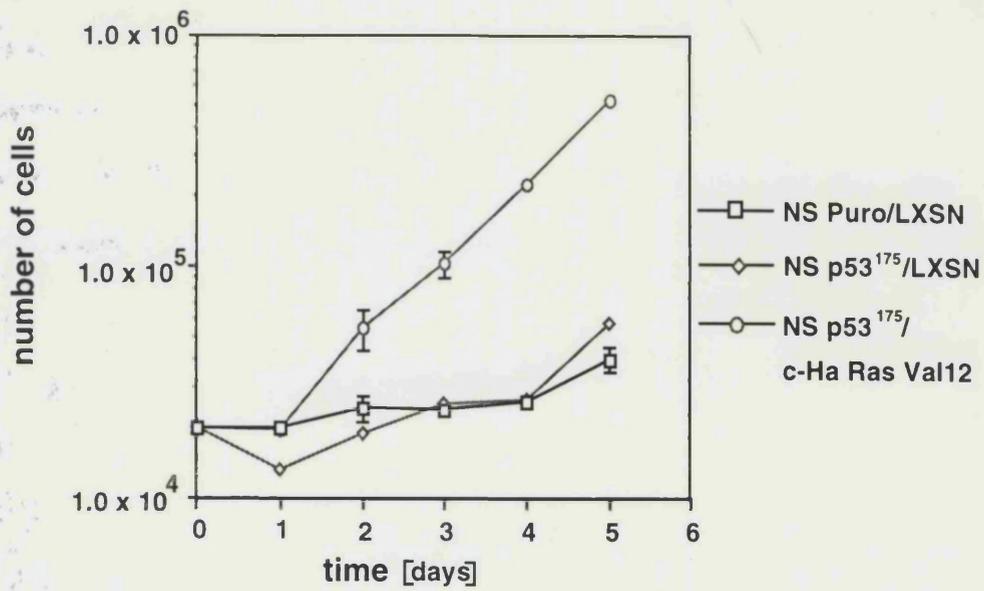
Figure IV.7: Morphological transformation of Schwann cells by mutant p53 or p53CT and c-Ha Ras Val12

Schwann cells were infected with pBabe Puro, pBabe Puro p53^{R175H} or pBabe Puro p53 CT$^{aa302-90}$ as described (Methods). After selection, these cells were infected with c-Ha Ras Val12-LXSN or the retroviral vector LXSN alone and selected for drug-resistant colonies. Fotos were taken at a magnification of 400x directly after outgrowth of colonies.

NS Puro/LNSX
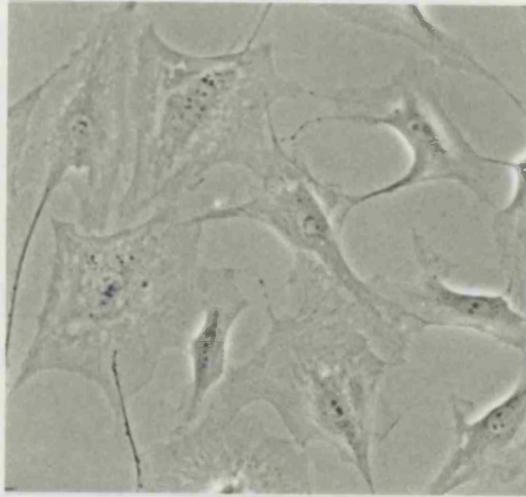
NS p53^{175}/LXSN
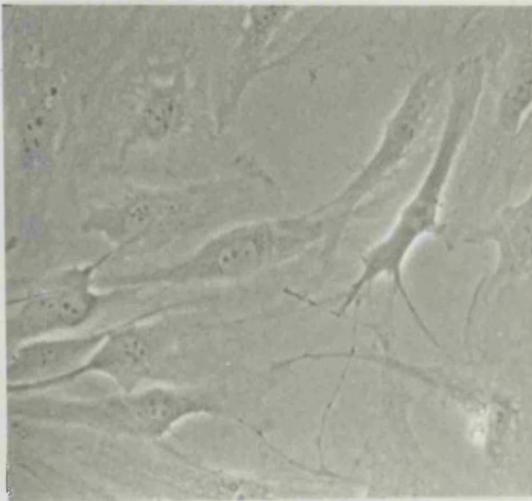
NS p53CT/LXSN
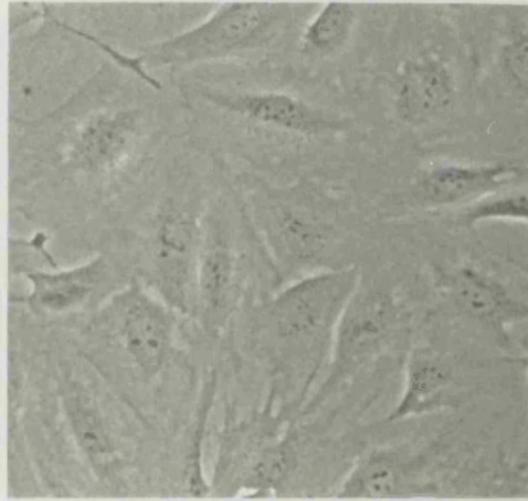
NS p53^{175}/c-Ha Ras Val12
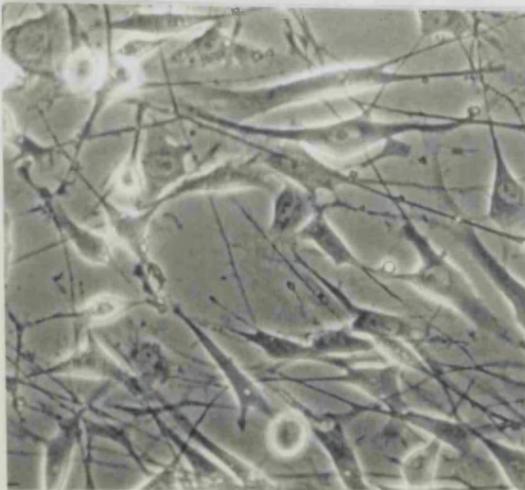
NS p53CT/c-Ha Ras Val12
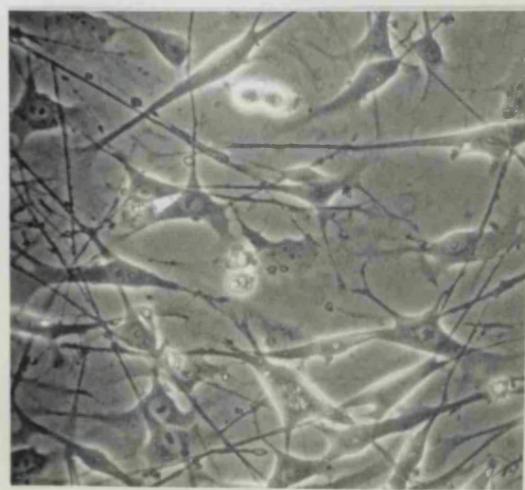
additional growth promoting factors, in particular glial growth factor and forskolin (Ridley et al., 1988; my own observation). In the absence of these growth promoting factors, untransformed Schwann cells did not grow at all or extremely slowly probably due to traces of growth promoting factors in serum.

Schwann cells NS Puro, NS mtp53^{175} or NS p53CT, or the same cells infected with c-Ha Ras Val12-LXSN, or the empty vector LXSN respectively, were seeded at 2×10^4 cells/well in medium without growth promoting factors. Cells were counted daily over a period of five days.

Schwann cells with c-Ha Ras Val12 and either mutant p53^{R175H} or the carboxy-terminus of p53 proliferated more rapidly compared to control cells (Figure IV.5 and IV.6). Cells with full-length mutant p53^{R175H}/c-Ha Ras Val12 had an increased proliferation rate compared to NS p53CT/c-Ha Ras Val12 cells. Schwann cells expressing either mutant p53^{R175H} or p53CT alone exhibited a similar growth rate to vector alone control cells (Figure IV.5 and IV.6). Schwann cells harbouring both mutant p53^{R175H} or p53CT and c-Ha Ras Val12 presented a highly refractile, spindular morphology as compared to cells containing only mutant p53^{R175H}/p53CT or vector alone (Figure IV.7). These data indicate that oncogenic Ras and mutant p53^{R175H} or the p53 carboxy-terminus are able to cooperate in growth factor-independent cell proliferation and morphological transformation (Figure IV.5, 6, 7) and thus, behave very similarly to Schwann cells expressing activated Ras and SV40 large T antigen (Ridley et al., 1988).

4.5   Mutant p53 cooperates with oncogenic Raf in Schwann cells

Oncogenic Ras and mutants of the tumour suppressor gene p53 co-coperate in Schwann cells as has been established in the previous sections. To further elucidate the cooperation between mutant p53 and oncogenic Ras, I sought to determine the signalling pathway downstream of Ras which was required for the cooperative action with mutant p53. Since oncogenic Ras connects to various downstream signalling pathways, I was interested in investigating whether Raf, one of the major downstream targets of Ras (Moodie et al., 1993; Van Aelst et al., 1993; Warne et al., 1993; Zhang et al., 1993; Vojtek et al., 1993), can also cooperate with mutant p53 in Schwann cell transformation. If activated Ras signals through Raf in order to cooperate with mutant p53, then constitutively active Raf molecules should also be capable of cooperating with mutant p53. The c-Raf mutant ΔRaf which has a deletion of the regulatory region-1 (CR-1) of Raf reportedly exhibited constitutive kinase activity (Leevers et al., 1994). Therefore, I used this constitutively active molecule expressed in the retroviral vector LNSX (Miller & Rosman, 1989) to explore the cooperation of mutant p53^{R175H} and oncogenic Raf as a downstream target of Ras in transforming Schwann cells. To test this hypothesis, the retroviral vectors ΔRaf-LXSN (Leevers et al., 1994) were introduced into low passage Schwann cells NSPuro and NSmtp53^{175}. Outgrowing colonies infected with ΔRaf alone were very small compared to colonies infected with ΔRaf and mutant p53^{175}. Furthermore, Schwann cells expressing ΔRaf on its own grew very poorly so that after more than three weeks not enough cells were available for the following experiment.

Transformation by cooperating ΔRaf and mutant p53^{R175H} were again measured as a function of cell proliferation of the pooled Schwann cell colonies in the absence of any growth-promoting factors. NSPuro and

Figure IV.8: Growth curve of NSp53^{175}/ΔRaf cells

Schwann cells were infected with either pBabe Puro or pBabe Puro mtp53^{R175H} as described (Methods). After selection, these cells were infected with either ΔRaf-LXSN or the retroviral vector LXSN alone and selected. Drug-resistant colonies were pooled and 2x10^4 cells were seeded into DMEM supplemented with 2% FCS but without the growth-promoting factors GGF and forskolin. Cells from two wells of a 6-well dish were trypsinised daily for six days and counted. The average cell number is plotted against time. The standard deviation of some points was too small to be seen in the presented graph.
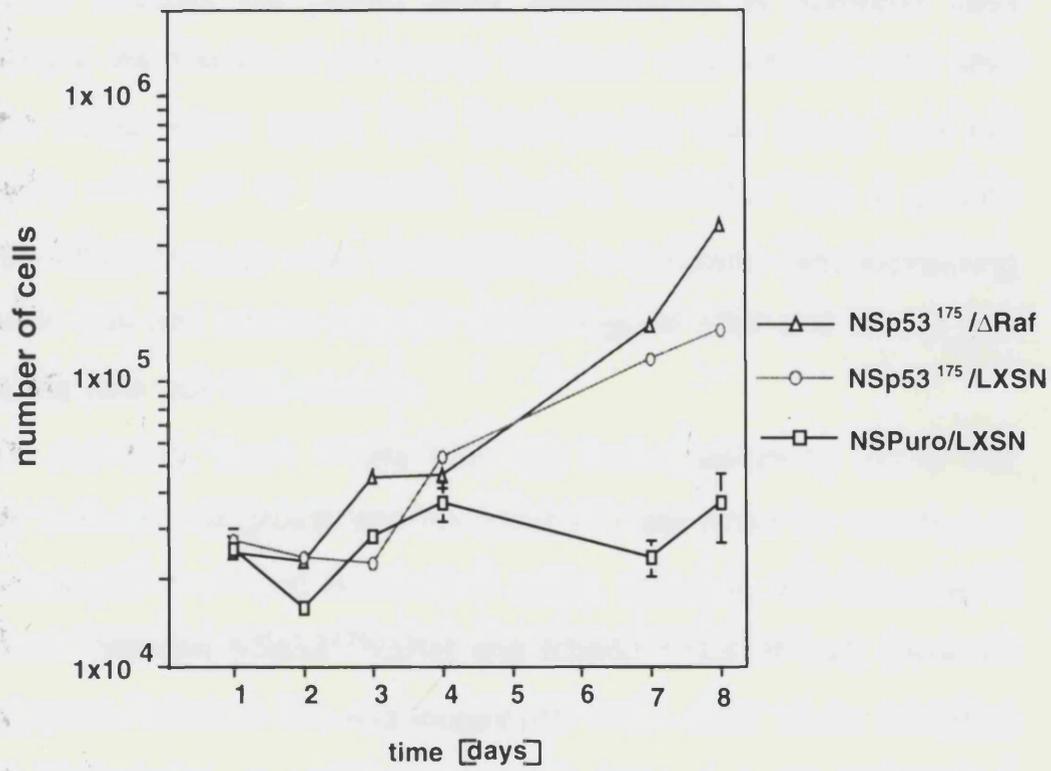
NSmtp53^{175} or the same cells infected with ΔRaf or the empty vector LXSN respectively were seeded at 2×10^4 cells/well in medium without growth promoting-factors. Cells were counted daily over a period of six days.

Schwann cells harbouring ΔRaf and mutant p53^{R175H} had an increased proliferation rate compared to control cells (Figure IV.8). Schwann cells expressing mutant p53 and an empty LXSN vector alone also showed an increased rate of cell proliferation, indicating that mutant p53 alone reduces the growth factor requirements in Schwann cells dramatically. As mentioned previously, Schwann cells infected with ΔRaf and the empty vector pBabe Puro were growing very slowly and cell confluency could not be achieved, indicating that ΔRaf had a growth-inhibitory effect on these cells. In comparison to Schwann cells expressing oncogenic Ras and mutant p53, the morphology of ΔRaf and mutant p53 expressing cells was less refractile.

In summary, these results indicated that constitutively active Raf strongly impeded cell growth and this effect was abolished by co-infection with mutant p53 in this cell proliferation assay. However, the difference in growth rate between NSp53^{175}/ΔRaf and NSp53^{175}/LXSN cells were not as profound as between Ras and mutant p53 which could be an indication for the involvement of additional Ras-dependent but Raf-independent pathways.

4.6   Mutant p53 abolishes ΔRafER induced growth arrest in Schwann cells

The previous experiments have provided evidence that mutants of the tumour suppressor gene p53 as well as the carboxy-terminus of p53 cooperate with activated Ras and activated Raf to transform Schwann cells.

However, to further investigate the mechanisms of cooperation between oncogenic Ras/Raf and mutant p53, these cells were only of very restricted use. Schwann cell clones 11ras2 and 10ras3 had to be grown in the presence of a cooperating oncogene at the permissive temperature prone to additional mutagenesis under these conditions. On the other hand, the generated Schwann cells harbouring mutant p53 and oncogenic Ras/Raf could not be regulated at all and a biochemical analysis of the same cells in the transformed and arrested state was impossible.

These restrictions did not apply to Schwann cells with an inducible form of ΔRaf (Lloyd et al., 1997). Oncogenic ΔRaf fused to the hormone-binding domain of the human oestrogen receptor (Samuels et al., 1993) was cloned into the high-titre retroviral vector LXSN (Miller & Rosman, 1989) in order to infect low-passage normal Schwann cells. In these Schwann cells, designated NSΔRafER, constitutive Raf-kinase activity had become strictly dependent on oestrogen or alternatively on the oestrogen analogue, 4-hydroxy tamoxifen (Lloyd et al., 1997).

NSΔRafER cells harbouring the inducible Raf-molecule offered various advantages for further investigation into the signalling pathways for the Ras/Raf cell-cycle arrest. Firstly, these Schwann cells could be grown in the absence of any cooperating oncogene and, secondly, induction of ΔRafER-dependent kinase activity was rapid, since induction occurred at the protein level. Kinetic studies regarding the activation of downstream targets of Raf and their contribution towards the growth arrest became technically possible.

Similar to Ras, activation of ΔRafER induces Schwann cells to arrest in G1 (Figure IV.9). This G1-phase arrest was independent of specific growth conditions (Lloyd et al., 1997). Furthermore the changes in cell morphology upon activation of Raf were indistinguishable from those

Figure IV.9: Growth arrest in NSΔRafER cells is abrogated by mutant p53

Subconfluent NSΔRafER, NSΔRafERp53[175] and NSempty control cells cells were assynchronously grown in the presence or absence of 1μM 4-hydroxy tamoxifen for 30 hours. Four hours prior trypsinisation, 10 μM bromo-desoxyuridine (BrdU) were added. Cells were examined for DNA content by propidium iodide staining and flow cytometry and analysed for BrdU uptake.

NSempty, -TM

G1   : 75.18 %
S   : 17.49 %
G2/M : 5.74 %

NSempty, +TM

G1   : 67.17 %
S   : 20.29 %
G2/M : 5.74 %

NSΔRafER, -TM

G1   : 78.34 %
S   : 17.13 %
G2/M : 2.10 %

NSΔRafER, +TM

G1   : 93.80 %
S   : 2.96 %
G2/M : 2.07 %

NSΔRafER p53 ^{175}, -TM

G1   : 66.88 %
S   : 29.60 %
G2/M : 3.17 %

NSΔRafER p53 ^{175}, +TM

G1   : 71.37 %
S   : 24.95 %
G2/M : 3.05 %
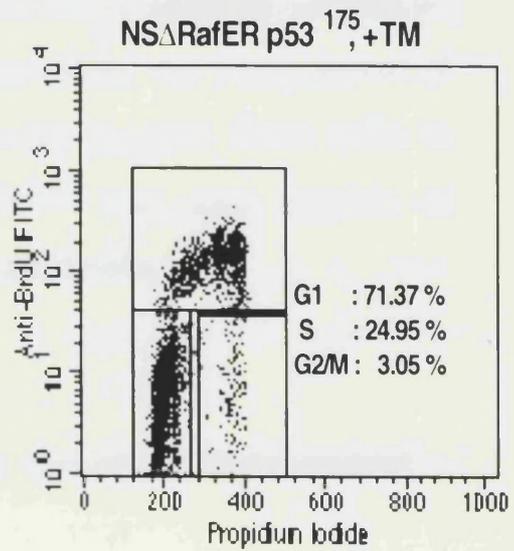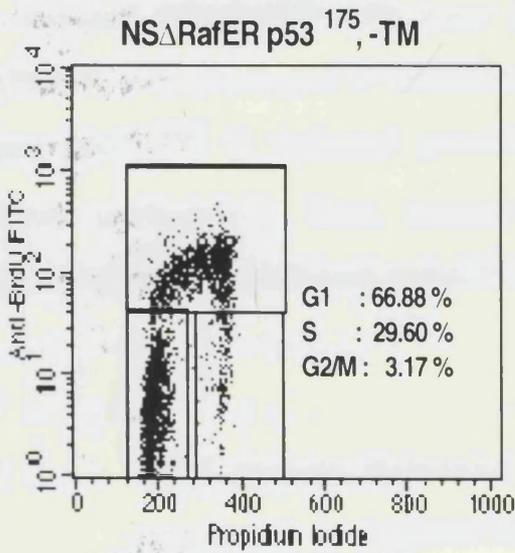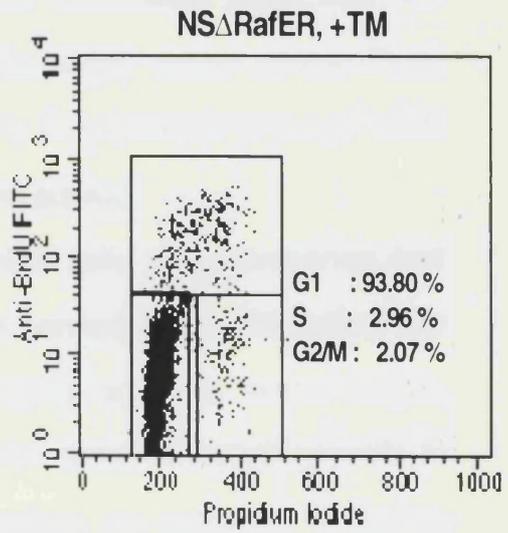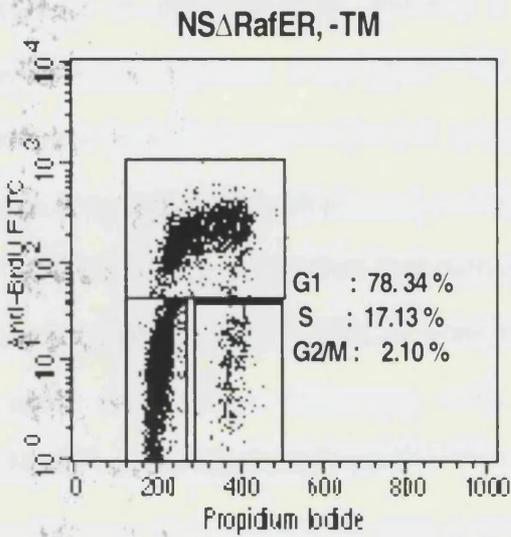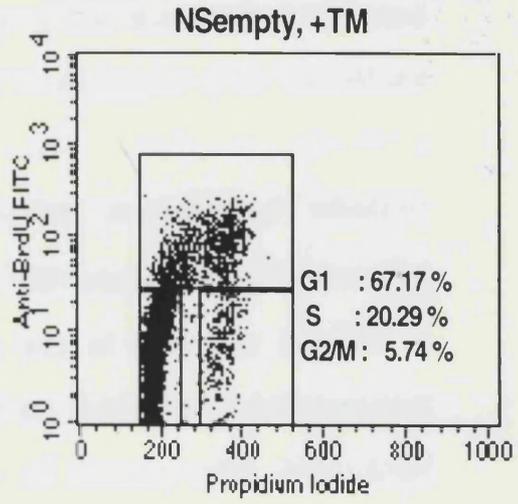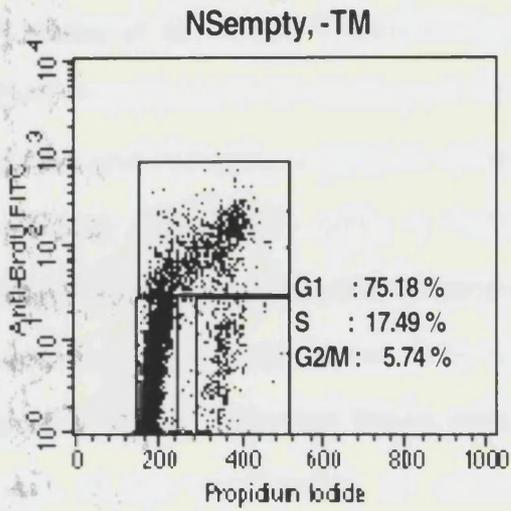
observed in Schwann cells expressing oncogenic Ras protein (Lloyd et al., 1997; Ridley et al., 1988). These findings indicated that the newly generated NSΔRafER cells could be employed for further investigation into the nature of the Ras and Raf induced growth arrest.

Using NSΔRafER cells, I therefore further investigated whether mutant p53^{R175H} was capable of cooperating with constitutively active Raf to overcome the growth arrest. This question was of particular interest in order to establish whether these cells could be used for a biochemical analysis of the mechanism of cooperation of mutant p53 and ΔRaf (described in chapter 5.0). Initially I infected NSΔRafER cells with the retroviral vector pBabe Puro p53^{R175H} and the empty vector pBabe Puro. Puromycin-resistant colonies were expanded in the absence of the ΔRafER inducer 4-hydroxy tamoxifen and subsequently pooled.

To test the proliferative behaviour of these cells in the presence and absence of active RafER, FACS analysis was carried out to determine the cell cycle distribution. NSempty cells containing only the vector LXSN, NSΔRafER and NSΔRafER p53^{R175H} cells were grown assynchronously in the presence or absence of 4-hydroxy tamoxifen. As indicated in Figure IV.9, induced NSΔRafER cells were arrested in G1 whereas uninduced NSΔRafER cells continued to proliferate. Interestingly, the coexpression of mutant p53^{R175H} in induced NSΔRafER cells enabled these cells to continue proliferating. Thus, mutant p53^{R175H} was also capable of overcoming the ΔRaf-induced arrest in NSΔRafER cells.

4.7   ΔRaf arrests Schwann cells through p53-dependent induction of p21$^{WAF1/Cip1}$

4.7.1   Introduction

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The generation of Schwann cells which arrested in a strictly hormone-dependent manner (NSΔRafER) (Figure IV.9) allowed investigations into the effects of activated Raf on members of the cell cycle machinery in particular cyclin/cdk complex kinase activity. Investigations into cyclin E, cyclin A and cdk2-dependent kinase activity revealed a strong decrease of activity in arrested Schwann cells, despite the fact that CDK2 complexed with cyclin A or cyclin E was mostly found in the CAK phosphorylated form understood to be the active form of these complexes (Lloyd et al., 1997). Since cyclin E, cyclin A and CDK2-dependent kinase activity is required for S-phase entry (Girard et al., 1991; Pagano et al., 1992; Zindy et al., 1992; Tsai et al., 1993; van den Heuvel & Harlow, 1993; Ohtsubo et al., 1995), the observed inhibition of cyclin/cdk kinase activity appeared to be sufficient to arrest Schwann cells in G1. In addition, my results showing that p53 mutants were capable of abrogating the ΔRaf-induced cell cycle arrest suggested a direct or indirect involvement of wild type p53 in mediating this cell cycle arrest. The nature of this interaction was however unclear at the time.

We considered the Raf-dependent and p53-dependent induction of a cdk-inhibitor as a possible explanation for our observations. As one possible candidate, the cdk-inhibitor p21[WAF1/Cip1] (Harper et al., 1993; Xiong et al., 1993; el-Deiry et al., 1993; Noda et al., 1994) not only binds to cyclin E/cdk2 complexes, cyclin A/cdk2 and cyclin A/cdc2 complexes and inhibits their specific kinase activity (Harper et al., 1993; Xiong et al., 1993; Gu et al., 1993). Moreover, p21[WAF1/Cip1] is also transcriptionally induced by wild type p53 (el-Deiry et al., 1993) and is able to mediate p53's growth-inhibitory function in various cell types. It was therefore intriguing to test the possibility that the Raf and p53-dependent induction of p21[WAF1/Cip1] was the underlying cause for the G1 cell cycle arrest in Schwann cells.

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4.7.2 ΔRafER induces cell cycle inhibitor p21$^{WAF1/Cip1}$ in Schwann cells

To analyse p21$^{WAF1/Cip1}$ protein level in Schwann cells, NSΔRafER cells were quiesced for two days prior to the addition of 4-hydroxy tamoxifen and mitogens. Protein samples were taken at indicated time points after induction of ΔRaf. The activation of ΔRafER in Schwann cells led to a strong induction of the cyclin/cdk inhibitor p21$^{WAF1/Cip1}$ initially visible at 10 hours and increasing over time (Figure IV.10). This induction coincided with the decrease of cyclinE/Cdk2 kinase activity at approximately 10 hours after activation of Raf (Figure IV.11). Furthermore, in parallel to the induction of p21$^{WAF1/Cip1}$ induction and the reduction of cyclin E-dependent kinase activity, the rate of DNA synthesis decreased steadily, indicating that cells were arrested in G1 (Figure IV.11). These findings provided first evidence that the induction of p21$^{WAF1/Cip1}$ could be the underlying cause for the Raf-induced G1 cell cycle arrest in Schwann cells.

Figure IV.10: ΔRafER induces p21$^{WAF1/Cip1}$ in Schwann cells

Asynchronously growing NSΔRafER cells were induced with 1 μM 4-hydroxy tamoxifen. Protein samples were prepared from quiescent NSΔRafER cells or induced or uninduced NSΔRafER cells at the indicated time points. 50 μg protein were analysed by western blot with the p21$^{WAF1/CIP1}$ specific antibody C36 (a kind gift by Wade Harper) and the ECL-detection system (Amersham International Ltd.).

Figure IV.11: Cyclin E dependent kinase activity and DNA synthesis decreases in response to constitutive Raf-activity

Asynchronously growing NSΔRafER cells were incubated in the presence of 1 μM 4-hydroxy tamoxifen or as a control ethanol. Protein lysates were prepared at indicated times and 300 μg protein were immunoprecipitated with an cyclin E antibody and assayed for histone H1 kinase activity. Incorporated γ-^{32}P was quantified using ImageQuant-software (Molecular Dynamics Ltd.) and are expressed as percentage of cyclin E-dependent kinase activity in untreated growing NSΔRafER cells. DNA synthesis was measured in a thymidine assay as described in Lloyd et al., 1997 and expressed as percentage of DNA synthesis in untreated cells.

I am indebt to Alison Lloyd for this figure.

p21

Q 0   - +   - +   - +   - +   - +   - +
       4 h   10 h   14 h   18 h   22 h   30 h

time after 4- hydroxy tamoxifen addition

—□— : cyclin E-dependent kinase activity

—✳— : DNA synthesis
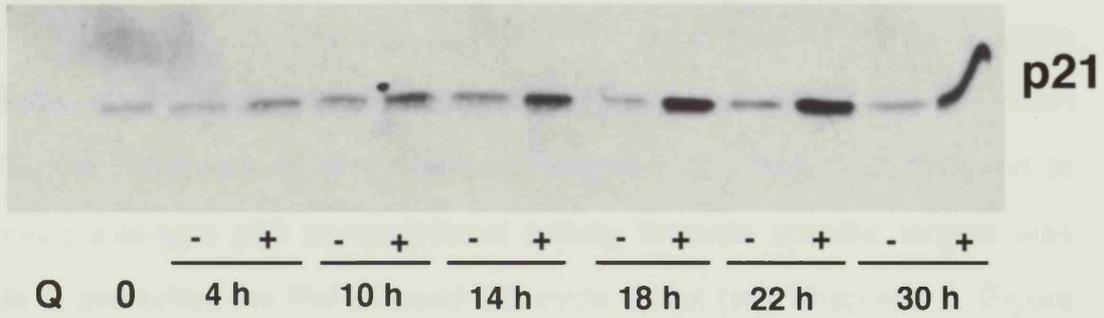
time after 4-hydroxy tamoxifen addition
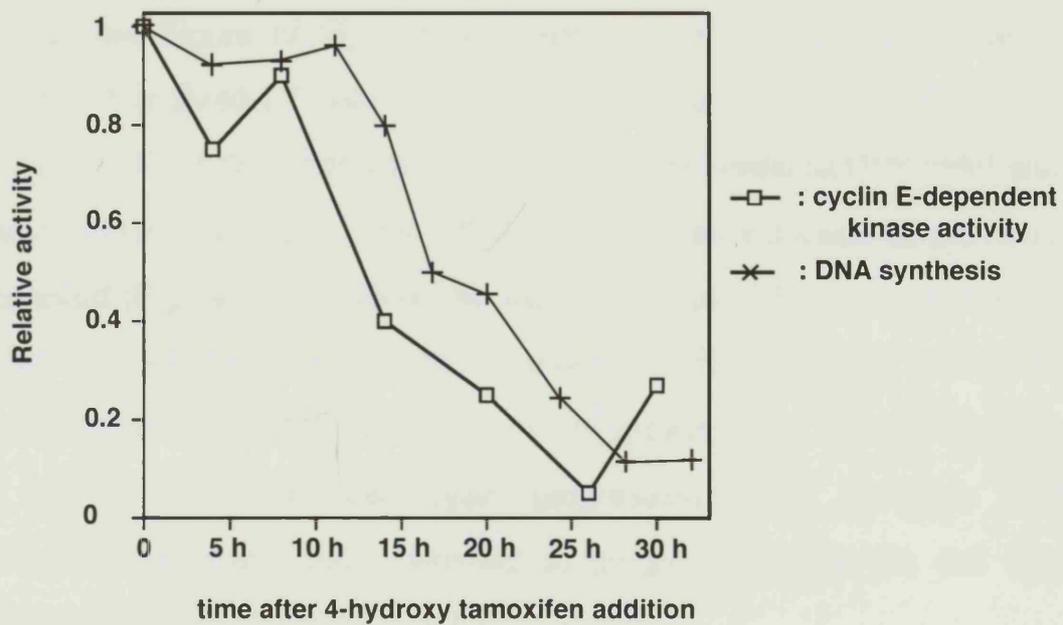
4.8　　　Mutant p53 abolishes p21$^{WAF1/Cip1}$ induction

The cell-cycle inhibitor p21$^{WAF1/Cip1}$ was discovered as a gene induced after activation of wild type p53 (el-Deiry et al., 1993). In NSΔRafER cells, the coexpression of a dominant negative p53 mutant understood to abolish wild-type p53 transcriptional activity towards specific targets was able to overcome the Raf-induced cell cycle arrest (see chapter 4.0, Figure IV.9). If the induction of p21$^{WAF1/Cip1}$ in response to activated Raf was p53-dependent, then NSΔRafER cells coexpressing mutant p53 should exhibit a clear decrease of p21$^{WAF1/Cip1}$ protein levels compared to arrested NSΔRafER cells.

To examine p21$^{WAF1/Cip1}$ protein levels in Schwann cells coexpressing dominant negative p53, NSΔRafER mtp53^{175}, NSΔRafER Puro and NSΔRafER SV40 LT cells were induced with 4-hydroxy tamoxifen. Protein lysates were prepared from induced and uninduced control cells 26 hours after induction, a time point at which the p21$^{WAF1/Cip1}$ protein level peaks (see Figure IV.10). Activated Raf in Schwann cells coexpressing p53^{R175H} or SV40 LT cells did, however, not induce p21$^{WAF1/Cip1}$ (Figure IV.12). In NSΔRafER Puro control cells the protein levels p21$^{WAF1/Cip1}$ were clearly induced 26 hours after 4-hydroxy tamoxifen induction as previously observed (Figure IV.12). Thus, the induction of p21$^{WAF1/Cip1}$ by activated ΔRaf occurred in a p53-dependent manner. This finding further established a correlation of p21$^{WAF1/Cip1}$ induction with growth arrest and the decrease of p21$^{WAF1/Cip1}$ with cell cycle progression since Schwann cells coexpressing mutant p53 continued to progress through the cell cycle regardless of Raf activation (see Figure IV.10).

Figure IV.12: Raf induces p21$^{WAF1/Cip1}$ by a p53-dependent mechanism

NSΔRafER Puro, NSΔRafER p53^{175} or NSΔRafER LT were grown for 30 hours in the presence or absence of 1 μM 4-hydroxy tamoxifen. Subsequently protein lysates were prepared. Cells were harvested after this time period and 50 μg of total cell protein were analysed on a SDS-PAGE. Western blotting analysis with an p21$^{WAF1/Cip1}$ specific antibody was carried out.

Figure IV.13: Cyclin D1 is induced in NSΔRafER p53^{175} cells in response to constitutively active Raf

NSΔRafER Puro, NSΔRafER p53^{175} and NSΔRafER LT cells were grown as specified above in the presence (+) or absence (-) of 1 μM 4-hydroxy tamoxifen. Cells were harvested after this time period and 50 μg of total cell protein were analysed on a SDS-PAGE. Western blotting analysis with an cyclin D1 specific antibody was carried out.

p21

TM   −   +   −   +   −   +

NSΔRafER Puro   NSΔRafER p53^{175} NSΔRafERLT

cyclin D1

TM   −   +   −   +   −   +

NSΔRafER Puro   NSΔRafER p53^{175} NSΔRafERLT
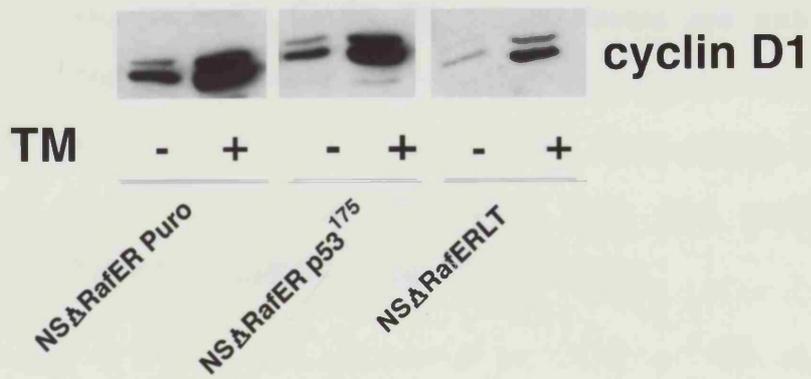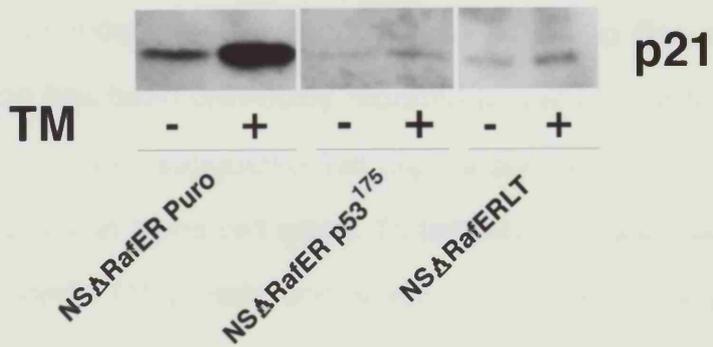
4.9 Cyclin D1 induction by Raf does not contribute to G1 cell cycle arrest in Schwann cells

The superinduction of cyclin D1 in response to Ras expression and transformation has been previously reported (Liu et al., 1995; Winston et al., 1996) and it has been suggested that superinduction of cyclin D1 can result in cell cycle arrest in some cell types. To test whether activated Raf can also superinduce cyclin D1 protein and whether this possible superinduction is correlated with cell cycle arrest in Schwann cells, cyclin D1 protein levels were examined in arrested and proliferating Schwann cells. NSΔRafER Puro, NSΔRafER p53[175] and NSΔRafER LT cells were grown in the presence or absence of 1 μM 4-hydroxy tamoxifen for 30 hours. Protein lysates were prepared and subsequently analysed by western blotting with a cyclin D1 specific antibody. Cyclin D1 protein levels are induced in response to activated Raf (Figure IV.13). However, the induction of cyclin D1 appears not to contribute to the G1 cell cycle arrest, since protein levels of cyclin D1 are similar in arrested NSΔRafER Puro and proliferating NSΔRafER p53[175] and NSΔRafER LT cells (Figure IV.13).

4.10 Raf induction and p53 transactivation activity

The induction of the cell-cycle inhibitor p21[WAF1/Cip1] is necessary and sufficient to arrest Schwann cells in the G1 phase of the cell-cycle (this thesis; Lloyd et al., 1997). This induction depends on p53 transactivation activity, since a dominant negative p53 mutant abrogates the p21[WAF1/Cip1] induction (this thesis; Lloyd et al., 1997). However, as the evidence was indirect, I sought a more direct approach to investigate whether p53's transactivation activity was actually increased after Raf activation.

To investigate a possible change in p53 transactivation activity in response to activated Raf, transient transfection experiments introducing p53-responsive reporter genes were performed. These experiments were not successful. Schwann cells can be only transfected with very low efficiency and did not grow any further after transfection. It has been reported that in eukaryotic cells p53 is activated after transient transfections and can cause growth arrest which may also occur in Schwann cells. To overcome these difficulties, I decided to construct a retroviral vector containing a p53-responsive reporter gene and infect NSΔRafER cells.

4.10.1   Construction of retroviral vector containing a
p53 binding site reporter gene

To directly measure p53 transactivation activity in NSΔRafER cells after activation of Raf, I constructed a reporter gene with a p53 binding consensus sequence. Since Schwann cells have to be infected for a successful introduction of genes, the luciferase reporter gene was cloned into a modified version of retroviral vector pBPSTR1 (Paulus et al.,1995). The DNA-sequences in pBPSTR1 responsible for the tetracycline regulation of the pBPSTR1 vector were substituted by a minimal thymidine kinase promoter and an adjacent luciferase gene. Oligomers containing a p53 consensus binding sequence (Chen et al., 1993) were cloned in front of the minimal thymidine kinase promoter and luciferase reporter gene (Figure IV.14) to measure luciferase gene expression in a p53-dependent way.

Figure IV.14: Retroviral P53-reporter construct pBPSTR
WTp53-tk-luc2F

To test p53 transactivation activity directly, the retroviral p53-reporter construct pBPSTR WTp53-tk-luc2F was constructed as described in Materials and Methods. A p53 DNA binding site from the Ribosomal Gene Cluster was integrated in front of a tk promoter and luciferase reporter gene into the retrovirus pBSTR.

pBSTR p53 tk-luc2F

AGG CCA GAC CTG CCC GGG CAA GCC TTG GCA

| LTR | *gag* | Puromycin R | SV40 | poly A | luciferase | tK | p53 binding site | LTR |

ATG
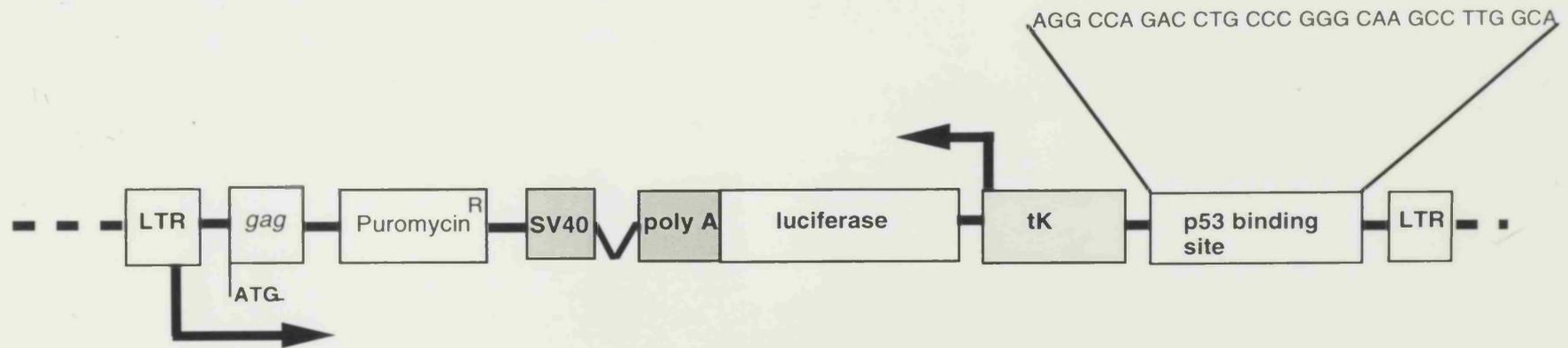
Figure IV.15A: p21$^{WAF1/Cip1}$ protein level in NSΔRafER wtp53

luc cells after UV-Irradiation and addition of 4-

hydroxy tamoxifen

NSΔRafER wtp53 luc cells were assynchronusly grown. Cells were

irradiated with UV for 20 seconds or alternatively 4-hydroxy tamoxifen was

added. Cell were harvested 20 hours after addition of 4-hydroxy tamoxifen

or UV-irradiation. 30μg total cell protein were separated on SDS-PAGE.

Western blot analysis was carried out with the p21 specific antibody.

Figure IV.15B: Luciferase activity in NSΔRafER wtp53 iuc ceils

Growing NSΔRafER wtp53 luc cells were either radiated with UV for 20

seconds or 1 μM 4-hydroxy tamoxifen was added. Cells were harvested

after 20 hours and luciferase activity was determined as described in

Materials.

p21^{WAF1/Cip1} →

-UV   +UV   +TM   -TM

NSΔRafER wtp53 luc

arbitary luciferase units

800

600

400

200

0

-UV   +UV   -TM   +TM   +pcDNA3 luc
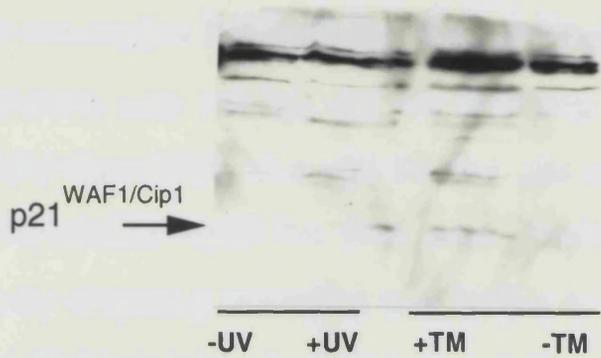
NSΔRafER wtp53 luc   NIH3T3
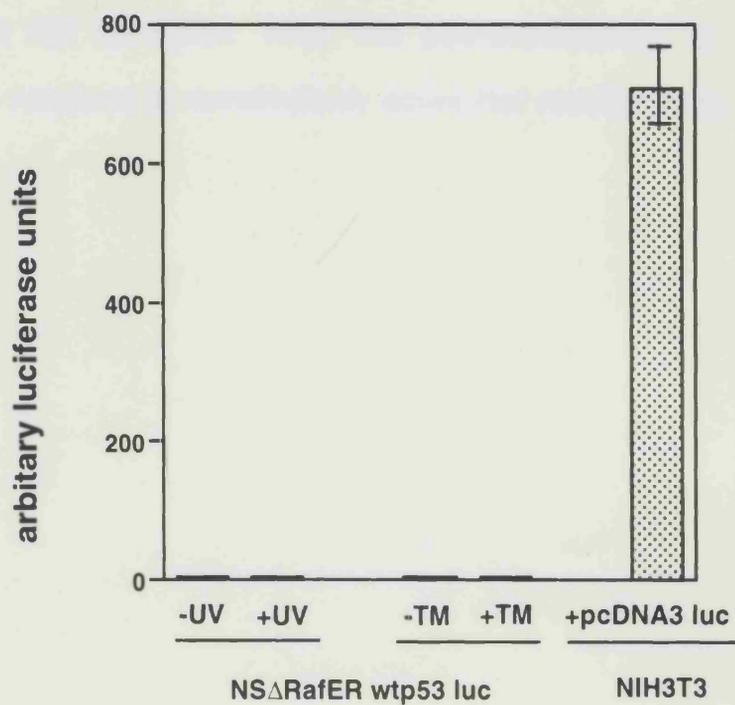
4.10.2   p53 transactivation activity after activation of Raf

To test whether Raf induces p53 transactivation activity in NSΔRafER cells, Schwann cells infected with the retrovirus containing the luciferase gene under the control of a p53 binding site were tested for luciferase activity. NSΔRafER wtp53 luc cells were subjected to UV-irradiation or alternatively 4-hydroxy tamoxifen was added to activate Raf. Cells were harvested 20 hours later and luciferase activity was determined. In parallel to measuring luciferase activity, I examined the induction of p21$^{WAF1/Cip1}$ protein.

The addition of 4-hydroxy tamoxifen as well as UV-irradiation which should clearly activated p53 did not result in measurable luciferase activity (Figure IV.15). Nevertheless, the chosen conditions activated the induction of p21$^{WAF1/Cip1}$ (Figure IV.15) which has been previously shown to be p53-dependent. Therefore, it seems likely that the retroviral p53 reporter gene construct is not functional. Thus, the p53-dependency of p21$^{WAF1/Cip1}$ induction in response to constitutively active Raf remains to be investigated.

CHAPTER 5.0

Involvement of MAP-kinase kinase and MAP-kinase in Raf-induced growth arrest in Schwann cells

5.0   Signalling pathway for ΔRaf-induced growth arrest in Schwann cells

5.1   Introduction

Activated Ras and Raf are sufficient to induce growth arrest in Schwann cells (Ridley et al., 1988; Lloyd et al., 1997). However, the pathway mediating the growth inhibitory signal in Schwann cells downstream of Ras and Raf is unknown.

The same members of the Ras-MAP-kinase signalling pathway can be utilised to mediate a mitogenic or alternatively a growth inhibitory signal. The activation of the Ras-MAP-kinase pathway has been implicated in mitogenesis in most cells (Feramisco et al., 1984; Morris et al., 1989; Meloche et al., 1992; Cowley et al., 1994; Mansour et al., 1994). Nevertheless, the activation of the MAP-kinase pathway has also been reported to be involved in differentiation (Cowley et al., 1994) in other cells. The mediation of different signals via the same signalling pathway could occur through differences in the kinetics of activation. Such a model has been proposed for phaeocytochroma PC12 cells (Traverse et al., 1992), in which members of the Ras-MAP-kinase pathway are involved in mitogenesis as well as differentiation. In PC12 cells the different biological responses after addition of epidermal growth factor (EGF) (mitogenesis) (Huff et al., 1981) or nerve growth factor (NGF) (growth arrest and differentiation) (Greene & Tischler, 1976) have been correlated with a difference in duration of the activation of the Ras-MAP-kinase pathway. The application of NGF led to a persistent increase of GTP-loaded Ras, in contrast to a much shorter increase in response to EGF (Muroya et al., 1992). Furthermore, a transient activation of MAP-kinase was observed after the addition of mitogens such as EGF, whereas a prolonged activation could be seen after addition of NGF, correlated with growth arrest and

differentiation (Heasley & Johnson, 1992; Traverse et al., 1992; Nguyen et al., 1993). In support of this idea of a prolonged versus transient activation of the Ras-MAP-kinase pathway being required for different biological responses, the overexpression of the insulin receptor, usually not involved in MAP-kinase activation and differentiation in PC12 cells (Ohmichi et al., 1993), resulted in MAP-kinase activation and differentiation (Dikic et al., 1994). Conversely, PC12 cells with a reduced number of the NGF-receptor TrkA responded to the addition of NGF with a transient MAP-kinase activation and proliferation instead of differentiation usually observed after NGF addition (Schlessinger & Bar-Sagi, 1995). Moreover, oncogenic Ras and Raf which cause the prolonged activation of the MAP-kinase pathway (Leevers & Marshall, 1992), can also stimulate neurite outgrowth, indicative for differentiation in PC12 cells (Noda et al., 1985; Wood et al., 1993).

Given the large body of evidence for an involvement of activated Ras and Raf in growth arrest and subsequential differentiation in PC12 cells, it was conceivable that a similar mechanism of transient versus prolonged signalling of the MAP-kinase pathway was the underlying mechanism for the Ras/Raf induced cell-cycle arrest in Schwann cells.

5.2 MAP-kinase activation in ΔRaf-induced growth arrest

To investigate whether MAP-kinase was indeed activated in the ΔRaf-induced growth arrest and whether a difference in the duration of MAP-kinase activation after mitogenic stimulation or Raf activation could explain the different biological responses, MAP-kinase p42^{ERK2} activation after induction of ΔRaf or addition of mitogens was examined. NSΔRafER cells were kept in the absence of growth promoting factors for two days and then induced with foetal calf serum (FCS) plus glial growth factor and forskolin

containing medium. Addition of mitogens to quiescent NSΔRafER cells resulted in a transient activation of MAP-kinase p42^{ERK2}. The activation of MAP-kinase which strictly depends on two phosphorylations within the kinase domain (Payne et al., 1991) was indicated by the appearance of a characteristic phosphorylation-dependent mobility shift (de Vries-Smits et al., 1992; Leevers et al.,1992). MAP-kinase activation was detected as early as ten minutes after addition of mitogens and remained elevated for up to four hours after stimulation (Figure V.1A). Individual experiments varied in that activation was observed between up to two to four hours after foetal calf serum addition. This showed that after stimulation with mitogens MAP-kinase p42^{ERK2} was transiently activated. In contrast, the induction of ΔRafER in the absence of added mitogens resulted in the prolonged activation of MAP-kinase p42^{ERK2}. In quiescent cells the induction of ΔRafER led to a continuous activation of MAP-kinase from fifteen minutes until the end of the observation period 48 hours after addition of 4-hydroxy tamoxifen (Figure V.1B).

The activation of p42^{ERK2} by activated ΔRaf was dominant over activation by mitogens. When both stimuli, mitogens and 4-hydroxy tamoxifen were added simultaneously, p42^{ERK2} remained activated over 48 hours (Figure V.1C), indicating that the reported induction of MAP-kinase specific phosphatases by foetal calf serum (Charles et al., 1993; Noguchi et al., 1993) was not sufficient to down-regulate the constitutive signal from ΔRaf. As a control, NSempty cells harbouring the retroviral vector LXSN showed only a very transient activation of MAP-kinase p42^{ERK2} upon addition of 4-hydroxy tamoxifen which disappeared already after 30 minutes (Figure V.1D) and these cells do not arrest (Lloyd et al., 1997). This result confirmed that activation of MAP-kinase after addition of 4-hydroxy

Figure V.1A: Stimulation of p42^{ERK2} kinase activation by

mitogens in NSΔRafER cells

Nearly confluent NSΔRafER cells were kept in DMEM medium plus 2% fetal calf serum for two days. Cells were stimulated with DMEM containing 3% fetal calf serum as well as GGF and forskolin. Cells were lysed at times indicated and 30 µg cell lysate were separated on a 15%SDS-PAGE. After transfer onto PVDF-membrane, the western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.1B: p42^{ERK2} kinase activation in induced NSΔRafER cells

Nearly confluent NSΔRafER cells were kept in DMEM medium plus 2% fetal calf serum for two days and then 1 µM 4-hydroxy tamoxifen was added. Western blotting was carried out as described in figure V.1A.

Figure V.1C: p42^{ERK2} kinase activation after addition of

mitogens and 4-hydroxy tamoxifen

Nearly confluent NSΔRafER cells were kept in DMEM medium plus 2% fetal calf serum for two days. DMEM medium with mitogens as described in figure V.1A was added as well as 1 µM 4-hydroxy tamoxifen. Cells were harvested at indicated times after stimulation and western blotting was carried out as described in figure V.1A.

Figure V.1D: p42^{ERK2} kinase activation in induced

NSempty cells

Nearly confluent NSempty cells were kept in DMEM medium plus 2% fetal calf serum for two days. Then 1 µM 4-hydroxy tamoxifen was added. Cells were harvested and analysed as described in figure V.1A.

A.

- 46 kD

PP →
p42^ERK2 →

0 5 10 15 30 1 h 2 h 4 h 6 h 12 h 24 h 48 h

Time after mitogen stimulation

B.

- 46 kD

PP →
p42^ERK2 →

0 5 10 15 30 1 h 2 h 4 h 6 h 24 h 48 h

Time after 4-hydroxy tamoxifen addition

C.

PP →
p42^ERK2 →

0 5 10 15 30 1 h 2 h 4 h 6 h 24 h 48 h

Time after 4-hydroxy tamoxifen addition

D.

