Ras/Raf signalling in Primary cells

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Abstract

Oncogenic activation of the Ras gene has been implicated in many human tumours. However, despite the ability of Ras to transform immortal cell lines, activated Ras is growth inhibitory in primary cells. We have previously shown that activation of Ras/Raf signalling in primary Schwann cells results in a proliferative arrest due to the induction of the cyclin dependent kinase inhibitor (CDKI) p21\textsuperscript{Cip1}. In this thesis I examine the mechanisms involved in p21\textsuperscript{Cip1} induction and the roles of other CDKIs in the Ras/Raf induced cell-cycle arrest. I show that Raf activation is also associated with the induction of the CDKI p15\textsuperscript{INK4b}, however, in contrast to other primary cell types, p16\textsuperscript{INK4a} levels decrease and the induction of p19\textsuperscript{ARF} is not associated with p53 stabilisation.

In certain cell types, the Ras induced proliferative arrest is associated with differentiation. I therefore investigated whether the Ras/Raf induced proliferative arrest in Schwann cells was associated with an induction of differentiation. Surprisingly in vitro I found that Raf/MAPK signalling blocks Schwann cell differentiation, as measured by the downregulation of differentiation markers. In addition, Raf is able to induce Schwann cell de-differentiation. In vivo, differentiated Schwann cells are found in the peripheral nervous system in association with axons and are capable of de-differentiating and proliferating in response to nerve damage throughout life. To investigate the effect of Ras/Raf activation on Schwann cell-neuron interactions I have set up a Schwann cell–dorsal root ganglion (DRG) co-culture system and I have generated transgenic mice expressing an inducible Raf protein in myelinating Schwann cells. Using the Schwann cell–DRG system I show that activation of Raf does not prevent the recognition or association of Schwann cells with axons. However, using time-lapse microscopy I have found that Raf activation results in subtle changes in the dynamics of Schwann cell-axon interactions.
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# Table of Contents

Abstract 2
Acknowledgements 3
Table of Contents 4
List of Figures and Tables 7

Chapter 1: Introduction 10

1.1. Regulation of G1-S phase transition 13
  Activation of Ras 16
  Ras signalling in tumorigenesis 19
  Ras signalling pathways 20
  P13K signalling 20
  Raf/MAPK signalling 21
  Regulation of G1-S phase cell cycle progression by Ras signalling 24

1.2. Ras signalling 16
  CDKI induction and function 25
  p53 activation and functions 27
    Regulation of p53 levels and stability 27
    Downstream signalling pathways from p53 30
    Regulation of p53 activity by p19ARF 31
  The mechanisms of the oncogenic Ras induced cell cycle arrest 33
    Mouse embryonic fibroblasts (MEFs) 33
    Human diploid fibroblasts (HDFs) 37
    Keratinocytes 38
    Schwann cells 39

1.3. Ras signalling in primary cells 24
  CDKI induction and function 25
  p53 activation and functions 27
    Regulation of p53 levels and stability 27
    Downstream signalling pathways from p53 30
    Regulation of p53 activity by p19ARF 31
  The mechanisms of the oncogenic Ras induced cell cycle arrest 33
    Mouse embryonic fibroblasts (MEFs) 33
    Human diploid fibroblasts (HDFs) 37
    Keratinocytes 38
    Schwann cells 39

1.4. Neurofibromatosis type 1 (NF1) 39
  Incidence and symptoms 39
  Neurofibroma structure and tumour progression 40
  Expression of NF1 and functions of Neurofibromin 42
  Neurofibromin as a tumour suppressor 44
  In vitro cellular studies 45
    Neurons 45
    Fibroblasts or perineural cells 46
    Schwann cells 46

1.5. Schwann cell differentiation 48
  Neural crest cell to Schwann cell precursor transition 50
    Specification of a glial fate 50
    Precursor Schwann cell formation and survival 51
  Schwann cell precursor to immature Schwann cell transition 51
  Immature Schwann cell to (non) myelinating Schwann cell transition 52
  Myelination and the components of the myelin sheath 53
    Compact myelin 57
    Components of non-compact myelin 59
  Transcriptional control of Schwann cell differentiation 61
    Pax-3 61
    Sox-10 63
    Oct-6 63
Chapter Two: Materials and Methods

2.1. Cell culture
- Primary Schwann cells
- NSARaf-1:ER cells
- GFP-NSARaf-1:ER cells
- NIH 3T3 cells, phoenix cells and perineural fibroblasts
- Dorsal root ganglia

2.2 Tissue culture assays
- DRG and Schwann cell or fibroblast co-cultures
- Time-lapse microscopy
- Differentiation assays
- MEK inhibitor assays
- DNA synthesis assay
- UV treatment of cells
- Transfection of DNA into Schwann cells
- Determination of the stability of p21\^{Cip1} protein using cyclohexamide

2.3 RNA/DNA manipulation
- Generation of the pRafTR IRES-EGFP transgenic construct
- Ligation of DNA fragments into vectors
- Bacterial Transformation
- Plasmid DNA extraction
- Genomic DNA extraction
- Southern Blotting
- RNA extraction
- Northern Blotting
- Probe labelling
- cDNA synthesis
- PCR
- Semi-quantitative RT-PCR

2.4 Other techniques
- Phase contrast microscopy
- Immunofluorescence
- Staining cells for \(\beta\)-galactosidase activity
- Protein extraction
- Western Blotting
- Statistical analysis

Chapter 3: Characterising the Raf induced cell cycle arrest and the effects of Raf activation on Schwann cell differentiation

Characterisation of the Raf induced arrest in primary Schwann cells.
Induction of cyclin dependent kinase inhibitors
Activation of Raf results in the induction of p21\^{Cip1} mRNA
Raf activation results in the induction of p19\^{ARF}, without increased p53 levels
p16\^{INK4a} and p19\^{INK4a} levels decrease with Raf activation.
Investigating the effect of Raf activation on Schwann cell differentiation
Activation of Raf does not result in Schwann cell differentiation
Raf activation can block or reverse PO expression
Raf activation causes downregulation of PO mRNA levels
Raf activation results in Schwann cell dedifferentiation
Raf activation can alter the expression of transcription factors associated with differentiation
Raf activation in differentiating or differentiated cells is associated with induction of cyclin dependent kinase inhibitors, cyclin D1 and p19ARF
Inhibition of the MAPK pathway prevents Schwann cell dedifferentiation in response to Raf activation.
Decreasing the activity of the MAPK pathway is insufficient to cause differentiation

Chapter 4: Examining the effects of Raf activation on Schwann cell–axon interactions
Schwann cells recognise and associate with DRG axons
Perineural fibroblasts do not recognise and associate with DRG axons over time
Confirming that the Schwann cells in DRG-Schwann cell co-cultures are NSRafER cells
Schwann cells with activated Raf are still able to rapidly associate with axons
The majority of Schwann cells with Raf activated prior to association remain stably associated with DRG axons
Raf activation in Schwann cells stably associated with axons does not cause the majority of these cells to lose axon contact
Individual Schwann cells with activated Raf can dissociate from DRG axons
Individual Schwann cells with activated Raf exhibit increased motility
Myelination assays

Chapter 5: Generation of RafTR transgenic mice
Cloning strategy and construct testing
PCR cloning RafTR into LXSN3
Subcloning RafTR into p IRES2-EGFP
Construction of the pPG6 RafTR IRES-EGFP expression vector
Examination of the RafTR transgenic mice
Generation of potential transgenic mice and screening for the presence of the transgene in gDNA
Investigation of transgene expression in the two lines of transgenic mice

Chapter 6: Discussion
Characterising the Raf induced arrest in primary Schwann cells
Examining the effects of Raf activation on Schwann cell differentiation
Examining the implications of Raf activation on Schwann cell behaviour during Wallerian degeneration
Examining the implications of Raf activation on Schwann cell behaviour during development and axon regeneration following injury
Potential relevance of Raf activation in Schwann cells for NF1

Chapter 7: References
List of Figures and Tables

Fig. 1.1. Genetic changes associated with Colorectal Tumorigenesis 10
Fig. 1.2. Regulation of the G1-S phase transition 15
Fig. 1.3. Ras activation and downstream signalling 18
Box. 1.1. Ras-mediated activation of Raf 23
Fig. 1.4. p53 activation and downstream targets 29
Table 1.1. Summary of the effects of Ras, Raf or MEK activation on proliferation of a number of primary cell types 34
Fig. 1.5. Induction of a cell cycle arrest by oncogenic Ras in primary fibroblasts 35
Fig. 1.6. The structure of normal peripheral nerves and neurofibromas of proteins 41
Fig. 1.7. Sequence similarity of Neurofibromin to the Ras-GAP family 41
Fig. 1.8. Diagram of the stages of Schwann cell differentiation showing characteristic changes in the expression patterns of developmental markers 49
Fig. 1.9. Diagrams of the structural regions and components of myelin 56
Fig. 1.10. Diagram to summarise the relationship between mutations in p0, PMP-22 and connexin 32 and inherited demyelinating neuropathies 57
Fig. 1.11. Summary of the expression patterns and functions of Schwann cell transcription factors during development 62
Fig. 1.12. Diagram of the main events that occur during Wallerian degeneration and axonal regeneration 70
Table 1.1. Primer sequences and reaction conditions used for PCR and RT-PCR 80
Table 2.1. Solutions used in genomic DNA extraction and Southern blotting 85
Table 2.2. Northern blotting solutions and wash conditions 87
Table 2.3. Origin of the DNA fragments used for probe labelling 88
Table 2.4. Antibodies used for Western blotting and immunofluorescence 93
Table 2.5. Solutions for protein extraction and Western blotting 94
Fig. 3.1. Activation of Raf results in a cell cycle arrest associated with the induction of cyclin dependent kinase inhibitors

Fig. 3.2. Activation of Raf results in increased p21^{Cip1} mRNA expression

Fig. 3.3. Induction of p19^{ARF} by Raf is not associated with increased levels of p53

Fig. 3.4. Raf activation is associated with decreased p16^{INK4a} and p19^{INK4d} protein levels, however p16^{INK4a} mRNA levels remain constant

Fig. 3.5. Raf activation does not cause p0 expression

Fig. 3.6. Raf activation can block p0 expression.

Fig. 3.7. Raf activation can reverse p0 expression

Fig. 3.8. Upregulation of p0 levels in primary Schwann cells is unaffected by the addition of tamoxifen or ß-estradiol

Fig. 3.9. Raf activation results in decreased p0 mRNA levels

Fig. 3.10. Raf activation can block or reverse Schwann cell differentiation - downregulation of myelination markers

Fig. 3.11. Expression patterns of some of the transcription factors involved in Schwann cell differentiation to the myelinating form

Fig. 3.12. The effect of Raf activation on transcription factors involved in Schwann cell differentiation

Fig. 3.13. The effect of Raf activation on the levels of cyclin D1, p19^{ARF} and the cyclin dependent kinase inhibitors p15^{INK4b} and p27^{KIP}

Fig. 3.14. Inhibition of the MAPK pathway is associated with Schwann cell differentiation and prevents Raf from blocking differentiation

Fig. 3.15. Raf activation is unable to reverse differentiation in the presence of a MEK inhibitor

Fig. 3.16. Decreasing the activity of the MAPK pathway is insufficient to cause differentiation

Fig. 4.1. Schwann cells recognise and associate with DRG axons

Fig. 4.2. Perineural fibroblasts do not associate with DRG axons over time

Fig. 4.3. The Schwann cells in DRG - Schwann cell co-cultures are NSRafER
Fig. 4.4. Schwann cells with activated Raf are still able to rapidly associate with axons

Fig. 4.5. The majority of Schwann cells with prior activation of Raf remain stably associated with axons

Fig. 4.6. Schwann cells remain associated with axons following Raf activation

Fig. 4.7. Schwann cells with activated Raf can dissociate from DRG axons

Fig. 4.8. Raf activation is associated with increased Schwann cell motility

Fig. 5.1. The RafTR is specifically activated by Tmx addition

Fig. 5.2. Diagram of the pRafTR IRES-EGFP cloning strategy and construct testing

Fig. 5.3. Diagram of the pPG6 RafTR IRES-EGFP cloning strategy

Fig. 5.4. The pPG6 RafTR IRES-EGFP is active in vitro under differentiating conditions

Fig. 5.5. pPG6 RafTR IRES-EGFP is detectable in 957 and 963 transgenic mice

Fig. 5.6. Germline transmission is observed in both parental lines

Fig. 5.7. The 963 line expresses RafTR mRNA

Fig. 6.1. Model of the mechanism of the oncogenic Ras/Raf induced cell cycle arrest in primary Schwann cells

Fig. 6.2. Models for the role of Raf/MAPK signalling during the development of myelinated peripheral nerves and Wallerian degeneration
Chapter 1: Introduction

Tumorigenesis is thought to be a multistep process, requiring several successive genetic changes for cellular transformation. This requirement has been most well documented for colorectal cancer, which usually develops over several decades and appear to require at least seven different genetic lesions, including those resulting in the activation of K-ras and loss of p53 function (reviewed by Kinzler and Vogelstein, 1996, see Fig. 1.1.). Moreover, these mutations appear to occur in a specific sequence and the number of lesions can be correlated with the stage of tumour development.

Fig. 1.1. Genetic changes associated with Colorectal Tumorigenesis

APC mutations initiate the neoplastic process with subsequent mutation of the indicated genes resulting in tumour progression. Mis-match repair (MMR) deficiency accelerates this process. K-Ras is activated by a single mutation. p53 inactivation may also only require one mutation since mutant proteins may act in a dominant negative manner to inhibit the remaining wild-type allele. The other genes indicated are tumour suppressor genes that require two genetic events (one per allele) for inactivation. The DCC, CPC4 and JV18 genes are candidate tumour suppressor genes for colorectal neoplasia that are located on chromosome 18q21. Other genetic changes have also been described in a small number of colorectal cancers. (Adapted Kinzler and Vogelstein, 1996.)

In vivo support for the multistep nature of tumorigenesis has been provided using transgenic mice models. For example, expression of either myc or oncogenic H-ras under the MMTV (mouse mammary tumour virus) promoter results in the formation of mammary adenocarcinomas, which develop after a lag period, form in a stochastic manner and are clonal in nature suggesting that additional genetic changes are required for tumour formation (Stewart et al., 1984; Sinn et al., 1987). Transgenic mice co-expressing myc and oncogenic H-ras in specific tissues have accelerated tumour formation, suggesting that the
molecules are acting synergistically. However, since the tumours are clonal and form in a stochastic manner this would suggest that deregulated expression of myc and oncogenic H-ras is still insufficient for tumour formation and additional changes are required prior to malignant tumour formation.

The requirement for multiple genetic changes in tumour formation is also supported by work in vitro using primary cells and immortalized cell lines. In primary cells, expression of a single oncogene is insufficient to cause cellular transformation (Land et al., 1983; Ruley, 1983). However, co-expression of at least two oncogenes, or a single oncogene in association with loss of a tumour suppressor gene function, facilitates transformation (Land et al., 1983; Ruley, 1983; Ridley et al., 1988; Tanaka et al., 1994; Serrano et al., 1997). For example oncogenic H-ras is unable to transform primary Schwann cells or fibroblasts, whereas oncogenic H-ras and myc, or oncogenic H-ras and loss of p53 function, cooperate to transform the primary cells. In contrast established cell lines can be transformed by a single oncogene due to the acquisition of a number of genetic changes during the immortalisation procedure. For example NIH 3T3 cells (an immortal mouse fibroblast cell line) are transformed by oncogenic Ras, due to the deletion of the \textit{INK4a} and \textit{INK4b} loci that encodes the cell cycle regulators \textit{p16}\textsuperscript{INK4a} and \textit{p15}\textsuperscript{INK4b} and the tumour suppressor \textit{p19}\textsuperscript{ARF} (Serrano et al., 1993; Quelle et al., 1995a; Kamijo et al., 1997; Sewing et al., 1997; Malumbres et al., 2000).

Rodent cells and human cells appear to differ in their susceptibility to transformation in vitro. Human cells are not transformed by two co-operating oncogenes and appear to require additional changes compared to rodent cells for successful transformation (Hahn and Weinberg, 2002). Recently Hahn and colleagues have achieved transformation of human cells in vitro by expressing SV40 large T antigen (LT), which inhibits the Rb and p53 pathways; the catalytic subunit of telomerase, hTERT; oncogenic Ras and SV40 small t antigen (ST), which inhibits protein phosphatases 2A (Hahn et al., 1999; Hahn et al., 2002). Thus transformation of human cells appears to require changes in at least five separate pathways.
The resistance of primary cells to transformation by a single oncogene has been proposed to act as a protective mechanism to prevent unregulated proliferation in response to a single genetic alteration. The mechanism of this resistance appears to vary between oncogenes, but it is becoming clear that the tumour promoting function of an oncogene is frequently balanced by an inhibitory effect, which must be overcome by additional mutations to allow tumorigenesis (Evan and Vousden, 2001). These inhibitory effects include induction of a cell cycle arrest or apoptosis. For example, Myc expression is tightly regulated in normal cells, where it promotes proliferation in the appropriate mitogenic environment, but deregulated Myc signalling is often observed in tumour cells. However, deregulated Myc expression is also able to promote apoptosis (Evan et al., 1992). Understanding of the mechanisms underlying the resistance of primary cells to transformation may enable us to reactivate these inhibitory pathways as a form of cancer therapy.

**Thesis Aims**

Many primary cell types, including Schwann cells, undergo a G1 cell cycle arrest in response to oncogenic Ras/Raf (Ridley et al., 1988; Lloyd et al., 1997). However, the mechanism underlying the resistance of primary cells to transformation by oncogenic Ras is still under examination and appears to differ between cells types and species (see below). In this thesis I shall present work aimed at investigating the mechanism of the p53 dependent Ras/Raf induced cell cycle arrest in primary Schwann cells. In addition, since oncogenic Ras/Raf activity is associated with the induction of a more differentiated phenotype in certain cell types (Bar-Sagi and Feramisco, 1985; Halfar et al., 2001; Roper et al., 2001), I have examined the effects of Raf activation on Schwann cell differentiation. We have chosen to use Schwann cells as a primary cell type for this research because Schwann cells are easily obtained from rat sciatic nerves and can be cultured in vitro for prolonged periods without undergoing transformation or senescence (Brockes et al., 1979; Mathon et al., 2001). In addition, defects in Ras signalling in Schwann cells have been implicated in tumour formation in patients with the genetic disorder Neurofibromatosis Type 1 (NF1), which predisposes sufferers to the development of Schwann cell derived tumours (reviewed by Lakkis and Tennekoon, 2000, see below).
In this introduction I shall begin by presenting a brief overview of the regulation of the G1-S-phase cell cycle transition. I will then summarise the mechanisms of Ras signalling, including the effects of Ras on cell cycle progression. As I am interested in the oncogenic Ras/Raf induced p53 dependent cell cycle arrest in primary Schwann cells, which is associated with CDKI induction, I shall review the regulation of p53 activity and CDKI regulation. I will then discuss the mechanisms involved in mediating the cell cycle arrest in response to oncogenic Ras/Raf signalling in a number of primary cells. I will then discuss the genetic disorder Neurofibromatosis type 1 (NF1) and conclude with an account of Schwann cell development, focusing on differentiation to the myelinating form and the roles of Schwann cells in peripheral nerve regeneration.

1.1. Regulation of G1-S phase transition

The somatic cell cycle is divided into four phases. DNA replication occurs during S-phase, with division of the cell and its components into two usually equal daughter cells during M-phase (mitosis). These phases are separated by two gap phases, G1 and G2, during which the cell accumulates the components required for DNA synthesis or mitosis and increases in size. Progression through the cell cycle is regulated at numerous stages by a series of checkpoints that prevent premature transition to the next phase or arrest the cell in response to abnormalities such as misalignment of chromosomes or DNA damage (reviewed by Malumbres and Pellicer, 1998).

Following mitogenic stimuli cells progress through G1 and, on passing the restriction point in late G1, become irreversibly committed to S-phase entry and a new round of division. Transition through the cell cycle is controlled by a number of cyclin dependent kinases (CDK), which function at different stages of the cell cycle. CDKs are serine/threonine kinases that require cyclins for their activity. The cyclin-CDK complexes are regulated by many mechanisms including cyclical changes in cyclin levels, and inhibitory and activating phosphorylation of the CDKs (Malumbres and Barbacid, 2001). Cyclin D-CDK4/6 and cyclin E-CDK2 activity are required to initiate S-phase entry, whilst cyclin A-CDK2 is active in late G1 and S-phases (Dulic et al., 1992; Matsushime et al., 1992; Pagano et al., 1992; Meyerson and Harlow, 1994). In line with their critical role in regulating G1-Sphase
transition the G1 cyclins and CDKs are frequently found to be deregulated in human cancers (Malumbres and Pellicer, 1998).

The activity of cyclin-CDK complexes is also regulated by cyclin dependent kinase inhibitors CDKIs, which are divided into two families of related molecules- the Cip/Kip proteins and the INK4 proteins (reviewed by Sherr and Roberts, 1995; Sherr, 1999). The CDKIs mediate the cell cycle arrest in primary cells in response to oncogenic Ras signalling (Lloyd et al., 1997; Serrano et al., 1997; Malumbres et al., 2000). The Cip/Kip family comprises p21\textsuperscript{Cip1}, p27\textsuperscript{Kip1} and p57\textsuperscript{Kip2}, which bind both the cyclin and CDK subunits to preferentially inhibit the activity of G1 cyclin-CDK complexes (Harper et al., 1993; Xiong et al., 1993; el-Deiry et al., 1994; Polyak et al., 1994a; Polyak et al., 1994b; Toyoshima and Hunter, 1994; Zhang et al., 1994b; Lee et al., 1995; Matsuoka et al., 1995; LaBaer et al., 1997). However, p21\textsuperscript{Cip1} and p27\textsuperscript{Kip1} have also been isolated in active CDK complexes and have been implicated in regulating the assembly and localisation of active type D cyclin-CDK4 complexes (Zhang et al., 1994a; LaBaer et al., 1997; Cheng et al., 1999). The INK4 family of CDKIs consists of p15\textsuperscript{INK4a}, p16\textsuperscript{INK4b}, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d}, which specifically bind to and inhibit CDK4/6, preventing their association with D type cyclins (Serrano et al., 1993; Hannon and Beach, 1994; Chan et al., 1995; Guan et al., 1994, 1996, Hirai et al., 1995). The INK4 CDKIs also induce the displacement of Cip/Kip proteins from active cyclin D-CDK complexes onto CDK2 containing complexes (McConnell et al., 1999).

The G1-S-phase transition is summarised in Fig.1.2. In the absence of mitogenic stimuli cells are quiescent (non-dividing) and cyclin D-CDK activity is low, allowing Rb to inhibit the activity of the E2F transcription factors to prevent the expression of genes required for cell cycle progression (Sherr, 1993), (Dyson, 1998) Mitogens induce cyclin D expression and the formation of cyclin D-CDK4/6 complexes (Sherr, 1993; Aktas et al., 1997; Cheng et al., 1998). These cyclin D-CDKs form active complexes with Cip/Kip proteins and are able to phosphorylate Rb (Zhang et al., 1994b; LaBaer et al., 1997). However, functional inactivation of Rb appears to require additional phosphorylation by cyclin E containing complexes (Lundberg and Weinberg, 1998). Phosphorylation of Rb by cyclin D1-CDK has
Fig. 1.2 Regulation of the G1-S phase transition.
Mitogenic signals promote the assembly of active cyclin D-CDK 4/6 complexes which sequester Cip/Kip proteins and phosphorylate Rb leading to a partial loss of repression of E2F and cyclin E gene expression. Active cyclin E-CDK2 complexes phosphorylate Cip/Kip proteins promoting their degradation. Rb phosphorylation by cyclin E-CDK2 and cyclin D CDK4/6 complexes inactivates Rb facilitating the transcription of genes required for phase entry by E2F. The degradation of the Cip/Kip proteins and induction of cyclins by E2F contribute to mitogen independence and the irreversibility of the transition (shaded box). (See text for details, adapted from Sherr and Roberts 1999.)
been proposed to displace histone deacetylase (HDAC) from the Rb-E2F complex and induce the partial activation of E2F transcription factors, resulting in the expression of specific genes such as cyclin E (Harbour et al., 1999; Zhang et al., 2000). The active cyclin E-CDK2 complexes then cooperate with cyclin D-CDK4/6 to fully phosphorylate and inactivate Rb, leading to the transcription of targets required for DNA replication and irreversible commitment to S-phase entry (Dyson, 1998; Lundberg and Weinberg, 1998). The cyclin D1-CDK4/6 complexes also facilitate cyclin E-CDK2 activity by sequestering Cip/Kip proteins, which are able to inhibit cyclin E-CDK2 activity (Harper et al., 1993; Polyak et al., 1994b; Cheng et al., 1998). In addition, phosphorylation of p27kip1 by Cyclin E-CDK2 complexes results in the targeting of p27kip1 for degradation (Sheaff et al., 1997).

1.2. Ras signalling

The ras genes were first discovered as the transforming agents of Harvey and Kirsten murine sarcoma viruses (reviewed by Malumbres and Pellicer, 1998). The Ras family consists of K-Ras A and K-Ras B, which are encoded by different exons for the last 25 amino acids, H-Ras, and N-Ras. The Ras proteins are highly homologous to each other across most of the protein sequence, however, the Ras proteins may have different cell specific activities since their expression patterns vary and different Ras molecules are specifically activated in certain types of tumour (Bos, 1989, see below; Malumbres and Pellicer, 1998). Moreover, N-Ras and H-Ras knockout mice are developmentally normal, whilst K-Ras nulls die from liver defects and anaemia in utero (Umanoff et al., 1995; Johnson et al., 1997).

Activation of Ras

Ras proteins are produced in the cytoplasm and become localised to the plasma membrane (PM) following a series of post-translational modifications (reviewed by Magee and Marshall, 1999). Cys 186 of a CAAX motif is farnesylated, followed by cleavage of the AAX residues, carboxy-terminal methylation and palmitoylation. The translocation of Ras to the PM is thought to be required for Ras signalling. However, a recent study by Chiu and colleagues suggests that Ras can signal to the MAPK pathway from other cellular membranes including the Golgi and ER (Chiu et al., 2002).
Ras proteins act as molecular switches, which are activated in response to extracellular stimuli and mediate signal transduction into the cytoplasm (Fig. 1.3). In response to mitogenic stimuli inactive GDP bound Ras is activated by the exchange of GDP for GTP, facilitating interaction with effector molecules and downstream signalling. Ras proteins have low intrinsic GTPase activity and the hydrolysis of GTP to GDP is promoted by interaction with GTPase activating proteins (GAPs), resulting in Ras inactivation (Trahey and McCormick, 1987; Adari et al., 1988; Scheffzek et al., 1997; Scheffzek et al., 1998). A number of RasGAPs have been identified in mammalian cells, including p120RasGAP and Neurofibromin (Trahey and McCormick, 1987; Adari et al., 1988; Ballester et al., 1990; Martin et al., 1990; Xu et al., 1990). The other group of Ras regulatory proteins are the guanine exchange factors (GEFs or guanine dissociation stimulators, GDS), which are thought to increase the dissociation of GDP by inducing a conformational change in Ras, facilitating the binding of GTP and thus promoting Ras activation (Quilliam et al., 1995; Boriack-Sjodin et al., 1998; Wittinghofer, 1998). The major mammalian GEFs are thought to be mSos 1 and 2, which are homologues of the Drosophila Son of Sevenless protein (Simon et al., 1991; Bonfini et al., 1992; Bowtell et al., 1992; Chardin et al., 1993).

Ras signalling is activated in response to a variety of extracellular stimuli including growth factors, hormones, cytokines and neurotransmitters that interact with cell surface receptors such as receptor tyrosine kinases (RTKs, for example the EGF receptor), non-RTK associated receptors or G-protein coupled seven transmembrane receptors (reviewed by Campbell et al., 1998). The pathway mediating Ras activation in response to growth factor signalling has been well characterised (Fig 1.2.). For example, binding of EGF to the EGF homodimer stimulates receptor autophosphorylation of tyrosine residues in the cytoplasmic domain, which form binding sites for the Src homology 2 domain (SH2) of the adaptor protein Grb2 (Lowenstein et al., 1992). Grb2 also contains two SH3 domains that bind a proline-rich motif in the carboxy-terminal of Sos and translocate the GEF to the PM to transiently activate Ras (Lowenstein et al., 1992; Chardin et al., 1993). Ras can also be activated in response to integrin signalling which is thought to be important for mediating
Fig.1.3. Ras activation and downstream signalling.
Ras activation in response to growth factor signalling is mediated by RTK activation of the adaptor proteins Shc and/or Grb2, which interact with the Ras GEF SOS. SOS promotes the exchange of GDP for GTP activating Ras, which interacts with a number of effector molecules to transduce signals into the cytoplasm and nucleus. Some examples of pathways downstream of the best characterised Ras effectors are shown (see text for details). Dashed arrows indicate potential interactions. (Adapted from Campbell et al., 1998.)
anchorage signals (Mainiero et al., 1997; Wary et al., 1998; reviewed Giancotti and Ruoslahti, 1999; Cullen and Lockyer, 2002).

**Ras signalling in tumorigenesis**

Ras activity is tightly regulated in the cell under normal conditions; however, mutations resulting in constitutive activation of Ras are frequently detected in human tumours. The incidence of Ras mutation varies between tumour types, for example over 90% of pancreatic tumours and about 50% of colon tumours have mutated Ras, compared to less than 10% of breast tumours (Bos, 1989). Mutations in specific Ras genes are also associated with different types of tumour. N-Ras is mutated in melanomas, whilst K-Ras mutations are found in adenocarcinomas of the lung, pancreas and colon. Moreover, a mouse model with somatic activation of oncogenic K-ras is predisposed to the development of early onset lung cancer, supporting a causal role for mutations of specific Ras genes in the formation of certain types of cancer (Johnson et al., 1997). In addition, in certain human tumours mutations have been detected in other components of the Ras signalling pathway, resulting in elevated levels of Ras signalling in the absence of Ras mutation. For example in the inherited genetic disorder Neurofibromatosis type 1 (NF1), mutation of the NFI gene, which encodes a putative RasGAP, results in elevated Ras signalling in Schwann cells and is associated with the development of benign neurofibromas and malignant peripheral nerve sheath tumours (Lakkis and Tennekoon, 2000). Overexpression of the neuregulin growth factor receptor erbB2, which can activate Ras signalling, is associated with tumours including breast, lung, pancreas and colon cancers (Yarden and Sliwkowski, 2001). Ras signalling is not only important for tumour formation, but is also required for tumour maintenance (Chin et al., 1999). In vitro, oncogenic Ras is able to transform immortal rodent cell lines, resulting in the loss of contact inhibition of growth, anchorage independent proliferation and mitogen independent proliferation (DeFeo et al., 1981; Inoue et al., 1996; Newbold and Overell, 1983). The oncogenic Ras induced loss of growth control is likely to be important for its role in tumour development.
Ras signalling pathways

Ras signalling has been implicated in numerous cellular processes including differentiation (Bar-Sagi and Feramisco, 1985; Freeman, 1998; Sternberg and Han, 1998; Jiang et al., 1999); cell growth (Prober and Edgar, 2000); cell cycle progression in response to mitogen and integrin signalling (Dobrowolski et al., 1994; Aktas et al., 1997; Giancotti and Ruoslahti, 1999); cell survival and apoptosis (Downward, 1998; Halfar et al., 2001). The ability of activated Ras to specify such a variety of different cellular outcomes may be mediated by the activation of different downstream effector pathways, of which the best characterised are the Raf/MAPK (mitogen activated protein kinase), PI3K (phosphoinositol 3-kinase) and RalGDS pathways (reviewed by Campbell et al., 1998, Fig.1.3.). RalGDS and the related proteins Rif and Rgl are GEFs for the GTPases Ral A and Ral B (Wolthuis et al., 1997; reviewed by Bos, 1998). Activated Ral has been shown to interact in vitro with Ral-binding protein (RalBP), which is a GAP for the Rho-like GTPases Cdc42 and Rac, and thus may have effects on the actin cytoskeleton. I shall now discuss Ras activation of Raf/MAPK and PI3K signalling in more detail.

PI3K signalling

PI3K consists of a p110 catalytic subunit and a p85 regulatory subunit (Campbell et al., 1998). PI3K interacts directly with Ras through the p110 catalytic subunit, which activates PI3K phosphorylation of inositol lipids to produce PI(3,4)P_2 and PI(3,4,5)P_3 in response to stimuli such as growth factors (Rodriguez-Viciana et al., 1994; Campbell et al., 1998). PI3K signalling has been implicated in a number of Ras induced cellular processes including cell cycle progression, transcription and cell survival (Downward, 1998) Datta et al., 1999; Medema et al., 2000; Jones and Kazlauskas, 2001). PI3K can also be activated independently of Ras signalling (Toker and Cantley, 1997, Kahn, 1998; Giancotti and Ruoslahti, 1999).

The PI3K effector AKT/PKB (protein kinase B) is suggested to regulate many of the cellular effects of PI3K (reviewed by Alessi and Cohen, 1998; Datta et al., 1999). AKT is activated by growth factors, such as PDGF, in a PI3K dependent manner (Franke et al., 1995). PI(3,4)P_2 and PI(3,4,5)P_3, appear to promote translocation of AKT to the PM where it is activated by phosphorylation by PDK1 (3-phosphoinositide dependent protein kinase
1) and possibly a modified form of PDK1 in complex with another kinase PRK2 (Alessi et al., 1996; Alessi et al., 1997; Stokoe et al., 1997; Datta et al., 1999). AKT targets include GSK3 (glycogen synthase kinase 3), which is inactivated by AKT phosphorylation leading to the activation of glycogen synthesis and the Forkhead transcription factors, which are inhibited by AKT, preventing the expression of pro-apoptotic or cell cycle arrest promoting proteins such as Fas and p27Kip1 respectively (Datta et al., 1999; Kops et al., 1999; Medema et al., 2000). AKT can also promote cell survival by phosphorylating Bad on Ser136, which inhibits the pro-apoptotic activity of Bad by inducing dissociation from BclXL and association with 14-3-3 (Datta et al., 1997; del Peso et al., 1997). PI3K activation of AKT is antagonized by the tumour suppressor PTEN (Phosphatases and Tensin homologue deleted on chromosome Ten) (Stambolic et al., 1998; Whang et al., 1998; reviewed by Yamada and Araki, 2001).