PP →
p42^ERK2 →

0 5 10 15 30 1 h 2 h 4 h 6 h 12 h 24 h

Time after 4-hydroxy tamoxifen addition
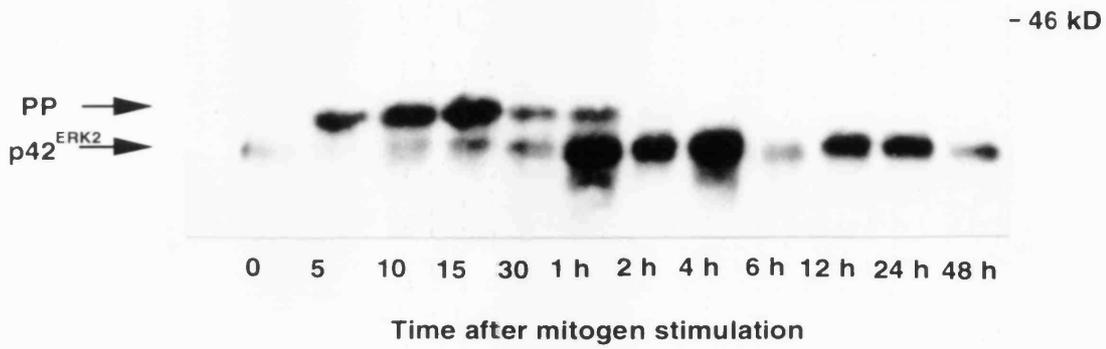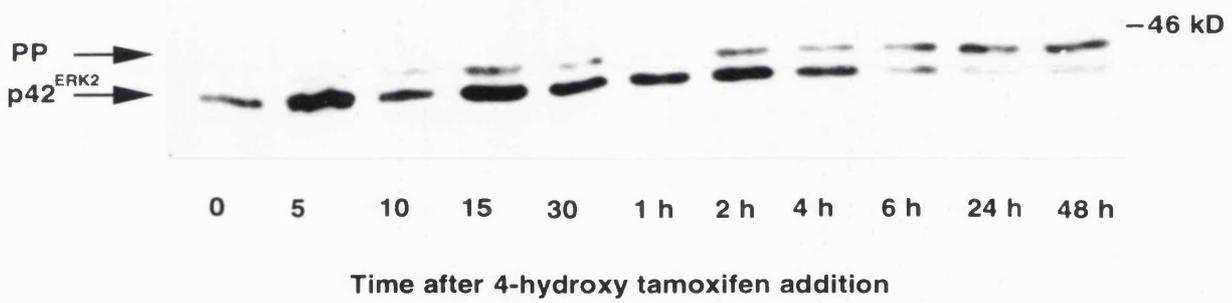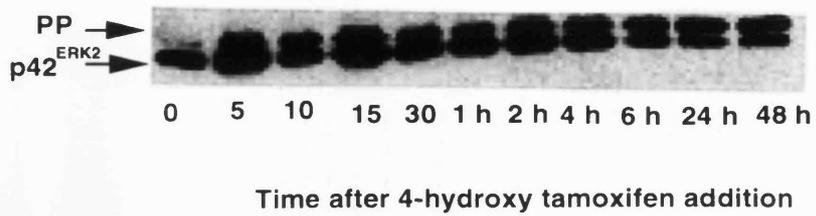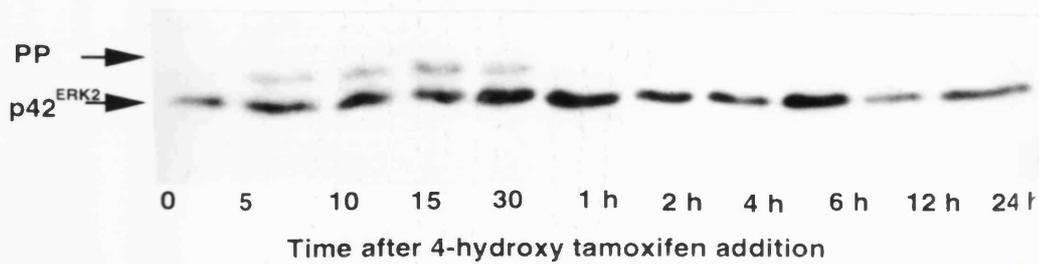
tamoxifen was specifically due to ΔRafER induction and not due to any unspecific activation of the MAP-kinase pathway.

The described kinetics of MAP-kinase activation after addition of mitogens or ΔRaf induction were congruent with a model in which the duration of the activation of the MAP-kinase pathway would determine the biological response to this signal in Schwann cells. As suggested for PC12 cells (Traverse et al., 1992), such a model for Schwann cells would mean that the prolonged activation of the MAP-kinase pathway by activated Raf would lead to growth arrest, regardless of surrounding growth conditions, whereas a transient activation would correlate with mitogenesis. In accordance with this hypothesis, cells with activated Raf plus added mitogens exhibited a prolonged activation of MAP-kinase and a G1 cell cycle arrest (Figure V.1C). Although it was plausible to explain the observed differences in MAP-kinase activation in Schwann cells through this described model, the presented data did not exclude other explanations.

5.2.1 MAP-kinase activation in NSΔRafER SV40 LT cells

Co-expression of SV40 large T antigen (SV40 LT) enabled Ras/Raf-arrested Schwann cells to overcome the cell cycle arrest (Ridley et al., 1988; Lloyd et al., 1997). Although it seemed likely that SV40LT rescued the cell-cycle arrest through its function to sequester p53 which then in turn would abolish p53-dependent p21$^{WAF1/Cip1}$ induction, I also sought to exclude the possibility that SV40LT had a direct or indirect effect on the prolonged activation of the MAP-kinase pathway upstream. An influence on the MAP-kinase pathway has been reported for SV40 small T antigen which inhibits phosphatases 2A required for the inactivation of the Ras-MAP-kinase pathway (Sontag et al.,1993).

Figure V.2A: p42^{ERK2} kinase activation in NSΔRafER LT cells

Nearly confluent NSΔRafER LT cells were kept in DMEM plus 2%FCS for two days. 1 µM 4-hydroxy tamoxifen was added at point 0 and cells were harvested at indicated times after stimulation. 30 µg cell lysate were separated on a 15%SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase and detected using the ECL-system.

Figure V.2B: Expression of SV40 LT antigen

50 µg NSΔRafER LT protein cell lysate used in the experiment above was analysed for SV40 large T expression with the monoclonal antibody 423 supernatant (0.5 µg /ml) and 1:2000 secondary antibody against mouse IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.2C: p42^{ERK2} kinase activation In mitogenically
stimulated NSΔRafER LT cells

Nearly confluent NSΔRafER LT cells were changed into 2% fetal calf serum in DMEM two days prior stimulation with 3% fetal calf serum in DMEM with GGF and forskolin. Cells were harvested at indicated times after stimulation. 30 µg cell lysate were separated on a 15%SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham International).

Figure V.2D: Expression of SV40 LT antigen

The expression of SV40 LT antigen in NSΔRafER LT cells used in the experiment figure V.3C were analysed as described for figure V.3B.

A.

PP →

p42^ERK2 →

| 0 | 5 | 10 | 15 | 30 | 1 h | 2 h | 4 h | 6 h | 12 h | 24 h | 48 h |

time after 4-hydroxy tamoxifen addition

B.

– 97.4 kD

| 0 | 5 | 10 | 15 | 30 | 1 h | 2 h | 4 h | 6 h | 12 h | 24 h | 48 h |

C.

← PP

← p42^ERK2

| 0 | 5 | 10 | 15 | 30 | 1 h | 2 h | 4 h | 6 h | 12 h | 24 h | 48 h |

time after mitogen addition

D.

– 97.4 kD

| 0 | 5 | 10 | 15 | 30 | 1 h | 2 h | 4 h | 6 h | 12 h | 24 h | 48 h |
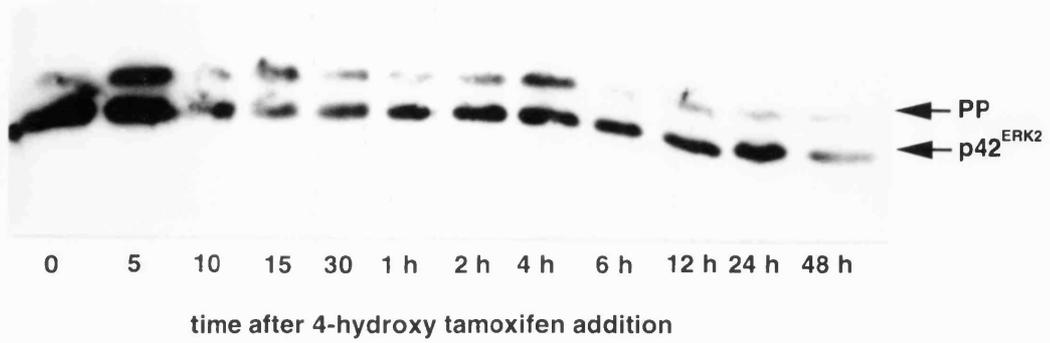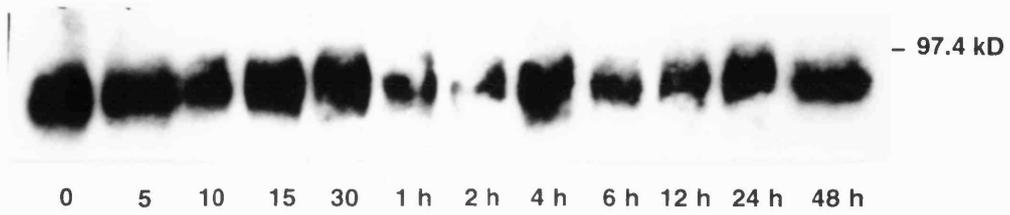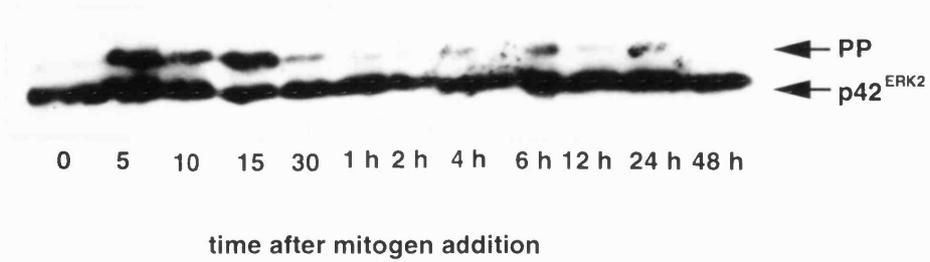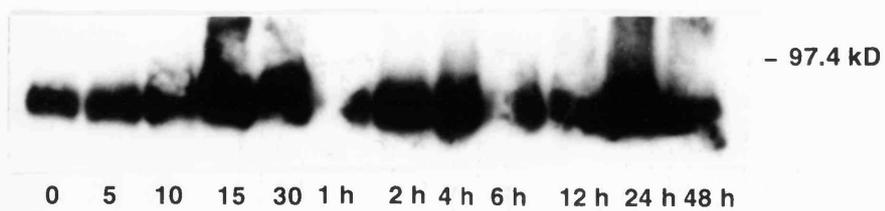
To investigate a possible interaction between SV40LT and the activation of Raf, MAP-kinase kinase or MAP-kinase, the kinetics of MAP-kinase activation after addition of mitogens or 4-hydroxy tamoxifen were analysed in NSΔRafER cells co-expressing SV40LT. The activation of ΔRafER in cells co-expressing large T-antigen (NSΔRafERLT) led also to the prolonged activation of MAP-kinase p42^{ERK2} with identical kinetics to those seen in NSΔRafER cells, independent of whether cells were quiesced or stimulated with mitogens (Figure V.2A & 2C). Therefore, the expression of SV40 LT has no effect on the activation of MAP-kinase p42^{ERK2}.

5.3 Dominant negative MAP-kinase kinase in the Raf-induced cell cycle arrest

5.3.1 Introduction

The previously described studies of MAP-kinase activation suggested that MAP-kinase may be a mediator of the ΔRaf-mediated growth arrest. Therefore, I examined whether MAP-kinase activity was indeed causally involved in the ΔRaf-induced growth arrest. If MAP-kinase activity was instrumental in establishing the cell-cycle arrest, then blocking of MAP-kinase activity should abolish or at least weaken the G1 cell cycle arrest mediated by activated Raf.

Inhibition of MAP-kinase activity could be achieved through either expression of a kinase-deficient MAP-kinase ERK2 protein which has a dominant negative function (Sally Leevers, personal communication) or through the expression of dominant negative mutants of the direct upstream activator MAP-kinase kinase (Cowley et al., 1994). In particular, the expression of dominant negative mutants of MAP-kinase kinase appeared to be promising for my investigations, since experiments in PC12 cells and

NIH3T3 cells did confirmed their potential of inhibiting biological responses mediated via the Ras-MAP-kinase pathway (Cowley et al., 1994).

5.3.2   Dominant negative MAP-kinase kinase mutants
in NSΔRafER cells

To address whether dominant negative mutants of MAP-kinase kinase were capable of inhibiting activation of MAP-kinase after ΔRaf activation, I subcloned wild-type MAP-kinase kinase (MKK1), MAP-kinase kinase mutant MKK1 LIDA (Ala-217) and MANA (Ala-221) into the retroviral vector pBabe Puro (Figure V.3A). Dominant negative mutants of MKK1, LIDA (Ala-217) and MANA (Ala-221) have point mutations in either one of the two phosphorylation site required for MAP-kinase kinase activity (Alessi et al., 1994).

NSΔRafER cells were infected with the described constructs and drug-selected, leading to pools of NSΔRafER MKK, NSΔRafER LIDA (Ala-217) and NSΔRafER MANA (Ala-221) cells. Initially, these cells were analysed for the expression of the exogenous MAP-kinase kinase proteins (Figure V.3B) and for their potential to block MAP-kinase activity after ΔRafER induction. In order to examine whether dominant negative mutants were sufficient to abrogate MAP-kinase activity after ΔRafER induction, cells were quiesced for two days and ΔRaf was induced. The co-expression of dominant negative mutants of MAP-kinase kinase was not sufficient to reduce the kinase activity of MAP-kinase compared to control cells (Figure V.3C).

In parallel, I examined whether the expression of dominant negative mutants of MAP-kinase kinase was sufficient to abolish the Raf-induced cell cycle arrest using FACS-analysis. As illustrated in Figure V.4A-D, the co-expression of dominant negative MAP-kinase kinase in NSΔRafER cells

Figure V.3A: Schematic representation of MAP-kinase kinase mutants

Figure V.3B: Expression of MAP-kinase kinase mutants in NS∆RafER cells

NS∆RafER Puro, NS∆RafER MKK, NS∆RafER MANA and NS∆RafER LIDA cells were examined for protein expression of the introduced MAP-kinase kinase mutants. 100 µg cell lysate were separated on a 12.5% SDS-PAGE gel and western blotted. Immunoreactive proteins with the antibody 9E10 in cells either quiesced (-), after addition of mitogens (+M) or after the addition of 4-hydroxy tamoxifen (+ TM) are indicated by an arrow.

Figure V.3C: MAP-kinase activity in NS∆RafER cells co-expressing MKK-mutants

NS∆RafER cells co-expressing MAP-kinase kinase mutants were quiesced prior the addition of 4-hydroxy tamoxifen. Cells were harvested 12 hours after addition of 4-hydroxy tamoxifen and 150 µg of cell lysates were used for an immunoprecipitation of the p42^{ERK2} specific antibody 122, followed by an *in-vitro* kinase assay with mylein basic protein as a substrate (MBP). 10 µl of the kinase assay were separated on a 15% SDS-PAGE gel.

A.

wt MKK

^{214}LIDSMANS^{221}

1   394

VII   9E10

MKK LIDA (Ala-217)

^{214}LIDAMANS^{221}

1   394

VII   9E10

MKK MANA (Ala-221)

^{214}LIDSMANA^{221}

1   394

VII   9E10

B.

   -   +M +TM   -   +M +TM   -   +M +TM   -   +M +TM

NSΔRafER Puro   NSΔRafER MKK   NSΔRafER MANA   NSΔRafER LIDA

C.

← MBP

NSΔRafER Puro
NSΔRafER MKK
NSΔRafER MANA
NSΔRafER LIDA
NSΔRafER Puro
NSΔRafER MKK
NSΔRafER MANA
NSΔRafER LIDA
NSΔRafER Puro
+ peptide

+ TM   -TM   + TM
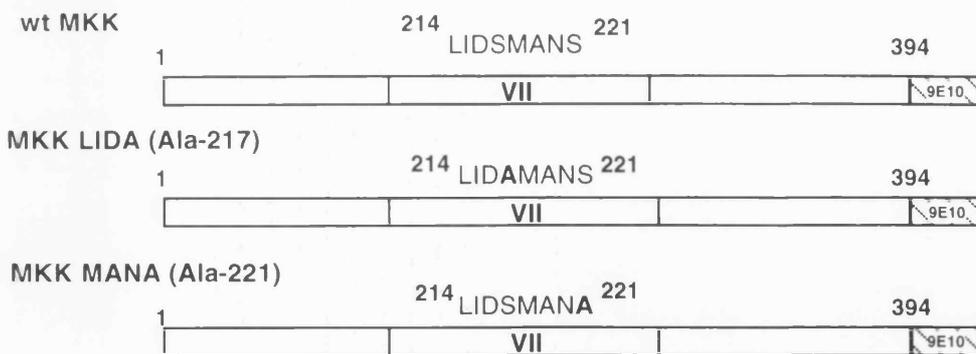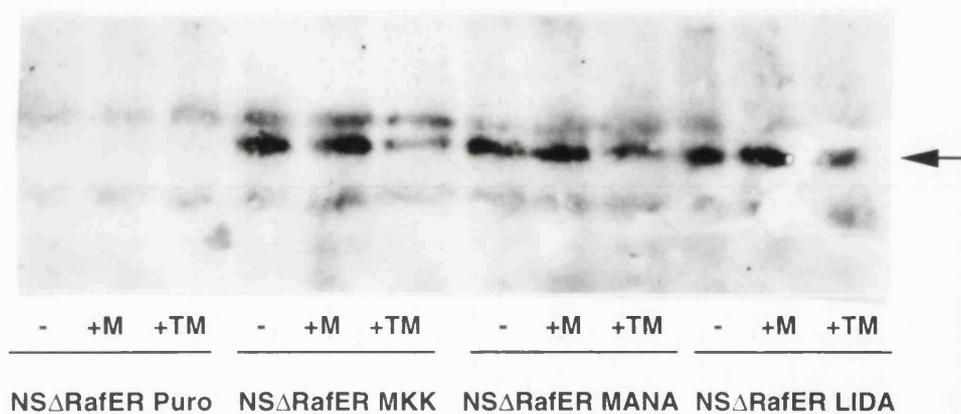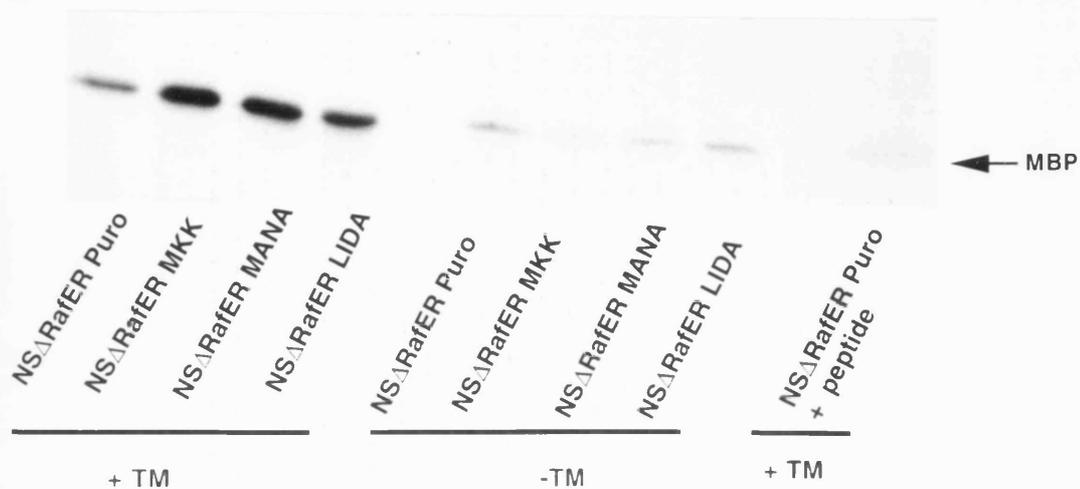
Figure V.4A-D: FACS-analysis of NSΔRafER cells expressing

MAP-kinase kinase mutants

NSΔRafER cells co-expressing mutants of MAP-kinase ERK2, ERK2 (A52) and ERK2 (AF) were assynchrously grown. 1 μM 4-hydroxy tamoxifen or ethanol as control were added. BrdU (10 μM) was added 26 hours after 4-hydroxy tamoxifen addition for 4 hours. The cell cycle distribution of these cells were measured by BrdU incorporation and DNA-content flow cytometry analysis. Cells were stained with FITC-anti-BrdU to detect BrdU incorporation and propidium iodide to detect total DNA. The upper box identifies cells incorporating BrdU (S-phase), the lower left box identifies G0/G1 cells and the lower right box displays G2/M cells.

A.

NSΔRafER Puro, - TM

Anti-BrdU FITC / Propidium Iodide

G1: 40.03 %
S: 43.43 %
G2/M: 12.22 %

NSΔRafER Puro, + TM

Anti-BrdU FITC / Propidium Iodide

G1: 62.16 %
S: 11.89 %
G2/M: 17.78 %

B.

NSΔRafER MKK, - TM

Anti-BrdU FITC / Propidium Iodide

G1: 38.16 %
S: 44.59 %
G2/M: 8.57 %

NSΔRafER MKK, + TM

Anti-BrdU FITC / Propidium Iodide

G1: 76.06 %
S: 7.22 %
G2/M: 10.19 %

C.

NSΔRafER LIDA (Ala-217), - TM

Anti-BrdU FITC / Propidium Iodide

G1: 48.51 %
S: 25.38 %
G2/M: 17.81 %

NSΔRafER LIDA (Ala-217), + TM

Anti-BrdU FITC / Propidium Iodide

G1: 57.67 %
S: 22.99 %
G2/M: 14.81 %

D.

NSΔRafER MANA (Ala-221), - TM

Anti-BrdU FITC / Propidium Iodide

G1: 47.20 %
S: 41.71 %
G2/M: 3.86 %

NSΔRafER MANA (Ala-221), + TM

Anti-BrdU FITC / Propidium Iodide

G1: 70.58 %
S: 14.93 %
G2/M: 7.48 %
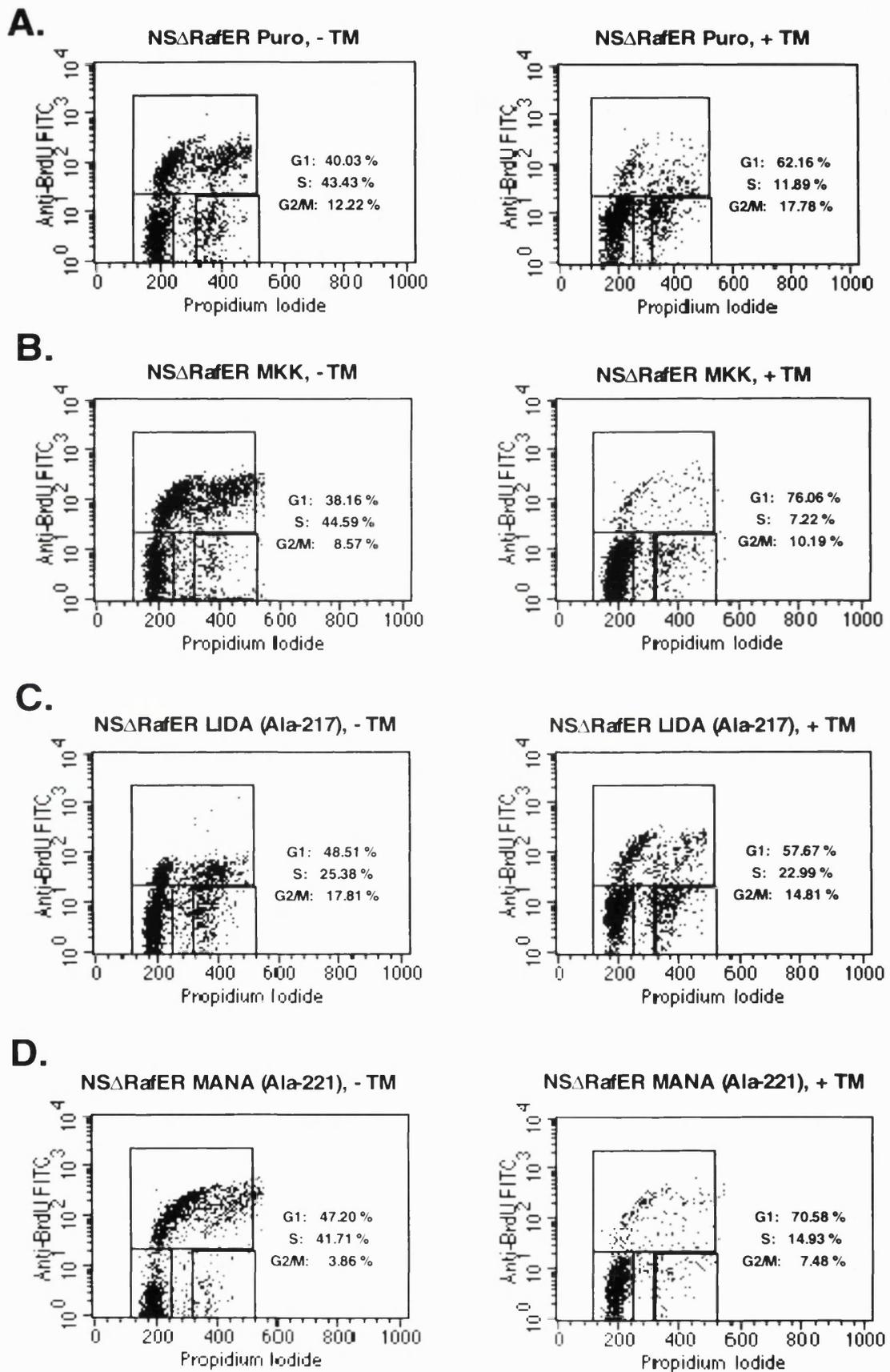
was not sufficient to abolish the Raf-induced G1 cell cycle arrest. Most cells expressing mutants showed a G1 cell cycle arrest upon addition of 4-hydroxy tamoxifen very similar to control cells expressing either wild-type MAP-kinase kinase or the vector alone. However, NSΔRafER cells expressing MAP-kinase kinase LIDA (Ala-217) showed only a slight reduction of cells in S-phase, although the number of S-phase cells in the absence of 4-hydroxy tamoxifen is only half of all other cells examined. This result may be explained by the effect of dominant negative MAP-kinase kinase in both mitogenesis and in blocking the growth arrest. In both situations, dominant negative MAP-kinase kinase negatively influence endogenous MAP-kinase kinase activity which could lead to a reduction of cells in S-phase in mitogenesis, whereas the inhibition of ΔRafER-induced G1 arrest will result in a higher proportion of cells remaining in S-phase. This result pointed to the possible involvement of MAP-kinase kinase in mediating the cell cycle arrest in response to activated Raf.

5.3.3 Dominant negative MAP-kinase mutants in NSΔRafER ceils

In addition to experiments with dominant negative mutants of MAP-kinase kinase, similar mutants of MAP-kinase were used to investigate the contribution of MAP-kinase in the Raf-induced cell cycle arrest. To address whether dominant negative mutants of MAP-kinase ERK2 were capable of inhibiting the ΔRaf-induced cell cycle arrest, I subcloned wild-type ERK2 and two mutants ERK2 (A52) and ERK2 (AF) into pBabe Puro. Mutant ERK2 (A52) exhibits no kinase activity due to a point mutation in the ATP-binding site of ERK2, whereas in mutant ERK2 (AF) both phosphorylation sites in the catalytic domain required for MAP-kinase activity are exchanged (Sally Leevers, personal communication).

Figure V.5A-C: FACS-analysis of cells expressing MAP-kinase mutants

NSΔRafER cells co-expressing mutants of MAP-kinase ERK2, ERK2 (A52) and ERK2 (AF) were assynchrously grown. 1 μM 4-hydroxy tamoxifen or ethanol as control were added. BrdU (10 μM) was added 26 hours after 4-hydroxy tamoxifen addition for 4 hours. The cell cycle distribution of NSΔRafER cellswas measured by BrdU incorporation and DNA-content flow cytometry analysis. Cells were stained with FITC-anti-BrdU to detect BrdU incorporation and propidium iodide to detect total DNA. The upper box identifies cells incorporating BrdU (S-phase), the lower left box identifies G0/G1 cells and the lower right box displays G2/M cells.

A.

NSΔRafER ERK2, - TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 36.32 %
S: 44.00 %
G2/M: 12.59 %

NSΔRafER ERK2, + TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 65.51 %
S: 11.28 %
G2/M: 18.71%

B.

NSΔRafER ERK2 (A52), - TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 38.80 %
S: 52.33 %
G2/M: 3.76 %

NSΔRafER ERK2 (A52), + TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 80.59 %
S: 5.64 %
G2/M: 9.26 %

C.

NSΔRafER ERK2 (AF), - TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 40.40 %
S: 44.79 %
G2/M: 7.25 %

NSΔRafER ERK2 (AF), + TM

- Y-axis: Anti-BrdU FITC (10^0 to 10^4)
- X-axis: Propidium Iodide (0 to 1000)

G1: 68.53 %
S: 19.56 %
G2/M: 6.67 %
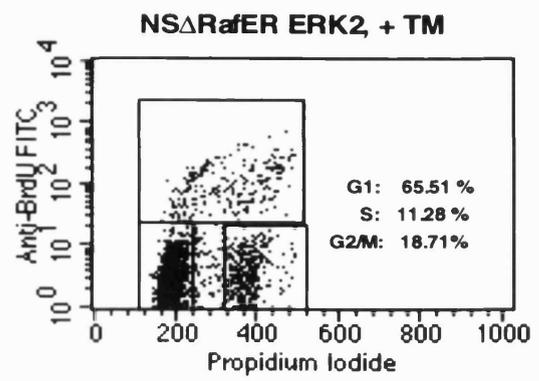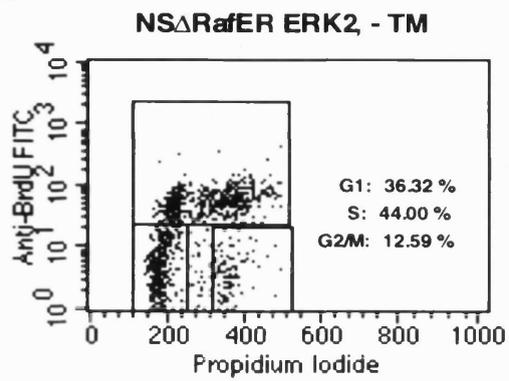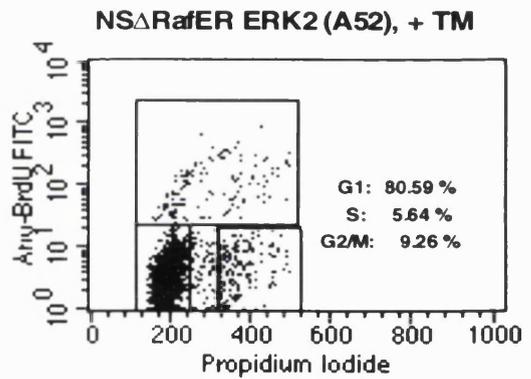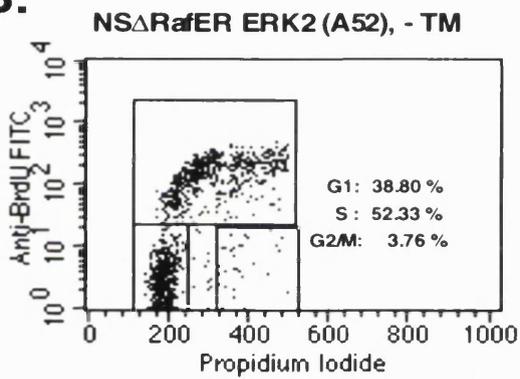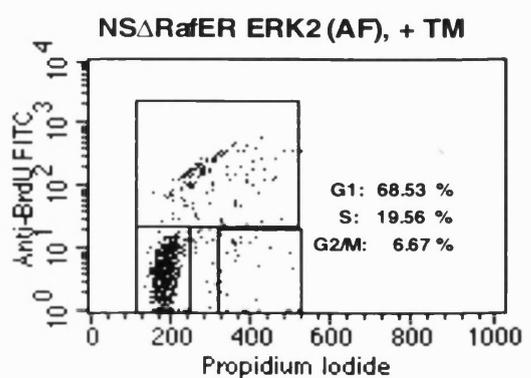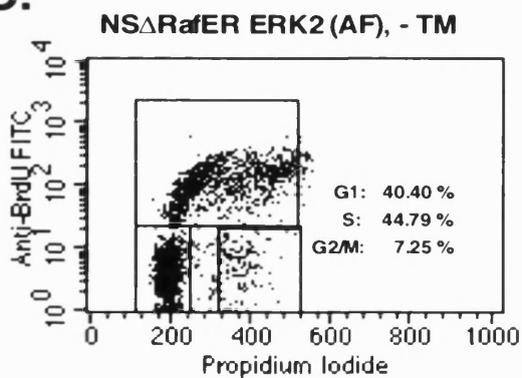
NSΔRafER cells were infected with the constructs described and drug-selected, leading to pools of NSΔRafER Puro, NSΔRafER ERK2, NSΔRafER ERK2 (A52), NSΔRafER ERK2 (AF) cells. I examined whether the expression of either of the dominant negative MAP-kinase mutants was sufficient to abolish the ΔRaf induced cell-cycle arrest using FACS-analysis. The coexpression of dominant negative MAP-kinase was not sufficient to abolish the Raf-induced G1 cell cycle arrest as illustrated in Figure V.5A-D. The mutant MAP-kinase expressing cells showed a G1 cell cycle arrest upon addition of 4-hydroxy tamoxifen very similar to control cells expressing wild-type MAP-kinase or the vector alone. However, the co-expression of ERK2 (AF) leaves more cells in S-phase after addition of 4-hydroxy tamoxifen (Figure V.5C) than in induced control cells (Figure V.5A) or ERK2 (A52) mutant cells (Figure V.5B), suggesting that MAP-kinase inhibition of this pathway by ERK2 (AF) is only partial and may be required for the growth inhibitory signalling pathway downstream of activated Raf.

5.3.4   Conclusions

The co-expression of the dominant negative MAP-kinase kinase mutant LIDA resulted in a reduction of cells in G1 phase with a higher number of cells remaining in S-phase in response to constitutive Raf-activity (see Figure V.4). This finding indicates the possibility that MAP-kinase kinase activity could be required for the mediation of the activated Raf-dependent cell cycle arrest. Interestingly, the same mutant reduces the number of cells in S-phase in the absence of constitutive Raf-activity. Constitutively active MAP-kinase kinase activity has been shown to be necessary and sufficient to induce S-phase in some cells (Cowley et al.,

1995). In line with these findings, a dominant negative mutant could negatively effect S-phase entry in Schwann cells.

The co-expression of dominant negative MAP-kinase molecules in NSΔRafER cells was not sufficient to inhibit MAP-kinase activity and subsequently the ΔRaf-induced cell cycle arrest. However, it can not be excluded that the protein expression achievable from a integrated retroviral constructs was too low to inhibit the MAP-kinase pathway. Since dominant negative mutants are understood to exhibit their inhibitory effects through competition with the wild-type molecule for the upstream activator. The possible low expression of these dominant negative mutants could have allowed the activation of the wild type molecule.

In this respect, it is also worth mentioning that the abrogation of biological effects by dominant negative MAP-kinase kinase mutants was achieved through either microinjection of protein into PC12 cells or injection of plasmid DNA into NIH3T3 cells (Cowley et al., 1994). Both methods will provide most certainly a higher protein concentration than achievable by the expression of a gene from an integrated retrovirus. Microinjections of either plasmid DNA or protein into Schwann cells have proven unsuccessful. Thus, it is not absolutely clear whether the lack of inhibition is due a possible low protein expression or reflects the fact that MAP-kinase is not involved in mediating the Raf-induced cell cycle arrest.

5.4 MAP-kinase kinase inhibitor PD98059 blocks p21$^{WAF1/Cip1}$ induction in Schwann cells

5.4.1 Introduction

The results utilising dominant negative mutants of MAP-kinase kinase or MAP-kinase were inconclusive due to the lack of strong inhibition by

these molecules in Schwann cells. Therefore, I tried to find alternative ways to block the MAP-kinase signalling pathway downstream of Raf in order to address the significance of this pathway in mediating the cell cycle arrest.

The recent discovery of a synthetic inhibitor for MAP-kinase kinases (MAPKKs) (Dudley et al., 1995) offered an independent approach to the usage of dominant negative MAP-kinase kinase or MAP-kinase molecules in assessing the contribution of MAP-kinase kinase and MAP-kinase in mediating the growth inhibitory signal in Schwann cells. The synthetic inhibitor for MAP-kinase kinase was isolated by testing a compound library for inhibitory substances in a coupled MAP-kinase cascade assay. The compound, PD98059 [2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one] (Figure V.6A) was identified as a specific inhibitor of MAP-kinase kinase 1 and 2 (Dudley et al., 1995). The compound PD98059 inhibits the activation of the unphoshorylated form of MAP-kinase kinase 1 & 2 (MAPKK1/MAPKK2) but was shown to be ineffective in inhibiting already phosphorylated and activated MAP-kinase kinase molecules (Dudley et al., 1995). PD98059 inhibits MAPKK2 at higher concentrations than MAPKK1. Additionally, PD98059 showed an inhibitory effect on a constitutively active mutant of MAPKK1(S217E, S221E) (Alessi et al., 1995; Cowely et al., 1994).

PD98059 has a very restricted specificity towards MAPKK1 and MAPKK2. Extensive studies were unable to reveal any inhibitory effects on a large number of highly related kinases over a wider range of concentrations *in vitro* and *in vivo*. Among other kinases, the activity of 18 protein Ser/Thr kinases, p70^{S6}-kinase, Jun N-terminal kinase (JNK), p38 kinase (RK), MAP-kinase kinase 4 (MKK4), MAP-kinase, MAPKAP kinase-1, Protein kinase A, Cyclin A/cdk2 (Alessi et al., 1995) and cAMP-dependent kinase, protein kinase C, v-src kinase, EGF-kinase, phosphatidylinositol-3-

kinase were unaffected by the inhibitor (Dudley et al., 1995). Most importantly for my investigations, PD98059 did not prevent the activation of c-Raf (Dudley et al., 1995) by such different stimuli as platelet-derived growth factor (PDGF), serum, insulin and phorbol esters in SW3T3 cells (Alessi et al., 1995).

The inhibition of MAP-kinase kinase 1 in *in vitro* assays has been confirmed by the inhibition of biological responses initiated by the Ras-MAP-kinase signalling pathway *in vivo*. For instance, PD98059 indirectly inhibits the activation of MAP-kinase, a direct downstream target of MAPKKs by growth factors *in vivo*. Consistent with the function of MAP-kinase in mediating mitogenic signals and differentiation, PC98059 is capable of blocking both processes. For example, PD98059 is capable of reversible inhibition of Ras-induced transformation in BALB 3T3 mouse fibroblasts and NRK rat kidney cells (Dudley et al., 1995). PD98059 can also inhibit nerve growth factor (NGF)-induced MAP-kinase activity and differentiation in phaeocytochroma cells (PC12) (Pang et al., 1995). These reports convincingly show that the inhibitory effect of PD98059 towards MAPKK1 & 2 *in vitro* can be reproduced *in vivo*.

5.4.2   MAP-kinase kinase inhibitor PD98059 in NSΔRafER cells

The inhibitor PD98059 with its well-characterised features in different cells (Dudley et al., 1995; Pang et al., 1995; Alessi et al., 1995, Lazar et al., 1995) offered an experimental tool for investigations into the involvement of MAP-kinase kinase in the Raf-induced growth arrest. The fact that PD98059 is not inhibiting c-Raf itself (Dudley et al., 1995), made PD98059 an interesting tool for my investigations into the MAP-kinase signalling pathway downstream of Raf. If MAP-kinase kinase activity was involved in the

ΔRafER induced growth arrest due to the induction of p21$^{WAF1/Cip1}$, then the application of PD98059 should abolish or at least delay the induction of p21$^{WAF1/Cip1}$ protein and the onset of the G1 cell cycle arrest. As a consequence, Schwann cells may enter into S-phase in the presence of PD98059.

5.4.3   Inhibitory effects of PD98059 in NSΔRafER cells

Initially I determined the inhibitory concentration of PD98059 for MAP-kinase kinase 1 & 2 activity after 4-hydroxy tamoxifen addition in a coupled assay using MAP-kinase and myelein basic protein as a substrate in Schwann cells. The inhibitory effects of PD98059 on MAPKK 1 & 2 were measured indirectly by monitoring the activity of MAP-kinase.

The inhibitor PD98059 can only act on unphosphorylated inactive MAPKK 1 & 2. Therefore, Schwann cells were kept in DMEM plus 2%FCS for two days to exclude any stimulation by growth factors and allow maximal inhibition. Prior to ΔRafER activation, various concentrations of PD98059 or as a negative control the solvent DMSO were added. Cells were harvested at indicated times and MAPKK1 & 2 activity was measured. Maximal inhibition of MAP-kinase kinase 1 & 2 activities were achieved at approximately 1.0 µM PD98059 (Figure V.6 B & C). However, the kinase activity of the direct downstream target MAP-kinase was completely inhibited at a higher concentration of PD98059 as indicated by the absence of the characteristic phosphorylation shift on the western blot (Figure V.7) paralleled by the lack of kinase activity (Figure V.7 B & C). This higher inhibitory concentration of 50 µM was used for all following experiments to ensure the blocking of MAP-kinase kinase and MAP-kinase activity. The inhibitor PD98059 showed no visible toxicity on NSΔRafER cells at any concentration tested.

Figure V.6A: Chemical structure of inhibitor PD98059

[2-(2´-amino-3´-methoxyphenyl)-oxanaphthalen-4-one]

Figure V.6B: PD98059 inhibits MAP-kinase kinase 1 and 2 in a dose-dependent manner

NSΔRafER cells were quiesced for two days in DMEM supplemented with 2% FCS. 30 minutes prior addition of 4-hydroxytamoxifen, the indicated concentration of PD98059 was added. Cells were harvested three hours after ΔRafER induction. 100 µg total cell lysate were used in a immunoprecipitation with antibodies against MKK1 and MKK2. Immunoprecipitates were used in a coupled assay with MAP kinase measuring kinase activity as radioactive phosphate incorporation into mylein basic protein as described (see Methods).

Figure V.6C: Quantification of MAP kinase kinase 1 & 2 inhibition

Incorporated $^{32}\gamma$ phosphate into mylein basic protein was quantified using ImageQuant-software (Molecular Diagnostics Ltd.). The inhibition of MAP-kinase kinase 1 & 2 is shown as percentage of inhibition plotted against concentration.

A.

B.

C.

Inhibition of MKK1/2 kinase activity

100%

75%

50%

25%

0

0   0.1   1.0   10   25   50

PD98059 (μM)

0   0.1   1.0   10   25   50   beads

PD98059 (μM)

NH$_2$

OCH$_3$
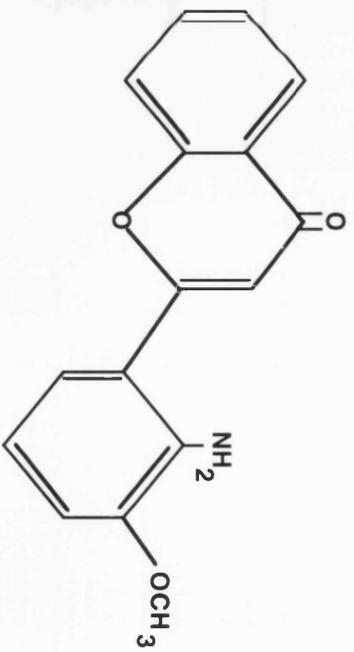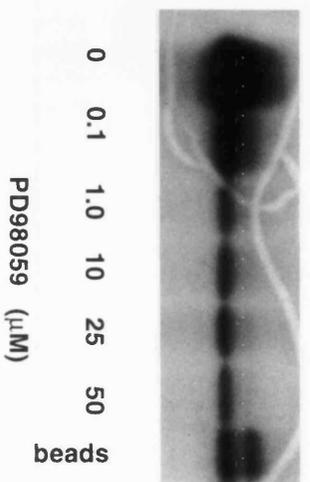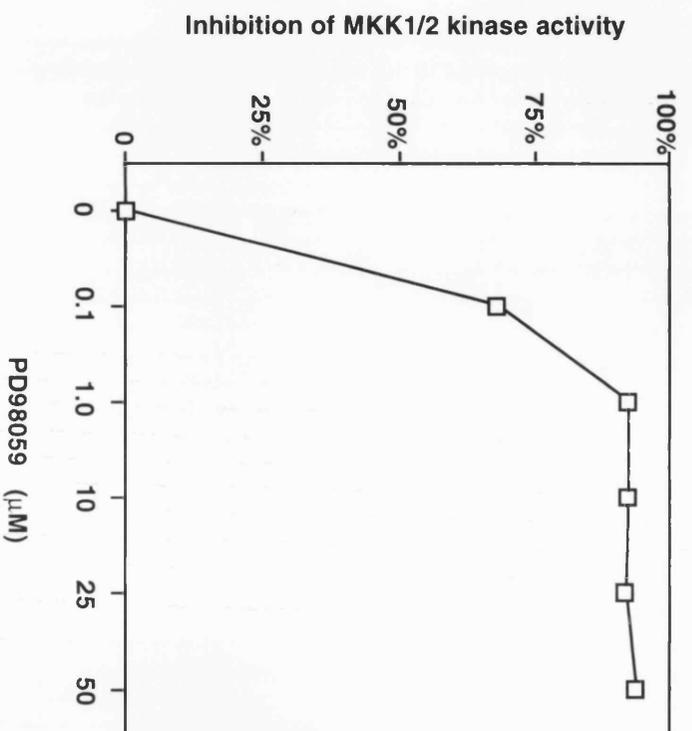
Figure V.7A: PD98059 blocks phosphorylation of p42^{ERK2} in a dose-dependent manner

Nearly confluent NSΔRafER cells were washed twice with pre-warmed PBSA and changed into 2% stripped fetal calf serum in DMEM without any growth promoting factors two days before the experiment. Indicated concentrations of PD98059 in DMSO were added 30 min prior induction of ΔRafER with 1 µM 4-hydroxy-tamoxifen. As a control the highest volume of DMSO used was added at point 0. Cells were harvested three hours after induction and 30 µg total protein were separated on a SDS-PAGE. The western blot was incubated with a 1:15000 dilution of Ab122, 1:2000 secondary antibody against rabbit IgGs coupled to horse raddish peroxidase. Immunoreactive bands were visualised using the ECL-system (Amersham Life Science).

Figure V.7B: PD98059 inhibits p42^{ERK2} kinase activity after ΔRafER induction

p42^{ERK2} was immunoprecipitated with 5 µl Ab 122 from 100 µg total protein of the same cell lysates used for the western blot above. Immunopreciptitation and the following *in-vitro* kinase assay were carried out as described (see Methods). 10 µl of the *in-vitro* kinase assay were separated on a 15% minigel and subject to autoradiography.

Figure V.7C: Quantification of p42^{ERK2} kinase activity

γ^{32}P-incorporation into myelin basic protein (MBP) from figure V.8B was quantified using Molecular Dynamics phosphoimager and ImageQuant software. Arbitary units are expressed as percentage inhibition of positive control and plotted against concentration of PD98059.

A.

p42 ERK2→   PP→

0   0.1   1.0   10   25   50

PD98059 (μM)

B.

0   0.1   1.0   10   25   50   peptide   beads

PD98059 (μM)

C.

Inhibition of p42 ERK2 kinase activity

100%
75%
50%
25%
0%

0   0.1   1.0   10   25   50
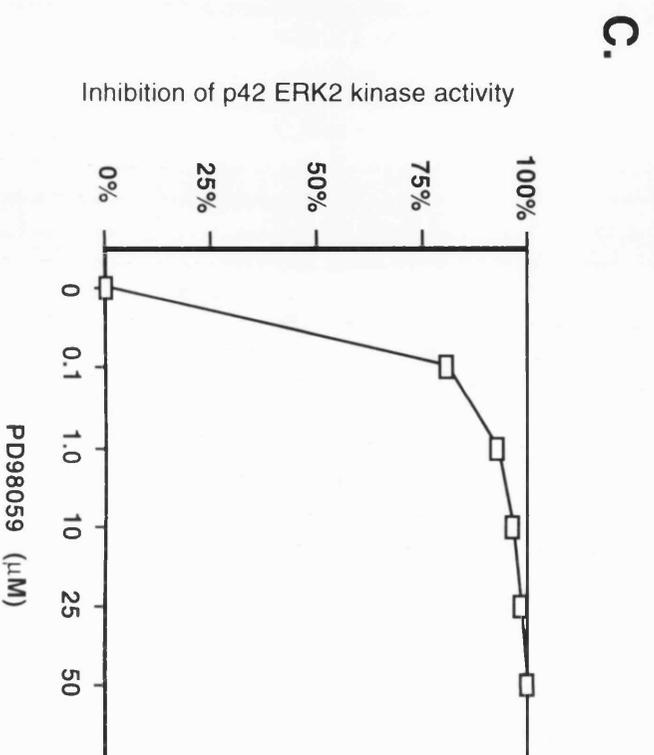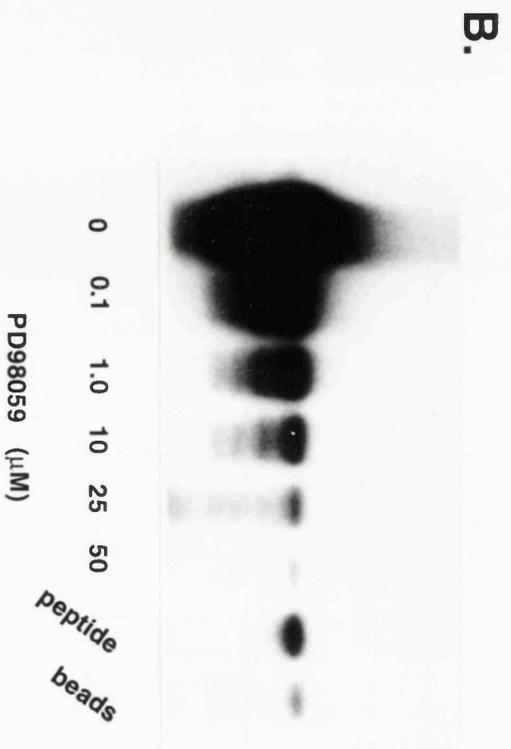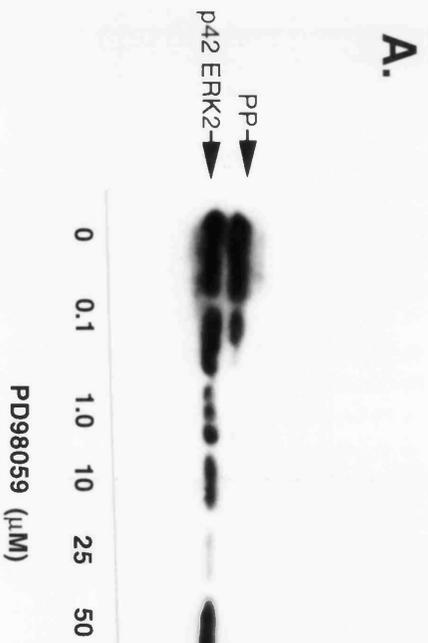
PD98059 (μM)

5.4.4 PD98059 delays p21$^{WAF1/Cip1}$ induction in NSΔRafER cells

The induction of ΔRafER caused a strong induction of the cell-cycle inhibitor p21$^{WAF1/Cip1}$ (Figure IV.10). Therefore, I was interested in investigating whether MAP-kinase kinase 1 & 2 are required for mediating the induction of p21$^{WAF1/Cip1}$. If MAP-kinase kinase 1 & 2 are mediating the signal towards p21$^{WAF1/Cip1}$ induction, the inhibition of MAPKK 1 & 2 by PD98059 should abolish or at least reduce the induction of p21$^{WAF1/Cip1}$.

To address these questions experimentally, NSΔRafER cells were quiesced for two days. Prior to the addition of mitogens and induction of ΔRafER, cells were treated with or without PD98059. A time-course over 48 hours was performed with cells harvested at indicated times. MAP-kinase p42^{ERK2} activation as characterised by the appearance of a phosphorylation shift was determined (Figure V.8). The same cell lysates were used to determine p21$^{WAF1/Cip1}$ induction in the presence and absence of PD98059 (Figure V.9).

Interestingly, p21$^{WAF1/Cip1}$ induction was strongly reduced at 24 hours in the presence of PD98059 arguing that MAP-kinase kinase was involved in mediating the growth inhibitory signal (Figure V.9). Correspondingly, at the same time the activation of MAP-kinase p42^{ERK2} was inhibited by PD98059, although the inhibition appeared less effective over a longer time period than 4 hours after Raf induction (Figure V.9). However, at 48 hours after induction no inhibitory effect on MAP-kinase activation nor p21$^{WAF1/Cip1}$ protein levels by PD98059 could be observed. This decrease of inhibition could be explained for instance through a gradual degradation of the inhibitor or an increased strength of the initiated signal from ΔRafER which has been previously reported (Samuels et al., 1994).

Figure V.8: Long-term effect of PD98059 on p42^{ERK2} activation in NSΔRafER cells

NSΔRafER cells were quiesced for two days. 30 minutes prior induction with 1 μM 4-hydroxy tamoxifen, 50 μM PD98059 or equal volumes of DMSO as control were added. Cells were harvested at indicated times after 4-hydroxy tamoxifen addition. 30 μg total cell protein were separated on SDS-PAGE, blotted and incubated with antibody 122. Immunoreactive bands were visualised with the ECL-system (Amersham International).

Figure V.9: PD98059 delays induction of p21$^{WAF1/CIP1}$ in NSΔRafER cells

30 μg total protein from each sample of the experiment described above were separated on a 15% SDS-PAGE, blotted and incubated with antibody CP36 against p21$^{WAF1/CIP1}$. Immunoreactive bands were visualised with the ECL-system (Amersham International). As a positive control NSΔRafER Puro cell lysate harvested 26 hours after 4-hydroxy tamoxifen induction was used.

PP ⟶

p42 ERK2 ⟶

—46 kD

—30 kD

PD98059   0   —   +   —   +   —   +   —   +

1 h   4 h   24 h   48 h

Time after 4-hydroxy tamoxifen addition

—30 kD

—21.5 kD

—14.3 kD

PD98059   0   —   +   —   +   —   +   —   +   positive control

1 h   4 h   24 h   48 h

Time after 4-hydroxy tamoxifen addition
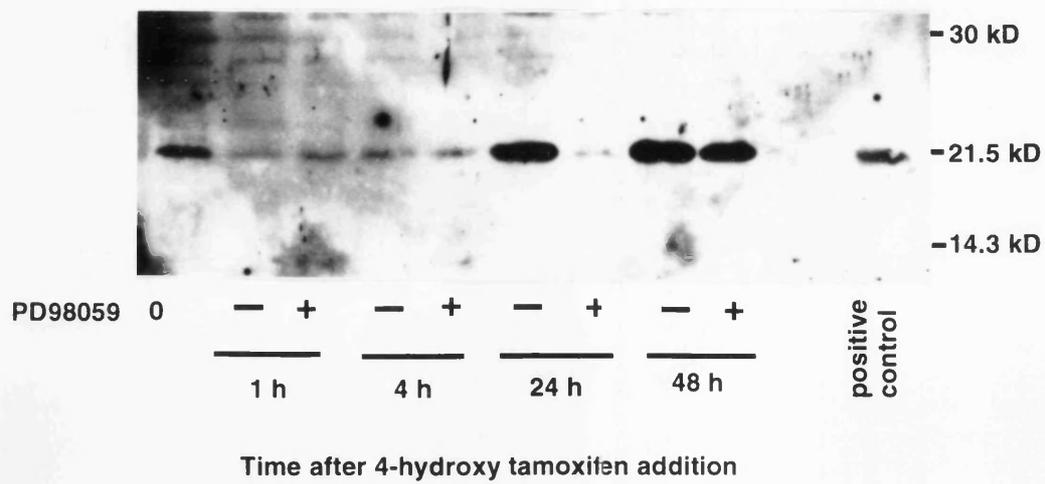
5.4.5 PD98059 delays signs of morphological transformation

In NSΔRafER cells the induction of ΔRaf resulted in morphological transformation characterised by highly refractile cells which became increasingly rounded up. The first morphological changes were detectable at approximately 12 hours but became more obvious at 24 hours after induction. Since PD98059 inhibited ΔRaf induced activation of MAP-kinase as a downstream target, I was interested to investigate whether the changes to morphological transformation could also be inhibited.

NSΔRafER cells pre-treated with PD98059 or the solvent DMSO were induced with 4-hydroxy tamoxifen. Photos were taken at indicated time points. The typical morphological changes in induced NSΔRafER cells were inhibited by PD98059 at 24 hours, whereas after 48 hours the morphological differences between PD98059 pre-treated and untreated cells were less obvious (Figure V.10). In conclusion, PD98059 delayed the morphological changes, although it was not able to block these changes fully.

5.5 Constitutively active MAP-kinase kinase in Schwann cells

5.5.1 Introduction

The MAP-kinase kinase inhibitor PD98059 is able to delay the activation of MAP-kinase for up to 24 hours and subsequential the induction of p21$^{WAF1/Cip1}$, as shown previously (chapter 5.4.4). This finding argued for an involvement of MAP-kinase kinase in the induction of p21$^{WAF1/Cip1}$. However, these experiments did not address whether activated MAP-kinase kinase is also sufficient in transmitting the signal for growth arrest.

Figure V.10: PD98059 delays morphological changes associated with ΔRafER induction

NSΔRafER cells were quiesced for 2 days. Either DMSO or 50 μM PD98059 were added 30 minutes prior addition of 4-hydroxy tamoxifen to NSΔRafER cells. Photos were taken 24 hours (upper photos) and 48 hours after 4-hydroxy tamoxifen addition (lower photos) (magnification 400x).

+ DMSO, 24 hours
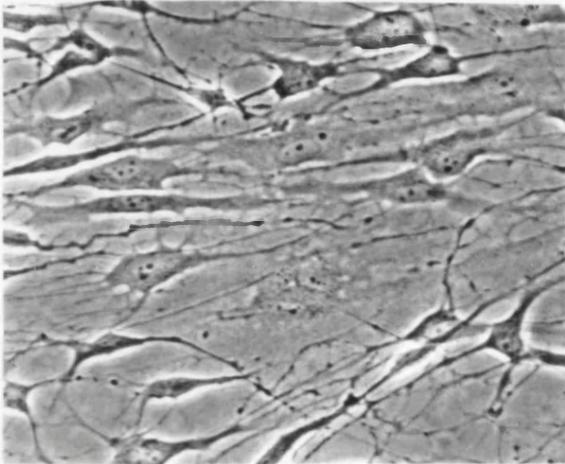
+ 50μM PD98059, 24 hours
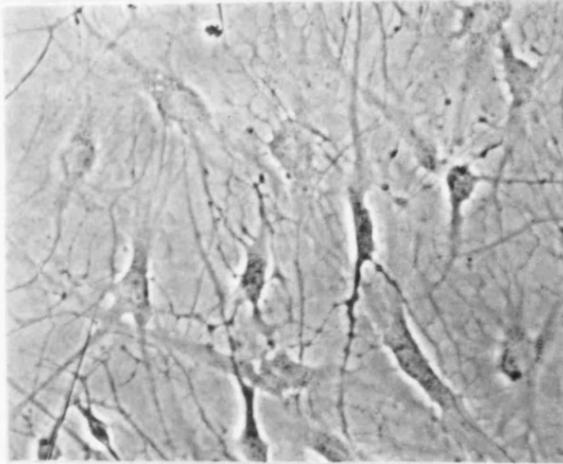
+ DMSO, 48 hours
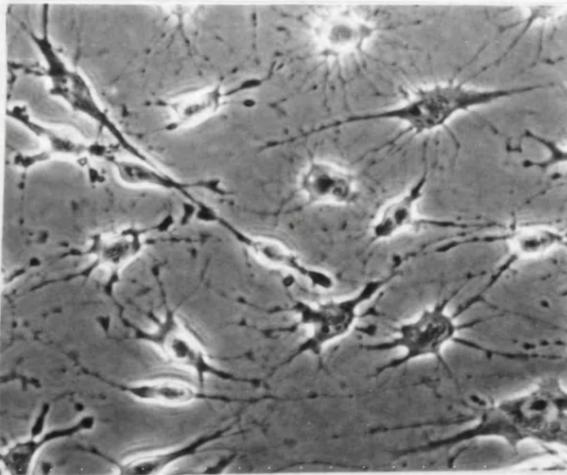
+ 50μM PD98059, 48 hours
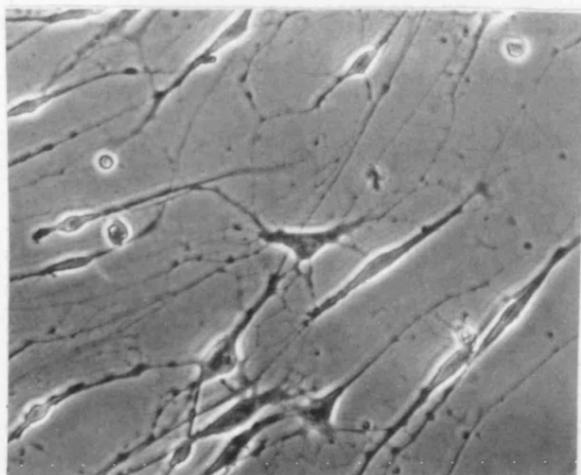
The generation of constitutively active mutants of MAP-kinase kinase enabled the analysis of the Ras-signalling pathway and its contribution in differentiation and mitogenesis in mammalian cells (Cowley et al., 1994; Mansour et al., 1994; Brunet et al., 1994). MAP-kinase kinase (MKK) also referred to as MAPK/ERK-kinase (MEK) is phosphorylated by c-Raf-1 (Alessi et al., 1994; Zheng & Guan, 1994), B-Raf (Papin et al., 1995) or v-Mos (Pham et al., 1995) at serine residues 218 and 222. These two phosphorylation events are necessary and sufficient to activate kinase activity towards its substrate, MAP-kinase. Constitutively active MAP-kinase kinase 1 (Glu 217/Glu 221) was generated by substitution of both phosphoacceptor residues to glutamic acid in the catalytic domain. These substitutions resulted in constitutive active MAP-kinase kinase (Cowley et al., 1994). Similarly, another constitutively active MAP kinase kinase was generated by substitution of serine 218 to glutamic acid and serine 222 to aspartic acid and a deletion of a predicted α-helical domain from amino acid 32 to 51 resulting in a even stronger constitutive MAP-kinase kinase, ΔMEK1(S218E,S222D) (Mansour et al., 1994).

The biological effects of constitutively active MAP-kinase kinase 1 (Glu-217/Glu-221) in mitogenesis and differentiation were examined in NIH3T3 cells and PC12 phaeochromocytoma cells, respectively. Constitutive active MAP-kinase kinase (S218D or S218D/S222D) decreased the growth factor requirement for DNA-synthesis in a fibroblast cell line (Brunet et al., 1994) and caused an increase of cells entering S-phase upon injection of constitutively active MAP-kinase kinase protein (Cowley et al., 1994). Furthermore, NIH3T3 cells transfected with MAP-kinase kinase 1 (Glu-217/Glu-221) resulted in about 15-30% of morphological transformed and drug resistant-colonies (Cowley et al., 1994). Individual clones were capable of anchorage-independent growth in

soft agar (Cowley et al., 1994; Mansour et al., 1994) and proliferated in serum-free conditions similarly to NIH3T3 cells transformed with oncogenic *ras* (Cowley et al., 1994; Marshall et al., 1985). Rapid tumour growth has been observed after the injection of NIH3T3 cells expressing MAP-kinase kinase 1 (Glu-217/Glu-221) into nude mice (Cowley et al., 1994; Mansour et al., 1994; Brunet et al., 1994) confirming *in vitro* data. As a model system for differentiation, PC12 phaeochromocytoma cells were employed since they differentiate in response to nerve growth factor (NGF) (Greene & Tischler, 1976), the expression of activated Ras (Noda et al., 1985) or activated Raf (Wood et al., 1993). Microinjections of MAP-kinase kinase 1 Glu-217/Glu-221 protein led to differentiation of more than 75% of injected PC12 cells, as indicated by the outgrowth of neurites (Cowley et al.1994).

In summary, these extensive investigations established that a constitutive active MAP-kinase kinase was capable of mimicking the biological responses previously observed with activated Ras and Raf. These studies showed further that MAP-kinase kinase was located downstream of Ras in the Ras-MAP-kinase signalling pathway, since blocking of Ras had no effect on the cellular responses initiated by MAP-kinase kinase (Cowley et al., 1994).

5.5.2 Constitutive MAP-kinase kinase does not inhibit cell growth in Schwann cells

The generation of constitutive active mutants of MAP-kinase kinase offered the possibility to further investigate the signalling pathway from Raf to the cell-cycle arrest via the p53-dependent p21$^{WAF1/Cip1}$ induction in Schwann cells. Provided that MAP-kinase kinase was part of the signalling pathway which led to the induction of p21$^{WAF1/Cip1}$, constitutive active MAP-kinase kinase should be capable of inducing p21$^{WAF1/Cip1}$ and arresting

Schwann cells. If a prolonged MAP-kinase signal is not only necessary but also sufficient to cause growth arrest in Schwann cells, a constitutive active MAP-kinase kinase should also be able to arrest Schwann cells.

The constitutive active ΔMEK1(S218E, S220D) mutant (Mansour et al., 1994), was fused to the hormone-binding domain of the mutated oestrogen receptor responsive to 4-hydroxy tamoxifen (a kind gift by Martin MacMahon). Constitutive MAP-kinase kinase 1 (ΔMEK1 (S218E, S222D):ER^{TM}) activity thereby became dependent on the presence of 4-hydroxy tamoxifen (Martin MacMahon, personal communication). This inducible constitutive active ΔMEK (S218E, S222D):ER^{TM} pBabe Puro construct was introduced through retroviral infection into Schwann cells. After selection puromycin-resistant Schwann cells were pooled.

To test whether the inducible ΔMEK(S218E, S222D):ER^{TM} was indeed activatible in Schwann cells, I analysed the kinetics of activation of the downstream target MAP-kinase after induction of ΔMEK(S218E, S222D) by 4-hydroxy tamoxifen in starved Schwann cells. NSΔMEK(S218E, S222D) cells were grown to confluency and starved for any exogenous stimuli for two days. Cells were stimulated with or without 4-hydroxy tamoxifen and tested for MAP-kinase activity at indicated times (Figure VI.11A). MAP-kinase activity was observed from 1 hours to 4 hours after addition of 4-hydroxy tamoxifen. However, in the absence of 4-hydroxy tamoxifen and mitogens MAP kinase activity as indicated by the phosphorylation shift was detectable in NSΔMEK(S218E, S222D) cells but not in NSΔRafER cells used as negative control cells. This result showed that the constitutively active MAP-kinase kinase is active even in the absence of 4-hydroxy tamoxifen and therefore is not fully regulatable by 4-hydroxy tamoxifen.

To test whether constitutive active MAP-kinase kinase was able to arrest Schwann cells similarly to activated ΔRafER, NSΔMEK (S218E, S222D):ER^TM cells were treated with mitogen plus and minus 4-hydroxy tamoxifen and S-phase entry was measured by ^{3}H-thymidine incorporation. As control NSΔRafER cells were also investigated for S-phase entry under identical conditions (Figure VI.11B,C). Induced constitutively active MAP-kinase kinase was not able to arrest Schwann cells as illustrated in Figure VI.11.BC & D. Schwann cells expressing inducible constitutive active MAP-kinase kinase entered S-phase as indicated by the incorporation of ^{3}H-thymidine. The increase of ^{3}H-thymidine incorporation with time suggested that under conditions which clearly arrested NSΔRafER cells (Figure VI.11B), NSΔMEK1 (S218E, S222D):ER^TM cells did not arrest but instead led to a slight stimulation of S-phase entry compared with uninduced control cells (Figure VI.11C). The continuous incorporation of ^{3}H-thymidine at all time periods suggested that NSΔMEK1 cells were not arrested in the absence of mitogens at the start of the experiment. These effects may be due to the observed MAP-kinase kinase activity in the absence of 4-hydroxy tamoxifen. Similarly, the FACS-analysis of NSΔMEK1 (S218E, S222D): ER^TM cells confirmed that the addition of 4-hydroxy tamoxifen to asynchronous growing cells did not result in a G1 cell cycle arrest but led to a slight increase in the population of S-phase cells (Figure VI.11B). This result was surprising, since the inhibition of Raf-induced MAP-kinase kinase activity by the specific inhibitor PD98059 did clearly delay the induction of p21^WAF1/Cip1 (Figure VI.9) which was instrumental to the cell cycle arrest (Lloyd et al., 1997). Although it has been reported that ΔMEK1 (S218E, S222D) exhibits a relative strong kinase activity in a number of assays (Mansour et al., 1994), this activity might be not strong enough to induce a cell cycle arrest in Schwann cells. As another possible explanation, due to

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Figure V.11A-D : Constitutive active MAP-kinase kinase in Schwann cells

A. ΔMEK1:ER^{TM} is active in the uninduced state

NSΔRafER cells were kept in the absence of mitogens for two days prior to the addition of 1μM 4-hydroxy tamoxifen. Control cells were left untreated in the absence of any mitogens. 30 μg cell lysate was subject to western blotting analysis. Immunoreactive bands with the p42^{ERK2} specific antibody 122 were detected using the ECL-system.

B. NSΔMEK1:ER^{TM} cells do not arrest in the presence of 4-hydroxy tamoxifen

NSΔMEK1:ER^{TM} cells were seeded at 2x10^4 cells per well. NSΔMEK1:ER^{TM} cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. ^{3}H-thymidine was added for indicated time periods. ^{3}H-thymidine incorporation was measured as described in Methods

C. NSΔRafER cells arrest in the presence of 4-hydroxy tamoxifen

NSΔRafER cells were seeded at 2x10^4 cells per well. NSΔRafER cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. ^{3}H-thymidine was added for indicated time periods.

D. FACS-analysis of NSΔMEK:ER^{TM} cells

NSΔMEK1:ER^{TM} cells were kept in the absence of mitogens for two days prior to the addition of mitogens. 1 μM 4-hydroxy tamoxifen or ethanol were added at point 0. BrdU was added 26 hours after induction for 4 hours and FACS-analysis was carried out.

A.

NSΔMEK1:ER^TM

◀ PP
◀ p42^ERK2

0   -   +   -   +

NSΔRAFER   1 h   4 h

time after 4-hydroxy tamoxifen addition

B.

NSΔMEK1:ER^TM

^{3}H-thymidine incorporation (cpm)

TM   -   +   -   +   -   +

0-17 h   17-35 h   35-52 h

C.

NSΔRafER

^{3}H-thymidine incorporation (cpm)

TM   -   +   -   +   -   +

0-17 h   17-35 h   35-52 h

D.

NSΔMEK1:ER^TM , - TM

Anti-BrdU FITC
Propidium Iodide

G1: 77.28 %
S: 16.61 %
G2/M: 3.02 %

NSΔMEK1:ER^TM , + TM

Anti-BrdU FITC
Propidium Iodide

G1: 72.89 %
S: 21.95 %
G2/M: 2.21 %
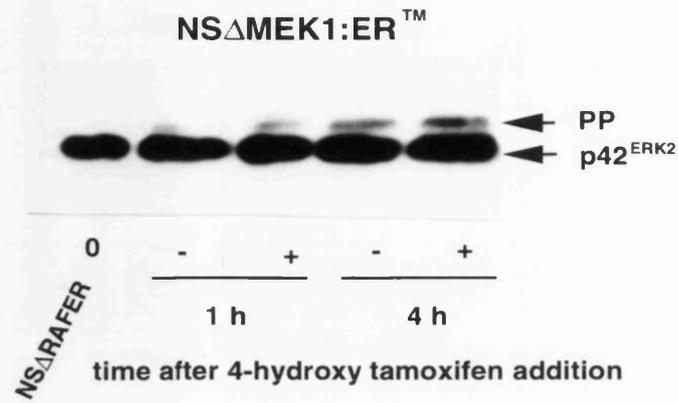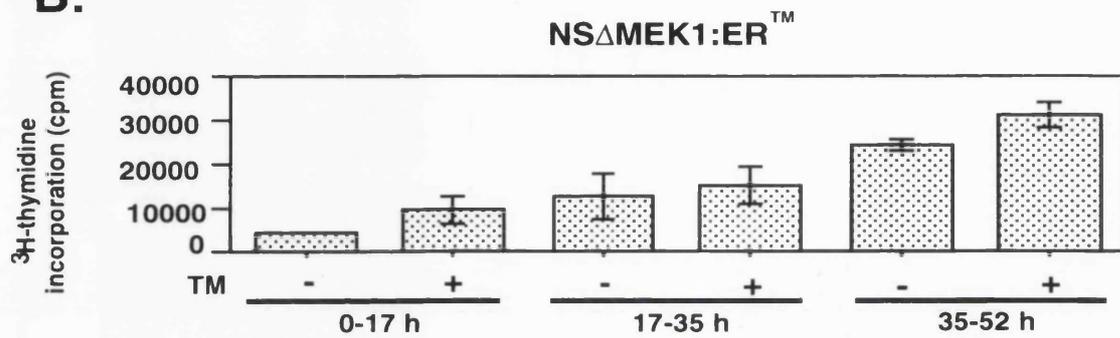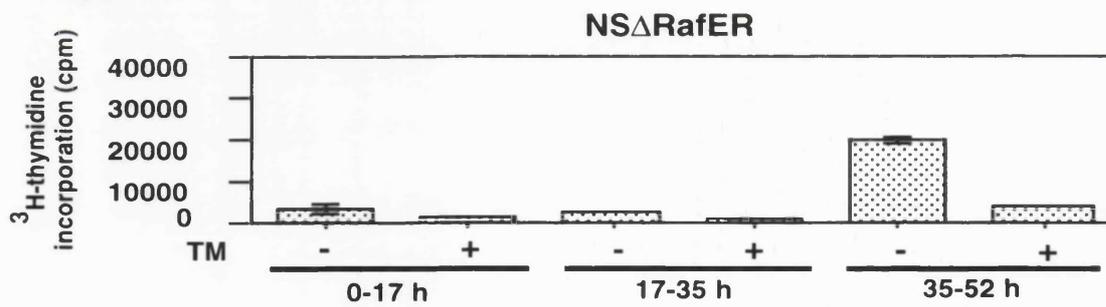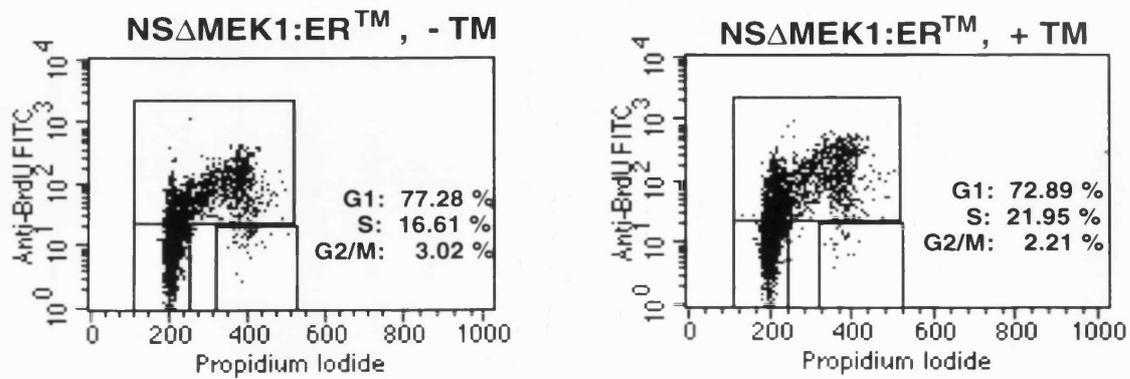
the activation of ΔMEK1 (S218E, S222D):ER^{TM} in the absence of 4-hydroxy tamoxifen individual cells which might have exhibited a strong kinase activity sufficient to arrest cells could have been selected against, thereby leaving only non-responsive cells. Further experimentation could address this possibility by testing single constitutively active MAP-kinase kinase expressing clones. Differences in inducibility and strength of MAP-kinase kinase activity could then be tested for p21$^{WAF1/Cip1}$ induction.

CHAPTER 6.0

The c-Jun amino-terminal kinase 1 (JNK) in
ΔRaf-induced growth arrest in Schwann cells

6.0 The c-Jun NH$_2$ terminal kinase 1 (JNK) in ΔRaf-induced growth arrest in Schwann cells

6.1 Introduction

The data presented so far have established a link between the prolonged activity of ΔRafER in Schwann cells and the growth arrest due to p53-dependent induction of p21$^{WAF1/Cip1}$. Inhibition by the MAPKKs specific inhibitor PD98059 (see chapter 5) has provided strong evidence for the mediation of the growth inhibitory signal by the Ras-MAP-kinase pathway downstream of Ras and Raf. However, various lines of evidence suggest a possible involvement of the JNK-kinase pathway in mediating the signal for the Ras/Raf-induced cell cycle arrest in Schwann cells.

The delay of p21$^{WAF1/Cip1}$ induction 10 hours after Raf induction (Chapter 4.0) could be explained by the secretion and the accumulative effect of a growth factor on Schwann cells. Interestingly, in NIH3T3 cells the induction of ΔRafER resulted in an enhanced expression and secretion of the heparin-binding epidermal growth factor (HB-EGF) (McCarthy et al., 1995). Furthermore, HB-EGF had a stimulatory effect on JNK-1 kinase activity in these cells (McCarthy et al., 1995). In particular, ΔRafER activation led to a delayed activation of JNK-1 kinase after 16 to 24 hours which was shown to be due to the effect of an autocrine loop in NIH3T3 cells (Minden et al., 1994; McCarthy et al., 1995). JNK-1 kinase has also been shown to be activatible by other factors such as the tumour necrosis factor (TNF-α) (Kyriakis et al., 1994) and possibly sphingomylein-based second messengers (Hannun et al., 1994). Thus, it was a possibility that ΔRaf-dependent production and secretion of a factor or factors could result in a possible activation of the JNK-kinase pathway in Schwann cells. In this respect, the suggestion that UV-activated JNK-1 kinase may phosphorylate

murine p53 *in vitro* and *in vivo* (Milne et al., 1995) was of interest, since the p21$^{WAF1/Cip1}$ induction in NSΔRafER cells is p53-dependent (chapter 5.0; Lloyd et al., 1997). Given these results, I sought to investigate whether JNK-1 kinase activity was firstly elevated in response to constitutive Raf-kinase activity in Schwann cells and secondly to establish whether JNK-kinase activity may contribute to the G1 cell cycle arrest.

6.2 JNK-1 kinase activity is induced by ΔRafER

To explore whether the JNK-pathway is activated in the ΔRafER-induced growth arrest in Schwann cells, I examined Jun N-terminal kinase 1 (JNK-1) activity in response to activated Raf. Confluent NSΔRafER cells were therefore induced with 4-hydroxy tamoxifen. Cells were harvested at indicated times. After immunoprecipitation with a JNK1-specific antibody, an *in-vitro* kinase assay with recombinant c-Jun amino acid 1-257 protein as substrate was performed.

Following induction of ΔRafER, JNK1-kinase activity increased dramatically (Figure VI.1A), while the addition of the solvent ethanol did not lead to a substantial increase of kinase activity. The quantification of incorporated ^{32}γ-phosphate showed a considerable increase of JNK-1 kinase activity 12 hours after induction, peaking at 14 hours with a 10-fold increase of kinase activity compared with uninduced cells. The JNK-1 kinase activity remained approximately 7-fold above background for at least 24 hours (Figure VI.1B). JNK-1 kinase activity after ΔRafER induction was tested in three independent experiments with very similar results to the results presented (Figure VI.1A). The protein expression of JNK-1 kinase remained relatively unchanged during this time course (Figure VI.2), suggesting that activation occurs via posttranslational modification such as

Figure VI.1A: JNK-1 kinase activity in induced NSΔRafER cells

NSΔRafER were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Cells were washed twice with prewarmed PBSA. Medium without mitogens was added for two days. Thereafter cells were stimulated with 1 μM 4-hydroxyl tamoxifen. Cells were harvested at indicated times. Cell lysates were prepared as described (see Methods) and 500 μg total protein was used for immunoprecipitation with monoclonal antibody C-17 against JNK-1 (Oncogene Science Ltd.). Immunoprecipitates were used in an *in-vitro* kinase assay with GST-c-Jun amino acids 1-257 as substrate (see Methods). 10 μl of the *in-vitro* kinase assay were separated on a 15% SDS-PAGE and subject to autoradiography.

**Figure VI.1B: Quantification of JNK-1 kinase activity in
                    NSΔRafER cells**

The incorporation of radioactive γ^{32}-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.). The JNK-1 kinase activity of various time points is referred to 1-fold of time point zero.

**Figure VI.2: Elevated JNK-1 kinase activity is independent of
                    protein expression**

30 μg total protein from cell lysates used for the kinase assay in figure VI.1A were separated on a 12.5% SDS-PAGE, blotted onto PVDF-membrane and incubated with JNK-1 specific antibody (C-17) (Oncogene Science Ltd.). Immunoreactive bands were visualised using the ECL-detection system (Amersham Ltd.).

A.

TM 0 | 2 h | 12 h | 14 h | 18 h | 24 h | peptide
— | + | — | + | — | + | — | + | — | +

B.

Fold kinase-activity
(0=1x)

0 | 2 h | 12 h | 14 h | 18 h | 24 h | peptide
— | + | — | + | — | + | — | + | — | +

TM 0 | 2 h | 12 h | 14 h | 18 h | 24 h
— | + | — | + | — | + | — | + | — | +
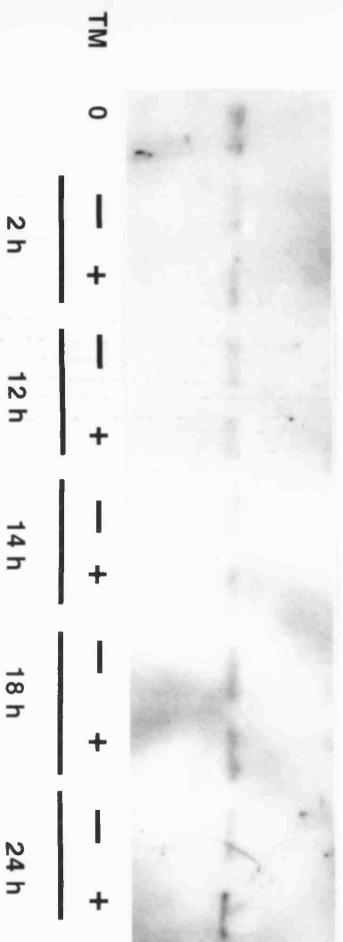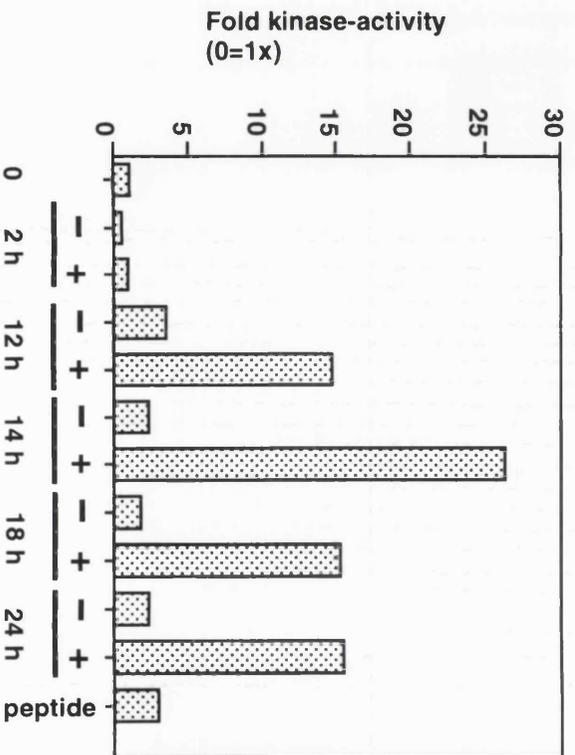
phosphorylation by MKK4/SEK1 (Yan et al., 1994) which is likely to be responsible for the increase in kinase activity.

The finding that JNK1-kinase activity strongly increases after ΔRafER induction in Schwann cells is surprising, since the weak activation of JNK-1 kinase by activated Ras has been explained by a Raf-independent pathway (Minden et al., 1994). However, similar to results in NIH3T3 cells (Minden et al., 1994; McCarthy et al., 1995), it is possible that the production of autocrine factors are responsible for the delayed JNK-1 kinase activation, given that high JNK-kinase activity coincides with the beginning of p21$^{WAF1/Cip1}$ induction (chapter 4.0) in Schwann cells.

6.3 MAP-kinase kinase inhibitor PD98059 indirectly inhibits JNK-1 kinase activity

The increase in JNK-1 kinase activity in response to activated Raf could have been the result of activation by a MAP-kinase kinase-dependent or independent signalling pathway. Previously, I have shown that the inhibition of MAP-kinase kinase by PD98059 led to a delayed induction of p21$^{WAF1/Cip1}$ (chapter 5.0). MAP-kinase kinase activation was therefore required for the induction of p21$^{WAF1/Cip1}$. Based on these results, I reasoned that if the activation of JNK-1 kinase was involved in mediating p21$^{WAF1/Cip1}$ induction, MAP-kinase kinase activation was necessary for enhanced JNK-1 kinase activity to occur in response to Raf.

To distinguish between a MAP-kinase kinase-dependent or independent activation of JNK-1 kinase, the specific MAP-kinase kinase inhibitor PD98059 was used. NSΔRafER cells were treated with or without 50 μM PD98059 prior to induction with 4-hydroxy tamoxifen. Cells were harvested at indicated times after ΔRafER induction. After

Figure VI.3A: PD98059 inhibits JNK1-kinase activity in NSΔRafER cells

NSΔRafER were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Medium with 2% FCS only was added for two days. One hour prior to stimulation with 1 μM 4-hydroxyl tamoxifen, 50 μM PD98059 or the solvent DMSO were added to the cells. Cells were harvested at indicated times and 500 μg total protein was used for immunoprecipitation with monoclonal antibody C-17 against JNK-1 (Oncogene Science Ltd.). Immunoprecipitates were used in an *in-vitro-*kinase assay with GST-c-Jun amino acids 1-257 as substrate (see Methods). 10 μl of the *in -vitro* kinase assay were separated on a 15% SDS-PAGE and subject to autoradiography.

Figure VI.3B: Quantification of JNK-1 kinase activity in PD98059 treated NSΔRafER cells

The incorporation of radioactive γ^{32}-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.).

A.

c-Jun aa 1-257 →

PD98059   −   −   −   +   −   +   −   +   −   +

GST   0   2 h   14 h   24 h   UV   peptide

Time after 4-hydroxy tamoxifen addition

B.

arbitary units

400

300

200

100

0

PD98059   −   −   −   +   −   +   −   +   −   +

GST   0   2 h   14 h   24 h   UV   peptide

Time after 4-hydroxy tamoxifen addition
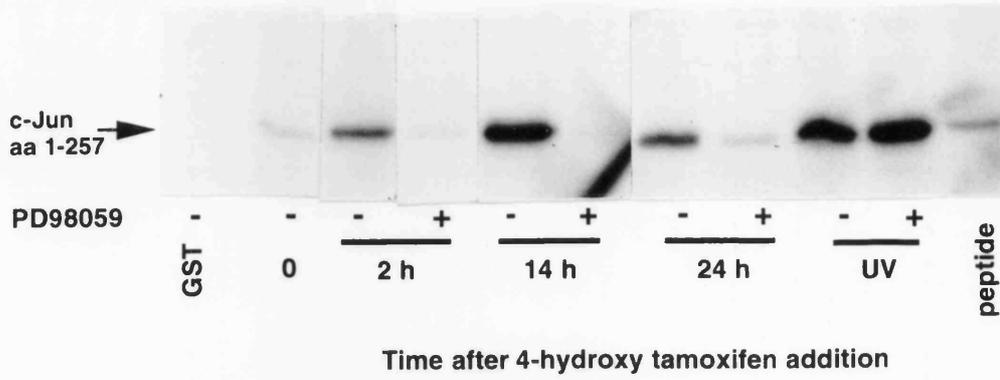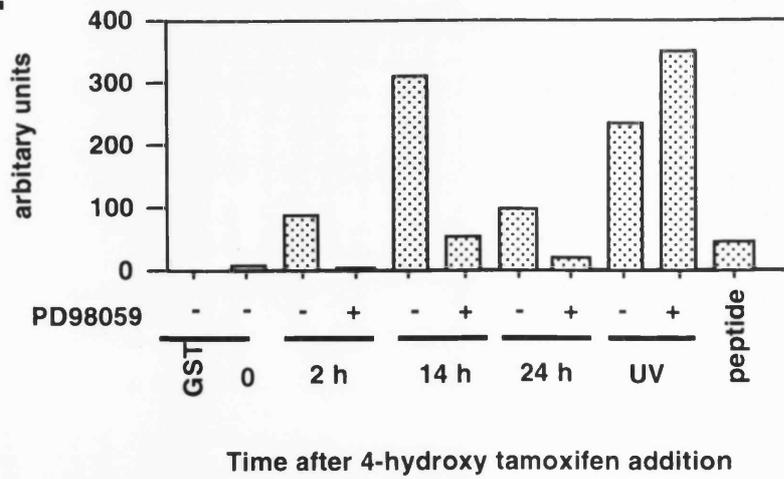
immunoprecipitation with a JNK1-specific antibody, an *in-vitro* kinase assay with recombinant c-Jun amino acids 1-257 protein as substrate was performed.

The MAP-kinase kinase inhibitor PD98059 had an inhibitory effect onto JNK-1 kinase activity at 14 hours and 24 hours (Figure VI.3A; VI.3B). This effect was not due to a direct inhibition of JNK-1 by PD98059, since JNK-1 kinase activation through UV-irradiation was not inhibited, confirming the lack of JNK-1 kinase inhibition by PD98059 previously reported (Alessi et al, 1995). Thus, the observed inhibition of JNK-1 kinase activity by PD98059 is likely due to inhibition of MAP-kinase kinase. Importantly, this result suggested the possibility that JNK-1 kinases could indeed be mediating the ΔRaf-induced growth arrest in Schwann cells.

6.4 Dominant negative Stress-activated protein kinase (SEK-1) in NSΔRafER cells

The inhibition of JNK-1 kinase activity by the MAP-kinase kinase inhibitor PD98059 suggests that the JNK-1 kinase is downstream of MAP-kinase kinase in the ΔRafER-induced signalling pathway (Figure VI.2A; VI.2B). Thus, the question arose whether JNK-kinase activity is causally involved in the induction of p21$^{WAF1/Cip1}$ and the following cell cycle arrest.

The generation of a dominant negative MAP-kinase kinase 4 (MKK4), also named Stress-activated protein kinase (SEK1) (Yan et al., 1994; Zanke et al., 1996), offered a genetic approach to address this question. It has been shown that MKK4/SEK1 is directly responsible for phosphorylation of JNK-1 kinase after activation by various stimuli (Yan et al., 1994). A dominant negative mutant of SEK1, designated SEK1-AL, was mutated to code for alanine and leucine at positions amino acids 220 and 224,

Figure VII.4A: Dominant negative SEK1-AL mutant reduces ΔRafER induced JNK-1 kinase activity

NSΔRafER, NSΔRafER SEK-1 and NSΔRafER SEK-1-AL cells were grown in medium containing 3% stripped fetal calf serum, GGF and forskolin to 80% confluence. Cells were washed twice with prewarmed PBSA. Medium with 2% FCS only was added for two days. Thereafter, cells were stimulated with either 1 μM 4 hydroxy tamoxifen or ethanol as a control. Cells were harvested at indicated times and 500 μg total protein was used for immunoprecipitation with a monoclonal antibody C-17 against JNK-1 (Oncogene Science). Immunoprecipitates were used in an *in-vitro* kinase assay with GST-c-Jun 1-257 as substrate (see Methods). 15 μl of the *in-vitro* kinase assay were separated on a 15% SDS-PAGE and subjected to autoradiography.

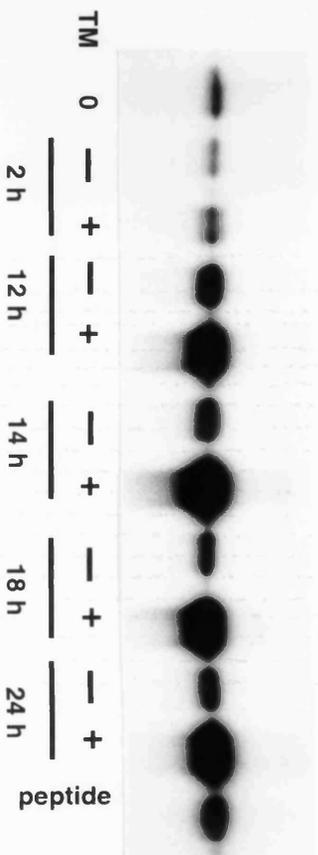## Figure VII.4B: Quantification of JNK-1 kinase activity

The incorporation of radioactive γ^{32}-phosphate was quantified with a PhosphorImager and ImageQuant software (Molecular Dynamics Ltd.) and is expressed in arbitary units.

Figure VII.5: p21$^{WAF1/CIP1}$ induction in NSΔRafER SEK1-AL cells

60 μg total protein from cell lysates used for the kinase assay in figure VII.4A were separated on a 15% SDS-PAGE, blotted onto PVDF-membrane and incubated with an antibody against rat p21$^{WAF1/CIP1}$ (Santa Cruz Ltd). Immunoreactive bands were visualised using the ECL-detection system (Amersham Ltd).

A.

	14 h 24 h	14 h 24 h	14 h 24 h		14 h 24 h	14 h 24 h	14 h 24 h
TM	+ +	+ +	+ +	peptide	− −	− −	− −
	Bleo	SEK-1	SEK-1 AL		Bleo	SEK-1	SEK-1 AL

B.

	14 h 24 h	14 h 24 h	14 h 24 h		14 h 24 h	14 h 24 h	14 h 24 h
TM	+ +	+ +	+ +	peptide	− −	− −	− −
	Bleo	SEK-1	SEK-1 AL		Bleo	SEK-1	SEK-1 AL

p21 $\rightarrow$ WAF-1

	14 h 24 h	14 h 24 h	14 h 24 h	14 h 24 h	14 h 24 h	14 h 24 h
TM	+ +	+ +	+ +	− −	− −	− −
	Bleo	SEK-1	SEK-1 AL	Bleo	SEK-1	SEK-1 AL
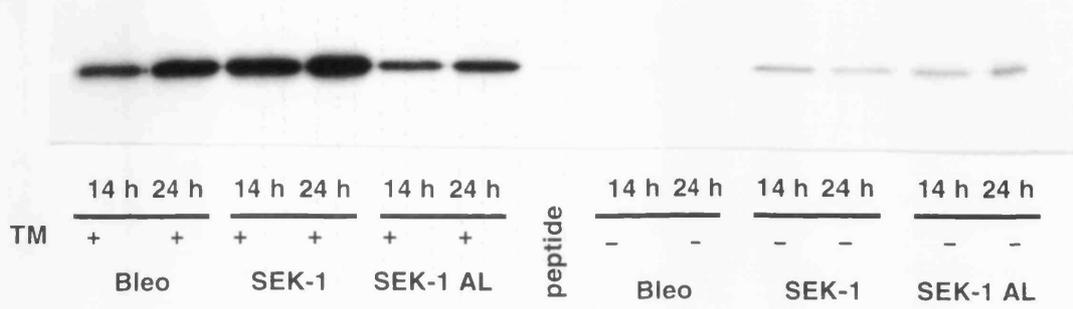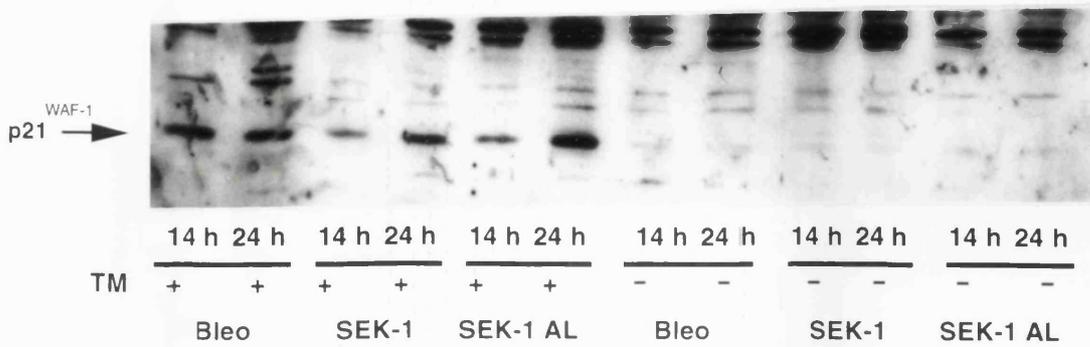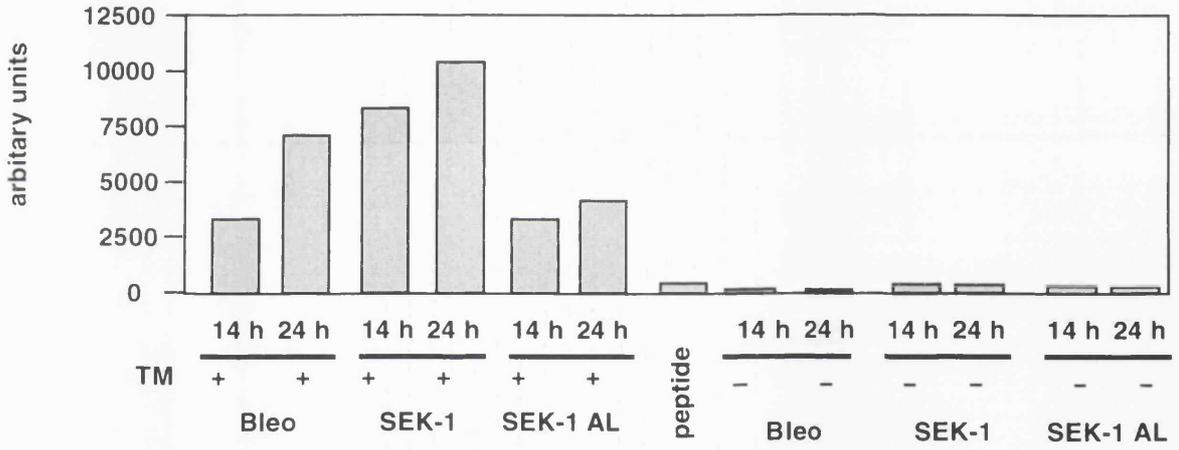
respectively (Yan et al., 1994). This mutant SEK1-kinase was capable of substantially reducing JNK-1 kinase activity mediated through SEK1 after heat-shock and the application of the DNA-damaging drug *cis*-platinum (Zanke et al., 1996).

I reasoned that if JNK-1 kinase activation is required and sufficient to induce p21$^{WAF1/Cip1}$, then the expression of a dominant negative mutant of SEK1 should inhibit or delay p21$^{WAF1/Cip1}$ induction and overcome the ΔRafER-induced growth arrest. To address this hypothesis, I cloned the dominant negative mutant SEK1-AL and wild type SEK1 (Zanke et al., 1996) into the retroviral vector pBabe Bleo. NSΔRafER cells were infected with pBabe Bleo SEK1 or pBabe Bleo SEK1-AL resulting in NSΔRafER SEK1 and NSΔRafER SEK1-AL cells. After selection, cells were pooled and expanded. Firstly, I tested whether Schwann cells expressing SEK1-AL were capable of reducing ΔRafER induced JNK-1 kinase activity. On the contrary, the expression of exogenous wild-type SEK1 should lead to a clear increase of JNK-1 kinase activity over control cells with only endogenous MKK4/SEK kinase. The JNK-1 kinase activity of these cells was compared to NSΔRafER cells harbouring the empty vector pBabe Bleo. NSΔRafER cells, NSΔRafER SEK1 cells and NSΔRafER SEK1-AL cells were induced with 4-hydroxy tamoxifen and harvested at indicated times (Figure VI.3A). After immunoprecipitation an *in-vitro* JNK-1 kinase assay was performed.

Expression of the dominant negative kinase (SEK-AL) reduced ΔRafER-induced JNK-1 kinase activity after 24 hours to about 40% of JNK-1 kinase activity observed in NSΔRafER Bleo control cells. Moreover, cells expressing exogenous wild-type SEK1 in addition to endogenous SEK1-kinase showed an approximately 45% increase of JNK-1 kinase activity at 14 and 24 hours after induction (Figure VI.3B). The observed JNK-1 kinase

activity after 4-hydroxy tamoxifen addition was not observed after the addition of the solvent ethanol (Figure VI.3B).

These results confirmed that infection with dominant negative SEK1 was sufficient to partially reduce ΔRafER-dependent JNK-1 kinase activity. As predicted, the introduction of exogenous SEK-1 led to an increase of JNK-1 kinase activity above that observed in control cells, arguing for a role of SEK1 in mediating ΔRafER-dependent induction of JNK-1 kinase activity. Given these results, I was interested to see whether the observed reduction of JNK-1 kinase activity in NSΔRafER SEK1-AL cells had an effect on the induction of p21$^{WAF1/Cip1}$ protein. Conversely, the increase of JNK-1 kinase activity in NSΔRafER SEK1 cells might also be reflected in a stronger induction of p21$^{WAF1/Cip1}$ in these cells.

To test whether such a correlation existed, I examined the p21$^{WAF1/Cip1}$ protein levels from the same cell lysates previously used for the JNK1-kinase assay (Figure VI.3A). Protein lysates were separated on an SDS-PAGE gel and analysed. Western blot analysis revealed that the co-expression of dominant negative SEK1-AL had no effect on the level of induced p21$^{WAF1/Cip1}$ protein. In agreement with this finding, the co-expression of wild-type SEK-1 did not increase the p21$^{WAF1/Cip1}$ protein level compared to that in induced control cells (Figure VI.4). In parallel, I examined whether NSΔRafER cells expressing the dominant negative mutant SEK1-AL were capable of rescuing the Raf-induced growth arrest by FACS-analysis. For this purpose, BrdU was added to assynchronously growing NSΔRafER SEK1-AL and SEK1 or control cells and 26 hours after Raf induction the cell cycle distribution of these cells was analysed. The FACS-analysis of NSΔRafER SEK1 and NSΔRafER SEK1-AL cells was inconclusive in that both NSΔRafER SEK1 and NSΔRafER SEK1-AL cells were inhibited to a similar degree in the presence of 4-hydroxy tamoxifen.

Figure VI.6A-B: FACS-analysis of NSΔRafER SEK1-AL cells and NSΔRafER SEK1

Assynchronously growing NSΔRafER SEK1-AL and NSΔRafER SEK1 cells were induced with 1 µM 4-hydroxy tamoxifen. After 26 hours 10 µM BrdU were added for 4 hours. The cell cycle distribution of NSΔRafER SEK1-AL and NSΔRafER SEK1 cells were measured by BrdU incorporation and DNA-content flow cytometry analysis. The upper box identifies cells incorporating BrdU (S-phase), the lower left-hand box represents Go/G1 cells and the lower right-hand box represents G2/M cells. The percentage of cells in G1, S or G2/M-phase of the cell cycle is given underneath the individual graph.

NSΔRafER Bleo, -TM
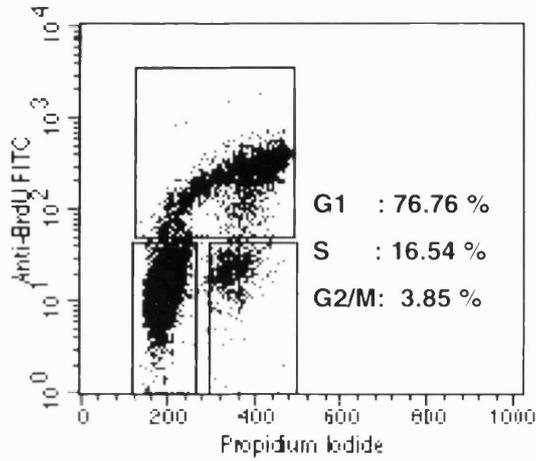
Anti-BrdU FITC vs Propidium Iodide

G1 : 76.76 %
S   : 16.54 %
G2/M: 3.85 %

NSΔRafER Bleo, +TM

G1 : 91.74 %
S   : 3.87 %
G2/M: 3.18 %

NSΔRafER SEK1, -TM

G1 : 79.08 %
S   : 15.41 %
G2/M: 3.46 %

NSΔRafER SEK1, +TM

G1 : 84.52 %
S   : 9.73 %
G2/M: 2.95 %

NSΔRafER SEK1-AL, -TM

G1 : 79.78 %
S   : 14.32 %
G2/M: 3.62 %

NSΔRafER SEK1-AL, +TM

G1 : 78.10 %
S   : 10.10 %
G2/M: 4.38 %
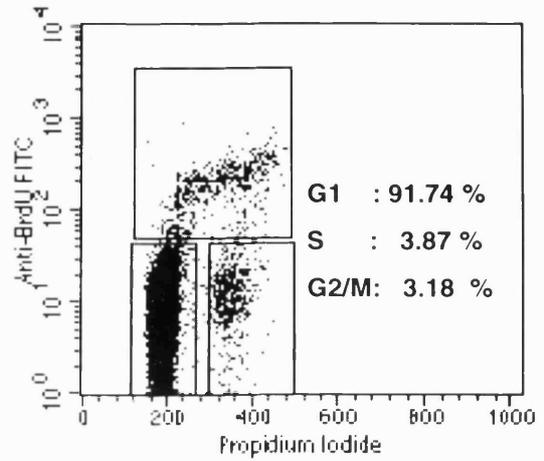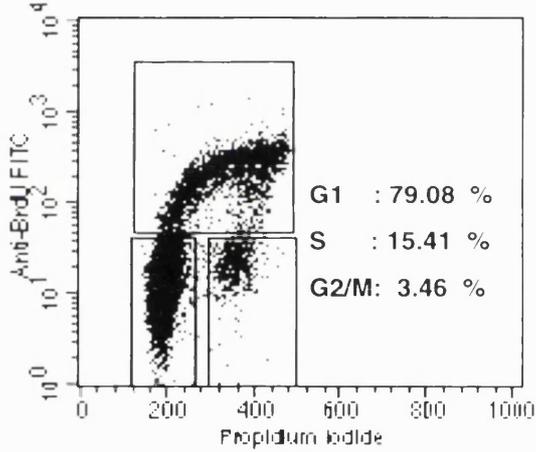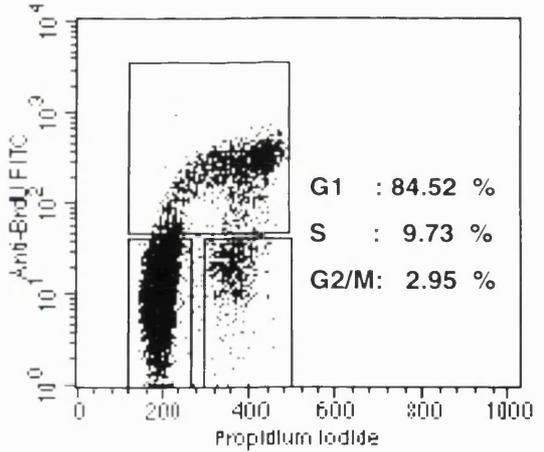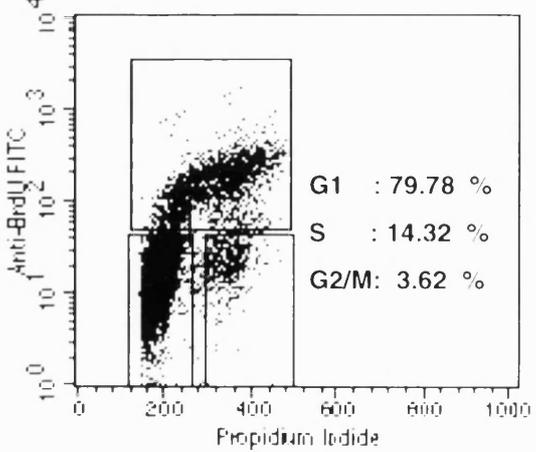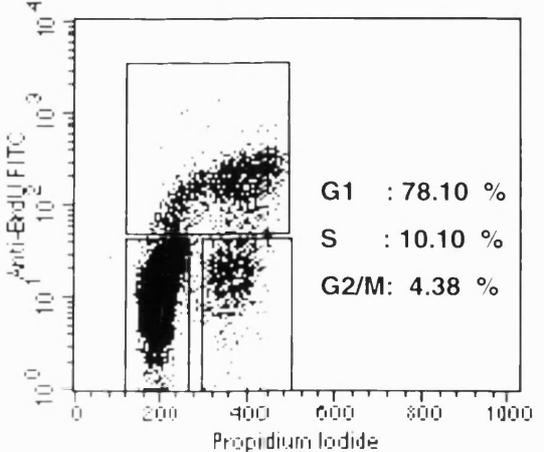
Surprisingly, the reduction of S-phase cells of the control NSΔRafER SEK1 cells was far less strong than that observed in NSΔRafER Bleo cells (Figure VI.5).

6.5 Discussion

The induction of constitutively active Raf-kinase activity led to a delayed MAP-kinase kinase-dependent increase in JNK-1 kinase activity. This enhanced JNK-1 kinase activity appears not to contribute directly to the Raf-induced cell cycle arrest in Schwann cells, since the co-expression of a dominant negative SEK-AL mutant did neither changed the amount of p21$^{WAF1/Cip1}$ protein induced by ΔRafER nor did it apparently alter the ability of NSΔRafER cells to arrest in the G1-phase of the cell-cycle arrest. Although these results seemed to indicate that the ΔRafER-dependent activation of JNK-1 kinase was not required for the induction of p21$^{WAF1/Cip1}$, it can not been excluded that the approximately 40% inhibition of JNK-1 kinase was not sufficient to abrogate the induction of p21$^{WAF1/Cip1}$ and the G1 cell cycle arrest. Alternative ways to inhibit JNK-1 kinase activity through a dominant negative JNK-1 molecule or experiments utilising antisense RNA may result in a stronger inhibition thereby clearly ruling out any possible involvement of JNK1-kinase in the induction of p21$^{WAF1/Cip1}$. The presented evidence currently suggest that it is unlikely that the observed JNK-1 kinase activity contributes to the induction of p21$^{WAF1/Cip1}$ in Schwann cells.

CHAPTER 7.0

Discussion

7.0   Discussion

7.1   Oncogene cooperation in Schwann cells

Mutants of the tumour suppressor p53 cooperate with activated Ras and activated Raf in transforming Schwann cells. Constitutive Raf-activity on its own results in G1 cell cycle arrest mediated by the induction of the cell cycle inhibitor p21$^{WAF1/Cip1}$ and a subsequent decrease in cyclin/cdk activity. The coexpression of dominant inhibitory mutants of p53 suppress the induction of p21$^{WAF1/Cip1}$ and enable Schwann cells to proliferate continuously. The cooperative potential of p53 mutants is unlikely to be due to a gain-of-function of p53 but rather reflects the reported dominant inhibitory function of mutant p53 over endogenous wild type p53 due to direct binding (Milner & Metcalf, 1991; Kern et al., 1992; Barnonetti et al., 1992). Thus, the suppression of the Raf-dependent induction of p21$^{WAF1/Cip1}$ by coexpression of mutant p53 offers a model of how cooperating oncogenes can overcome cell cycle control mechanisms.