Raf/MAPK signalling

The Raf family consists of the serine/threonine kinases Raf-1, A-Raf and B-Raf, which interact directly with the effector domain of Ras-GTP (Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993). Raf signalling has been shown genetically to specifically mediate a number of Ras activities including differentiation in the Drosophila eye and vulval development in C.elegans, demonstrating the importance of Raf as a Ras effector molecule (Dickson et al., 1992; Han et al., 1993; Freeman, 1998; Sternberg and Han, 1998). Ras induced Raf signalling has also been implicated in regulating transcription and mammalian cell differentiation, proliferation, apoptosis and survival (Kolch et al., 1991; Bruder et al., 1992; Wood et al., 1993; Marshall, 1995; Coolican et al., 1997; Downward, 1998; Mikula et al., 2001; Huser et al., 2001). In addition, interaction between the Raf and PI3K pathways has been shown to co-operatively mediate Ras signalling under certain circumstances, whilst in others the two pathways can have opposing effects (Kolch et al., 1991; Datta et al., 1997; del Peso et al., 1997; Fang et al., 1999; Rommel et al., 1999; Zimmermann and Moelling, 1999; Jones and Kazlauskas, 2001).

Raf activation is a multistep process that requires the co-ordinated interaction of a number of proteins and is summarised in Box 1.1. Recently mammalian scaffolding proteins such as JNK interacting protein (JIP-1) and MEK protein 1(MP1) have been suggested to
localise molecules involved in MAPK signalling, facilitating their interaction and potentially regulating the specificity of signalling in response to particular stimuli (Schaeffer et al., 1998; Stewart et al., 1999; Whitmarsh et al., 1998). These signalling complexes may also contain regulatory molecules such as Raf kinase inhibitor protein (RKIP) (Yeung et al., 1999).

Ras induced activation of Raf initiates a MAPK cascade whereby Raf (a MAPKK kinase) phosphorylates and activates the MAPK kinases, MEK1/2, which then phosphorylate ERK1/2 (MAPK) (Howe et al., 1992; Moodie et al., 1993). However, it has also been reported that Raf can signal independently of MEK (Mikula et al., 2001; Huser et al., 2001). Activated ERK1/2 can translocate to the nucleus and regulate transcription by phosphorylating a number of targets including the Ets transcription factors (Wasylyk et al., 1998), the MAPK interacting kinases MNK1/2 (Fukunaga and Hunter, 1997; Waskiewicz et al., 1997) and the MAPK activated protein kinase (MAPKAPs), which phosphorylate a number of substrates such as CREB and CBP (Tan et al., 1996; Xing et al., 1996; Dalby et al., 1998). In addition, phosphorylation of the anti-proliferative protein Tob by ERK1/2 has recently been implicated in Ras mediated proliferation and transformation (Suzuki et al., 2002).

Interestingly, Ras/Raf/MAPK signalling can have different effects in the same cell type depending on the intensity and duration of signalling. For example in PC12 cells (a phaeochromocytoma cell line) EGF addition is associated with transient MAPK activation, which is mediated by Ras/Raf signalling and results in proliferation (Bar-Sagi and Feramisco, 1985; Wood et al., 1993; Cowley et al., 1994) Marshall, 1995; York et al., 1998; Kao et al., 2001). In contrast, prolonged MAPK activation in response to NGF addition or oncogenic Ras expression induces the development of a differentiated, neuronal phenotype.
Box 1.1. Ras-mediated activation of Raf.
Conserved region 1 (CR1) of Raf (shown in colour) contains two Ras binding domains, the RBD, and the cysteine rich domain (CRD), which is obscured by 14-3-3 binding, stabilizing the inactive conformation. The kinase domain is located in CR3, which constitutively associates with the heat-shock proteins Hsp90 and p50 (Schulte 1995). Ras activation facilitates interaction with the Raf RBD and translocation of Raf to the PM, which may result in a conformational change that displaces 14-3-3 and exposes the CRD to Ras binding. 14-3-3 proteins then interact with the Raf carboxy terminal kinase domain and may be required to stabilise Raf in an active conformation (Tzivion 1998). Phospholipids (PS), Ksr (kinase suppressor of Ras) and CNK (connector enhancer of KSR) may also be involved in Raf activation and/or downstream signalling (Thierren et al., 1995, 1996; Stokoe and McCormick 1997; Denouel-Galy et al., 1998; Thierren et al., 1998; Yu et al., 1998; Muller et al., 2001). Activation of Raf may also involve phosphorylation by Src family kinases, Janus activated kinases or PAK3 (JAKs, Marais et al., 1995; Xia et al., 1996; King et al., 1998). (Adapted from Morrison and Cutler 1997; Campbell et al., 1998.)
Regulation of G1-S phase cell cycle progression by Ras signalling

Stimulation of quiescent cells by mitogens, such as PDGF, and integrins results in rapid Ras activation and cell cycle entry (Satoh et al., 1990; Giancotti and Ruoslahti, 1999). Ras activity is required at several points during the G1-S-phase interval to inactivate Rb (Mulcahy et al., 1985; Dobrowolski et al., 1994; Mittnacht et al., 1997; Peeper et al., 1997; Takuwa and Takuwa, 1997). During early G1, mitogenic stimulation of Ras activates the Raf/MEK/ERK pathway leading to transcription of the cyclin D1 gene and assembly of cyclin D1-CDK4/6 complexes (Aktas et al., 1997; Kerkhoff and Rapp, 1997; Peeper et al., 1997; Cheng et al., 1998). During the later stages of G1, however, elevated PI3K levels are required for cell cycle progression (Gille and Downward, 1999; Jones and Kazlauskas, 2001). Ras dependent PI3K activation of AKT has been demonstrated to stabilize cyclin D1 by inhibiting the phosphorylation of cyclin D1 on Thr 286 by GSK3β, preventing nuclear export and degradation of cyclin D1 (Diehl et al., 1998). Thus mitogenic stimulation of Ras results in elevated levels of active cyclin D1 complexes, which facilitates cell cycle progression (Peeper et al., 1997; Cheng et al., 1998). Moreover, Ras activation reduces the expression of p27^{kip1} via PI3K/AKT induced inhibition of AFX-like Forkhead transcription factor activity and promotes p27^{kip1} degradation, which may be mediated by cyclin E-CDK2 phosphorylation of p27^{kip1} (Aktas et al., 1997; Takuwa and Takuwa, 1997; Medema et al., 2000).

1.3. Ras signalling in primary cells

Oncogenic Ras is able to transform immortal cell lines, however, in primary cells oncogenic Ras causes a cell cycle arrest and a second co-operating oncogene or loss of a tumour suppressor gene function is required for transformation (Ridley et al., 1988; Lloyd et al., 1997; Serrano et al., 1997; Zindy et al., 1998). In many primary cell types the oncogenic Ras induced cell cycle arrest is caused by the induction of the CDKIs p16^{INK4a} and p21^{Cip1}, with p21^{Cip1} induction occurring in a p53 dependent manner. This response has been proposed to act as a protective mechanism to prevent unregulated proliferation in response to a single genetic alteration (Lloyd et al., 1997; Serrano et al., 1997; Malumbres et al., 2000). Oncogenic Ras is able to cooperate with inactivation of the p53 pathway or CDKI loss to transform primary cells in vitro and this may reflect a requirement to
overcome the oncogenic Ras induced cell cycle arrest during tumorigenesis in vivo (Kinzler and Vogelstein, 1996; Lloyd et al., 1997; Serrano et al., 1997; Kamijo et al., 1997). I will now discuss the regulation of the CDKIs in response to oncogenic Ras/Raf signalling and their involvement in human tumour formation. I will then present an overview of the regulation of p53 activity and discuss the current ideas regarding the mechanisms involved in the induction of the cell cycle arrest in a number of primary cell types.

CDKI induction and function

p21^Cipl has been implicated in mediating the induction of a G1 cell cycle arrest, and/ or a G2/M arrest, in response to DNA damage in a p53 dependent manner (Xiong et al., 1993; el-Deiry et al., 1994; Chan et al., 2000). In addition, in many primary cell types, oncogenic Ras/Raf expression results in the induction of p21^Cipl, leading to a cell cycle arrest (Lloyd et al., 1997; Serrano et al., 1997). The oncogenic Ras induced cell cycle arrest is p53 dependent in some primary cell types, which may reflect a requirement for p53-mediated transcription of p21^Cipl (Lloyd et al., 1997; Serrano et al., 1997; Roper et al., 2001). However, oncogenic Ras/Raf can also induce p21^Cipl independently of p53 under certain circumstances and this process may be negatively regulated by Rho signalling (Sewing et al., 1997; Woods et al., 1997, Olson et al., 1998; Kivinen et al., 1999; Gartel et al., 2000). In addition, p21^Cipl is expressed independently of p53 during development and may be involved in mediating the cell cycle arrest preceding terminal differentiation (Macleod et al., 1995; Zhang et al., 1995; Di Cunto F, 1998; Harvat et al., 1998).

Mutations resulting in the complete loss of p21^Cipl function are rarely observed in human cancers; however, mutations in p53 are very common and are associated with reduced levels of p21^Cipl (Gao et al., 1995; Hollstein et al., 1991; Tanaka et al., 1996; Sherr, 1999). In agreement with these findings in humans, p21^Cipl /- mice are not predisposed to spontaneous tumour development, unlike p53/- mice (Donehower et al., 1992; Deng et al., 1995). However, p21^Cipl/- keratinocytes have an increased proliferative potential and are susceptible to transformation by oncogenic Ras, suggesting that p21^Cipl is also able to act as a tumour suppressor under certain circumstances, although it is unclear whether this function also exists in human cells (Missero et al., 1996). In addition, Zhou and colleagues
have recently shown that some cancers associated with erbB2 mutations may overcome the anti-proliferative effects of high levels of p21\textsuperscript{cip1} by promoting translocation of p21\textsuperscript{cip1} to the cytoplasm (Zhou et al., 2001).

The signals regulating the INK4 proteins remain unclear. p15\textsuperscript{INK4b} is expressed in young mice and has been implicated in mediating the TGF\(\beta\) induced cell cycle arrest, although p15\textsuperscript{INK4b} is not essential for this process (Hannon and Beach, 1994; Zindy et al., 1997b; Zindy et al., 1997a; Latres et al., 2000). p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d} are expressed during development and may be involved in terminal differentiation (Zindy et al., 1997b; Zindy et al., 1997a; Phelps, 1998). p16\textsuperscript{INK4a} is not expressed during development in the mouse, but is widely expressed at increasing levels in older animals (Zindy et al., 1997a; Zindy et al., 1997b). In vitro p16\textsuperscript{INK4a} is induced with progressive cell population doublings and has been suggested to form part of a mechanism that prevents unlimited cellular replication by inducing a cell cycle arrest (Alcorta et al., 1996).

p16\textsuperscript{INK4a} has also been implicated in the induction of a cell cycle arrest in response to oncogenic Ras activation in some cell primary types (Serrano et al., 1997; Zhu, 1998; Lin and Lowe, 2001). However, the importance of p16\textsuperscript{INK4a} as a mediator of the Ras induced arrest appears to vary between cell types. For example, p16\textsuperscript{INK4a} null MEFs are still capable of undergoing a cell cycle arrest in response to oncogenic Ras, whilst in p16\textsuperscript{INK4a} null HDFs this arrest is abrogated (Serrano et al., 1996; Kamijo et al., 1997; Krimpenfort et al., 2001; Sharpless et al., 2001; Brookes et al., 2002). Induction of p16\textsuperscript{INK4a} in response to Ras/Raf/MAPK activation in human diploid fibroblasts (HDFs) is p53 independent (Wei et al., 2001) and may be mediated by the balance between the transcriptional activators Ets1/2 and their repressor Id1 (Ohtani et al., 2001; reviewed by Zebedee and Hara, 2001). Another possible regulator of transcription at the INK4a locus is the transcriptional repressor bmi-1, which may prevent p16\textsuperscript{INK4a} expression during development (Jacobs et al., 1999). In addition, JunB is able to bind the p16\textsuperscript{INK4a} promoter and may be involved in mediating a G1 cell cycle arrest by transactivation of p16\textsuperscript{INK4a} expression (Passegue and Wagner, 2000).
The \textit{INK4a} locus, which encodes p16\textsuperscript{INK4a}, is unusual in that it also encodes another protein, p19\textsuperscript{ARF}, in an alternative reading frame (Quelle \textit{et al.}, 1995b, see below). p16\textsuperscript{INK4a} and p19\textsuperscript{ARF} are encoded by exon 1\textalpha{} and 1\textbeta{} respectively, but share a common exon 2. The \textit{INK4a} locus is frequently mutated in human cancers or inactivated by methylation, although it can be difficult to determine whether these changes affect p16\textsuperscript{INK4a} or p19\textsuperscript{ARF} activity (reviewed by Ruas and Peters, 1998; Sharpless and DePinho, 1999). Germline mutations in \textit{p16 INK4a} have been identified in familial melanoma and pancreatic adenocarcinoma patients. \textit{INK4a mutant} mice, which produce an unstable p16\textsuperscript{INK4a} protein, do not form tumours within the first year of life, however \textit{INK4a mutant} mice that are also heterozygous for p19\textsuperscript{ARF} are susceptible to developing melanomas, suggesting that p19\textsuperscript{ARF} is haplo-insufficient for tumour suppression in this genetic environment (Krimpenfort \textit{et al.}, 2001). In contrast, \textit{INK4a ex1\alpha{}-/} (p16\textsuperscript{INK4a} null) mice have an increased incidence of spontaneous tumours and are susceptible to tumour formation following treatment with carcinogens (Sharpless \textit{et al.}, 2001). In addition, \textit{INK4a ex1\alpha{}-/} MEFs are immortalized \textit{in vitro} at an accelerated rate. In contrast, p18\textsuperscript{INK4c} and p19\textsuperscript{INK4d} are rarely mutated in human cancers (Ruas and Peters, 1998). In mice loss of p19\textsuperscript{INK4d} function does not appear to be associated with tumour formation (Zindy \textit{et al.}, 2000), however p18\textsuperscript{INK4c}-/ mice develop pituitary hyperplasia, amongst other tumours, and may exhibit gigantism and organomegaly (Franklin \textit{et al.}, 1998; Latres \textit{et al.}, 2000). p15\textsuperscript{INK4b} mutations also occur in human cancers, but are usually associated with a large homozygous deletion that also affect the \textit{INK4a} locus (Ruas and Peters, 1998). p15\textsuperscript{INK4b}-/ mice have a have a slight increase in spontaneous tumour formation supporting the role of p15\textsuperscript{INK4b} as a tumour suppressor (Latrese \textit{et al.}, 2000).

\textbf{p53 activation and functions}

\textbf{Regulation of p53 levels and stability}

\textit{p53} is one of the most frequently mutated genes in human tumours (Hollstein \textit{et al.}, 1991). Both Li-Fraumeni patients, who inherit mutations in \textit{p53}, and \textit{p53} null mice are predisposed to tumour formation, supporting the role of \textit{p53} as a tumour suppressor (Malkin \textit{et al.}, 1990; Donehower \textit{et al.}, 1992). Moreover, mice expressing a stable mutant of \textit{p53} in the active wild-type conformation have an enhanced resistance to tumorigenesis
(Tyner et al., 2002). p53 function may also be inactivated in the absence of p53 mutation in some tumours by overexpression of mdm-2, deletion of p19ARF, p53 mislocalisation and viral oncoproteins including adenovirus E1, SV40 LT and the papillomavirus E6 protein (Vogelstein et al., 2000). The p53 family also includes p63 and p73 (reviewed by Yang et al., 2002).

p53 is maintained at low levels in the cell under normal circumstances by the interaction of mdm-2 with the p53 transcriptional activator domain, which inhibits p53 activity and targets p53 for proteosomal degradation (Momand et al., 1992; Oliner et al., 1993; Honda et al., 1997, Haupt et al., 1997; Kubbutat et al., 1997; Roth et al., 1998; Tao and Levine, 1999a). Yap and colleagues have recently demonstrated that mdm-2 inhibits the apoptotic and trans-repressor functions of p53 mainly by targeting p53 for degradation, whilst this is not necessary for the inhibition of p53 transcriptional activity by mdm-2 (Yap et al., 2000). Moreover, since mdm-2 is a transcriptional target of p53, this interaction creates a negative feedback loop whereby p53 activation results in increased levels of mdm-2, promoting p53 degradation and thus returning p53 levels to basal (Barak et al., 1993; Wu et al., 1993; Haupt et al., 1997; Kubbutat et al., 1997). Mdm-2 expression can also be regulated independently of p53 by the Ras/Raf/MAPK pathway (Ries et al., 2000).

p53 is activated by many stimuli, in addition to oncogenic stress, including DNA damage, hypoxia and rNTP depletion (summarised in Fig. 1.4, reviewed by Giaccia and Kastan, 1998; Abraham, 2001). These stimuli induce a range of post-translational modifications that stabilise and activate p53, in part by regulating the interaction between mdm-2 and p53 (Avantaggiati et al., 1997; Gu et al., 1997; Shieh et al., 1997; Banin et al., 1998; Canman et al., 1998; Sakaguchi et al., 1998; Waterman et al., 1998; Gostissa et al., 1999; Liu et al., 1999; Rodriguez et al., 1999; Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000; Ito et al., 2001). Interestingly, the interaction of mdm-2 with Rb appears to inhibit the effects of mdm-2 on p53 stabilisation and apoptosis, without relieving the repression of p53 transcriptional activity (Xiao et al., 1995; Hsieh et al., 1999; Yap et al., 1999).
Fig. 1.4. p53 activation and downstream targets.

p53 is maintained in a transcriptionally inactive state in the cell by interaction with mdm-2, which also targets p53 for proteosomal degradation. Following stimuli such as DNA damage or oncogenic stress p53 is covalently modified by acetylation, sumoylation and/or phosphorylated by a number of protein kinases depending on the nature of the stimuli. This results in p53 activation and/or stabilisation. Examples of molecules that are proposed to modify p53 are shown. Mdm-2 may also be inactivated by interaction with p19ARF or phosphorylation. Active p53 transactivates expression of a variety of target genes leading to a diverse array of potential outcomes, depending on the original stimulus and cell type. Selected examples of target genes are shown. Dashed arrows indicate potential interactions. See text for details. (Adapted from Giaccia and Kastan 1999; Vogelstein et al., 2000.)
Oncogenic stress has been shown to stabilise and activate p53 by an alternative mechanism involving the induction of p19ARF. For example, in primary mouse embryonic fibroblasts (MEFs) oncogenic Ras expression induces a p53 dependent cell cycle arrest, which also requires p19ARF activity (Kamijo et al., 1997; Palmero et al., 1998, see below). PML, the product of the PML gene of promyelocytic leukaemia, has also been implicated in regulating p53 activity in response to oncogenic Ras and DNA damage (Guo et al., 2000; Ferbeyre, 2000; Pearson et al., 2000). In primary fibroblasts oncogenic Ras upregulates PML expression and PML overexpression is able to induce a p53 dependent cell cycle arrest, similar to oncogenic Ras (Ferbeyre, 2000; Pearson et al., 2000). In MEFs, oncogenic Ras has been found to induce the re-localisation of p53 and CBP to PML nuclear bodies, where they form a trimeric complex with PML that may be involved in mediating p53 activation by promoting acetylation on lysine 382 (Pearson et al., 2000). Moreover, the oncogenic Ras induced cell cycle arrest and acetylation of p53 are impaired in PML -/- MEFs, confirming the importance of this protein in mediating the Ras induced p53 dependent arrest. Interestingly oncogenic Ras expression in PML -/- MEFs is still able to induce similar levels of p53 although this is associated with reduced expression of the p53 transcriptional target p21Cip1, suggesting that PML is required for p53 activation but not stabilisation. In contrast to MEFs, in human diploid fibroblasts (HDFs), it has been suggested that Ras induced upregulation of PML results in p53 activation by promoting phosphorylation of serine 15 (Ferbeyre, 2000). The histone deacetylase SIRT1, which is recruited to PML nuclear bodies following oncogenic Ras expression, has recently been found to antagonise PML induced acetylation of p53 and repress p53-mediated transactivation (Vaziri et al., 2001; Langley et al., 2002).

**Downstream signalling pathways from p53**

Activation of p53 by oncogenic Ras in primary cells is associated with the induction of the p53 transcriptional target p21Cip1, resulting in a G1 cell cycle arrest (el-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993; Lloyd et al., 1997; Serrano et al., 1997). Activation of p53 in response to stimuli other than oncogenic stress has a variety of potential effects including G1 and/or G2 cell cycle arrest, DNA repair, inhibition of blood vessel formation and apoptosis (summarised in Fig.1.4, reviewed by Vogelstein et al., 2000; Bargonetti and
Manfredi, 2002). Interestingly, c-Jun has recently been shown to negatively regulate p53 association with the p21<sup>Cip1</sup> promoter following UV treatment, and thus may downregulate p21<sup>Cip1</sup> expression to allow cell cycle re-entry after successful DNA repair (Shaulian et al., 2000). The effect of p53 activation appears to vary between cell type and stimuli to some extent, but it remains unclear how the outcome of p53 activation is determined (Lane, 2001). However, the identification of ASPP1/2 (apoptosis stimulating protein of p53) suggests that accessory proteins may target p53 activity to specific promoters to induce apoptosis (Samuels-Lev et al., 2001).

**Regulation of p53 activity by p19<sup>ARF</sup>**

Mutations of the INK4a locus that selectively affect p19<sup>ARF</sup> (or p14<sup>ARF</sup> in humans) are much less common than mutations affecting both proteins or just p16INK4a, although a recent study suggests that the p19<sup>ARF</sup> promoter can also be inactivated by methylation (Robertson and Jones, 1998; Ruas and Peters, 1998; Zhang and Xiong, 1999). However, the importance of p19<sup>ARF</sup> as a tumour suppressor has been clearly demonstrated by p19<sup>ARF</sup>-/- mice, which are predisposed to spontaneous tumour formation (Kamijo et al., 1997). p19<sup>ARF</sup> expression is induced by hyperproliferative signals including overexpression of myc, adenovirus E1a, v-Abl, resulting in apoptosis (Radfar et al., 1998; de Stanchina et al., 1998; Zindy et al., 1998). In contrast, p19<sup>ARF</sup> overexpression or induction of p19<sup>ARF</sup> by oncogenic Ras or overexpression of β-catenin is associated with a cell cycle arrest (Quelle et al., 1995b; Palmero et al., 1998).

The induction of p19<sup>ARF</sup> in response to oncogenic stress and certain types of DNA damage is thought to promote p53 activation (Quelle et al., 1995b; Kamijo et al., 1997; Palmero et al., 1998; Stott, 1998; Damalas et al., 2001; Khan et al., 2000). Overexpression of p19<sup>ARF</sup> in mouse fibroblasts is associated with p53 stabilisation and transactivation of p21<sup>Cip1</sup>, resulting in a G1 and/or G2/M cell cycle arrest (Quelle et al., 1995b; Kamijo et al., 1997; Stott, 1998). p19<sup>ARF</sup> interacts with mdm-2 and by antagonising mdm-2 function is able to stabilise p53 levels and promote p53 activity (Pomerantz et al., 1998; Zhang, 1998; Honda and Yasuda, 1999). p19<sup>ARF</sup> is thought to sequester mdm-2 in the nucleolus, preventing ubiquitination and export of p53 and facilitating p53 activation (Honda and Yasuda, 1999;
Tao and Levine, 1999b; Weber et al., 1999). However, nucleolar sequestration of mdm-2 is not required or sufficient for p53 activation by p19ARF (Llanos et al., 2001; Korgaonkar et al., 2002). Furthermore, p53 stabilisation may not be essential for p19ARF-mediated activation of p53 (Korgaonkar et al., 2002). Thus the importance of p19ARF localisation during the p53 dependent cell cycle arrest still remains to be clarified. p19ARF has also been suggested to act independently of p53 to induce a cell cycle arrest, although the identity of other p19ARF targets is unclear (Eischen et al., 1999; Carnero, 2000; Weber et al., 2000; Korgaonkar et al., 2002).

Expression of p19ARF in response to hyperproliferative stimuli may be mediated by the transcription factor DMP1, which is able to bind to the p19ARF promoter in vitro and induce a p19ARF dependent arrest mediated by p53 (Inoue et al., 1999). p19ARF is also induced by abnormally high levels of E2F1, which may provide a link between aberrant mitogenic stimuli and the induction of p19ARF induced cell cycle arrest or apoptosis (Bates et al., 1998). In addition DAP kinase, which is frequently inactivated in human cancers, induces apoptosis by p53 in a p19ARF dependent manner (Raveh et al., 2001). p53 has been implicated in repressing of p19ARF expression, which, in contrast, may be positively regulated by mdm-2 (Robertson and Jones, 1998; Stott, 1998; Weber et al., 2000). Other potential repressors of p19ARF include bmi-1 (Jacobs et al., 1999, see above); Twist, which is thought to inhibit the p53 tumour suppressor pathway by reducing p19ARF levels (Maestro et al., 1999), and TBX2, which is amplified in some breast cancers and can repress p19ARF induction by myc, E2F1 and oncogenic Ras (Jacobs et al., 2000). These repressors may be involved inhibiting expression of p19ARF and/or p16INK4a during development. p19ARF expression has also been implicated in replicative senescence in vitro, and negative regulation of p19ARF expression by JunD is able to protect cells from p53 dependent arrest and apoptosis (Kamijo et al., 1997; Carnero, 2000; Weitzman and Yaniv, 1999). However, p19ARF is not necessarily required for p53-mediated apoptosis in response to oncogenic stress (Tolbert et al., 2002).
The mechanisms of the oncogenic Ras induced cell cycle arrest

In many primary cell types oncogenic Ras induces a cell cycle arrest, which is caused by the induction of CDKIs (Lloyd et al., 1997; Serrano et al., 1997; Malumbres et al., 2000). Since the Ras induced arrest is mediated by activation of the Raf/MAPK pathway, a number of groups have used inducible Raf proteins or active MAPK proteins to investigate the mechanism of the arrest (Lloyd et al., 1997, Lin, 1998; Zhu, 1998). However, although activation of the Ras/Raf/MAPK pathway results in a common outcome in a number of primary cell types, the mechanisms responsible for the arrest appear to differ between cell types. Moreover, in some cases the effects of Ras/MEK and Raf activation appear to differ within a single cell type (Serrano et al., 1997; Lin, 1998; Zhu, 1998; Lin and Lowe, 2001; Roper et al., 2001; Wei et al., 2001). The effects of oncogenic Ras/Raf/MAPK expression in certain primary cell types are summarised in Table 1.1.

Mouse embryonic fibroblasts (MEFs)

The mechanism of the Ras induced arrest has been most well characterised in primary MEFs due to the advantages of the availability of knockout cells to determine the importance of potential mediators of the arrest. In primary MEFs oncogenic Ras induces a cell cycle arrest associated with a senescent phenotype that is mediated by the expression of the CDKIs p15^{INK4b}, p16^{INK4a} and p21^{Cip1} (Serrano et al., 1997; Malumbres et al., 2000, Fig.1.5.). The oncogenic Ras induced cell cycle arrest is dependent on the presence of Rb family members, which are maintained in an active state by the inhibition of G1 cyclin-CDK complexes following CDKI induction. However, loss of Rb or p107 and p130 function is insufficient to overcome the Ras induced arrest, probably due to functional compensation by the other family members, and loss of all three proteins is required for abrogation of the oncogenic Ras induced arrest (Sage et al., 2000).

The mechanism responsible for the induction of the CDKIs by oncogenic Ras remains unclear, although a number of studies have demonstrated the importance of p53 for the Ras induced arrest in MEFs (Serrano et al., 1997; Lin, 1998). The Ras induced arrest is p53 dependent and has been shown to result in elevated levels of active p53, which is thought to transcriptionally activate p21^{Cip1} expression. Oncogenic Ras expression is also associated
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Oncogenic Ras</th>
<th>Conditional Raf</th>
<th>Gain of function MEK mutant</th>
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<tr>
<td><strong>Primary rat Schwann cells</strong> Ridley et al., 1988 Lloyd et al., 1997</td>
<td>cell cycle arrest LT → transformed myc → transformed E1a → transformed</td>
<td>cell cycle arrest ↑ p21&lt;sup&gt;G1&lt;/sup&gt;, p53 dependent dn p53 → no arrest p21&lt;sup&gt;G1&lt;/sup&gt; antisense RNA → partial abrogation of arrest</td>
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<td><strong>Primary mouse Keratinocytes</strong> Missero 1996 Tremain et al., 2000 Lin and Lowe 2001 Roper et al., 2001 Paramio et al., 2001</td>
<td>cell cycle arrest with ↑ differentiation markers ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p21&lt;sup&gt;G1&lt;/sup&gt;, p19&lt;sup&gt;ARF&lt;/sup&gt;, p53 p19&lt;sup&gt;ARF&lt;/sup&gt; → no arrest p53 → no arrest p21&lt;sup&gt;G1&lt;/sup&gt; → no arrest *senescence p21&lt;sup&gt;G1&lt;/sup&gt; → arrest INK4a&lt;sup&gt;α2&lt;/sup&gt; → no arrest p21&lt;sup&gt;G1&lt;/sup&gt; + INK4a&lt;sup&gt;α2&lt;/sup&gt; → no arrest **senescence ↑TGFβ, p15&lt;sup&gt;INK4b&lt;/sup&gt;, TGFβ → no arrest</td>
<td>cell cycle arrest with ↑ differentiation markers ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p53 p19&lt;sup&gt;ARF&lt;/sup&gt; → arrest p53&lt;sup&gt;−&lt;/sup&gt; → no arrest p21&lt;sup&gt;G1&lt;/sup&gt; → no arrest</td>
<td></td>
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<td><strong>Primary MEFs</strong> Serrano et al., 1996 Kamijo et al., 1997 Serano et al., 1997 Sewing et al., 1997 Woods et al., 1997 Lin et al., 1998 Palmero et al., 1998 Pantozza et al., 1999 Malumbres et al., 2000 Pearson et al., 2000 Ries et al., 2000 Krimpenfort et al., 2001 Sharpless et al., 2001</td>
<td>cell cycle arrest – senescence ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p15&lt;sup&gt;INK4b&lt;/sup&gt;, p21&lt;sup&gt;G1&lt;/sup&gt;, p19&lt;sup&gt;ARF&lt;/sup&gt;, p53, PML p53 → no arrest INK4a&lt;sup&gt;α2&lt;/sup&gt; → no arrest p19&lt;sup&gt;ARF&lt;/sup&gt; → no arrest p16&lt;sup&gt;INK4a&lt;/sup&gt; → arrest p15&lt;sup&gt;INK4b&lt;/sup&gt; → no arrest p53 → no arrest p21&lt;sup&gt;G1&lt;/sup&gt; → arrest PML → no arrest</td>
<td>cell cycle arrest–senescence ↑mdm2 (p53 independent), p21&lt;sup&gt;G1&lt;/sup&gt;, p19&lt;sup&gt;ARF&lt;/sup&gt; p53&lt;sup&gt;−&lt;/sup&gt; → arrest (↑p21 occurs independently of p53) p21&lt;sup&gt;G1&lt;/sup&gt; → no arrest</td>
<td>cell cycle arrest–senescence ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p21&lt;sup&gt;G1&lt;/sup&gt;, p53 INK4a&lt;sup&gt;α2&lt;/sup&gt; → no arrest p53&lt;sup&gt;−&lt;/sup&gt; → no arrest</td>
</tr>
<tr>
<td><strong>Human fibroblasts (including IMR90)</strong> Serrano et al., 1997 Lin et al., 1998 Zhu et al., 1998 Ferbeyre et al., 2000 Wei et al., 2001 Brookes et al., 2002</td>
<td>cell cycle arrest – senescence ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p21&lt;sup&gt;G1&lt;/sup&gt;, p53, PML MEK inhibition → no arrest E1a → no arrest dn p53 → arrest Rb/p16 pathway inhibited → arrest p21&lt;sup&gt;G1&lt;/sup&gt; → arrest p53 → arrest p16&lt;sup&gt;INK4a&lt;/sup&gt; → no arrest*</td>
<td>cell cycle arrest – senescence ↑p21&lt;sup&gt;G1&lt;/sup&gt; (p53 dependent), p16&lt;sup&gt;INK4a&lt;/sup&gt;, no change in p53 levels E6 → arrest (no ↑ p21&lt;sup&gt;G1&lt;/sup&gt;) MEK inhibition → no arrest</td>
<td>cell cycle arrest–senescence ↑p16&lt;sup&gt;INK4a&lt;/sup&gt;, p21&lt;sup&gt;G1&lt;/sup&gt;, p53</td>
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Table 1.1. Summary of the effects of Ras, Raf or MEK activation on proliferation of a number of primary cells. (See text for details) dn p53, dominant negative p53; E1a, Adenovirus protein that inhibits Rb; p53 and Rb; E6, human papilloma virus protein that inhibits p53; * and ** alternative studies.
Fig. 1. 5. Induction of a cell cycle arrest by oncogenic Ras in primary fibroblasts.

A) MEFs and B) HDFs. It is not clear whether CBP/p300 interact with p53 and PML in HDFs under these conditions. See text for details. PNB, PML nuclear body; P, Ser-15 phosphorylation; Ac, acetylation on lysines 320 and 385. (Adapted from McMahon and Woods 2001; Wei et al., 2001.)
with increased levels of p19ARF, which is required for the establishment of the cell cycle arrest and is thought to be involved in stabilising and activating p53 (Kamijo et al., 1997, Palmero et al., 1998, see above). p19ARF induction by oncogenic Ras/Raf may be mediated by the transcription factors myc, E2F and DMP1 (Zindy et al., 1998; Dimri et al., 2000; Inoue et al., 1999; Inoue et al., 2000). PML has also been implicated in mediating the Ras induced arrest, since this arrest is associated with increased PML expression and overexpression of PML results in the induction of p19ARF and p53 activation (Pearson et al., 2000, see above). PML is thought to facilitate p53 activation by promoting p53 acetylation by the histone acetyl transferases CBP/p300 in PML nuclear bodies (Guo et al., 2000; Pearson et al., 2000). However, PML mediated induction of p19ARF may also contribute to p53 activation.

The oncogenic Ras induced arrest in primary MEFs is p53 dependent, however, p21Cip1/-MEFs still arrest in response to oncogenic Ras, suggesting that alternative transcriptional targets of p53 also contribute to the arrest in this cell type (Serrano et al., 1997; Kamijo et al., 1997; Pantoja, 1999). p16INK4a is not required for the Ras induced arrest in MEFs, although p15INK4b/- MEFS are susceptible to transformation by oncogenic Ras (Malumbres et al., 2000; Latres et al., 2000; Krimpenfort et al., 2001; Sharpless et al., 2001). Recently hDRIL1 has been identified as a factor that is able to overcome the Ras induced arrest, despite increased levels of p53, p19ARF, p16INK4a and p21Cip1 (Peeper et al., 2002). hDRIL1 is thought to act downstream of these molecules and bypasses the cell cycle arrest by increasing cyclin E levels.