7.2   Raf induces the CDK-inhibitor p21$^{WAF1/Cip1}$

Constitutive Raf-activity induces the cell cycle inhibitor p21$^{WAF1/Cip1}$ in Schwann cells (this thesis, Lloyd et al., 1997). p21$^{WAF1/Cip1}$ has been shown to interact (Nakanishi et al., 1995; Chen et al., 1996; Ball et al., 1997) with a number of G1 cyclin-dependent kinase complexes (Xiong et al., 1993). In particular, CDK2, CDK3, CDK4 and CDK6-dependent kinase activities are inhibited by p21$^{WAF1/Cip1}$ (Xiong et al., 1993). In addition, p21$^{WAF1/Cip1}$ prevents the activating phosphorylation steps of CDK2 and CDK6 by CDK-activating kinase (CAK) (Aprelikova et al., 1995). No direct interaction between CAK and p21$^{WAF1/Cip1}$ could be detected, suggesting

that the binding of p21$^{WAF1/Cip1}$ to CDK subunits renders CDK inaccessible to CAK-dependent phosphorylation (Aprelikova et al., 1995).

The inhibition of CDK-complex activity requires the binding of several p21$^{WAF1/Cip1}$ molecules to CDKs (Harper et al., 1995) which is in agreement with the finding that p21$^{WAF1/Cip1}$ protein levels are significantly increased in arrested Schwann cells and cyclin E and cyclin A-dependent kinase activity strongly reduced (this thesis; Lloyd et al., 1997). Since p21$^{WAF1/Cip1}$ is part of Cyclin-CDK complexes in growing cells (Xiong et al., 1993; Zhang et al., 1994a, 1994b; Harper et al., 1995), this finding did suggest that the number of bound p21$^{WAF1/Cip1}$ molecules determines whether CDK-activity is inhibited or not (Harper et al., 1995). Indeed, at low protein concentrations p21$^{WAF1/Cip1}$ facilitates the assembly of CDK4/Cyclin D complexes by decreasing the dissociation rate of already formed complexes and supporting the localisation of CDK4 and Cyclin D protein to the nucleus (LaBaer et al., 1997).

In arrested human fibroblasts high p21$^{WAF1/Cip1}$ protein levels can be observed compared to cells in S-phase (Nakanishi et al., 1995; Li et al., 1994) and overexpression of p21$^{WAF1/Cip1}$ leads to G1 cell cycle arrest (Harper et al., 1995). The coexpression of antisense p21$^{WAF1/Cip1}$ RNA results in abrogation of the growth arrest and S-phase entry (Nakanishi et al., 1995). Similarly, in arrested Schwann cells, lowering of p21$^{WAF1/Cip1}$ protein levels by coexpression of p21$^{WAF1/Cip1}$ antisense RNA leads to the abrogation of G1 cell cycle arrest (Lloyd et al., 1997), arguing that the p21$^{WAF1/Cip1}$ induction is causal to the G1 cell cycle arrest.

7.3 The p53-dependency of p21$^{WAF1/Cip1}$ induction in Schwann cells

In Schwann cells the abrogation of p53 function abolishes the induction of p21$^{WAF1/Cip1}$ protein levels and allows activated Raf to transform these cells (this thesis). The p21$^{WAF1/Cip1}$ gene has been described as a transcriptional target of p53 (El-Deiry et al., 1993), a notion supported by the existence of two conserved p53-binding sites in the p21$^{WAF1/Cip1}$ promoter (Dulic et al., 1994; Michieli et al., 1994; Macleod et al., 1995). Although abrogation of p21$^{WAF1/Cip1}$ induction by coexpressing mutant p53 suggests that p53 activates the p21$^{WAF1/Cip1}$ gene in Schwann cells, direct evidence for increased p53 transactivation activity could not be obtained. Experiments carried out so far could not clearly establish an increase of p21$^{WAF1/Cip1}$ transcription in arrested Schwann cells (A.Lloyd pers. comm.). Apart from transcriptional mechanisms, posttranslational mechanisms regulating the protein half-life may regulate p21$^{WAF1/Cip1}$. These mechanisms may also be occurring in response to constitutively active Raf in Schwann cells. In this respect, the recent discovery of a p73 protein which has a strong structural homology with p53, in particular with the DNA-binding domain (Kaghad et al., 1997) may possibly contribute towards Raf-induced p21$^{WAF1/Cip1}$ induction in Schwann cells. The overexpression of p73 led to the induction of p21$^{WAF1/Cip1}$ and effectively blocked colony formation of transformed cells (Kaghad et al., 1997; Jost et al., 1997). Since an interaction between p73β, a splice variant and p53 has been reported *in vitro* (Kaghad et al., 1997), a possible functional interaction *in vivo* could be negatively intercepted by mutant p53. The exact nature of p53-dependency and possible contribution of p73 in the induction of p21$^{WAF1/Cip1}$ in Schwann cells remains to be further investigated.

Various studies have revealed that the p21$^{WAF1/Cip1}$ induction is regulated in either p53-dependent or p53-independent ways. The regulation of p21$^{WAF1/Cip1}$ in a p53-independent manner has been observed in tissue development (Macleod et al., 1995), cell differentiation (Macleod et al., 1995; Steinman et al., 1994; Halevy et al., 1995; Skapek et al., 1995; Parker et al., 1995; Zeng & El-Deiry, 1996; Liu et al., 1996) and senescence (Noda et al., 1994; Serrano et al., 1997). Furthermore, p21$^{WAF1/Cip1}$ induction has been observed in response to serum stimulation (Macleod et al., 1995; Jiang et al., 1994; Sheikh et al., 1994; Li et al., 1994), interferon γ (Chin et al., 1996) and TGF-β application (Datto et al., 1995). Although it is not in all cases absolutely clear whether p53 is involved or not in p21$^{WAF1/Cip1}$ induction by the above mentioned stimuli, the induction in a p53-dependent manner has been mainly described after DNA damage by ionizing radiation or DNA-damaging agents (Dulic et al., 1994; Xiong et al., 1993a, b; El-Deiry et al., 1993, 1994; Di Leonardo et al., 1994; Brugarolas et al., 1995).

In Schwann cells is seems unlikely that constitutive Raf-activity results in DNA-damage since there are no signs of apoptosis in G1-arrested cells (Lloyd et al., 1997), although it can not be fully excluded. Furthermore, the p53-dependent induction of p21$^{WAF1/Cip1}$ and subsequent G1 cell cycle arrest does not appear to resemble the phenotype of differentiated Schwann cells nor is there any evidence for the induction of the P0 protein (A. Lloyd, unpublished observation) which is strictly associated with differentiation in Schwann cells (Owens & Boyd, 1991). It has been reported, that in fibroblasts oncogenic Ras results in a permanent G1 cell cycle arrest and these cells are phenotypically not distinguishable from senescent cells (Serrano et al., 1997). Although senescence markers have not been tested in G1-arrested Schwann cells, cells are viable but

permanently arrested as long as the constitutive Raf signal persists which suggests that premature senescence could have occurred. It has been suggested that since high p21$^{WAF1/Cip1}$ protein levels occur in senescent as well as DNA damaged cells, the progressively shortening of telomeres in senescence could be interpreted as a form of DNA damage, leading to a similar arrest mechanism (Stein & Dulic, 1995). However, there is evidence for a direct involvement of p53, since mutant p53 directly allows fibroblasts to escape from senescence (Bond et al., 1994; 1995).

The p53-dependent induction of p21$^{WAF1/Cip1}$ as a response to the constitutive action of a single oncogene may be a new feature of p53´s tumour suppressor function in that mitogenic signals required for cell proliferation are monitored by p53 and aberrant signals provoke a p53-dependent cell cycle arrest or senescence. In this respect, the growth factor-mediated transient stimulation of Raf activity may result in a form of p53 activation which allows cell-cycle progression, whereas continuous Raf-activity could strongly increase p53 transactivation activity which in turn would lead to the induction of target genes such as p21$^{WAF1/Cip1}$.

Interestingly, the p21$^{WAF1/Cip1}$ gene itself is rarely lost or mutated in tumours or cancer cell lines from various cell types (Shiohara et al.; 1994; Li et al., 1995), whereas mutations or deletions of the p53 gene occur within tumours with a high frequency (Hollstein et al., 1996). This finding has been supported by investigations of p53 or p21$^{WAF1/Cip1}$ nullizygous mice, respectively. Mice nullizygous for p21$^{WAF1/Cip1}$ develop normally (Deng et al., 1995), however, in these mice the G1 cell cycle arrest is strongly impaired in response to DNA damage (Deng et al., 1995). In contrast, p53 nullizygous mice are highly susceptible to tumorigenesis and die due to multiple tumours at an early stage (Donehower et al., 1992). Similarly, primary murine fibroblasts nullizygous for p53 are transformed by activated

Ras alone (Tanaka et al., 1994). Surprisingly, keratinocytes from p21$^{WAF1/Cip1}$ nullizygous mice are also aggressively transformed by activated Ras alone (Missero et al., 1996).

The apparent conflicting result that p21$^{WAF1/Cip1}$ mice can be easily transformed by introduction of activated Ras, although mutations of p21$^{WAF1/Cip1}$ in human cancer are relatively sporadic in comparison with p53 mutations could be explained through the multiple regulatory functions of p53. The inactivation of p53 will not only affect the transactivation activity and thereby the p21$^{WAF1/Cip1}$ induction, moreover, mutant p53 abrogates p53-dependent apoptosis which could be the limiting factor in progression of tumour growth. Other functions regarding the genomic stability such as a p53-dependent spindle checkpoint could also be affected (Cross et al., 1995) offering an explanation for the observed differences.

7.4 Signalling pathways regulating the induction of p21$^{WAF1/Cip1}$

The result that activated Ras and activated Raf cause cell cycle arrest in the G1 phase of the cell cycle in primary cells (this thesis; Lloyd et al., 1997; Serrano et al., 1997) raises the question of normal Ras and Raf function in cell proliferation and signal specificity. As shown in this thesis, MAP-kinase kinase activity is required to induce p21$^{WAF1/Cip1}$, arguing for the involvement of the Ras-MAP-kinase module in mediating the G1 cell cycle arrest. However, the activity of the Ras-MAP-kinase kinase module is also required for cell cycle progression in many cells (Mulcahy et al., 1985; Feig & Cooper, 1988; Moodie et al., 1993; Cowley et al., 1994) and appears to be also important for cell cycle progression in Schwann cells, since the expression of a dominant negative MAP-kinase kinase reduces the

percentage of cells in S-phase (this thesis). It is, therefore, surprising that the same signalling pathway appears to be sufficient for S-phase entry (Cowely et al., 1994) and can activated p53 and p21$^{WAF1/Cip1}$ resulting in G1 cell cycle arrest (this thesis, Lloyd et al., 1997). The question how presumably the same signalling pathway can mediate a mitogenic and a growth inhibitory effect could be explained by different kinetics of Raf activity, in particular duration and strength of the signal.

7.4.1   Duration of the signal initiated by activated Raf

The finding that members of the Ras-MAP-kinase signalling pathway can mediate a mitogenic or inhibitory cellular response could be explained by a difference in the duration of Raf-activity. MAP-kinase kinase, a downstream target of Raf-kinase, is required for the induction of p21$^{WAF1/Cip1}$, since inhibition of this kinase effects the induction of p21$^{WAF1/Cip1}$ (this thesis). Experiments with the MAP-kinase kinase specific inhibitor PD98059 suggest that the Raf-dependent induction of p21$^{WAF1/Cip1}$ is mediated via MAP-kinase kinase and the downstream target MAP-kinase. Furthermore, studies of the kinetics of MAP-kinase activation have shown that the induction of constitutively active Raf results in a continuous activation of MAP-kinase correlated with the induction of p21$^{WAF1/Cip1}$, whereas mitogenic stimulation is associated with a temporary activation for up to four hours and much lower protein levels of p21$^{WAF1/Cip1}$ (this thesis). Although prolonged MAP-kinase activation after Raf induction coincides with the G1 cell cycle arrest, it can, however, not be excluded that other so far unknown targets of MAP-kinase kinase mediate the induction of p21$^{WAF1/Cip1}$ in Schwann cells.

The phenomenon that a prolonged activation of a signalling pathway has a different biological outcome compared to a short activation is not

unprecedented. The differences in duration of activation of the Ras-MAP-kinase pathway has been suggested as an explanation for the different biological response of PC12 cells to either epidermal growth factor (EGF) or nerve growth factor (NGF) (Traverse et al., 1992). Addition of NGF or the prolonged activation of the Ras-MAP-kinase pathway by expression of activated Ras (Noda et al., 1985), activated Raf (Wood et al., 1993) and activated MAP-kinase kinase (Cowley et al., 1994) results in growth arrest followed by neurite outgrowth whereas a transient activation led to cell proliferation (Traverse et al., 1992).

7.4.2   Strength of the signal initiated by activated Raf

Differences in signal strength could be another possible explanation for the opposing cellular responses to oncogenic Raf and mitogenically activated Raf. For instance, constitutively active Raf leads to a mitogenic response in NIH3T3 cells (Kolch et al., 1991; Smith et al., 1990; Pritchard et al., 1995). Nevertheless, it seems that the strength of the signal is significant for this cellular response. In NIH3T3 cells, a strong Raf signal leads to G1 cell cycle arrest due to the induction of p21$^{WAF1/Cip1}$ accompanied by inhibition of cyclin E and D-dependent kinase complexes (Sewing et al., 1997; Woods et al., 1997). However, the induction of p21$^{WAF1/Cip1}$ by constitutively active Raf is p53-independent, since it also occurs in p53$^{-/-}$ mouse embryo fibroblasts (Sewing et al., 1997). The strength of the inducible Raf-signal has been correlated with the degree of MAP-kinase activation with a weak signal sufficient for proliferation while a strong Raf-signal causes G1- growth arrest (Sewing et al., 1997). In support for this hypothesis, the induction of ΔA-RafER in NIH3T3 cells led to a weak activation of MAP-kinase activity and entry into DNA synthesis, whereas ΔRaf-1ER and ΔB-RafER strongly activate MAP-kinase but were insufficient

to promote the entry of the NIH3T3 cells into DNA synthesis (Pritchard et al.,

1995). Interestingly, it has been reported that the strength of the signal

initiated by activated ΔRafER increases 10fold over 16 hours in NIH3T3

cells (Samuels et al., 1993). This increase of signal has been partly

attributed to the selective stabilisation of the ΔRaf-1ER fusion protein

(Samuels et al., 1993) or the ΔB-Raf and ΔA-RafER fusion proteins

(Pritchard et al., 1995).

Different cellular responses to a strong or weak signal have been

reported in other cell systems. In A431 human squamous carcinoma cells

picomolar EGF concentrations stimulate cell proliferation whereas

nanomolar EGF concentrations inhibit cell proliferation (Fan et al., 1995;

Jakus & Yeudall, 1996). The EGF-mediated inhibition of cell proliferation is

correlated with a rapid p21$^{WAF1/Cip1}$ induction (Fan et al., 1995), increased

binding to cyclin-CDK complexes and subsequent inhibition of CDK2/6-

dependent kinase activity as well as cyclin A and cyclin E associated kinase

activity (Jakus & Yeudall, 1996; Fan et al., 1995). Since A431 cells express

a mutant p53 (Somers et al., 1992), the EGF-mediated p21$^{WAF1/Cip1}$

induction appears to be p53-independent.

In developmental processes, the strength of a signal can determine

the cell fate of precursor cells. The Ras involvement in a number of

developmental systems suggests that the strength of Ras activity mediates

different responses. For instance, in the fungus *Aspergillus nidulans* high

Ras activity is associated with nuclear division but the development stops at

an early step of germ tube formation. At intermediate Ras activity aerial

hypha formation is inhibited and low Ras activity inhibits the formation of

conidiophores (Som & Kolaparthi, 1994).

The distinction between a strong signal and a prolonged signal may

,however, be artificial. The generation of a strong signal, for instance, due to

the overexpression of a receptor may lead to a stronger signal which at the same time may be prolonged when measured downstream of the receptor, since the inactivation mechanisms, for example, through phosphatase activity may not be sufficient to down-regulated a stronger signal. For example, the introduction of exogenous EGF-receptors into NIH3T3 cells increases cell proliferation and the transformation frequency in response to EGF (Velu et al., 1989). In PC12 cells, additional EGF-receptors convert a proliferative response into differentiation, usually only observed after NGF addition (Traverse et al., 1994).

7.5　　　Cyclin D1 induction in response to activated Raf

In Schwann cells, constitutive active Raf strongly induces cyclin D1 protein levels as early as 3 hours after Raf-induction (Lloyd et al., 1997). The induction of cyclin D has also been previously reported in cells expressing activated Ras (Liu et al., 1995; Winston et al., 1996). Although cyclin D1 induction by activated Ras via the MAP-kinase pathway (Lavoie et al., 1996) has been correlated with cell cycle progression in that it shortens the G1-phase (Filmus et al., 1994; Liu et al., 1995; Winston et al., 1996), in Schwann cells cyclin D1 induction does not prevent the G1 cell cycle arrest (Lloyd et al., 1997).

The strong induction of cyclin D1 on its own (Baldin et al., 1993; Pagano et al., 1994; Atajada et al., 1995) or in conjunction with p21$^{WAF1/Cip1}$ (DeSal et al., 1996) has also been associated with growth inhibition. In Schwann cells, however, the Raf-induced up-regulation of cyclin D1 is not likely to contribute to the G1 cell cycle arrest, since arrested Schwann cells and proliferating Schwann cells coexpressing mutant p53 exhibit comparable levels of cyclin D1 protein in response to Raf activity

(this thesis; Lloyd et al., 1997), also indicating that cyclin D1 induction is independent of p53 activity. The G1 cell cycle arrest in Schwann cells is characterised by the inhibition of cyclin E-dependent kinase activity through binding by the CDK-inhibitor p21$^{WAF1/Cip1}$ (Lloyd et al., 1997). The increase in p21$^{WAF1/Cip1}$ protein may also be sufficient for the inhibition of cyclin D1-dependent kinase activity so that the induction of cyclin D1 does not result in an increase of cyclin D-dependent kinase activity.

7.6   Autocrine factors and the JNK-kinase pathway

The p53-dependent induction of p21$^{WAF1/Cip1}$ is characterised by a delayed increase of p21$^{WAF1/Cip1}$ protein levels (this thesis). This observation may point to the involvement of a feedback loop of a secreted autocrine factor. As observed in Schwann cells, in NIH3T3 cells MAP-kinase kinase and MAP-kinase were rapidly activated by ΔRaf-1ER induction (Samuels et al., 1993), whereas the strong activation of JNK-kinase was delayed (McCarty et al., 1995). In NIH3T3 cells, the delayed activation of JNK-kinase was due to the ΔRafER-dependent increase of the heparin-binding epidermal growth factor (HB-EGF) secretion, since antibodies against HB-EGF abolish the activation of JNK-kinase (McCarthy et al., 1995).

In Schwann cells, my results have shown that JNK-1 kinase activity is strongly enhanced in response to ΔRafER induction and it is possible that this delayed activation of JNK-1 kinase is also due to the secretion of HB-EGF or another factor. In general, Schwann cells have been reported to secrete a number of factors (Rogister et al., 1993; Eccleston et al., 1991, Krystosek et al., 1984) and constitutively active Raf leads to the secretion of

an unknown factor or factors with a positive effect on cell proliferation of Swiss3T3 but not on Schwann cells (A.Lloyd, unpublished observation).

JNK-1 kinase has been reported to phosphorylate p53 (Milne et al., 1995). Furthermore, the p53-dependent induction of p21$^{WAF1/Cip1}$ coincides with the strongest JNK-1 kinase activity in Schwann cells (this thesis). Despite this intriguing coincidence, a direct cause-effect relationship between these two events has not been established. The co-expression of dominant negative JNK-1 molecules reduces endogenous JNK-1 kinase activity but failed to delay or reduce p21$^{WAF1/Cip1}$ induction (this thesis). In summary, it cannot be excluded that the induction of p21$^{WAF1/Cip1}$ is mediated via JNK-1 kinase since the experimental approaches used do not sufficiently address this question.

7.7   Cellular responses to a single oncogene or cooperating oncogenes

The presented results in Schwann cells provide important evidence for a crucial role for CDK-inhibitors in protecting against the growth promoting effects of single oncogenes in primary cells. The induction of a cell cycle inhibitor in response to oncogenes is not restricted to Schwann cells but has been found in other cell types via the induction of different cell cycle inhibitors. For instance, constitutively Ras-activity arrests primary human fibroblasts via the induction of p16 and p53 (Serrano et al., 1997). Interestingly, the observed arrest of human fibroblasts in response to activated Ras is indistinguishable from cellular senescence (Serrano et al., 1997), suggesting that senescence may not entirely dependent on the lifespan of cells (Stein & Dulic, 1995) but can be also occur as a response to a single oncogene such as *Ha-ras* (Serrano et al., 1997). Similarly,

oncogenic Raf may also lead to senescence in Schwann cells, although the expression of senescence markers has not been tested.

The cooperation between oncogenic Raf and mutant p53 due to the suppression of p21$^{WAF1/Cip1}$ protein induction in Schwann cells allows cellular transformation to occur. Similarly, the suppression of other CDK-inhibitors enable cooperating oncogenes to transform other cell types, indicating that the described molecular mechanism of cooperation may be of general relevance. For instance, the adenovirus E1A protein has been found to block the growth inhibitory effects of the CDK-inhibitor p27^{Kip1} through direct binding (Mai et al., 1996). The adenovirus E1A protein can also overcome the suppression of cell transformation by the CDK-inhibitor p16^{INK4A} (Serrano et al., 1995). Moreover, Ras or Raf alone can transform primary fibroblasts which have lost the INK4A tumour suppressor locus, understood to encode p16^{INK4A} (Serrano et al., 1996) or in keratinocytes, the p21$^{WAF1/Cip1}$ gene (Missero et al., 1996). These examples suggest that similarly to Raf and mutant p53 in Schwann cells, a reduction in cyclin/CDK inhibitory protein levels reveals the transforming potential of Raf or Ras. Thus, the induction of cellular senescence or cell cycle arrest can be understood as mechanism by which organisms can protect themselves from uncontrolled proliferation initiated by activated oncogenes with additional mutations required for uncontrolled cell proliferation to occur.

7.8   Schwann cells as model for neurofibromatosis

Neurofibromatosis type 1 is a human genetic disorder affecting the peripheral nervous system and various organs (Riccardi 1992). NF-1 patients are susceptible to developing benign neurofibromas which consist mainly of Schwann cells and fibroblasts. The loss of the neurofibromin

gene, a GTPase activating protein of Ras (Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990a, b) has been associated with the development of neurofibromas and neurofibrosarcomas (Wallace et al., 1990; Li et al., 1992; The et al., 1993; Shannon et al., 1994). In fact, malignant Schwann cell lines derived from NF1-patients exhibit very low protein levels of neurofibromin which has been shown to correlate with an increase of Ras in the active state (Basu et al., 1992; DeClue et al., 1992). Furthermore, murine Schwann cells deficient in *NF-1* are angiogenic and invasive (Sheela et al., 1990; Kim et al., 1997). These studies suggest that the lack of neurofibromin function and increase of Ras activity in Schwann cells contribute to the formation of neurofibromas.

The absence of the *NF-1* gene in primary murine Schwann cells, however, results in elevated Ras activity and inhibition of cell proliferation (Kim et al., 1995). Similarly, high Ras/Raf activity in primary Schwann cells inhibit their growth (Ridley et al., 1988; Lloyd et al., 1997; this thesis). In contrast, the introduction of activated Ras into the Schwann cell line RN-22 (Kim et al., 1995) as well as high Ras activity malignant Schwannomas in cells from NF-1 patients increase cell proliferation (Basu et al., 1992; DeClue et al., 1992). This increase in cell proliferation was inhibited by lowering the amount of active Ras by injection of an neutralising Ras-antibody or the increase of Ras-GAP activity (Basu et al., 1992; DeClue et al., 1992). The difference in cell growth to constitutively Ras activity could be explained by the accumulation of additional mutations in Schwannomas cells or the Schwann cell line RN-22 which will alter the cellular response to Ras. For instance, mutations in the tumour suppressor gene *p53* alter the cellular response to constitutive Ras or Raf activity towards increased cell proliferation and transformation (this thesis). In support of this idea, p53 mutations have been described in neurofibrosarcomas from patients with

neurofibromatosis type 1 (Menon et al., 1990). Moreover, a cooperative effect between the loss of the *NF-1* gene, leading to high Ras activity (Kim et al., 1995), and mutants of p53 has been shown in a transgenic mice model (Cichowski et al., 1996). Likewise, a SV40 LT transgene expressed in Schwann cells led to the development of neurocristopathies which resemble neurofibromatosis type 1, indicating that LT's various function, such as inactivating p53, can contribute towards tumour development in Schwann cells (Mazarakis et al., 1996). The expression of SV40 LT in our Schwann cell model also cooperates strongly with activated Ras and Raf (Ridley et al., 1988; Lloyd et al., 1997). Thus, Schwann cells expressing inducible activated Raf will be useful in further investigations addressing the molecular mechanisms of neurofibromatosis.

Bibliography

Addison C, Jenkins JR & Sturzbecher HW: The p53 nuclear localisation signal is structurally linked to a p34cdc2 kinase motif. Oncogene 1990, 5:423-6.

Agoff SN, Hou J, Linzer DI & Wu B: Regulation of the human hsp70 promoter by p53. Science 1993, 259:84-7.

Ahn NG, Robbins DJ, Haycock JW, Seger R, Cobb MH & Krebs EG: Identification of an activator of the microtubule-associated protein 2 kinases ERK1 and ERK2 in PC12 cells stimulated with nerve growth factor or bradykinin. J Neurochem 1992, 59:147-56.

Alessi DR, Smythe C & Keyse SM: The human CL100 gene encodes a Tyr/Thr-protein phosphatase which potently and specifically inactivates MAP kinase and suppresses its activation by oncogenic ras in Xenopus oocyte extracts. Oncogene 1993, 8:2015-20.

Alessi DR, Saito Y, Campbell DG, Cohen P, Sithanandam G, Rapp U, Ashworth A, Marshall CJ & Cowley S: Identification of the sites in MAP kinase kinase-1 phosphorylated by p74raf-1. Embo J 1994, 13:1610-9.

Alessi DR, Cohen P, Ashworth A, Cowley S, Leevers SJ & Marshall CJ: Assay and expression of mitogen-activated protein kinase, MAP kinase kinase, and Raf. Methods Enzymol 1995, 255:279-90.

Alessi DR, Cuenda A, Cohen P, Dudley DT & Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 1995, 270:27489-94.

Alessi DR, Gomez N, Moorhead G, Lewis T, Keyse SM & Cohen P: Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr Biol 1995, 5:283-95.

Alessi DR, Cuenda A, Cohen P, Dudley DT & Saltiel AR: PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. Journal of Biological Chemistry 1995, 270:27489-94.

Allen JD & Berns A: Complementation tagging of cooperating oncogenes in knockout mice. Seminars in Cancer Biology 1996, 7:299-306.

Almog N, Li R, Peled A, Schwartz D, Wolkowicz R, Goldfinger N, Pei H & Rotter V: The murine C'-terminally alternatively spliced form of p53 induces attenuated apoptosis in myeloid cells. Mol Cell Biol 1997, 17:713-22.

Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N & Perucho M: Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988, 53:549-54.

Alvarez E, Northwood IC, Gonzalez FA, Latour DA, Seth A, Abate C, Curran T & Davis RJ: Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. Characterization of the phosphorylation of c-myc and c-jun proteins by an epidermal growth factor receptor threonine 669 protein kinase. J Biol Chem 1991, 266:15277-85.

Am dS, Burgering BM, Leevers SJ, Marshall CJ & Bos JL: Involvement of p21ras in activation of extracellular signal-regulated kinase 2. Nature 1992, 357:602-4.

Ammerer G: Sex, stress and integrity: the importance of MAP kinases in yeast. Current Opinion in Genetics & Development 1994, 4:90-5.

Anderson NG, Maller JL, Tonks NK & Sturgill TW: Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. Nature 1990, 343:651-3.

Anderson NG, Li P, Marsden LA, Williams N, Roberts TM & Sturgill TW: Raf-1 is a potential substrate for mitogen-activated protein kinase in vivo. Biochem J 1991, 345-49.

Aprelikova O, Xiong Y & Liu ET: Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase. Journal of Biological Chemistry 1995, 270:18195-7.

Arai N, Nomura D, Yokota K, Wolf D, Brill E, Shohat O & Rotter V: Immunologically distinct p53 molecules generated by alternative splicing. Mol Cell Biol 1986, 6:3232-9.

Aroian RV, Koga M, Mendel JE, Ohshima Y & Sternberg PW: The let-23 gene necessary for Caenorhabditis elegans vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. Nature 1990, 348:693-9.

Aronheim A, Engelberg D, Li N, al AN, Schlessinger J & Karin M: Membrane targeting of the nucleotide exchange factor Sos is sufficient for activating the Ras signaling pathway. Cell 1994, 78:949-61.

Askew DS, Ihle JN & Cleveland JL: Activation of apoptosis associated with enforced myc expression in myeloid progenitor cells is dominant to the suppression of apoptosis by interleukin-3 or erythropoietin. Blood 1993, 82:2079-87.

Attardi LD, Lowe SW, Brugarolas J & Jacks T: Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis. Embo J 1996, 15:3693-701.

Aziz N, Wu J, Dubendorff JW, Lipsick JS, Sturgill TW & Bender TP: c-Myb and v-Myb are differentially phosphorylated by p42mapk in vitro. Oncogene 1993, 8:2259-65.

Bacus SS, Yarden Y, Oren M, Chin DM, Lyass L, Zelnick CR, Kazarov A, Toyofuku W, Gray BJ, Beerli RR, Hynes NE, Nikiforov M, Haffner R, Gudkov A & Keyomarsi K: Neu differentiation factor (Heregulin) activates a p53-dependent pathway in cancer cells. Oncogene 1996, 12:2535-47.

Baker SJ, Markowitz S, Fearon ER, Willson JK & Vogelstein B: Suppression of human colorectal carcinoma cell growth by wild-type p53. Science 1990, 249:912-5.

Baker SJ, Kerppola TK, Luk D, Vandenberg MT, Marshak DR, Curran T & Abate C: Jun is phosphorylated by several protein kinases at the same sites that are modified in serum-stimulated fibroblasts. Mol Cell Biol 1992, 12:4694-705.

Baldin V, Lukas J, Marcote MJ, Pagano M & Draetta G: Cyclin D1 is a nuclear protein required for cell cycle progression in G1. Genes & Development 1993, 7:812-21.

Ball KL, Lain S, Fahraeus R, Smythe C & Lane DP: Cell-cycle arrest and inhibition of Cdk4 activity by small peptides based on the carboxy-terminal domain of p21WAF1. Curr Biol 1997, 7:71-80.

Ballester R, Marchuk D, Boguski M, Saulino A, Letcher R, Wigler M & Collins F: The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. Cell 1990, 63:851-9.

Baltensperger K, Kozma LM, Cherniack AD, Klarlund JK, Chawla A, Banerjee U & Czech MP: Binding of the Ras activator son of sevenless to insulin receptor substrate-1 signaling complexes. Science 1993, 260:1950-2.

Bar SD & Feramisco JR: Microinjection of the ras oncogene protein into PC12 cells induces morphological differentiation. Cell 1985, 42:841-8.

Bar SD, Rotin D, Batzer A, Mandiyan V & Schlessinger J: SH3 domains direct cellular localization of signaling molecules. Cell 1993, 74:83-93.

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Barak Y & Oren M: Enhanced binding of a 95 kDa protein to p53 in cells undergoing p53-mediated growth arrest. Embo J 1992, 11:2115-21.

Barak Y, Juven T, Haffner R & Oren M: mdm2 expression is induced by wild type p53 activity. Embo J 1993, 12:461-8.

Barak Y, Gottlieb E, Juven GT & Oren M: Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes Dev 1994, 8:1739-49.

Barbeau D, Marcellus RC, Bacchetti S, Bayley ST & Branton PE: Quantitative analysis of regions of adenovirus E1A products involved in interactions with cellular proteins. Biochemistry & Cell Biology 1992, 70:1123-34.

Bardeesy N, Beckwith JB & Pelletier J: Clonal expansion and attenuated apoptosis in Wilms' tumors are associated with p53 gene mutations. Cancer Res 1995, 55:215-9.

Bargonetti J, Friedman PN, Kern SE, Vogelstein B & Prives C: Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell 1991, 65:1083-91.

Bargonetti J, Reynisdottir I, Friedman PN & Prives C: Site-specific binding of wild-type p53 to cellular DNA is inhibited by SV40 T antigen and mutant p53. Genes Dev 1992, 6:1886-98.

Bargonetti J, Manfredi JJ, Chen X, Marshak DR & Prives C: A proteolytic fragment from the central region of p53 has marked sequence-specific DNA-binding activity when generated from wild-type but not from oncogenic mutant p53 protein. Genes Dev 1993, 7:2565-74.

Barnard D, Diaz B, Hettich L, Chuang E, Zhang XF, Avruch J & Marshall M: Identification of the sites of interaction between c-Raf-1 and Ras-GTP. Oncogene 1995, 10:1283-90.

Bartek J, Iggo R, Gannon J & Lane DP: Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. Oncogene 1990, 5:893-9.

Basu TN, Gutmann DH, Fletcher JA, Glover TW, Collins FS & Downward J: Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients . Nature 1992, 356:713-5.

Baudier J, Delphin C, Grunwald D, Khochbin S & Lawrence JJ: Characterization of the tumor suppressor protein p53 as a protein kinase C substrate and a S100b-binding protein. Proc Natl Acad Sci U S A 1992, 89:11627-31.

Bayle JH, Elenbaas B & Levine AJ: The carboxyl-terminal domain of the p53 protein regulates sequence-specific DNA binding through its nonspecific nucleic acid-binding activity. Proc Natl Acad Sci U S A 1995, 92:5729-33.

Beer RP, Glass S & Rolfe M: Antisense targeting of E6AP elevates p53 in HPV-infected cells but not in normal cells. Oncogene 1997, 14:595-602.

Beitel GJ, Clark SG & Horvitz HR: Caenorhabditis elegans ras gene let-60 acts as a switch in the pathway of vulval induction. Nature 1990, 348:503-9.

Beitel GJ, Tuck S, Greenwald I & Horvitz HR: The Caenorhabditis elegans gene lin-1 encodes an ETS-domain protein and defines a branch of the vulval induction pathway. Genes Dev 1995, 9:3149-62.

Berns A: Tumorigenesis in transgenic mice: identification and characterization of synergizing oncogenes. Journal of Cellular Biochemistry 1991, 47:130-5.

Bienz B, Zakut HR, Givol D & Oren M: Analysis of the gene coding for the murine cellular tumour antigen p53. Embo J 1984, 3:2179-83.

Biggs Wr, Zavitz KH, Dickson B, van dSA, Brunner D, Hafen E & Zipursky SL: The Drosophila rolled locus encodes a MAP kinase required in the sevenless signal transduction pathway. Embo J 1994, 13:1628-35.

Bischoff JR, Friedman PN, Marshak DR, Prives C & Beach D: Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2. Proc Natl Acad Sci U S A 1990, 87:4766-70.

Blagosklonny MV, Wu GS, Omura S & el DW: Proteasome-dependent regulation of p21WAF1/CIP1 expression. Biochem Biophys Res Commun 1996, 227:564-9.

Blenis J, Chung J, Erikson E, Alcorta DA & Erikson RL: Distinct mechanisms for the activation of the RSK kinases/MAP2 kinase/pp90rsk and pp70-S6 kinase signaling systems are indicated by inhibition of protein synthesis. Cell Growth Differ 1991, 2:279-85.

Bodrug SE, Warner BJ, Bath ML, Lindeman GJ, Harris AW & Adams JM: Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. Embo Journal 1994, 13:2124-30.

Boguski MS & McCormick F: Proteins regulating Ras and its relatives. Nature 1993, 366:643-54.

Bollag G & McCormick F: GTPase activating proteins. Semin Cancer Biol 1992, 3:199-208.

Bollag G, McCormick F & Clark R: Characterization of full-length neurofibromin: tubulin inhibits Ras GAP activity. Embo J 1993, 12:1923-7.

Bollag G, Clapp DW, Shih S, Adler F, Zhang YY, Thompson P, Lange BJ, Freedman MH, McCormick F, Jacks T & Shannon K: Loss of NF1 results in activation of the Ras signaling pathway and leads to aberrant growth in haematopoietic cells. Nat Genet 1996, 12:144-8.

Bond JA, Wyllie FS & Wynford TD: Escape from senescence in human diploid fibroblasts induced directly by mutant p53. Oncogene 1994, 9:1885-9.

Bond JA, Blaydes JP, Rowson J, Haughton MF, Smith JR, Wynford TD & Wyllie FS: Mutant p53 rescues human diploid cells from senescence without inhibiting the induction of SDI1/WAF1. Cancer Research 1995, 55:2404-9.

Bos JL: ras oncogenes in human cancer: a review. Cancer Research 1989, 49:4682-9.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH & Yancopoulos GD: ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 1991, 65:663-75.

Bowman T, Symonds H, Gu L, Yin C, Oren M & Van DT: Tissue-specific inactivation of p53 tumor suppression in the mouse. Genes Dev 1996, 10:826-35.

Braithwaite AW, Sturzbecher HW, Addison C, Palmer C, Rudge K & Jenkins JR: Mouse p53 inhibits SV40 origin-dependent DNA replication. Nature 1987, 329:458-60.

Braithwaite A, Nelson C, Skulimowski A, McGovern J, Pigott D & Jenkins J: Transactivation of the p53 oncogene by E1a gene products. Virology 1990, 177:595-605.

Braithwaite AW, Blair GE, Nelson CC, McGovern J & Bellett AJ: Adenovirus E1b-58 kD antigen binds to p53 during infection of rodent cells: evidence for an N-terminal binding site on p53. Oncogene 1991, 6:781-7.

Brondello JM, McKenzie FR, Sun H, Tonks NK & Pouyssegur J: Constitutive MAP kinase phosphatase (MKP-1) expression blocks G1 specific gene transcription and S-phase entry in fibroblasts. Oncogene 1995, 10:1895-904.

Brondello JM, Brunet A, Pouyssegur J & McKenzie FR: The dual specificity mitogen-activated protein kinase phosphatase-1 and -2 are induced by the p42/p44MAPK cascade. J Biol Chem 1997, 272:1368-76.

Brugarolas J, Chandrasekaran C, Gordon JI, Beach D, Jacks T & Hannon GJ: Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature 1995, 377:552-7.

Brunet A, Pages G & Pouyssegur J: Constitutively active mutants of MAP kinase kinase (MEK1) induce growth factor-relaxation and oncogenicity when expressed in fibroblasts. Oncogene 1994, 9:3379-87.

Brunet A, Pages G & Pouyssegur J: Growth factor-stimulated MAP kinase induces rapid retrophosphorylation and inhibition of MAP kinase kinase (MEK1). Febs Lett 1994, 346:299-303.

Brunet A & Pouyssegur J: Identification of MAP kinase domains by redirecting stress signals into growth factor responses. Science 1996, 272:1652-5.

Brunner D, Oellers N, Szabad J, Biggs Wr, Zipursky SL & Hafen E: A gain-of-function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signaling pathways. Cell 1994, 76:875-88.

Bryant SS, Briggs S, Smithgall TE, Martin GA, McCormick F, Chang JH, Parsons SJ & Jove R: Two SH2 domains of p120 Ras GTPase-activating protein bind synergistically to tyrosine phosphorylated p190 Rho GTPase-activating protein. Journal of Biological Chemistry 1995, 270:17947-52.

Buckbinder L, Talbott R, Velasco MS, Takenaka I, Faha B, Seizinger BR & Kley N: Induction of the growth inhibitor IGF-binding protein 3 by p53. Nature 1995, 377:646-9.

Buday L & Downward J: Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2 adapter protein, and Sos nucleotide exchange factor. Cell 1993, 73:611-20.

Buday L & Downward J: Epidermal growth factor regulates the exchange rate of guanine nucleotides on p21ras in fibroblasts. Mol Cell Biol 1993, 13:1903-10.

Buday L, Egan SE, Rodriguez VP, Cantrell DA & Downward J: A complex of Grb2 adaptor protein, Sos exchange factor, and a 36-kDa membrane-bound tyrosine phosphoprotein is implicated in ras activation in T cells. J Biol Chem 1994, 269:9019-23.

Buday L, Warne PH & Downward J: Downregulation of the Ras activation pathway by MAP kinase phosphorylation of Sos. Oncogene 1995, 11:1327-31.

Burgering BM, de VSA, Medema RH, van WP, Tertoolen LG & Bos JL: Epidermal growth factor induces phosphorylation of extracellular signal-regulated kinase 2 via multiple pathways. Mol Cell Biol 1993, 13:7248-56.

Burgering BM & Coffer PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature 1995, 376:599-602.

Canman CE, Wolff AC, Chen CY, Fornace AJ & Kastan MB: The p53-dependent G1 cell cycle checkpoint pathway and ataxia-telangiectasia. Cancer Res 1994, 54:5054-8.

Canman CE, Gilmer TM, Coutts SB & Kastan MB: Growth factor modulation of p53-mediated growth arrest versus apoptosis. Genes Dev 1995, 9:600-11.

Carnero A, Cuadrado A, del PL & Lacal JC: Activation of type D phospholipase by serum stimulation and ras-induced transformation in NIH3T3 cells. Oncogene 1994, 9:1387-95.

Carpino N, Wisniewski D, Strife A, Marshak D, Kobayashi R, Stillman B & Clarkson B: p62(dok): a constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell 1997, 88:197-204.

Carthew RW & Rubin GM: seven in absentia, a gene required for specification of R7 cell fate in the Drosophila eye. Cell 1990, 63:561-77.

Chang F & Herskowitz I: Phosphorylation of FAR1 in response to alpha-factor: a possible requirement for cell-cycle arrest. Molecular Biology of the Cell 1992, 3:445-50.

Chang HC, Karim FD, O'Neill EM, Rebay I, Solomon NM, Therrien M, Wassarman DA, Wolff T & Rubin GM: Ras signal transduction pathway in Drosophila eye development. Cold Spring Harbor Symposia on Quantitative Biology 1994, 59:147-53.

Chang HC & Rubin GM: 14-3-3 epsilon positively regulates Ras-mediated signaling in Drosophila. Genes & Development 1997, 11:1132-9.

Chardin P, Camonis JH, Gale NW, van AL, Schlessinger J, Wigler MH & Bar SD: Human Sos1: a guanine nucleotide exchange factor for Ras that binds to GRB2. Science 1993, 260:1338-43.

Charles CH, Sun H, Lau LF & Tonks NK: The growth factor-inducible immediate-early gene 3CH134 encodes a protein-tyrosine-phosphatase. Proc Natl Acad Sci U S A 1993, 90:5292-6.

Chen RH, Sarnecki C & Blenis J: Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Molecular & Cellular Biology 1992, 12:915-27.

Chen RH, Abate C & Blenis J: Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc Natl Acad Sci U S A 1993, 90:10952-6.

Chen J, Marechal V & Levine AJ: Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol 1993, 13:4107-14.

Chen JY, Funk WD, Wright WE, Shay JW & Minna JD: Heterogeneity of transcriptional activity of mutant p53 proteins and p53 DNA target sequences. Oncogene 1993, 8:2159-66.

Chen J, Lin J & Levine AJ: Regulation of transcription functions of the p53 tumor suppressor by the mdm-2 oncogene. Mol Med 1995, 1:142-52.

Chen X, Bargonetti J & Prives C: p53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. Cancer Res 1995, 55:4257-63.

Chen J, Jackson PK, Kirschner MW & Dutta A: Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. Nature 1995, 374:386-8.

Chen RH, Juo PC, Curran T & Blenis J: Phosphorylation of c-Fos at the C-terminus enhances its transforming activity. Oncogene 1996, 12:1493-502.

Chen J, Wu X, Lin J & Levine AJ: mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol Cell Biol 1996, 16:2445-52.

Chen X, Ko LJ, Jayaraman L & Prives C: p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. Genes Dev 1996, 10:2438-51.

Chen J, Saha P, Kornbluth S, Dynlacht BD & Dutta A: Cyclin-binding motifs are essential for the function of p21CIP1. Mol Cell Biol 1996, 16:4673-82.

Cherniack AD, Klarlund JK & Czech MP: Phosphorylation of the Ras nucleotide exchange factor son of sevenless by mitogen-activated protein kinase. J Biol Chem 1994, 269:4717-20.

Chin KV, Ueda K, Pastan I & Gottesman MM: Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. Science 1992, 255:459-62.

Chin YE, Kitagawa M, Su WC, You ZH, Iwamoto Y & Fu XY: Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1. Science 1996, 272:719-22.

Cho Y, Gorina S, Jeffrey PD & Pavletich NP: Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. Science 1994, 265:346-55.

Chou SY, Baichwal V & Ferrell JJ: Inhibition of c-Jun DNA binding by mitogen-activated protein kinase. Mol Biol Cell 1992, 3:1117-30.

Chowdary DR, Dermody JJ, Jha KK & Ozer HL: Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. Mol Cell Biol 1994, 14:1997-2003.

Christofori G, Naik P & Hanahan D: A second signal supplied by insulin-like growth factor II in oncogene-induced tumorigenesis. Nature 1994, 369:414-8.

Christofori G, Naik P & Hanahan D: Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during beta-cell tumorigenesis. Nature Genetics 1995, 10:196-201.

Chu Y, Solski PA, Khosravi FR, Der CJ & Kelly K: The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity in vivo toward the ERK2 sevenmaker mutation. J Biol Chem 1996, 271:6497-591.

Chumakov AM, Miller CW, Chen DL & Koeffler HP: Analysis of p53 transactivation through high-affinity binding sites. Oncogene 1993, 8:3005-11.

Chung J, Chen RH & Blenis J: Coordinate regulation of pp90rsk and a distinct protein-serine/threonine kinase activity that phosphorylates recombinant pp90rsk in vitro. Mol Cell Biol 1991, 11:1868-74.

Chung J, Pelech SL & Blenis J: Mitogen-activated Swiss mouse 3T3 RSK kinases I and II are related to pp44mpk from sea star oocytes and participate in the regulation of pp90rsk activity. Proc Natl Acad Sci U S A 1991, 88:4981-5.

Chung J, Kuo CJ, Crabtree GR & Blenis J: Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell 1992, 69:1227-36.

Chung J, Grammer TC, Lemon KP, Kazlauskas A & Blenis J: PDGF- and insulin-dependent pp70S6k activation mediated by phosphatidylinositol-3-OH kinase. Nature 1994, 370:71-5.

Cichowski K, Shih TS & Jacks T: Nf1 gene targeting: toward models and mechanisms.]Seminars in Cancer Biology 1996, 7:291-8.

Clark SG, Stern MJ & Horvitz HR: C. elegans cell-signalling gene sem-5 encodes a protein with SH2 and SH3 domains. Nature 1992, 356:340-4.

Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML & Wyllie AH: Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993, 362:849-52.

Clarke AR: The role of the tumor suppressor genes p53 and Rb-1 in development and cell death. Birth Defects Orig Artic Ser 1996, 30:261-8.

Clore GM, Omichinski JG, Sakaguchi K, Zambrano N, Sakamoto H, Appella E & Gronenborn AM: High-resolution structure of the oligomerization domain of p53 by multidimensional NMR. Science 1994, 265:386-91.

Conklin DS, Galaktionov K & Beach D: 14-3-3 proteins associate with cdc25 phosphatases. Proc Natl Acad Sci U S A 1995, 92:7892-6.

Cook SJ & McCormick F: Inhibition by cAMP of Ras-dependent activation of Raf. Science 1993, 262:1069-72.

Corbalan GS, Yang SS, Degenhardt KR & Bar SD: Identification of the mitogen-activated protein kinase phosphorylation sites on human Sos1 that regulate interaction with Grb2. Mol Cell Biol 1996, 16:5674-82.

Cordon CC, Zhang ZF, Dalbagni G, Drobnjak M, Charytonowicz E, Hu SX, Xu HJ, Reuter VE & Benedict WF: Cooperative effects of p53 and pRB alterations in primary superficial bladder tumors. Cancer Res 1997, 57:1217-21.

Cowell JK: The nuclear oncoproteins: RB and p53. Seminars in Cancer Biology 1990, 1:437-46.

Cowley S, Paterson H, Kemp P & Marshall CJ: Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 1994, 77:841-52.

Cox LS, Hupp T, Midgley CA & Lane DP: A direct effect of activated human p53 on nuclear DNA replication. Embo J 1995, 14:2099-105.

Crespo P, Xu N, Simonds WF & Gutkind JS: Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature 1994, 369:418-20.

Crespo P, Schuebel KE, Ostrom AA, Gutkind JS & Bustelo XR: Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product. Nature 1997, 385:169-72.

Crews CM, Alessandrini AA & Erikson RL: Mouse Erk-1 gene product is a serine/threonine protein kinase that has the potential to phosphorylate tyrosine. Proc Natl Acad Sci U S A 1991, 88:8845-9.

Crews CM, Alessandrini A & Erikson RL: The primary structure of MEK, a protein kinase that phosphorylates the ERK gene product. Science 1992, 258:478-80.

Crews CM, Alessandrini A & Erikson RL: Erks: their fifteen minutes has arrived. Cell Growth Differ 1992, 3:135-42.

Crook T, Marston NJ, Sara EA & Vousden KH: Transcriptional activation by p53 correlates with suppression of growth but not transformation. Cell 1994, 79:817-27.

Cross SM, Sanchez CA, Morgan CA, Schimke MK, Ramel S, Idzerda RL, Raskind WH & Reid BJ: A p53-dependent mouse spindle checkpoint. Science 1995, 267:1353-6.

Cross MJ, Roberts S, Ridley AJ, Hodgkin MN, Stewart A, Claesson WL & Wakelam M: Stimulation of actin stress fibre formation mediated by activation of phospholipase D. Curr Biol 1996, 6:588-97.

Dameron KM, Volpert OV, Tainsky MA & Bouck N: Control of angiogenesis in fibroblasts by p53 regulation of thrombospondin-1. Science 1994, 265:1582-4.

Datta K, Franke TF, Chan TO, Makris A, Yang SI, Kaplan DR, Morrison DK, Golemis EA & Tsichlis PN: AH/PH domain-mediated interaction between Akt molecules and its potential role in Akt regulation. Mol Cell Biol 1995, 15:2304-10.

Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y & Wang XF: Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A 1995, 92:5545-9.

Deb S, Jackson CT, Subler MA & Martin DW: Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. Journal of Virology 1992, 66:6164-70.

Deb SP, Munoz RM, Brown DR, Subler MA & Deb S: Wild-type human p53 activates the human epidermal growth factor receptor promoter. Oncogene 1994, 9:1341-9.

DeClue JE, Cohen BD & Lowy DR: Identification and characterization of the neurofibromatosis type 1 protein product. Proc Natl Acad Sci U S A 1991, 88:9914-8.

DeClue JE, Stone JC, Blanchard RA, Papageorge AG, Martin P, Zhang K & Lowy DR: A ras effector domain mutant which is temperature sensitive for cellular transformation: interactions with GTPase-activating protein and NF-1. Mol Cell Biol 1991, 11:3132-8.

DeClue JE, Zhang K, Redford P, Vass WC & Lowy DR: Suppression of src transformation by overexpression of full-length GTPase-activating protein (GAP) or of the GAP C terminus. Mol Cell Biol 1991, 11:2819-25.

DeClue JE, Papageorge AG, Fletcher JA, Diehl SR, Ratner N, Vass WC & Lowy DR: Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell 1992, 69:265-73.

DeClue JE, Vass WC, Johnson MR, Stacey DW & Lowy DR: Functional role of GTPase-activating protein in cell transformation by pp60v-src. Mol Cell Biol 1993, 13:6799-809.

Deffie A, Wu H, Reinke V & Lozano G: The tumor suppressor p53 regulates its own transcription. Mol Cell Biol 1993, 13:3415-23.

Del SG, Murphy M, Ruaro E, Lazarevic D, Levine AJ & Schneider C: Cyclin D1 and p21/waf1 are both involved in p53 growth suppression. Oncogene 1996, 12:177-85.

DeLeo AB, Jay G, Appella E, Dubois GC, Law LW & Old LJ: Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci U S A 1979, 76:2420-4.

Deng C, Zhang P, Harper JW, Elledge SJ & Leder P: Mice lacking p21CIP1/WAF1 undergo normal development, but are defective in G1 checkpoint control. Cell 1995, 82:675-84.

Denhardt DT: Signal-transducing protein phosphorylation cascades mediated by Ras/Rho proteins in the mammalian cell: the potential for multiplex signalling. Biochemical Journal 1996, 987-994.

Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM & Sturgill TW: Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 1992, 257:1404-7.

Dent P, Jelinek T, Morrison DK, Weber MJ & Sturgill TW: Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. Science 1995, 268:1902-6.

Derijard B, Hibi M, Wu IH, Barrett T, Su B, Deng T, Karin M & Davis RJ: JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. Cell 1994, 76:1025-37.

Derijard B, Raingeaud J, Barrett T, Wu IH, Han J, Ulevitch RJ & Davis RJ: Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. Science 1995, 267:682-5.

Dhand R, Hara K, Hiles I, Bax B, Gout I, Panayotou G, Fry MJ, Yonezawa K, Kasuga M & Waterfield MD: PI 3-kinase: structural and functional analysis of intersubunit interactions. Embo Journal 1994, 13:511-21.

Di LA, Linke SP, Clarkin K & Wahl GM: DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev 1994, 8:2540-51.

Dickson B, Sprenger F, Morrison D & Hafen E: Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature 1992, 360:600-3.

Dickson BJ, Dominguez M, van dSA & Hafen E: Control of Drosophila photoreceptor cell fates by phyllopod, a novel nuclear protein acting downstream of the Raf kinase. Cell 1995, 80:453-62.

Dickson BJ, van dSA, Dominguez M & Hafen E: Mutations Modulating Raf signaling in Drosophila eye development. Genetics 1996, 142:163-71.

Dikic I, Schlessinger J & Lax I: PC12 cells overexpressing the insulin receptor undergo insulin-dependent neuronal differentiation. Current Biology 1994, 4:702-8.

Dimri GP, Nakanishi M, Desprez PY, Smith JR & Campisi J: Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expressing or lacking a functional retinoblastoma protein. Mol Cell Biol 1996, 16:2987-97.

Dittmer D, Pati S, Zambetti G, Chu S, Teresky AK, Moore M, Finlay C & Levine AJ: Gain of function mutations in p53. Nat Genet 1993, 4:42-6.

Dobrowolski S, Harter M & Stacey DW: Cellular ras activity is required for passage through multiple points of the G0/G1 phase in BALB/c 3T3 cells. Mol Cell Biol 1994, 14:5441-9.

Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CJ, Butel JS & Bradley A: Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature 1992, 356:215-21.

Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D & Varmus HE: Deficiency of p53 accelerates mammary tumorigenesis in Wnt-1 transgenic mice and promotes chromosomal instability. Genes Dev 1995, 9:882-95.

Donehower LA, Godley LA, Aldaz CM, Pyle R, Shi YP, Pinkel D, Gray J, Bradley A, Medina D & Varmus HE: The role of p53 loss in genomic instability and tumor progression in a murine mammary cancer model. Prog Clin Biol Res 1996, 395:1-11.