In primary cells, the oncogenic Ras induced cell cycle arrest is mediated by the Raf/MAPK pathway, with MEK activation in MEFs resulting in increased levels of p53, p21Cip1 and p16INK4a, leading to a cell cycle arrest that is p53, and probably p19ARF, dependent (Lin, 1998). However, although high intensity Raf activation in primary MEFs is also associated with the induction of p21Cip1 and p19ARF, the resulting arrest is p21Cip1 and p53 independent (Sewing et al., 1997; Woods et al., 1997). This apparent paradox may be due to differences in the kinetics of MAPK pathway activation by Raf and oncogenic Ras/activated MEK. Interestingly oncogenic Raf expression in NIH3T3 cells or p53MEFS is able to induce a
p21\textsuperscript{Cip1} dependent arrest in a p53 independent manner (Sewing et al., 1997; Woods et al., 1997). Under these circumstances p21\textsuperscript{Cip1} expression may be mediated by the transcription factors E2F-1 or Sp1 and Sp3 (Kivinen et al., 1999; Gartel et al., 2000). In addition, Rho has been implicated in repressing the induction of p21\textsuperscript{Cip1} expression by oncogenic Ras, but the mechanism remains unclear (Olson et al., 1998).

**Human diploid fibroblasts (HDFs)**

In primary human fibroblasts the oncogenic Ras induced cell cycle arrest appears to be mediated by a different mechanism to primary MEFs, since loss of p53 is insufficient to overcome the arrest and the absence of a detectable induction of p19\textsuperscript{ARF} suggests the p19\textsuperscript{ARF}/p53 pathway is not activated in these cells (Serrano et al., 1997; Ferbeyre, 2000; Wei et al., 2001; Brookes et al., 2002). However, similar to MEFs, oncogenic Ras signalling in HDFs results in the induction of increased levels of p16\textsuperscript{INK4a}, p53 and p21\textsuperscript{Cip1}, (Serrano et al., 1997; Lin, 1998; Zhu, 1998; Wei et al., 2001, Fig.1.5.). Oncogenic Ras has also been implicated in the induction of PML in HDFs, but in contrast to MEFS, PML is thought to promote activation of p53 by phosphorylation on Ser15 (Ferbeyre, 2000). Moreover, overexpression of PML is able to induce an arrest associated with increased levels of p16\textsuperscript{INK4a}, p21\textsuperscript{Cip1} and p53, although it remains unclear how the induction of p16\textsuperscript{INK4a} is mediated by PML.

p16\textsuperscript{INK4a} has been suggested to be an important mediator of the oncogenic Ras/Raf induced cell cycle arrest in HDFs (Zhu, 1998; Brookes et al., 2002). Induction of p16\textsuperscript{INK4a} by oncogenic Ras is associated with a cell cycle arrest in p53\textsuperscript{-/-} or p21\textsuperscript{Cip1/-} HDFs, which are unable to induce p21\textsuperscript{Cip1}, demonstrating that p16\textsuperscript{INK4a} induction is sufficient to cause the arrest (Serrano et al., 1997; Wei et al., 2001). However, oncogenic Ras expression in HDFs with an abrogated p16\textsuperscript{INK4a}/Rb pathway is still able to induce a cell cycle arrest, demonstrating that the p53/p21\textsuperscript{Cip1} pathway is also able to induce the arrest (Serrano et al., 1997). In agreement with these findings co-expression of oncogenic Ras and E1A is able to overcome the arrest demonstrating that Ras induced transformation of HDFs requires inhibition of both the Rb and p53 pathways (Serrano et al., 1997). In contrast, a recent study by Brookes and colleagues, using HDFs from a patient with a homozygous deletion
in the \textit{INK4a} locus exon 2 that appears to specifically inactivate \(p16^{\text{INK4a}}\), find that loss of \(p16^{\text{INK4a}}\) is sufficient to overcome the Ras induced arrest (Brookes \textit{et al.}, 2002). However, this work does not completely demonstrate the absence of additional mutations, although p53 and p19\textsuperscript{ARF} can still be induced successfully by other stimuli. The apparent importance of \(p16^{\text{INK4a}}\) in mediating the Ras induced arrest in HDFs is in contrast to MEFs where \(p16^{\text{INK4a}}\) is not required for the oncogenic Ras induced arrest (Krimpenfort \textit{et al.}, 2001; Sharpless \textit{et al.}, 2001).

Ras activation in HDFs has been reported to induce increased p53 levels, however, the study by Brookes and colleagues was unable to detect this (Serrano \textit{et al.}, 1997; Brookes \textit{et al.}, 2002). In addition, Raf activation in HDFs does not result in increased p53 levels, although induction of \(p16^{\text{INK4a}}\) and \(p21^{\text{Cipl}}\) are still observed in association with a cell cycle arrest (Zhu, 1998). Since the induction of \(p21^{\text{Cipl}}\) in response to Ras/Raf activation is p53 dependent, p53 activity may be increased in the absence of p53 stabilisation and this may be mediated by interaction with PML (Zhu, 1998; Ferbeyre, 2000).

\textbf{Keratinocytes}

In primary mouse keratinocytes oncogenic Ras/Raf signalling induces a cell cycle arrest associated with the induction of differentiation markers and increased \(p21^{\text{Cipl}}\), \(p15^{\text{INK4b}}\) and p53 levels in a p53 dependent manner (Tremain, 2000; Lin and Lowe, 2001; Roper \textit{et al.}, 2001). However, reports differ as to whether \(p16^{\text{INK4a}}\) and p19\textsuperscript{ARF} are induced, with the oncogenic Ras induced arrest being p19\textsuperscript{ARF} dependent, whilst the activated Raf induced arrest is p19\textsuperscript{ARF} independent. Thus the study by Lin and Lowe suggests a model similar to primary MEFs whereby oncogenic Ras induces both \(p16^{\text{INK4a}}\) and p19\textsuperscript{ARF}/\(p53/ p21^{\text{Cipl}}\) signalling pathways, whilst Roper and colleagues suggest that activated Raf causes a p19\textsuperscript{ARF} independent arrest mediated by p53 induction of \(p21^{\text{Cipl}}\) and demonstrate that the arrest is \(p21^{\text{Cipl}}\) dependent (Lin and Lowe, 2001; Roper \textit{et al.}, 2001). The situation is further complicated by the findings of Paramio and colleagues that loss of both \(p21^{\text{Cipl}}\) and the INK4 locus are required to overcome the Ras induced arrest, whilst Tremain and colleagues propose a role for TGF\(\beta\) signalling in mediating the arrest (Paramio \textit{et al.}, 2001; Tremain, 2000). Moreover, since keratinocyte differentiation is not normally associated with elevated levels of p19\textsuperscript{ARF} and p53, the oncogenic Ras/Raf induced expression of differentiation
markers is probably a protective mechanism against unregulated proliferation that is distinct from terminal differentiation, but shares some of the same phenotypic characteristics.

Schwann cells

Oncogenic Ras/Raf expression in primary Schwann cells results in rapid and prolonged MAPK activation, leading to the p53 dependent induction of the CDKI p21\(^{Cip1}\) and a G1 cell cycle arrest (Ridley et al., 1988; Lloyd et al., 1997). The Raf induced arrest is associated with inhibition of the specific activity of cyclin E -CDK, which occurs with similar kinetics to p21\(^{Cip1}\) induction, suggesting that p21\(^{Cip1}\) may be involved in mediating this arrest. Moreover, p21\(^{Cip1}\) antisense RNA is able to partially abrogate the Raf induced arrest confirming the importance of p21\(^{Cip1}\) in mediating the arrest. However, other CDKIs may be involved in mediating the oncogenic Ras/Raf induced arrest in Schwann cells, similar to other primary cell types. The Raf induced increase in p21\(^{Cip1}\) levels occurs after a lag period of about 14 hrs suggesting that p21\(^{Cip1}\) induction is indirect. However, the mechanism of p21\(^{Cip1}\) induction in response to Ras/Raf activation and the identity of any other CDKIs involved in mediating the arrest have yet to be determined.

1.4. Neurofibromatosis type 1 (NF1)

Incidence and symptoms

Neurofibromatosis Type 1 (NF1) is an inherited genetic disorder associated with an increased incidence of tumour formation that affects 1 in 3500 individuals worldwide. Patients diagnosed with NF1 characteristically develop numerous benign, Schwann cell derived, peripheral nerve sheath tumours (neurofibromas), café-au-lait spots (due to defects in pigmentation) and Lisch nodules (benign lesions of the iris) (reviewed by Huson, 1994, Weistler, 1994). Plexiform neurofibromas are found in ~10% of affected individuals and carry an additional risk of transformation into malignant peripheral nerve sheath tumours (MPNSTs). NF1 patients are also predisposed to developing astrocytic brain tumours, phaeochromocytoma and myeloid leukaemia. Other symptoms can include learning disabilities in 40-60% of cases, bone dysplasias, and shortness of stature. The
major defects associated with this disease are predominantly found in neural crest-derived
tissues although mesodermal and neural tube derived tissues have also been implicated.

**Neurofibroma structure and tumour progression**

Peripheral nerves are complex structures which are composed of a number of interacting
cell types (Fig. 1.6.A). Schwann cells associate with peripheral nerves fibres in an ECM,
becoming myelinating or non-myelinating cells depending on signals from the nerve
(Aguayo *et al.*, 1976a). Several nerve fibres with associated Schwann cells are bundled into
a fascicle and surrounded by a layer of perineurial cells, which act as a diffusion barrier to
prevent inappropriate exposure to circulating growth factors and hormones (Low, 1976;
Landon and Hall, 1976). Multiple fascicles in connective tissue form an individual nerve.
This organisation is disrupted in neurofibromas (Fig.1.6.B, reviewed by Cichowski and
Jacks, 2001), where large numbers of unencapsulated Schwann cells and fibroblasts are
scattered in a collagen rich ECM, with mast cells and axons (Waggener, 1966; Poirier *et
al.*, 1968; Stefansson *et al.*, 1982). The most abundant cell types are thought to be Schwann
cells, which form 60-80% of the cell population, and fibroblasts (Peltonen *et al.*, 1988). The
Schwann cells however, are often observed to occur without apparent nerve contact,
although myelinated and non-myelinated axons are also present at low numbers (Waggener,
1966; Poirier *et al.*, 1968; Stefansson *et al.*, 1982).

Discrete neurofibromas are found at a single site, within a defined area, and may involve
cutaneous or subcutaneous nerves (Korf, 1999; Wiestler and Radner, 1994). In contrast,
plexiform neurofibromas develop much less frequently, in about 10% of NF1 sufferers, and
extend along the length of deep peripheral nerves. These tumours may be congenital and
can grow to be large and debilitating, potentially involving multiple nerve branches. In
addition, plexiform neurofibromas have been shown to contain cytogenetic abnormalities
not found in dermal neurofibromas and there is thought to be a 4-5% life-time risk of
transformation of plexiform neurofibromas into MPNSTs (Lakkis and Tennekeon, 2000;
Wallace *et al.*, 2000). *p53* mutations have been detected in human MPNSTs, but not in
benign neurofibromas, and *NF1+/-* and *p53 +/-* cis mice develop MPNSTs associated with
LOH at both loci, implicating *p53* mutation in MPNST formation (Menon *et al.*, 1990,
Fig. 1.6. The structure of normal peripheral nerves and neurofibromas

(A) Peripheral nerves are normally composed of Schwann cells in association with axons, which are surrounded by a perineurial sheath to form a nerve fascicle. Several fascicles are grouped to form a nerve fibre. (B) In neurofibromas increased numbers of Schwann cells are found in a collagen rich ECM in the absence of axonal contact. The perineurium is also disrupted. Top, longitudinal section; below, transverse sections of nerve fibre. (Adapted from Rosenbaum et al., 1997 and Cichowski and Jacks 2001.)

Fig. 1.7. Sequence similarity of Neurofibromin to the RAS-GAP family of proteins.

Neurofibromin has sequence similarity to Drosophila NF1, p120RasGAP and the yeast RasGAP proteins IRA1 and IRA2. Homology with the IRA proteins (and Drosophila NF1) extends beyond the GAP-related domain. (Adapted from Cichowski and Jacks, 2001; Kim and Tamanoi, 1998.)
Legius et al., 1994, Cichowski et al., 1999; Vogel et al., 1999). Mutations in the INK4a locus, involving deletion of exon 2 and leading to INK4a LOH, have also been reported in MPNSTs, but are not detected in neurofibromas (Kourea et al., 1999b; Nielsen et al., 1999). Moreover, the cellular localisation of the CDKI p27 Kip1 is altered from nuclear (in neurofibromas), to cytoplasmic (in MPNSTs), and cyclin E expression is elevated, potentially resulting in increased proliferation (Kourea et al., 1999a). Thus MPNSTs formation appears to involve multiple changes in a number of regulatory genes, in addition to NF1 LOH found in neurofibromas. In addition, changes in the expression patterns of receptors and mitogens may also enhance proliferation. For example, increased levels of angiogenic factors, such as midkine are found in neurofibromas (Mashour et al., 2001) and high levels of stem cell factor and its receptor KIT are associated with loss of Neurofibromin in neurofibroma and MPNST-derived Schwann cells (Badache et al., 1998).

Epigenetic effects have also been implicated in tumour formation in NF1 (reviewed by Ratner and Daston, 2001; Lakkis and Tennekoon, 2000). For example, increased tumour growth is associated with puberty and pregnancy suggesting that altered hormone levels are important, however, the molecular basis for this response is unknown. In addition, increased neurofibroma growth has been reported following accidental trauma or surgery. As a result it has been suggested that NF1 patients undergo an altered response to normal nerve repair signals, resulting in the initiation of neurofibroma formation. In support of this idea, NF1 +/- mice have abnormal responses to wounding and Neurofibromin expression is increased following normal nerve damage (Wrabetz et al., 1995; Atit et al., 1999). Moreover, sciatic nerve injury in NF1/- mice is associated with rare tumour formation and hyperpigmentation around the wound (Ratner and Daston, 2001).

**Expression of NF1 and functions of Neurofibromin**

The NF1 gene is expressed ubiquitously during early development (Gutmann et al., 1995), but in adult tissues, expression is most abundant in cells of the nervous system, including neurons, oligodendrocytes and Schwann cells, and the adrenal gland (Daston et al., 1992). Following peripheral nerve damage the levels of the NF1 gene product, Neurofibromin, increase in dedifferentiating Schwann cells (Wrabetz et al., 1995). However, interpretation
of studies of NF1 expression are complicated by the existence of several isoforms of Neurofibromin, with differing RasGAP capabilities ((Andersen et al., 1993; Gutmann et al., 1993; Viskochil, 1999).

The product of the NF1 gene, Neurofibromin contains a region of homology corresponding to the catalytic domain of GTPase-activating proteins (GAPs) (DeClue et al., 1991; Xu et al., 1990b). This family includes p120RasGAP and the yeast RasGAP proteins IRA1 and IRA2, which share an extended region of homology with Neurofibromin (Fig.1.7, Ballester et al., 1990, Xu et al., 1990b; Kim and Tamanoi, 1998). The ability of Neurofibromin to act as a RasGAP in vivo and vitro has been confirmed by several groups (Martin et al., 1990, Ballester et al., 1990 and Xu et al., 1990a). Neurofibromin has a much higher affinity for Ras than p120RasGAP, suggesting that Neurofibromin is a significant regulator of Ras activity, especially at lower Ras concentrations (Martin et al., 1990). In addition, Neurofibromin appears to be a more an important regulator of Ras activity in certain tissues than p120 RasGAP (Bollag and McCormick, 1991).

The importance of elevated Ras-GTP levels in NF1- associated tumours has been demonstrated using Schwann cell lines derived from MPNSTs. These cell lines have decreased levels of Neurofibromin expression associated with elevated levels of activated Ras, which are critical for the maintenance of a transformed phenotype (DeClue et al., 1992; Basu et al., 1992). Since p120RasGAP levels and activity are normal in the MPNST derived cell lines this also suggests that Ras activity is primarily regulated by Neurofibromin in Schwann cells. In addition elevated Ras signalling has recently been implicated in mediating the learning defects associated with NF1+/- mice, suggesting that constitutive activation of Ras signalling in response to loss of Neurofibromin function may also be responsible for these defects in NF1 patients (Costa et al., 2002; Costa et al., 2001).

NF1 has been suggested to have additional non-Ras related functions. This idea is supported by work in Drosophila that has implicated Neurofibromin in the regulation of cAMP activated pathways (The et al., 1997; Guo et al., 1997; Guo et al., 2000). In contrast to flies with activated Ras1 or 2, NF1-/- flies are small, with defects in voltage-gated
potassium channel function, learning and short-term memory and a reduced escape response (Fortini et al., 1992; The et al., 1997; Guo et al., 1997; Guo et al., 2000). Defects in NFI-/− flies are rescued by increased PKA activity rather than reduced Ras signalling, suggesting that Neurofibromin has an important role in activating the adenylyl cyclase pathway in Drosophila (The et al., 1997; Guo et al., 1997; Guo et al., 2000). However, NFI-/− flies also manifest a defect in circadian rhythm related locomotor activity, which is rescued by reducing Ras/Raf/MAPK activity (Williams et al., 2001). Therefore in Drosophila Neurofibromin is implicated in both Ras/Raf/MAPK and cAMP activated pathways. A recent study by Tong and colleagues has shown that human Neurofibromin can rescue the defects in cAMP signalling in NFI-/− flies (Tong et al., 2002). In addition, since cAMP levels are significantly lower in NFI-/− neurons in vitro compared to NFI+/+ cultures, Neurofibromin may also regulate the cAMP pathway in mammalian cells. Moreover, the inhibition of Ras activity in NFI-/− mouse Schwann cells is unable to inhibit Schwann cell invasiveness, despite blocking proliferative defects, supporting non-Ras dependent functions for Neurofibromin (Kim et al., 1997). However, in contrast to the findings in NFI-/− Drosophila and NFI-/− mouse neurons, Kim and colleagues have shown that NFI-/− Schwann cells have elevated levels of cAMP (Kim et al., 2001).

**Neurofibromin as a tumour suppressor**

Knudson’s two-hit hypothesis proposes that tumour formation requires mutations in both alleles of a tumour suppressor (Knudson, 1971; Knudson et al., 1975). Individuals with an inherited tumour suppressor gene mutation only require inactivation of the remaining wild-type allele prior to the initiation of tumour formation and are thus predisposed to developing cancer at an earlier age than the rest of the population. NFI+/+ mice are also predisposed to tumour development, supporting the idea that Neurofibromin is a tumour suppressor (Jacks et al., 1994). The tumours developed include phaeochromocytoma and myeloid leukaemia, which are common in NFI patients and are associated with the loss of the remaining wild-type NFI allele. However, the NFI+/− mice do not develop neurofibromas, probably due to the requirement for LOH at the NFI locus in Schwann cells, and also lack pigmentation defects. In support of this idea NFI-/− chimaeric mice are susceptible to neurofibroma development, demonstrating that bi-allelic inactivation of NFI
is required for neurofibroma formation (Vogel et al., 1999; Cichowski et al., 1999). Moreover, Zhu and colleagues have recently demonstrated that loss of heterozygosity (LOH) at the \textit{NFl} locus in Schwann cells specifically may be involved in causing neurofibroma formation in an \textit{NFl+/-} background (Zhu et al., 2002). This study provides support for the role of Schwann cells as the initiating cell type for these tumours, which has been suggested by the examination of human neurofibroma derived cells (Serra et al., 2000) Wallace et al., 2000, Kluwe et al., 1999). However, although neurofibroma derived or \textit{NFl-/-} mouse neurons, mast cells and fibroblasts do not appear to undergo \textit{NFl} LOH, these cell types also have altered behaviour \textit{in vitro} (see below) and are though to be involved in producing an abnormal cellular environment that promotes tumour formation or progression. This model is also supported by the work of Zhu and colleagues who demonstrate that \textit{NFl} haplo-insufficiency of other cell types (in addition to LOH in Schwann cells), does contribute to neurofibroma formation \textit{in vivo} (Zhu et al., 2002).

\textit{In vitro} cellular studies

Several animal models have been developed to allow investigation of the formation of neurofibromas or MPNSTs and Neurofibromin function \textit{in vivo} and \textit{in vitro} (see above). The phenotypes of Schwann cells, neurons and perineural cells or fibroblasts derived from these animal models and human neurofibromas or MPNSTs have been investigated by a number of groups. These studies have shown that although LOH of \textit{NFl} has only been detected in Schwann cells; neurons and perineural cells also have an abnormal phenotype \textit{in vitro}, suggesting that the altered environmental provided by these cells may enhance tumour progression \textit{in vivo}, a view that is supported by the findings of Zhu and colleagues that are discussed above (Zhu et al., 2002).

\textbf{Neurons}

\textit{NFl-/-} sympathetic and sensory neurons are capable of surviving and extending neurites \textit{in vitro} in the absence of neurotrophins, whereas wild type neurons die under the same conditions (Atit et al., 1999; Vogel et al., 1995). In addition neurotrophin independent survival by \textit{NFl-/-} neurons is dependent on Ras/PI3K signalling (Klesse and Parada, 1998). It is unclear how this defect is associated with tumorigenesis in NFl patients. However,
neurotrophin independence may result in the aberrant acquisition of nerve connections, which could be involved in the learning difficulties associated with NF1 patients and *NF1*−/− *Drosophila* (reviewed by Cichowski and Jacks, 2001; Guo *et al.*, 2000).

**Fibroblasts or perineural cells**

To investigate the effect of Neurofibromin loss on nerve fascicle formation, fibroblasts from *NF1*−/− and *NF1*+/+ animals were added to *NF1*−/− or *NF1*+/+ DRG-Schwann cell co-cultures respectively (Rosenbaum *et al.*, 1995). *NF1*−/− fibroblasts produce a reduced number of fascicles, which are associated with an abnormal morphology. This defect is also observed with *NF1*−/− fibroblasts in wild-type Schwann cell-DRG cultures and *NF1*+/− fibroblasts have an intermediate phenotype between wild-type and *NF1* null cells. In addition, some *NF1*−/− fibroblasts have an increased rate of proliferation in culture. Moreover, following wounding *NF1*−/− fibroblasts undergo abnormal collagen deposition and proliferation in response to wound cytokines (Atit *et al.*, 1999). Thus *NF1* deficient fibroblasts have defects in proliferation and perineurium formation and as a result may contribute to neurofibroma formation *in vivo* by exposing the other cell types to an altered local environment. However, loss of Neurofibromin function in fibroblasts does not result in increased levels of Ras activity, suggesting that these defects are caused by deregulation of other non-Ras related Neurofibromin functions (Sherman *et al.*, 2000a).

**Schwann cells**

Schwann cells in human neurofibromas are frequently observed without nerve contact and have been shown to undergo LOH at the *NF1* locus, which is sufficient to initiate neurofibroma formation in a *NF1*+/− mouse model (Waggener, 1966; Poirier *et al.*, 1968; Stefansson *et al.*, 1982; Serra *et al.*, 2000, Wallace *et al.*, 2000, Kluwe *et al.*, 1999; Zhu *et al.*, 2002). In addition, Sherman *et al.*, have demonstrated that a subpopulation of neurofibroma-derived Schwann cells, probably corresponding to *NF1*+ cells, have elevated Ras-GTP levels, which suggests that Neurofibromin functions as a RasGAP in these cells and that increased levels of Ras activity may be a rate limiting requirement for neurofibroma formation (Sherman *et al.*, 2000a). Human neurofibroma-derived Schwann cells are angiogenic and invasive *in vitro*, although they do not cause progressive tumours.
when injected into nude mice (Sheela et al., 1990). Moreover, the neurofibromas formed by this procedure contain nerve bundles, which are probably of mouse origin, suggesting that the neurofibroma-derived Schwann cells can associate with host axons successfully in vivo.

Schwann cells derived from \( NF1^{+/-} \) and \( NF1^{-/-} \) mice have been used extensively to examine the effects of Neurofibromin loss on Schwann cell function in vitro. \( NF1^{-/-} \) Schwann cells are angiogenic and invasive in vitro, similar to human neurofibroma-derived Schwann cells, and have elevated levels of Ras-GTP compared to wild type cells (Sheela et al., 1990; Kim et al., 1997; Kim et al., 1995). In addition, \( NF1^{-/-} \) Schwann cells have an altered morphology (elongated and hyper-refractile with long processes similar to oncogenic Ras expressing cells), and a decreased rate of proliferation in response to GGF addition or axon derived signals (Kim et al., 1995, Ridley et al., 1988, Lloyd et al., 1997). \( NF1^{+/-} \) Schwann cells have an intermediate phenotype, demonstrating that loss of even one \( NF1 \) allele has effects on Schwann cell behaviour. Importantly, both oncogenic Ras expressing and \( NF1^{-/-} \) Schwann cells are able to associate normally with DRG axons in co-cultures (with serum containing medium) (Kim et al., 1995) and \( NF1^{-/-} \) Schwann cells are able to myelinate \( NF1^{+/-} \) axons in co-cultures (Rosenbaum, 1999). However, Schwann cell–axon co-cultures of \( NF1^{-/-} \) Schwann cells and \( NF1^{-/-} \) axons or \( NF1^{+/-} \) Schwann cells and \( NF1^{+/-} \) axons have decreased myelination, suggesting that the loss or reduction of Neurofibromin function in the surrounding cell types may provide an altered environment that could be important for the development of the peripheral nerve defects associated with NF1. This idea is supported by the recent work of Zhu and colleagues (Zhu et al., 2002).

However, since NF1 patients are though to have Schwann cell hyperplasia, Kim and colleagues examined whether the \( NF1^{-/-} \) Schwann cells have enhanced proliferative capabilities under different culture conditions (Kim et al., 1997). Interestingly they found that \( NF1^{-/-} \) Schwann cells are hyperproliferative in the presence of serum free medium containing elevated cAMP, unlike both wild- type and \( NF1^{+/-} \) cells. Thus altering the cellular environment can have a striking affect on \( NF1^{-/-} \) Schwann cell behaviour. In addition, in the absence of serum (and without cAMP elevation) some \( NF1^{-/-} \) Schwann cells exhibit growth factor independence, morphological changes, hyperplasia and
formation of foci, suggesting that they have become transformed by additional mutation(s). Moreover, under these conditions \textit{NF1/-} transformed cells arise and proliferate in the absence of contact with axons in DRG- \textit{NF1/-} Schwann cell co-cultures, resulting in a similar phenotype to that observed in neurofibromas (Waggener, 1966; Poirier \textit{et al.}, 1968; Kim \textit{et al.}, 1997).

Some of the effects of Neurofibromin loss in \textit{NF1/-} Schwann cells can be reverted by preventing Ras activation (Kim \textit{et al.}, 1997). However, neither invasiveness of \textit{NF1/-} Schwann cells nor hyperproliferation in response to cAMP elevation are prevented by inhibition of Ras activity, suggesting that Neurofibromin mediates these effects independently of Ras signalling, possibly using the cAMP responsive pathways implicated in \textit{NF1/-} Drosophila (Guo \textit{et al.}, 1997; Kim \textit{et al.}, 1997). However, in contrast to the \textit{Drosophila NF1/-} model and the experiments of Tong and colleagues using \textit{NF1/-} neurons, \textit{NF1/-} Schwann cells appear to have elevated levels of cAMP and constitutively express cyclin D1, suggesting that Neurofibromin usually antagonises the activity of cAMP pathways and reduces cyclin D1 expression in these cells (Guo \textit{et al.}, 1997; The 1997; Guo \textit{et al.}, 2000; Kim \textit{et al.}, 2001; Tong \textit{et al.}, 2002). Interestingly, \textit{NF1/-} and oncogenic Ras expressing Schwann cells appear to constitutively express P0 \textit{in vitro} (Rosenbaum, 1999). In contrast, transformed \textit{NF1/-} Schwann cells have lost constitutive P0 expression and have lost cAMP induction of P0 expression, suggesting that these cells have a defect in differentiation (Kim \textit{et al.}, 1997).

\subsection*{1.5. Schwann cell differentiation}

Schwann cells are derived from neural crest cells, which migrate into developing peripheral nerves and form Schwann cell precursors by E14/15 in the rat (Fig. 1.8.). These cells associate with numerous axons and proliferate in response to axon-derived signals. Immature Schwann cells develop by E16/17 and bundle the axons into progressively smaller groups until the axons are associated with myelinating or non-myelinating Schwann cells (reviewed by Mirsky and Jessen, 1996). Myelinating Schwann cells associate with a single axon, which stimulates the expression of myelination associated transcription factors and myelin genes, resulting in the formation of a myelin sheath. Non-myelinating Schwann
Myelinating Schwann cell
Associated with one axon
S100+, p75 NGFR, Oct-6+, Krox-20+, GalC+, myelin genes expressed

Pro-myelin Schwann cell
Myelin sheath formation
S100+, p75 NGFR, SCIP++, SCIP-, Krox-20+, GalC+, ↑ myelin gene expression

Neural-crest cell
Migrate to peripheral nerves
NRG-1 does not promote survival
p0-, GAP-43-, p75 NGFR+

Schwann-cell precursor
Associated with many axons
Dependent on axons for survival via NRG-1 production
p0* (low levels), GAP-43*, p75 NGFR*, S100-, GFAP-
High motility
Flattened, sheet-like morphology in vitro

Immature Schwann cells
Associated with multiple axons
S100+, GFAP+, O4 antigen*, GalC*, p75NG-
GAP-43+
Low motility
Bi- or tri-polar in vitro

Non-myelinating Schwann cell

Fig. 1.8. Diagram of the stages of Schwann cell differentiation showing characteristic changes in the expression patterns of developmental markers. (See text for details.) Schwann cell differentiation is reversible (dashed arrows) during Wallerian degeneration. E, embryonic day (rat). The mouse transitions occur approx. 2 days earlier than the rat. The formation of myelinating and non-myelinating cells begins around birth. Insets A and B - EM pictures of transverse sections from an adult rat sciatic nerve. (A) A myelinating Schwann cell forms a myelin sheath (M) around a single, large axon (Ax). (B) A non-myelinating Schwann cell (N-M) is shown ensheathing 13 axons or a single axon (A*). (C) Myelin sheath of nearby axon. (C) collagen-rich extracellular space. Bar, 0.5μm. (Adapted from Zorick and Lemke 1996; Jessen and Mirsky 1999; Mirsky and Jessen 2001.)
cells usually ensheathe many axons, which are smaller in diameter than the myelinated axons. Axon derived signals are thought to determine and maintain Schwann cell differentiation. Importantly Schwann cell are a regenerative cell type that respond to nerve damage by dedifferentiating and re-entering the cell cycle, and this behaviour is thought to be important for successful nerve regeneration.

Schwann cell development is regulated by a number of changing nerve derived cues, which are poorly understood. One family of factors, however, has been shown to be important in several stages of Schwann cell development. The Neuregulins (NRGs) are a family of factors with homology to EGF, which signal via the receptor tyrosine kinases of the ErbB family (reviewed by Yarden and Sliwkowski, 2001, Olayioye et al., 2000). The NRG family includes four genes, of which NRG-1 has been implicated in Schwann cell development (reviewed by Garratt et al., 2000a; Adlkofer and Lai, 2000). NRG-1 exists in several isoforms that can be secreted or transmembrane and include: (1) Neu differentiation factor, (NDF, Heregulin, acetylcholine receptor inducing activity (ARIA), type I NRG-1); (2) Glial growth factor (GGF, type II), and (3) sensory and motor neuron-derived factor (SMDF, cysteine rich domain (CRD-NRG-1), type III). ErbB2 and ErbB3 heterodimers appear to be important in mediating proliferative and survival responses in Schwann cells (Levi et al., 1995; Carroll et al., 1997). Schwann cell development involves three main stages of transition that I will now discuss, focusing on myelination in particular.

**Neural crest cell to Schwann cell precursor transition**

**Specification of a glial fate**

Neural crest cells are multipotent stem cells that can give rise to a number of lineages, including neurons, glia and melanocytes (reviewed by Anderson, 1997, Le Douarin et al., 1991). Schwann cells and satellite cells are derived from the glial lineage. Although the mechanisms regulating the specification of a glial fate remain unclear, addition of GGF (a NRG-1 isoform) to an in vitro clonal neural crest culture system results in a single population of glial cells, with no apparent neuronal cell development or death (Shah et al., 1994), suggesting that GGF is able to direct the neural crest stem cells along a glial lineage. However, NRG-1 signalling is not essential for specification of a glial lineage (Meyer and
Birchmeier, 1995). In contrast, the transcription factor Sox-10 (see below) may play a more important role in glial fate determination in vivo since Sox-10 null mice lack both Schwann cells and satellite cells in the peripheral nervous system, which may be due to defects in Notch-1 and NRG-1 signalling (Morrison et al., 2000; Wakamatsu et al., 2000; Britsch et al., 2001). However, overexpression of Sox-10 in neural crest cells in vitro is insufficient to specify a glial fate (Paratore et al., 2001).

**Precursor Schwann cell formation and survival**

Neural crest cells migrate into the embryonic peripheral nerve and become Schwann cell precursors by E14/15 in the rat and about two days earlier in the mouse (Jessen et al., 1994, Dong et al., 1999). Schwann cell precursors grow in vitro in flattened sheets of cells, which are highly motile, and dependent on axon derived signals for survival, whereas Schwann cells grow as single, less motile, bi-polar or tri-polar cells and can be cultured in vitro for prolonged periods in the absence of axon derived signals (Brockes et al., 1979; Jessen et al., 1994; Mathon et al., 2001). The dependency of Schwann cell precursors on axon derived signals may be important in matching Schwann cell numbers to axons during development. The neuronally derived factor responsible for precursor Schwann cell survival and developmental progression has been identified as Neu differentiation factor (NDF), an isoform of NRG-1 (Dong et al., 1995).

**Schwann cell precursor to immature Schwann cell transition**

The precursor to immature Schwann cell transition occurs rapidly in vivo in association with a sharp increase in S100+ cells at E16/17 in the rat (Jessen et al., 1994) and E14/15 in the mouse (Dong et al., 1999). Following this transition, immature Schwann cells sort axons from the large bundles formed by precursors into smaller groups. In vitro immature Schwann cells can be cultured at high density in the absence of neurons or NRG-1, suggesting that the immature cells have developed an autocrine survival loop (Jessen et al., 1994; Cheng et al., 1998; Dong et al., 1999; Meier et al., 1999). The autocrine loop has been shown to involve the activity of neurotrophin-3 (NT-3), insulin-like growth factor (IGF) and platelet derived growth factor BB (PDGF-BB) and is thought to facilitate
Schwann cell survival during Wallerian degeneration in the absence of axon derived signals.

**Immature Schwann cell to (non) myelinating Schwann cell transition**

The transition from immature to mature Schwann cell begins around birth in rodents with the final sorting of axons into either a one to one relationship with a Schwann cell which subsequently differentiates and surround the axon in a myelin sheath, or the many to one relationship of smaller axons with non-myelinating Schwann cells (Mirsky and Jessen, 1996; Fig.1.8). Schwann cell differentiation is accompanied by cell cycle withdrawal (Stewart *et al.*, 1993) and it takes several weeks for the completion of myelination. Immature Schwann cells are capable of differentiating into either myelinating or non-myelinating Schwann cells depending on the associated axon. This process is reversible during Wallerian degeneration in response to nerve injury when Schwann cells dedifferentiate in the absence of contact with axons to the proliferative immature stage and can be easily cultured *in vitro*. Moreover, the dedifferentiated cells retain the ability to re-differentiate along either fate depending on the associated axon (Aguayo *et al.*, 1976b; Weinberg and Spencer, 1976). The axon-derived signals regulating Schwann cell differentiation are still unknown, however, larger axons (>1μm) are usually myelinated, whilst smaller axons are grouped in shallow membrane troughs by non-myelinating Schwann cells.

Non-myelinating Schwann cells have not been studied as extensively as myelinating ones due to the difficulty of distinguishing them from immature Schwann cells, which express many of the same markers (Lobsiger *et al.*, 2002). Immature and non-myelinating Schwann cells can be separated by the absence or presence of GalC or sGalC in the Schwann cell membrane respectively, however, myelinating Schwann cells also express these markers (Jessen *et al.*, 1985). In contrast, myelinating Schwann cells undergo a co-ordinated induction of myelination associated proteins and downregulate expression of the immature Schwann cell markers neural cell adhesion molecule (NCAM), L1, p75-NGFR and glial fibrillary acidic protein (GFAP) (Mirsky and Jessen, 2001).

52
Myelination and the components of the myelin sheath

Myelination is dependent on axon derived signals which result in the upregulation of expression of myelination-associated genes, such as P0, that peak in expression (mRNA) at ~14 days after birth and then fall to a lower maintenance level in the adult PNS (Lemke and Axel, 1985; Lemke and Chao, 1988; Trapp et al., 1988). The timing of myelination appears to be determined by a balance of positive and negative signals regulating gene expression. This has been clearly shown for P0, the main component of compact myelin, which is expressed at low levels in rat neural crest cells and immature Schwann cells and is upregulated during differentiation in myelinating Schwann cells, whilst levels are reduced in non-myelinating cells in response to axon derived signals (Cheng and Mudge, 1996, Lee et al., 1997). The axon-derived signals involved in the induction of myelin gene expression remain unclear. However, in vitro, expression of myelination markers can be induced by the elevation of cAMP levels in the absence of cell division, whereas in the presence of other factors elevated cAMP results in Schwann cell proliferation (Lemke and Brockes, 1984; Lemke and Chao, 1988, Morgan et al., 1991). As a result cAMP has been proposed to form part of the axon-Schwann cell signal involved in stimulating proliferation of immature Schwann cells and myelination by differentiating cells that have withdrawn from the cell cycle, however, the evidence for this remains controversial (Poduslo et al., 1995; Howe and McCarthy, 2000; see results Chapters 3 and 4 for more details).

Other factors that may be involved in regulating myelination include transforming growth factor β1 (TGFβ1), fibroblast growth factor (FGF2), NRG-1 and the neurotrophins brain derived neurotrophic factor (BDNF) and neurotrophin–3 (NT-3) (Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995; Maurel and Salzer, 2000; Chan et al., 2001; Zanazzi et al., 2001). In vitro TGFβ1 and FGF2 are able to induce Schwann cell proliferation (in co-operation with cAMP elevators) and can inhibit myelination, suggesting that they may be involved in preventing premature myelination during development and peripheral nerve regeneration (Davis and Stroobant, 1990; Ridley et al., 1989; Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995; Zanazzi et al., 2001). The idea that downregulation of these factors may be required for myelination to proceed is supported by the observation that TGFβ1 levels decrease during postnatal development as P0 levels rise
GGF is a Schwann cell mitogen that stimulates proliferation by activation of the PI3K pathway (Lemke and Brockes, 1984; Maurel and Salzer, 2000). Inhibition of the PI3K pathway in Dorsal root ganglion (DRG) -Schwann cell co-cultures under myelinating conditions blocks Schwann cell elongation and myelination; however, once myelination has begun inhibition of PI3K activity has no effect on myelination. Thus PI3K activity is required for the initial events of myelination. A positive role for NRG-1 signalling in myelination is also suggested by mice with a conditional knockout of ErbB2 in Schwann cells that have hypomyelinated axons (Garratt et al., 2000b). However, addition of GGF to DRG -Schwann cell co-cultures under myelinating conditions blocks myelination and the Schwann cells appear to be maintained in a pre-myelinating state (Zanazzi et al., 2001). A possible explanation for the discrepancy of these results may be that PI3K signalling by NRG-1 is required to establish Schwann cell-axon interactions prior to myelination, but that reduced NRG-1 signalling is required for myelination and the maintenance of myelination, since GGF is also able to cause demyelination of myelinated co-cultures (Zanazzi et al., 2001). In support of this idea, neuregulin signalling appears to be downregulated in the adult during myelination (Grinspan et al., 1996; Kwon et al., 1997; Sherman et al., 2000b). The effects of NRG-1 signalling on myelination could potentially be mediated by regulation of Id1 and Id3, which antagonise basic helix-loop-helix transcription factors and have been implicated in the prevention of myelin gene expression (Thatikunta et al., 1999). Neurotrophins have also been implicated in regulating myelination (Chan et al., 2001). NT-3 has been found to inhibit myelination of DRG -Schwann cell co-cultures and sciatic nerves in vivo, whereas addition of BDNF promotes myelination. In addition, during development the levels of NT-3 decrease prior to the onset of myelination.

During differentiation the Schwann cell associates with a single axon to be myelinated and ceases proliferation (Scherer, 1997). The Schwann cell membrane then begins to wrap around the axon in a spiralling manner and, with the expression of a characteristic set of myelin proteins and lipids, the myelin sheath is formed. The structure of a myelinating Schwann cell, in association with an axon, is shown in Fig. 1.9. The myelin sheath is composed of areas of compact and non-compact myelin, which have different molecular
Compact myelin is formed by the apposition of membrane wraps around the axon and the exclusion of cytoplasm. The apposition of intracellular surfaces forms major dense lines, whilst the apposition of opposing surfaces forms intraperiod lines. Compact myelin consists of the proteins P0, myelin basic protein (MBP), PMP22 and the lipids GalC/sGalC. Non-compact myelin is found at the paranodes, Schmidt-Lanterman incisures (SLI) and the inner and outer collars of the myelin sheath. Non-compact myelin components include periaxin and the integrins α6β1,α6β4, which interact with the basal lamina directly or indirectly, and connexin 32, myelin associated glycoprotein (MAG) and E-cadherin. The axon–Schwann cell units are surrounded by a continuous basal lamina secreted by the Schwann cells, which is required for myelination to proceed successfully (Fernandez-Valle et al., 1993). At the edge of each Schwann cell-axon unit is an area of naked axon called the node of Ranvier, which is important in signal conductance along the nerves due to the concentration of Sodium–gated ion channels (reviewed by Ellisman et al., 2001). Myelination of axons results in an increase in velocity of impulse conductance.

I shall now discuss the components of the myelin sheath and their importance for successful myelination with reference to mutations associated with peripheral neuropathies and the phenotypes of any naturally occurring or specifically generated mouse models. Fig.1.10 provides an overview of the peripheral neuropathies concerned and summarises the associated mutations. It is important to note that changes in several genes can have similar phenotypic outcomes, raising the possibility of interaction of some of the factors. In addition, mutations in the same gene can have very different effects, leading to the suggestion that the correct gene dosage is required for normal myelination and the maintenance of the myelin sheath (reviewed by Wrabetz et al., 2001). This gene dosage model is supported by research into neuropathies associated with mutations in P0 and PMP22 (see below).
Fig. 1.9. Diagrams of the structural regions and components of myelin.
(A) A myelinating Schwann cell associated with an axon forms regions of compact and non-compact myelin. Areas containing non-compact myelin are the paranodes, the inner collar, outer collar and Schmidt-Lantermann incisures (SLI). The node of Ranvier consists of the naked axon between two Schwann cell membranes and is separated from the endoneurial space by the basal lamina. The components of compact and non-compact myelin are shown in (B) and (C) respectively. Cx32, connexin 32. (D) Extracellular matrix interactions occur between laminin-2 in the basal lamina and the integrin α6β4 or the dystroglycan complex, via DRP2 and periaxin. (Adapted from Scherer 1997, see text for details.)
Compact myelin

PO is a member of the Ig superfamily and consists of a single transmembrane region, a large glycosylated hydrophobic extracellular domain and a smaller intracellular domain (Lemke and Axel, 1985; Lemke et al., 1988). PO is the most abundant myelin sheath protein in the PNS and has been implicated in myelin compaction. This is thought to occur via a combination of homophilic interactions of the extracellular domains across membranes to form intraperiod lines and the interactions of the intracellular domains with the lipid bilayer to form major dense lines (Lemke and Axel, 1985; Filbin et al., 1990, Ding and Brunden, 1994). X-ray crystallography suggests that PO forms tetramers that interact with other PO tetramers in the opposing membrane (Shapiro et al., 1996). PO has also been implicated in mediating adherence junction formation (Menichella et al., 2001) and has been suggested to regulate the expression of some myelination-associated genes (Xu et al., 2000).

Fig.1.10. Diagram to summarise the relationship between mutations in PO, PMP22 and connexin 32 and inherited demyelinating neuropathies.

Charcot-Marie-Tooth disease type 1 (CMT1) is an autosomal dominantly inherited demyelinating neuropathy, characterised by demyelination and remyelination resulting in progressive distal muscle weakness (reviewed by Suter, et al., 1995; Wrabetz, et al., 2001). DSS (Dejerine-Sottas syndrome) patients have a more severe phenotype than those with CMT1. HNPP (Hereditary neuropathy with liability to pressure palsies) is characterised by episodic, multiple focal myelin thickenings (tomaculi), which result in mild demyelination. (Adapted from Scherer, 1997, see main text for details.)
Mutations in \( P0 \) have been detected in CMT1B, DSS and congenital hypomyelination (CH) (Warner et al., 1996), with the differences in phenotype being attributed to the type of mutation and the resulting effect of protein function (see Fig. 1.10.). \( P0 \) null mice have hypomyelinated peripheral nerves and reduced membrane compaction, resembling DSS, whilst \( P0 +/- \) mice have a phenotype similar to CMT1B associated with initial myelination followed by progressive demyelination after a few months (Giese et al., 1992; Martin et al., 1995). These results confirm that \( P0 \) is essential for stable myelination and show that although a single copy of \( P0 \) is sufficient for myelination to occur, two \( P0 \) genes are required for the maintenance of the myelin sheath. Patients with \( P0 \) mutations are normally heterozygotes, but can have severe symptoms similar to \( P0 \) null mice, suggesting that some mutations may have a dominant gain-of-function (Warner et al., 1996). This has also been observed \textit{in vitro} where truncated forms of \( P0 \) have been shown to prevent the adhesion of transfected CHO cells by wild type \( P0 \) (Wong and Filbin, 1996).

PMP22 is a tetraspan membrane protein that forms 2-5% of compact myelin in the PNS (reviewed by Naef and Suter, 1998; Muller, 2000). The importance of PMP22 in the PNS was confirmed by the finding of a 1.5mega base duplication of a region containing the \( PMP22 \) gene in patients with CMT1A (Lupski et al., 1991; Valentijn et al., 1992; Patel et al., 1992; Timmerman et al., 1992; Matsunami et al., 1992), whilst loss of 1 copy of \( PMP22 \) is associated with the milder phenotype of HNPP (Chance et al., 1993). Patients with inherited duplication of \( PMP22 \) from both parents have a more severe demyelinating neuropathy, DSS (Lupski et al., 1991). The use of spontaneous (\textit{Trembler, Trembler-J} mice) and specifically generated animal models has confirmed the causative role for altered PMP22 expression in peripheral neuropathies (Suter et al., 1992, Adlkofer et al., 1997; Adlkofer et al., 1995; Sereda et al., 1996; Magyar 1996; reviewed by Nave, 2001). PMP22 deficient mice undergo hypermyelination followed by severe demyelination, whereas heterozygotes are less severely affected with a comparable phenotype to HNPP (Adlkofer et al., 1997; Adlkofer et al., 1995). Increasing the copy number of \( PMP22 \) in a transgenic rat results in a CMT-like neuropathy associated with peripheral hypomyelination (Sereda et al., 1996). Possible results of altered PMP22 levels include increased PMP22 degradation.
via upregulation of the lysosomal pathway (Notterpek et al., 1997) and impaired trafficking (D’Urso et al., 1998), potentially leading to instability of the myelin sheath.

The functions of PMP22 in compact myelin remain unclear, but PMP22 has recently been suggested to interact with P0 to form complexes that may be involved in stabilising membrane compaction (D’Urso et al., 1999). This finding may explain why mutations in P0 and PMP22 can result in phenotypically similar demyelinating neuropathies. PMP22 overexpression has also been implicated in the regulation of cell proliferation and apoptosis, although opposing results have been obtained using Schwann cells from a transgenic rat overexpressing PMP22 (Zoidl et al., 1995; Fabbretti et al., 1995; Sereda et al., 1996). Thus the relevance of these potential functions in the development of peripheral neuropathies associated with altered levels of PMP22 remains unclear.

Myelin basic protein (MBP) is a small positively charged membrane associated molecule that is expressed in compact myelin in both the PNS (where it forms 10-20% protein by mass) and CNS (Nave, 2001). MBP has been implicated in myelin compaction in the PNS since in the absence of P0, MBP mutation results in the loss of major dense lines (Martin et al., 1995). MBP is expressed following P0 during myelination and thus may play a role in the stabilisation or maintenance of the major dense lines, whereas P0 has been suggested to be important for early myelin compaction (Giese et al., 1992, Martin et al., 1995). GalC (galactocerebroside) and its sulphated derivative sGalC are the major myelin-associated lipids in the PNS and CNS, which have been implicated in maintaining the stability of the myelin sheath and the generation of normal action potentials in the PNS (Coetzee et al., 1996; Dupree et al., 1999). However, GalC and sGalC are also expressed by non-myelinating Schwann cells (Jessen et al., 1985).

Components of non-compact myelin

E-Cadherin is a calcium-dependent adhesion molecule, which is expressed in Schwann cells in Schmidt-Lanterman incisures (SLI) and appears to be involved in forming adherens junctions between the adjacent loops of non-compact myelin at the paranodes (Fannon et al., 1995). Myelin-associated glycoprotein (MAG) is a member of the Ig superfamily of
proteins that is expressed in myelinating glia and may be involved in myelin sheath maintenance (Owens and Bunge, 1991; Li et al., 1994; Montag et al., 1994; Martini and Carenini, 1998). Connexin 32 is a member of the connexin family of four-transmembrane protein that oligomerize to form gap junctions. Connexin 32 is expressed at high levels in Schwann cells and is concentrated in the nodes of Ranvier and SLI where it forms gap junctions and may facilitate the transfer of ions across the layers of the myelin sheath (Bergoffen et al., 1993; Scherer et al., 1995a). Mutations in connexin 32 are associated with the X- linked CMT in humans (Bergoffen et al., 1993). Integrins mediate interactions between the extracellular environment and cytoplasm and have been implicated in peripheral nerve development (reviewed by Previtali et al., 2001). In Schwann cells, the integrins α6β1 and α6β4 interact with laminin-2, the major component of the Schwann cell basal lamina. Examination of transgenic mice with disrupted expression of the β1 subunit specifically in Schwann cells prior to myelination has suggested that β1 is important for neuron sorting and for promyelinating cells to initiate myelination (Feltri et al., 2002).

Periaxin is a myelination -associated protein, which is initially expressed in pro-myelinating Schwann cells of the peripheral nervous system during early myelination, before P0 and MBP, in response to axon derived stimuli (Gillespie et al., 1994; Scherer et al., 1995b). Periaxin exists in two forms: L- periaxin and S-periaxin, which both contain a PDZ protein-binding domain that has been found in proteins interacting with the cytoplasmic tail of plasma membrane proteins (Dytrych et al., 1998). L-periaxin, unlike S-periaxin, is targeted to the membrane of myelinating Schwann cells and is found in non-compact myelin (Gillespie et al., 1994; Dytrych et al., 1998). Periaxin has been implicated in stabilizing the mature myelin sheath since periaxin−/− mice undergo demyelination, despite normal initial myelination (Gillespie et al., 2000). Recently it has been shown that periaxin interacts with dystrophin related protein 2 (DRP2) in a dystroglycan complex that is probably involved in linking the basal lamina to the myelinating Schwann cell cytoskeleton (Sherman et al., 2001). This complex has been proposed to play a role in signalling to regulate myelin sheath thickness and stability. Moreover, mutations in the periaxin gene in humans are associated with an autosomal recessive demyelinating
neuropathy CMT4F and Dejerine Sottas syndrome, confirming the importance of periaxin in the maintenance of the myelin sheath (Boerkoel et al., 2001; Guilbot et al., 2001).

**Transcriptional control of Schwann cell differentiation**

Myelin gene expression is controlled at the mRNA level in response to axon-derived signals (Trapp et al., 1988; Lemke and Chao, 1988). The nature of these signals remain unclear, however, a number of transcription factors have been implicated in Schwann cell development and differentiation and I will now discuss them in some detail (Wegner, 2000; Topilko and Meier, 2001, Fig 1.11).

**Pax-3**

Pax-3 is a member of the Pax family of proteins that are characterised by the presence of a paired domain (PD) (reviewed by Chi and Epstein, 2002). In addition, several Pax proteins, including Pax-3, also contain a homeodomain DNA binding domain, allowing bipartite binding to target DNA sequences. Mutations of the human homologue of Pax-3, HuP2, have been detected in Waardenburg syndrome (WS), and are associated with defects in neural crest derived tissues (Baldwin, 1992; Tassabehji et al., 1992).

The pattern of Pax-3 expression remains to be established conclusively in the Schwann cell lineage, with both biphasic and continuous expression patterns being reported from around E12 in the mouse (Goulding et al., 1991; Kioussi et al., 1995; Blanchard et al., 1996). Pax-3 expression appears to be maintained in non-myelinating Schwann cells, whilst expression decreases prior to myelination in myelinating cells. During Wallerian degeneration, Pax-3 levels transiently increase in dedifferentiating myelinating Schwann cells in association with decreased levels of myelin sheath proteins and are downregulated in myelinating Schwann cells following nerve contact (Kioussi et al., 1995). Pax-3 may be involved in preventing the expression of myelin genes in immature Schwann cells and in non-myelinating Schwann cells, and downregulation of Pax-3 in pro-myelinating cells appears to be required for myelination. Pax-3 activity may be regulated by interaction with other transcription factors, including Sox-10 (Lang et al., 2000).
Fig. 1.11. Summary of the expression patterns and functions of Schwann cell transcription factors during development.
(A) Diagram of the expression patterns of transcription factors implicated in Schwann cell development. (Adapted from Topilko and Meijer 2001.)
(B) Summary of the interactions and targets of the Schwann cell transcription factors Sox-10, Oct-6 and Krox-20 during Schwann cell development. (See text for details.) Dashed lines represent potential interactions. MSE, Myelinating Schwann cell element; ?, unknown target(s).
Sox-10

Sox-10 is a member of the Sox subgroup of HMG (high mobility group) proteins (reviewed by Wegner, 2000; Topilko and Meier, 2001). In Schwann cells, Sox-10 has been shown to interact synergistically with Oct-6 \textit{in vitro} and may modulate the activity of the transcription factors Pax-3 and Krox-20 (Kuhlbrodt et al., 1998; Bondurand \textit{et al.}, 2001). These findings suggest that Sox-10 may function \textit{in vivo} to regulate the activity, and perhaps specificity, of other transcription factors during development. In the developing PNS, Sox-10 expression is first detected in emerging neural crest cells, before being preferentially expressed in neural crest cells that form melanocyte and PNS (Schwann cell; enteric, sensory and sympathetic ganglia) lineages (Kuhlbrodt \textit{et al.}, 1998; Southard-Smith \textit{et al.}, 1998; Britsch \textit{et al.}, 2001). During later stages of development Sox-10 expression appears to be confined to glial cells (Britsch \textit{et al.}, 2001).

The importance of Sox-10 in the development of PNS glia has been demonstrated by \textit{Sox-10/-} mice, which lack both Schwann cells and satellite cells (Britsch \textit{et al.}, 2001). In addition, naturally occurring Sox-10 loss-of-function mutations in heterozygote people (Waardenburg-Hirschsprung syndrome) and mice (\textit{Dominant megacolon} model) are associated with defects in neural crest derived lineages (Herbarth \textit{et al.}, 1998; Pingault \textit{et al.}, 1998; Southard-Smith \textit{et al.}, 1998). Dominant negative mutations of Sox-10 have also been identified in people, resulting in additional defects in peripheral and central nervous system myelination (Inoue \textit{et al.}, 1999). The effects of Sox-10 on glial cell specification may be mediated by regulation of Notch -1 signalling and ErbB3 levels (Britsch \textit{et al.}, 2001). Other potential targets of Sox-10 include the microphthalmia protein (MITF) in the melanocyte lineage (Lee \textit{et al.}, 2000) and, in cooperation with Pax-3, the enteric nervous system transcription factor c-Ret (Lang \textit{et al.}, 2000). Sox-10 has been shown to positively regulate expression of PO \textit{in vitro} and \textit{vivo} (Peirano \textit{et al.}, 2000) and acts synergistically with Krox-20 in the expression of Connexin 32 (Bondurand \textit{et al.}, 2001).

Oct-6

Oct-6/ SCIP/ Tst-1 is a member of a family of nuclear proteins characterised by a highly conserved POU DNA binding domain (reviewed by Topilko and Meier, 2001). Oct-6 is expressed in myelinating glia of both the PNS and CNS (Monuki \textit{et al.}, 1989). In the PNS,
Oct-6 is first detected in Schwann cell precursors and immature Schwann cells, with levels increasing around birth (Blanchard et al., 1996). Oct-6 expression peaks in pro-myelinating Schwann cells and may persist in myelinating Schwann cells for the first two postnatal weeks before being downregulated as the nerve matures (Blanchard et al., 1996; Scherer et al., 1994; Zorick et al., 1996; Arroyo et al., 1998). In non-myelinating Schwann cells Oct-6 has been reported to be expressed transiently (Zorick et al., 1996) or permanently (Blanchard et al., 1996), depending on the study. Following nerve damage Oct-6 is re-expressed when the regenerating axons make contact with Schwann cells (Scherer et al., 1994). However, in contrast to during normal development, Oct-6 levels remain high for over a month following Wallerian degeneration, suggesting that although re-differentiation recapitulates development in many ways the two processes are not completely equivalent (Scherer et al., 1994) Zorick et al., 1996; Arroyo et al., 1998).

Since Oct-6 expression is induced by elevated levels of cAMP and neuronal contact prior to myelin gene expression, Oct-6 was suggested to play a role in regulating myelination in Schwann cells (Monuki et al., 1989, Scherer et al., 1994). However, it was observed that in co-transfection assays Oct-6 repressed the activity of P0 and MBP reporter constructs (He et al., 1991; Monuki et al., 1993; Monuki et al., 1990), suggesting that Oct-6 may instead act to regulate the timing of myelination by repressing the expression of myelin genes. Based on these findings a transgenic mouse model was generated expressing ΔSCIP, under the control of the P0 promoter, to investigate the function of Oct-6 during Schwann cell development in vivo (Weinstein et al., 1995). The ΔSCIP construct contains the functional POU domain of Oct-6 and was found to act as a dominant negative Oct-6 in reporter assays. ΔSCIP mice have hypermyelinated peripheral nerves with increased levels of P0 expression. Moreover, occasional Schwann cells are observed myelinating several axons, suggesting that myelination is occurring prematurely, prior to the segregation of axons into a one-to-one relationship with Schwann cells. Thus, in support of the in vitro data, the ΔSCIP model suggests that Oct-6 represses myelination in vivo.

However, the co-transfection experiments are no longer thought to provide information that is representative of the situation in vivo and recent re-examination of the ΔSCIP model has
raised doubts about the precise function of the \( \triangle \)SCIP construct (Gondre et al., 1998; Wu et al., 2001, in discussion). When \( \triangle \)SCIP mice were crossed with Oct-6\(^{-/-}\) mice the resulting offspring were found to have normal levels of myelin gene expression and protein, suggesting that the \( \triangle \)SCIP construct was acting as a positive regulator of transcription via the POU domain, rather than as a dominant repressor of wild-type Oct-6 function. Thus the observed phenotype in \( \triangle \)SCIP mice is probably due to the increased number of copies of the POU domain present resulting in hypermyelination.

Two potentially more informative and easily interpretable mouse models have been generated by disruption of the Oct-6 gene by the insertion of a \( \beta \)-galactosidase-neomycin fusion gene (Bermingham et al., 1996; Jaegle et al., 1996). The phenotypes of the Oct-6\(^{-/-}\) mice suggest that Oct-6 has a positive effect on the expression of myelin sheath components. During early development Oct-6\(^{-/-}\) peripheral nerves appear normal, but by postnatal day 5 the surviving Oct-6\(^{-/-}\) animals have tremors due to a delay in myelination, which is caused by the transient arrest of Oct-6\(^{-/-}\) Schwann cells at the promyelinating stage for approximately two weeks (Bermingham et al., 1996; Jaegle et al., 1996). However, other as yet unidentified transcription factors appear to be able to compensate for the absence of Oct-6 expression during Schwann cell development and once this delay is overcome myelination appears to proceed as normal. In addition, in one model Oct-6\(^{-/-}\) sciatic nerves show a reduction in expression of myelin sheath components compared to Oct-6\(^{+/+}\) and wild-type animals during the first two weeks following birth (Jaegle et al., 1996) Thus, although Oct-6 is not required for myelination, Oct-6 appears to be involved in promoting the promyelinating to myelinating transition and may positively regulate myelin gene expression.

One potential target of Oct-6 is Krox-20, which is expressed 24-36hrs after Oct-6 and has been implicated in regulating transition through the promyelinating stage (Topilko et al., 1994; Bermingham et al., 1996; Jaegle et al., 1996; Zorick et al., 1996). Krox-20 expression is delayed in Oct-6\(^{+/-}\) cells (Topilko and Meier, 2001). Moreover, the myelinating Schwann cell element (MSE) in the Krox-20 promoter contains several potential Oct-6 binding sites and is activated in an Oct-6 dependent manner, suggesting that
Oct-6 is positively involved in regulating the expression of Krox-20 (Ghislain et al., 2002). Another potential target for Oct-6 regulation is Oct-6 itself (Arroyo et al., 1998; Jaegle and Meijer, 1998). The downregulation of Oct-6 during myelination appears to require functional Oct-6, suggesting that Oct-6 may be involved in downregulating expression of the Oct-6 gene and by implication may also be involved in downregulating other genes as well. Krox-20 has also been implicated in, but is not sufficient for, the downregulation of Oct-6 levels (Jaegle and Meijer, 1998; Zorick, 1999, see below).

**Krox-20 and Krox-24**

Krox-20 (Egr-2) and Krox-24 (Egr1) are members of the early growth response gene family, which are characterised by the presence of a highly conserved DNA binding domain containing three zinc finger motifs (reviewed by Topilko and Meier, 2001). Krox-20 and Krox-24 are transcription factors, which bind identical DNA sequences and have a conserved inhibitory region, the R1 domain. The activity of both factors can be modified by interaction with the co-repressor proteins NAB1 and 2 via the R1 domain, leading to transcriptional repression or activation (Russo et al., 1995, Svaren et al., 1996; Swirnoff et al., 1998; Sevetson et al., 2000). NAB2 is induced by Krox-20 expression in Schwann cells in vitro (Nagarajan et al., 2001) and both NAB 1 and 2 promoters have multiple Krox-20 binding sites (Mechta-Grigoriou et al., 2000). The importance of these interactions has been implied by the discovery of a recessive Krox-20 R1 domain mutation (I1268N) in a patient with a hypomyelinating neuropathy (Warner et al., 1998; Warner et al., 1999).

In the PNS at E10.5 (mouse) Krox-24 is expressed in Schwann cell precursors lining the cranial and spinal nerves, whilst Krox-20 in expressed in a population of glial cells close to the neural tube (Topilko et al., 1997; Topilko et al., 1994; Murphy et al., 1996; Zorick et al., 1996). At E15.5, Krox-20 mRNA is detected in a subpopulation of immature Schwann cells lining the peripheral nerves and Krox-24 expression is downregulated. Krox-20 expression peaks approximately 24hrs after Oct-6 and is maintained in myelinating Schwann cells (Blanchard et al., 1996; Topilko et al., 1997; Zorick et al., 1996). In contrast, in non-myelinating cells Krox-20 expression is downregulated concomitant with Krox-24 re-expression.
Krox-20 expression is dependent on nerve contact (Murphy 1996). During Wallerian degeneration, dedifferentiating Schwann cells rapidly upregulate Krox-24 expression, whilst Krox-20 levels are reduced (Zorick et al., 1996; Topilko et al., 1997). These expression patterns are reversed in myelinating Schwann cells following successful nerve repair as Schwann cells re-differentiate in a similar manner to during normal development. Thus Krox 20 and Krox-24 appear to be expressed in a mutually exclusive manner.

The importance of Krox-20 in regulating myelination in the PNS has been demonstrated by the phenotype of Krox-20 -/- mice, which have impaired Schwann cell differentiation, with cells permanently arrested at the promyelinating stage unable to form a compact myelin sheath (Topilko et al., 1994). Decreased levels of P0, MBP and PMP-22 were also observed. In contrast, Krox-24 -/- mice do not have a reported Schwann cell phenotype (Lee et al., 1996; Topilko et al., 1998). Krox-20 mutations have also been implicated in the peripheral neuropathies Charcot-Marie-Tooth disease type 1 (CMT1) and congenital hypomyelinating neuropathy (CHN) (Warner et al., 1998). These mutations are mainly dominant and affect the DNA binding domain of Krox-20 to abolish Krox-20 function. However, since Krox-20 +/- mice lack PNS defects (Topilko et al., 1994) mutant Krox-20 proteins may act in a dominant negative manner, leading to a significant reduction in expression of myelin genes and peripheral neuropathy. Investigation of the functional capabilities of neuropathy-derived mutants supports this idea (Nagarajan et al., 2001).

Potential Krox-20 targets include Oct-6, NAB 2, the myelin proteins MAG, P0, PMP22, connexin 32 (in synergy with Sox-10), periaxin and MBP, and proteins involved in myelin lipid assembly (Zorick, 1999; Bondurand et al., 2001; Nagarajan et al., 2001) In addition, a Krox-20 target may be involved in cell cycle exit prior to myelination since some Krox-20 -/- Schwann cells do not withdraw from the cell cycle, but continue to proliferate (Zorick, 1999). However, the continued proliferation is associated with increased apoptosis, resulting in a relatively conserved number of Schwann cells in Krox-20 -/--sciatic nerves.
Schwann cell-axon interactions

Schwann cell-axon interactions are required for the survival and differentiation of Schwann cells during development. However, these interactions are reciprocal, with neurons showing dependence on Schwann cells for survival, perineurium formation and development of the nodes of Ranvier, for example (reviewed by Mirsky and Jessen, 2001). Parmantier and colleagues have shown that secretion of Desert hedgehog by Schwann cells is required for formation of the perineurial sheath, which surrounds peripheral nerves and acts as a diffusion barrier (Parmantier et al., 1999). Schwann cells have also been implicated in regulating the clustering of Sodium channels to the nodes of Ranvier during development (Dugandzija-Novakovic et al., 1995). The importance of Schwann cells in supporting neuronal survival has been demonstrated in erbB3-/- mice and the rescued erbB2-/- mice which have an approximately 80% loss of sensory and motor neurons by E18, associated with the absence of Schwann cells (Riethmacher et al., 1997; Woldeyesus et al., 1999). Moreover, neuronal death is also seen in Sox-10-/- mice that do not produce Schwann cell precursors (Britsch et al., 2001). The increase in neuronal death is probably due to the absence of neurotrophins, such as NT-3, which are produced by Schwann during development (Meier et al., 1999). Secretion of neurotrophins by Schwann cells has also been implicated in peripheral nerve repair, which I will now discuss in more detail.

Schwann cell-axon interactions during peripheral nerve degeneration and regeneration

A characteristic series of events, collectively termed Wallerian degeneration, occur in the distal nerve stump following nerve damage (reviewed by Stoll and Muller, 1999; Scherer and Salzer, 2001, Fig.1.12.) Distal to the site of injury the damaged axons degenerate and leak axoplasm. Schwann cells dedifferentiate, re-enter the cell cycle and secrete neurotrophins that support nerve regrowth (Aguayo et al., 1976b; Heumann et al., 1987; LeBlanc and Poduslo, 1990; Zhang et al., 2000). The myelin sheath is degraded and phagocytosed by Schwann cells expressing MAC2, a galactose specific lectin that binds sGalC and GaC in myelin debris, and then by infiltrating macrophages (Stoll et al., 1989; Reichert et al., 1994). The Schwann cells proliferate within the basal lamina to form the
band of Büngner, which consists of a linear array of Schwann cells that provide a conduit for nerve regrowth. The damaged nerves produce nerve sprouts proximal to the site of injury, which grow along the band of Büngner to re-innervate the target. As the regenerating nerve grows towards its target Schwann cells re-associate with the axons and remyelination occurs in a similar manner to during development (LeBlanc and Poduslo, 1990). The regenerative capability of Schwann cells following nerve damage is thought to be important for successful nerve repair (see below). Importantly, both nerve crush and axotomy activate Wallerian degeneration, however, loss of a continuous basal lamina following nerve transection often impedes regeneration.