Dosaka AH, Hu SX, Fujino M, Harada M, Kinoshita I, Xu HJ, Kuzumaki N, Kawakami Y & Benedict WF: Altered retinoblastoma protein expression in nonsmall cell lung cancer: its synergistic effects with altered ras and p53 protein status on prognosis. Cancer 1997, 79:1329-37.

Downward J: KSR: a novel player in the RAS pathway. Cell 1995, 83:831-4.

Drechsel DN, Hyman AA, Cobb MH & Kirschner MW: Modulation of the dynamic instability of tubulin assembly by the microtubule-associated protein tau. Mol Biol Cell 1992, 3:1141-54.

Drewes G, Lichtenberg KB, Doring F, Mandelkow EM, Biernat J, Goris J, Doree M & Mandelkow E: Mitogen activated protein (MAP) kinase transforms tau protein into an Alzheimer-like state. Embo J 1992, 11:2131-8.

Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR & Greenberg ME: Regulation of neuronal survival by the serine-threonine protein kinase Akt. Science 1997, 275:661-5.

Dudley DT, Pang L, Decker SJ, Bridges AJ & Saltiel AR: A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proceedings of the National Academy of Sciences of the United States of America 1995, 92:7686-9.

Dulic V, Drullinger LF, Lees E, Reed SI & Stein GH: Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. Proc Natl Acad Sci U S A 1993, 90:11034-8.

Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ & Reed SI: p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994, 76:1013-23.

Dutta A, Ruppert JM, Aster JC & Winchester E: Inhibition of DNA replication factor RPA by p53. Nature 1993, 365:79-82.

Eccleston PA, Mirsky R & Jessen KR: Spontaneous immortalisation of Schwann cells in culture: short-term cultured Schwann cells secrete growth inhibitory activity. Development 1991, 112:33-42.

Egan SE, Giddings BW, Brooks MW, Buday L, Sizeland AM & Weinberg RA: Association of Sos Ras exchange protein with Grb2 is implicated in tyrosine kinase signal transduction and transformation. Nature 1993, 363:45-51.

el DW, Kern SE, Pietenpol JA, Kinzler KW & Vogelstein B: Definition of a consensus binding site for p53. Nat Genet 1992, 1:45-9.

el DW, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW & Vogelstein B: WAF1, a potential mediator of p53 tumor suppression. Cell 1993, 75:817-25.

el DW, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y & et al: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994, 54:1169-74.

Eliyahu D, Raz A, Gruss P, Givol D & Oren M: Participation of p53 cellular tumour antigen in transformation of normal embryonic cells. Nature 1984, 312:646-9.

Eliyahu D, Michalovitz D, Eliyahu S, Pinhasi KO & Oren M: Wild-type p53 can inhibit oncogene-mediated focus formation. Proc Natl Acad Sci U S A 1989, 86:8763-7.

Ellis RE, Yuan JY & Horvitz HR: Mechanisms and functions of cell death. Annu Rev Cell Biol 1991, 7:663-98.

Elson A, Deng C, Campos TJ, Donehower LA & Leder P: The MMTV/c-myc transgene and p53 null alleles collaborate to induce T-cell lymphomas, but not mammary carcinomas in transgenic mice. Oncogene 1995, 11:181-90.

Erikson E & Maller JL: Purification and characterization of ribosomal protein S6 kinase I from Xenopus eggs. Journal of Biological Chemistry 1991, 266:5249-55.

Errede B & Ammerer G: STE12, a protein involved in cell-type-specific transcription and signal transduction in yeast, is part of protein-DNA complexes. Genes & Development 1989, 3:1349-61.

Errede B, Gartner A, Zhou Z, Nasmyth K & Ammerer G: MAP kinase-related FUS3 from S. cerevisiae is activated by STE7 in vitro. Nature 1993, 362:261-4.

Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ & Hancock DC: Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992, 69:119-28.

Ewen ME, Sluss HK, Sherr CJ, Matsushime H, Kato J & Livingston DM: Functional interactions of the retinoblastoma protein with mammalian D-type cyclins. Cell 1993, 73:487-97.

Fabian JR, Daar IO & Morrison DK: Critical tyrosine residues regulate the enzymatic and biological activity of Raf-1 kinase. Mol Cell Biol 1993, 13:7170-9.

Fabian JR, Morrison DK & Daar IO: Requirement for Raf and MAP kinase function during the meiotic maturation of Xenopus oocytes. J Cell Biol 1993, 122:645-52.

Fabian JR, Vojtek AB, Cooper JA & Morrison DK: A single amino acid change in Raf-1 inhibits Ras binding and alters Raf-1 function. Proc Natl Acad Sci U S A 1994, 91:5982-6.

Fakharzadeh SS, Trusko SP & George DL: Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. Embo J 1991, 10:1565-9.

Fan Z, Lu Y, Wu X, DeBlasio A, Koff A & Mendelsohn J: Prolonged induction of p21Cip1/WAF1/CDK2/PCNA complex by epidermal growth factor receptor activation mediates ligand-induced A431 cell growth inhibition. Journal of Cell Biology 1995, 131:235-42.

Fanidi A, Harrington EA & Evan GI: Cooperative interaction between c-myc and bcl-2 proto-oncogenes. Nature 1992, 359:554-6.

Fantl WJ, Muslin AJ, Kikuchi A, Martin JA, MacNicol AM, Gross RW & Williams LT: Activation of Raf-1 by 14-3-3 proteins. Nature 1994, 371:612-4.

Farmer G, Bargonetti J, Zhu H, Friedman P, Prywes R & Prives C: Wild-type p53 activates transcription in vitro. Nature 1992, 358:83-6.

Farrar MA, Alberol I & Perlmutter RM: Activation of the Raf-1 kinase cascade by coumermycin-induced dimerization. Nature 1996, 383:178-81.

Fearon ER & Vogelstein B: A genetic model for colorectal tumorigenesis. Cell 1990, 61:759-67.

Feig LA & Cooper GM: Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 1988, 8:3235-43.

Feramisco JR, Gross M, Kamata T, Rosenberg M & Sweet RW: Microinjection of the oncogene form of the human H-ras (T-24) protein results in rapid proliferation of quiescent cells. Cell 1984, 38:109-17.

Fields S & Herskowitz I: The yeast STE12 product is required for expression of two sets of cell-type specific genes. Cell 1985, 42:923-30.

Fields S & Jang SK: Presence of a potent transcription activating sequence in the p53 protein. Science 1990, 249:1046-9.

Filhol O, Baudier J, Chambaz EM & Cochet C: Casein kinase 2 inhibits the renaturation of complementary DNA strands mediated by p53 protein. Biochem J 1996, 987-1005.

Filmus J, Robles AI, Shi W, Wong MJ, Colombo LL & Conti CJ: Induction of cyclin D1 overexpression by activated ras. Oncogene 1994, 9:3627-33.

Finlay CA, Hinds PW, Tan TH, Eliyahu D, Oren M & Levine AJ: Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. Mol Cell Biol 1988, 8:531-9.

Finlay CA, Hinds PW & Levine AJ: The p53 proto-oncogene can act as a suppressor of transformation. Cell 1989, 57:1083-93.

Fiscella M, Ullrich SJ, Zambrano N, Shields MT, Lin D, Lees MS, Anderson CW, Mercer WE & Appella E: Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. Oncogene 1993, 8:1519-28.

Flaman JM, Waridel F, Estreicher A, Vannier A, Limacher JM, Gilbert D, Iggo R & Frebourg T: The human tumour suppressor gene p53 is alternatively spliced in normal cells. Oncogene 1996, 12:813-8.

Flores RH, Kelman Z, Dean FB, Pan ZQ, Harper JW, Elledge SJ, O'Donnell M & Hurwitz J: Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc Natl Acad Sci U S A 1994, 91:8655-9.

Foster R, Hu KQ, Shaywitz DA & Settleman J: p190 RhoGAP, the major RasGAP-associated protein, binds GTP directly. Mol Cell Biol 1994, 14:7173-81.

Foster R, Hu KQ, Lu Y, Nolan KM, Thissen J & Settleman J: Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. Mol Cell Biol 1996, 16:2689-99.

Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR & Tsichlis PN: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. Cell 1995, 81:727-36.

Franke TF, Kaplan DR, Cantley LC & Toker A: Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate. Science 1997, 275:665-8.

Franza BJ, Maruyama K, Garrels JI & Ruley HE: In vitro establishment is not a sufficient prerequisite for transformation by activated ras oncogenes. Cell 1986, 44:409-18.

Freed E, McCormick F & Ruggieri R: Proteins of the 14-3-3 family associate with Raf and contribute to its activation. Cold Spring Harb Symp Quant Biol 1994, 59:187-93.

Frey AB & Levine AJ: p53-plus-ras-transformed rat embryo fibroblasts express tumor-specific transplantation antigen activity which is shared by independently transformed cells. J Virol 1989, 63:5440-4.

Friedlander P, Haupt Y, Prives C & Oren M: A mutant p53 that discriminates between p53-responsive genes cannot induce apoptosis. Mol Cell Biol 1996, 16:4961-71.

Friedman PN, Kern SE, Vogelstein B & Prives C: Wild-type, but not mutant, human p53 proteins inhibit the replication activities of simian virus 40 large tumor antigen. Proc Natl Acad Sci U S A 1990, 87:9275-9.

Fritsche M, Haessler C & Brandner G: Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. Oncogene 1993, 8:307-18.

Fu L, Minden MD & Benchimol S: Translational regulation of human p53 gene expression. Embo J 1996, 15:4392-401.

Fuchs B, O'Connor D, Fallis L, Scheidtmann KH & Lu X: p53 phosphorylation mutants retain transcription activity. Oncogene 1995, 10:789-93.

Fujita YJ, Shirouzu M, Ito Y, Hattori S, Furuyama S, Nishimura S & Yokoyama S: A constitutive effector region on the C-terminal side of switch I of the Ras protein. J Biol Chem 1995, 270:4661-7.

Fukasawa K, Choi T, Kuriyama R, Rulong S & Vande WG: Abnormal centrosome amplification in the absence of p53. Science 1996, 271:1744-7.

Funk WD, Pak DT, Karas RH, Wright WE & Shay JW: A transcriptionally active DNA-binding site for human p53 protein complexes. Mol Cell Biol 1992, 12:2866-71.

Galaktionov K & Beach D: Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. Cell 1991, 67:1181-94.

Galaktionov K, Lee AK, Eckstein J, Draetta G, Meckler J, Loda M & Beach D: CDC25 phosphatases as potential human oncogenes. Science 1995, 269:1575-7.

Galaktionov K, Jessus C & Beach D: Raf1 interaction with Cdc25 phosphatase ties mitogenic signal transduction to cell cycle activation. Genes Dev 1995, 9:1046-58.

Galaktionov K, Chen X & Beach D: Cdc25 cell-cycle phosphatase as a target of c-myc. Nature 1996, 382:511-7.

Gale NW, Kaplan S, Lowenstein EJ, Schlessinger J & Bar SD: Grb2 mediates the EGF-dependent activation of guanine nucleotide exchange on Ras. Nature 1993, 363:88-92.

Gannon JV & Lane DP: p53 and DNA polymerase alpha compete for binding to SV40 T antigen. Nature 1987, 329:456-8.

Gannon JV, Greaves R, Iggo R & Lane DP: Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. Embo J 1990, 9:1595-602.

Gao Q, Hauser SH, Liu XL, Wazer DE, Madoc JH & Band V: Mutant p53-induced immortalization of primary human mammary epithelial cells. Cancer Res 1996, 56:3129-33.

Gardner AM, Vaillancourt RR, Lange CC & Johnson GL: MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity. Mol Biol Cell 1994, 5:193-201.

Gautier J, Solomon MJ, Booher RN, Bazan JF & Kirschner MW: cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. Cell 1991, 67:197-211.

Ghosh S & Bell RM: Identification of discrete segments of human Raf-1 kinase critical for high affinity binding to Ha-Ras. J Biol Chem 1994, 269:30785-8.

Ghosh S, Xie WQ, Quest AF, Mabrouk GM, Strum JC & Bell RM: The cysteine-rich region of raf-1 kinase contains zinc, translocates to liposomes, and is adjacent to a segment that binds GTP-ras. J Biol Chem 1994, 269:10000-7.

Ghosh S, Strum JC, Sciorra VA, Daniel L & Bell RM: Raf-1 kinase possesses distinct binding domains for phosphatidylserine and phosphatidic acid. Phosphatidic acid regulates the translocation of Raf-1 in 12-O-tetradecanoylphorbol-13-acetate-stimulated Madin-Darby canine kidney cells. J Biol Chem 1996, 271:8472-80.

Gille H, Sharrocks AD & Shaw PE: Phosphorylation of transcription factor p62TCF by MAP kinase stimulates ternary complex formation at c-fos promoter. Nature 1992, 358:414-7.

Gille H, Strahl T & Shaw PE: Activation of ternary complex factor Elk-1 by stress-activated protein kinases. Curr Biol 1995, 5:1191-200.

Gille H, Kortenjann M, Thomae O, Moomaw C, Slaughter C, Cobb MH & Shaw PE: ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation. Embo J 1995, 14:951-62.

Ginsberg D, Mechta F, Yaniv M & Oren M: Wild-type p53 can down-modulate the activity of various promoters. Proc Natl Acad Sci U S A 1991, 88:9979-83.

Girard F, Strausfeld U, Fernandez A & Lamb NJ: Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell 1991, 67:1169-79.

Givol I, Givol D, Rulong S, Resau J, Tsarfaty I & Hughes SH: Overexpression of human p21waf1/cip1 arrests the growth of chicken embryo fibroblasts transformed by individual oncogenes. Oncogene 1995, 11:2609-18.

Godley LA, Kopp JB, Eckhaus M, Paglino JJ, Owens J & Varmus HE: Wild-type p53 transgenic mice exhibit altered differentiation of the ureteric bud and possess small kidneys. Genes Dev 1996, 10:836-50.

Gonos ES, Burns JS, Mazars GR, Kobrna A, Riley TE, Barnett SC, Zafarana G, Ludwig RL, Ikram Z, Powell AJ & Jat PS: Rat embryo fibroblasts immortalized with simian virus 40 large T antigen undergo senescence upon its inactivation. Mol Cell Biol 1996, 16:5127-38.

Gonzalez FA, Seth A, Raden DL, Bowman DS, Fay FS & Davis RJ: Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. J Cell Biol 1993, 122:1089-101.

Gorina S & Pavletich NP: Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science 1996, 274:1001-5.

Gorospe M, Cirielli C, Wang X, Seth P, Capogrossi MC & Holbrook NJ: p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells. Oncogene 1997, 14:929-35.

Gottlieb E, Haffner R, von RT, Wagner EF & Oren M: Down-regulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. Embo J 1994, 13:1368-74.

Gottlieb E, Lindner S & Oren M: Relationship of sequence-specific transactivation and p53-regulated apoptosis in interleukin 3-dependent hematopoietic cells. Cell Growth Differ 1996, 7:301-10.

Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW & Giaccia AJ: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996, 379:88-91.

Graham SM, Vojtek AB, Huff SY, Cox AD, Clark GJ, Cooper JA & Der CJ: TC21 causes transformation by Raf-independent signaling pathways. Mol Cell Biol 1996, 16:6132-40.

Gray BJ, Zalvide J, Fox MP, Knickerbocker CJ, DeCaprio JA & Keyomarsi K: Cyclin E, a redundant cyclin in breast cancer. Proceedings of the National Academy of Sciences of the United States of America 1996, 93:15215-20.

Greenblatt MS, Bennett WP, Hollstein M & Harris CC: Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res 1994, 54:4855-78.

Greene LA & Tischler AS: Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. Proceedings of the National Academy of Sciences of the United States of America 1976, 73:2424-8.

Greenhalgh DA, Wang XJ, Donehower LA & Roop DR: Paradoxical tumor inhibitory effect of p53 loss in transgenic mice expressing epidermal-targeted v-rasHa, v-fos, or human transforming growth factor alpha. Cancer Res 1996, 56:4413-23.

Griffiths SD, Clarke AR, Healy LE, Ross G, Ford AM, Hooper ML, Wyllie AH & Greaves M: Absence of p53 permits propagation of mutant cells following genotoxic damage. Oncogene 1997, 14:523-31.

Groom LA, Sneddon AA, Alessi DR, Dowd S & Keyse SM: Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. Embo J 1996, 15:3621-32.

Grossfeld GD, Ginsberg DA, Stein JP, Bochner BH, Esrig D, Groshen S, Dunn M, Nichols PW, Taylor CR, Skinner DG & Cote RJ: Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. J Natl Cancer Inst 1997, 89:219-27.

Gruda MC, Kovary K, Metz R & Bravo R: Regulation of Fra-1 and Fra-2 phosphorylation differs during the cell cycle of fibroblasts and phosphorylation in vitro by MAP kinase affects DNA binding activity. Oncogene 1994, 9:2537-47.

Grumont RJ, Rasko JE, Strasser A & Gerondakis S: Activation of the mitogen-activated protein kinase pathway induces transcription of the PAC-1 phosphatase gene. Mol Cell Biol 1996, 16:2913-21.

Gu Y, Turck CW & Morgan DO: Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. Nature 1993, 366:707-10.

Guan KL & Butch E: Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. J Biol Chem 1995, 270:7197-203.

Guillouf C, Rosselli F, Krishnaraju K, Moustacchi E, Hoffman B & Liebermann DA: p53 involvement in control of G2 exit of the cell cycle: role in DNA damage-induced apoptosis. Oncogene 1995, 10:2263-70.

Gupta S, Seth A & Davis RJ: Transactivation of gene expression by Myc is inhibited by mutation at the phosphorylation sites Thr-58 and Ser-62. Proc Natl Acad Sci U S A 1993, 90:3216-20.

Gurley KE & Kemp CJ: p53 induction, cell cycle checkpoints, and apoptosis in DNAPK-deficient scid mice. Carcinogenesis 1996, 17:2537-42.

Hainaut P & Milner J: Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. Cancer Res 1993, 53:4469-73.

Hainaut P, Rolley N, Davies M & Milner J: Modulation by copper of p53 conformation and sequence-specific DNA binding: role for Cu(II)/Cu(I) redox mechanism. Oncogene 1995, 10:27-32.

Hainaut P, Soussi T, Shomer B, Hollstein M, Greenblatt M, Hovig E, Harris CC & Montesano R: Database of p53 gene somatic mutations in human tumors and cell lines: updated compilation and future prospects. Nucleic Acids Res 1997, 25:151-7.

Halazonetis TD & Kandil AN: Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. Embo J 1993, 12:5057-64.

Halazonetis TD, Davis LJ & Kandil AN: Wild-type p53 adopts a 'mutant'-like conformation when bound to DNA. Embo J 1993, 12:1021-8.

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Hale AJ, Smith CA, Sutherland LC, Stoneman VE, Longthorne VL, Culhane AC & Williams GT: Apoptosis: molecular regulation of cell death. Eur J Biochem 1996, 236:1-26.

Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D & Lassar AB: Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 1995, 267:1018-21.

Hall PA, McKee PH, Menage HD, Dover R & Lane DP: High levels of p53 protein in UV-irradiated normal human skin. Oncogene 1993, 8:203-7.

Hall PA, Kearsey JM, Coates PJ, Norman DG, Warbrick E & Cox LS: Characterisation of the interaction between PCNA and Gadd45. Oncogene 1995, 10:2427-33.

Hall SR, Campbell LE & Meek DW: Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation. Nucleic Acids Res 1996, 24:1119-26.

Hall M & Peters G: Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. Advances in Cancer Research 1996, 68:67-108.

Han M & Sternberg PW: let-60, a gene that specifies cell fates during C. elegans vulval induction, encodes a ras protein. Cell 1990, 63:921-31.

Han M & Sternberg PW: Analysis of dominant-negative mutations of the Caenorhabditis elegans let-60 ras gene. Genes Dev 1991, 5:2188-98.

Han KA & Kulesz MM: Alternatively spliced p53 RNA in transformed and normal cells of different tissue types. Nucleic Acids Res 1992, 20:1979-81.

Han J, Sabbatini P, Perez D, Rao L, Modha D & White E: The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. Genes Dev 1996, 10:461-77.

Hanahan D: Heritable formation of pancreatic beta-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. Nature 1985, 315:115-22.

Hannun YA: The sphingomyelin cycle and the second messenger function of ceramide. Journal of Biological Chemistry 1994, 269:3125-8.

Hansen R, Reddel R & Braithwaite A: The transforming oncoproteins determine the mechanism by which p53 suppresses cell transformation: pRb-mediated growth arrest or apoptosis. Oncogene 1995, 11:2535-45.

Hansen RS & Braithwaite AW: The growth-inhibitory function of p53 is separable from transactivation, apoptosis and suppression of transformation by E1a and Ras. Oncogene 1996, 13:995-1007.

Hao M, Lowy AM, Kapoor M, Deffie A, Liu G & Lozano G: Mutation of phosphoserine 389 affects p53 function in vivo. J Biol Chem 1996, 271:29380-5.

Harlow E, Williamson NM, Ralston R, Helfman DM & Adams TE: Molecular cloning and in vitro expression of a cDNA clone for human cellular tumor antigen p53. Mol Cell Biol 1985, 5:1601-10.

Harper JW, Adami GR, Wei N, Keyomarsi K & Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993, 75:805-16.

Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell CL, Swindell E & et al: Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell 1995, 6:387-400.

Harrington EA, Bennett MR, Fanidi A & Evan GI: c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. Embo Journal 1994, 13:3286-95.

Harvey JJ & East J: Biological activity and separation of a leukaemogenic virus from murine sarcoma virus-Harvey (MSV-H). International Journal of Cancer 1969, 4:655-65.

Harvey DM & Levine AJ: p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. Genes Dev 1991, 5:2375-85.

Harvey M, McArthur MJ, Montgomery CJ, Butel JS, Bradley A & Donehower LA: Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. Nat Genet 1993, 5:225-9.

Harvey M, Vogel H, Morris D, Bradley A, Bernstein A & Donehower LA: A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice. Nat Genet 1995, 9:305-11.

Haupt Y, Alexander WS, Barri G, Klinken SP & Adams JM: Novel zinc finger gene implicated as myc collaborator by retrovirally accelerated lymphomagenesis in E mu-myc transgenic mice. Cell 1991, 65:753-63.

Haupt Y, Rowan S & Oren M: p53-mediated apoptosis in HeLa cells can be overcome by excess pRB. Oncogene 1995, 10:1563-71.

Hawes BE, van BT, Koch WJ, Luttrell LM & Lefkowitz RJ: Distinct pathways of Gi- and Gq-mediated mitogen-activated protein kinase activation. J Biol Chem 1995, 270:17148-53.

Haycock JW, Ahn NG, Cobb MH & Krebs EG: ERK1 and ERK2, two microtubule-associated protein 2 kinases, mediate the phosphorylation of tyrosine hydroxylase at serine-31 in situ. Proc Natl Acad Sci U S A 1992, 89:2365-9.

Haystead TA, Dent P, Wu J, Haystead CM & Sturgill TW: Ordered phosphorylation of p42mapk by MAP kinase kinase. Febs Lett 1992, 306:17-22.

He Z, Brinton BT, Greenblatt J, Hassell JA & Ingles CJ: The transactivator proteins VP16 and GAL4 bind replication factor A. Cell 1993, 73:1223-32.

Heasley LE & Johnson GL: The beta-PDGF receptor induces neuronal differentiation of PC12 cells. Molecular Biology of the Cell 1992, 3:545-53.

Hecker D, Page G, Lohrum M, Weiland S & Scheidtmann KH: Complex regulation of the DNA-binding activity of p53 by phosphorylation: differential effects of individual phosphorylation sites on the interaction with different binding motifs. Oncogene 1996, 12:953-61.

Heisermann GJ, Wiley HS, Walsh BJ, Ingraham HA, Fiol CJ & Gill GN: Mutational removal of the Thr669 and Ser671 phosphorylation sites alters substrate specificity and ligand-induced internalization of the epidermal growth factor receptor. Journal of Biological Chemistry 1990, 265:12820-7.

Henderson S, Rowe M, Gregory C, Croom CD, Wang F, Longnecker R, Kieff E & Rickinson A: Induction of bcl-2 expression by Epstein-Barr virus latent membrane protein 1 protects infected B cells from programmed cell death. Cell 1991, 65:1107-15.

Hermeking H & Eick D: Mediation of c-Myc-induced apoptosis by p53. Science 1994, 265:2091-3.

Herskowitz I: MAP kinase pathways in yeast: for mating and more. Cell 1995, 80:187-97.

Hibi M, Lin A, Smeal T, Minden A & Karin M: Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 1993, 7:2135-48.

Hicks GG, Egan SE, Greenberg AH & Mowat M: Mutant p53 tumor suppressor alleles release ras-induced cell cycle growth arrest. Molecular & Cellular Biology 1991, 11:1344-52.

Hiebert SW, Packham G, Strom DK, Haffner R, Oren M, Zambetti G & Cleveland JL: E2F-1:DP-1 induces p53 and overrides survival factors to trigger apoptosis. Mol Cell Biol 1995, 15:6864-74.

Hill RJ & Sternberg PW: The gene lin-3 encodes an inductive signal for vulval development in C. elegans. Nature 1992, 358:470-6.

Hill RJ & Sternberg PW: Cell fate patterning during C. elegans vulval development. Dev Suppl 1993, 1993:9-18.

Hinds P, Finlay C & Levine AJ: Mutation is required to activate the p53 gene for cooperation with the ras oncogene and transformation. J Virol 1989, 63:739-46.

Hinds PW, Finlay CA, Quartin RS, Baker SJ, Fearon ER, Vogelstein B & Levine AJ: Mutant p53 DNA clones from human colon carcinomas cooperate with ras in transforming primary rat cells: a comparison of the "hot spot" mutant phenotypes. Cell Growth Differ 1990, 1:571-80.

Hinds PW, Mittnacht S, Dulic V, Arnold A, Reed SI & Weinberg RA: Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. Cell 1992, 70:993-1006.

Hinds PW & Weinberg RA: Tumor suppressor genes. Curr Opin Genet Dev 1994, 4:135-41.

Hinds PW, Dowdy SF, Eaton EN, Arnold A & Weinberg RA: Function of a human cyclin gene as an oncogene. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:709-13.

Hinds PW: The retinoblastoma tumor suppressor protein. Curr Opin Genet Dev 1995, 5:79-83.

Hoever M, Clement JH, Wedlich D, Montenarh M & Knochel W: Overexpression of wild-type p53 interferes with normal development in Xenopus laevis embryos. Oncogene 1994, 9:109-20.

Hofer F, Fields S, Schneider C & Martin GS: Activated Ras interacts with the Ral guanine nucleotide dissociation stimulator. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:11089-93.

Hofmann F, Rex G, Aktories K & Just I: The ras-related protein Ral is monoglucosylated by Clostridium sordellii lethal toxin. Biochem Biophys Res Commun 1996, 227:77-81.

Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith SB, Montesano R & Harris CC: Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res 1994, 22:3551-5.

Hollstein M, Shomer B, Greenblatt M, Soussi T, Hovig E, Montesano R & Harris CC: Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. Nucleic Acids Res 1996, 24:141-6.

Horikoshi N, Usheva A, Chen J, Levine AJ, Weinmann R & Shenk T: Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. Mol Cell Biol 1995, 15:227-34.

Houweling A, van, den, Elsen, Pj, van, der, Eb & Aj: Partial transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. Virology 1980, 105:537-50.

Howe LR, Leevers SJ, Gomez N, Nakielny S, Cohen P & Marshall CJ: Activation of the MAP kinase pathway by the protein kinase raf. Cell 1992, 71:335-42.

Howe LR & Marshall CJ: Lysophosphatidic acid stimulates mitogen-activated protein kinase activation via a G-protein-coupled pathway requiring p21ras and p74raf-1. J Biol Chem 1993, 268:20717-20.

Howes KA, Ransom N, Papermaster DS, Lasudry JG, Albert DM & Windle JJ: Apoptosis or retinoblastoma: alternative fates of photoreceptors expressing the HPV-16 E7 gene in the presence or absence of p53. Genes & Development 1994, 8:1300-10.

Hsu JC & Perrimon N: A temperature-sensitive MEK mutation demonstrates the conservation of the signaling pathways activated by receptor tyrosine kinases. Genes Dev 1994, 8:2176-87.

Huang W, Alessandrini A, Crews CM & Erikson RL: Raf-1 forms a stable complex with Mek1 and activates Mek1 by serine phosphorylation. Proceedings of the National Academy of Sciences of the United States of America 1993, 90:10947-51.

Hubbert NL, Sedman SA & Schiller JT: Human papillomavirus type 16 E6 increases the degradation rate of p53 in human keratinocytes. J Virol 1992, 66:6237-41.

Huff K, End D & Guroff G: Nerve growth factor-induced alteration in the response of PC12 pheochromocytoma cells to epidermal growth factor. Journal of Cell Biology 1981, 88:189-98.

Huibregtse JM, Scheffner M & Howley PM: A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. Embo J 1991, 10:4129-35.

Hundley JE, Koester SK, Troyer DA, Hilsenbeck SG, Subler MA & Windle JJ: Increased tumor proliferation and genomic instability without decreased apoptosis in MMTV-ras mice deficient in p53. Molecular & Cellular Biology 1997, 17:723-31.

Hunter T: Oncoprotein networks. Cell 1997, 88:333-46.341. Hupp TR, Meek DW, Midgley CA & Lane DP: Regulation of the specific DNA binding function of p53. Cell 1992, 71:875-86.

Hupp TR, Meek DW, Midgley CA & Lane DP: Activation of the cryptic DNA binding function of mutant forms of p53. Nucleic Acids Res 1993, 21:3167-74

Hupp TR & Lane DP: Allosteric activation of latent p53 tetramers. Curr Biol 1994, 4:865-75.

Hupp TR, Sparks A & Lane DP: Small peptides activate the latent sequence-specific DNA binding function of p53. Cell 1995, 83:237-45.

Hupp TR & Lane DP: Two distinct signaling pathways activate the latent DNA binding function of p53 in a casein kinase II-independent manner. J Biol Chem 1995, 270:18165-74.

Huwiler A, Brunner J, Hummel R, Vervoordeldonk M, Stabel S, van dBH & Pfeilschifter J: Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. Proc Natl Acad Sci U S A 1996, 93:6959-63.

Ikram Z, Norton T & Jat PS: The biological clock that measures the mitotic life-span of mouse embryo fibroblasts continues to function in the presence of simian virus 40 large tumor antigen. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:6448-52.

Illenberger S, Drewes G, Trinczek B, Biernat J, Meyer HE, Olmsted JB, Mandelkow EM & Mandelkow E: Phosphorylation of microtubule-associated proteins MAP2 and MAP4 by the protein kinase p110mark. Phosphorylation sites and regulation of microtubule dynamics. J Biol Chem 1996, 271:10834-43.

Irie K, Gotoh Y, Yashar BM, Errede B, Nishida E & Matsumoto K: Stimulatory effects of yeast and mammalian 14-3-3 proteins on the Raf protein kinase. Science 1994, 265:1716-9.

Ishido S, Muramatsu S, Fujita T, Iwanaga Y, Tong WY, Katayama Y, Itoh M & Hotta H: Wild-type, but not mutant-type, p53 enhances nuclear accumulation of the NS3 protein of hepatitis C virus. Biochem Biophys Res Commun 1997, 230:431-6.351.

Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT & Weinberg RA: Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994, 4:1-7.

Jackson P, Bos E & Braithwaite AW: Wild-type mouse p53 down-regulates transcription from different virus enhancer/promoters. Oncogene 1993, 8:589-97.

Jackson P, Ridgway P, Rayner J, Noble J & Braithwaite A: Transcriptional regulation of the PCNA promoter by p53. Biochem Biophys Res Commun 1994, 203:133-40.

Jackson P, Shield M, Buskin J, Hawkes S, Reed M, Perrem K, Hauschka SD & Braithwaite A: p53-dependent activation of the mouse MCK gene promoter: identification of a novel p53-responsive sequence and evidence for cooperation between distinct p53 binding sites. Gene Expr 1995, 5:19-33.

Jakus J & Yeudall WA: Growth inhibitory concentrations of EGF induce p21 (WAF1/Cip1) and alter cell cycle control in squamous carcinoma cells. Oncogene 1996, 12:2369-76.

Jamal S & Ziff EB: Raf phosphorylates p53 in vitro and potentiates p53-dependent transcriptional transactivation in vivo. Oncogene 1995, 10:2095-101.

Janknecht R, Ernst WH, Pingoud V & Nordheim A: Activation of ternary complex factor Elk-1 by MAP kinases. Embo J 1993, 12:5097-104.

Janknecht R & Nordheim A: MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. Biochem Biophys Res Commun 1996, 228:831-7.

Janknecht R & Hunter T: Activation of the Sap-1a transcription factor by the c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase. J Biol Chem 1997, 272:4219-24.

Jayaraman J & Prives C: Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. Cell 1995, 81:1021-9.

Jayaraman L, Murthy KG, Zhu C, Curran T, Xanthoudakis S & Prives C: Identification of redox/repair protein Ref-1 as a potent activator of p53. Genes Dev 1997, 11:558-70.

Jeffrey PD, Gorina S & Pavletich NP: Crystal structure of the tetramerization domain of the p53 tumor suppressor at 1.7 angstroms. Science 1995, 267:1498-502.

Jelinek T, Dent P, Sturgill TW & Weber MJ: Ras-induced activation of Raf-1 is dependent on tyrosine phosphorylation. Mol Cell Biol 1996, 16:1027-34.

Jen J, Powell SM, Papadopoulos N, Smith KJ, Hamilton SR, Vogelstein B & Kinzler KW: Molecular determinants of dysplasia in colorectal lesions. Cancer Research 1994, 54:5523-6.

Jiang H, Lin J, Su ZZ, Collart FR, Huberman E & Fisher PB: Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene 1994, 9:3397-406.

Jiang H, Luo JQ, Urano T, Frankel P, Lu Z, Foster DA & Feig LA: Involvement of Ral GTPase in v-Src-induced phospholipase D activation. Nature 1995, 378:409-12.

Johnson MR, Look AT, DeClue JE, Valentine MB & Lowy DR: Inactivation of the NF1 gene in human melanoma and neuroblastoma cell lines without impaired regulation of GTP.Ras. Proc Natl Acad Sci U S A 1993, 90:5539-43.

Johnson MR, DeClue JE, Felzmann S, Vass WC, Xu G, White R & Lowy DR: Neurofibromin can inhibit Ras-dependent growth by a mechanism independent of its GTPase-accelerating function. Mol Cell Biol 1994, 14:641-5.

Johnson NL, Gardner AM, Diener KM, Lange CC, Gleavy J, Jarpe MB, Minden A, Karin M, Zon LI & Johnson GL: Signal transduction pathways regulated by mitogen-activated/extracellular response kinase kinase kinase induce cell death. J Biol Chem 1996, 271:3229-37.

Jones SN, Roe AE, Donehower LA & Bradley A: Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995, 378:206-8.

Jones CJ, Shaw JJ, Wyllie FS, Gaillard N, Schlumberger M & Wynford TD: High frequency deletion of the tumour suppressor gene P16INK4a (MTS1) in human thyroid cancer cell lines. Mol Cell Endocrinol 1996, 116:115-9.

Jones DL & Munger K: Analysis of the p53-mediated G1 growth arrest pathway in cells expressing the human papillomavirus type 16 E7 oncoprotein. J Virol 1997, 71:2905-12.

Jost CA, Marin MC & Kaelin WJ: p73 is a human p53-related protein that can induce apoptosis . Nature 1997, 389:191-4.

Juven T, Barak Y, Zauberman A, George DL & Oren M: Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the mdm2 gene. Oncogene 1993, 8:3411-6.

Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalon P, Lelias JM, Dumont X, Ferrara P, McKeon F & Caput D: Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell 1997, 90:809-19.

Kastan MB, Onyekwere O, Sidransky D, Vogelstein B & Craig RW: Participation of p53 protein in the cellular response to DNA damage. Cancer Res 1991, 657-666.

Kastan MB, Zhan Q, el DW, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B & Fornace AJ: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. Cell 1992, 71:587-97.

Katz WS, Hill RJ, Clandinin TR & Sternberg PW: Different levels of the C. elegans growth factor LIN-3 promote distinct vulval precursor fates. Cell 1995, 82:297-307.

Kauffmann RC, Li S, Gallagher PA, Zhang J & Carthew RW: Ras1 signaling and transcriptional competence in the R7 cell of Drosophila. Genes Dev 1996, 10:2167-78.

Kauffmann ZA, Rodriguez VP, Ulrich E, Gilbert C, Coffer P, Downward J & Evan G: Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature 1997, 385:544-8.

Kemp CJ, Burns PA, Brown K, Nagase H & Balmain A: Transgenic approaches to the analysis of ras and p53 function in multistage carcinogenesis. Cold Spring Harbor Symposia on Quantitative Biology 1994, 59:427-34.

Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN & Hay N: The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. Genes & Development 1997, 11:701-13.

Kern SE, Kinzler KW, Baker SJ, Nigro JM, Rotter V, Levine AJ, Friedman P, Prives C & Vogelstein B: Mutant p53 proteins bind DNA abnormally in vitro. Oncogene 1991, 6:131-6.

Kern SE, Pietenpol JA, Thiagalingam S, Seymour A, Kinzler KW & Vogelstein B: Oncogenic forms of p53 inhibit p53-regulated gene expression. Science 1992, 256:827-30.

Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, Lorincz AT, Hedrick L & Cho KR: Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proceedings of the National Academy of Sciences of the United States of America 1993, 786-794.

Keyomarsi K, O'Leary N, Molnar G, Lees E, Fingert HJ & Pardee AB: Cyclin E, a potential prognostic marker for breast cancer. Cancer Research 1994, 54:380-5.

Keyomarsi K, Conte DJ, Toyofuku W & Fox MP: Deregulation of cyclin E in breast cancer. Oncogene 1995, 11:941-50.

Keyse SM & Emslie EA: Oxidative stress and heat shock induce a human gene encoding a protein-tyrosine phosphatase. Nature 1992, 359:644-7.

Keyse SM: An emerging family of dual specificity MAP kinase phosphatases. Biochim Biophys Acta 1995, 1265:152-60.

Khosravi FR, Chrzanowska WM, Solski PA, Eva A, Burridge K & Der CJ: Dbl and Vav mediate transformation via mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. Mol Cell Biol 1994, 14:6848-57.

Khosravi FR, Solski PA, Clark GJ, Kinch MS & Der CJ: Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Molecular & Cellular Biology 1995, 15:6443-53.

Khosravi FR, White MA, Westwick JK, Solski PA, Chrzanowska WM, Van AL, Wigler MH & Der CJ: Oncogenic Ras activation of Raf/mitogen-activated protein kinase-independent pathways is sufficient to cause tumorigenic transformation. Molecular & Cellular Biology 1996, 16:3923-33.

Kikuchi A, Demo SD, Ye ZH, Chen YW & Williams LT: ralGDS family members interact with the effector loop of ras p21. Molecular & Cellular Biology 1994, 14:7483-91.

Kim HA, Rosenbaum T, Marchionni MA, Ratner N & DeClue JE: Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. Oncogene 1995, 11:325-35.

Kim HA, Ling B & Ratner N: Nf1-deficient mouse Schwann cells are angiogenic and invasive and can be induced to hyperproliferate: reversion of some phenotypes by an inhibitor of farnesyl protein transferase. Molecular & Cellular Biology 1997, 17:862-72.

Kinzler KW & Vogelstein B: Lessons from hereditary colorectal cancer. Cell 1996, 87:159-70.

Kirsten WH, Somers KD & Mayer LA: Multiplicity of cell response to a murine erythroblastosis virus. Bibliotheca Haematologica 1968, 30:64-5.

Klarlund JK, Cherniack AD, McMahon M & Czech MP: Role of the Raf/mitogen-activated protein kinase pathway in p21ras desensitization. J Biol Chem 1996, 271:16674-7.

Knippschild U, Oren M & Deppert W: Abrogation of wild-type p53 mediated growth-inhibition by nuclear exclusion. Oncogene 1996, 12:1755-65.

Knippschild U, Milne D, Campbell L & Meek D: p53 N-terminus-targeted protein kinase activity is stimulated in response to wild type p53 and DNA damage. Oncogene 1996, 13:1387-93.

Ko LJ & Prives C: p53: puzzle and paradigm. Genes Dev 1996, 10:1054-72.

Kockel L, Vorbruggen G, Jackle H, Mlodzik M & Bohmann D: Requirement for Drosophila 14-3-3 zeta in Raf-dependent photoreceptor development. Genes & Development 1997, 11:1140-7.

Kodaki T, Woscholski R, Hallberg B, Rodriguez VP, Downward J & Parker PJ: The activation of phosphatidylinositol 3-kinase by Ras. Curr Biol 1994, 4:798-806.

Koide H, Satoh T, Nakafuku M & Kaziro Y: GTP-dependent association of Raf-1 with Ha-Ras: identification of Raf as a target downstream of Ras in mammalian cells. Proc Natl Acad Sci U S A 1993, 90:8683-6.

Kolch W, Heidecker G, Lloyd P & Rapp UR: Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. Nature 1991, 349:426-8.

Kornfeld K, Hom DB & Horvitz HR: The ksr-1 gene encodes a novel protein kinase involved in Ras-mediated signaling in C. elegans. Cell 1995, 83:903-13.

Kornfeld K, Guan KL & Horvitz HR: The Caenorhabditis elegans gene mek-2 is required for vulval induction and encodes a protein similar to the protein kinase MEK. Genes Dev 1995, 9:756-68.

Korsmeyer SJ: Regulators of cell death. Trends Genet 1995, 11:101-5.

Kramer H, Cagan RL & Zipursky SL: Interaction of bride of sevenless membrane-bound ligand and the sevenless tyrosine-kinase receptor. Nature 1991, 352:207-12.

Krek W, Ewen ME, Shirodkar S, Arany Z, Kaelin WJ & Livingston DM: Negative regulation of the growth-promoting transcription factor E2F-1 by a stably bound cyclin A-dependent protein kinase. Cell 1994, 78:161-72.

Krengel U, Schlichting L, Scherer A, Schumann R, Frech M, John J, Kabsch W, Pai EF & Wittinghofer A: Three-dimensional structures of H-ras p21 mutants: molecular basis for their inability to function as signal switch molecules. Cell 1990, 62:539-48.

Krystosek A & Seeds NW: Peripheral neurons and Schwann cells secrete plasminogen activator. Journal of Cell Biology 1984, 98:773-6.

Kubbutat MH & Vousden KH: Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. Mol Cell Biol 1997, 17:460-8.

Kuerbitz SJ, Plunkett BS, Walsh WV & Kastan MB: Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc Natl Acad Sci U S A 1992, 89:7491-5.

Kulesz MM, Lisafeld B, Huang H, Kisiel ND & Lee L: Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal cells. Mol Cell Biol 1994, 14:1698-708.

Kumagai A & Dunphy WG: The cdc25 protein controls tyrosine dephosphorylation of the cdc2 protein in a cell-free system. Cell 1991, 64:903-14.

Kuo CJ, Chung J, Fiorentino DF, Flanagan WM, Blenis J & Crabtree GR: Rapamycin selectively inhibits interleukin-2 activation of p70 S6 kinase. Nature 1992, 358:70-3.

Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ & Pavletich NP: Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain [comment]. Science 1996, 274:948-53.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR & Avruch J: Raf-1 activates MAP kinase-kinase. Nature 1992, 358:417-21.

Kyriakis JM, Force TL, Rapp UR, Bonventre JV & Avruch J: Mitogen regulation of c-Raf-1 protein kinase activity toward mitogen-activated protein kinase-kinase. J Biol Chem 1993, 268:16009-19.

Kyriakis JM, Banerjee P, Nikolakaki E, Dai T, Rubie EA, Ahmad MF, Avruch J & Woodgett JR: The stress-activated protein kinase subfamily of c-Jun kinases. Nature 1994, 369:156-60.

LaBaer J, Garrett MD, Stevenson LF, Slingerland JM, Sandhu C, Chou HS, Fattaey A & Harlow E: New functional activities for the p21 family of CDK inhibitors. Genes & Development 1997, 11:847-62.

Lackner MR, Kornfeld K, Miller LM, Horvitz HR & Kim SK: A MAP kinase homolog, mpk-1, is involved in ras-mediated induction of vulval cell fates in Caenorhabditis elegans. Genes Dev 1994, 8:160-73.

Lammie GA, Smith R, Silver J, Brookes S, Dickson C & Peters G: Proviral insertions near cyclin D1 in mouse lymphomas: a parallel for BCL1 translocations in human B-cell neoplasms. Oncogene 1992, 7:2381-7.

Land H, Parada LF & Weinberg RA: Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature 1983, 304:596-602.

Land H, Chen AC, Morgenstern JP, Parada LF & Weinberg RA: Behavior of myc and ras oncogenes in transformation of rat embryo fibroblasts. Mol Cell Biol 1986, 6:1917-25.

Lane DP & Crawford LV: T antigen is bound to a host protein in SV40-transformed cells. Nature 1979, 278:261-3.

Lane DP & Benchimol S: p53: oncogene or anti-oncogene? Genes Dev 1990, 4:1-8.

Langdon WY, Harris AW & Cory S: Acceleration of B-lymphoid tumorigenesis in E mu-myc transgenic mice by v-H-ras and v-raf but not v-abl. Oncogene Research 1989, 4:253-8.

Lange CC, Pleiman CM, Gardner AM, Blumer KJ & Johnson GL: A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. Science 1993, 260:315-9.431.

Lange CC & Johnson GL: Ras-dependent growth factor regulation of MEK kinase in PC12 cells. Science 1994, 265:1458-61.

Latham KM, Eastman SW, Wong A & Hinds PW: Inhibition of p53-mediated growth arrest by overexpression of cyclin-dependent kinases. Mol Cell Biol 1996, 16:4445-55.

Lavoie JN, L'Allemain G, Brunet A, Muller R & Pouyssegur J: Cyclin D1 expression is regulated positively by the p42/p44MAPK and negatively by the p38/HOGMAPK pathway. J Biol Chem 1996, 271:20608-16.

Lazar DF, Wiese RJ, Brady MJ, Mastick CC, Waters SB, Yamauchi K, Pessin JE, Cuatrecasas P & Saltiel AR: Mitogen-activated protein kinase kinase inhibition does not block the stimulation of glucose utilization by insulin. Journal of Biological Chemistry 1995, 270:20801-7.

Leach FS, Elledge SJ, Sherr CJ, Willson JK, Markowitz S, Kinzler KW & Vogelstein B: Amplification of cyclin genes in colorectal carcinomas. Cancer Research 1993, 53:1986-9.

Lechner MS, Mack DH, Finicle AB, Crook T, Vousden KH & Laimins LA: Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. Embo J 1992, 11:3045-52.

Lee RM, Cobb MH & Blackshear PJ: Evidence that extracellular signal-regulated kinases are the insulin-activated Raf-1 kinase kinases. J Biol Chem 1992, 267:1088-92.

Lee JM, Abrahamson JL, Kandel R, Donehower LA & Bernstein A: Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene 1994, 9:3731-6.

Lee S, Elenbaas B, Levine A & Griffith J: p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion/deletion mismatches. Cell 1995, 81:1013-20.

Lee S, Cavallo L & Griffith J: Human p53 binds Holliday junctions strongly and facilitates their cleavage. J Biol Chem 1997, 272:7532-9.

Lees MS, Sakaguchi K, Ullrich SJ, Appella E & Anderson CW: Human DNA-activated protein kinase phosphorylates serines 15 and 37 in the amino-terminal transactivation domain of human p53. Mol Cell Biol 1992, 12:5041-9.

Leevers SJ & Marshall CJ: Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. Embo J 1992, 11:569-74.

Leevers SJ, Paterson HF & Marshall CJ: Requirement for Ras in Raf activation is overcome by targeting Raf to the plasma membrane. Nature 1994, 369:411-4.

Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A & Pouyssegur J: Growth factors induce nuclear translocation of MAP kinases (p42mapk and p44mapk) but not of their activator MAP kinase kinase (p45mapkk) in fibroblasts. J Cell Biol 1993, 122:1079-88.

Levine AJ: Tumor suppressor genes. Bioessays 1990, 12:60-6.

Levine AJ, Momand J & Finlay CA: The p53 tumour suppressor gene. Nature 1991, 351:453-6.

Levine AJ: The p53 tumour suppressor gene and product. Cancer Survey 1992, 12:59-79.

Levine AJ: p53, the cellular gatekeeper for growth and division. Cell 1997, 88:323-31.

Levy DB, Smith KJ, Beazer BY, Hamilton SR, Vogelstein B & Kinzler KW: Inactivation of both APC alleles in human and mouse tumors. Cancer Research 1994, 54:5953-8.

Lewis T, Groom LA, Sneddon AA, Smythe C & Keyse SM: XCL100, an inducible nuclear MAP kinase phosphatase from Xenopus laevis: its role in MAP kinase inactivation in differentiated cells and its expression during early development. J Cell Sci 1995, 987-993.

Li FP & Fraumeni JJ: Rhabdomyosarcoma in children: epidemiologic study and identification of a familial cancer syndrome. J Natl Cancer Inst 1969, 43:1365-73.

Li Y, Bollag G, Clark R, Stevens J, Conroy L, Fults D, Ward K, Friedman E, Samowitz W, Robertson M & et al: Somatic mutations in the neurofibromatosis 1 gene in human tumors. Cell 1992, 69:275-81.

Li BQ, Kaplan D, Kung HF & Kamata T: Nerve growth factor stimulation of the Ras-guanine nucleotide exchange factor and GAP activities. Science 1992, 256:1456-9.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar SD, Margolis B & Schlessinger J: Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 1993, 363:85-8.

Li S & Sedivy JM: Raf-1 protein kinase activates the NF-kappa B transcription factor by dissociating the cytoplasmic NF-kappa B-I kappa B complex. Proc Natl Acad Sci U S A 1993, 90:9247-51.

Li R, Waga S, Hannon GJ, Beach D & Stillman B: Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature 1994, 371:534-7.

Li S, Janosch P, Tanji M, Rosenfeld GC, Waymire JC, Mischak H, Kolch W & Sedivy JM: Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. Embo J 1995, 14:685-96.

Lill NL, Tevethia MJ, Eckner R, Livingston DM & Modjtahedi N: p300 family members associate with the carboxyl terminus of simian virus 40 large tumor antigen. J Virol 1997, 71:129-37.

Lin LL, Wartmann M, Lin AY, Knopf JL, Seth A & Davis RJ: cPLA2 is phosphorylated and activated by MAP kinase. Cell 1993, 72:269-78.

Lin A, Minden A, Martinetto H, Claret FX, Lange CC, Mercurio F, Johnson GL & Karin M: Identification of a dual specificity kinase that activates the Jun kinases and p38-Mpk2. Science 1995, 268:286-90.

Lin J, Teresky AK & Levine AJ: Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene 1995, 10:2387-90.

Lin J, Reichner C, Wu X & Levine AJ: Analysis of wild-type and mutant p21WAF-1 gene activities. Mol Cell Biol 1996, 16:1786-93.

Lin SC, Skapek SX & Lee EY: Genes in the RB pathway and their knockout in mice. Seminars in Cancer Biology 1996, 7:279-89.

Linke SP, Clarkin KC, Di LA, Tsou A & Wahl GM: A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev 1996, 10:934-47.

Linzer DI, Maltzman W & Levine AJ: The SV40 A gene product is required for the production of a 54,000 MW cellular tumor antigen. Virology 1979, 98:308-18.

Linzer DI & Levine AJ: Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell 1979, 17:43-52.

Littlewood TD, Hancock DC, Danielian PS, Parker MG & Evan GI: A modified oestrogen receptor ligand-binding domain as an improved switch for the regulation of heterologous proteins. Nucleic Acids Res 1995, 23:1686-90.

Liu X, Miller CW, Koeffler PH & Berk AJ: The p53 activation domain binds the TATA box-binding polypeptide in Holo-TFIID, and a neighboring p53 domain inhibits transcription. Mol Cell Biol 1993, 13:3291-300.

Liu JJ, Chao JR, Jiang MC, Ng SY, Yen JJ & Yang YH: Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Mol Cell Biol 1995, 15:3654-63.

Liu X & Berk AJ: Reversal of in vitro p53 squelching by both TFIIB and TFIID. Mol Cell Biol 1995, 15:6474-8.471. Liu JJ, Chao JR, Jiang MC, Ng SY, Yen JJ & Yang YH: Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. Molecular & Cellular Biology 1995, 15:3654-63.

Liu Y, Martindale JL, Gorospe M & Holbrook NJ: Regulation of p21WAF1/CIP1 expression through mitogen-activated protein kinase signaling pathway. Cancer Res 1996, 56:31-5.

Livingstone LR, White A, Sprouse J, Livanos E, Jacks T & Tlsty TD: Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 1992, 70:923-35.

Lloyd AC, Obermuller F, Staddon S, Barth CF, McMahon M & Land H: Cooperating oncogenes converge to regulate cyclin/cdk complexes. Genes Dev 1997, 11:663-77.

Lohrum M & Scheidtmann KH: Differential effects of phosphorylation of rat p53 on transactivation of promoters derived from different p53 responsive genes. Oncogene 1996, 13:2527-39.

Lovec H, Sewing A, Lucibello FC, Muller R & Moroy T: Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. Oncogene 1994, 9:323-6.

Lovec H, Grzeschiczek A, Kowalski MB & Moroy T: Cyclin D1/bcl-1 cooperates with myc genes in the generation of B-cell lymphoma in transgenic mice. Embo Journal 1994, 13:3487-95.

Lowe SW, Ruley HE, Jacks T & Housman DE: p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993, 74:957-67.

Lowe SW, Schmitt EM, Smith SW, Osborne BA & Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993, 362:847-9.

Lowe SW & Ruley HE: Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis. Genes Dev 1993, 7:535-45.

Lowe SW, Jacks T, Housman DE & Ruley HE: Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. Proc Natl Acad Sci U S A 1994, 91:2026-30.

Lowenstein EJ, Daly RJ, Batzer AG, Li W, Margolis B, Lammers R, Ullrich A, Skolnik EY, Bar SD & Schlessinger J: The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 1992, 70:431-42.

Lowy DR & Willumsen BM: Function and regulation of ras. Annu Rev Biochem 1993, 62:851-91.

Lu X, Park SH, Thompson TC & Lane DP: Ras-induced hyperplasia occurs with mutation of p53, but activated ras and myc together can induce carcinoma without p53 mutation. Cell 1992, 70:153-61.

Lu X, Chou TB, Williams NG, Roberts T & Perrimon N: Control of cell fate determination by p21ras/Ras1, an essential component of torso signaling in Drosophila. Genes Dev 1993, 7:621-32.

Lu X & Lane DP: Differential induction of transcriptionally active p53 following UV or ionizing radiation: defects in chromosome instability syndromes? Cell 1993, 75:765-78.

Lu H & Levine AJ: Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proc Natl Acad Sci U S A 1995, 92:5154-8.

Lu X, Burbidge SA, Griffin S & Smith HM: Discordance between accumulated p53 protein level and its transcriptional activity in response to u.v. radiation. Oncogene 1996, 13:413-8.

Ludes MJ, Subler MA, Shivakumar CV, Munoz RM, Jiang P, Bigger JE, Brown DR, Deb SP & Deb S: Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol 1996, 16:6009-19.

Ludlow JW & Skuse GR: Viral oncoprotein binding to pRB, p107, p130, and p300. Virus Research 1995, 35:113-21.

Luo ZJ, Zhang XF, Rapp U & Avruch J: Identification of the 14.3.3 zeta domains important for self-association and Raf binding. J Biol Chem 1995, 270:23681-7.