**Regulation of Schwann behaviour: dedifferentiation, demyelination and re-differentiation**

Schwann cell differentiation is regulated and maintained by nerve signals (Aguayo et al., 1976b; Lemke and Chao, 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990). Following injury induced loss of nerve contact Schwann cells dedifferentiate, downregulating expression of a number of myelin related genes including P0, MBP, PMP22, connexin 32 and periaxin, whilst upregulating expression of genes associated with the immature and non-myelinating phenotype such as p75-NGFR, N-CAM and L1 (Taniuchi et al., 1986; Lemke and Chao, 1988; Trapp et al., 1988; LeBlanc and Poduslo, 1990; Scherer et al., 1995a; Scherer et al., 1995b). The transcription factors Pax-3 and Krox-24 are also induced, whilst Krox-20 levels decrease (Kioussi et al., 1995; Topilko et al., 1997). Concomitant with these changes Schwann cells distal to the site of injury re-enter the cell cycle and proliferate (Aguayo et al., 1976b; Clemence et al., 1989). Following successful re-innervation the Schwann cells re-associate with axons and re-differentiate in a similar manner to during development, although regeneration does not completely recapitulate development (LeBlanc and Poduslo, 1990; Scherer et al., 1994; Scherer et al., 1995a; Scherer et al., 1995b; Topilko et al., 1997).
Fig. 1. Diagram of the main events that occur during Wallerian degeneration and axonal regeneration
(a) An uninjured myelinating axon. Following nerve injury the basal lamina surrounding the myelinating Schwann cell-axon unit is left intact. (b) A few days after injury the axons and myelin sheath degenerate distal to the site of injury. Macrophages invade the basal lamina and phagocytose myelin debris. The Schwann cells dedifferentiate and proliferate. A growth cone forms proximal to the site of injury and proceeds to regenerate along the bands of Bungner. (c) A few weeks after injury the axon has regenerated further down the bands of Bungner and the more proximal portion has been re-ensheathed and myelinated by Schwann cells. (Adapted from Scherer and Salzer, 2001)

The signals responsible for Schwann cell de-differentiation have yet to be determined, however a number of factors have been implicated in regulating Schwann cell-axon interactions during development and following nerve injury, including TGFβ, NRG-1 and FGF2 (Morgan et al., 1994; Einheber et al., 1995, Carroll et al., 1997; Zanazzi et al., 2001). TGFβ1-3 are potent Schwann cell mitogens, which are expressed at low levels in Schwann cells during post-natal development (Ridley et al., 1989; Scherer et al., 1993). However, following nerve injury TGFβ1 levels increase in the distal stump and remain high until Schwann cell-axon contact is resumed. In vitro TGFβ1 is able to block the induction of P0 and myelination by Schwann cells in response to elevated cAMP levels or axon derived signals respectively (Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995). Moreover, in vitro TGFβ has been found recently to reduce the ‘basal’ RNA levels and attenuate the induction by elevated cAMP of a number of myelination associated proteins including P0, MBP, PMP22, Krox-20 and, in contrast to Einheber et al., Oct-6
(Einheber et al., 1995; Awatramani et al., 2002). Thus TGFβ1 may be involved in preventing premature myelination during development and peripheral nerve regeneration and may stimulate Schwann cell proliferation following nerve damage. However, since TGFβ1 is unable to cause demyelination it is unlikely that this factor is involved in mediating the initial events of Wallerian degeneration (Zanazzi et al., 2001). In addition, the majority of TGFβ1 produced in vitro by Schwann cells, neurons or co-cultures is in the latent form and the source of active TGFβ1 in the peripheral nerve following damage is unclear (Einheber et al., 1995).

FGF2 levels also increase in the sciatic nerve following damage (Meisinger and Grothe, 1997). In vitro FGF2 is a Schwann cell mitogen that prevents the expression of P0 by cAMP elevation and induces Schwann cell proliferation (Davis and Stroobant, 1990; Morgan et al., 1994; Cheng and Mudge, 1996). Similar to TGFβ1, FGF2 addition blocks myelination, but does not cause demyelination of myelinated Schwann cell–axon co-cultures (Zanazzi et al., 2001).

In the peripheral nerve, expression of the NRG-1 isoform GGF and receptors ErbB2 and 3 are elevated during Wallerian degeneration concomitant with the onset of Schwann cell proliferation (Carroll et al., 1997). In Schwann cells distal to the nerve injury ErbB2 receptor phosphorylation (Y1248) has been reported and may result in activation of the Ras/Raf/MAPK pathway, stimulating proliferation (Kwon et al., 1997). In addition, GGF stimulates the production of neurotrophins by Schwann cells and has been shown to increase Schwann cell motility, which may facilitate Schwann cell migration during nerve repair (Mahanthappa et al., 1996). Interestingly, addition of GGF to myelinated DRG-Schwann cell co-cultures results in demyelination associated with Schwann cell dedifferentiation to the promyelinating stage, suggesting that upregulation of GGF levels following nerve injury may cause demyelination and Schwann cell dedifferentiation (Zanazzi et al., 2001). GGF may also be involved in regulating the timing of myelination during development and remyelination following nerve damage (Zanazzi et al., 2001). In contrast to these results Garratt and colleagues has shown that NRG-1 signalling is essential for myelination using a conditional ErbB2 knockout in Schwann cells (Garratt et al.,
Moreover, Maurel and Salzer find that the PI3K pathway, a NRG-1 activated pathway, is required for the initial events of myelination (Maurel and Salzer, 2000). Thus NRG-1 signalling may be required for the early establishment of Schwann cell-axon interactions, however, at later stages of development or nerve regeneration NRG-1 signalling may be downregulated to allow myelination to proceed (see myelination section as well).

The importance of Schwann cells in peripheral nerve regeneration
The behaviour of Schwann cells during Wallerian degeneration is thought to be important for successful nerve repair. Proliferating Schwann cells produce a variety of neurotrophins including nerve growth factor (NGF), NT-3, glial derived neurotrophic factor (GDNF) and leukaemia inhibitory factor (LIF), which are though to support neuronal survival and regrowth (reviewed by Terenghi, 1999). Both NGF and BDNF are upregulated following nerve damage and their levels decrease during nerve regeneration, (Taniuchi et al., 1986; Heumann et al., 1987; Zhang et al., 2000). Schwann cell proliferation however, is not essential for nerve regeneration, since in cyclin D1 -/- mice re-innervation occurs in the absence of proliferation (Kim et al., 2000). In addition, this model suggests that Schwann cell proliferation during Wallerian degeneration uses a different pathway to during development and is cyclin D1 dependent. Peripheral nerve regeneration can occur in the absence of Schwann cells if the basal lamina remains intact, although the rate of regeneration is reduced (Sketelj et al., 1989). However, the accuracy of re-innervation is dependent, at least in part, on the Schwann cell generated bands of Büngner which form within an intact basal lamina and act as a conduit for nerve regrowth (Scherer and Salzer, 2001).

During Wallerian degeneration denuverated Schwann cells have also been implicated in the increased expression of ECM molecules in the basal lamina, including laminin, fibronectin and F-spondin, which provide a suitable environment for nerve regrowth (Lefcort et al., 1992; Burstyn-Cohen et al., 1998; Agius and Cochard, 1998). Interactions between the ECM and Schwann cells or axons are mediated by a number of cell adhesion molecules (CAM) from the integrin, cadherin and Ig-like CAM families (e.g. L1, NCAM). Both β1
containing integrins, which bind laminin-2 in the ECM, and α4 containing integrins, which bind fibronectin, have been implicated in facilitating neurite outgrowth (Toyota et al., 1990; Agius and Cochard, 1998; Vogelezang et al., 2001). Fibronectin levels are upregulated during Wallerian degeneration, although the pattern of α4 integrin expression on the damaged nerves is unaltered (Lefcort et al., 1992; Mathews and Ffrench-Constant, 1995; Vogelezang et al., 2001). In addition, during Wallerian degeneration the pattern of fibronectin splicing reverts to the embryonic forms, which may facilitate nerve growth during development and nerve regeneration (Mathews and Ffrench-Constant, 1995; Vogelezang et al., 2001; Vogelezang et al., 1999). However, during nerve re-regrowth fibronectin splicing does not revert to the adult pattern. The ECM can also be modified following nerve damage by the incorporation of factors that are normally separated from nerves by an intact perineurium and vasculature. Recent work by Akassoglou and colleagues has shown that fibrinogen from the blood can be deposited in the ECM as fibrin and that clearance of this molecule from the ECM correlates with remyelination (Akassoglou et al., 2002).
Chapter Two: Materials and Methods

Tissue culture dishes were obtained from Nunc, culture medium from Gibco BRL, plastic ware from Falcon or Sterilin and reagents from Sigma, unless otherwise specified. All kits were used according to the manufacturers instructions and solutions made up with MilliQ deionised water unless specified. $\alpha^{32}$P dCTP (3000Ci/mmole, Amersham) was used in all applications involving radioactivity, except DNA synthesis assays.

2.1. Cell culture

Primary Schwann cells

Primary Schwann cells were extracted from postnatal day 7 Sprague Dawley rat sciatic nerves and purified by sequential immunopanning, such that the resulting cultures were greater than 99.9% pure by immunofluorescence for antigenic markers (Cheng et al., 1995). Cells were expanded at 37°C (10% CO$_2$) on poly-D-lysine (2.4µg/ml) coated dishes using DMEM minus phenol red with 1000mg/ml glucose, supplemented with 3% charcoal stripped FCS (Autogen Bioclear), 4mM glutamine (ICN), kanomycin (100µg/ml), gentamycin (2µg/ml), 1µM forskolin (Calbiochem), and GGF (R and D systems).

NSARaf-1:ER cells

Primary Schwann cells were infected by co-cultivation at a 1:2 ratio with a mitomycin C pre-treated producer line expressing Arf-1:ER containing retrovirus (Lloyd et al., 1997). After a few days the cultures were transferred to selective medium containing 400µg/ml G418 and the resulting drug-resistant colonies were pooled and expanded. The Raf-1:ER was activated by the addition of 100nm tamoxifen (Tmx) in ethanol.

GFP-NSARaf-1:ER cells

NSARaf-1:ER cells were serially infected with a GFP-expressing retrovirus (pBabe-CME-EGFP) in which the puromycin resistance gene had been replaced by the coding sequence of EGFP (a gift from the Raff lab) making selection of positive cells impossible, but ensuring that the infected cells expressed high levels of EGFP under the control of the CMV promoter.
Transient viral production was obtained using phoenix cells (a producer cell line) transfected with pBabe-CME-EGFP using Lipofectamine Plus (Gibco BRL). 3x10^6 phoenix cells were seeded per 90mm dish and left to grow overnight. 5µg of plasmid was mixed with 500µl of serum free medium followed by 17.5µl of PLUS reagent (solution A), and left at room temperature for 15 minutes. 25µl of LipofectAMINE was mixed with 500µl of serum free medium in a separate tube and added to solution A for a further 15 minutes. The cells were changed to 4mls of serum free medium and the DNA/lipid mix was added gently. After 4hrs incubation under normal growth conditions (37°C, 10% CO_2) the medium was replaced with 10% FCS containing growth medium (see below).

After 24 hours the medium was replaced with 5mls of fresh medium to concentrate the virus overnight. The following morning the medium was removed and replaced with another 5mls of medium. The removed medium was treated with 8µg/ml polybrene and passed through a 0.45µM filter unit (Gelman Sciences) to remove any contaminating phoenix cells. The viral supernatant was added to actively dividing NSARaf-1:ER cells, which had been seeded the day before at a density of 7x10^5 per 90mm dish. After 3-4hrs the cells were fed into normal 3% FCS medium for a 2hr recovery period, before a second infection was carried out. This procedure was carried out on 3 separate occasions to obtain cells that were about 80% GFP positive. The selection stage was omitted with this construct due to the absence of a selectable marker.

**NIH 3T3 cells, phoenix cells and perineural fibroblasts**

NIH 3T3 cells and phoenix cells were cultured in a humidified incubator at 37°C (10% CO_2) in phenol red free DMEM supplemented with 10%FCS (Autogen Bioclear), 4mM glutamine (ICN), 100µg/ml kanomycin (Gibco BRL) and 2µg/ml gentamycin (Gibco BRL). NIH 3T3 cells were used to test RafTR LXSN3 function and were infected with construct containing virus produced by the phoenix packaging cell line, in the manner described above for the generation of EGFP-NSARaf-1:ER cells. Infected cells were selected in medium containing 400µg/ml G418. NIH3T3 cells were also used to test the pRafTR IRES-EGFP construct. Perineural fibroblasts were a kind gift from N.Mathon and were cultured under the same conditions as NIH3T3 cells.
**Dorsal root ganglia**

Dorsal root ganglia (DRG) were extracted from postnatal day 1 or 2 Sprague Dawley rats and stored in cold (4°C) L-15 medium before plating on poly-D-lysine (2.4μg/ml) and laminin (50μg/ml, Sigma) coated coverslips in 160μl of defined medium supplemented with 50ng/ml NGF (Alomone labs). The defined medium consisted of 1:1 F-12: phenol red free DMEM with glucose, supplemented with 100μg/ml BSA (Gibco BRL), 60ng/ml progesterone, 16μg/ml putrescine, 50ng/ml thyroxine, 50ng/ml triiodothyrine, 40ng/ml selenium, 10μg/ml insulin (Gibco BRL) and 100μg/ml transferrin. Cultures were incubated overnight at 37°C with 5% CO₂, before the addition of 10⁻⁷M cytosine β-D-arabinofuranoside, a DNA synthesis inhibitor, for 2 days in a final volume of 400μl. A second pulse of inhibitor was added after a recovery period of 2 days, to ensure that all endogenous Schwann cells were killed. Cultures were allowed to grow for around a week before use.

**2.2 Tissue culture assays**

**DRG and Schwann cell or fibroblast co-cultures**

NSARaf-1:ER cells were either treated with 100nM Tmx for 24hrs prior to addition to DRG cultures, or allowed to associate with axons before the addition of Tmx for 24-48hrs. In both cases cells were trypsinised and resuspended in 3% serum containing growth medium to inhibit trypsin activity. The cells were then centrifuged at 1000rpm and resuspended in the DRG defined medium containing NGF. The number of cells present was determined using a coulter counter (Beckman Coulter) and 1.5x10⁴ cells / coverslip were prepared in a volume of 500μl defined medium plus NGF, supplemented with 1/5 Schwann cell conditioned defined medium to provide additional survival factors. The cells were added to the edges of the axon network (where the network is sparser) and at low density to allow accurate observation of their interactions with the axons. Optimal Schwann cell density was determined by titration. NSARaf-1:ER cells that had already been treated with Tmx, were plated in the presence of fresh Tmx for the duration of the experiment. Control experiments were carried out using perineural fibroblast- DRG co-cultures. Co-cultures were fixed at 30 minutes or 24-48hrs after the addition of Schwann cells or fibroblast.
**Time-lapse microscopy**

DRG-Schwann cell co-cultures were maintained in slide bottom flasks to allow maintenance of the CO₂ balance. The inverted time-lapse microscope (Zeiss Axioscope) has a 37°C, 10% CO₂ chamber and is connected to a digital camera system running Open Lab software. The co-cultures were placed in the heated chamber either within 15 minutes of adding the Schwann cells or after the cells had been allowed to associate for 2 days. A frame was taken every 400 seconds and the images were compacted after 50 frames. The data was analysed for the speed of cell movement using Openlab software and converted to a Quicktime movie using a Sorenson video compressor, with a regular time interval of 0.09 seconds between frames.

**Differentiation assays**

Primary Schwann and NSΔRaf-1:ER cells were cultured to confluency, washed three times in serum free defined medium then left in serum free defined medium for the duration of the experiment. The defined medium consisted of phenol red free DMEM with glucose, supplemented with 100μg/ml BSA (Gibco BRL), 60ng/ml progesterone, 16μg/ml putrescine, 50ng/ml thyroxine 50ng/ml triiodothyrine, 40ng/ml selenium, 10μg/ml insulin (Gibco BRL) and 100μg/ml transferring. Cells were treated with 1mM dibutyryl cAMP to induce differentiation as required.

**MEK inhibitor assays**

U0126 (Promega) was made up to a stock concentration of 10mM in DMSO and the dose required to inhibit MAPK activity over a period of 2 days was determined. NSΔRaf-1:ER cells were treated with varying concentrations of inhibitor 15 minutes before the addition of Tmx, which strongly activates the MAPK pathway in these cells. The cells were fed with fresh inhibitor-containing medium after 24hrs and harvested for analysis by Western blotting after another 24hrs. The lowest dose (30μM), which completely inhibited ERK1/2 phosphorylation, was subsequently used, unless stated otherwise.
DNA synthesis assay
Schwann cells were plated in 6-well dishes and treated in triplicate with Tmx or control solvent for 24hrs. 0.5μCi/ml ³H thymidine (Amersham) was added and the cells were lysed with 1% SDS after 6hrs. 3 ml of ice-cold 15% TCA was added to the cell lysates on ice and incubated for more than 5 minutes after thorough mixing. The lysates were transferred to filter paper using a vacuum pump and washed with 20mls of ice-cold 5% TCA. The filters were washed with absolute ethanol, allowed to dry and transferred to scintillation vials for counting on a liquid scintillation counter (Packard).

UV treatment of cells
Medium was removed from the cells and retained. The cells were exposed to 50μJ/cm² x100 (50J/m²) UV on a UV Stratalinker (Stratagene), the medium replaced and the dishes returned to the incubator for the required time.

Transfection of DNA into Schwann cells
60mm dishes of primary Schwann cells were seeded at a density of 1.8x10⁵ in 5mls of Schwann cell medium. Cells were allowed to reach confluency and transferred to defined medium with/without 1mM dibutyryl cAMP (as detailed for the differentiation assays) for 2 days, to allow the upregulation of P0 expression prior to transfection. 7μl of FuGene 6 transfection reagent (Roche) was added to 230μl of DMEM, and left for 5 minutes at room temperature. 2.5μg of DNA in total (2.0μg of pRafTR IRES-EGFP or pPG6RafTR IRES-EGFP DNA with 0.5μg PCDNA3.1 lacZ, or 2.5μg PCDNA3.1 vector control) was added to the reaction and incubated for 15 minutes at room temperature. The reaction was then added directly to dishes containing 3mls of the defined culture medium. The medium was changed after 4hrs. After days EGFP positive cells were counted using an inverted fluorescence microscope (Leica). The dishes were then fixed in 4% paraformaldehyde for 10 minutes and stained for β-galactosidase activity (see below). The number of β-galactosidase positive cells was used to equalise the transfection efficiency across the dishes.
Determination of the stability of p21\textsuperscript{Cip1} protein using cyclohexamide

NSA Raf-1:ER cells were treated with control solvents or Tmx for 30hrs before addition of 20μg/ml cyclohexamide (a protein synthesis inhibitor). The cells were collected at the stated times and analysed by Western blotting with an anti-p21\textsuperscript{Cip1} antibody. The results were quantified using a densitometer and Quantity One software (both Biorad) and equalised to control time (t) = 0.

2.3 RNA/DNA manipulation.

Generation of the pRafTR IRES- EGFP transgenic construct

PCR cloning was used to amplify the RafTR from pRafTR LXSP3. The RafTR B and D primers were designed with BamHI adaptors at the 5’ ends to facilitate subsequent cloning steps. (See Table 2.1. for the primer sequence and reaction conditions and Figs.5.2 and 5.3. for schematic diagrams of the cloning procedure.) The PCR product was digested with BamHI to generate suitable ends for ligation into BamHI and calf intestinal phosphatase (CIP) treated pLXSN3 and pIRES2-EGFP vectors (Clontech). (Prior to CIP treatment, endocuclease reactions were heat inactivated at 65°C for 10 minutes, then 1μl of CIP was added and the reaction incubated at 37°C for another 30 minutes to dephosphorylate the vector ends.) Sequencing of pRafTR LXSN3 was carried out by Oswel (Southampton). The potential pRafTR IRES-EGFP plasmids were screened for the presence of a 2.2kb insert using BamHI digests and PCR with the RafTR C and Raf 25 primers. The correct orientation of RafTR with respect to the IRES-EGFP was determined by PCR using the Raf 25 and EGFP AS primers. The orientation was also confirmed using Raf13 and EGFP primers, which detect RafTR fragments in the wrong orientation in the pIRES2 EGFP vector.
<table>
<thead>
<tr>
<th>Reaction (10μ M Primers)</th>
<th>Primer sequence</th>
<th>Annealing temp. °C</th>
<th>Cycle(s) used</th>
<th>Use</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Krox 24 S Krox 24A S</td>
<td>GAGATGATGCTGCTGAG GTAAGGTGGTCACTACGACTG CAAC</td>
<td>52</td>
<td>25 and 27</td>
<td>SQ-RT-PCR^</td>
<td>1kb</td>
</tr>
<tr>
<td>Krox 20 ~Krox 20 S</td>
<td>ATAGCCCGGCTTGGGG TCCGATACCTGCCTGC</td>
<td>43</td>
<td>24 and 27</td>
<td>SQ-RT-PCR^</td>
<td>450bp</td>
</tr>
<tr>
<td>GAPDH S GAPDH AS</td>
<td>AAAAGGGTCATCATCTCCGC GGATGCAGGGGATGTTCT</td>
<td>58</td>
<td>30</td>
<td>To confirm cDNA synthesis SQ-RT-PCR^</td>
<td>350bp</td>
</tr>
<tr>
<td>p21^Cip1^ S p21^Cip1^ AS</td>
<td>AGTATGCCGCTGCTGCTGTTCCG ACACCAGAGTGCAAGACAGCG</td>
<td>52 hot start</td>
<td>22 and 24</td>
<td>SQ-RT-PCR^</td>
<td>350bp</td>
</tr>
<tr>
<td>+Peraixin S +Peraixin AS</td>
<td>CCTGAATTCCACCTTTCTCCTCC ACGTCACCAGTGAGTGAGCCACGC GGTC</td>
<td>56 - 45secs</td>
<td>24 and 27</td>
<td>SQ-RT-PCR^</td>
<td>500bp</td>
</tr>
<tr>
<td>MBP S MBP AS</td>
<td>ATGTGATGGCATCACAGAAGAGAC GTCTGCTCTAACTAGCTATTGG</td>
<td>47 hot start</td>
<td>22 and 24</td>
<td>SQ-RT-PCR^</td>
<td>500bp</td>
</tr>
<tr>
<td>*PO S *PO AS</td>
<td>TGGTGCTGCTGTTGCTTCC TTGGTGCTCTGGCTGTTGGTCC</td>
<td>49 hot start</td>
<td>20 and 22</td>
<td>SQ-RT-PCR^</td>
<td>300bp</td>
</tr>
<tr>
<td>*PMP22 S *PMP22 AS</td>
<td>CCCAACTCCCAGCCACCA TGCTCAGTCTGTAGTGAGGC TGCC</td>
<td>52</td>
<td>20 and 22</td>
<td>SQ-RT-PCR^</td>
<td>500bp</td>
</tr>
<tr>
<td>*SCIP S *SCIP AS</td>
<td>ATTCCTCCGCGAATGAGCAGAAGAGAGGAGGAG TCCCTGGAGAGAGAGGGGCGAGAATA AAGTGAC</td>
<td>62 hot start</td>
<td>22 and 24</td>
<td>SQ-RT-PCR^</td>
<td>100bp</td>
</tr>
<tr>
<td>P19^REF^</td>
<td>GCCACTGCTGGAGAGTTCGGC GTGCAGTACTACCAGAGTG</td>
<td>50</td>
<td>24 and 26</td>
<td>SQ-RT-PCR^</td>
<td>380bp</td>
</tr>
<tr>
<td>RAT p 19 5' P16 AS</td>
<td>CTCCGAGAGGAGGCGAACCCTCG GTGCAGTACTACCAGAGTG</td>
<td>50</td>
<td>24 and 26</td>
<td>SQ-RT-PCR^</td>
<td>350bp</td>
</tr>
<tr>
<td>Reaction (10 μM Primers)</td>
<td>Primer sequence</td>
<td>Annealing temp. °C</td>
<td>Cycle(s) used</td>
<td>Use</td>
<td>Product size</td>
</tr>
<tr>
<td>--------------------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>--------------</td>
<td>-----</td>
<td>-------------</td>
</tr>
<tr>
<td>RafTR B, RafTR D**</td>
<td>CACAGGATCCCGATCCCTCCCTTTATCCAG TGTGGGATCCCTAACTGACACACACACATTC CC</td>
<td>52, 4 minutes elongation</td>
<td>15 ***</td>
<td>Cloning of RafTR from LXSP3 vector</td>
<td>2.2kb</td>
</tr>
<tr>
<td>RafTR S, RafTR AS</td>
<td>GCAATGAAGAGGCTGGTAGCTG GAACCAGCTCCCTATCTGCTAGG</td>
<td>55 hot start</td>
<td>30, 20 for SQ-RT-PCR ^)</td>
<td>Screening for presence of transgenic construct</td>
<td>475bp</td>
</tr>
<tr>
<td>Raf 25 EGFP AS (correct orientation)</td>
<td>CTGCTCCAACACTCTCTACCG GTTCACCTTGATGCCGTCTTC</td>
<td>48, 4 minutes elongation</td>
<td>25</td>
<td>Confirming orientation of RafTR with respect to IRES-EGFP vector</td>
<td>2.3kb</td>
</tr>
<tr>
<td>Raf 13 EGFP AS (incorrect orientation)</td>
<td>AGCTTTAATCTCTCTG TCCACG GTTCACCTTGATGCCGTCTTC</td>
<td>48, 4 minutes elongation</td>
<td>25</td>
<td>Presence of RafTR in pPG6 vector</td>
<td>1.2Kb</td>
</tr>
<tr>
<td>Raf 25 RAFTR C</td>
<td>CTGCTCCAACACTCTCTACCG AGCCGGATCCAGATCTGACGAATTC</td>
<td>48</td>
<td>25</td>
<td>Confirming orientation of RafTR IRES-EGFP with respect to pPG6</td>
<td>1.2Kb</td>
</tr>
<tr>
<td>T7, Raf13 (correct orientation)</td>
<td>AATACGACTCATATAG AGCTTGATCTCTCTG TCCACG</td>
<td>48</td>
<td>20</td>
<td>Presence of RafTR in pPG6 vector</td>
<td>1.2Kb</td>
</tr>
<tr>
<td>T3, Raf13 (incorrect orientation)</td>
<td>AGCTTTAATCTCTCTG TCCACG ATTAACCCCTACTAAAG</td>
<td>48</td>
<td>20</td>
<td>Confirming orientation of RafTR IRES-EGFP with respect to pPG6</td>
<td>1.4Kb</td>
</tr>
</tbody>
</table>

- * Primer sequence obtained from Blanchard et al., 1996, + a kind gift from Dr. D. Parkinson.
- ^ Semi-quantitative RT-PCR (SQ-RT-PCR)
- ** BamHI adaptors are in bold, ***Pfu polymerase was used in this reaction

Table 2.1. Primer sequences and reaction conditions used for PCR and RT-PCR/ SQ-RT-PCR.

Primers are listed in the order sense, antisense. (See text for more details of primer usage.)
pRafTR IRES-EGFP was digested with Sac II and Not I and the reaction inactivated by heating at 65°C for 10 minutes, prior to treatment with Klenow fragment to blunt and fill in the relevant DNA overhangs. The pRafTR IRES EGFP DNA fragment was diluted in 1x reaction buffer to a volume of 50 μl with 1μl of Klenow, in the absence of dNTPs, and incubated at for 15 minutes at 37°C to blunt the 3' Sac II overhang. 1μl 10mM dNTPs was then added and the reaction incubated at 37°C for 30 minutes to fill in the 5' Not I overhang. The resulting fragment was gel purified and extracted using the Qiaex II kit (Qiagen, see below).

The pPG6 vector (a gift from Greg Lemke, Salk Institute, CA) was digested with Asp718, an isoschizomer of Kpn I. A Sac II adaptor oligonucleotide was designed to be complementary to the ends generated by Asp718 digestion. The oligonucleotide (GTACACCGCGGT, Sac II site in bold) was heated at 70°C for 10 minutes to denature the DNA and then allowed to cool to facilitate binding of complementary sequences to form an oligonucleotide dimer. The Sac II oligonucleotide dimer was ligated into the Asp718 digested pPG6 vector and the products of this reaction were then digested with Asp718, to linearise resealed empty vectors, before bacterial transformation. Isolated plasmid DNA was screened using Sac II restriction endonuclease treatment for the excision of a 2.3kb band. The new pPG6-Sac II adaptor plasmid was digested with EcoRV and treated with CIP, prior to ligation of the RafTR IRES-EGFP blunt ended fragment. The presence of the RafTR in plasmid DNA was determined by PCR (as above) and Sac II digest that yielded a 5.8kb fragment. The orientation of RafTR IRES-EGFP with respect to the pPG6 vector was confirmed by PCR using T7 /Raf13 primers (correct orientation), and T3 /Raf 13 primers (incorrect orientation).

**Ligation of DNA fragments into vectors**

DNA fragments were obtained by restriction digests of plasmids or PCR products and purified to remove contaminants using the Quiex II kit (Qiagen) as described below. A sample of insert and vector DNA was separated on an agarose gel and the ratios of molecules in each sample estimated. The ligation reaction was set up at a number of ratios of insert to vector and either left at room temperature overnight (blunt ends) or incubated at
16°C overnight (insert with overhanging DNA ends). The reaction consisted of 1x T4 ligase buffer, 3U/μl of T4 DNA polymerase, DNA (vector and insert) and water to a final volume of 10μl. Control ligations with vector or insert alone were also carried out. The reactions were transformed into competent bacteria (Nova blue singles, Novagen) to allow amplification and extraction of plasmid DNA, followed by screening for successful ligations.

**Restriction enzyme digests**

All buffers and restriction enzymes were obtained from Promega. A total volume of 30μl was normally used. The reaction mixture consisted of:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>3μl of miniprep DNA (or the required number of μg)</td>
</tr>
<tr>
<td>Buffer (1x)</td>
<td>3μl</td>
</tr>
<tr>
<td>Bovine serum albumin (100x)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>Restriction enzyme(s)</td>
<td>0.5μl each (normally 5U) or more as required.</td>
</tr>
<tr>
<td>RNase 10mg/ml</td>
<td>0.5μl</td>
</tr>
<tr>
<td>Water</td>
<td>to 30μl</td>
</tr>
</tbody>
</table>

The reactions were incubated at 37°C for 1-2 hours before visualising on agarose (Anachem) gels, using 1X TAE running buffer (40mM Tris acetate, 2mM EDTA pH8), and/or purification for subsequent cloning reactions. Purification was carried out using Quiaxe II kit (Qiagen). Briefly a slice of gel containing the DNA of interest was melted and mixed with a silica matrix under high salt conditions to bind DNA to the matrix. The DNA was washed to remove the non-nucleic acid impurities and excess salts and eluted under low salt conditions in water or TE.

**Bacterial Transformation**

10μl of ligation mixture (or 20ng of purified plasmid) was added to 100ul of competent bacteria (NovaBlue singles, Novagen, K-12 strain recA and endA mutant) and incubated on ice for 45 minutes, prior to 2 minutes heat-shock at 42°C. After less than 5 minutes on ice 1ml of L- broth without antibiotics (10g tryptone, 5g yeast extract, 5g NaCl, 1ml IN NaOH,
to final volume of 1 litre in water) was added to each reaction and incubated at 37°C for 45 minutes. The bacterial mixture was subsequently collected by centrifugation at 6000rpm for 2 minutes in a microfuge (Sigma) and about three quarters of the supernatant removed. The bacteria were resuspended in the remaining L-broth and spread over agar plates containing the relevant antibiotic selection. Plates were incubated at 37°C overnight and the resulting single colonies were subsequently used to inoculate L-broth (with antibiotics) to obtain cultures for plasmid extraction. Plates were made by melting L-broth agar (10g tryptone, 5g yeast extract, 5g NaCl, 1ml IN NaOH, 15g agar or agarose, to final volume of 1 litre in water) and once cooled sufficiently, adding the correct antibiotic selection prior to pouring. Ampicillin was used at a final concentration of 0.1mg/ml. Kanamycin was used at a final concentration of 30μg/ml to select for pIRES-EGFP and pRafTR IRES-EGFP plasmids.

**Plasmid DNA extraction**

Midi- and maxi-preps were carried out using QIAfilter kits (Qiagen). Small amounts (mini-preps) of DNA were obtained from 2ml bacterial cultures inoculated as described above and grown overnight at 37°C. Cultures were collected by centrifugation at 6000rpm for 3 minutes and the supernatant removed. The bacterial pellets were resuspended in 100μl solution 1 (50mM glucose, 25mM Tris-Cl pH8, 10mM EDTA pH8), prior to the addition of 200μl of solution 2 (0.2M NaOH, 1% SDS) and incubation on ice for 5 minutes while cell lysis occurred. 150μl of cold solution 3 (5M Potassium acetate pH 4.8) was added and the tubes incubated on ice for a further 5 minutes to precipitate chromosomal DNA and protein. After collecting the cellular debris by centrifugation, the supernatant was added to 1ml of absolute ethanol in a new set of eppendorf tubes and incubated for a few minutes to precipitate the plasmid DNA. The DNA was then collected, washed with 1ml of 70% ethanol to remove excess salts and resuspended in 30μl of TE buffer (10mM Tris-Cl pH 7.5-8, 1mM EDTA pH8) or water.

**Genomic DNA extraction**

500μl lysis buffer (see Table 2.2.) and 5μl proteinase K (Roche) were added to 0.8-1cm of tail from 10-14 day old mice and the reactions were incubated at 55°C overnight with mixing. Tubes were then centrifuged to pellet debris and the supernatant was added to a
new tube. (For quick screening of tails supernatant could be frozen at this stage to precipitate SDS, centrifuged and used directly in PCR reactions.) For good quality genomic DNA, two phenol (equilibrated with Tris pH 7) extractions were carried out, followed by two chloroform extractions. The gDNA was precipitated with 1ml absolute ethanol (BDH) plus 50µl 3M sodium acetate pH 5.2 at -20°C for 2 hours, and washed in 70% ethanol. The resulting gDNA was resuspended in 100µl TE.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>100mM Tris-HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>10mg/ml proteinase K in 50% glycerol, 50% TE,</td>
</tr>
<tr>
<td>Depurination solution</td>
<td>0.25M HCL</td>
</tr>
<tr>
<td>Denaturing solution</td>
<td>0.5M NaOH, 1.5M NaCl</td>
</tr>
<tr>
<td>Neutralising solution</td>
<td>3M NaCl, 0.5M Tris-HCl pH 7.0</td>
</tr>
<tr>
<td>10X TBE</td>
<td>890mM Tris Base, 890mM Boric acid, 20mM EDTA pH 8.0</td>
</tr>
</tbody>
</table>

Table 2.2. Solutions used in genomic DNA extraction and Southern blotting.

Southern Blotting

5 µg genomic DNA was digested with excess units of XmnI overnight at 37°C to generate DNA fragments for detection. A negative control sample of parent strain genomic DNA was included. Samples were re-suspended with DNA loading dye to final volume of 20µl and separated on a 0.9-1% TBE gel, containing 5µl of Ethidium bromide (10mg/ml) for several hours at 60V or overnight at 30V in1X TBE (see Table 2.2).

Prior to transfer the gel was depurinated in 0.25M HCL for about 15 minutes at room temperature to nick the genomic DNA and improve the transfer of large DNA fragments. The gel was then soaked in denaturing solution for 1hr, rinsed in deionised water twice, then soaked in a neutralising solution for 1hr. The genomic DNA was transferred to
Hybond N membrane (Amersham) by capillary action using 20XSSC overnight, before baking at 80°C for 2 hrs.

The Southern blot was pre-hybridised (using the Denhardt based buffer described in Table 2.3) at 65°C for several hours, before hybridisation with an α³²-P labelled RafTR IRES-EGFP fragment as a probe (see below). The probe was boiled at 94°C for 5 minutes and cooled on ice prior to use. Washes were carried out as described for Northern blotting below. Blots were exposed to X-Omat AR film (Kodak) for the required time at room temperature or -80°C if overnight or longer and developed in an Agfa automatic processor.

RNA extraction

Cells were treated with Tmx, UV, dibutyryl cAMP or control solvents as described in the results section, before being lysed in denaturing solution and the RNA extracted using RNAgent's total RNA isolation system (Promega). Briefly the lysates were phenol chloroform extracted (phenol: chloroform: isoamyl alcohol 125:24:1, buffered with 42mM Sodium citrate pH 4.3-4.7) to remove DNA and protein, which were retained in the interphase and phenol phase. The aqueous, RNA containing, phase was removed, and the RNA precipitated with isopropanol and washed in 70% ethanol to remove excess salt. The concentration of RNA obtained was determined using a spectrophotometer (Beckman) at OD 260nm using the equation: $A_{260} = 40 \mu g/ml RNA$

Northern Blotting

RNase free water (BDH) was used for all solutions. 12.5μl deionised formamide, 2.5μl 10X MOPS and 4μl formaldehyde (37%) were added to each sample of 10-20μg of RNA. The RNA samples were denatured at 65°C for 5mins and then chilled on ice for 3 minutes. 2.5μl of RNA loading dye was added to each tube and 0.5μl of Ethidium Bromide (10mg/ml) was added specifically to the RNA markers (Gibco BRL). The gel was prepared from 108mls RNase free water with 1.5g agarose (Anachem), in a bottle that had been previously baked at 200°C. Upon cooling to around 60°C, 26mls of 37% formaldehyde and 15mls of 10X MOPS were added and the gel poured. Samples were run overnight in at 20V
or for about 5hrs at 60V in 1X MOPS buffer using a Hybaid gel tank with buffer recirculation capabilities. (See Table 2.3.)

Gels were transferred by capillary action with 20X SSC over night onto nylon membrane (Hybond N, Amersham). Membranes were baked at 80°C for 2hrs, before being pre-hybridised for at least 2 hrs at 42°C in glass tubes in a Hybaid rotating oven. Denatured radiolabelled probe (see below) was added to fresh hybridisation solution and used to replace the pre-hybridisation solution overnight. Washes were carried out in the oven, using the integral rocking platform, at various temperatures and concentrations, depending on the stringency of the wash required.

<table>
<thead>
<tr>
<th>RNA Solution</th>
<th>Components and additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>20X SSC</td>
<td>3M NaCl, 0.3M Na citrate 2H₂O, pH 7 with HCl</td>
</tr>
<tr>
<td>10X MOPS</td>
<td>0.4M MOPS pH.7, 0.1M Sodium acetate pH.7, 0.1M EDTA pH.8.</td>
</tr>
<tr>
<td>10X RNA loading dye</td>
<td>50% glycerol (v/v), 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol.</td>
</tr>
<tr>
<td>Hybridisation solutions</td>
<td>5XSSC, 50% deionised formamide, 5X Denhardt solution, 0.5% SDS, salmon sperm at 100µg/ml.</td>
</tr>
<tr>
<td>100X Denhardt solution</td>
<td>2% w/v Ficoll 400, 20mg/ml BSA (pentax fraction V), 2% w/v polyvinylpyrrolidone.</td>
</tr>
<tr>
<td>Wash solutions</td>
<td>2XSSC, 0.1%SDS (15minutes at room temperature)</td>
</tr>
<tr>
<td></td>
<td>0.5XSSC, 0.1%SDS (15minutes at 42°C -low stringency wash)</td>
</tr>
<tr>
<td></td>
<td>0.3XSSC, 0.1%SDS (15minutes at 55°C - medium stringency)</td>
</tr>
<tr>
<td></td>
<td>0.2XSSC, 0.1%SDS (15minutes at 65°C -high stringency)</td>
</tr>
</tbody>
</table>

Table 2.3. Northern blotting solutions and wash conditions
Probe labelling

DNA for probe labelling was obtained by restriction digests of the relevant plasmids (Table 2.4). These fragments were separated on agarose gels and purified using a Quiaex II gel purification kit (Qiagen). DNA was labelled with $\alpha^{32}$P dCTP using an oligolabelling kit (Amersham Pharmacia Biotech). 25-50ng of DNA was dissolved in a maximum of 34μl water and denatured at 95°C for 2-3minutes, before placing on ice for 2 minutes. The DNA was added to a fresh tube containing 10μl reaction mix, 5μl (50μCi) of $\alpha^{32}$P dCTP and water to a final volume of 49 μl. 1μl Klenow fragment (8U/μl) was added and the reaction incubated at 37°C for 30-60mins.

The probe was purified using microspin columns (Amersham). These were pre-equilibrated at 3.5 x1000g in a Sigma microfuge for 1 minute precisely, before the probe was added. After 2 minutes centrifugation in a fresh tube the purified probe had collected at the bottom of the tube, whilst any contaminants were retained in the purification resin. 1μl of probe was counted in a liquid scintillation counter (Packard) to calculate the efficiency of labelling and determine the volume required to obtain 1x10⁶cpm/ml hybridisation buffer. Typical probe labelling efficiencies were around 1x10⁶cpm/μg. The probe was boiled for 5 minutes at 95°C and cooled on ice before adding to warm hybridisation solution.

<table>
<thead>
<tr>
<th>Probe name</th>
<th>Origin</th>
<th>Use</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21&lt;sup&gt;Cipl&lt;/sup&gt;</td>
<td>SmaI/EcoRV fragment of pMT2FM - rat p21&lt;sup&gt;Cipl&lt;/sup&gt;, a gift from P.Jat.</td>
<td>Northern blotting</td>
<td>420bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>PCR product</td>
<td>Northern blotting</td>
<td>350bp</td>
</tr>
<tr>
<td>P0</td>
<td>EcoR1/PstI digest of P0-pGEM, a gift from G.Lemke.</td>
<td>Northern blotting</td>
<td>600bp</td>
</tr>
<tr>
<td>RafTR</td>
<td>BamH1 digest of pRafTR LXSN3.</td>
<td>Southern blotting</td>
<td>2.2kb</td>
</tr>
</tbody>
</table>

Table 2.4. Origin of the DNA fragments used for probe labelling
cDNA synthesis

The Superscript pre-amplification system for 1st strand synthesis (Gibco BRL) was used with random primers to produce cDNA for RT-PCR and semi-quantitative RT-PCR applications. Samples of RNA from the RafTR transgenics were treated with 1µl DNase (10U/µl, Amersham) for 20 minutes at 37°C to ensure that contaminating DNA was removed prior to analysis. Samples were then phenol: chloroform extracted, precipitated with 2.5 volumes of 100% ethanol and 1/10 volume of 3M sodium acetate pH 5.2 at –20°C for 2 hours, and washed in 70% ethanol. The RNA was resuspended in DEPC treated water prior to cDNA synthesis and the RNA concentration re-calculated.

Reactions were prepared containing 3µg RNA and 2µl random hexamers (50ng/µl stock), made up to a final volume of 12µl in DEPC treated water. Every reaction was set up in duplicate to allow treatment +/- reverse transcriptase (RT) for each RNA sample. The reactions were incubated at 70°C for 10 minutes (to remove secondary structure) then placed on ice for more than 1 minute. A reaction mix was prepared containing 2µl of 10x PCR buffer, 2µl of 25mM MgCl₂, 1µl of 10mM dNTP mix and 2µl of 0.1M DTT for n+1 reactions (n, number of reactions). 7µl of this reaction mix was added to each sample and incubated at 25°C for 5 minutes. 1µl of RT (200U/µl) was added to each tube (except -RT tubes) and incubated at 25°C for 10 minutes, then 42°C for 50 minutes. The reactions were terminated at 70°C for 15 minutes then chilled on ice. 1µl RNase H (2U/µl) was added to each tube and incubated at 37°C for 20 minutes to degrade the RNA. The successful production of cDNA (and the absence of product in the – RT tubes) was confirmed by PCR for GAPDH.

PCR

Reactions were set up using 1-2µl of cDNA or very small amounts of plasmid DNA (about 30ng) or genomic DNA. The primers and their uses are detailed in Table 2.1.

50µl reactions were set up as follows with all components supplied by Promega: 5µl 10X PCR buffer, 3µl 25mM MgCl₂, 1µl 10mM dNTP mix, 1 µl each of sense and antisense primers (10µM), DNA and water to 49µl. 0.5µl taq DNA polymerase (5U/µl) was added.
before the reaction was transferred to the PCR machine (Techne), unless a hot start was required. In this case the taq polymerase was added after an initial 3 minutes at 94°C. A reaction without DNA was included as a negative control. Reactions were based on following programme and altered as required by the primer melting temperature and product size: Denaturation 94°C 1 minutes
Annealing 52°C 1 minute
Elongation 72°C 1 minute

The number of cycles used was determined by the requirement for sequence accuracy (e.g. for cloning a lower number of cycles and Pfu polymerase were used, which has proof reading capabilities), avoiding product levels reaching a plateau (e.g. semi-quantitative RT-PCR) and the abundance of the target. Mineral oil was used to prevent cross contamination of tubes where a hot start was required.

**Semi-quantitative RT-PCR**

The RNA of interest was converted to cDNA as described above. PCR reactions were set with GAPDH primers and samples of the reaction were removed at 20 and 22 cycles. The reaction mix was the same as above, but with the presence of 0.3μl of α32P dCTP per reaction. One reaction lacking cDNA was included as a negative control.

The reactions were separated on a 4% acrylamide gel made up in 0.5x TBE with 300μl of 10% APS and 25μl of TEMED (BioRad) as polymerising agents. The gel was pre-run for 30 minutes at 60V. Wells were loaded with 5μl of the PCR reaction mixed with loading dye (20% w/v Ficoll 400, 1% w/v SDS, 0.25% w/v Bromophenol blue and 0.25% w/v Xylene cyanol) and electrophoresed at 60V in 0.5X TBE until the blue loading dye was near the end of the gel. The gel was soaked in destain solution (7% v/v acetic acid, 5% v/v methanol, 88% v/v water) for 20 minutes, before being transferred to sheet of Whatman 3MM filter paper (Whatman) and dried on a gel drier (BioRad) at 80°C for 1-2hrs. The gel was exposed to film or a phosphoimager detection screen for the required time and quantified using an FX phosphoimager and Quantity-One quantification software (both Biorad).
The cDNA samples were equalised and the GAPDH PCR was repeated to confirm that equal levels of cDNA were being used across samples, before reactions were carried out with other primers. In each case the number of cycles required for a particular reaction was determined by taking out aliquots of the PCR reactions over a wide range of cycles and visualising them on a gel, as above. Two cycles were chosen prior to the reaction reaching a plateau. Results were considered acceptable if fold differences between samples were maintained across the cycles. Each reaction was carried out on several occasions with cDNA obtained from at least two separate experiments.

2.4 Other techniques

Phase contrast microscopy

Phase contrast pictures of cell were taken using an inverted microscope (Leica) and the images were processed using Openlab software.

Immunofluorescence

DRG-Schwan cell/fibroblast co-cultures were fixed on coverslips with 4% paraformaldehyde for 30 minutes before washing carefully in PBSA. An acid alcohol (5% acetic acid in methanol for 10 minutes at -20°C) step was included to maintain neurofilament structure and improve antibody binding. Anti-RT97 antibody (a gift from Dr. John Wood, see Table 2.5) was diluted to 1:1000 in 0.1% BSA, 0.1% azide, PBS. 80μl was added to each coverslip (apart from the secondary controls) and incubated at room temperature in the dark for 1hr. After each treatment the coverslips were washed nine times in PBSA and the relevant secondary was incubated with the coverslips for 45 minutes. The cells were then permeabilised with ice-cold methanol for 10 minutes at -20°C and washed in PBSA before incubating with 1:400 anti-S100 antibody for 1hr. To amplify the S-100 signal anti-rabbit biotin, (1:100), was added for 30 minutes followed by streptavidin-flouescein, (1:100) for 30 minutes. Hoescht was used at 1:6000 for 20 minutes to detect the cell nuclei. The coverslips were then fixed with ice cold 5% acetic acid in ethanol for 10 minutes at -20°C, mounted using Citifluor (Citifluor) and sealed with nail varnish. Coverslips were visualised using a Zeiss Axioskop microscope with Openlab software.
This protocol was adapted for anti-Thy-1 staining of fibroblasts. Cells were fixed for 10 minutes only in the absence of DRG. See Table 2.5 for the complete list of antibodies used for immunofluorescence and Western blotting.

**Staining cells for β-galactosidase activity**

Schwann cells were transfected using Fugene 6 reagent as described above and fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were rinsed three times with PBSA and incubated in staining solution (5mM K$_3$Fe(CN)$_6$, 5mM K$_4$Fe(CN)$_6$.3H$_2$O, 2mM MgCl$_2$ in PBSA with 1mg/ml X-gal) overnight at 37°C. The number of blue cells in a number of fields per dish were counted using an inverted microscope and used to equalise the efficiency of transfection across dishes.

**Protein extraction**

Cells were treated with Tmx, UV, dibutyryl cAMP or control solvents as described in the results chapters. The cells were washed in 1X PBSA and scraped off the dish in 1ml PBSA with a rubber bung. Cells were collected using a chilled microfuge (Heraus) at 13,00rpm and either frozen in liquid nitrogen for later use or lysed in IN or RIPA buffer (Table 2.6), depending on the protein(s) of interest. The cell debris was collected by centrifugation and the protein content of the sample determined using a Bradford assay (BioRad) at OD 595nm. Sample buffer was added to equal amounts of protein and boiled for 5 minutes, prior to separation by SDS-PAGE. 15% gels were used for small proteins (up to about 30kDa), with periaxin requiring a 7.5% gel and phospho-ERK1/2 and α-tubulin being separated on 10% gels.

Periaxin is a large protein that is easily degraded. To look at periaxin protein levels in my experiments I harvested the cells in 200μl of sample buffer directly and boiled the samples at 95°C before storing at -20°C. The samples were disrupted by sonication (Branson sonifier) and the cellular debris removed before use. An equal volume of each sample was separated on a mini protein gel and the levels of α-tubulin determined by Western blotting.
to allow equalisation of protein concentration across samples. The equalised samples were then separated on a maxi protein gel and analysed by Western blotting as described below.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Origin of antibody</th>
<th>Dilution</th>
<th>Development of signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin D1</td>
<td>Santa Cruz</td>
<td>1/1000</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p15 p27kip1</td>
<td>Santa Cruz</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, TBST, ECL plus</td>
</tr>
<tr>
<td>p16 p21Cip1</td>
<td>Santa Cruz</td>
<td>1/200</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p18 p19ARF</td>
<td>Santa Cruz</td>
<td>1/200</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19 p21Cip1</td>
<td>Santa Cruz</td>
<td>1/1000</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19 p27kip1</td>
<td>Santa Cruz</td>
<td>1/1000</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19 p21Cip1</td>
<td>AbCAM for our lab.</td>
<td>1/1000</td>
<td>Anti-rabbit HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19 p21Cip1</td>
<td>Calbiochem</td>
<td>1/20</td>
<td>Anti-mouse HRP, PBST, ECL plus</td>
</tr>
<tr>
<td>p19 p21Cip1</td>
<td>Gift from J. Archelos</td>
<td>1/100</td>
<td>Anti-mouse HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>Oct-6</td>
<td>Gift from D. Meijer,</td>
<td>1/30,000</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td></td>
<td>Erasmus University</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFP</td>
<td>AbCAM</td>
<td>1/500</td>
<td>PBST, ECL normal</td>
</tr>
<tr>
<td>Raf</td>
<td>Santa Cruz</td>
<td>1/400</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>ER</td>
<td>Santa Cruz</td>
<td>1/500</td>
<td>Anti-rabbit HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>P-ERK1/2</td>
<td>Sigma</td>
<td>1/5000</td>
<td>Anti-mouse HRP, TBST, ECL plus</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>Sigma</td>
<td>1/60,000</td>
<td>Anti-mouse HRP, PBST, ECL normal</td>
</tr>
<tr>
<td>S100</td>
<td>Dako</td>
<td>1/400</td>
<td>Anti-rabbit biotin</td>
</tr>
<tr>
<td>Neurofilament (RT97)</td>
<td>Gift from J. Woods, UCL</td>
<td>1/1000</td>
<td>Anti-mouse Cy3</td>
</tr>
<tr>
<td>Anti-rabbit HRP</td>
<td>Amersham</td>
<td>1/2000</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse HRP</td>
<td>Amersham</td>
<td>1/2000</td>
<td></td>
</tr>
<tr>
<td>Anti-goat HRP</td>
<td>Dako</td>
<td>1/2000</td>
<td></td>
</tr>
<tr>
<td>Streptavidin-fluorescein</td>
<td>Amersham</td>
<td>1/100</td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit Biotin</td>
<td>Amersham</td>
<td>1/100</td>
<td></td>
</tr>
<tr>
<td>Anti-mouse Cy3</td>
<td>Jackson Immunological</td>
<td>1/1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>research labs</td>
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</tr>
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Table 2.5. Antibodies used for Western blotting and immunofluorescence.
<table>
<thead>
<tr>
<th>Solutions</th>
<th>Components and additional information</th>
</tr>
</thead>
<tbody>
<tr>
<td>1N lysis buffer</td>
<td>1% NP-40, 150mM NaCl, 50mM Tris pH 8, 20mM NaF, 100μg/ml PMSF, 15μg/ml aprotinin and 1mM Na₂VO₄.</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>1% Triton X-100, 0.5% Sodium deoxycholate, 50mM Tris pH7.5, 100mM NaCl, 1mM EGTA pH8, 20mM NaF, 100μg/ml PMSF, 15μg/ml aprotinin, 1mM Na₂VO₄.</td>
</tr>
<tr>
<td>(Use BioRad DC protein</td>
<td>assay)</td>
</tr>
<tr>
<td>4X Sample buffer</td>
<td>200mM Tris pH 6.8, 8% SDS (BioRad), 40% glycerol, 400mM DTT, 0.25% bromophenol blue.</td>
</tr>
<tr>
<td>10X Running buffer</td>
<td>2.5M glycine (BDH), 250mM Tris, 1% SDS.</td>
</tr>
<tr>
<td>10X Transfer buffer</td>
<td>200mM Tris, 1.5M glycine, 20% methanol (BDH)</td>
</tr>
<tr>
<td>Milk block</td>
<td>5% milk, 0.05% Tween -20 (BioRad) in PBS</td>
</tr>
<tr>
<td>Stripping Buffer</td>
<td>200mM glycine , pH2.5, 0.4% SDS</td>
</tr>
<tr>
<td>1X PBSA Tween wash</td>
<td>0.05% Tween-20 in PBS</td>
</tr>
<tr>
<td>20x TBS Tween</td>
<td>200mM Tris, pH8, 3M NaCl 1% Tween -20</td>
</tr>
<tr>
<td>PBSA</td>
<td>137mM NaCl, 2.7mM KCl, 1.47mM KH₂PO₄, 8.1mM Na₂HPO₄</td>
</tr>
<tr>
<td>20X TBS</td>
<td>200mM Tris pH 8, 3M NaCl.</td>
</tr>
</tbody>
</table>

**Table 2.6 Solutions for protein extraction and Western blotting**

**Western Blotting**

Proteins were transferred to PVDF membrane (Immobilon P, Millipore) using transfer apparatus (see Table 2.6). Membranes were blocked overnight at 4°C or for 45 minutes at room temperature. Blots that were being probed for periaxin protein levels were blocked overnight with a modified block solution consisting of 1% milk (Merck), 0.05% Tween -20 in PBSA. (Blots for re-probing were washed in stripping buffer for 30 minutes and then blocked in milk.) Membranes were incubated in primary antibody in plastic bags at room temperature on a rocking platform (Stuart Scientific). The antibody was diluted in the remaining block to the required concentration (see Table 2.5). After 1hr the blot was washed in either PBST or TBST (higher stringency) solutions for three sets of 5 minutes. The relevant secondary antibody was incubated with the blots for 1hr and the washing stage
repeated. A final wash was made in PBS or TBS alone and the blot half-dried using Whatman 3MM paper. ECL solution (ECL normal or plus depending on the antibody, Amersham) was carefully pipetted onto the protein side of the blot and incubated at room temperature for 5 minutes. After developing the blot was dried as before and wrapped in cling film. The blot was exposed to film (Biomax ML, Kodak) for the required time in a dark room and the films developed using an Agfa automatic film processor.

**Statistical analysis**

Results were analysed using the student’s two-sample T-test to test whether measurements made on two populations were different from each other (Ennos, 2000). The null hypothesis proposed that the two populations were the same in every case.
Chapter 3: Characterising the Raf induced cell cycle arrest and
the effects of Raf activation on Schwann cell differentiation

Oncogenic activation of Ras is a frequent event in the development of certain human
tumours (Bos, 1989). In vitro, oncogenic Ras expression can transform immortal cell lines,
however, activated Ras alone is insufficient to transform primary cells. Transformation of
primary cells by activated Ras requires additional changes, including loss of p53 function
or the presence of SV40 LT (Ridley et al., 1988, Lloyd et al., 1997, Serrano et al., 1997).
Oncogenic Ras expression in many primary cell types has been shown to result in a cell-

cycle arrest associated with the induction of cyclin dependent kinase inhibitors (CDKIs)
(Serrano et al., 1997; Lloyd et al., 1997; Malumbres et al., 2000; Lin and Lowe, 2001). In
primary MEFS for example, Ras activation results in the induction of a senescent
phenotype associated with increased levels of p21$^{Cip1}$, p15$^{INK4b}$ and p16$^{INK4a}$. The situation is
similar in human diploid fibroblasts (HDFs, Wei et al., 2001) and keratinocytes (Roper et
al., 2001, Lin and Lowe, 2001). This response to oncogenic Ras expression has been
proposed to act as a protective mechanism to prevent unregulated proliferation in response
to a single genetic alteration.

In primary Schwann cells, Ras/Raf activation results in a G1 cell-cycle arrest associated
with the induction of p21$^{Cip1}$ (Ridley et al., 1988, Lloyd et al., 1997). The importance of
p21$^{Cip1}$ induction has been demonstrated using p21$^{Cip1}$ antisense RNA, which was able to
partially abrogate the Raf induced arrest. p21$^{Cip1}$ induction is p53 dependent and associated
with inhibition of cyclin A- and cyclin E- dependent kinase activity. However, the
mechanism of p21$^{Cip1}$ induction and whether other CDKIs are involved in the Raf induced
cell-cycle arrest remains to be determined.

Characterisation of the Raf induced arrest in primary Schwann cells.

Induction of cyclin dependent kinase inhibitors

To investigate the mechanism of the Ras induced cell-cycle arrest in primary Schwann cells
I used cells expressing an inducible Raf protein, the ΔRaf-1:ER, since we have previously
shown that the Raf/MAPK pathway mediates the Ras induced cell cycle arrest in these
primary cells (Lloyd et al., 1997). The ΔRaf-1:ER consists of the kinase domain of Raf-1 fused to the estrogen receptor hormone-binding domain (Samuels et al., 1993). This construct is reversibly activated by the addition of β-estradiol (or Tamoxifen (Tmx), an estrogen analogue), resulting in rapid MAPK activation and transformation of immortal cells or cell-cycle arrest in primary cells (Samuels et al., 1993; Lloyd et al., 1997, Lin, 1998). Moreover, since the ΔRaf-1:ER construct is inducible we are able to routinely culture Schwann cells in the absence of Raf activation. In the work described in this thesis I predominantly used pools of primary Schwann cells expressing the ΔRaf-1:ER construct (NSRafER cells).

I initially confirmed that Raf activation in NSRafER cells resulted in a cell cycle arrest. I treated NSRafER cells with Tmx for 30hrs. 3H- thymidine was added for the last 6hrs and the degree of DNA incorporation of this labelled nucleotide was analysed as a measure of DNA synthesis. As shown in Fig.3.1, Raf activation resulted in a strong inhibition of DNA synthesis. This was associated with a strong induction (7-fold) of p21Cip1, as expected from previous results (Lloyd et al., 1997).

We have previously demonstrated the importance of p21Cip1 in mediating the Raf induced cell-cycle arrest in primary Schwann cells (Lloyd et al., 1997). However, since p21Cip1 antisense RNA was only able to partially abrogate the Raf induced arrest, other CDKIs may also be involved in establishing this arrest. The Ras induced arrest in primary MEFs is associated with the induction of the CDKIs p15Ink4b, p16Ink4a and p21Cip1 and is p15Ink4b dependent (Pantoja, 1999; Malumbres et al., 2000; Krimpenfort et al., 2001; Sharpless et al., 2001). In contrast, in primary keratinocytes, induction of p21Cip1 is essential for the Raf induced arrest, but it is unclear whether p16Ink4a is also involved (Roper et al., 2001; Lin and Lowe, 2001; Paramio et al., 2001). In primary Schwann cells, Raf activation also resulted in an increase in p15Ink4b levels, which could be involved in mediating the cell cycle arrest in these cells in co-operation with p21Cip1 (Fig. 3.1.B).
Fig. 3.1 Activation of Raf results in a cell cycle arrest associated with the induction of cyclin dependent kinase inhibitors.

(A) Primary Schwann cells infected with retrovirus containing ΔRaf-1:ER (NSRafER) were treated with 100nM of Tamoxifen (TMX) or ethanol as a control (C) for 30hrs in triplicate. $^3$H-Thymidine was added between 24-30hrs. Lysates were assayed for $^3$H-Thymidine incorporation as described in Materials and Methods. Representative mean values of incorporation and SD are shown. (B) NSRafER cells were treated with ethanol or TMX for 30hrs, before being lysed and Western blotted for p21$^{Cip1}$ or with the antibodies specified.
Activation of Raf results in the induction of p21\textsuperscript{Cip1} mRNA

p21\textsuperscript{Cip1} is induced in response to Ras/Raf activation in primary Schwann cells and has been shown to play an important role in mediating the resulting cell cycle arrest (Lloyd \textit{et al.}, 1997). Although the mechanism responsible for p21\textsuperscript{Cip1} induction by Raf is unclear, the kinetics of p21\textsuperscript{Cip1} expression suggest that p21\textsuperscript{Cip1} induction is occurring indirectly, since there is a delay of about 14hrs following Raf activation prior to the increase in p21\textsuperscript{Cip1} levels. The Raf induced arrest in primary Schwann cells is p53 dependent and this might suggest that p21\textsuperscript{Cip1} is a transcriptional target of p53 under these conditions. In primary MEFS, the Ras induced arrest is thought to induce p21\textsuperscript{Cip1} expression in this manner (Serrano \textit{et al.}, 1997). However, in other cell types p21\textsuperscript{Cip1} expression is p53 independent (Halevy \textit{et al.}, 1995, Missero \textit{et al.}, 1996, Sewing \textit{et al.}, 1997; Woods \textit{et al.}, 1997; Gartel \textit{et al.}, 2000; reviewed by McMahon and Woods, 2001). For example, activation of Raf in NIH3T3 cells (which lack the INK4a/ARF locus) or p53\textsuperscript{-/} MEFS results in a cell cycle arrest associated with elevated p21\textsuperscript{Cip1} levels (Sewing \textit{et al.}, 1997) Woods \textit{et al.}, 1997), which may be mediated by the transcription factors E2F-1 and Sp1/Sp3 (Kivinen \textit{et al.}, 1999; Gartel \textit{et al.}, 2000). In contrast, in a number of cell types stabilisation of p21\textsuperscript{Cip1} protein has been implicated in the elevation of p21\textsuperscript{Cip1} levels in association with modest mRNA increases (Macleod \textit{et al.}, 1995; Timchenko \textit{et al.}, 1996). In order to determine the mechanism of the Raf induced increase in the levels of p21\textsuperscript{Cip1}, I decided to investigate the effect of Raf activation on both p21\textsuperscript{Cip1} mRNA levels and protein stability.

To determine whether p21\textsuperscript{Cip1} induction in response to Raf activation in primary Schwann cells was caused by increased mRNA levels, I analysed p21\textsuperscript{Cip1} mRNA levels by Northern blotting following treatment of NSRafER cells with Tmx. UV was used as a positive control since it induces the stabilization of p53 in response to DNA damage and results in p21\textsuperscript{Cip1} transcription in certain cell types (Lu and Lane, 1993; Poon \textit{et al.}, 1996). I found that Raf activation resulted in a 2.5-fold induction of p21\textsuperscript{Cip1} mRNA (Fig.3.2.A.). This result was confirmed using semi-quantitative RT-PCR. (Fig.3.2B, C). Thus Raf activation results in p21\textsuperscript{Cip1} induction at the mRNA level, which could be responsible for the observed increase in protein levels. However, since the fold mRNA induction is small compared to
the 7-fold increase in p21^{Cip1} protein, this may suggest that p21^{Cip1} levels are also regulated post-transcriptionally.

To measure whether the stability of p21^{Cip1} was altered by Raf activation we inhibited protein synthesis using cyclohexamide and analysed the half-life of p21^{Cip1} by Western blotting. The half-life of p21^{Cip1} has previously been shown to be short, varying between about 30 minutes in HT1 cells (Timchenko et al., 1996) to 90 minutes in NIH 3T3 cells (Kivinen et al., 1999). NSRafER cells were treated with Tmx for 30hrs, before the addition of 20µg/ml cyclohexamide. Cells were lysed at various times between t = 0 and 160 minutes post cyclohexamide addition and analysed for p21^{Cip1} levels by Western blotting. The results were equalised to t = 0 using a densitometer. As shown in Figure 3.2 D, the rate of degradation of p21^{Cip1} in cells treated with/ without Tmx remained similar, with a half-life of around 60 minutes, suggesting that Raf activation did not alter the stability of p21^{Cip1} protein. This finding, taken with the increase in mRNA levels, suggests that the Raf induced induction of p21^{Cip1} occurs at the level of mRNA, and possibly by an as yet undetermined post-transcriptional mechanism, to induce the 7-fold increase in p21^{Cip1} protein levels detected.

**Raf activation results in the induction of p19^{ARF}, without increased p53 levels**

In primary MEFS, the Ras induced arrest is p53 dependent and associated with the induction of p19^{ARF}, which results in the stabilization of p53 protein levels (Serrano et al., 1997; Palmero et al., 1998; Pomerantz et al., 1998; Zhang, 1998). The importance of p19^{ARF} induction in the p53 response to Ras was clearly demonstrated using p19^{ARF} null MEFs which no longer induce p53 or arrest in response to oncogenic Ras expression (Kamijo et al., 1997; Palmero et al., 1998). In primary keratinocytes Ras/Raf induced differentiation is also p53 dependent, although it remains debatable whether p19^{ARF} is involved (Roper et al., 2001, Lin and Lowe, 2001, Paramio et al., 2001). In contrast in HDFs, the expression of oncogenic Ras results in a p53 independent arrest without a detectable increase in p19^{ARF} levels, suggesting that the arrest is p19^{ARF} independent (Wei et al., 2001). Since the Raf induced cell cycle arrest in primary Schwann cells was previously shown to be p53 dependent (Lloyd et al., 1997), I decided to investigate
Fig. 3.2. Activation of Raf results in increased p21Cipl mRNA expression.
NSRafER cells were treated with 50μj/cm² x 100 UV (UV), a control solvent or
100nM Tmx and lysed after 6hrs or 30hrs. The lysates were analysed by
Northern blotting (A) or semi-quantitative RT-PCR for p21Cipl mRNA
expression (B). (C) Quantification of the results in (B) obtained using BioRad
phospho-imaging software. For more details see Materials and Methods. (D)
Cells were treated with control solvents or Tmx for 30hrs before the addition of
cyclohexamide (protein synthesis inhibitor) at 20 μg/ml. Dishes were harvested
between t = 0 and 160 minutes and analysed for p21Cipl protein levels by
Western blotting. The change in p21Cipl levels was quantified over time using a
densitometer and compared to t = 0.

101
whether p53 stabilisation was occurring in these cells with a similar mechanism to the primary MEFs.

I treated NSRafER cells with Tmx prior to analysing p19ARF levels by Western blotting. I found that Raf activation strongly induced p19ARF expression (Fig. 3.3A), suggesting that p19ARF may also be involved in the Raf induced arrest in primary Schwann cells. The induction of p19ARF protein was associated with a 3.5-fold increase in p19ARF mRNA levels (Fig. 3.3 C, D). (This work was carried out using semi-quantitative RT-PCR as the levels of p19ARF and p16ink4a mRNA expressed were below the level of detection by Northern blotting.) The oncogenic Ras induced arrest in primary MEFs is p19ARF dependent (Palmero et al., 1998). In order to investigate the importance of p19ARF induction on the Raf induced cell cycle arrest in primary Schwann cells I decided to use a construct to express p19ARF antisense RNA. Unfortunately the antisense construct was unable to reduce p19ARF levels during Raf activation, although Carnero et al., successfully managed to downregulate p19ARF levels using a similar construct (Carnero, 2000). I was therefore unable to ascertain the importance of p19ARF induction in primary Schwann cells in response to Raf activation.

In primary MEFs, oncogenic Ras expression results in increased p19ARF levels, leading to the stabilisation of p53 (Palmero et al., 1998). In primary Schwann cells p21Cip1 induction is p53 dependent (Lloyd et al., 1997), however, I was unable to detect any changes in p53 levels in response to Raf activation (Fig. 3.3B). There are several possible explanations for this result. p53 activity may be increased by Raf activation in the absence of stabilisation. In NIH 3T3 cells mutants of p19ARF, lacking the ability to stabilise p53 levels, are still capable of inducing p53 activity in reporter assays (Korgaonkar et al., 2002). Alternatively, in the absence of a Raf induced increase in p53 activity, basal levels of p53 may still be required for the transactivation of target genes by other transcription factors. For example, in response to γ-irradiation, transactivation of the p21Cip1 promoter by IRF-1 is dependent on the presence of p53 (Tanaka et al., 1996).
Fig. 3.3. Induction of p19^{ARF} by Raf is not associated with increased levels of p53.