Luo Z, Tzivion G, Belshaw PJ, Vavvas D, Marshall M & Avruch J: Oligomerization activates c-Raf-1 through a Ras-dependent mechanism. Nature 1996, 383:181-5.

Luongo C, Moser AR, Gledhill S & Dove WF: Loss of Apc+ in intestinal adenomas from Min mice. Cancer Research 1994, 54:5947-52.

MacCallum DE, Hupp TR, Midgley CA, Stuart D, Campbell SJ, Harper A, Walsh FS, Wright EG, Balmain A, Lane DP & Hall PA: The p53 response to ionising radiation in adult and developing murine tissues. Oncogene 1996, 13:2575-87.

Mack DH, Vartikar J, Pipas JM & Laimins LA: Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. Nature 1993, 363:281-3.

Macleod KF, Sherry N, Hannon G, Beach D, Tokino T, Kinzler K, Vogelstein B & Jacks T: p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev 1995, 9:935-44.

Macleod KF, Hu Y & Jacks T: Loss of Rb activates both p53-dependent and independent cell death pathways in the developing mouse nervous system. Embo J 1996, 15:6178-88.

Malkin D, Li FP, Strong LC, Fraumeni JJ, Nelson CE, Kim DH, Kassel J, Gryka MA, Bischoff FZ, Tainsky MA & et al: Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science 1990, 250:1233-8.

Maltzman W & Czyzyk L: UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol Cell Biol 1984, 4:1689-94.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande WG & Ahn NG: Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 1994, 265:966-70.

Marais R, Wynne J & Treisman R: The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 1993, 73:381-93.

Marais R, Light Y, Paterson HF & Marshall CJ: Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. Embo J 1995, 14:3136-45.

Marais R, Light Y, Paterson HF, Mason CS & Marshall CJ: Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J Biol Chem 1997, 272:4378-83.

Marechal V, Elenbaas B, Piette J, Nicolas JC & Levine AJ: The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes. Mol Cell Biol 1994, 14:7414-20.

Margolis B, Hu P, Katzav S, Li W, Oliver JM, Ullrich A, Weiss A & Schlessinger J: Tyrosine phosphorylation of vav proto-oncogene product containing SH2 domain and transcription factor motifs. Nature 1992, 356:71-4.

Marklund U, Brattsand G, Shingler V & Gullberg M: Serine 25 of oncoprotein 18 is a major cytosolic target for the mitogen-activated protein kinase. J Biol Chem 1993, 268:15039-47.

Marshall CJ, Vousden K & Ozanne B: The involvement of activated ras genes in determining the transformed phenotype. Proceedings of the Royal Society of London   Series B: Biological Sciences 1985, 226:99-106.

Marshall CJ & Leevers SJ: Mitogen-activated protein kinase activation by scrape loading of p21ras. Methods Enzymol 1995, 255:273-9.

Marshall CJ: Ras effectors. Current Opinion in Cell Biology 1996, 8:197-204.

Marte BM, Rodriguez VP, Wennstrom S, Warne PH & Downward J: R-Ras can activate the phosphoinositide 3-kinase but not the MAP kinase arm of the Ras effector pathways. Curr Biol 1997, 7:63-70.

Martin GA, Viskochil D, Bollag G, McCabe PC, Crosier WJ, Haubruck H, Conroy L, Clark R, O'Connell P, Cawthon RM & et al: The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. Cell 1990, 63:843-9.

Martin DW, Munoz RM, Subler MA & Deb S: p53 binds to the TATA-binding protein-TATA complex. J Biol Chem 1993, 268:13062-7.

Martinez J, Georgoff I, Martinez J & Levine AJ: Cellular localization and cell cycle regulation by a temperature-sensitive p53 protein. Genes Dev 1991, 5:151-9.

Massimi P & Banks L: Repression of p53 transcriptional activity by the HPV E7 proteins. Virology 1997, 227:255-9.

Matlashewski G, Pim D, Banks L & Crawford L: Alternative splicing of human p53 transcripts. Oncogene Res 1987, 1:77-85.

Matsuda S, Gotoh Y & Nishida E: Phosphorylation of Xenopus mitogen-activated protein (MAP) kinase kinase by MAP kinase kinase kinase and MAP kinase. J Biol Chem 1993, 268:3277-81.

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW & Elledge SJ: p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev 1995, 9:650-62.

Mazarakis ND, Yannoutsos N, el JJ, Hatton W, Fletcher R & Grosveld F: Neurocristopathy resembling neurofibromatosis type 1 in an NGF-SV40 transgenic line. Genes to Cells 1996, 1:125-37.

Mazars GR & Jat PS: Expression of p24, a novel p21Waf1/Cip1/Sdi1-related protein, correlates with measurement of the finite proliferative potential of rodent embryo fibroblasts. Proc Natl Acad Sci U S A 1997, 94:151-6.

McCarthy SA, Samuels ML, Pritchard CA, Abraham JA & McMahon M: Rapid induction of heparin-binding epidermal growth factor/diphtheria toxin receptor expression by Raf and Ras oncogenes. Genes & Development 1995, 9:1953-64.

McCurrach ME, Connor TM, Knudson CM, Korsmeyer SJ & Lowe SW: bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. Proc Natl Acad Sci U S A 1997, 94:2345-9.

McDonnell TJ, Deane N, Platt FM, Nunez G, Jaeger U, McKearn JP & Korsmeyer SJ: bcl-2-immunoglobulin transgenic mice demonstrate extended B cell survival and follicular lymphoproliferation. Cell 1989, 57:79-88.

McGlade J, Brunkhorst B, Anderson D, Mbamalu G, Settleman J, Dedhar S, Rozakis AM, Chen LB & Pawson T: The N-terminal region of GAP regulates cytoskeletal structure and cell adhesion. Embo J 1993, 12:3073-81.

McMasters KM, Montes dOLR, Pena JR & Lozano G: mdm2 deletion does not alter growth characteristics of p53-deficient embryo fibroblasts. Oncogene 1996, 13:1731-6.

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Medema RH, de LW, Martin GA, McCormick F & Bos JL: GTPase-activating protein SH2-SH3 domains induce gene expression in a Ras-dependent fashion. Mol Cell Biol 1992, 12:3425-30.

Meek DW & Eckhart W: Mutation of the serine 312 phosphorylation site does not alter the ability of mouse p53 to inhibit simian virus 40 DNA replication in vivo. J Virol 1990, 64:1734-44.

Meek DW, Simon S, Kikkawa U & Eckhart W: The p53 tumour suppressor protein is phosphorylated at serine 389 by casein kinase II. Embo J 1990, 9:3253-60.

Meek DW: Post-translational modification of p53. Semin Cancer Biol 1994, 5:203-10.

Meloche S, Seuwen K, Pages G & Pouyssegur J: Biphasic and synergistic activation of p44mapk (ERK1) by growth factors: correlation between late phase activation and mitogenicity. Molecular Endocrinology 1992, 6:845-54.

Menon AG, Anderson KM, Riccardi VM, Chung RY, Whaley JM, Yandell DW, Farmer GE, Freiman RN, Lee JK, Li FP & et al: Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. Proceedings of the National Academy of Sciences of the United States of America 1990, 87:5435-9.531.

Mercer WE, Amin M, Sauve GJ, Appella E, Ullrich SJ & Romano JW: Wild type human p53 is antiproliferative in SV40-transformed hamster cells. Oncogene 1990, 5:973-80.

Mercer WE, Shields MT, Amin M, Sauve GJ, Appella E, Romano JW & Ullrich SJ: Negative growth regulation in a glioblastoma tumor cell line that conditionally expresses human wild-type p53. Proc Natl Acad Sci U S A 1990, 87:6166-70.

Metcalfe AM, Dixon RM & Radda GK: Wild-type but not mutant p53 activates the hepatocyte growth factor/scatter factor promoter. Nucleic Acids Res 1997, 25:983-6.

Michalovitz D, Fischer FL, Vesco C, Pipas JM & Oren M: Activated Ha-ras can cooperate with defective simian virus 40 in the transformation of nonestablished rat embryo fibroblasts. J Virol 1987, 61:2648-54.

Michalovitz D, Halevy O & Oren M: Conditional inhibition of transformation and of cell proliferation by a temperature-sensitive mutant of p53. Cell 1990, 62:671-80.

Michaud NR, Fabian JR, Mathes KD & Morrison DK: 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are biologically activated in a 14-3-3- and Ras-independent manner. Mol Cell Biol 1995, 15:3390-7.

Michieli P, Chedid M, Lin D, Pierce JH, Mercer WE & Givol D: Induction of WAF1/CIP1 by a p53-independent pathway. Cancer Research 1994, 54:3391-5.

Midgley CA, Owens B, Briscoe CV, Thomas DB, Lane DP & Hall PA: Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type in vivo. J Cell Sci 1995, .876-890.

Mietz JA, Unger T, Huibregtse JM & Howley PM: The transcriptional transactivation function of wild-type p53 is inhibited by SV40 large T-antigen and by HPV-16 E6 oncoprotein. Embo Journal 1992, 11:5013-20.

Miller AD & Rosman GJ: Improved retroviral vectors for gene transfer and expression. Biotechniques 1989, 7:980-2.

Miller ML, Andringa A, Cody T, Dixon K & Albert RE: Cell proliferation and nuclear abnormalities are increased and apoptosis is decreased in the epidermis of the p53 null mouse after topical application of benzo[a]pyrene. Cell Prolif 1996, 29:561-76.

Miller SD, Moses K, Jayaraman L & Prives C: Complex formation between p53 and replication protein A inhibits the sequence-specific DNA binding of p53 and is regulated by single-stranded DNA. Mol Cell Biol 1997, 17:2194-201.

Milne DM, Palmer RH & Meek DW: Mutation of the casein kinase II phosphorylation site abolishes the anti-proliferative activity of p53. Nucleic Acids Res 1992, 20:5565-70.

Milne DM, Palmer RH, Campbell DG & Meek DW: Phosphorylation of the p53 tumour-suppressor protein at three N-terminal sites by a novel casein kinase I-like enzyme. Oncogene 1992, 7:1361-9.

Milne DM, Campbell DG, Caudwell FB & Meek DW: Phosphorylation of the tumor suppressor protein p53 by mitogen-activated protein kinases. J Biol Chem 1994, 269:9253-60.

Milne DM, Campbell LE, Campbell DG & Meek DW: p53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. J Biol Chem 1995, 270:5511-8.

Milne DM, McKendrick L, Jardine LJ, Deacon E, Lord JM & Meek DW: Murine p53 is phosphorylated within the PAb421 epitope by protein kinase C in vivo, even after stimulation with the phorbol ester o-tetradecanoylphorbol 13-acetate. Oncogene 1996, 13:205-11.

Milner J & Watson JV: Addition of fresh medium induces cell cycle and conformation changes in p53, a tumour suppressor protein. Oncogene 1990, 5:1683-90.

Milner J, Cook A & Mason J: p53 is associated with p34cdc2 in transformed cells. Embo J 1990, 9:2885-9.

Milner J & Medcalf EA: Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. Cell 1991, 65:765-74.

Milner J, Medcalf EA & Cook AC: Tumor suppressor p53: analysis of wild-type and mutant p53 complexes. Molecular & Cellular Biology 1991, 11:12-9.

Minden A, Lin A, McMahon M, Lange CC, Derijard B, Davis RJ, Johnson GL & Karin M: Differential activation of ERK and JNK mitogen-activated protein kinases by Raf-1 and MEKK. Science 1994, 266:1719-23.

Minden A, Lin A, Smeal T, Derijard B, Cobb M, Davis R & Karin M: c-Jun N-terminal phosphorylation correlates with activation of the JNK subgroup but not the ERK subgroup of mitogen-activated protein kinases. Mol Cell Biol 1994, 14:6683-8.

Minden A, Lin A, Claret FX, Abo A & Karin M: Selective activation of the JNK signaling cascade and c-Jun transcriptional activity by the small GTPases Rac and Cdc42Hs. Cell 1995, 81:1147-57.

Missero C, Di CF, Kiyokawa H, Koff A & Dotto GP: The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. Genes & Development 1996, 10:3065-75.

Miyashita T & Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995, 80:293-9.

Moll UM, LaQuaglia M, Benard J & Riou G: Wild-type p53 protein undergoes cytoplasmic sequestration in undifferentiated neuroblastomas but not in differentiated tumors. Proceedings of the National Academy of Sciences of the United States of America 1995, 92:4407-11.

Momand J, Zambetti GP, Olson DC, George D & Levine AJ: The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992, 69:1237-45.

Momand J & Zambetti GP: Analysis of the proportion of p53 bound to mdm-2 in cells with defined growth characteristics. Oncogene 1996, 12:2279-89.

Montes dOLR, Wagner DS & Lozano G: Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995, 378:203-6.

Moodie SA, Willumsen BM, Weber MJ & Wolfman A: Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 1993, 260:1658-61.

Moodie SA, Paris M, Villafranca E, Kirshmeier P, Willumsen BM & Wolfman A: Different structural requirements within the switch II region of the Ras protein for interactions with specific downstream targets. Oncogene 1995, 987-995.

Morgenbesser SD, Williams BO, Jacks T & DePinho RA: p53-dependent apoptosis produced by Rb-deficiency in the developing mouse lens. Nature 1994, 371:72-4.

Morgenstern JP & Land H: Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. Nucleic Acids Res 1990, 18:3587-96.

Moroy T, Verbeek S, Ma A, Achacoso P, Berns A & Alt F: E mu N- and E mu L-myc cooperate with E mu pim-1 to generate lymphoid tumors at high frequency in double-transgenic mice. Oncogene 1991, 6:1941-8.

Morris JD, Price B, Lloyd AC, Self AJ, Marshall CJ & Hall A: Scrape-loading of Swiss 3T3 cells with ras protein rapidly activates protein kinase C in the absence of phosphoinositide hydrolysis. Oncogene 1989, 4:27-31.

Morris GF, Bischoff JR & Mathews MB: Transcriptional activation of the human proliferating-cell nuclear antigen promoter by p53. Proc Natl Acad Sci U S A 1996, 93:895-9.

Morrison DK & Cutler RE: The complexity of Raf-1 regulation. Curr Opin Cell Biol 1997, 9:174-9.

Motokura T, Bloom T, Kim HG, Juppner H, Ruderman JV, Kronenberg HM & Arnold A: A novel cyclin encoded by a bcl1-linked candidate oncogene. Nature 1991, 350:512-5.

Mott HR, Carpenter JW, Zhong S, Ghosh S, Bell RM & Campbell SL: The solution structure of the Raf-1 cysteine-rich domain: a novel ras and phospholipid binding site. Proc Natl Acad Sci U S A 1996, 93:8312-7.

Mulcahy LS, Smith MR & Stacey DW: Requirement for ras proto-oncogene function during serum-stimulated growth of NIH 3T3 cells. Nature 1985, 313:241-3.

Mulligan LM, Matlashewski GJ, Scrable HJ & Cavenee WK: Mechanisms of p53 loss in human sarcomas. Proc Natl Acad Sci U S A 1990, 87:5863-7.

Mummenbrauer T, Janus F, Muller B, Wiesmuller L, Deppert W & Grosse F: p53 Protein exhibits 3'-to-5' exonuclease activity. Cell 1996, 85:1089-99.

Muroya K, Hattori S & Nakamura S: Nerve growth factor induces rapid accumulation of the GTP-bound form of p21ras in rat pheochromocytoma PC12 cells. Oncogene 1992, 7:277-81.

Murphy M, Hinman A & Levine AJ: Wild-type p53 negatively regulates the expression of a microtubule-associated protein. Genes Dev 1996, 10:2971-80.

Naik P, Christofori G & Hanahan D: Insulin-like growth factor II is focally up-regulated and functionally involved as a second signal for oncogene-induced tumorigenesis. Cold Spring Harbor Symposia on Quantitative Biology 1994, 59:459-70.

Nakamura T, Pichel JG, Williams SL & Westphal H: An apoptotic defect in lens differentiation caused by human p53 is rescued by a mutant allele. Proc Natl Acad Sci U S A 1995, 92:6142-6.

Nakanishi M, Robetorye RS, Adami GR, Pereira SO & Smith JR: Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. Embo J 1995, 14:555-63.

Nakayama T, Toguchida J, Wadayama B, Kanoe H, Kotoura Y & Sasaki MS: MDM2 gene amplification in bone and soft-tissue tumors: association with tumor progression in differentiated adipose-tissue tumors. Int J Cancer 1995, 64:342-6.

Nakielny S, Cohen P, Wu J & Sturgill T: MAP kinase activator from insulin-stimulated skeletal muscle is a protein threonine/tyrosine kinase. Embo J 1992, 11:2123-9.

Nedbal W, Frey M, Willemann B, Zentgraf H & Sczakiel G: Mechanistic insights into p53-promoted RNA-RNA annealing. J Mol Biol 1997, 266:677-87.

Neiman AM & Herskowitz I: Reconstitution of a yeast protein kinase cascade in vitro: activation of the yeast MEK homologue STE7 by STE11. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:3398-402.

Newbold RF & Overell RW: Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature 1983, 304:648-51.

Nguyen TT, Scimeca JC, Filloux C, Peraldi P, Carpentier JL & Van OE: Co-regulation of the mitogen-activated protein kinase, extracellular signal-regulated kinase 1, and the 90-kDa ribosomal S6 kinase in PC12 cells. Distinct effects of the neurotrophic factor, nerve growth factor, and the mitogenic factor, epidermal growth factor. Journal of Biological Chemistry 1993, 268:9803-10.

Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA & et al: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1995, 376:37-43.

Noda M, Ko M, Ogura A, Liu DG, Amano T, Takano T & Ikawa Y: Sarcoma viruses carrying ras oncogenes induce differentiation-associated properties in a neuronal cell line. Nature 1985, 318:73-5.

Noguchi T, Metz R, Chen L, Mattei MG, Carrasco D & Bravo R: Structure, mapping, and expression of erp, a growth factor-inducible gene encoding a nontransmembrane protein tyrosine phosphatase, and effect of ERP on cell growth. Mol Cell Biol 1993, 13:5195-205.

Northwood IC, Gonzalez FA, Wartmann M, Raden DL & Davis RJ: Isolation and characterization of two growth factor-stimulated protein kinases that phosphorylate the epidermal growth factor receptor at threonine 669. J Biol Chem 1991, 266:15266-76.

Nunez G, Hockenbery D, McDonnell TJ, Sorensen CM & Korsmeyer SJ: Bcl-2 maintains B cell memory. Nature 1991, 353:71-3.

O'Connor DJ, Lam EW, Griffin S, Zhong S, Leighton LC, Burbidge SA & Lu X: Physical and functional interactions between p53 and cell cycle co-operating transcription factors, E2F1 and DP1. Embo J 1995, 14:6184-92.

O'Neill EM, Rebay I, Tjian R & Rubin GM: The activities of two Ets-related transcription factors required for Drosophila eye development are modulated by the Ras/MAPK pathway. Cell 1994, 78:137-47.

Ohmichi M, Pang L, Ribon V & Saltiel AR: Divergence of signaling pathways for insulin in PC-12 pheochromocytoma cells. Endocrinology 1993, 133:46-56.

Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM & Pagano M: Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Molecular & Cellular Biology 1995, 15:2612-24.

Okamoto K & Beach D: Cyclin G is a transcriptional target of the p53 tumor suppressor protein. Embo J 1994, 13:4816-22.

Okamoto K, Kamibayashi C, Serrano M, Prives C, Mumby MC & Beach D: p53-dependent association between cyclin G and the B' subunit of protein phosphatase 2A. Mol Cell Biol 1996, 16:6593-602.

Okazaki K & Sagata N: The Mos/MAP kinase pathway stabilizes c-Fos by phosphorylation and augments its transforming activity in NIH 3T3 cells. Embo J 1995, 14:5048-59.

Oliner JD, Pietenpol JA, Thiagalingam S, Gyuris J, Kinzler KW & Vogelstein B: Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature 1993, 362:857-60.

Olivier JP, Raabe T, Henkemeyer M, Dickson B, Mbamalu G, Margolis B, Schlessinger J, Hafen E & Pawson T: A Drosophila SH2-SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. Cell 1993, 73:179-91.

Olson DC, Marechal V, Momand J, Chen J, Romocki C & Levine AJ: Identification and characterization of multiple mdm-2 proteins and mdm-2-p53 protein complexes. Oncogene 1993, 8:2353-60.

Olson DC & Levine AJ: The properties of p53 proteins selected for the loss of suppression of transformation. Cell Growth Differ 1994, 5:61-71.

Olson MF, Ashworth A & Hall A: An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science 1995, 269:1270-2.

Oltvai ZN, Milliman CL & Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993, 74:609-19.

Oren M & Levine AJ: Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. Proc Natl Acad Sci U S A 1983, 80:56-9.

Osifchin NE, Jiang D, Ohtani FN, Fujita T, Carroza M, Kim SJ, Sakai T & Robbins PD: Identification of a p53 binding site in the human retinoblastoma susceptibility gene promoter. J Biol Chem 1994, 269:6383-9.

Owen SL, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang WW, Kruzel E & et al: Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. Mol Cell Biol 1995, 15:3032-40.

Owens GC & Boyd CJ: Expressing antisense P0 RNA in Schwann cells perturbs myelination. Development 1991, 112:639-49.

Ozbun MA & Butel JS: Tumor suppressor p53 mutations and breast cancer: a critical analysis. Advances in Cancer Research 1995, 66:71-141.

Pagano M, Pepperkok R, Verde F, Ansorge W & Draetta G: Cyclin A is required at two points in the human cell cycle. Embo Journal 1992, 11:961-71.

Pagano M, Theodoras AM, Tam SW & Draetta GF: Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. Genes & Development 1994, 8:1627-39.

Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S & Pouyssegur J: Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A 1993, 90:8319-23.

Pages G, Brunet A, L'Allemain G & Pouyssegur J: Constitutive mutant and putative regulatory serine phosphorylation site of mammalian MAP kinase kinase (MEK1). Embo J 1994, 13:3003-10.

Paggi MG, Baldi A, Bonetto F & Giordano A: Retinoblastoma protein family in cell cycle and cancer: a review. Journal of Cellular Biochemistry 1996, 62:418-30.

Pai EF, Krengel U, Petsko GA, Goody RS, Kabsch W & Wittinghofer A: Refined crystal structure of the triphosphate conformation of H-ras p21 at 1.35 A resolution: implications for the mechanism of GTP hydrolysis. Embo Journal 1990, 9:2351-9.

Pan H & Griep AE: Altered cell cycle regulation in the lens of HPV-16 E6 or E7 transgenic mice: implications for tumor suppressor gene function in development. Genes & Development 1994, 8:1285-99.

Pan ZQ, Reardon JT, Li L, Flores RH, Legerski R, Sancar A & Hurwitz J: Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. J Biol Chem 1995, 270:22008-16.

Papin C, Eychene A, Brunet A, Pages G, Pouyssegur J, Calothy G & Barnier JV: B-Raf protein isoforms interact with and phosphorylate Mek-1 on serine residues 218 and 222. Oncogene 1995, 10:1647-51.

Parada LF, Land H, Weinberg RA, Wolf D & Rotter V: Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation. Nature 1984, 312:649-51.

Parker SB, Eichele G, Zhang P, Rawls A, Sands AT, Bradley A, Olson EN, Harper JW & Elledge SJ: p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. Science 1995, 267:1024-7.

Paulus W, Baur I, Boyce FM, Breakefield XO & Reeves SA: Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. Journal of Virology 1996, 70:62-7.

Pavletich NP, Chambers KA & Pabo CO: The DNA-binding domain of p53 contains the four conserved regions and the major mutation hot spots. Genes Dev 1993, 7:2556-64.

Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ & Sturgill TW: Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). Embo Journal 1991, 10:885-92.

Peacock JW, Matlashewski GJ & Benchimol S: Synergism between pairs of immortalizing genes in transformation assays of rat embryo fibroblasts. Oncogene 1990, 5:1769-74.

Peeper DS, Upton TM, Ladha MH, Neuman E, Zalvide J, Bernards R, DeCaprio JA & Ewen ME: Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. Nature 1997, 386:177-81.

Pennica D, Goeddel DV, Hayflick JS, Reich NC, Anderson CW & Levine AJ: The amino acid sequence of murine p53 determined from a c-DNA clone. Virology 1984, 134:477-82.

Perkins ND, Felzien LK, Betts JC, Leung K, Beach DH & Nabel GJ: Regulation of NF-kappaB by cyclin-dependent kinases associated with the p300 coactivator. Science 1997, 275:523-7.

Perrem K, Rayner J, Voss T, Sturzbecher H, Jackson P & Braithwaite A: p53 represses SV40 transcription by preventing formation of transcription complexes. Oncogene 1995, 11:1299-307.

Perrimon N: Signalling pathways initiated by receptor protein tyrosine kinases in Drosophila. Current Opinion in Cell Biology 1994, 6:260-6.

Perry ME, Piette J, Zawadzki JA, Harvey D & Levine AJ: The mdm-2 gene is induced in response to UV light in a p53-dependent manner. Proc Natl Acad Sci U S A 1993, 90:11623-7.

Peter M, Gartner A, Horecka J, Ammerer G & Herskowitz I: FAR1 links the signal transduction pathway to the cell cycle machinery in yeast. Cell 1993, 73:747-60.

Peter M & Herskowitz I: Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. Science 1994, 265:1228-31.

Peterson SN, Trabalzini L, Brtva TR, Fischer T, Altschuler DL, Martelli P, Lapetina EG, Der CJ & White Gn: Identification of a novel RalGDS-related protein as a candidate effector for Ras and Rap1. Journal of Biological Chemistry 1996, 271:29903-8.

Peto R, Roe FJ, Lee PN, Levy L & Clack J: Cancer and ageing in mice and men. British Journal of Cancer 1975, 32:411-26.

Pham CD, Arlinghaus RB, Zheng CF, Guan KL & Singh B: Characterization of MEK1 phosphorylation by the v-Mos protein. Oncogene 1995, 10:1683-8.

Pietenpol JA, Tokino T, Thiagalingam S, el DW, Kinzler KW & Vogelstein B: Sequence-specific transcriptional activation is essential for growth suppression by p53. Proc Natl Acad Sci U S A 1994, 91:1998-2002.

Pietenpol JA, Lengauer C, Jordan J, Kinzler KW & Vogelstein B: Mammalian cells resistant to tumor suppressor genes. Proc Natl Acad Sci U S A 1996, 93:8390-4.

Poon R, Jiang W, Toyoshima H & Hunter T: Cyclin-dependent kinases are inactivated by a combination of p21 and Thr-14/Tyr-15 phosphorylation after UV-induced DNA damage. J Biol Chem 1996, 271:13283-91.

Porfiri E & McCormick F: Regulation of epidermal growth factor receptor signaling by phosphorylation of the ras exchange factor hSOS1. J Biol Chem 1996, 271:5871-7.

Prendergast GC, Khosravi FR, Solski PA, Kurzawa H, Lebowitz PF & Der CJ: Critical role of Rho in cell transformation by oncogenic Ras. Oncogene 1995, 10:2289-96.

Price MA, Cruzalegui FH & Treisman R: The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light. Embo J 1996, 15:6552-63.

Pritchard CA, Samuels ML, Bosch E & McMahon M: Conditionally oncogenic forms of the A-Raf and B-Raf protein kinases display different biological and biochemical properties in NIH 3T3 cells. Molecular & Cellular Biology 1995, 15:6430-42.

Pronk GJ, Medema RH, Burgering BM, Clark R, McCormick F & Bos JL: Interaction between the p21ras GTPase activating protein and the insulin receptor. J Biol Chem 1992, 267:24058-63.

Pronk GJ, Polakis P, Wong G, de VSA, Bos JL & McCormick F: Association of a tyrosine kinase activity with GAP complexes in v-src transformed fibroblasts. Oncogene 1992, 7:389-94.

Pronk GJ, de VSA, Ellis C & Bos JL: Complex formation between the p21ras GTPase-activating protein and phosphoproteins p62 and p190 is independent of p21ras signalling. Oncogene 1993, 8:2773-80.

Pronk GJ, de VSA, Buday L, Downward J, Maassen JA, Medema RH & Bos JL: Involvement of Shc in insulin- and epidermal growth factor-induced activation of p21ras. Mol Cell Biol 1994, 14:1575-81.

Pulciani S, Santos E, Long LK, Sorrentino V & Barbacid M: ras gene Amplification and malignant transformation. Molecular & Cellular Biology 1985, 5:2836-41.

Pulverer BJ, Kyriakis JM, Avruch J, Nikolakaki E & Woodgett JR: Phosphorylation of c-jun mediated by MAP kinases. Nature 1991, 353:670-4.

Qin XQ, Livingston DM, Kaelin WJ & Adams PD: Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:10918-22.

Qin XQ, Livingston DM, Ewen M, Sellers WR, Arany Z & Kaelin WJ: The transcription factor E2F-1 is a downstream target of RB action. Mol Cell Biol 1995, 15:742-55.

Qiu RG, Chen J, Kirn D, McCormick F & Symons M: An essential role for Rac in Ras transformation. Nature 1995, 374:457-9.

Rabault B, Roussel MF, Quang CT & Ghysdael J: Phosphorylation of Ets1 regulates the complementation of a CSF-1 receptor impaired in mitogenesis. Oncogene 1996, 13:877-81.

Radziwill G, Steinhusen U, Aitken A & Moelling K: Inhibition of Raf/MAPK signaling in Xenopus oocyte extracts by Raf-1-specific peptides. Biochem Biophys Res Commun 1996, 227:20-6.

Raff MC, Barres BA, Burne JF, Coles HS, Ishizaki Y & Jacobson MD: Programmed cell death and the control of cell survival: lessons from the nervous system. Science 1993, 262:695-700.

Ragimov N, Krauskopf A, Navot N, Rotter V, Oren M & Aloni Y: Wild-type but not mutant p53 can repress transcription initiation in vitro by interfering with the binding of basal transcription factors to the TATA motif. Oncogene 1993, 8:1183-93.

Rainwater R, Parks D, Anderson ME, Tegtmeyer P & Mann K: Role of cysteine residues in regulation of p53 function. Mol Cell Biol 1995, 15:3892-903.

Rapp UR, Goldsborough MD, Mark GE, Bonner TI, Groffen J, Reynolds FJ & Stephenson JR: Structure and biological activity of v-raf, a unique oncogene transduced by a retrovirus. Proceedings of the National Academy of Sciences of the United States of America 1983, 80:4218-22.

Rapp UR, Heidecker G, Huleihel M, Cleveland JL, Choi WC, Pawson T, Ihle JN & Anderson WB: raf family serine/threonine protein kinases in mitogen signal transduction. Cold Spring Harbor Symposia on Quantitative Biology 1988, 1:173-84.

Rapp UR: Role of Raf-1 serine/threonine protein kinase in growth factor signal transduction. Oncogene 1991, 6:495-500.

Rassoulzadegan M, Cowie A, Carr A, Glaichenhaus N, Kamen R & Cuzin F: The roles of individual polyoma virus early proteins in oncogenic transformation. Nature 1982, 300:713-8.

Rassoulzadegan M, Naghashfar Z, Cowie A, Carr A, Grisoni M, Kamen R & Cuzin F: Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines. Proceedings of the National Academy of Sciences of the United States of America 1983, 80:4354-8.

Raycroft L, Wu HY & Lozano G: Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science 1990, 249:1049-51.

Ready DF: A multifaceted approach to neural development. Trends in Neurosciences 1989, 12:102-10.

Rebay I & Rubin GM: Yan functions as a general inhibitor of differentiation and is negatively regulated by activation of the Ras1/MAPK pathway. Cell 1995, 81:857-66.

Reed M, Woelker B, Wang P, Wang Y, Anderson ME & Tegtmeyer P: The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. Proc Natl Acad Sci U S A 1995, 92:9455-9.

Reich NC & Levine AJ: Specific interaction of the SV40 T antigen-cellular p53 protein complex with SV40 DNA. Virology 1982, 117:286-90.

Reihsaus E, Kohler M, Kraiss S, Oren M & Montenarh M: Regulation of the level of the oncoprotein p53 in non-transformed and transformed cells. Oncogene 1990, 5:137-45.

Reinke R & Zipursky SL: Cell-cell interaction in the Drosophila retina: the bride of sevenless gene is required in photoreceptor cell R8 for R7 cell development. Cell 1988, 55:321-30.

Resnicoff M, Sell C, Rubini M, Coppola D, Ambrose D, Baserga R & Rubin R: Rat glioblastoma cells expressing an antisense RNA to the insulin-like growth factor-1 (IGF-1) receptor are nontumorigenic and induce regression of wild-type tumors. Cancer Research 1994, 54:2218-22.

Riccardi VM: Type 1 neurofibromatosis and the pediatric patient. Current Problems in Pediatrics 1992, 22:66-106.

Richard S, Yu D, Blumer KJ, Hausladen D, Olszowy MW, Connelly PA & Shaw AS: Association of p62, a multifunctional SH2- and SH3-domain-binding protein, with src family tyrosine kinases, Grb2, and phospholipase C gamma-1. Mol Cell Biol 1995, 15:186-97.

Ridley AJ, Paterson HF, Noble M & Land H: Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. Embo J 1988, 7:1635-45.

Ridley AJ, Davis JB, Stroobant P & Land H: Transforming growth factors-beta 1 and beta 2 are mitogens for rat Schwann cells. J Cell Biol 1989, 345-352.

Ridley AJ & Hall A: The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 1992, 70:389-99.

Ridley AJ, Self AJ, Kasmi F, Paterson HF, Hall A, Marshall CJ & Ellis C: rho family GTPase activating proteins p190, bcr and rhoGAP show distinct specificities in vitro and in vivo. Embo J 1993, 12:5151-60.

Ridley AJ & Hall A: Signal transduction pathways regulating Rho-mediated stress fibre formation: requirement for a tyrosine kinase. Embo J 1994, 13:2600-10.

Ridley AJ: Signal transduction through the GTP-binding proteins Rac and Rho. J Cell Sci Suppl 1994, 18:127-31.

Rime H, Huchon D, De SV, Thibier C, Galaktionov K, Jessus C & Ozon R: Microinjection of Cdc25 protein phosphatase into Xenopus prophase oocyte activates MPF and arrests meiosis at metaphase I. Biol Cell 1994, 82:11-22.

Robinson MJ, Harkins PC, Zhang J, Baer R, Haycock JW, Cobb MH & Goldsmith EJ: Mutation of position 52 in ERK2 creates a nonproductive binding mode for adenosine 5'-triphosphate. Biochemistry 1996, 35:5641-6.

Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV & Lane DP: p53 mutations in colorectal cancer. Proc Natl Acad Sci U S A 1990, 87:7555-9.

Rodriguez VP, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD & Downward J: Phosphatidylinositol-3-OH kinase as a direct target of Ras. Nature 1994, 370:527-32.

Rodriguez VP, Warne PH, Vanhaesebroeck B, Waterfield MD & Downward J: Activation of phosphoinositide 3-kinase by interaction with Ras and by point mutation. Embo J 1996, 15:2442-51.

Rodriguez VP, Warne PH, Khwaja A, Marte BM, Pappin D, Das P, Waterfield MD, Ridley A & Downward J: Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell 1997, 89:457-67.

Rogister B, Delree P, Leprince P, Martin D, Sadzot C, Malgrange B, Munaut C, Rigo JM, Lefebvre PP, Octave JN & et al: Transforming growth factor beta as a neuronoglial signal during peripheral nervous system response to injury. Journal of Neuroscience Research 1993, 34:32-43.

Rohan PJ, Davis P, Moskaluk CA, Kearns M, Krutzsch H, Siebenlist U & Kelly K: PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. Science 1993, 259:1763-6.

Rolley N & Milner J: Specific DNA binding by p53 is independent of mutation at serine 389, the casein kinase II site. Oncogene 1994, 9:3067-70.

Rolley N, Butcher S & Milner J: Specific DNA binding by different classes of human p53 mutants. Oncogene 1995, 11:763-70.

Rotter V, Schwartz D, Almon E, Goldfinger N, Kapon A, Meshorer A, Donehower LA & Levine AJ: Mice with reduced levels of p53 protein exhibit the testicular giant-cell degenerative syndrome. Proc Natl Acad Sci U S A 1993, 90:9075-9.

Rowan S, Ludwig RL, Haupt Y, Bates S, Lu X, Oren M & Vousden KH: Specific loss of apoptotic but not cell-cycle arrest function in a human tumor derived p53 mutant. Embo J 1996, 15:827-38.

Rozakis AM, McGlade J, Mbamalu G, Pelicci G, Daly R, Li W, Batzer A, Thomas S, Brugge J, Pelicci PG & et al: Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. Nature 1992, 360:689-92.

Rozakis AM, Fernley R, Wade J, Pawson T & Bowtell D: The SH2 and SH3 domains of mammalian Grb2 couple the EGF receptor to the Ras activator mSos1. Nature 1993, 363:83-5.

Rozakis AM, van dGP, Mbamalu G & Pawson T: MAP kinase phosphorylation of mSos1 promotes dissociation of mSos1-Shc and mSos1-EGF receptor complexes. Oncogene 1995, 11:1417-26.

Ruley HE: Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. Nature 1983, 304:602-6.

Russell M, Lange CC & Johnson GL: Direct interaction between Ras and the kinase domain of mitogen-activated protein kinase kinase kinase (MEKK1). J Biol Chem 1995, 270:11757-60.

Sabbatini P, Lin J, Levine AJ & White E: Essential role for p53-mediated transcription in E1A-induced apoptosis. Genes Dev 1995, 9:2184-92.

Sabbatini P, Chiou SK, Rao L & White E: Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19K protein. Mol Cell Biol 1995, 15:1060-70.

Sager R, Tanaka K, Lau CC, Ebina Y & Anisowicz A: Resistance of human cells to tumorigenesis induced by cloned transforming genes. Proceedings of the National Academy of Sciences of the United States of America 1983, 80:7601-5.

Sah VP, Attardi LD, Mulligan GJ, Williams BO, Bronson RT & Jacks T: A subset of p53-deficient embryos exhibit exencephaly. Nat Genet 1995, 10:175-80.

Samuels ML, Weber MJ, Bishop JM & McMahon M: Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1 protein kinase. Molecular & Cellular Biology 1993, 13:6241-52.

Sanchez I, Hughes RT, Mayer BJ, Yee K, Woodgett JR, Avruch J, Kyriakis JM & Zon LI: Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun. Nature 1994, 372:794-8.

Sandig V, Brand K, Herwig S, Lukas J, Bartek J & Strauss M: Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. Nat Med 1997, 3:313-9.

Sangfelt O, Erickson S, Einhorn S & Grander D: Induction of Cip/Kip and Ink4 cyclin dependent kinase inhibitors by interferon-alpha in hematopoietic cell lines. Oncogene 1997, 14:415-23.

Sato T, Tanigami A, Yamakawa K, Akiyama F, Kasumi F, Sakamoto G & Nakamura Y: Allelotype of breast cancer: cumulative allele losses promote tumor progression in primary breast cancer. Cancer Res 1990, 50:7184-9.

Schaap D, van dWJ, Howe LR, Marshall CJ & van BW: A dominant-negative mutant of raf blocks mitogen-activated protein kinase activation by growth factors and oncogenic p21ras. J Biol Chem 1993, 268:20232-6.

Scheffner M, Werness BA, Huibregtse JM, Levine AJ & Howley PM: The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990, 63:1129-36.

Scheffner M, Munger K, Huibregtse JM & Howley PM: Targeted degradation of the retinoblastoma protein by human papillomavirus E7-E6 fusion proteins. Embo J 1992, 11:2425-31.

Scheffner M, Takahashi T, Huibregtse JM, Minna JD & Howley PM: Interaction of the human papillomavirus type 16 E6 oncoprotein with wild-type and mutant human p53 proteins. J Virol 1992, 66:5100-5.

Scheffner M, Huibregtse JM, Vierstra RD & Howley PM: The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 1993, 75:495-505.

Schlichting I, Almo SC, Rapp G, Wilson K, Petratos K, Lentfer A, Wittinghofer A, Kabsch W, Pai EF, Petsko GA & et al: Time-resolved X-ray crystallographic study of the conformational change in Ha-Ras p21 protein on GTP hydrolysis. Nature 1990, 345:309-15.

Schott AF, Apel IJ, Nunez G & Clarke MF: Bcl-XL protects cancer cells from p53-mediated apoptosis. Oncogene 1995, 11:1389-94.

Schuuring E, Verhoeven E, Mooi WJ & Michalides RJ: Identification and cloning of two overexpressed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene 1992, 7:355-61.

Sedlak TW, Oltvai ZN, Yang E, Wang K, Boise LH, Thompson CB & Korsmeyer SJ: Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci U S A 1995, 92:7834-8.

Sehgal PB & Margulies L: Cell-type- and promoter-dependent ts phenotype of p53 Val135. Oncogene 1993, 8:3417-9.

Selvakumaran M, Lin HK, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B & Liebermann D: Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene 1994, 9:1791-8.

Sentman CL, Shutter JR, Hockenbery D, Kanagawa O & Korsmeyer SJ: bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. Cell 1991, 67:879-88.

Serrano M, Gomez LE, DePinho RA, Beach D & Bar SD: Inhibition of ras-induced proliferation and cellular transformation by p16INK4. Science 1995, 267:249-52.

Serrano M, Lee H, Chin L, Cordon CC, Beach D & DePinho RA: Role of the INK4a locus in tumor suppression and cell mortality. Cell 1996, 85:27-37.

Serrano M, Lin AW, McCurrach ME, Beach D & Lowe SW: Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 1997, 88:593-602.

Seth A, Alvarez E, Gupta S & Davis RJ: A phosphorylation site located in the NH2-terminal domain of c-Myc increases transactivation of gene expression. J Biol Chem 1991, 266:23521-4.

Seth A, Gonzalez FA, Gupta S, Raden DL & Davis RJ: Signal transduction within the nucleus by mitogen-activated protein kinase. J Biol Chem 1992, 267:24796-804.

Seto E, Usheva A, Zambetti GP, Momand J, Horikoshi N, Weinmann R, Levine AJ & Shenk T: Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc Natl Acad Sci U S A 1992, 89:12028-32.

Settleman J, Albright CF, Foster LC & Weinberg RA: Association between GTPase activators for Rho and Ras families. Nature 1992, 359:153-4.

Settleman J, Narasimhan V, Foster LC & Weinberg RA: Molecular cloning of cDNAs encoding the GAP-associated protein p190: implications for a signaling pathway from ras to the nucleus. Cell 1992, 69:539-49.

Sewing A, Wiseman B, Lloyd AC & Land H: High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Molecular & Cellular Biology 1997, 17:5588-97.

Shannon KM, O'Connell P, Martin GA, Paderanga D, Olson K, Dinndorf P & McCormick F: Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders . New England Journal of Medicine 1994, 330:597-601.

Shapiro GI, Park JE, Edwards CD, Mao L, Merlo A, Sidransky D, Ewen ME & Rollins BJ: Multiple mechanisms of p16INK4A inactivation in non-small cell lung cancer cell lines. Cancer Res 1995, 55:6200-9.

Shaulian E, Zauberman A, Ginsberg D & Oren M: Identification of a minimal transforming domain of p53: negative dominance through abrogation of sequence-specific DNA binding. Mol Cell Biol 1992, 12:5581-92.

Shaulian E, Zauberman A, Milner J, Davies EA & Oren M: Tight DNA binding and oligomerization are dispensable for the ability of p53 to transactivate target genes and suppress transformation. Embo J 1993, 12:2789-97.

Shaulian E, Haviv I, Shaul Y & Oren M: Transcriptional repression by the C-terminal domain of p53. Oncogene 1995, 10:671-80.

Shaulsky G, Goldfinger N, Ben ZeA & Rotter V: Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. Mol Cell Biol 1990, 10:6565-77.

Shaulsky G, Goldfinger N, Tosky MS, Levine AJ & Rotter V: Nuclear localization is essential for the activity of p53 protein. Oncogene 1991, 6:2055-65.

Shaw P, Freeman J, Bovey R & Iggo R: Regulation of specific DNA binding by p53: evidence for a role for O-glycosylation and charged residues at the carboxy-terminus. Oncogene 1996, 12:921-30.

Sheela S, Riccardi VM & Ratner N: Angiogenic and invasive properties of neurofibroma Schwann cells. Journal of Cell Biology 1990, 111:645-53.

Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP & Green DR: Role for c-myc in activation-induced apoptotic cell death in T cell hybridomas. Science 1992, 257:212-4.

Shibata D, Schaeffer J, Li ZH, Capella G & Perucho M: Genetic heterogeneity of the c-K-ras locus in colorectal adenomas but not in adenocarcinomas. Journal of the National Cancer Institute 1993, 85:1058-63.

Shibuya EK, Boulton TG, Cobb MH & Ruderman JV: Activation of p42 MAP kinase and the release of oocytes from cell cycle arrest. Embo J 1992, 11:3963-75.

Shin TH, Paterson AJ & Kudlow JE: p53 stimulates transcription from the human transforming growth factor alpha promoter: a potential growth-stimulatory role for p53. Mol Cell Biol 1995, 15:4694-701.

Shiohara M, el DW, Wada M, Nakamaki T, Takeuchi S, Yang R, Chen DL, Vogelstein B & Koeffler HP: Absence of WAF1 mutations in a variety of human malignancies. Blood 1994, 84:3781-4.

Shirouzu M, Koide H, Fujita YJ, Oshio H, Toyama Y, Yamasaki K, Fuhrman SA, Villafranca E, Kaziro Y & Yokoyama S: Mutations that abolish the ability of Ha-Ras to associate with Raf-1. Oncogene 1994, 9:2153-7.

Shivakumar CV, Brown DR, Deb S & Deb SP: Wild-type human p53 transactivates the human proliferating cell nuclear antigen promoter. Mol Cell Biol 1995, 15:6785-93.

Shou C, Farnsworth CL, Neel BG & Feig LA: Molecular cloning of cDNAs encoding a guanine-nucleotide-releasing factor for Ras p21. Nature 1992, 358:351-4.

Simon MA, Dodson GS & Rubin GM: An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. Cell 1993, 73:169-77.

Simon MA: Signal transduction during the development of the Drosophila R7 photoreceptor. Dev Biol 1994, 166:431-42.

Simons PJ, Bassin RH & Harvey JJ: Transformation of hamster embryo cells in vitro by Murine Sarcoma Virus (Harvey). Proceedings of the Society for Experimental Biology & Medicine 1967, 125:1242-6.

Sinn E, Muller W, Pattengale P, Tepler I, Wallace R & Leder P: Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell 1987, 49:465-75.

Sjolander A, Yamamoto K, Huber BE & Lapetina EG: Association of p21ras with phosphatidylinositol 3-kinase. Proceedings of the National Academy of Sciences of the United States of America 1991, 88:7908-12.

Skladanowski A & Larsen AK: Expression of wild-type p53 increases etoposide cytotoxicity in M1 myeloid leukemia cells by facilitated G2 to M transition: implications for gene therapy. Cancer Res 1997, 57:818-23.

Skolnik EY, Lee CH, Batzer A, Vicentini LM, Zhou M, Daly R, Myers MJ, Backer JM, Ullrich A, White MF & et al: The SH2/SH3 domain-containing protein GRB2 interacts with tyrosine-phosphorylated IRS1 and Shc: implications for insulin control of ras signalling. Embo J 1993, 12:1929-36.

Skolnik EY, Batzer A, Li N, Lee CH, Lowenstein E, Mohammadi M, Margolis B & Schlessinger J: The function of GRB2 in linking the insulin receptor to Ras signaling pathways. Science 1993, 260:1953-5.

Smith MR, Heidecker G, Rapp UR & Kung HF: Induction of transformation and DNA synthesis after microinjection of raf proteins. Molecular & Cellular Biology 1990, 10:3828-33.

Smith ML, Chen IT, Zhan Q, Bae I, Chen CY, Gilmer TM, Kastan MB, O'Connor PM & Fornace AJ: Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science 1994, 266:1376-80.

Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM & Fornace AJ: Antisense GADD45 expression results in decreased DNA repair and sensitizes cells to u.v.-irradiation or cisplatin. Oncogene 1996, 13:2255-63.

Smith ML, Kontny HU, Bortnick R & Fornace AJ: The p53-regulated cyclin G gene promotes cell growth: p53 downstream effectors cyclin G and Gadd45 exert different effects on cisplatin chemosensitivity. Exp Cell Res 1997, 230:61-8.

Som T & Kolaparthi VS: Developmental decisions in Aspergillus nidulans are modulated by Ras activity. Molecular & Cellular Biology 1994, 14:5333-48.

Somasundaram K & El DW: Inhibition of p53-mediated transactivation and cell cycle arrest by E1A through its p300/CBP-interacting region. Oncogene 1997, 14:1047-57.

Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M & Mumby M: The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell 1993, 75:887-97.

Sorensen TS, Girling R, Lee CW, Gannon J, Bandara LR & La TN: Functional interaction between DP-1 and p53. Mol Cell Biol 1996, 16:5888-95.

Spaargaren M & Bischoff JR: Identification of the guanine nucleotide dissociation stimulator for Ral as a putative effector molecule of R-ras, H-ras, K-ras, and Rap. Proceedings of the National Academy of Sciences of the United States of America 1994, 91:12609-13.

Srivastava S, Zou ZQ, Pirollo K, Blattner W & Chang EH: Germ-line transmission of a mutated p53 gene in a cancer-prone family with Li-Fraumeni syndrome. Nature 1990, 348:747-9.

Srivastava S, Tong YA, Devadas K, Zou ZQ, Sykes VW, Chen Y, Blattner WA, Pirollo K & Chang EH: Detection of both mutant and wild-type p53 protein in normal skin fibroblasts and demonstration of a shared 'second hit' on p53 in diverse tumors from a cancer-prone family with Li-Fraumeni syndrome. Oncogene 1992, 7:987-91.761.

Srivastava S, Wang S, Tong YA, Pirollo K & Chang EH: Several mutant p53 proteins detected in cancer-prone families with Li-Fraumeni syndrome exhibit transdominant effects on the biochemical properties of the wild-type p53. Oncogene 1993, 8:2449-56.

Stacey DW & Kung HF: Transformation of NIH 3T3 cells by microinjection of Ha-ras p21 protein. Nature 1984, 310:508-11.

Stacey DW, Roudebush M, Day R, Mosser SD, Gibbs JB & Feig LA: Dominant inhibitory Ras mutants demonstrate the requirement for Ras activity in the action of tyrosine kinase oncogenes. Oncogene 1991, 6:2297-304.

Stacey DW, Feig LA & Gibbs JB: Dominant inhibitory Ras mutants selectively inhibit the activity of either cellular or oncogenic Ras. Mol Cell Biol 1991, 11:4053-64.

Stein GH & Dulic V: Origins of G1 arrest in senescent human fibroblasts. Bioessays 1995, 17:537-43.

Steinman RA, Hoffman B, Iro A, Guillouf C, Liebermann DA & el HM: Induction of p21 (WAF-1/CIP1) during differentiation. Oncogene 1994, 9:3389-96.

Stenger JE, Tegtmeyer P, Mayr GA, Reed M, Wang Y, Wang P, Hough PV & Mastrangelo IA: p53 oligomerization and DNA looping are linked with transcriptional activation. Embo J 1994, 13:6011-20.

Stern MJ, Marengere LE, Daly RJ, Lowenstein EJ, Kokel M, Batzer A, Olivier P, Pawson T & Schlessinger J: The human GRB2 and Drosophila Drk genes can functionally replace the Caenorhabditis elegans cell signaling gene sem-5. Mol Biol Cell 1993, 4:1175-88.

Sternberg PW, Golden A & Han M: Role of a raf proto-oncogene during Caenorhabditis elegans vulval development. Philos Trans R Soc Lond B Biol Sci 1993, 340:259-65.

Stewart N, Hicks GG, Paraskevas F & Mowat M: Evidence for a second cell cycle block at G2/M by p53. Oncogene 1995, 10:109-15.

Stokoe D, Campbell DG, Nakielny S, Hidaka H, Leevers SJ, Marshall C & Cohen P: MAPKAP kinase-2; a novel protein kinase activated by mitogen-activated protein kinase. Embo J 1992, 11:3985-94.

Stokoe D, Macdonald SG, Cadwallader K, Symons M & Hancock JF: Activation of Raf as a result of recruitment to the plasma membrane. Science 1994, 264:1463-7.

Strahl T, Gille H & Shaw PE: Selective response of ternary complex factor Sap1a to different mitogen-activated protein kinase subgroups. Proc Natl Acad Sci U S A 1996, 93:11563-8.

Strasser A, Harris AW, Bath ML & Cory S: Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. Nature 1990, 348:331-3.

Strausfeld U, Labbe JC, Fesquet D, Cavadore JC, Picard A, Sadhu K, Russell P & Doree M: Dephosphorylation and activation of a p34cdc2/cyclin B complex in vitro by human CDC25 protein. Nature 1991, 351:242-5.

Stuart ET, Haffner R, Oren M & Gruss P: Loss of p53 function through PAX-mediated transcriptional repression. Embo J 1995, 14:5638-45.

Sturzbecher HW, Maimets T, Chumakov P, Brain R, Addison C, Simanis V, Rudge K, Philp R, Grimaldi M, Court W & et al: p53 interacts with p34cdc2 in mammalian cells: implications for cell cycle control and oncogenesis. Oncogene 1990, 5:795-81.

Sturzbecher HW, Brain R, Addison C, Rudge K, Remm M, Grimaldi M, Keenan E & Jenkins JR: A C-terminal alpha-helix plus basic region motif is the major structural determinant of p53 tetramerization. Oncogene 1992, 7:1513-23.

Subler MA, Martin DW & Deb S: Inhibition of viral and cellular promoters by human wild-type p53. J Virol 1992, 66:4757-62.

Subler MA, Martin DW & Deb S: Overlapping domains on the p53 protein regulate its transcriptional activation and repression functions. Oncogene 1994, 9:1351-9.

Suen KL, Bustelo XR, Pawson T & Barbacid M: Molecular cloning of the mouse grb2 gene: differential interaction of the Grb2 adaptor protein with epidermal growth factor and nerve growth factor receptors. Molecular & Cellular Biology 1993, 13:5500-12.

Suen KL, Bustelo XR & Barbacid M: Lack of evidence for the activation of the Ras/Raf mitogenic pathway by 14-3-3 proteins in mammalian cells. Oncogene 1995, 11:825-31.

Sun H, Charles CH, Lau LF & Tonks NK: MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 1993, 75:487-93.

Sun H, Tonks NK & Bar SD: Inhibition of Ras-induced DNA synthesis by expression of the phosphatase MKP-1. Science 1994, 266:285-8.

Sun Y, Bian J, Wang Y & Jacobs C: Activation of p53 transcriptional activity by 1,10-phenanthroline, a metal chelator and redox sensitive compound. Oncogene 1997, 14:385-93.

Sundaram M & Han M: The C. elegans ksr-1 gene encodes a novel Raf-related kinase involved in Ras-mediated signal transduction. Cell 1995, 83:889-901.

Sundaram M, Yochem J & Han M: A Ras-mediated signal transduction pathway is involved in the control of sex myoblast migration in Caenorhabditis elegans. Development 1996, 122:2823-33.

Sutherland C, Campbell DG & Cohen P: Identification of insulin-stimulated protein kinase-1 as the rabbit equivalent of rskmo-2. Identification of two threonines phosphorylated during activation by mitogen-activated protein kinase. Eur J Biochem 1993, 212:581-8.

Symonds H, Krall L, Remington L, Saenz RM, Lowe S, Jacks T & Van DT: p53-dependent apoptosis suppresses tumor growth and progression in vivo. Cell 1994, 78:703-11.

Takenaka I, Morin F, Seizinger BR & Kley N: Regulation of the sequence-specific DNA binding function of p53 by protein kinase C and protein phosphatases. J Biol Chem 1995, 270:5405-11.

Takishima K, Griswold PI, Ingebritsen T & Rosner MR: Epidermal growth factor (EGF) receptor T669 peptide kinase from 3T3-L1 cells is an EGF-stimulated "MAP" kinase. Proc Natl Acad Sci U S A 1991, 88:2520-4.

Tanaka K, Nakafuku M, Satoh T, Marshall MS, Gibbs JB, Matsumoto K, Kaziro Y & Toh eA: S. cerevisiae genes IRA1 and IRA2 encode proteins that may be functionally equivalent to mammalian ras GTPase activating protein. Cell 1990, 60:803-7.

Tanaka K, Lin BK, Wood DR & Tamanoi F: IRA2, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proceedings of the National Academy of Sciences of the United States of America 1991, 88:468-72.

Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW & Taniguchi T: Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell 1994, 77:829-39.

Tanaka N, Ishihara M, Lamphier MS, Nozawa H, Matsuyama T, Mak TW, Aizawa S, Tokino T, Oren M & Taniguchi T: Cooperation of the tumour suppressors IRF-1 and p53 in response to DNA damage. Nature 1996, 382:816-8.

Tarunina M & Jenkins JR: Human p53 binds DNA as a protein homodimer but monomeric variants retain full transcription transactivation activity. Oncogene 1993, 8:3165-73.

Taylor SJ & Shalloway D: Cell cycle-dependent activation of Ras. Curr Biol 1996, 6:1621-7.

Tegtmeyer P, Schwartz M, Collins JK & Rundell K: Regulation of tumor antigen synthesis by simain virus 40 gene A. Journal of Virology 1975, 16:168-78.

Terry LA, Boyd J, Alcorta D, Lyon T, Solomon G, Hannon G, Berchuck A, Beach D & Barrett JC: Mutational analysis of the p21/WAF1/CIP1/SDI1 coding region in human tumor cell lines. Mol Carcinog 1996, 16:221-8.

Therrien M, Chang HC, Solomon NM, Karim FD, Wassarman DA & Rubin GM: KSR, a novel protein kinase required for RAS signal transduction. Cell 1995, 83:879-88.

Therrien M, Michaud NR, Rubin GM & Morrison DK: KSR modulates signal propagation within the MAPK cascade. Genes & Development 1996, 10:2684-95.

Thompson TC, Southgate J, Kitchener G & Land H: Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted organ. Cell 1989, 56:917-30.

Thorburn J, Carlson M, Mansour SJ, Chien KR, Ahn NG & Thorburn A: Inhibition of a signaling pathway in cardiac muscle cells by active mitogen-activated protein kinase kinase. Mol Biol Cell 1995, 6:1479-90.

Thut CJ, Chen JL, Klemm R & Tjian R: p53 transcriptional activation mediated by coactivators TAFII40 and TAFII60. Science 1995, 267:100-4.

Timchenko NA, Wilde M, Nakanishi M, Smith JR & Darlington GJ: CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. Genes Dev 1996, 10:804-15.

Traverse S, Gomez N, Paterson H, Marshall C & Cohen P: Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. Biochem J 1992, .445-450.

Traverse S, Cohen P, Paterson H, Marshall C, Rapp U & Grand RJ: Specific association of activated MAP kinase kinase kinase (Raf) with the plasma membranes of ras-transformed retinal cells. Oncogene 1993, 8:3175-81.

Traverse S, Seedorf K, Paterson H, Marshall CJ, Cohen P & Ullrich A: EGF triggers neuronal differentiation of PC12 cells that overexpress the EGF receptor. Curr Biol 1994, 4:694-701.

Treisman R, Novak U, Favaloro J & Kamen R: Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature 1981, 292:595-600.

Treisman R: Regulation of transcription by MAP kinase cascades. Curr Opin Cell Biol 1996, 8:205-15.

Truant R, Xiao H, Ingles CJ & Greenblatt J: Direct interaction between the transcriptional activation domain of human p53 and the TATA box-binding protein. J Biol Chem 1993, 268:2284-7.

Truant R, Antunovic J, Greenblatt J, Prives C & Cromlish JA: Direct interaction of the hepatitis B virus HBx protein with p53 leads to inhibition by HBx of p53 response element-directed transactivation. J Virol 1995, 69:1851-9.

Tsai LH, Lees E, Faha B, Harlow E & Riabowol K: The cdk2 kinase is required for the G1-to-S transition in mammalian cells. Oncogene 1993, 8:1593-602.

Tsuda L, Inoue YH, Yoo MA, Mizuno M, Hata M, Lim YM, Adachi YT, Ryo H, Masamune Y & Nishida Y: A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila. Cell 1993, 72:407-14.

Tsurumi C, Ishida N, Tamura T, Kakizuka A, Nishida E, Okumura E, Kishimoto T, Inagaki M, Okazaki K, Sagata N & et al: Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. Mol Cell Biol 1995, 15:5682-7.

Ueda H, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Feitelson MA & Jay G: Functional inactivation but not structural mutation of p53 causes liver cancer. Nature Genetics 1995, 9:41-7.

Ueda K & Ganem D: Apoptosis is induced by N-myc expression in hepatocytes, a frequent event in hepadnavirus oncogenesis, and is blocked by insulin-like growth factor II. Journal of Virology 1996, 70:1375-83.

Ueki K, Matsuda S, Tobe K, Gotoh Y, Tamemoto H, Yachi M, Akanuma Y, Yazaki Y, Nishida E & Kadowaki T: Feedback regulation of mitogen-activated protein kinase kinase kinase activity of c-Raf-1 by insulin and phorbol ester stimulation. J Biol Chem 1994, 269:15756-61.

Unger T, Mietz JA, Scheffner M, Yee CL & Howley PM: Functional domains of wild-type and mutant p53 proteins involved in transcriptional regulation, transdominant inhibition, and transformation suppression. Molecular & Cellular Biology 1993, 13:5186-94.

Urano T, Emkey R & Feig LA: Ral-GTPases mediate a distinct downstream signaling pathway from Ras that facilitates cellular transformation. Embo J 1996, 15:810-6.

Valentinis B, Bhala A, DeAngelis T, Baserga R & Cohen P: The human insulin-like growth factor (IGF) binding protein-3 inhibits the growth of fibroblasts with a targeted disruption of the IGF-I receptor gene. Mol Endocrinol 1995, 9:361-7.

van LM, Verbeek S, Krimpenfort P, Domen J, Saris C, Radaszkiewicz T & Berns A: Predisposition to lymphomagenesis in pim-1 transgenic mice: cooperation with c-myc and N-myc in murine leukemia virus-induced tumors. Cell 1989, 56:673-82.

van LM, Verbeek S, Scheijen B, Wientjens E, van, der, Gulden, H & Berns A: Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. Cell 1991, 65:737-52.

Van, Vactor, D.L., Jr, Cagan RL, Kramer H & Zipursky SL: Induction in the developing compound eye of Drosophila: multiple mechanisms restrict R7 induction to a single retinal precursor cell. Cell 1991, 67:1145-55.

Van AL, Barr M, Marcus S, Polverino A & Wigler M: Complex formation between RAS and RAF and other protein kinases. Proc Natl Acad Sci U S A 1993, 90:6213-7.

van, den, Heuvel, S & Harlow E: Distinct roles for cyclin-dependent kinases in cell cycle control. Science 1993, 262:2050-4.

Van AL, White MA & Wigler MH: Ras partners. Cold Spring Harbor Symposia on Quantitative Biology 1994, 59:181-6.

van BT, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, Sakaue M, Luttrell LM & Lefkowitz RJ: Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. Nature 1995, 376:781-4.

van, der, Lugt, Nm, Domen J, Verhoeven E, Linders K, van, der, Gulden, H, Allen J & Berns A: Proviral tagging in E mu-myc transgenic mice lacking the Pim-1 proto-oncogene leads to compensatory activation of Pim-2. Embo Journal 1995, 14:2536-44.

Vaux DL, Cory S & Adams JM: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 1988, 335:440-2.

Velu TJ, Vass WC, Lowy DR & Beguinot L: Functional heterogeneity of proto-oncogene tyrosine kinases: the C terminus of the human epidermal growth factor receptor facilitates cell proliferation. Molecular & Cellular Biology 1989, 9:1772-8.

Verbeek S, van LM, van, der, Valk, M, Domen J, Kraal G & Berns A: Mice bearing the E mu-myc and E mu-pim-1 transgenes develop pre-B-cell leukemia prenatally. Molecular & Cellular Biology 1991, 11:1176-9.

Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM & Bos JL: Genetic alterations during colorectal-tumor development. New England Journal of Medicine 1988, 319:525-32.

Vogelstein B & Kinzler KW: The multistep nature of cancer. Trends in Genetics 1993, 9:138-41.

Vojtek AB, Hollenberg SM & Cooper JA: Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell 1993, 74:205-14.

Vousden KH: Human papillomavirus oncoproteins. Semin Cancer Biol 1990, 1:415-24.

Waga S, Hannon GJ, Beach D & Stillman B: The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Science 1994, 4:358-361.

Wagner AJ, Small MB & Hay N: Myc-mediated apoptosis is blocked by ectopic expression of Bcl-2. Molecular & Cellular Biology 1993, 13:2432-40.

Waldman T, Kinzler KW & Vogelstein B: p21 is necessary for the p53-mediated G1 arrest in human cancer cells. Cancer Research 1995, 55:5187-90.

Walker KK & Levine AJ: Identification of a novel p53 functional domain that is necessary for efficient growth suppression. Proc Natl Acad Sci U S A 1996, 93:15335-40.

Wallace MR, Marchuk DA, Andersen LB, Letcher R, Odeh HM, Saulino AM, Fountain JW, Brereton A, Nicholson J, Mitchell AL & et al: Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients, Science, 1990,3:244-249.

Wang EH, Friedman PN & Prives C: The murine p53 protein blocks replication of SV40 DNA in vitro by inhibiting the initiation functions of SV40 large T antigen. Cell 1989, 57:379-92.

Wang Y, You M, Reynolds SH, Stoner GD & Anderson MW: Mutational activation of the cellular Harvey ras oncogene in rat esophageal papillomas induced by methylbenzylnitrosamine. Cancer Res 1990, 50:1591-5.

Wang Y & Eckhart W: Phosphorylation sites in the amino-terminal region of mouse p53. Proc Natl Acad Sci U S A 1992, 89:4231-5.

Wang Y, Reed M, Wang P, Stenger JE, Mayr G, Anderson ME, Schwedes JF & Tegtmeyer P: p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes Dev 1993, 7:2575-86.

Wang P, Reed M, Wang Y, Mayr G, Stenger JE, Anderson ME, Schwedes JF & Tegtmeyer P: p53 domains: structure, oligomerization, and transformation. Mol Cell Biol 1994, 14:5182-91.

Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR & Harris CC: Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. Proc Natl Acad Sci U S A 1994, 91:2230-4.

Wang Y & Prives C: Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases. Nature 1995, 376:88-91.

Wang Y, Schwedes JF, Parks D, Mann K & Tegtmeyer P: Interaction of p53 with its consensus DNA-binding site. Mol Cell Biol 1995, 15:2157-65.

Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly JM, Wang Z, Freidberg EC, Evans MK, Taffe BG & et al: p53 modulation of TFIIH-associated nucleotide excision repair activity. Nat Genet 1995, 10:188-95.

Wang XW, Gibson MK, Vermeulen W, Yeh H, Forrester K, Sturzbecher HW, Hoeijmakers JH & Harris CC: Abrogation of p53-induced apoptosis by the hepatitis B virus X gene. Cancer Res 1995, 55:6012-6.

Wang XW, Vermeulen W, Coursen JD, Gibson M, Lupold SE, Forrester K, Xu G, Elmore L, Yeh H, Hoeijmakers JH & Harris CC: The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. Genes Dev 1996, 10:1219-32.

Wang A, Yoshimi N, Ino N, Tanaka T & Mori H: WAF1 expression and p53 mutations in human colorectal cancers. J Cancer Res Clin Oncol 1997, 123:118-23.

Warbrick E, Lane DP, Glover DM & Cox LS: A small peptide inhibitor of DNA replication defines the site of interaction between the cyclin-dependent kinase inhibitor p21WAF1 and proliferating cell nuclear antigen. Curr Biol 1995, 5:275-82.

Ward Y, Gupta S, Jensen P, Wartmann M, Davis RJ & Kelly K: Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. Nature 1994, 367:651-4.

Warne PH, Viciana PR & Downward J: Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. Nature 1993, 364:352-5.

Wartmann M & Davis RJ: The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex. J Biol Chem 1994, 269:6695-701.

Wartmann M, Hofer P, Turowski P, Saltiel AR & Hynes NE: Negative modulation of membrane localization of the Raf-1 protein kinase by hyperphosphorylation. J Biol Chem 1997, 272:3915-23.

Wassarman DA, Solomon NM, Chang HC, Karim FD, Therrien M & Rubin GM: Protein phosphatase 2A positively and negatively regulates Ras1-mediated photoreceptor development in Drosophila. Genes Dev 1996, 10:272-8.

Waters SB, Chen D, Kao AW, Okada S, Holt KH & Pessin JE: Insulin and epidermal growth factor receptors regulate distinct pools of Grb2-SOS in the control of Ras activation. J Biol Chem 1996, 271:18224-30.

Weinberg WC, Azzoli CG, Chapman K, Levine AJ & Yuspa SH: p53-mediated transcriptional activity increases in differentiating epidermal keratinocytes in association with decreased p53 protein. Oncogene 1995, 10:2271-9.

Weintraub H, Hauschka S & Tapscott SJ: The MCK enhancer contains a p53 responsive element. Proc Natl Acad Sci U S A 1991, 88:4570-1.
863.

Werness BA, Levine AJ & Howley PM: Association of human papillomavirus types 16 and 18 E6 proteins with p53. Science 1990, 248:76-9.
864.

Whalen AM, Galasinski SC, Shapiro PS, Nahreini TS & Ahn NG: Megakaryocytic differentiation induced by constitutive activation of mitogen-activated protein kinase kinase. Mol Cell Bioi 1997, 17:1947-58.

White MA, Nicolette C, Minden A, Polverino A, Van AL, Karin M & Wigler MH: Multiple Ras functions can contribute to mammalian cell transformation. Cell 1995, 80:533-41.

Whiteway MS, Wu C, Leeuw T, Clark K, Fourest LA, Thomas DY & Leberer E: Association of the yeast pheromone response G protein beta gamma subunits with the MAP kinase scaffold Ste5p. Science 1995, 269:1572-5.

Wigler M, Sweet R, Sim GK, Wold B, Pellicer A, Lacy E, Maniatis T, Silverstein S & Axel R: Transformation of mammalian cells with genes from procaryotes and eucaryotes. Cell 1979, 16:777-85.

Will K, Warnecke G, Bergmann S & Deppert W: Species- and tissue-specific expression of the C-terminal alternatively spliced form of the tumor suppressor p53. Nucleic Acids Res 1995, 23:4023-8.

Williams BO, Remington L, Albert DM, Mukai S, Bronson RT & Jacks T: Cooperative tumorigenic effects of germline mutations in Rb and p53. Nature Genetics 1994, 7:480-4.

Winston JT, Coats SR, Wang YZ & Pledger WJ: Regulation of the cell cycle machinery by oncogenic ras. Oncogene 1996, 12:127-34.

Wittinghofer-A N-NH-GH-CS-AM-F: TI: The 2.2 A crystal structure of the Ras-binding domain of the serine/threonine kinase c-Raf1 in complex with Rap1A and a GTP analogue. Nature.; 375(6532): 554-60.

Wolkowicz R, Peled A, Elkind NB & Rotter V: Augmented DNA-binding activity of p53 protein encoded by a carboxyl-terminal alternatively spliced mRNA is blocked by p53 protein encoded by the regularly spliced form. Proc Natl Acad Sci U S A 1995, 92:6842-6.

Wood KW, Sarnecki C, Roberts TM & Blenis J: ras mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. Cell 1992, 68:1041-50.

Wood KW, Qi H, D'Arcangelo G, Armstrong RC, Roberts TM & Halegoua S: The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: a potential role for cellular raf kinases in neuronal growth factor signal transduction. Proc Natl Acad Sci U S A 1993, 90:5016-20.

Woods D, Parry D, Cherwinski H, Bosch E, Lees E & McMahon M: Raf-induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by p21Cip1. Molecular & Cellular Biology 1997, 17:5598-611.

Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ & Sturgill TW: Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. Science 1993, 262:1065-9.

Wu J, Harrison JK, Dent P, Lynch KR, Weber MJ & Sturgill TW: Identification and characterization of a new mammalian mitogen-activated protein kinase kinase, MKK2. Mol Cell Biol 1993, 13:4539-48.

Wu J, Harrison JK, Vincent LA, Haystead C, Haystead TA, Michel H, Hunt DF, Lynch KR & Sturgill TW: Molecular structure of a protein-tyrosine/threonine kinase activating p42 mitogen-activated protein (MAP) kinase: MAP kinase kinase. Proc Natl Acad Sci U S A 1993, 90:173-7.

Wu X, Bayle JH, Olson D & Levine AJ: The p53-mdm-2 autoregulatory feedback loop. Genes Dev 1993, 7:1126-32.

Wu J, Lau LF & Sturgill TW: Rapid deactivation of MAP kinase in PC12 cells occurs independently of induction of phosphatase MKP-1. Febs Lett 1994, 353:9-12.

Wu Y & Han M: Suppression of activated Let-60 ras protein defines a role of Caenorhabditis elegans Sur-1 MAP kinase in vulval differentiation. Genes Dev 1994, 8:147-59.

Wu X & Levine AJ: p53 and E2F-1 cooperate to mediate apoptosis. Proc Natl Acad Sci U S A 1994, 91:3602-6.

Wu Y, Liu Y, Lee L, Miner Z & Kulesz MM: Wild-type alternatively spliced p53: binding to DNA and interaction with the major p53 protein in vitro and in cells. Embo J 1994, 13:4823-30.

Wu Y, Han M & Guan KL: MEK-2, a Caenorhabditis elegans MAP kinase kinase, functions in Ras-mediated vulval induction and other developmental events. Genes Dev 1995, 9:742-55.

Wu L, Bayle JH, Elenbaas B, Pavletich NP & Levine AJ: Alternatively spliced forms in the carboxy-terminal domain of the p53 protein regulate its ability to promote annealing of complementary single strands of nucleic acids. Mol Cell Biol 1995, 15:497-504.

Wyllie FS, Haughton MF, Bond JA, Rowson JM, Jones CJ & Wynford TD: S phase cell-cycle arrest following DNA damage is independent of the p53/p21(WAF1) signalling pathway. Oncogene 1996, 12:1077-82.

Xiao ZX, Chen J, Levine AJ, Modjtahedi N, Xing J, Sellers WR & Livingston DM: Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature 1995, 375:694-8.

Xiong Y, Zhang H & Beach D: Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. Genes Dev 1993, 7:1572-83.

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R & Beach D: p21 is a universal inhibitor of cyclin kinases. Nature 1993, 366:701-4.

Xu GF, Lin B, Tanaka K, Dunn D, Wood D, Gesteland R, White R, Weiss R & Tamanoi F: The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S. cerevisiae. Cell 1990, 63:835-41.

Xu GF, O'Connell P, Viskochil D, Cawthon R, Robertson M, Culver M, Dunn D, Stevens J, Gesteland R, White R & et al: The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell 1990, 62:599-608.

Yamanashi Y & Baltimore D: Identification of the Abl- and rasGAP-associated 62 kDa protein as a docking protein, Dok. Cell 1997, 88:205-11.

Yan M, Dai T, Deak JC, Kyriakis JM, Zon LI, Woodgett JR & Templeton DJ: Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1. Nature 1994, 372:798-800.

Yang BS, Hauser CA, Henkel G, Colman MS, Van BC, Stacey KJ, Hume DA, Maki RA & Ostrowski MC: Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. Mol Cell Biol 1996, 16:538-47.

Yao B, Zhang Y, Delikat S, Mathias S, Basu S & Kolesnick R: Phosphorylation of Raf by ceramide-activated protein kinase. Nature 1995, 378:307-10.

Yao R & Cooper GM: Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science 1995, 267:2003-6.

Yin Y, Tainsky MA, Bischoff FZ, Strong LC & Wahl GM: Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell 1992, 70:937-48.

Yin XM, Oltvai ZN & Korsmeyer SJ: Heterodimerization with Bax is required for Bcl-2 to repress cell death. Curr Top Microbiol Immunol 1995, 194:331-8.

Yin C, Knudson CM, Korsmeyer SJ & Van DT: Bax suppresses tumorigenesis and stimulates apoptosis in vivo. Nature 1997, 385:637-40.

Yoshida K, Murohashi I & Hirashima K: p53-independent induction of p21 (WAF1/CIP1) during differentiation of HL-60 cells by tumor necrosis factor alpha. Int J Hematol 1996, 65:41-8.

Zambetti GP, Bargonetti J, Walker K, Prives C & Levine AJ: Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. Genes Dev 1992, 6:1143-52.

Zambetti GP & Levine AJ: A comparison of the biological activities of wild-type and mutant p53. Faseb J 1993, 7:855-65.

Zanke BW, Boudreau K, Rubie E, Winnett E, Tibbles LA, Zon L, Kyriakis J, Liu FF & Woodgett JR: The stress-activated protein kinase pathway mediates cell death following injury induced by cis-platinum, UV irradiation or heat. Current Biology 1996, 6:606-13.

Zariwala M, Liu E & Xiong Y: Mutational analysis of the p16 family cyclin-dependent kinase inhibitors p15INK4b and p18INK4c in tumor-derived cell lines and primary tumors. Oncogene 1996, 12:451-5.

Zauberman A, Barak Y, Ragimov N, Levy N & Oren M: Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53 - MDM2 complexes. Embo J 1993, 12:2799-808.

Zauberman A, Flusberg D, Haupt Y, Barak Y & Oren M: A functional p53-responsive intronic promoter is contained within the human mdm2 gene. Nucleic Acids Res 1995, 23:2584-92.

Zauberman A, Lupo A & Oren M: Identification of p53 target genes through immune selection of genomic DNA: the cyclin G gene contains two distinct p53 binding sites. Oncogene 1995, 10:2361-6.

Zeng YX & el DW: Regulation of p21WAF1/CIP1 expression by p53-independent pathways. Oncogene 1996, 12:1557-64.

Zhan Q, Carrier F & Fornace AJ: Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol Cell Biol 1993, 13:4242-50.

Zhan Q, Bae I, Kastan MB & Fornace AJ: The p53-dependent gamma-ray response of GADD45. Cancer Res 1994, 54:2755-60.

Zhan Q, Fan S, Smith ML, Bae I, Yu K, Alamo IJ, O'Connor PM & Fornace AJ: Abrogation of p53 function affects gadd gene responses to DNA base-damaging agents and starvation. Dna Cell Biol 1996, 15:805-15.

Zhang K, DeClue JE, Vass WC, Papageorge AG, McCormick F & Lowy DR: Suppression of c-ras transformation by GTPase-activating protein. Nature 1990, 346:754-6.

Zhang XF, Settleman J, Kyriakis JM, Takeuchi SE, Elledge SJ, Marshall MS, Bruder JT, Rapp UR & Avruch J: Normal and oncogenic p21ras proteins bind to the amino-terminal regulatory domain of c-Raf-1. Nature 1993, 364:308-13.

Zhang H, Xiong Y & Beach D: Proliferating cell nuclear antigen and p21 are components of multiple cell cycle kinase complexes. Mol Biol Cell 1993, 4:897-906.

Zhang F, Strand A, Robbins D, Cobb MH & Goldsmith EJ: Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. Nature 1994, 367:704-11.

Zhang H, Hannon GJ, Casso D & Beach D: p21 is a component of active cell cycle kinases. Cold Spring Harb Symp Quant Biol 1994, 59:21-9.

Zhang H, Hannon GJ & Beach D: p21-containing cyclin kinases exist in both active and inactive states. Genes Dev 1994, 8:1750-8.

Zhang Q, Gutsch D & Kenney S: Functional and physical interaction between p53 and BZLF1: implications for Epstein-Barr virus latency. Molecular & Cellular Biology 1994, 14:1929-38.

Zhang SY, Liu SC, Goodrow T, Morris R & Klein SA: Increased expression of G1 cyclins and cyclin-dependent kinases during tumor progression of chemically induced mouse skin neoplasms. Mol Carcinog 1997, 18:142-52.

Zhang Y, Yao B, Delikat S, Bayoumy S, Lin XH, Basu S, McGinley M, Chan HP, Lichenstein H & Kolesnick R: Kinase suppressor of Ras is ceramide-activated protein kinase. Cell 1997, 89:63-72.

Zhao J, Schmieg FI, Simmons DT & Molloy GR: Mouse p53 represses the rat brain creatine kinase gene but activates the rat muscle creatine kinase gene. Mol Cell Biol 1994, 14:8483-92.

Zhao J, Schmieg FI, Logsdon N, Freedman D, Simmons DT & Molloy GR: p53 binds to a novel recognition sequence in the proximal promoter of the rat muscle creatine kinase gene and activates its transcription. Oncogene 1996, 13:293-302.

Zheng CF & Guan KL: Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases. J Biol Chem 1993, 268:23933-9.

Zheng CF & Guan KL: Cloning and characterization of two distinct human extracellular signal-regulated kinase activator kinases, MEK1 and MEK2. J Biol Chem 1993, 268:11435-9.

Zheng CF & Guan KL: Dephosphorylation and inactivation of the mitogen-activated protein kinase by a mitogen-induced Thr/Tyr protein phosphatase. Journal of Biological Chemistry 1993, 268:16116-9.

Zheng CF, Ohmichi M, Saltiel AR & Guan KL: Growth factor induced MEK activation is primarily mediated by an activator different from c-raf. Biochemistry 1994, 33:5595-9.

Zheng CF & Guan KL: Activation of MEK family kinases requires phosphorylation of two conserved Ser/Thr residues. Embo Journal 1994, 13:1123-31.

Zindy F, Lamas E, Chenivesse X, Sobczak J, Wang J, Fesquet D, Henglein B & Brechot C: Cyclin A is required in S phase in normal epithelial cells. Biochemical & Biophysical Research Communications 1992, 182:1144-54.

Zipursky SL & Rubin GM: Determination of neuronal cell fate: lessons from the R7 neuron of Drosophila. Annual Review of Neuroscience 1994, 17:373-97.

Cooperating oncogenes converge to regulate cyclin/cdk complexes

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The cooperation of oncogenes in the transformation of primary rat Schwann cells is a strikingly synergistic process. We have explored the molecular mechanisms involved. Activation of an inducible Raf kinase results in morphologically transformed cells that are arrested in G_1 via the induction of p21^{Cip1} and subsequent inhibition of cyclin/cdk activity. In contrast, coexpression of SV40 large T (LT) or a dominant-negative mutant of p53 abolishes p21^{Cip1} induction and alleviates the growth arrest. Moreover in this scenario, Raf activation results in an increase in the specific activity of cyclin/cdk complexes with Raf and LT cooperating to superinduce cyclin A/cdk2 activity and stimulate proliferation in the absence of mitogens. Thus, signaling by Raf and its cooperating partners converges at the regulation of cyclin/cdk complexes, with the cellular responses to Raf modulated by p53.

Tumorigenesis is a multistep process involving the accumulation of genetic defects that contribute to the phenotype of the tumor. This is reflected by a large number of in vitro and in vivo studies that have shown that more than one oncogenic lesion is required to fully transform most primary cell types (for review, see Hunter 1991). However, the contribution of each oncogene to the transformed phenotype and the molecular mechanisms that underlie oncogene cooperation are poorly understood.

Primary rat Schwann cells are particularly suitable to study oncogene cooperation as homogenous cultures of these cells isolated from neonatal sciatic nerves can be cultured successfully in vitro for extended periods (Brockes et al. 1979). Previously we have shown that the cooperation of Ras and simian virus 40 large T antigen (LT) in the transformation of primary Schwann cells is a synergistic process in which the cellular response to Ras is dependent on the presence of LT (Ridley et al. 1988). Introduction of activated Ras alone results in morphologically transformed cells that are growth-arrested and only when coexpressed with LT does Ras induce the formation of highly proliferative, anchorage-independent cultures. In contrast, LT alone lowers the growth factor requirement of cells that otherwise exhibit normal behavior.

The Ras signaling pathway in Schwann cells is of particular interest as activation of the Ras pathway through the loss of the GTPase activating protein neurofibromin

has been implicated in the common genetic disorder neurofibromatosis type 1, a disease primarily affecting Schwann cells (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a,b). Patients with this disorder frequently develop multiple benign neurofibromas, which are composed mainly of Schwann cells. They also have an increased risk of developing malignant tumors derived from Schwann cells or other neural crest-derived cell types. Inhibition of the Ras pathway in tumor cells derived from these patients, using either neutralizing antibodies to Ras or by increasing Ras–GAP activity, results in a reversion of the tumor cells, confirming the role of Ras in tumor formation in this disease (Basu et al. 1992; DeClue et al. 1992). In addition, transgenic animals in which LT expression is directed to Schwann cells develop neurocristopathies that resemble neurofibromatosis type I, demonstrating that LT expression can also contribute to the development of tumors in this cell type in vivo (Mazarakis et al. 1996).

To explore the molecular basis for the cooperation between Ras and LT we have used an inducible Raf protein in which an activated Raf kinase has been fused to the estrogen receptor hormone-binding domain (ΔRaf-1:ER) (Samuels et al. 1993). Raf has been shown to act directly downstream of Ras (Moodie et al. 1993; Van Aelst et al. 1993; Vojtek et al. 1993; Warne et al. 1993) and elicits a similar phenotype in some cell types; for example, in PC12 cells both activated Ras and Raf proteins are capable of inducing neurite outgrowth (Wood et al. 1993). In primary Schwann cells we show that the effects of Raf are indistinguishable from those of Ras. The use of ΔRaf-

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1:ER has allowed the biochemical analysis of signaling events in Schwann cells following Raf activation in the presence and absence of LT. We show that Raf and LT cooperate to regulate cyclin/cdk activity. Raf alone induces the cdk inhibitor p21^{Cip1} by a p53-dependent mechanism, resulting in a cell cycle arrest. In cells in which p21^{Cip1} induction is counteracted by the expression of dominant-negative p53 or LT, Raf increases the specific activity of cyclin/cdk complexes with Raf and LT signals cooperating to superinduce cyclin A/cdk2 activity and stimulate proliferation of the transformed cells.

Results

Raf alters Schwann cell morphology and causes G_1 arrest

To facilitate the analysis of Ras and LT cooperation in primary Schwann cells we exploited the ΔRaf-1:ER system. This Raf/estrogen receptor fusion protein (ΔRaf-1:ER) exhibits kinase and transforming activity in a strictly hormone-dependent fashion (Samuels et al. 1993). Low passage normal rat Schwann cells were infected with the retroviral vector LXSN (Miller and Rosman 1989) or its derivative LXSN–ΔRaf-1:ER. Several hundred G418 resistant colonies infected with the empty vector (NSE) or the vector encoding the inducible Raf protein (NSΔRafER) were pooled and expanded. The growth properties of these cells were maintained over several months, such that the cells did not appear to enter crisis or show any significant changes in their ability to quiesce or respond to mitogenic signals. Early passage NSΔRafER cells were then infected with a second retroviral vector, Babe-Puro (Morgenstern and Land 1990) or with the Babe-Puro vector expressing SV40 LT. Puromycin-resistant colonies expressing both ΔRaf-1:ER and LT (NSΔRafERLT) or ΔRaf-1:ER and the empty vector (NSΔRafERBp) were pooled and expanded.

The addition of 4-hydroxy-tamoxifen (TMX) to the NSΔRafER cells resulted in a dramatic change in cell morphology (Fig. 1A). When viewed by time-lapse video microscopy, elongation of the normally flat cells could be seen as early as 6 hr. In addition to the cells exhibiting a highly refractile phenotype, they also extended processes and became more motile. This motility was not inhibited by cell–cell contact as the cells would move across each other. These morphological changes were indistinguishable from those seen when Schwann cells are injected with Ras protein (Ridley et al. 1988) or those reported in Schwann cells isolated from late-stage embryos with a homozygous deletion of the NF1 gene (Kim et al. 1995). The activation of Raf in the cells coexpressing LT (NSΔRafERLT) led to similar morphological changes and increased cell motility, whereas the addition of TMX to Schwann cells expressing the empty LXSN vector (NSE) had no detectable effects on the morphology of the cells (data not shown).

In order to address the effects of the activation of ΔRaf-

1:ER on the proliferation of normal Schwann cells, the cells were analyzed for bromodeoxyuridine (BrdU) incorporation and DNA content by flow cytometry. The addition of TMX to the NSΔRafER cells resulted in a cell cycle arrest in the G_1 phase of the cell cycle, whereas TMX had no effect on the cell cycle profile of the control NSE cells (Fig. 1B). This inhibition of cell growth was confirmed by ^{3}H-thymidine uptake assays, which showed a ~80% decrease in DNA synthesis (Fig. 1C, right). When the cells were followed by time-lapse microscopy a complete cessation of cell division was observed within 30 hr. This was not associated with any observable cell death during the 72 hr of the experiment (data not shown). Our previous results showed that Ras induced both a G_1 and G_2 arrest in Schwann cells (Ridley et al. 1988). However, these experiments were carried out in cells coexpressing a temperature-sensitive LT and it is possible that the G_2 arrest is attributable to the removal of LT from cells cultured habitually in the presence of LT (Gonos et al. 1996). In contrast to cells expressing ΔRaf-1:ER alone, cells coexpressing LT (NSΔRafERLT) were not inhibited in response to the activation of ΔRaf-1:ER; indeed, ΔRaf-1:ER increased the rate of DNA synthesis (Fig. 1C, right). The mitogenic effect of Raf in the NSΔRafERLT cells was seen more clearly when the cells were incubated in Dulbecco's modified Eagle medium (DMEM) containing 2% FCS without glial growth factor (GGF) or forskolin, conditions in which the NSΔRafER cells quiesce and the LT cells grow more slowly (Fig. 1C, left). Under these conditions, addition of TMX to the NSΔRafERLT cells led to a greater than three-fold increase in the rate of DNA synthesis as measured by ^{3}H-thymidine uptake. The activation of ΔRaf-1:ER had no significant effect on DNA synthesis in the quiescent NSΔRafER cells (Fig 1C, left).

Thus, Raf behaves similarly to Ras in Schwann cells by inducing distinct morphological changes and a cell cycle arrest. As with Ras, the cell cycle block induced by Raf is overcome by coexpressing LT and in this scenario the Raf signal is reinterpreted as a mitogenic stimulus. This would suggest that in Schwann cells it is the Raf signaling pathway that is responsible for both Ras-induced morphological transformation and the effects of Ras on the cell cycle.

Constitutive activation of MAP kinase by ΔRaf-1:ER in Schwann cells

As an indicator of the activation of signaling pathways downstream from Raf, we compared the kinetics of mitogen-activated protein (MAP) kinase activation following mitogen stimulation in the presence and absence of activated ΔRaf-1:ER. Treatment of quiescent NSΔRafER cells with mitogens resulted in a transient activation of p42–ERK-2, as measured by either a phosphorylation-dependent mobility shift that has been shown to be characteristic for MAP kinase activity (Fig. 1D) or an in vitro kinase assay (data not shown). In contrast, the addition of TMX to the NSΔRafER cells in the presence (Fig. 1D) or absence (not shown) of exogenous mitogens resulted
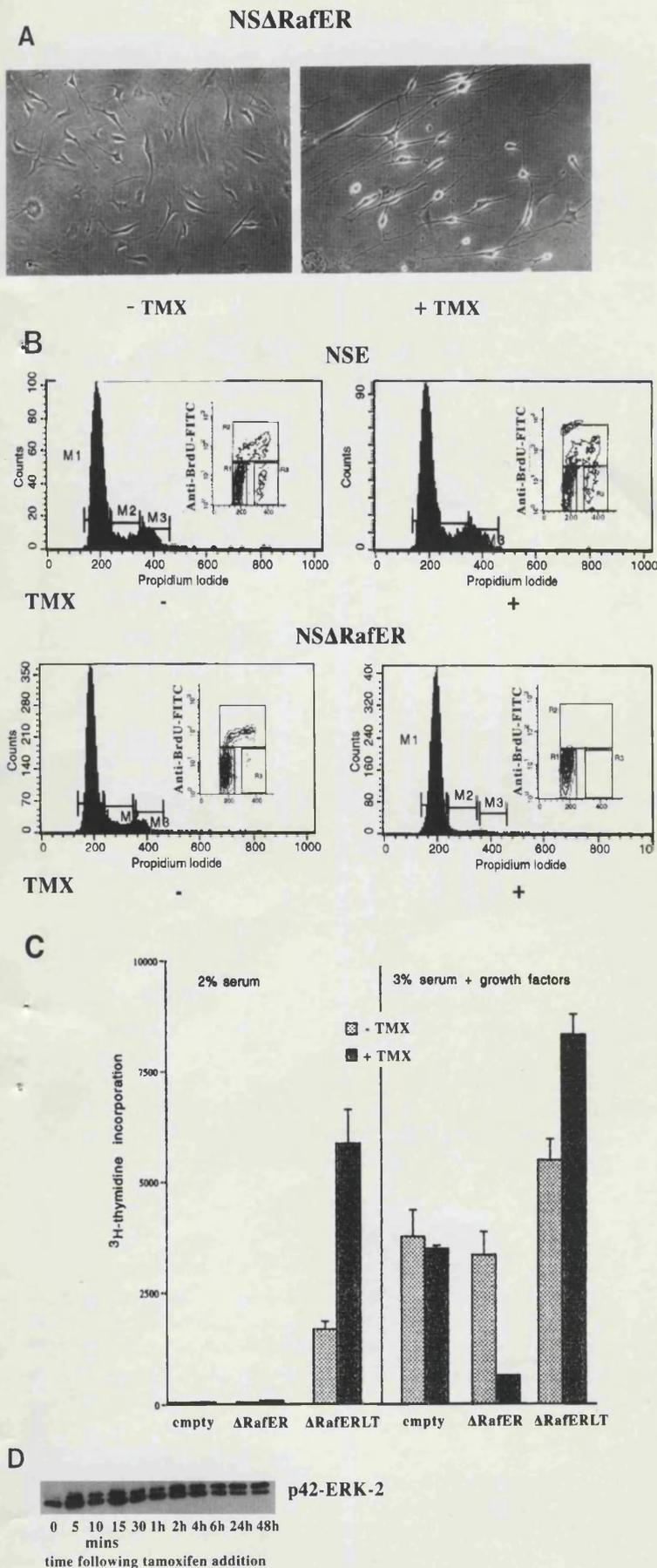
A NS∆RafER

- TMX   + TMX

B NSE

NS∆RafER

C

D

Figure 1. Activation of Raf in primary Schwann cells leads to the constitutive activation of MAP kinase, morphological alterations, and a G_1-specific cell cycle arrest. (A) Phase-contrast micrographs of pools of primary Schwann cells infected with a retrovirus encoding ∆Raf-1:ER, grown in the presence or absence of 200 nM TMX for 30 hr. (B) Pools of asynchronously growing NS∆RafER cells and control NSE cells were cultured in the presence or absence of 200 nM TMX for 30 hr. Cells were trypsinized and examined for DNA content by propidium iodide staining and flow cytometry and also analyzed for BrdU uptake in the 4 hr prior to lysis (see insets). (C) NSE, NS∆RafER and NS∆RafERLT were seeded into DMEM supplemented with 2% FCS. Forty-eight hours later the cells were treated with TMX or ethanol (–TMX) in the absence (left) or presence (right) of fresh medium supplemented with 3% FCS, forskolin, and GGF. [³H]Thymidine uptake was measured between 12 and 28 hr following the addition of hormone. (D) NS∆RafER cells were quiesced for 48 hr in DMEM supplemented with 2% FCS. The cells were lysed at the indicated time points following the addition of fresh medium supplemented with GGF and forskolin and 200 nM TMX. The lysates were equalized for protein content and then Western blotted using the anti-ERK-2 polyclonal antiserum 122.

in the prolonged activation of p42–ERK-2. It therefore appears that the constitutive activity of ΔRaf-1:ER, which results in a cell cycle arrest, is overriding the growth-promoting signals by the mitogens. The activation of ΔRaf1-1:ER in cells coexpressing LT (NSΔRafERLT) also led to the prolonged activation of p42–ERK-2, with kinetics indistinguishable from those seen in the NSΔRafER cells (not shown). Thus depending on the presence of LT, a constitutive Raf signal has opposing effects on the cell cycle.

Raf arrests Schwann cells prior to induction of cyclin A

To investigate the mechanism by which Raf causes a cell cycle arrest and how this is overcome in the NSΔRafERLT cells, we decided to study the effects of Raf on cyclin/cdk activity. In an initial experiment, we addressed whether the activation of Raf was capable of blocking NSΔRafER cells from entering the first S phase following growth factor stimulation of quiescent cells, as this would enable us to analyze the effects of Raf in a synchronized cell population. Subconfluent NSΔRafER cells were rendered quiescent by incubating them in medium containing 2% FCS for 2 days. Pulse-labeling with ^{3}H-thymidine showed that entry into S phase occurred 16–20 hr following mitogen addition with a peak of DNA synthesis between 20 and 28 hr (Fig. 2A). The coaddition of TMX resulted in a ~80% decrease in the proportion of cells reaching the first S phase. This inhibition was not attributable to a delay in the cells entering S phase because further incubation did not lead to a significant increase in the incorporation of ^{3}H-thymidine in cells treated with TMX, whereas the untreated cells continued to cycle (not shown).

To determine whether this ΔRaf-induced arrest was linked to changes in cyclin or cdk expression, cell lysates were prepared at various time points and analyzed by immunoblotting. Cyclin D1 levels were very low in the quiescent cells (Fig. 2B) and were induced as the cells entered G_1. In the presence of TMX, the levels of cyclin D1 were superinduced. This induction is not dependent on the presence of growth factors as it can be seen in the absence of factor addition with similar kinetics (data not shown). Other studies have reported that Ras and Raf expression and transformation leads to a marked elevation of cyclin D1 levels in immortalized rodent fibroblasts (Liu et al. 1995; Winston et al. 1996). Our results demonstrate that the activation of the Raf pathway is sufficient to induce cyclin D1 in nonestablished cells. Cyclin E levels appeared to be invariant as the cells progressed from the quiescent state through G_1 and were unaffected by the activation of ΔRaf-1:ER, suggesting that modulation of cyclin E levels is not the principal mechanism controlling cell cycle progression in primary Schwann cells. Cyclin A protein, although present in significant amounts in the quiescent cells, was induced as the cells progressed toward and entered S phase. However, in the cells treated with TMX, this induction of cyclin A was not seen (Fig. 2B). Cdk4 expression in-
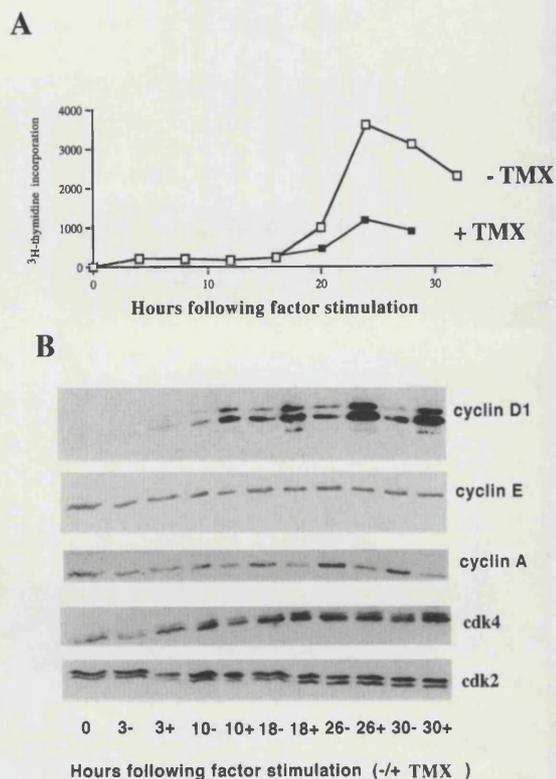

Figure 2. Raf arrests Schwann cells in G_1 prior to the induction of cyclin A. (A) NSΔRafER cells were quiesced for 48 hr in DMEM supplemented with 2% FCS. The cells were stimulated with fresh medium supplemented with forskolin and GGF in the presence or absence of 200 nM TMX. The cells were pulse-labeled with [^{3}H]thymidine at four hourly intervals and TCA-precipitable material was filtered and counted in a scintillation counter. (B) Protein lysates were prepared at the time points indicated and were analyzed by Western blotting with antibodies specific for the indicated proteins.

creased slightly during G_0/G_1 progression, whereas cdk2 levels remained relatively unchanged. However, neither cdk4 nor cdk2 levels appeared to be affected by ΔRaf-1:ER activation. These results suggest that activation of ΔRaf-1:ER leads to a cell cycle arrest in G_1 prior to the induction of cyclin A.

Raf inhibits cyclin E- and cyclin A-dependent kinase activity

The finding that the block in G_1 appeared to occur prior to the induction of cyclin A was also reflected by the low levels of cyclin A protein observed when growing cells were arrested by activation of ΔRaf-1:ER (Fig. 3A, top left). Immunoprecipitation of cyclin A complexes from these cells showed that there was a corresponding decrease in cyclin A/cdk2 complexes (Fig. 3A, top right) and cyclin A-dependent kinase activity (Fig. 3A, bottom). It appears that in Schwann cells, virtually all the cdk2 associated with cyclin A is the faster migrating isoform of cdk2, which has been shown to correspond to the ac-
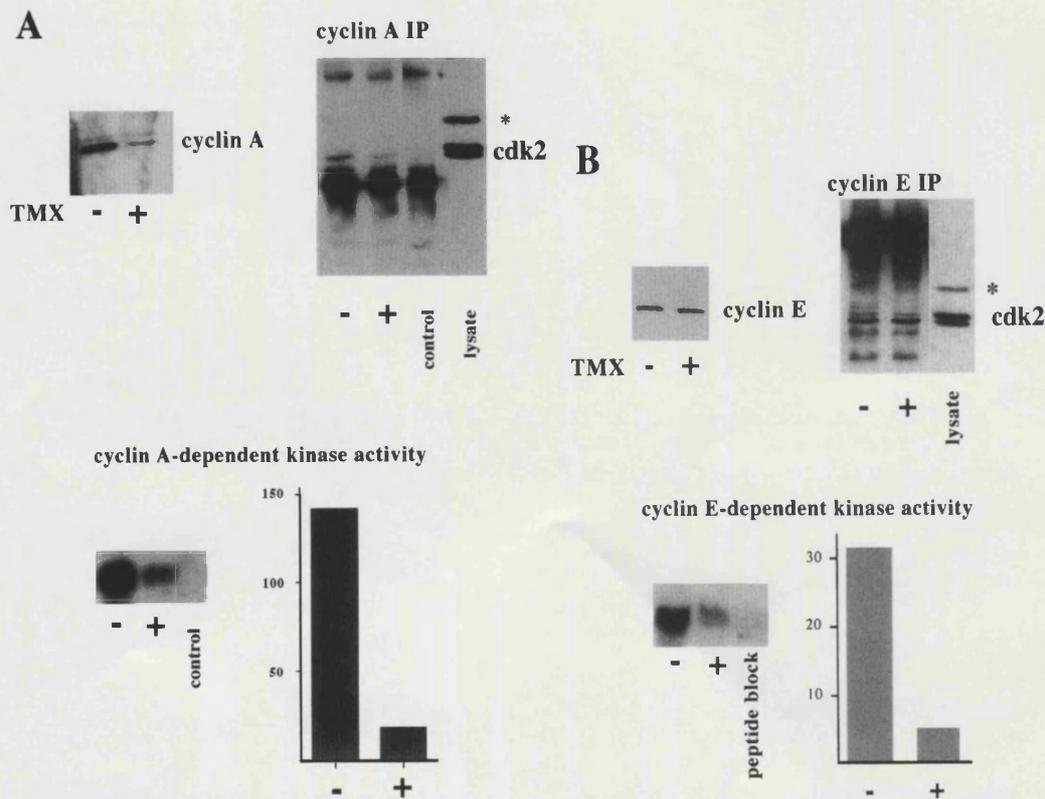
Figure 3. Raf activation inhibits cyclin A- and cyclin E-dependent kinase activity. Asynchronously growing NSΔRafER cells were cultured in the presence (+) or absence (−) of 200 nM TMX for 30 hr. Protein lysates were prepared and standardized for protein content. (*A,B topleft*) (*left*) Western analysis for cyclin A (*A*) and cyclin E (*B*). Lysates (100–300μg) were subjected to immunoprecipitation with a cyclin A monoclonal antibody (*A*) or a cyclin E antibody (*B*). The controls were protein G–Sepharose beads alone for the cyclin A antibody or a peptide block for the cyclin E antibody. The immunoprecipitates were either Western blotted with a cdk2 antibody (*top right*) or assayed for histone H1 kinase activity (*bottom*). (*) Alternative spliced form of cdk2 seen in rodent cells (Matsushime et al. 1994).

tive form phosphorylated on residue Thr-160 (Gu et al. 1992). This may reflect high CDK-activating kinase (CAK) activity in Schwann cells or possibly a preferential association of cyclin A with the activated kinase.

Unlike cyclin A, cyclin E levels were unaffected by activation of ΔRaf-1:ER (Fig. 3B, top left). Moreover, immunoprecipitation of cyclin E complexes from both growing and arrested NSΔRafER cells showed that there were similar levels of cyclin E/cdk2 complexes in the growing and arrested cells (Fig. 3B, top right). The cdk2 complexed to cyclin E, as for the cyclin A complexes, was mostly the CAK–phosphorylated form. Interestingly, however, the cyclin E-dependent kinase activity associated with these complexes was reduced almost to background levels in the arrested cells (Fig. 3B, bottom). Thus, activation of Raf leads to a dramatic decrease in the specific activity of cyclin E/cdk complexes. The loss of both cyclin E and cyclin A-dependent kinase activities in the arrested cells was reflected by a similar inhibition of cdk2 precipitable H1 kinase activity (data not shown). Cyclin E, cyclin A, and cdk2 activity have each been shown to be required for entry into S phase (Girard et al. 1991; Pagano et al. 1992; Zindy et al. 1992; Tsai et al. 1993; van den Heuvel and Harlow 1993; Ohtsubo et al.

1995). Therefore it is likely that the inhibition of these cyclin/cdk complexes is sufficient to arrest the cells in G_1.

Raf induces p21^{Cip1} expression

Because cyclin E/cdk2 activity was reduced without a concomitant decrease in the expression of the proteins, we investigated whether Raf induction affected the expression of cdk inhibitors, such as p21^{Cip1} (el-Deiry et al. 1993; Harper et al. 1993; Xiong et al. 1993; Noda et al. 1994) and p27^{Kip1} (Polyak et al. 1994; Toyoshima and Hunter 1994). Lysates were prepared from NSΔRafER cells grown in the presence or absence of TMX and the inhibitor levels were analyzed by Western blotting. Activation of Raf had no effect on p27^{Kip1} levels (Fig. 4A). However, Raf activation induced a large increase in the levels of p21^{Cip1} (Fig. 4B). The addition of TMX to the control NSE cells had no effect on the levels of p21^{Cip1} (data not shown). Samples of each lysate were also immunoprecipitated with cyclin E antibodies and the precipitated proteins were immunoblottted with p21^{Cip1} antibodies. Figure 4C shows that the induction of Raf in the NSΔRafER cells resulted in a corresponding increase
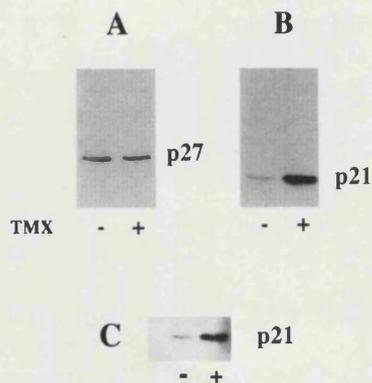
Figure 4. Activation of Raf leads to an increase in p21^{Cip1}. NSΔRafER cells were grown for 30 hr in the presence of TMX(+) or the solvent control (–). Thirty micrograms of protein lysates was subjected to immunoblot analysis with an anti-p27 antibody (A) or an anti-p21 antibody (B). Three hundred micrograms of the lysates was immunoprecipitated with the cyclin E antibody and Western blotted with an anti-p21 antibody.

in the amount of p21^{Cip1} associated with cyclin E complexes.

To further address whether the induction of p21^{Cip1} is responsible for the suppression of cyclin E-dependent kinase activity and the inhibition of DNA synthesis in Raf-arrested cells, we determined the kinetics of these three events. The rate of the ΔRaf-1:ER-induced inhibition of DNA synthesis was analyzed by measuring the incorporation of ^{3}H-thymidine in NSΔRafER cells pulse-labeled at various time points. These experiments showed that the growth inhibition induced by ΔRaf-1:ER commenced after a 12-hr lag period with significant decreases in [^{3}H]thymidine uptake apparent after 14 hr and complete inhibition after 24 hr (Fig. 5A). The late onset of the growth arrest induced by ΔRaf-1:ER was paralleled by a similar delay in the induction of p21^{Cip1}, with a small increase seen at 10 hr and maximal induction at ~22 hr (Fig. 5B). This delayed appearance of p21^{Cip1} was in contrast to the rapid induction of cyclin D1, which could be seen as early as 3 hr following TMX addition to growing (not shown) or quiescent cells (Fig. 2B). The decrease in cyclin E-dependent kinase activity coincided with the induction of p21^{Cip1} and preceded the inhibition of DNA synthesis (Fig. 5A), arguing that p21^{Cip1} is responsible for the inhibition of the kinase activity that results in the growth arrest of the cells.

Raf induction of p21^{Cip1} and cell cycle arrest are p53 dependent

Schwann cells coexpressing ΔRaf-1:ER and LT (NSΔRafERLT) no longer arrest in response to ΔRaf-1:ER activation. Although it has been reported that LT can counteract the inhibitory effect of p21^{Cip1} in cotransfection experiments (Harper et al. 1993), we were interested to explore whether ΔRaf-1:ER would induce p21^{Cip1} in these cells. Pools of NSΔRafERLT cells and control

NSΔRafERBp cells were grown for 30 hr in the presence or absence of TMX. Lysates prepared from these cells were then subjected to Western blot analysis to detect p21^{Cip1}. We found that although ΔRaf-1:ER induced p21^{Cip1} in the control cells, LT coexpression resulted in both a decrease in the basal level of p21^{Cip1} expression and an apparent loss of p21^{Cip1} induction (Fig. 6A). Thus, in LT expressing cells, activation of ΔRaf-1:ER can no longer elevate p21^{Cip1} levels.