(A) NSRafER cells were treated as described in Fig.3.2. with control solvents or Tmx for 30hrs before being analysed by Western blotting using anti- p19^{ARF} and α-tubulin antibodies. (B) Protein lysates were made from cells treated with control solvents, Tmx or UV for 6hrs or 30hrs and Western blotted with anti- p53 antibody. (C) Cells were treated as in (B) before being lysed for analysis by semi-quantitative RT-PCR of p19^{ARF} expression. (D) Quantification of the results shown in (C).
To investigate whether Raf activation resulted in an increase in p53 transcriptional activity, I planned to assay p53 activity using a series of CAT reporter constructs under the control of p53 target promoters. Unfortunately transient transfection of the NSRafER cells resulted in p53 induction, in response to the transfection procedure, and thus reporter construct activation. This induction of p53 prevented the detection of any increase in p53 transcription caused by Raf activation.

In summary, p19arf is induced in response to Raf activation in primary Schwann cells and may serve as an important mediator of the cell cycle arrest via interaction with p53. However, p53 protein levels do not appear to increase in response to Raf activation raising the possibility that p19arf is acting independently of p53 or is activating p53 in the absence of protein stabilisation (Korgaonkar et al., 2002). The basis of the p53 dependence of the Raf induced arrest in primary Schwann cells remains to be determined.

**p16INK4a and p19INK4d levels decrease with Raf activation.**

In primary fibroblasts the Ras/Raf induced arrest is associated with increased levels of p16INK4a (Serrano et al., 1997, Zhu, 1998, Wei et al., 2001). This induction, however, does not appear to be essential for the arrest in primary MEFs, as p16INK4a-/- cells still senesce in response to oncogenic Ras/Raf activity (Krimpenfort et al., 2001, Sharpless et al., 2001). In contrast in primary human fibroblasts, the Ras/Raf induced arrest is associated with p16INK4a induction, in the absence of increased levels of p19arf (Serrano et al., 1997; Zhu, 1998; Wei et al., 2001; Brookes et al., 2002). Since the arrest is both p53 and p21Cip1 independent, this suggests that in human fibroblasts p16INK4a may be an important mediator of the Ras/Raf induced arrest. This idea is supported by the recent observation that human fibroblasts with mutated p16INK4a derived from a familial melanoma patient no longer arrest in response to oncogenic Ras (Brookes et al., 2002).

As p16INK4a induction is associated with the Ras/Raf induced arrest in both primary HDFs and MEFs, I examined whether Raf activation in primary Schwann cells resulted in increased p16INK4a levels. I also investigated the effect of Raf activation on the levels of the CDKIs p18INK4c and p19INK4d. Surprisingly, in contrast to other primary cell types, p16INK4a
levels decreased to undetectable levels in response to Raf activation (Fig. 3.4 A). In addition, p19\textsuperscript{INK4a} levels decreased in response to Raf activation, whilst p18\textsuperscript{INK4c} levels remained constant. Thus p16\textsuperscript{INK4a} is not a mediator of the cell cycle arrest in primary Schwann cells in response to Raf activation. This suggests that the Raf induced arrest is due to the increased levels of the CDKIs, p15\textsuperscript{INK4b} and p21\textsuperscript{Cip1}.

In order to investigate the mechanism of the decrease in p16\textsuperscript{INK4a} protein levels I decided to use semi-quantitative RT-PCR to determine whether Raf induced a corresponding decrease in p16\textsuperscript{INK4a} mRNA levels. I found that p16\textsuperscript{INK4a} mRNA levels remained constant following Raf activation in primary Schwann cells (Fig. 3.4 B and C). Therefore Raf does not cause decreased p16\textsuperscript{INK4a} protein levels by reducing mRNA levels, suggesting that Raf is acting post-transcriptionally, perhaps by increasing the rate of protein degradation.

Thus, in contrast to primary fibroblasts, Ras/Raf activation in primary Schwann cells results in a decrease in p16\textsuperscript{INK4a} protein levels. However, downregulation of the levels of the CDKI p16\textsuperscript{INK4a} may be important for Ras/Raf to act as a proliferative signal under different circumstances in primary Schwann cells. Since the Ras/Raf induced decrease in p16\textsuperscript{INK4a} protein levels occurs in the absence of a corresponding decrease in mRNA levels, I decided to investigate whether Raf activation resulted in reduced protein stability. I planned to analyse the half-life of p16\textsuperscript{INK4a} protein in response to Raf activation in a similar manner to that described for p21\textsuperscript{Cip1} in Fig. 3.2C. However, I have been unable to determine the mechanism responsible for the reduction in p16\textsuperscript{INK4a} levels to date due to the lack of a suitable antibody. In the future, expression of a tagged p16\textsuperscript{INK4a} protein should enable me to examine the effects of Raf activation on p16\textsuperscript{INK4a} protein stability. This would allow me to perform a pulse-chase experiment to quantify any Raf induced changes in the half-life of p16\textsuperscript{INK4a} protein using immunoprecipitation of exogenous p16\textsuperscript{INK4a} by an antibody against the attached protein tag.
Fig. 3.4. Raf activation is associated with decreased p16^INK4a and p19^INK4d protein levels, however p16^INK4a mRNA levels remain constant.

(A) NSRafER cells were treated with 100nM Tmx or a control solvent for 30hrs before being analysed by Western blotting with the specified antibodies. (B) NSRafER cells were treated with control solvents or Tmx as described in Fig.3.2, before analysis by semi-quantitative RT-PCR for p16^INK4a expression. (C) Quantification of the results shown in (B).
In summary, Raf activation in primary Schwann cells results in a p53 dependent cell cycle arrest associated with the induction of p15^{INK4b}, p21^{CIP1} and p19^{ARF}, similar to the Ras induced arrest in primary MEFs. However, in contrast to primary fibroblasts and keratinocytes, Raf activation in primary Schwann cells results in reduced p16^{INK4a} levels and is not associated with p53 stabilisation. Thus, although in many primary cell types activation of Ras/Raf results in a cell-cycle arrest associated with the induction of CDKIs, the mechanism responsible for mediating the arrest differs between cell types.

**Investigating the effect of Raf activation on Schwann cell differentiation**

Ras activation has been implicated in both terminal differentiation and proliferation in a number of cell types. In primary keratinocytes the induction of differentiation markers is associated with the Ras/Raf induced cell-cycle arrest (Lin and Lowe, 2001, Roper et al., 2001). In PC12 cells, oncogenic Ras expression, or NGF addition, results in prolonged and sustained MAPK activity, leading to cell cycle exit and the development of a neuronal phenotype (Bar-Sagi and Feramisco, 1985, Wood et al., 1993; Traverse et al., 1992, Qui and Green, 1992, Nguyen et al., 1993, Marshall, 1995). In contrast, EGF addition results in a transient Raf/MAPK signal in PC12 cells and is associated with proliferation. Thus the same pathway is capable of regulating two independent outcomes, depending on signal strength and duration (Traverse et al., 1992, Qui and Green, 1992, Nguyen et al., 1993, Marshall, 1995). *In vivo* studies on Drosophila eye development have also provided support for the hypothesis that different thresholds of MAPK activity are responsible for mediating different Ras functions, with high levels of Ras/Raf/MAPK activity inducing premature differentiation of R1-7 photoreceptors (Halfar et al., 2001). However, in contrast to both keratinocytes and PC12 cells, prolonged activation of the Ras/Raf/MAPK pathway in skeletal myoblast cells inhibits myoblast fusion and the induction of muscle-specific gene products (Olson et al., 1987; Coolican et al., 1997; Dorman and Johnson, 1999). Since Ras/Raf signalling can induce differentiation of other cell types, I decided to investigate whether the oncogenic Ras/Raf induced cell cycle arrest in primary Schwann cells was associated with differentiation.
Schwann cell differentiation is a complex process that is regulated by multiple Schwann cell-axon interactions (Mirsky and Jessen, 2001). (See Introduction, Chapter 1 and Fig. 1.8. for more details.) Schwann cells are derived from neural crest cells that migrate from the neural tube and associate with embryonic nerve bundles. In the rat by E14/15 these cells have become Schwann cell precursors and are dependent on axon derived signals for survival and proliferation. Immature Schwann cells (E17 to birth) sort axons into smaller groups depending on axon size and develop an autocrine survival loop which enables them to survive independently of axon derived signals during nerve repair. As the Schwann cells mature further, two populations arise: non-myelinating Schwann cells which wrap many smaller calibre axons for each Schwann cell and myelinating Schwann cells which form 1:1 relationships with larger axons, wrapping them in a myelin sheath.

The maintenance of Schwann cell differentiation is dependant on axon-derived signals. When axon interactions are disrupted, Schwann cells dedifferentiate and re-enter the cell cycle in a process called Wallerian degeneration (reviewed by Scherer and Salzer, 2001; see Introduction, Chapter 1). The regenerative response of Schwann cells to nerve injury is thought to be important for successful nerve regeneration. Moreover, this behavioural response to nerve damage can be manipulated to obtain Schwann cells for culture in vitro (Brockes et al., 1979). These cells are isolated by dissociation of rat sciatic nerves and can be cultured in vitro for prolonged periods without transformation (Mathon et al., 2001). Cells isolated in this manner can be used to investigate Schwann cell survival, proliferation and differentiation, since these cells can be induced to re-differentiate and myelinate axons under appropriate culture conditions.

An in vitro assay has been developed that can mimic Schwann cell differentiation to the myelinating form in the absence of neurons. Treatment of Schwann cells with growth factors and forskolin results in proliferation and is routinely used to expand cells in vitro. However, elevation of cAMP levels, by addition of forskolin or dibutyryl cAMP, in the absence of mitogens is associated with the induction of myelination-associated markers, including P0, PMP22 and MBP (Lemke and Chao, 1988, Morgan et al., 1991, (Fig. 3.5. A). Since both elevated cAMP levels in vitro and axon derived signals are capable of
stimulating Schwann cell proliferation and differentiation under different conditions, it has been suggested that cAMP may be involved in signalling between axons and Schwann cells during development. This assay has been used extensively to investigate the regulation of induction of myelin sheath components and myelination-associated transcription factors, with comparable results to those obtained in vivo.

Activation of Raf does not result in Schwann cell differentiation

In order to investigate whether Raf activation resulted in Schwann cell differentiation I decided to use the assay described above to study the mature myelinating Schwann cell, selecting expression of PO (the major myelin sheath protein) as a marker of differentiation. I confirmed that addition of the cAMP elevator, dibutyryl cAMP (dcAMP) was able to cause a strong induction of PO in NSRafER cells in the absence of mitogens (Figure 3.5. B). (PO is detected as a doublet (Archelos et al., 1993). The lower band is a degradation product of the 30kDa protein.) Activation of Raf in these cells did not result in increased PO levels, although p21Cip1 levels increased as expected. Moreover, the levels of PO were reduced to below basal levels by Raf activation. These results demonstrate that Raf activation in primary Schwann cells does not cause differentiation to the myelinating form, despite prolonged MAPK activation. Thus MAPK activation in primary Schwann cells is insufficient to cause differentiation. This is in contrast to primary keratinocytes and PC12 cells, which undergo differentiation in response to oncogenic Ras/Raf activation.

Raf activation can block or reverse PO expression

In the previous experiment I found that Raf activation in primary Schwann cells did not result in differentiation, but was associated with a reduction in PO levels to below basal levels (Fig. 3.5. B). In view of this observation I decided to investigate whether Raf activation was able to block Schwann cell differentiation. NSRafER cells were treated with Tmx, dcAMP or dcAMP and Tmx simultaneously. The resulting cellular morphology is shown in Fig. 3.6.B. Differentiated cells were enlarged and flattened compared to control cells, whilst cells with activated Raf were elongated, refractive and showed loss of contact inhibition of movement. Raf activation in Schwann cells treated with dcAMP resulted in a similar morphology to cells with Raf activation alone. Interestingly, in Schwann cells under
Abundant myelin sheath proteins e.g. ↑ P0, MBP, PMP22, periaxin.

Changes in transcription factor expression - e.g. ↑ Krox 20, Oct-6.

Fig. 3.5. Raf activation does not cause P0 expression.
(A) Schematic diagram of the differentiation assay, showing characteristic changes in the expression patterns of differentiation associated proteins. (B) NSRafER cells were treated with either 1 mM dibutyryl cAMP (D), control solvents (C) or Tmx for 48hrs. Protein lysates were Western blotted with antibodies for the specified proteins.
differentiating conditions activated Raf prevented the induction of P0 expression and reduced P0 levels to below basal (Fig. 3.6.A), whilst increasing p21\textsuperscript{Cip1} levels. Thus Raf activation appears to block Schwann cell differentiation.

One major advantage of the RafER system is that Raf activity is rapidly inducible by the addition of tamoxifen (Tmx), whereas in the absence of Tmx NSRafER cells behave as normal. Thus Schwann cells can be allowed to differentiate in response to elevated cAMP levels prior to activation of Raf. In order to investigate the effect of Raf activation in differentiated cells, I treated cells with dcAMP to allow differentiation to occur, before activating Raf in one set of differentiated cells. Activation of Raf resulted in downregulation of P0 expression and a corresponding induction of p21\textsuperscript{Cip1} (Fig. 3.7), suggesting that Raf activation was reversing the differentiated phenotype.

To confirm that Tmx addition was not responsible for decreasing P0 levels independently of Raf activation, I repeated the experiments described above using Schwann cells lacking the ΔRaf-1:ER construct. Addition of Tmx to differentiating cells had no effect on the expression of P0 (Fig. 3.8.A), with P0 levels comparable to dcAMP alone. In differentiated cells, addition of Tmx or β-estradiol was unable to alter P0 levels (Fig.3.8.B), thus proving that the observed downregulation of P0 (Fig. 3.6. and 3.7.) was indeed due to the presence of activated Raf.

**Raf activation causes downregulation of P0 mRNA levels**

In Schwann cells during differentiation in vivo, or following treatment with cAMP elevators in vitro, the expression of P0 increases at both the mRNA and protein levels (Lemke and Chao, 1988; Morgan et al., 1994). Downregulation of P0 mRNA and protein levels have been implicated in the process of Wallerian degeneration, which is associated with Schwann cell dedifferentiation (Scherer and Salzer, 2001). I have shown that activation of Raf in primary Schwann cells was able to reverse P0 expression or block P0 induction in cells under differentiating conditions. Since P0 expression is regulated at the mRNA level in response to axon derived stimuli and dcAMP (Lemke and Chao, 1988, Desarnaud et al., 1998), I decided to investigate the mechanism of the Raf induced
Fig. 3.6. Raf activation can block p0 expression.

(A) NSRafER cells were treated with either control solvents (C), 1mM dibutyryl cAMP (D), or dibutyryl cAMP and Tmx (D + Tmx) for 72hrs, or Tmx alone for 48hrs. 30μg of protein lysate was Western blotted with the stated antibodies. (B) Phase micrographs of NSRafER cells treated with control solvents (i), dcAMP (ii), Tmx (iii) or dcAMP and Tmx (iv). Scale bar, 20μm.
Fig. 3.7. Raf activation can reverse p0 expression.
NSRafER cells were treated with dibutylryl cAMP or control solvents for 72hrs, then TMX (D-> D+Tmx) was added where stated, for 48hrs. Western blots were carried out as described in Fig. 3.6. A.
Fig. 3.8. Upregulation of pO levels in primary Schwann cells is unaffected by the addition of tamoxifen or β-estradiol.

(A) Primary Schwann cells were treated with dibutyryl cAMP (D), control solvents (C) or dibutyryl cAMP and Tmx (D+Tmx) for 72hrs, or Tmx alone for 48hrs prior to Western blotting with the specified antibody.

(B) Primary Schwann cells were treated with dibutyryl cAMP or control solvents for 72hrs, then TMX (D→ D+Tmx) or 500nM β-estradiol (D→ D+E) were added where stated, for 48hrs. 30μg of protein lysate was Western blotted with anti-p0 antiserum.
downregulation of PO protein levels by looking at the effect of Raf activation on PO mRNA levels.

NSRafER cells were treated with Tmx, dcAMP or dcAMP and Tmx. In parallel a second set of cells were allowed to differentiate, prior to the activation of Raf. The levels of PO mRNA were analysed by Northern blotting. In both cases activation of Raf resulted in decreased expression of PO mRNA to below basal levels (Fig.3.9.). Therefore Raf is capable of downregulating PO at the mRNA level. Since cAMP elevation in Schwann cells has been shown to result in increased PO mRNA via increased activity at the PO promoter (Desarnaud et al., 1998), Raf activation may be associated with a reduction in transcription of PO.

**Raf activation results in Schwann cell dedifferentiation**

To examine whether Raf activation was inducing Schwann cell differentiation I chose to use the myelin sheath protein PO as a marker of differentiation. However, since Raf activation was able to downregulate PO levels in differentiated cells, I decided to look at the effect of Raf activation on the myelin-associated proteins periaxin, MBP and PMP22 to determine whether Raf was causing Schwann cell dedifferentiation. PO is the most abundant myelin sheath protein (Lemke and Axel, 1985), however, MBP and PMP22 are also expressed at significant levels in compact myelin in response to axon derived signals during development and by elevated cAMP levels *in vitro* (Lemke and Chao, 1988; Desarnaud et al., 1998). Periaxin is expressed exclusively in Schwann cells of the peripheral nervous system during early myelination in response to axon derived stimuli and has been implicated in stabilizing Schwann cell-axon interactions (Scherer et al., 1995; Gillespie et al., 2000).

Due to the lack of suitable antibodies for Western blotting (and the low levels of expression of some of the factors investigated later) most of this work was carried out using semi-quantitative RT-PCR. The results presented are representative of two independent experiments, with each PCR being carried out at least twice. PO mRNA levels were also investigated using this technique to confirm that the results were similar to those obtained
Fig. 3.9. Raf activation results in decreased PO mRNA levels.

(A) NSRafER cells were treated with control solvents, dibutyryl cAMP and Tmx as described in Fig. 3.6. 10μg mRNA was extracted and analysed by Northern blotting for PO levels. (B) Quantification of the results shown in (A).
by Northern blotting (Fig. 3.9.B and Fig. 3.10.B). I found that activation of Raf in NSRafER cells under differentiating conditions blocked the induction of both MBP and PMP22 mRNA and resulted in a reduction of mRNA levels to below detectable and basal levels respectively (Fig. 3.10.B and C). In addition, activating Raf in differentiated cells downregulated MBP and PMP22 mRNA to undetectable levels.

Activation of Raf in NSRafER cells under differentiating conditions reduced periaxin mRNA to undetectable levels, with Raf activation in differentiated cells having a similar effect (Figure 3.10.). Furthermore, Raf activation in Schwann cells under differentiating conditions greatly reduced the induction of periaxin protein. However, activation of Raf in differentiated cells did not alter periaxin protein levels. These findings could suggest that periaxin protein has a long half-life and that prolonged activation of Raf in differentiated cells could downregulate periaxin protein levels. Examination of the expression patterns of periaxin mRNA and protein during development support this idea (Gillespie et al., 1994).

In the rat sciatic nerve, periaxin mRNA levels peak at postnatal day 15, before falling sharply to just above background by day 21. In comparison, periaxin protein levels peak within a similar time frame and then decrease gradually, with a substantial decrease in protein levels only occurring 30-150 days after birth (as the animals reach maturity). As a result there is a lag period between the reduction in periaxin mRNA levels and the resulting decrease in protein expression. This could also explain the presence of slightly increased levels of periaxin protein, despite reduced mRNA levels, in cells with Raf activation under differentiating conditions. If the effect of Raf activation on periaxin and P0 mRNA levels occurred after a lag period, elevated cAMP levels could induce myelin gene mRNA expression prior to inhibition by downstream effectors of Raf and since periaxin has a long half-life, the resulting protein would not be rapidly degraded. In contrast, I have shown that P0 protein levels decrease rapidly, within 48hrs, in response to a Raf induced reduction in mRNA levels (Fig. 3.7. and 3.9 A.), suggesting that P0 protein has a shorter half-life than periaxin.
Fig. 3.10. Raf activation can block or reverse Schwann cell differentiation - downregulation of myelination markers.

(A) Western blot analysis of periaxin levels in response to Raf activation. (B) Semi-quantitative RT-PCR analysis of expression patterns of the specified myelination markers. See Fig. 3.6. and 3.7. for details of experimental conditions. (C) Quantification of the results shown in (B).
Fig. 3.10. cont.
(C) Quantification of the semi-quantitative RT-PCR analysis shown in (B).
These results indicate that Raf activation is capable of regulating the expression of a number of myelination-associated proteins, demonstrating that Raf is changing the differentiation state of the Schwann cells, rather than having a specific effect on P0 alone. Thus Raf activation is able both to block the differentiation of primary Schwann cells and cause Schwann cell dedifferentiation.

**Raf activation can alter the expression of transcription factors associated with differentiation**

Differentiation from immature Schwann cells to mature myelinating or non-myelinating Schwann cells involves characteristic changes in gene expression in response to axon-derived signals. Several transcription factors have been implicated in the development of the myelinating phenotype, including Oct-6, Krox-24 and Krox-20. Oct-6 expression is transiently induced in differentiating Schwann cells in response to axon-derived signals (Scherer *et al.*, 1994). In addition, *Oct 6/-* mouse Schwann cells undergo a transient arrest at the promyelinating stage, delaying final myelination by about two weeks, suggesting a positive role for Oct-6 in the expression of myelination-associated genes (Jaegle *et al.*, 1996). In *vivo* Krox-20 levels are upregulated prior to the induction of myelin gene expression (Zorick *et al.*, 1996) and reduced in Wallerian degeneration in association with Schwann cell dedifferentiation (Topilko *et al.*, 1997). Krox-20 has been shown to activate P0 reporter constructs *in vitro* (Zorick, 1999). Moreover, mutations in the *Krox-20* gene have been implicated in human neuropathies (Warner *et al.*, 1998) and the resulting mutant proteins have lost the ability to transactivate myelin gene expression *in vitro* (Zorick, 1999; Nagarajan *et al.*, 2001). In addition, *Krox-20 -/-* mice have impaired Schwann cell differentiation (Topilko *et al.*, 1994). Thus Krox-20 appears to play an important role in regulating myelination. In contrast, Krox-24 expression is confined to Schwann cell precursors and downregulation of Krox-24 levels may be important for myelination (Topilko *et al.*, 1997). Since transcriptional control has been implicated in the expression of myelin sheath components and differentiation (Lemke and Chao, 1988; Desarnaud *et al.*, 1998), I decided to examine whether Raf activation was able to alter the expression of Oct-6, Krox-24 and Krox-20 as a mechanism of regulating differentiation. The expression
patterns of these transcription factors are summarised in Fig. 3.11. (A more detailed account is presented in the Introduction, Chapter 1.)

In order to investigate the mechanism of the downregulation of myelination-associated proteins by Raf, I examined the effects of Raf activation on the expression of the differentiation-associated transcription factors Oct-6, Krox-20 and Krox-24. As previously reported (Monuki et al., 1989), Oct-6 protein expression was induced in differentiating cells (Fig. 3.12.A). Raf activation blocked the induction of Oct-6 protein in cells treated with dCAMP and downregulated Oct-6 expression in differentiated cells. This pattern was repeated at the mRNA level (Fig. 3.12.B). Raf activation had a similar effect on Krox-20 mRNA expression, reducing levels to below detectable in both differentiated cells and cells under differentiating conditions. In contrast Krox-24 mRNA expression was induced by Raf activation in cells under differentiating conditions and in differentiated cells. However, Tmx alone did not induce Krox-20 expression, suggesting that synergy between cAMP and Raf signalling is required for Krox-24 induction. Since Krox-24 is not expressed in myelinating cells, but in precursor and immature Schwann cells this result is consistent with the idea that Raf activation is altering the differentiation state of the cell by reversing the phenotype to an earlier developmental stage. This pattern of reduced Krox-20 and elevated Krox-24 levels is similar to that seen during Wallerian degeneration, where the Schwann cells dedifferentiate to an earlier proliferative stage to aid nerve repair. Upon re-innervation and re-myelination the levels of Krox-20 increase and Krox-24 decrease in a similar manner to during normal development (Topilko et al., 1997).

**Raf activation in differentiating or differentiated cells is associated with induction of cyclin dependent kinase inhibitors, cyclin D1 and p19ARF**

Induction of CDKIs is associated with differentiation in a number of primary cell types, including keratinocytes and oligodendrocytes, and is thought to be important to ensure cell cycle withdrawal. In keratinocytes, for example, increased levels of p21Cip1, p27Kip1 and p16INK4a are associated with, but not sufficient for, differentiation (Harvat et al., 1998). Raf activation in proliferating Schwann cells results in a cell cycle arrest associated with the induction of cyclin D1, p19ARF and the CDKIs p21Cip1 and p15INK4b (Lloyd et al., 1997 and...
Fig. 3.11. Expression patterns of some of the transcription factors involved in Schwann cell differentiation to the myelinating form. See text and Introduction for details. PO is used to represent the expression of the myelin sheath proteins. However, PO is also expressed at low levels prior to myelination in the Schwann cell lineage (Lee et al., 1997). Abundance refers to each transcription factor independently. Sc, Schwann cell.
Fig. 3.12 The effect of Raf activation on transcription factors involved in Schwann cell differentiation.

(A) Western blot analysis of Oct-6 levels in response to Raf activation. (B) Semi-quantitative RT-PCR analysis of expression patterns of the myelination-associated transcription factors Krox-20, Krox-24 and Oct-6. See Fig.3.6. and 3.7. for details of experimental conditions. (C) Quantification of the results shown in (B).
Fig. 3.12 cont.

(C) Quantification of the semi-quantitative RT-PCR analysis shown in (B).
results described above). In view of the association of CDKI induction with differentiation in other cell types and the induction of CDKIs, with cyclin D1 and p19\textsuperscript{ARF}, in response to Raf activation in primary Schwann cells, I decided to examine whether differentiation of these cells was associated with induction of p19\textsuperscript{ARF}, Cyclin D1, and CDKIs and the effect of Raf activation on the expression of these proteins.

Elevation of cAMP levels in the absence of mitogens resulted in increased expression of p21\textsuperscript{Cip1}, but this increase was small in comparison to the levels of p21\textsuperscript{Cip1} induced by Raf activation (compare Fig.3.15. lanes C and D). p27\textsuperscript{Kip} induction is associated with differentiation of both keratinocytes and oligodendrocytes (Missero et al., 1995; Durand et al., 1997; Durand 1998). Moreover, p27\textsuperscript{Kip} is thought to form part of the timing mechanism involved in regulating oligodendrocyte differentiation (Durand et al., 1998). Differentiation of primary Schwann cells resulted in increased p27\textsuperscript{Kip} levels (Fig. 3.13.). Raf activation in these cells resulted in a downregulation of p27\textsuperscript{Kip} levels, providing further support for the idea that Raf activation is causing Schwann cell dedifferentiation. In addition, activation of Raf in cells under differentiating conditions or in differentiated cells resulted in the induction of cyclin D1, p21\textsuperscript{Cip1}, p15\textsuperscript{NK4b} and p19\textsuperscript{ARF} (Fig. 3.6, 3.7. and 3.13.). Thus the Raf signal is dominant over the differentiation signal provided by elevated cAMP signalling.

In summary, activation of Raf in primary Schwann cells blocks differentiation. In addition, Raf activation is able to induce Schwann cell dedifferentiation. The Raf induced reduction in myelination-associated protein expression is associated with decreased levels of their mRNA, and may be mediated by downregulation of Krox-20 and Oct-6 levels. In contrast, Krox-24 is induced in response to Raf activation in differentiated Schwann cells or cells under differentiating conditions. Since Krox-24 is expressed by precursor and immature Schwann cells, this finding suggests that Raf activation is causing Schwann cell dedifferentiation to this earlier stage of development.
Fig. 3.13. The effect of Raf activation on the levels of cyclin D1, p19ARF and the cyclin dependent kinase inhibitors p15INK4b and p27KIP1.

(A) NSRafER cells were treated with either 1mM dibutyryl cAMP, control solvents or dibutyryl cAMP and Tmx for 72hrs, or Tmx alone for 48hrs. 30µg of protein lysate was Western blotted with the stated antibodies. (B) NSRafER cells were treated with dibutyryl cAMP or control solvents for 72hrs, then TMX was added to dishes for 48hrs. Western blots were carried out as in (A).
These results are of interest for a number of reasons. Peripheral nerve damage triggers a process called Wallerian degeneration, whereby mature, axon associated Schwann cells downregulate the expression of myelination associated genes and undergo dedifferentiation to an earlier, proliferative, developmental stage (Scherer and Salzer, 2001). This regenerative ability of Schwann cells is thought to be important for successful nerve repair. Moreover, Wallerian degeneration is also associated with the upregulation of the transcription factor Krox-24, which is expressed in Schwann cells prior to development of a pro-myelinating phenotype. However, the pathways regulating the regenerative capacity of Schwann cells are poorly understood. My findings raise the possibility that Raf signalling is involved in mediating the signals responsible for this process. The ability of Raf activation to prevent and reverse differentiation also has implications for tumour biology. Activation of this oncogenic pathway appears to push the cells towards a less differentiated, potentially more proliferative state. These findings may have important implications for the genetic disorder NF type1. Sufferers of this disease are predisposed to developing tumours, including benign peripheral nerve neurofibromas in which Schwann cells are frequently observed in the absence of nerve contact. The observed disruption of Schwann cell-axon interactions in neurofibromas could be caused by high levels of Ras/Raf signalling, resulting in altered Schwann cell differentiation.

**Inhibition of the MAPK pathway prevents Schwann cell dedifferentiation in response to Raf activation.**

To address whether the effects of Raf activation on Schwann cell differentiation are occurring via activation of the MAPK pathway, I carried out a series of experiments using the MEK inhibitor, U0126. Using this inhibitor at 30µM reduced the level of MAPK activity, as measured by ERK1/2 phosphorylation, to below detectable levels (Figure 3.14, see Materials and Methods for more details). NSRafER cells were treated with Tmx under differentiating conditions or following differentiation in the presence/absence of 30µM U0126. To ensure that MEK inhibition occurred prior to activation of the MAPK pathway by Raf, U0126 was added 15 minutes prior to the Tmx.
Addition of the MEK inhibitor to Schwann cells under differentiating conditions did not prevent P0 induction, suggesting that MAPK activity is not required for differentiation (Fig. 3.14.). In agreement with this finding, inhibition of the MAPK pathway during myelination in Schwann cell-dorsal root ganglion (DRG) co-cultures has no effect on the development of myelinated axon segments (Maurel and Salzer, 2000). In the presence of U0126 and dcAMP, Raf activation was unable to block the induction of P0. This demonstrates that the MAPK pathway is mediating the Raf induced downregulation of P0 levels. Moreover, in differentiated cells, Raf activation resulted in reduced P0 expression, except in the presence of the MEK inhibitor (Fig. 3.15.). Thus signalling via the MAPK pathway is necessary for the dedifferentiation of myelinating Schwann cells in response to Raf activation.

Decreasing the activity of the MAPK pathway is insufficient to cause differentiation

The inhibitor experiments described above show that the MAPK pathway is required to mediate the effects of Raf activation on Schwann cell differentiation. In addition we made two interesting observations. (1) The differentiation of primary Schwann cells by cAMP was associated with decreased levels of MAPK activity (Fig. 3.14.), raising the possibility that inhibition of the MAPK pathway was required for differentiation to occur. (2) In previously differentiated cells, inhibition of the MAPK pathway resulted in elevated levels of P0 compared to cells treated with dcAMP alone (Fig. 3.15), suggesting that P0 expression, and thus Schwann cell differentiation, are negatively regulated by the MAPK pathway. As a result we decided to investigate whether inhibition of the MAPK pathway was sufficient to drive Schwann cell differentiation.

Primary Schwann cells were treated with increasing concentrations of MEK inhibitor with the aim of decreasing MAPK activity from basal to undetectable in the absence of dcAMP (Fig. 3.16. A). If the MAPK pathway was negatively regulating P0 expression then inhibition of this pathway should result in either dose dependent changes in P0 levels or strong induction of P0 once MAPK activity has fallen below the threshold required for differentiation. Inhibition of the MAPK pathway, however, had little effect on P0 levels, except to cause a slight decrease at higher concentrations (Fig.3.16. A). Thus reducing the
Fig. 3.14. Inhibition of the MAPK pathway is associated with Schwann cell differentiation and prevents Raf from blocking differentiation.

(A) NSRafER cells were treated with control solvents, dibutyryl cAMP, Tmx or dibutyryl cAMP and Tmx for 48hrs in the presence or absence of 30μM MEK inhibitor, U0126. The Tmx was added 15 mins after U0126. The dishes were fed into the same conditions after 24hrs and lysed after a total of 48hrs. 30μg of each lysate was analysed by Western blotting with the specified antibodies.
Fig. 3.15. Raf activation is unable to reverse differentiation in the presence of a MEK inhibitor.

NSRafER cells were treated with control solvents or dibutyryl cAMP for 72hrs. 30μM U0126 was then added to one set of dishes with fresh control solvent or dibutyryl cAMP, along with Tmx, where stated. The Tmx was added 15mins after U0126. The dishes were fed into the same conditions after 24hrs and protein lysates were made after a total of 48hrs, for analysis by Western blotting with the specified antibodies.
Fig. 3. Decreasing the activity of the MAPK pathway is insufficient to cause differentiation.

(A) NSRafER cells were treated with the specified dose of U0126, a MEK inhibitor, for 24hrs. Dishes were then fed with fresh inhibitor and medium for a further 24hrs, before lysing. Western blots were carried out using anti-phospho (P) - MAPK, p0 and α- tubulin antibodies. (B) NSRafER cells were treated as in (A) with the addition of 1mM dibutyryl cAMP for the duration of the experiment.
activity of the MAPK pathway is insufficient to cause differentiation of primary Schwann
cells. We then decided to investigate whether inhibition of the MAPK pathway under
differentiating conditions was able to increase the expression of differentiation markers, as
suggested in Fig. 3.15. However, inhibition of the MAPK pathway, in the presence of
dcAMP, did not increase P0 expression compared to dcAMP alone (Fig.3.16.B).

In summary, activation of the MAPK pathway is responsible for mediating the inhibitory
effects of Raf activation on Schwann cell differentiation. Schwann cell differentiation is
associated with reduced MAPK activity, although inhibition of the MAPK pathway alone is
insufficient to induce differentiation. This result, however, does not exclude the possibility
that reduced MAPK activity is required to allow differentiation and under certain
conditions inhibition of the MAPK pathway is observed to result in increased P0
expression.
Chapter 4: Examining the effects of Raf activation on Schwann cell–axon interactions.