One of the properties of LT is that it can bind to and sequester p53 (Lane and Crawford 1980; Maltzman et al. 1981). p53 is known to control the regulation of p21^{Cip1} expression (el-Deiry et al. 1993; Li et al. 1994; Liu et al. 1995; Macleod et al. 1995) and in recent experiments it has been shown that embryonic fibroblasts isolated from p21–/– mice and a colon cancer cell line with a homozygous deletion of the p21 gene are significantly or com-
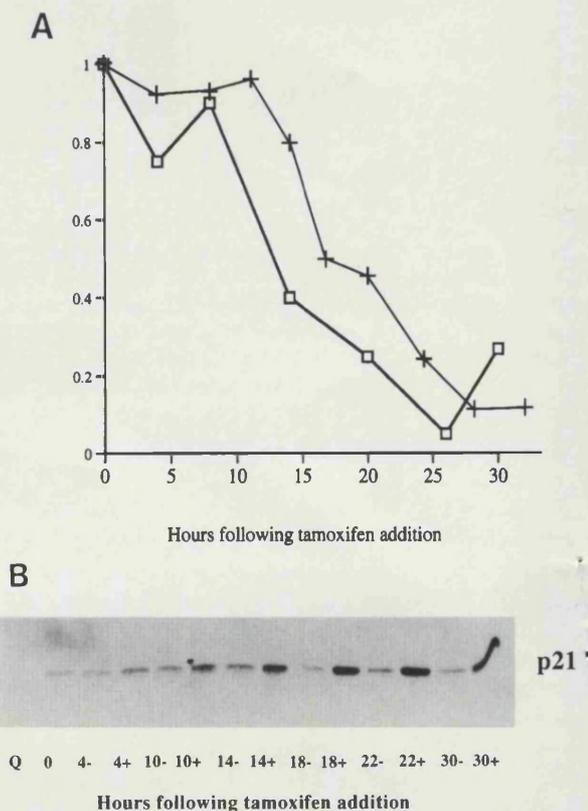

Figure 5. The kinetics of p21^{Cip1} induction, cyclin E-dependent kinase activity, and DNA synthesis following Raf activation. Asynchronously growing NSΔRafER cells were incubated in the presence of TMX (+) or control solvent (–). (A) (□) Protein lysates were prepared at the times indicated. Three hundred micrograms of the lysates was immunoprecipitated with an anti-cyclin E antibody and assayed for histone H1 kinase activity. (+) DNA synthesis was measured by the uptake of [^{3}H]thymidine added between the times indicated. The results are expressed as the amount of activity relative to the corresponding control activity. (B) Protein lysates were prepared at the indicated time points or from quiescent NSΔRafER cells (Q) and were subjected to immunoblotting analysis with an anti-p21 antibody.
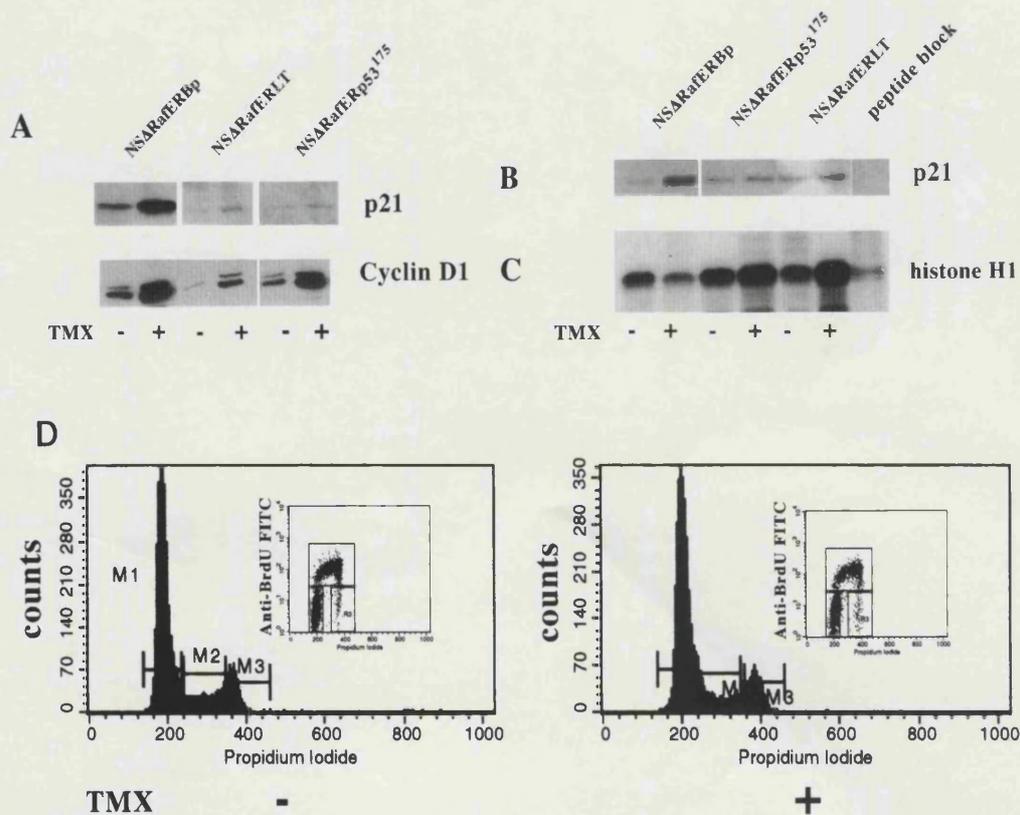
Figure 6. The induction of p21^{Cip1} in Raf-arrested cells is p53-dependent. NSΔRafER cells were infected with retroviral vectors expressing a dominant-negative form of p53 (p53^{175}). Pools of NSΔRafERBp, NSΔRafERp53^{175}, and NSΔRafERLT cells were grown in the presence of TMX (+) or the control solvent (−) for 30 hr. (*A*) Protein lysates were prepared and analyzed by Western blotting with an anti-p21 antibody or an anti-cyclin D1 antibody as indicated. (*B,C*) Three hundred micrograms of lysates was immunoprecipitated with an anti-cyclin E antibody and then (*B*) analyzed by Western blotting with an anti-p21^{Cip1} antibody or (*C*) assayed for histone H1 kinase activity. (*D*) The NSΔRafERp53^{175} cells were trypsinized and analyzed for DNA content by propidium iodide staining and flow cytometry and for BrdU incorporation in the 4 hr prior to trypsinization (see insets).

pletely deficient in their ability to arrest in G$_1$ in response to p53-dependent growth inhibitory signals (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). Therefore, we were interested in whether the induction of p21^{Cip1} in response to Raf was p53-dependent and whether inactivation of p53 was capable of abolishing the ΔRaf-1:ER-induced growth arrest. NSΔRafER cells were infected with a Babe-Puro retroviral vector constructed to encode a dominant negative mutant (dn) of p53 (p53^{175}), which has been shown to inhibit the activity of endogenous p53 (Kern et al. 1992). Puromycin-resistant colonies were pooled and expanded. Proliferating NSΔRafERp53^{175} were incubated in the presence or absence of TMX. Lysates were prepared and FACS analysis was carried out on the cells 30 hr after the addition of hormone. Immunoblot analysis of the lysates showed that as with the LT-expressing cells, the basal levels of p21^{Cip1} expression were reduced in the cells expressing a dominant-negative mutant of p53 (dn–p53) and ΔRaf-1:ER activation no longer stimulated a significant induction of p21^{Cip1} (Fig. 6A). Consistent with this finding, equivalent levels of p21^{Cip1} were precipitated by cyclin E antibodies from the same lysates (Fig. 6B). Simi-

lar results were obtained with NSΔRafER cells infected with a retrovirus expressing the carboxy-terminal oligomerization domain of p53 (data not shown). Thus ΔRaf-1:ER induction of p21^{Cip1} in Schwann cells is p53 dependent.

Cyclin D1 induction by ΔRaf-1:ER, unlike p21^{Cip1}, does not appear to be p53-dependent. Immunoblot analysis of lysates of the NSΔRafERp53^{175} cells showed that Raf stimulated a large induction of cyclin D1, similar to that seen in the parental cells (Fig. 6A). This observation demonstrates that p53 is required only for a specific subset of Raf signals and suggests that the levels of cyclin D1 induced by Raf are unlikely to be involved in the growth arrest of these cells. Cyclin D1 levels are lower in NSΔRafERLT cells, a finding consistent with other reports that have shown that RB-binding proteins such as LT down-regulate cyclin D1 expression and these cells no longer require cyclin D1 function to cycle (Lukas et al. 1994). Raf activation is still able to stimulate cyclin D1 expression in these cells but only to levels found in the uninduced NSΔRafER cells (Fig. 6A), indicating that the pathway required for cyclin D1 induction remains active in LT-expressing cells.

Cyclin E-dependent kinase assays performed on aliquots of lysates from NSΔRafERp53[175] showed that in the presence of dn–p53, ΔRaf-1:ER no longer inhibited the kinase activity but instead stimulated a two-fold increase in kinase activity (Fig. 6C). Likewise, in the NSΔRafERLT cells Raf also stimulated an increase in cyclin E-dependent kinase activity. Thus, in the absence of p21[Cip1] induction, signals from Raf increase cyclin E-dependent kinase activity.

FACS analysis of the NSΔRafERp53[175] cells showed that their cycling was unaffected by ΔRaf-1:ER activation (Fig. 6D), demonstrating that the Raf-induced growth arrest is dependent on normal p53 function. This finding indicates that the increase in cyclin E-dependent kinase activity stimulated by Raf is not the result of increased cell cycling and is thus likely to be a more direct effect of Raf activation. These results would also suggest that the elvation in cyclin D levels and the increased cyclin E-dependent kinase activity are not sufficient to effect the cell cycle distribution of these cells.

Induction of p21[Cip1] is necessary for Raf-induced cell cycle arrest

We have shown that the induction of p21[Cip1] by Raf is p53-dependent. As it has been shown previously that p21[Cip1] is at least partly responsible for the G_1 arrest in response to p53-dependent radiation damage (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995), we compared the levels of p21[Cip1] induced by ΔRaf-1:ER and γ-irradiation in primary Schwann cells. NSΔRafER cells were treated with either 5 Gy γ-irradiation or TMX. [3H]thymidine-uptake assays showed that this led to a 75% and 80% inhibition of DNA synthesis, respectively. As expected, NSΔRafERp53[175] cells were mostly protected from the inhibition of DNA synthesis induced by γ-irradiation or TMX with only a 24% and 3% inhibition of DNA synthesis seen in these cells, respectively. Western analysis of lysates prepared from parallel plates showed that the levels of p21[Cip1] induced by ΔRaf-1:ER were equivalent to those induced by γ-irradiation (Fig. 7A) and that the induction was reduced greatly in the cells expressing dn–p53. Thus the levels of p21[Cip1] induced by Raf are likely to be sufficient to arrest the cells.

To further test the role of p21[Cip1] in the growth arrest elicited by Raf we attempted to reduce the induction of p21[Cip1] by expression of antisense RNA. NSΔRafER cells were infected with the Babe-Puro retrovirus constructed to express rat p21 antisense RNA. Puromycin-resistant colonies were picked and expanded. At all times the clones were grown in conditioned medium from confluent dishes of Schwann cells, as this allowed the expansion of the clones. Two out of 10 clones tested, 7 and 9, showed a greatly reduced ability to arrest in response to Raf activation as measured by [3H]-thymidine uptake assays compared with clones that arrested (Fig. 7B) or cells infected with the empty vector (not shown), despite being morphologically transformed. Western blot analysis of clones 7 and 9 showed that both expressed ΔRaf-1:ER at levels comparable to those of clones that arrested,
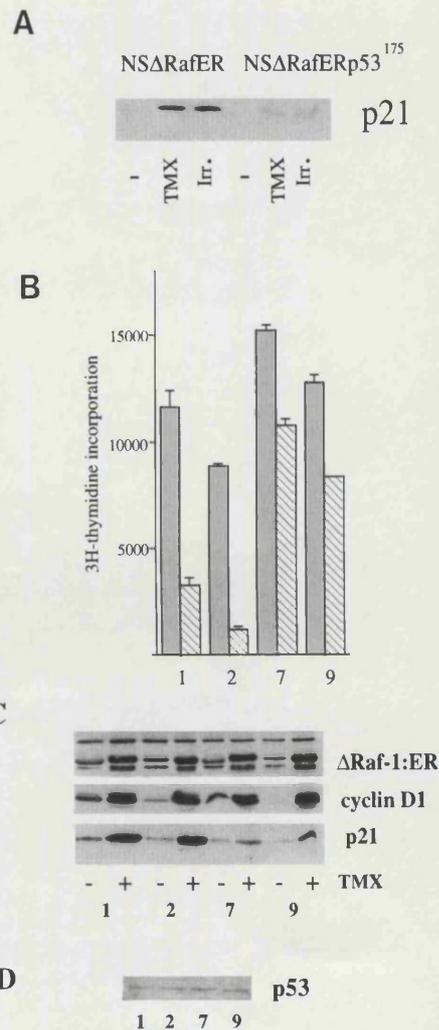

Figure 7. Induction of p21[Cip1] is responsible for the G_1 arrest. (A) Preconfluent NSΔRafER and NSΔRafERp53[175] cells were treated with either 200 nM TMX or 5 Gy of γ-irradiation. Lysates were prepared 24 hr following stimulation. Thirty micrograms of protein lysates were subjected to immunoblot analysis with an anti-p21 antibody. (B) NSΔRafER cells were infected with the Babe-Puro retroviral vector constructed to express antisense rat p21 mRNA. Clones were picked and expanded. [3H]Thymidine was added 20 hr following tamoxifen addition. At 30 hr the cells were lysed. TCA precipitated material was filtered and counted. The results for clones 1,2,7, and 9 are shown. (C) Cell lysates of clones 1,2,7, and 9 were prepared 26 hr following the addition of ethanol (–) or TMX (+) to growing cells. Thirty micrograms of protein was analyzed by Western blotting with an anti-hER, anti-cyclin D1, or anti-p21 antibody as indicated. (D) Cell lysates of clones 1,2,7, and 9 (30 μg protein) were analyzed by Western blotting with an anti-p53 antibody.

with the levels being induced to a similar extent by the addition of tamoxifen (Fig. 7C) as reported previously (Samuels et al. 1993). Cyclin D1 expression was also induced to a similar extent in clones 7 and 9 when compared with arresting clones (Fig. 7C) or with cells expressing empty vector (not shown). These results dem-

onstrate that Raf appears to be similarly active in all four clones in response to tamoxifen. However, in both clones 7 and 9 the ability of ΔRaf-1:ER activation to induce p21^{Cip1} levels was specifically attenuated (Fig. 7C). This phenotype is similar to cells expressing mutant p53. We therefore compared the p53 status of the four clones. As shown in Figure 7D, the clones express similar levels of p53. Because mutant p53 proteins are more stable than their wild-type counterparts (Finlay et al. 1988; Harvey and Levine 1991), this indicates that all clones express normal p53. Taken together, these results support the idea that p21^{Cip1} induction is responsible for the G$_1$ cell cycle arrest induced by Raf, although we cannot rule out other mechanisms contributing to the partial inhibition still seen in clones 7 and 9.

Raf and LT cooperate to increase cyclin A/cdk2 activity and induce DNA synthesis

Although Schwann cells expressing dn–p53 do not arrest in response to ΔRaf-1:ER, it is only in LT-expressing Schwann cells that ΔRaf-1:ER causes an increase in cell cycling. This indicates that in addition to inhibiting p53 activity, LT has additional effects on the cell cycle (Fig. 8A). It is unlikely that this involves the ΔRaf-1:ER-induced increase in cyclin E-dependent kinase activity, as similar levels of kinase activity were found in cells expressing dn–p53 or LT (Fig. 6D). In contrast, cyclin A-dependent kinase activity was found to be consistently higher in the LT cells (Fig. 8B). Western blot analysis showed that NSΔRafERLT cells had elevated levels of cyclin A and cdk2 when compared with NSΔRafER or NSΔRafERp53^{175} cells (Fig. 8B), whereas cyclin E levels were unaffected by LT expression (not shown). Cyclin A and cdk2 levels were unaffected by the removal of mitogens (not shown) or by the activation of Raf (Fig. 7B), demonstrating that the increase in expression levels does not correlate with the proliferative rate of the cells and thus appears to be the direct result of LT expression. These findings are consistent with those reported by Oshima et al. (1993), showing that the expression of LT in primary rat lung epithelial cells resulted in a dramatic increase in the expression of cyclin A and cdc2 proteins.

To address whether the elevated levels of cyclin A complexes induced by LT may contribute to the ability of Raf to stimulate DNA synthesis in the NSΔRafER cells, we infected NSΔRafERp53^{175} cells with a Babe-Hygro retroviral vector constructed to encode cyclin A. Consistent with a role of cyclin A in the increased proliferative response to ΔRaf-1:ER, polyclonal populations of these cells showed an increased proliferative response to ΔRaf-1:ER, whereas Babe-Hygro-infected control cells behaved as the parental cells (Fig. 8A, right). These results show that the induction of cyclin A by LT is likely to contribute to the increased proliferation seen in response to Raf, although as LT is a multifunctional protein, other factors are likely to be involved as well.

When we measured cyclin A-dependent kinase activity in NSΔRafERLT cells we found that in the complete absence of exogenous mitogens, the complexes were mostly inactive, even though the levels of the complexes were similar to those seen in the presence of mitogens (Fig. 8C). Activation of ΔRaf-1:ER in these conditions stimulated a 3- to 4-fold increase in the proportion of cells in S phase, as measured by FACS analysis or [^{3}H]thymidine uptake (not shown) and resulted in a 8- to 20-fold increase in kinase activity (Fig. 8C, top). This activation was not associated with a detectable change in the levels of cyclin A/cdk2 complexes (Fig. 8C, bottom) and thus represents an increase in the specific activity of the complexes. Addition of mitogens also increased the cyclin A-dependent kinase activity and subsequent activation of Raf resulted in a further two- to fivefold stimulation of kinase activity (Fig. 8C), although a small increase (less than twofold) in cdk2 levels was seen upon Raf activation under these conditions. Activation of cdk2-dependent kinase activity was stimulated to a similar extent (not shown), indicating the increase in kinase activity was at the level of the cyclin A/cdk2 complexes.

Thus Raf signaling and LT function not only converge to regulate cyclin E/cdk2 activity but also cooperate to increase cyclin A/cdk2 activity with LT-inducing cyclin A/cdk2 levels and Raf increasing the specific activity of these complexes. This effect is most apparent in the absence of exogenous mitogens, suggesting that Raf and mitogens may activate the complexes by a similar mechanism. Thus cooperation between Raf and LT involves both the loss of a cell cycle inhibitor protein and the synergistic activation of cyclin A complexes.

Discussion

Cooperating oncogenes target cyclin/cdk inhibitors

We have shown that cooperating oncogenes can regulate cyclin/cdk complexes by distinct inhibitory and activating mechanisms. Such regulation appears to play a key role in determining the specificity of the cellular response to the Ras/Raf pathway. Activation of an inducible Raf protein alone results in a G$_1$-specific cell cyle arrest mediated by an induction of p21^{Cip1} and the concomitant inhibition of cyclin/cdk activity. In the presence of LT or dominant-negative mutants of p53, however, the p21^{Cip1} induction is suppressed, the ability of Raf to increase the specific activity of cyclin-dependent kinases is revealed, and the growth arrest abolished (Fig. 9). This effect is most evident in cells containing LT, as they express higher levels of cyclin A/cdk2 complexes and in these cells Raf activation is sufficient to stimulate proliferation in the absence of exogenous growth factors.

Our experiments support the idea that the p21^{Cip1} induction is at least in part responsible for the Raf-dependent cell cycle arrest. As such, its suppression by cooperating oncogenes plays an essential role in cellular transformation. Indeed, it appears that the suppression of cdk inhibitor proteins is a common property of immortalizing oncogenes that cooperate with Ras and/or Raf. E1A is able to bind to and block the inhibitory effects of
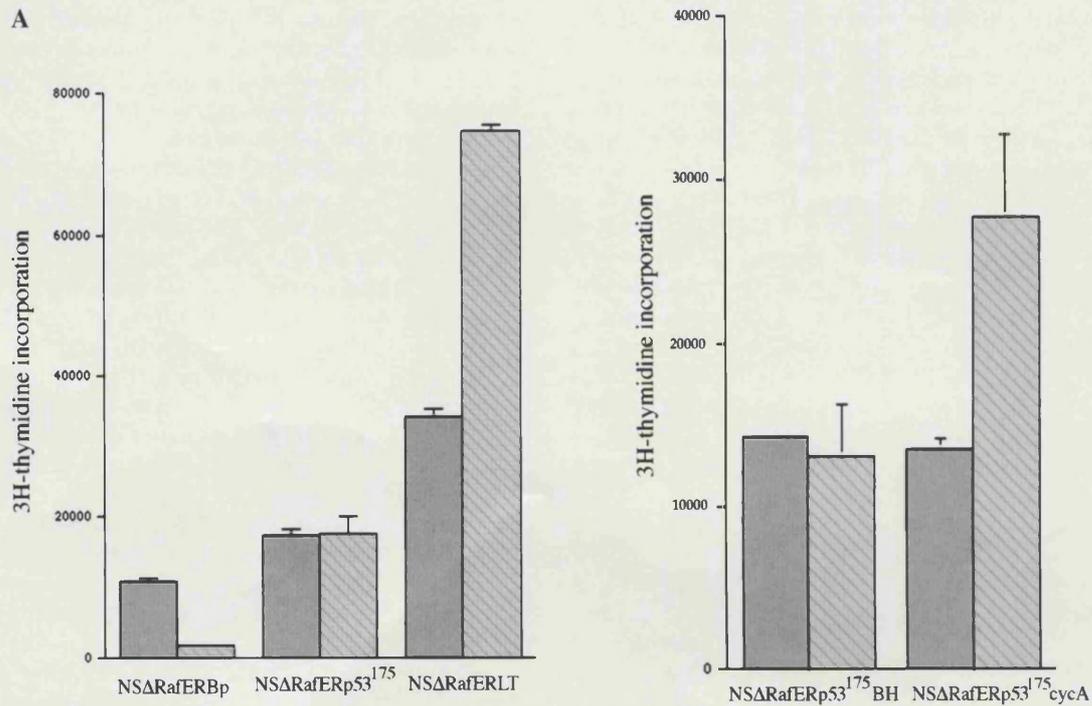
A

B

C

Figure 8. Raf and LT cooperate to induce high levels of cyclin A-dependent kinase activity and DNA synthesis. (*A, left*) Equal numbers of NSΔRafERBp, NSΔRafERp53^{175}, NSΔRafERLT cells were seeded into DMEM supplemented with 2% FCS. Forty-eight hours later the cells were stimulated with fresh medium supplemented with 3% FCS, forskolin, and GGF in the presence of TMX (+) or the control solvent (–). [^{3}H]Thymidine was added 12 hr later and the cells were harvested after a further 18 hr. TCA precipitable material was filtered and counted. (*Right*) NSΔRafERp53^{175} cells were infected with the Babe hygro retroviral vector constructed to express the cyclin A gene or the empty vector control to generate NSΔRafERp53^{175}cycA and NSΔRafERp53^{175}BH cells, respectively. Hygromycin-resistant colonies were pooled and expanded. DNA synthesis assays were performed as described above. (*B*) Protein lysates were prepared from growing NSΔRafER, NSΔRafERp53^{175} and NSΔRafERLT cells. Thirty micrograms of protein was analyzed by Western blotting with either an anti-cyclin A or anti-cdk2 antibody as indicated (*top* and *middle* panels). One hundred micrograms of the lysates was immunoprecipitated with an anti-cyclin A antibody and assayed for histone H1 kinase activity (*bottom*). (*C*) NSΔRafERLT cells were seeded into DMEM supplemented with SATO mix, a serum-free medium supplement (Brockes et al. 1979), (–mitogens) or DMEM supplemented with 3% FCS, GGF, and forskolin (+ mitogens) for 48 hr prior to the experiment. The cells were then treated with TMX (+) or control solvent (–) for 24 hr and protein lysates were prepared. Two hundred micrograms of protein was immunoprecipitated with an anti-cyclin A antibody and kinase assays were performed with the precipitates. The kinase reactions were subjected to SDS-PAGE and then transferred onto a PVDF membrane. The filter was exposed to film (*top*) and then Western blotted with an anti-cdk2 antibody (*bottom*).

p27^{Kip1} (Mai et al. 1996) and p16^{INK4A} (Serrano et al. 1995). Similarly, Myc can overcome cell cycle inhibiton by p27^{Kip1} (Vlach et al. 1996; I. Perez-Roger, D.L.C. Solomon, and H. Land, in prep.), p21^{Cip1} (A. Sewing and H. Land, in prep.), and p16^{INK4A} (B. Amati, pers. comm.; S. Shellard and H. Land, unpubl.) in fibroblasts via yet unknown mechanisms. In addition, fibroblasts isolated from mice carrying a targeted deletion of the INK4a tumor suppressor locus, which encodes the cdk4/cdk6 inhibitory protein p16^{INK4A}, can be transformed by Ras or Raf alone (Serrano et al. 1996). This suggests that, similarly to Raf in Schwann cells, a reduction in the level of cyclin/cdk inhibitory proteins reveals the transforming potential of Ras in fibroblasts.

DNA damage-independent role of p53

The activity of p53 is pivotal to the switch in Raf from a growth-inhibitory to a stimulatory signal. Previous work has demonstrated a critical role of p53 in G$_1$ arrest and apoptosis induced by DNA damage after UV irradiation (Kastan et al. 1992; Kuerbitz et al. 1992). Here we show that the activation of the Raf pathway arrests Schwann cells in a p53-dependent fashion in the absence of DNA damage. Thus, it is tempting to speculate that the ability of p53 to modulate the proliferative response to signals such as Ras/Raf activation may play an important role in its function as a tumor suppressor gene. It is possible that the constitutive activation of the Raf pathway may
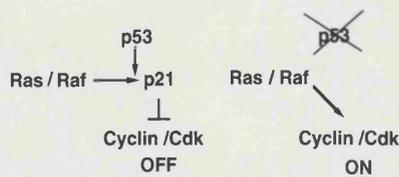
Figure 9. Signaling by cooperating oncogenes converges at the regulation of cyclin/cdk complexes. In Schwann cells Ras or Raf oncogenes induce a G_1-specific cell cycle arrest via induction of p21^{Cip1} and the concomitant inhibition of cyclin/cdk activity. In the absence of functional p53, however, the p21^{Cip1} induction is suppressed and the growth arrest abolished. In the absence of p21^{Cip1} the ability of Raf to activate cyclin/cdk activity is revealed. Thus Raf elicits either growth inhibitory or stimulatory signals depending on the presence of functional p53.

mimic a damage signal and therefore be sensed by p53 as inappropriate. However, the expression of dn–p53 mutants reduces the growth factor requirement of Schwann cells in the absence of activated Raf (Fig. 7A; data not shown), which suggests a function for p53 in the regulation of normal proliferative signals.

Raf induces p21^{Cip1} by a p53-dependent mechanism

The ΔRaf-induced G_1 arrest is preceded by a loss of cyclin E- and cyclin A-dependent kinase activity. The inhibition of cyclin A-dependent kinase activity appears to result from a corresponding decrease in the levels of cyclin A/cdk2 complexes, reflecting a block prior to the induction of cyclin A expression. In contrast, the suppression of cyclin E-dependent kinase is attributable to an inhibition of the specific activity of cyclin E/cdk2 complexes that is likely to be a result of increased expression and binding of the inhibitor protein p21^{Cip1} to the complex. The arrested state induced by Raf is very different from the quiescent state resulting from the removal of growth factors in terms of the balance of cyclin/cdk complexes and inhibitor levels. In addition to elevated p21^{Cip1} expression, Raf-arrested cells also overexpress cyclin D1, whereas p21^{Cip1} and cyclin D1 are barely detectable in quiescent cells. Moreover, in quiescent cells the amount of cdk2 bound to cyclin E is very low, whereas the levels of cyclin E/cdk2 complexes in Raf-arrested cells are similar to those in proliferating cells (A. Lloyd and H. Land, unpubl.).

It has been shown previously that overexpression of p21^{Cip1} leads to a cell cycle arrest in G_1 (Harper et al. 1995). Moreover, p53-dependent G_1 arrest in response to DNA damage is largely dependent on p21^{Cip1} (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). Our observation that γ-irradiation or Raf activation involve equivalent levels of p21^{Cip1} in Schwann cells (Fig. 7A) is thus consistent with our view that p21^{Cip1} is causally involved in the Raf-induced growth arrest.

Similar to the experiments shown here, others have investigated cyclin/cdk activity following various stimuli that lead to a G_1 arrest associated with the induction of cdk inhibitory proteins. p53-dependent G_1 arrest in response to DNA damage (Dulic et al. 1994), which is known to be significantly dependent on p21^{Cip1}, or the induction of the G_1 inhibitory proteins p21^{Cip1} and p27^{Kip1} following the detachment of cells from the substratum (Fang et al. 1996) each results in a G_1 arrest associated with a decrease in both cyclin E and cyclin A-dependent kinase activity. Interestingly, in both cases as well as in our system, this involves an inhibition of the specific activity of cyclin E kinases, resulting in a block in G_1 prior to the induction of cyclin A. Thus, cyclin E/cdk2 complexes appear to be a target for p21^{Cip1} and p27^{Kip1} when induced by various mechanisms. This is consistent with cyclin E/cdk2 activity being required for the induction of cyclin A expression (Rudolph et al. 1996). It is possible that p21^{Cip1} is also inhibiting cyclin D-dependent kinase activity, although because of the technical difficulties in measuring cyclin D-dependent kinase activity in these cells, this was not further investigated.

The regulation of p21^{Cip1} expression appears complex. DNA damage signals are known to result in the induction of p21^{Cip1} in a p53-dependent fashion via two conserved p53-binding sites in the promoter (Dulic et al. 1994; Michieli et al. 1994; Macleod et al. 1995). The G_1 arrest associated with these signals has been shown to be partly or fully dependent upon p21^{Cip1} (Brugarolas et al. 1995; Deng et al. 1995; Waldman et al. 1995). However, p21^{Cip1} expression is induced as an immediate early gene, by various mitogens and differentiation agents and by the growth inhibitory peptide transforming growth factor-β (TGF-β), in a p53-independent fashion (Jiang et al. 1994; Li et al. 1994; Michieli et al. 1994; Steinman et al. 1994) and the response to mitogens can be blocked or mimicked by inhibitors or activators of the MAP kinase pathway (Liu et al. 1996). The role of the induction of p21^{Cip1} as cells enter the cycle is unclear, although it has been proposed that p21^{Cip1} may act as an assembly factor of cyclin/cdk complexes (Zhang et al. 1994). Elevated levels of p21^{Cip1} expression are also associated with the differentiated state of specific tissues and have been postulated to be involved in the maintenance of the quiescent differentiated state (Halevy et al. 1995; Macleod et al. 1995). The latter has also been shown to be a p53-independent mechanism. In Schwann cells, we show that the Raf-dependent induction of p21^{Cip1} is a p53-dependent process and thus differs from the immediate early response seen following mitogen stimulation. It will be of interest to dissect the mechanisms involved in this p53-dependent induction.

In contrast to the rapid induction of cyclin D1 in response to Raf activation, the induction of p21^{Cip1} is delayed. This suggests that the mechanism by which Raf stimulates p21^{Cip1} expression may be indirect. The kinetics of the induction, however, may explain how activation of the same pathway, that is, the MAP-kinase pathway, can result in opposing effects on the cell cycle. If constitutive activation of the MAP kinase pathway for several hours is required to induce p21^{Cip1}, this may partly explain why transient activation of the same pathway by mitogens stimulates rather than inhibits the cell cycle.

Raf activates cyclin/cdk activity in presence of a cooperating partner

In the absence of normal p53 function, Raf activation results in an increase in cyclin/cdk activity, presumably by a mechanism that can be suppressed by p21^{Cip1}. This demonstrates that the effects of an oncogene cannot be gauged solely by the introduction of a single oncogene into a primary cell, as the specificity of the cellular response can depend on the expression of other cooperating genes. In addition to inhibiting p53, LT leads to the constitutive overexpression of cyclin A/cdk2 complexes with Raf activation causing a superinduction of the kinase activity. In this context it is noteworthy that although loss of functional p53 abolishes the Raf-induced cell cycle arrest, only cells expressing LT respond to Raf activation with increased proliferation. The elevation of the cyclin A complexes appears to be at least partly responsible for the ability of Raf to stimulate proliferation in these cells, as p53-defective cells infected with a cyclin A-carrying retrovirus are similarly induced to cycle by Raf. Preliminary experiments indicate that the mechanism of this activation does not involve further alterations in the levels of p21^{Cip1} or p27^{kip-1} or cdc25-dependent dephosphorylation of cdk2 (A. Lloyd and H. Land, unpubl.). As the activation of cyclin-dependent kinase activity appears to be induced in a similar fashion by growth factors (Fig. 8C) it will be important to identify the mechanisms involved in this process.

Implications for neurofibromatosis

The model system that we have developed demonstrates how progressive genetic changes contribute to the transformed phenotype and describe some of the molecular mechanisms involved. Raf alone leads to a change in the morphology of the cells, coincident with an increase in cell motility and the induction of growth factor secretion, all of which can be imagined to disrupt the microenvironment of the cell. However, these cells are growth-arrested as a result of the induction of p21^{Cip1}. Inactivation of p53 abolishes the growth arrest, while retaining the other Raf-dependent properties. The coexpression of LT, in addition, results in the cooperative activation of cyclin A-dependent kinases, and these cells acquire the ability to proliferate in response to the Raf signal in the absence of exogenous mitogens.

In neurofibromatosis type 1, the Ras pathway is activated via inactivation of neurofibromin, a Ras–GAP (Ballester et al. 1990; Martin et al. 1990; Xu et al. 1990a,b). Inhibition of the Ras pathway in tumor cells derived from these patients results in a reversion of the tumor cells, confirming the role of Ras in tumor formation in this disease (Basu et al. 1992; DeClue et al. 1992). We have shown that activation of Raf in primary Schwann cells, as reported previously for Ras (Ridley et al. 1988), results in a growth arrest of the cells. In addition, a recent report described the isolation of Schwann cells from NF–/– embryos. These Schwann cells were shown to have elevated levels of Ras–GTP, resembled Ras-infected Schwann cells, and grew much more slowly than the wild-type cells (Kim et al. 1995), demonstrating that the observable NF–/– phenotype is mimicked by activation of the Ras/Raf pathway. The poor growth properties of primary Schwann cells in which the Ras pathway is activated suggest that further genetic events are required for tumor formation in this cell type. It will be of great interest to investigate whether the benign and malignant tumors found in patients suffering from neurofibromatosis type I have further genetic defects that result in the suppression of the effects of p21^{Cip1}. These may involve the loss of p21^{Cip1} or p53 expression, mutations that affect cyclin/p21^{Cip1} interaction, or increases in the levels of cyclin/cdk complexes. Interestingly, reports of p53 mutations in neurofibrosarcomas from neurofibromatosis type 1 patients have been reported previously (Menon et al. 1990).

Oncogene cooperation in cell regulation

Cooperating oncogenic lesions are expected to increase the proliferative advantage of tumor cells. However, it is remarkable that as part of this process they enhance the oncogenic potential of each other. This applies not only to the cooperation between Raf and LT or loss of p53 function, but also to the cooperation between Myc and Bcl-2 where the latter blocks Myc-induced apoptosis and reveals the ability of Myc to induce cell cycle entry (Fanidi et al. 1992). The Ras/Raf pathway is involved in many cellular responses to peripheral signals and in the experimental system used here activates two mutually exclusive cellular programs: cell cycle progression and cell cycle arrest. The decision of whether to progress through the cell cycle or to arrest, however, is determined by multiple signals converging at the regulation of cyclin/cdk complexes. How cellular signaling networks specify cellular decisions is of central importance to the understanding of cell regulation. Models in which the cooperation of oncogenes or other signaling molecules can be studied at the molecular level will provide a powerful tool to explore this question.

Materials and methods

Cell culture

Schwann cells were purified from 2- to 3-day old Wistar rats, as described previously (Brockes et al. 1979; Ridley et al. 1988). The Schwann cells were cultured routinely at 37°C (10% CO_2) in DMEM with 1.5 mg/ml glucose, supplemented with 3% FCS, 1 μM forskolin (Calbiochem) and GGF (a kind gift from Mark Noble, University of Utah, Salt Lake City), on dishes precoated with poly-L-lysine (Sigma). Throughout all the experiments phenol red-minus medium and charcoal-stripped serum were used. To render the cells quiescent, the cells were washed twice in DMEM and incubated in either DMEM supplemented with 2% FCS or B/S medium, a mitogen-free supplement (Raff et al. 1983), for 48 hr.

Retroviral vectors

The XhoI–ClaI(blunted) fragment encoding ΔRaf-1:ER (Samuels et al. 1993) was subcloned into the XhoI–BamHI(blunted) site of

the retroviral vector (Miller and Rosman 1989). The *Bam*HI fragment encoding SV40LT (Jat et al. 1986) and a *Bam*HI fragment encoding human p53[175] (provided by David Lane) (Vojtesek et al. 1992) were subcloned into the *Bam*HI site of the Babe-Puro retroviral (Morgenstern and Land 1990). A sequenced PCR fragment encoding amino acids 302–390 of murine p53 (p53[CT]) was subcloned into the *Bam*HI–*Eco*RI site of Babe-Puro (a kind gift of Trevor Littlewood, ICRF, London, UK). A *Bam*HI–*Sal*I fragment encoding the human cyclin A gene (a kind gift from Jonathan Pines, Wellcome/CRC Institute, Cambridge, UK) was subcloned into the *Bam*HI–*Sal*I sites of the Babe-Hygro retroviral vector (Morgenstern and Land 1990). A *Xho*I–*Bgl*II fragment encoding rat p21 cDNA (R. Mazars and P. Jat, unpubl.) was subcloned into the *Xho*I–*Bam*HI sites of the pBabe-Puro/2 vector. Each of the constructs was transfected, using the standard calcium phosphate method, into the packaging cell line GP+E (Markowitz et al. 1990), and G418, hygromycin, or puromycin colonies were pooled and expanded.

Infection of Schwann cells

Schwann cells were infected by cocultivation, at a 1:2 ratio, with the producer cell lines, which had been pretreated for 2 hr with 20 µg/ml of mitomycin C (Sigma). Two to three days after plating the cultures were transferred into selective medium containing 400 µg/ml G418 (GIBCO) or 0.4 µg/ml puromycin (Sigma) as appropriate. Drug-resistant colonies were pooled and expanded.

FACS analysis and DNA synthesis assays

Cells (1×10^6 to 2×10^6) were preincubated for 4 hr with 10 µM BrdU(Sigma), trypsinized and then fixed in 80% ethanol. The fixed cells were then incubated with fluorescein isothiocyanate-cojugated anti-BrdU antibodies (Becton-Dickinson) and stained with propidium iodide containing RNase (20 µg/ml). Replicative DNA synthesis and DNA content were analyzed using bivariate flow cytometry. For DNA synthesis assays, 5×10^4 cells were seeded in triplicate into six-well dishes in conditions as described in the figure legends. [³H]thymidine was used at a concentration of 0.5 µCi/ml. At the indicated times the cells were lysed in 1% SDS and the trichloroacetic acid (TCA)-precipitable material was filtered and counted.

Western blot analysis

The cells were lysed in buffer A (1% NP-40, 50 mM Tris at pH 8, 150 mM NaCl 10 µg/ml of aprotinin, leupeptin, and pepstatin, 20 mM NaF, 1 mM Na₃VO₄ 100 µg/ml of PMSF). Protein concentration was determined using the Biorad protein assay. Thirty micrograms of lysate were resolved by SDS-PAGE and electroblotted onto Immobilon P membranes (Millipore). The following antibodies were used: anti-p42 ERK-2(122), provided by Chris Marshall (Leevers and Marshall 1992); anti-cyclin D1, provided by Gordon Peters (Bates et al. 1994); anti-cyclin E (Santa Cruz, sc-481); anti-cyclin A-E-23, provided by Julian Gannon and Tim Hunt (ICRF, South Mimms, UK); anti-cdk4 (Santa Cruz, sc-749); anti-cdk2 (Santa Cruz, sc-163); anti-p27 (Santa Cruz, sc-528); anti-p21(CP36), provided by Wade Harper (Baylor College of Medicine, Houston, TX), anti-hER (Samuels et al. 1993), and anti-p53 (Santa Cruz, sc-1313). Immunoreactive bands were visualized using enhanced chemiluminescence detection (Amersham International PLC).

Immunoprecipitations and kinase assays

Cells were lysed in buffer A. Three hundred micrograms of lysate was incubated with 10 µg of antibody [cyclin E-Santa Cruz 481, cyclin A-E72, provided by Julian Gannon and Tim Hunt (Slingerland et al. 1994), cdk-2-Santa Cruz 163] for 1 hr at 37°C, followed by incubation for an additional hour with protein A– or protein G–sepharose, as appropriate. The beads were washed five times in buffer A and subjected either to Western blot analysis or to kinase assays. For kinase assays the beads were washed a further two times in kinase buffer (50 mM Tris at pH 7.5, 10 mM MgCl₂, 1 mM dithiothreitol) and then resuspended in 50 µl of kinase buffer supplemented with 50 µM ATP, 5 µCi [³²P]ATP and 10 µg of histone H1 (Boehringer Mannheim) for 30 min at 37°C. The samples were resolved by SDS-PAGE and exposed to Kodak X-OMAT AR. The kinase assays were quantified using ImageQuant by Molecular Dynamics.

References

Ballester, R., D. Marchuk, M. Boguski, A. Saulino, R. Letcher, M. Wigler, and F. Collins. 1990. The NF1 locus encodes a protein functionally related to mammalian GAP and yeast IRA proteins. *Cell* **63**: 851–859.

Basu, T.N., D.H. Gutmann, J.A. Fletcher, T.W. Glover, F.S. Collins, and J. Downward. 1992. Aberrant regulation of ras proteins in malignant tumour cells from type 1 neurofibromatosis patients. *Nature* **356**: 713–715.

Bates, S., D. Parry, L. Bonetta, K. Vousden, C. Dickson, and G. Peters. 1994. Absence of cyclin D/cdk complexes in cells lacking functional retinoblastoma protein. *Oncogene* **9**: 1633–1640.

Brockes, J.P., K.L. Fields, and M.C. Raff. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165**: 105-118.

Brugarolas, J., C. Chandrasekaran, J.I. Gordon, D. Beach, T. Jacks, and G.J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**: 552–557.

DeClue, J.E., A.G. Papageorge, J.A. Fletcher, S.R. Diehl, N. Ratner, W.C. Vass, and D.R. Lowy. 1992. Abnormal regulation of mammalian p21ras contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. *Cell* **69**: 265–273.

Deng, C., P. Zhang, J.W. Harper, S.J. Elledge, and P. Leder. 1995. Mice lacking p21[Cip1/WAF1] undergo normal development, but are defective in G1 checkpoint control. *Cell* **82**: 675–684.

Dulic, V., W.K. Kaufmann, S.J. Wilson, T.D. Tlsty, E. Lees, J.W. Harper, S.J. Elledge, and S.I. Reed. 1994. p53-dependent in-

hibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell* **76:** 1013–1023.

el-Deiry, W., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817–825.

Fang, F., G. Orend, N. Watanabe, T. Hunter, and E. Ruoslahti. 1996. Dependence of cyclin E-CDK2 kinase activity on cell anchorage. *Science* **271:** 499–502.

Fanidi, A., E.A. Harrington, and G.I. Evan. 1992. Cooperative interaction between c-myc and bcl-2 proto-oncogenes. *Nature* **359:** 554–556.

Finlay, C.A., P.W. Hinds, T.H. Tan, D. Eliyahu, M. Oren, and A.J. Levine. 1988. Activating mutations for transformation by p53 produce a gene product that forms an hsc70–p53 complex with an altered half-life. *Mol. Cell. Biol.* **8:** 531–539.

Girard, F., U. Strausfeld, A. Fernandez, and N.J. Lamb. 1991. Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* **67:** 1169–1179.

Gonos, E.S., J.S. Burna, G.R. Mazars, A. Kobrna, T.E. Riley, S.C. Barnett, G. Zafarana, R.L. Ludwig, Z. Ikram, A.J. Powell, and P.S. Jat. 1996. Rat embryo fibroblasts immortalised with simian virus large T antigen undergo senescence upon its inactivation. *Mol. Cell. Biol.* **16:** 5127–5138.

Gu, Y., J. Rosenblatt, and D.O. Morgan. 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. *EMBO J.* **11:** 3995–4005.

Halevy, O., B.G. Novitch, D.B. Spicer, S.X. Skapek, J. Rhee, G.J. Hannon, D. Beach, and A.B. Lassar. 1995. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. *Science* **267:** 1018–1021.

Harper, J.W., G.R. Adami, N. Wei, K. Keyomarsi, and S.J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75:** 805–816.

Harper, J.W., S.J. Elledge, K. Keyomarsi, B. Dynlacht, L.H. Tsai, P. Zhang, S. Dobrowolski, C. Bai, C.L. Connell, E. Swindell, et al. 1995. Inhibition of cyclin-dependent kinases by p21. *Mol. Biol. Cell* **6:** 387–400.

Harvey, D.W. and A.J. Levine. 1991. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes & Dev.* **5:** 2375–2385.

Hunter, T. 1991. Cooperation between oncogenes. *Cell* **64:** 249–270.

Jat, P.S., C.L. Cepko, R.C. Mulligan, and P.A. Sharp. 1986. Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. *Mol. Cell. Biol.* **6:** 1204–1217.

Jiang, H., J. Lin, Z.Z. Su, F.R. Collart, E. Huberman, and P.B. Fisher. 1994. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. *Oncogene* **9:** 3397–3406.

Kastan, M.B., Q. Zhan, W.S. el-Deiry, F. Carrier, T. Jacks, W.V. Walsh, B.S. Plunkett, B. Vogelstein, and A.J. Fornace. 1992. A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* **71:** 587–597.

Kern, S.E., J.A. Pietenpol, S. Thiagalingam, A. Seymour, K.W. Kinzler, and B. Vogelstein. 1992. Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science* **256:** 827–830.

Kim, H.A., T. Rosenbaum, M.A. Marchionni, N. Ratner, and J.E. DeClue. 1995. Schwann cells from neurofibromin deficient mice exhibit activation of p21ras, inhibition of cell proliferation and morphological changes. *Oncogene* **11:** 325–335.

Kuerbitz, S.J., B.S. Plunkett, V.W. Walsh, and M.B. Kastan. 1992. Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci.* **89:** 7491–7495.

Lane, D.P. and L.V. Crawford. 1980. The complex between simian virus 40 T antigen and a specific host protein. *Proc. R. Soc. Lond. B. Biol. Sci.* **210:** 451–463.

Leevers, S.J. and C.J. Marshall. 1992. Activation of extracellular signal-regulated kinase, ERK2, by p21ras oncoprotein. *EMBO J.* **11:** 569–574.

Li, Y., C.W. Jenkins, M.A. Nichols, and Y. Xiong. 1994. Cell cycle expression and p53 regulation of the cyclin-dependent kinase inhibitor p21. *Oncogene* **9:** 2261–2268.

Liu, J.J., J.R. Chao, M.C. Jiang, S.Y. Ng, J.J. Yen, and Y.H. Yang. 1995. Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. *Mol. Cell. Biol.* **15:** 3654–3663.

Liu, Y., J.L. Martindale, M. Gorospe, and N.J. Holbrook. 1996. Regulation of p21$^{\text{WAF1-CIP1}}$ expression through mitogen-activated protein kinase signaling pathway. *Cancer Res.* **56:** 31–35.

Lukas, J., H. Muller, J. Bartkova, D. Spitkovsky, A.A. Kjerulff, D.P. Jansen, M. Strauss, and J. Bartek. 1994. DNA tumor virus oncoproteins and retinoblastoma gene mutations share the ability to relieve the cell's requirement for cyclin D1 function in G1. *J. Cell Biol.* **125:** 625–638.

Macleod, K.F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein, and T. Jacks. 1995. p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. *Genes & Dev.* **9:** 935–944.

Mai, A., R.Y.C. Poon, P.H. Howe, H. Toyoshima, T. Hunter, and M.L. Harter. 1996. Inactivation of p27$^{\text{Kip1}}$ by the viral E1A oncoprotein in TGFβ-treated cells. *Nature* **380:** 262–265.

Maltzman, W., M. Oren, and A.J. Levine. 1981. The structural relationships between 54,000-molecular-weight cellular tumor antigens detected in viral- and nonviral-transformed cells. *Virology* **112:** 145–156.

Markowitz, D., C. Hesdorffer, M. Ward, S. Goff, and A. Bank. 1990. Retroviral gene transfer using safe and efficient packaging cell lines. *Ann. N.Y. Acad. Sci.* **612:** 407–414.

Martin, G.A., D. Viskochil, G. Bollag, P.C. McCabe, W.J. Crosier, H. Haubruck, L. Conroy, R. Clark, P. O'Connell, R.M. Cawthon, et al. 1990. The GAP-related domain of the neurofibromatosis type 1 gene product interacts with ras p21. *Cell* **63:** 843–849.

Matsushime, H., D.E. Quelle, S.A. Shurtleff, M. Shibuya, C.J. Sherr, and J.Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* **14:** 2066–2076.

Mazarakis, N.D., N. Yannoutsos, J.N. El-Jabbour, W. Hatton, R. Fletcher, and F. Grosveld. 1996. Neurocristopathy resembling neurofibromatosis type 1 in an NGF-SV40 transgenic line. *Genes Cells* **1:** 125–137.

Menon, A.G., K.A. Anderson, V.M. Riccardi, R.Y. Chung, J.M. Whaley, D.W. Yandell, G.E. Farmer, R.N. Freiman, J.K. Lee, F.P. Li, et al. 1990. Chromosome 17p deletions and p53 gene mutations associated with the formation of malignant neurofibrosarcomas in von Recklinghausen neurofibromatosis. *Proc. Natl. Acad. Sci.* **87:** 5435–5439.

Michieli, P., M. Chedid, D. Lin, J.H. Pierce, W.E. Mercer, and D. Givol. 1994. Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res.* **54:** 3391–3395.

Miller, A.D. and G.J. Rosman. 1989. Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7:** 980–982.

Moodie, S.A., B.M. Willumsen, M.J. Weber, and A. Wolfman. 1993. Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. *Science* **260:** 1658–1661.

Morgenstern, J.P. and H. Land. 1990. Advanced mammalian

gene transfer: High titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18:** 3587–3596.

Noda, A., Y. Ning, S.F. Venable, S.O. Pereira, and J.R. Smith. 1994. Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211:** 90–98.

Ohtsubo, M., A.M. Theodoras, J. Schumacher, J.M. Roberts, and M. Pagano. 1995. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol. Cell. Biol.* **15:** 2612–2624.

Oshima, J., K.E. Steinmann, J. Campisi, and R. Schlegel. 1993. Modulation of cell growth, p34cdc2 and cyclin A levels by SV-40 large T antigen. *Oncogene* **8:** 2987–2993.

Pagano, M., R. Pepperkok, F. Verde, W. Ansorge, and G. Draetta. 1992. Cyclin A is required at two points in the human cell cycle. *EMBO J.* **11:** 961–971.

Polyak, K., J.Y. Kato, M.J. Solomon, C.J. Sherr, J. Massague, J.M. Roberts, and A. Koff. 1994. p27^{Kip1}, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & Dev.* **8:** 9–22.

Raff, M.C., R.H. Miller, and M. Noble. 1983. A glial progenitor cell that develops in vitro into an astrocyte or an oligodendrocyte depending on culture medium. *Nature* **303:** 390–396.

Ridley, A.J., H.F. Paterson, M. Noble, and H. Land. 1988. Ras-mediated cell cycle arrest is altered by nuclear oncogenes to induce Schwann cell transformation. *EMBO J.* **7:** 1635–1645.

Rudolph, B., R. Saffrich, J. Zwicker, B. Henglein, R. Muller, W. Ansorge, and M. Eilers. 1996. Activation of cyclin-dependent kinases by Myc mediates induction of cyclin A, but not apoptosis. *EMBO J.* **15:** 3065–3076.

Samuels, M.L., M.J. Weber, J.M. Bishop, and M. McMahon. 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human Raf-1 protein kinase. *Mol. Cell. Biol.* **13:** 6241–6252.

Serrano, M.L., L.E. Gomez, R.A. DePinho, D. Beach, and S.D. Bar. 1995. Inhibition of ras-induced proliferation and cellular transformation by p16INK4. *Science* **267:** 249–252.

Serrano, M., H.-W. Lee, L. Chin, C. Cordon-Cardo, D. Beach, and R.A. DePinho. 1996. Role of the INK4a locus in tumor suppression and cell mortality. *Cell* **85:** 27–37.

Slingerland, J.M., L. Hengst, C.H. Pan, D. Alexander, M.R. Stampfer, and S.I. Reed. 1994. A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor beta-arrested epithelial cells. *Mol. Cell. Biol.* **14:** 3683–3694.

Steinman, R.A., B. Hoffman, A. Iro, C. Guillouf, D.A. Liebermann, and M.E. el-Houseini. 1994. Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9:** 3389–3396.

Toyoshima, H. and T. Hunter. 1994. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* **78:** 67–74.

Tsai, L.H., E. Lees, B. Faha, E. Harlow, and K. Riabowol. 1993. The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* **8:** 1593–1602.

Van Aelst, L., M. Barr, S. Marcus, A. Polverino, and M. Wigler. 1993. Complex formation between RAS and RAF and other protein kinases. *Proc. Natl. Acad. Sci.* **90:** 6213–6217.

van den Heuvel, S. and E. Harlow. 1993. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* **262:** 2050–2054.

Vlach, J., S. Hennecke, K. Alevizopoulos, D. Conti, and B. Amati. 1996. Growth arrest by the cyclin-dependent kinase inhibitor p27^{Kip1} is abrogated by c-Myc. *EMBO J.* **15:** 6595–6604.

Vojtek, A.B., S.M. Hollenberg, and J.A. Cooper. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* **74:** 205–214.

Vojtesek, B., J. Bartek, C.A. Midgley, and D.P. Lane. 1992. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *J. Immunol. Methods* **151:** 237–244.

Waldman, T., K.W. Kinzler, and B. Vogelstein. 1995. p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.* **55:** 5187–5190.

Warne, P.H., P.R. Viciana, and J. Downward. 1993. Direct interaction of Ras and the amino-terminal region of Raf-1 in vitro. *Nature* **364:** 352–355.

Winston, J.T., S.R. Coats, Y.Z. Wang, and W.J. Pledger. 1996. Regulation of the cell cycle machinery by oncogenic ras. *Oncogene* **12:** 127–134.

Wood, K.W., H. Qi, G. D'Arcangelo, R.C. Armstrong, T.M. Roberts, and S. Halegoua. 1993. The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: A potential role for cellular raf kinases in neuronal growth factor signal transduction. *Proc. Natl. Acad. Sci.* **90:** 5016–5020.

Xiong, Y., G.J. Hannon, H. Zhang, D. Casso, R. Kobayashi, and D. Beach. 1993. p21 is a universal inhibitor of cyclin kinases. *Nature* **366:** 701–704.

Xu, G.F., B. Lin, K. Tanaka, D. Dunn, D. Wood, R. Gesteland, R. White, R. Weiss, and F. Tamanoi. 1990a. The catalytic domain of the neurofibromatosis type 1 gene product stimulates ras GTPase and complements ira mutants of S. cerevisiae. *Cell* **63:** 835–841.

Xu, G.F., P. O'Connell, D. Viskochil, R. Cawthon, M. Robertson, M. Culver, D. Dunn, J. Stevens, R. Gesteland, R. White, and et al. 1990b. The neurofibromatosis type 1 gene encodes a protein related to GAP. *Cell* **62:** 599–608.

Zhang, H., G.J. Hannon, and D. Beach. 1994. p21-containing cyclin kinases exist in both active and inactive states. *Genes & Dev.* **8:** 1750–1758.

Zindy, F., E. Lamas, X. Chenivesse, J. Sobczak, J. Wang, D. Fesquet, B. Henglein, and C. Brechot. 1992. Cyclin A is required in S phase in normal epithelial cells. *Biochem. Biophys. Res. Commun.* **182:** 1144–1154.