The regenerative capacity of Schwann cells is thought to be essential for successful nerve repair, although the signalling pathways mediating this process are poorly understood (reviewed by Scherer and Salzer, 2001). In the previous chapter, I demonstrated that activation of the Raf/MAPK pathway in primary Schwann cells in vitro blocked their ability to differentiate. Moreover, in differentiated cells, Raf activation resulted in Schwann cell dedifferentiation. These findings were obtained using an in vitro assay where high cAMP levels drive Schwann cell differentiation. However, the role of cAMP levels in regulating the myelination process in vivo is controversial. Elevating cAMP levels is capable of stimulating Schwann cell proliferation and the expression of differentiation markers in a similar manner to axon derived signals, implicating cAMP as a potential mediator of neuronal signals during Schwann cell development (Lemke and Chao, 1988, Morgan et al., 1991). In support of a role for cAMP in regulating Schwann cell myelination, inhibition of cAMP dependent protein kinase (PKA) has been shown to inhibit myelination in response to axon derived stimuli in DRG–Schwann cell co-cultures (Howe and McCarthy, 2000). However, elevation of cAMP levels in normal, crushed or transected sciatic nerve explants is insufficient to induce expression of P0 and MBP (Poduslo et al., 1995). In addition, following nerve injury cAMP levels increase with slower kinetics than the induction of P0 making it unlikely that cAMP is involved in initial myelin gene expression, although this does not rule out a role in the later stages of myelination, possibly in myelin sheath maintenance. Thus the actual role of cAMP in differentiation remains unclear and other pathways are likely to be involved.

My work has implicated activation of the Raf/MAPK pathway in Schwann cell dedifferentiation and is potentially of relevance for Wallerian degeneration and tumour formation by Schwann cells. As cAMP is not necessarily the differentiation signal in vivo, I chose to make use of two more physiological experimental systems that will enable me to investigate the effects of activation of the MAPK pathway on Schwann cell differentiation.
in response to axon derived signals. The two approaches I have taken are Schwann cell-neuron co-cultures and the development of a transgenic mouse model.

Schwann cell differentiation is thought to involve a complex series of axon–Schwann cell interactions (Mirsky and Jessen, 2001). Initially, Schwann cells precursors recognise axons and migrate towards them. They then associate and proliferate in response to neuronal stimuli to populate the axon network, becoming immature Schwann cells over time. Finally the Schwann cells cease proliferation and differentiate. Axon derived signals determine whether the Schwann cells will become non- myelinating or myelinating (Aguayo et al., 1976). Smaller axons are bundled into groups by single non-myelinating Schwann cells, whilst larger axons associate with myelinating Schwann cells in a 1:1 relationship and are ensheathed in myelin. Schwann cell differentiation, however, is reversible. Nerve damage results in the alteration or disruption of Schwann cell–axon interactions, causing Schwann cell dedifferentiation and proliferation in a process called Wallerian degeneration. As nerve repair proceeds, the Schwann cells re-associate with axons and differentiate in a manner similar to that during development (Scherer and Salzer, 2001). Thus neuronal signals play an important role in initiating and maintaining Schwann cell differentiation. However, the nature and mechanisms of action of these signals are poorly understood, although several transcription factors (Scherer et al., 1994; Murphy et al., 1996; Topilko et al., 1997) and potential mitogens (Morgan et al., 1994; Einheber et al., 1995, Guenard et al., 1995; Zanazzi et al., 2001) have been implicated.

An *in vitro* assay system has been developed which allows investigation of Schwann cell-axon interactions. This system is based on the culture of rat dorsal root ganglion (DRG) neurons, which produce complex axon networks in response to NGF (Wood and Bunge, 1975). Endogenous fibroblasts and Schwann cells can be killed by treatment with mitotic inhibitors to leave a bare axon network, which can be repopulated with exogenous Schwann cells as required. Myelination of the axons by the Schwann cells is facilitated by the addition of ascorbic acid in the presence of serum, which enables basal lamina formation. This has been shown to be essential for formation of myelin sheath segments by myelination inducing axons (Fernandez-Valle et al., 1993).
DRG-Schwann cell co-cultures have been used extensively to examine the interactions between Schwann cells and axons leading to myelination, with the results being comparable to \textit{in vivo} and the \textit{in vitro} cAMP elevation assay. This system has been used to investigate the transcription factors Krox-20 and Oct-6 and the induction of myelin sheath gene expression in response to axon derived signals (Scherer \textit{et al.}, 1994; Murphy \textit{et al.}, 1996; Fernandez-Valle \textit{et al.}, 1993). In addition, co-cultures have enabled the isolation of factors capable of downregulating myelin gene expression, which may play important roles in preventing premature differentiation during development and following Wallerian degeneration. These include TGFβ1-3 (Einheber \textit{et al.}, 1995; Guenard \textit{et al.}, 1995) and GGF (Zanazzi \textit{et al.}, 2001). The PI3K pathway has also been implicated in the initial events of myelination using this system (Maurel and Salzer, 2000).

Since activation of Raf in Schwann cells \textit{in vitro} can block differentiation and cause dedifferentiation, I decided to use the DRG-Schwann cell co-culture system to investigate the effects of Raf activation at each stage of axon-Schwann cell interaction. These include:

- the initial recognition and migration to the axons by the Schwann cells;
- association of the Schwann cells with axons, followed by proliferation and migration across the axon network, then cell cycle exit and a more permanent association with individual or groups of axons;
- formation of a myelin sheath by Schwann cells associated with myelination inducing axons.

At each stage I planned to investigate the effects of Raf activation on the establishment and maintenance of these interactions. In the assays described below I looked at recognition and association at the edges of axon networks, where I can focus on interactions between small numbers of Schwann cells and individual axons.

**Schwann cells recognise and associate with DRG axons**

The factors involved in the recognition and association of Schwann cells with axons are poorly understood, but it has been suggested that GGF secretion by the peripheral nerves may be involved (Mahanthappa \textit{et al.}, 1996). In the following experiments Schwann cells
were counted as associated if they were aligned along an axon (with the nucleus on/next to the axon) or if they were spread specifically over several axons in areas of axon crossing. Schwann cells at 30 minutes post-addition were counted as associated if the nuclei were situated on the axons. Schwann cells found in close proximity to axons were only counted as associated if the Schwann cell body was in contact with the axon.

To confirm that the co-culture system was working successfully, I decided to look at the recognition and association of Schwann cells with axons in the absence of activated Raf signalling. I dissected DRG from postnatal day zero rats and cultured them as explants, with NGF, in the absence of serum for a week, with two pulses of treatment with the mitotic inhibitor cytosine β-D-arabinofuranoside (AraC) to kill any endogenous proliferating cells and ensure that the axon network was bare. Addition of NSRafER cells, in the absence of Tmx, resulted in a rapid recognition of the axons by these cells, followed by association of the Schwann cells with axons within a period of a few hours. This work was carried out using time-lapse microscopy and immunofluorescence of fixed co-cultures (Fig. 4.1, see Movie ‘Controls before’). At 30 minutes post-plating the Schwann cells were randomly spread with relation to the axon network (Fig. 4.1.A, E), but with some Schwann cells settling on or near to axons. In contrast, nearly 100% of the Schwann cells were associated with the axons by 48hrs (Fig. 4.1.B, E) and in many cases had assumed an elongated morphology along a single axon (Fig. 4.1.C). However, the Schwann cells remained motile, migrating over the axon network and proliferating in response to axon derived stimuli. Thus under normal culture conditions NSRafER cells associate with axons rapidly and remain associated over time, although the cells continue to be motile for prolonged periods.
**Fig. 4.1. Schwann cells recognise and associate with DRG axons.**

DRG explants were cultured in defined medium with (A, B, C) or without (D) NSRafER cells. The co-cultures were fixed at 0.5hrs (A) or 48hrs (B, C, D) post Schwann cell addition and stained for neuron neurofilaments (mAb RT-97, red), nuclei (Hoescht, blue) (A, B, D) and Schwann cells (anti-S100 antibody, green) (C). Scale bar, 20μm. (E) The mean percentage association of Schwann cells increases significantly over time (p<0.001), with SEM of 0.7% and 5.7% for 0.5hrs and 48hrs respectively. n=358 cells (0.5hrs), n=598 cells (48hrs) over 4 different experiments.
Perineural fibroblasts do not recognise and associate with DRG axons over time

The peripheral nerve is a highly complex structure with Schwann cell-axon units in an ECM surrounded by fibroblasts and perineural cells to form fascicles, several of which form a nerve fibre (Landon and Hall, 1976; Low, 1976). During sciatic nerve dissection and dissociation to obtain the pure Schwann cell cultures used in these experiments, fibroblasts are removed by immunopanning (Cheng et al., 1998) and can be maintained in culture. Perineural fibroblasts were isolated in this manner and added to bare DRG axons to provide a negative control for association, since in vivo perineural fibroblasts do not interact directly with axons. Fibroblasts were considered to be associated if an axon was found along the length of the cell with the nucleus on or near to the axon.

I added perineural fibroblasts to DRG cultures, then fixed the co-cultures after 30 minutes or 48hrs and analysed the number of associated cells by immunofluorescence. At 30 minutes post-addition, a few fibroblasts were randomly associated with axons (Fig.4.2). However, in contrast to Schwann cells, by 48hrs the majority of the perineural fibroblasts had not associated with the DRG axons. If cultures were allowed to continue for longer than 48hrs, the fibroblasts rapidly formed a confluent monolayer without apparent reference to the axons. In contrast, Schwann cells, left to proliferate in response to axon derived signals over a week or more, were seen to be preferentially associated with the axon network, with areas of empty coverslip next to dense patches of Schwann cells aligned with axons. Thus the Schwann cells recognised and migrated to the axons, prior to specific association, whereas the fibroblasts showed no preference for axons.

Confirming that the Schwann cells in DRG-Schwann cell co-cultures are NSRafER cells

To ensure that the Schwann cells under investigation in the following experiments were NSRafER cells (rather than endogenous Schwann cells), I serially infected NSRafER
**Fig. 4.2. Perineural fibroblasts do not associate with DRG axons over time.**

DRG explants were cultured with perineural fibroblasts for 0.5hrs (A) or 48hrs (B). Cocultures were stained with anti-Thy-1 antibody (axons and fibroblasts, red) and Hoescht (nuclei, blue). Scale bar, 20μm. (C) The mean association is shown as a percentage of total for both fibroblasts and Schwann cells over time with n >400 cells, over 3 separate experiments. The SEM are ±3%, ±7% for the fibroblasts and ±6%, ±5% for the Schwann cells at 0.5hrs and 48hrs respectively. The percentage association was significantly higher at 48hrs than 0.5hrs in both fibroblasts (p<0.001) and Schwann cells (p<0.001).
cells with an EGFP expressing retrovirus to obtain a Schwann cell population that was about 80% EGFP positive. Addition of these cells to DRG axons allowed confirmation that the cells counted in these experiments were not endogenous cells that had proliferated in the absence of mitotic inhibitor to populate the DRG axon network. One such example is shown in Fig.4.3, where NSRafER EGFP cells were allowed to associate with axons. The majority of the cells are EGFP positive. In addition, the non-EGFP positive cells still probably derive from the NSRafER EGFP cells since control cultures remained uniformly free of Schwann cells around the edges of the axon network (Fig.4.1.D). An occasional contaminating Schwann cell was observed close to the DRG explant, but this did not effect assay results as counting was confined to the edge of the axon network. Thus the Schwann cells under investigation in the following experiments were the added NSRafER cells.

**Schwann cells with activated Raf are still able to rapidly associate with axons**

I decided to investigate the effect of Raf activation on Schwann cell-axon interactions using the DRG-Schwann cell co-culture system. This assay allows investigation of the effects of Raf activation on the establishment and maintenance of Schwann cell-axon interactions. To examine the effects of Raf activation on the establishment of these interactions, I used NSRafER cells that had been previously treated with Tmx for 24 hours to activate Raf prior to addition to DRG cultures. This time period is sufficient for the full morphological changes associated with Raf activation to become apparent. NSRafER cells that had been allowed to associate with axons for 48hrs, before Raf activation, were used to investigate the maintenance of Schwann cell-axon interactions. The movement of the Schwann cells over time was monitored using time-lapse phase contrast microscopy and immunofluorescence of fixed co-cultures.

To investigate whether activation of Raf in primary Schwann cells prevented recognition of the axons and association by the Schwann cells, I treated NSRafER cells with Tmx for 24hrs, before addition to the DRG culture. Tmx was maintained in the culture for the duration of the experiment. Initially the cells settled randomly, but rapidly produced
Fig. 4.3. The Schwann cells in DRG - Schwann cell co-cultures are NSRafER.
NSRafER cells were serially infected with an EGFP-expressing retrovirus such that greater than 80% cells were EGFP-positive. Cells were added to DRG cultures and allowed to associate. (A) Phase contrast, (B) fluorescence and (C) merged images. Scalebar 100μm.
membrane protrusions and began moving in a confined area (Fig.4.4.A). Following recognition of an axon, the Schwann cells began migrating along the axon network. Recognition and initial association occurred rapidly since by 1.5hrs post-addition Schwann cells that had landed on or very near to axons had already associated (Fig.4.4B.), whilst Schwann cells that landed further away from the axons took a further 3-4 hours to associate (Fig. 4.4.C). (This can be clearly seen in the initial few seconds of the time-lapse Movie ‘Before’) In all cases, however, the presence of an activated Raf signal was unable to prevent recognition and initial association of the Schwann cells with the axon network.

The majority of Schwann cells with Raf activated prior to association remain stably associated with DRG axons

To investigate whether Raf activation altered the association of Schwann cells with axons, I allowed the Tmx treated Schwann cells to associate with axons, as described above, and fixed the cultures 24hrs later to look at the establishment of a stable association (Fig. 4.5.). Schwann cells with activated Raf, which had successfully recognised and associated with the axons, did not dissociate from the axons in substantial numbers over time (Fig.4.4.B, C, D), although a small reduction in the percentage of associated cells was observed compared to control cultures. However, during further examination of the fixed and stained co-cultures it was clear that a greater number of Schwann cells with activated Raf appeared to be less well associated with the axons compared to control cultures (compare Fig.4.1.C. and Fig. 4.5.C). These Schwann cells appeared to be in the process of moving between axons, whereas Schwann cells without activated Raf were more uniformly aligned or clearly spread over a point where several axons crossed. An example of this is shown in Fig.4.5.C, where the marked Schwann cell is not fully associated with an axon and the cell in the top right hand corner also appears to be caught in the process of moving between axons. This is also reflected in the small, but significant (p<0.001) decrease in the number of associated cells compared to control cells over the 24hrs. These findings suggest that, although Raf activation does
Fig. 4.4. Schwann cells with activated Raf are still able to rapidly associate with axons.

NSRafER cells were treated with 100nM Tmx for 24hrs prior to addition to DRG cultures, and again treated with fresh Tmx. Co-cultures were observed using time-lapse microscopy over a period of 30hrs and analysed using Openlab software. Scalebar, 40μm. (A) 15 minutes, (B) 1.5hrs and (C) 4hrs post Schwann cell addition. Three examples of associating Schwann cells are marked with arrows.
**Fig. 4.5. The majority of Schwann cells with prior activation of Raf remain stably associated with axons.**

NSRafER cells were treated with Tmx (B, C) or control solvents (A) for 24hrs in order to activate Raf prior to addition to DRG axons. Tmx treatment was continued for a further 24hrs post DRG addition (B, C). Co-cultures were fixed and stained with mAb RT97 (axons, red), Hoescht (nuclei, blue) (A,B) and anti-S100antibody (Schwann cells, green) (C). Note that the marked Schwann cell (*) in (C) appears to be poorly associated with the axon and may be in the process of moving between two axons. Scale bar, 20μm. (D) Raf activation results in a significant reduction in association (p<0.001). The mean percentage association is shown for n >600 cells per condition over 4 independent experiments with the SEM being 0.05 and 0.2 for Control (C, control solvents) and Tmx respectively.
not prevent the recognition and initial association of the Schwann cells with axons, Raf signalling is reducing the number of fully associated cells.

**Raf activation in Schwann cells stably associated with axons does not cause the majority of these cells to lose axon contact**

I then decided to investigate whether Raf activation was able to disrupt the maintenance of stable Schwann cell-axon interactions. The RafER system has a major advantage over using Schwann cells with disrupted expression of Neurofibromin or continual oncogenic Ras expression, in that association and differentiation can be allowed to proceed as normal prior to rapid activation of Raf signalling by Tmx addition. I allowed NSRafER cells to associate with DRG axons for 2 days prior to addition of Tmx for 30-48hrs. Activation of Raf in nerve associated Schwann cells resulted in a small, but significant (p< 0.001 at 30hrs, p< 0.05 at 48hrs) decrease in Schwann cell association (Fig. 4.6.). However, only a small number of non-associated cells and very few poorly associated Schwann cells were observed, in comparison to activation of Raf prior to addition to the axon network (Fig. 4.5. C and D, compared to Fig.4.6. C and D). Thus it appears that prior association of Schwann cells with axons is able to partially overcome the effect of Raf activation, resulting in reduced Schwann cell dissociation from the axon network.

**Individual Schwann cells with activated Raf can dissociate from DRG axons**

I have shown that activation of Raf does not affect the ability of Schwann cells to recognise and associate with axons or disrupt the maintenance of an established association. However, under both experimental conditions a small, but significant, number of cells were found to be poorly associated with the axon network as a result of Raf activation. In addition, I made two interesting observations by examining the time-lapse data generated by these experiments. Firstly, I noticed that a few individual Schwann cells dissociated from the axon network under both experimental conditions (Fig.4.7. and time-lapse Movies ‘Before’, ‘After’, ‘Control before’ and ‘Control after’). Secondly, the Schwann cells appeared to be more motile following Raf activation.
Schwann cells remain associated with axons following Raf activation.

NSRafER cells were allowed to associate with axons for 48hrs prior to the addition of control solvents (A) or 100nM Tmx (B, C) for for 30hrs. Co-cultures were fixed and stained with mAb RT97 (axons, red), Hoescht (nuclei, blue) (A,B) and anti-S100 antibody (Schwann cells, green) (C). Scale bar, 20μm. (D) A significant number of Schwann cells with activated Raf lose axonal contact (p< 0.001, 30hrs Tmx and p< 0.05 48hrs). The mean percentage association is shown with n > 600 for control (C) and Tmx 30hrs and n= 200 for Tmx 48hrs, over four separate experiments. The SEM were 5%, 4% ,5% respectively.
Fig. 4.7. Schwann cells with activated Raf can dissociate from DRG axons. (A,B) NSRafER cells were treated with Tmx for 24hrs prior to addition to the DRG cultures and then with fresh Tmx for a further 24hrs. Co-cultures were filmed and the resulting time-lapse data analysed using Openlab software. (A) After 4hrs the Schwann cells have associated with the axons. (B) Schwann cells can be seen leaving the axons in this frame, taken at 17hrs. One such example is marked with an arrow. Scale bar, 40µm.
Fig. 4.7. cont. (C,D) NSRafER cells were allowed to associate with axons for 48hrs (C) prior to the addition of 100mM Tmx for 48hrs (D). In this frame, taken at 19hrs after Tmx addition, some cells (marked with arrows) have dissociated from the axons. Scale bar, 40μm.
This was especially noticeable with activation of Raf in previously associated cells (see Movie ‘After’).

Schwann cells with Raf activation prior to addition to the DRG culture (Movie ‘Before’), or with Raf activation following a few days of axon association (Movie ‘After’), were seen moving rapidly between axons, resulting in a distortion of the axon network in that area. During this movement the majority of Schwann cells remained in contact with one axon whilst reaching out and making contact with the next axon, then rapidly aligned themselves with the new axon; or alternatively travelled along the axon network directly. However, in some cases the Schwann cells lost contact with one axon before reaching the next, resulting in a brief period of axon independent movement (Fig.4.7.). Occasionally the Schwann cells remained dissociated for longer periods. In contrast to Schwann cells with activated Raf, control cells associated with axons rapidly and did not dissociate over time, despite initially moving fairly rapidly around the axon network (See time-lapse Movies ‘Control before’ and ‘Control after’ for initial axon association by Schwann cells and movement following several days of association.)

Thus, although Raf activation did not prevent Schwann cell association or cause mass dissociation of previously associated cells, Schwann cells with activated Raf were observed to move independently of the axon network for varying lengths of time. The majority of cells, however, remained associated with the axon network and dissociated cells usually became re-associated over time.

**Individual Schwann cells with activated Raf exhibit increased motility**

I also observed that as the effects of Raf signalling became apparent, the Schwann cells appeared to migrate at a faster rate across the axons with the result that the entire axon network shifted under the force of their movement. This was especially noticeable in previously associated Schwann cells (Movie ‘After’). In comparison, Schwann cells in control cultures were either relatively motile or fairly stationary depending on the age of the co-culture (see Movies ‘Control before’ and ‘Control after’), but did not have a
similar effect on the axon network or appear to move at the same rate as cells with activated Raf signalling.

As many Schwann cells showed long periods of inactivity I decided to compare the more active cells in a population to investigate whether the speed (μm/hr) of Schwann cell motility was altered by Raf activation. Since individual Schwann cells with activated Raf often moved at varying speeds over the duration of a time-lapse movie, I determined the maximum speed for each selected cell as well as the average and minimum speeds of these cells. To calculate the speed (μm/hr) I plotted the distances travelled for each selected cell over a series of set time intervals and examined several time-lapse movies per experimental condition. An example of this analysis is shown in Fig. 4.8.A. and the results are shown in Fig. 4.8. B-F.

Raf activation in Schwann cells before addition to DRG cultures (Fig.4.8.C) resulted in a range of maximum speeds that were similar to control cells, although the most active cell with activated Raf had a maximum speed that was 1.4 fold greater than the equivalent control cell. In addition, the range of average speeds of the most active cells with prior Raf activation completely overlapped with the equivalent control cells (Fig.4.8.E) and neither the maximum or average speeds of these cells were significantly faster than control cells. In contrast, Schwann cells with Raf activation following axon association showed a significant increase in maximum speed compared to control cells (Fig.4.8.D, p<0.05) and were also significantly faster (p<0.05) on average than control cells (Fig.4.8.F). The difference in results between Schwann cells with Raf activation prior to or following axon association may be partly due to the relatively fast movement of control Schwann cells during recognition and initial association with axons, whereas cells which have been allowed to associate for several days frequently move at a much slower rate (see Movies ‘Control before’ and ‘Control after’). These cells may be about to permanently associate with axons, prior to myelination with the appropriate culture conditions.
**Fig. 4.8. Raf activation is associated with increased Schwann cell motility.**

(A) Sample timelapse micrograph marked with the paths and selection of speeds (μm/hr) for two NSRaf:ER cells. The cells had been treated with Tmx for 24hrs prior to addition to the DRG culture. t = 15 minutes post-Schwann cell addition. The lines divide the cell paths into hours and the marked speeds are in μm/hr. Scale bar, 40μm. (B) Table to show the speeds of the five most active cells for each condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Maximum speed (μm/hr)</th>
<th>Minimum speed (μm/hr)</th>
<th>Average speed (μm/hr)</th>
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</thead>
<tbody>
<tr>
<td>NSRaf:ER added to DRG after 24hrs</td>
<td>170</td>
<td>64</td>
<td>102</td>
</tr>
<tr>
<td>Tmx</td>
<td>151</td>
<td>11</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>151</td>
<td>11</td>
<td>62</td>
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<tr>
<td></td>
<td>158</td>
<td>6</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>245</td>
<td>1</td>
<td>64</td>
</tr>
<tr>
<td>Control minus Tmx</td>
<td>178</td>
<td>17</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>112</td>
<td>9</td>
<td>56</td>
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<td></td>
<td>153</td>
<td>9</td>
<td>63</td>
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<td>115</td>
<td>27</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>92</td>
<td>7</td>
<td>64</td>
</tr>
<tr>
<td>NSRaf:ER with Tmx added after association</td>
<td>240</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>134</td>
<td>27</td>
<td>92</td>
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<tr>
<td></td>
<td>154</td>
<td>4</td>
<td>75</td>
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<td></td>
<td>462</td>
<td>3</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>154</td>
<td>2</td>
<td>75</td>
</tr>
<tr>
<td>Control minus Tmx</td>
<td>121</td>
<td>58</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>77</td>
<td>21</td>
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<td></td>
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</table>
Fig. 4.8. cont. (C-F) Presentation of the results shown in (B). (C, D) Maximum speeds of the five most active Schwann cells treated with/without Tmx before addition to DRG culture (C) or with/without Tmx added after association (D). The maximum speed of the most active Schwann cells with Raf activation following association (D) were significantly faster than the equivalent control cells (p<0.05). (E-F) Average speeds of the same cells over the duration of the time-lapse recording with Raf activated before (E) or after (F) addition to the DRG culture. Schwann cells with activation of Raf after axonal associated (F) were also faster on average than control cells (p< 0.05).
In summary, activation of the Raf/MAPK pathway does not affect the ability of Schwann cells to recognise and associate with axons. In addition, activation of Raf in Schwann cells following previous axon association does not result in the dissociation of the majority of the Schwann cell population. However, under both experimental conditions a number of individual Schwann cells with activated Raf were able to dissociate from the axon network for varying periods of time. Moreover, Raf activation in Schwann cells previously allowed to associate with axons resulted in a significant increase in the speed of some cells. Thus Raf activation in Schwann cells prior to or following axon association has a number of subtle effects on Schwann cell behaviour. The resulting changes in Schwann cell-axon interactions could be important for understanding the mechanisms involved in neurofibroma development and Wallerian degeneration.

Myelination assays

I have shown that Raf activation results in Schwann cell dedifferentiation and blocks Schwann cell differentiation via the MAPK pathway. However, to investigate the effects of Raf activation under more physiological conditions I have chosen to use a Schwann cell-DRG co-culture system where Schwann cells can be stimulated to differentiate in response to neuronal signals. This system will enable me to examine the effects of Raf activation on both the establishment and maintenance of myelination, by examining any effects on the number and structure of myelin sheath segments present. I have yet to complete these experiments as I am currently optimising the dissociated DRG culture conditions. However, if Raf has a similar effect on Schwann cell differentiation under these conditions to that observed using the cAMP elevation assay, I might expect that Raf activation in Schwann cells under myelinating conditions would block the expression of myelin sheath components and thus interfere with myelin sheath assembly. Following Raf activation in differentiated cells I might expect to see a reduction in myelin sheath protein expression, which could destabilise existing myelin segments and result in demyelination.
Chapter 5: Generation of RafTR transgenic mice

In the previous chapters I showed that Raf/MAPK activation results in Schwann cell dedifferentiation, with potential implications for Schwann cell behaviour during Wallerian degeneration and in the genetic disorder NF1. In order to determine the effect of Raf activation in Schwann cells differentiated in response to neuronal signals, I decided to use a DRG-Schwann cell co-culture system and a transgenic mouse model. In this chapter I will detail the generation of a transgenic mouse expressing an inducible Raf molecule specifically in myelinating Schwann cells. This will enable me to investigate whether activation of the Raf/MAPK pathway leads to dedifferentiation in vivo.

In order to prevent the premature activation of Raf by estrogen in transgenic embryos I have used a modified form of the RafER that only responds to the estrogen antagonist tamoxifen. The Raf TR consists of the kinase domain of human Raf-1 fused to the mouse estrogen receptor hormone-binding domain, which has a point mutation resulting in the amino acid substitution G525R that renders the construct unresponsive to estrogen (Fig.5.1.A) (Danielian et al., 1993); (Littlewood et al., 1995). A similar construct, c-MycER™ (c-mycTR), has been used successfully to generate transgenic mice (Pelengaris et al., 1999).

I decided to target RafTR expression to myelinating Schwann cells using the pPG6 vector, which has been used successfully by a number of groups for this purpose (Messing et al., 1992; Giovannini et al., 1999); see Fig.5.3.). This construct consists of a 1.1kb fragment of the rat P0 promoter with a rabbit β-globin 3’ splicing and polyadenylation signal flanking a multiple cloning site with a pBluescript vector backbone (Messing et al., 1992). The pPG6 vector has been shown to regulate transgene expression in myelinating Schwann cells in a comparable manner to wild-type P0; which is mainly expressed in myelinating Schwann cells, although low levels have been detected in neural crest cells and embryonic Schwann cells during development (Lemke and Axel, 1985; Messing et al., 1992; Lee et al., 1997; Giovannini et al., 1999).
Cloning strategy and construct testing

PCR cloning RafTR into LXSN3

RafTR LXSP3 was a kind gift from H. Land. I was unable to excise RafTR from RafTR LXSP3 for subcloning due to the absence of suitable restriction endonuclease sites, therefore I decided to use PCR to clone the RafTR sequence. The RafTR was amplified using primers with BamHI adaptors and the purified fragment subcloned into the BamHI site of the LXSN3 vector to allow confirmation that the PCR cloned RafTR sequence was functional, prior to generation of the transgenic construct (Fig.5.1.A, see Materials and Methods for details). To confirm that PCR cloning had not introduced any sequence errors the construct was sequenced and tested in a functional assay. As shown in Fig.5.1B-E, NIH 3T3 cells infected with RafTR LXSN3 were unresponsive to β-estradiol addition, but responded to Tmx confirming the specificity of the construct. In addition, sequencing of the construct confirmed that no errors had been introduced during PCR amplification.

Subcloning RafTR into p IRES2-EGFP

In order to follow the expression of RafTR, we decided to construct a vector that would express EGFP concomitantly with RafTR. To do this RafTR was subcloned into the pIRES2-EGFP vector (Clontech), which contains a multiple cloning site upstream of an IRES-EGFP sequence. Upon expression of this vector a single mRNA transcript is produced containing the subcloned gene of interest and IRES-EGFP.

The RafTR was excised from RafTR LXSN3 and subcloned into the BamHI site in the polylinker of the pIRES2-EGFP vector to generate pRafTR IRES-EGFP (Fig.5.2.). I confirmed co-expression of RafTR and EGFP by transfection of NIH 3T3 cells with pRafTR IRES-EGFP and Western blotting for the Raf and TR proteins (Fig.5.2.B.). The NIH 3T3 cells transfected with pRafTR IRES-EGFP were EGFP positive and upon treatment with Tmx appeared elongated and refractile, demonstrating that RafTR and EGFP were being co-expressed. I was unable to show co-expression of EGFP and RafTR.
**Fig. 5.1. The RafTR is specifically activated by Tmx addition.**

(A) Diagram of the structure of the RafTR construct following PCR cloning into LXSN3. The construct consists of the catalytic domain (CR3) of human Raf-1 fused to the mouse Estrogen Receptor hormone binding domain by a short linker sequence (bright green). The relevant amino acids (aa) encoded by the Raf/ER sequences used are shown below the construct. The ER has been converted to TR (only activated by Tmx addition) by the aa substitution G525R. The BamHI containing adaptors used in subsequent cloning steps are shown in yellow. (B-E) NIH 3T3 cells were retrovirally infected with LXSN3 vector (B,C) or RafTR LXN3 (D,E) before treatment with β-estradiol (B,D) or Tmx (C,E) for 30hrs. Scale bar, 40 μm.
Fig. 5.2. Diagram of the pRafTR IRES-EGFP cloning strategy and construct testing.

(A) A BamHI fragment containing the RafTR was subcloned into the BamHI site in the target vector, pIRES2-EGFP, to form pRafTR IRES-EGFP. Following confirmation of the correct orientation using a combination of PCR and restriction digests, a SacII / NotI fragment containing RafTR IRES-EGFP was generated by restriction endonuclease treatment. The fragment was treated with Klenow DNA polymerase to generate a blunt ended product, destroying the SacII / NotI sites. (MCS) multiple cloning site. (B) NIH 3T3 cells were transfected with the pRafTR IRES-EGFP construct. 2 days post transfection, the cells were treated with / without Tmx for 30hrs, then analysed for RafTR expression by Western blotting using anti-Raf and anti-ER antibodies. (See Materials and Methods for more details.)
by immunofluorescence due to the presence of endogenous Raf in NS cells and the lack of a suitable anti-ER antibody. Thus to confirm RafTR expression I analysed the transfected cells by Western blotting and detected elevated levels of Raf in cells transfected with pRafTR IRES-EGFP compared to vector alone (Fig. 5.2 B). In addition, these cells contained detectable levels of ER, which is not expressed endogenously in NIH 3T3 cells, suggesting that the TR was also expressed. In conclusion, using the pRafTR IRES-EGFP vector I was able to achieve co-expression of RafTR and EGFP in NIH 3T3 cells.

Construction of the pPG6 RafTR IRES-EGFP expression vector
Due to the lack of a suitable 5' restriction site for excision of the final transgenic construct prior to microinjection, I inserted a Sac II containing oligonucleotide adaptor into the Kpn I site in the pPG6 vector to provide a 5' restriction site that was not also present in the construct (Fig.5.3). Subsequently RafTR IRES-EGFP was excised and blunt cloned into the Eco RV site of the pPG6 polylinker to generate pPG6 RafTR IRES-EGFP.

To confirm that pPG6 RafTR IRES-EGFP was expressed specifically under differentiating conditions I tested the construct in vitro in non-differentiated and differentiated primary Schwann cells. The efficiency of transfection was monitored using β- galactosidase expression. NS cells transfected with pRafTR IRES-EGFP were EGFP positive in both the presence and absence of dcAMP (Fig.5.4.A, B). In contrast, in dishes of pPG6 RafTR IRES-EGFP transfected cells without dcAMP no EGFP positive cells were detected, whereas with dcAMP treatment EGFP positive cells were observed (Fig.5.4.C, D, E). Thus EGFP and the RafTR are expressed specifically in differentiated Schwann cells transfected with pPG6 RafTR IRES EGFP.
Fig. 5.3. Diagram of the pPG6 RafTR IRES-EGFP cloning strategy. The transgenic construct was made by inserting the RafTR IRES-EGFP fragment into the the EcoRV site in the MCS (multiple cloning site) of the pPG6 vector. Prior manipulation of the pPG6 construct inserted a second Sac II site (in place of Kpn I) to allow excision of the full construct, containing the RafTR IRES-EGFP flanked by the rat P0 promoter and the rabbit β-globin 3’ splicing and polyadenylation signal. (See text and Materials and Methods for more details.)
Fig. 5.4. The pPG6 RafTR IRES-EGFP construct is active in vitro under differentiating conditions.

NS cells were grown to confluency under normal growth conditions, before being transferred to defined medium with dcAMP or control solvents for 2 days. Cells were then transfected with PCDNA3.1 lac Z and RafTR-IRES EGFP (A, B) or PCDNA3.1 lac Z and pPG6 RafTR -IRES EGFP (C, D) and treated with (B, D) or without (A, C) dcAMP (as before) for a further 2 days. Scale bar 40μm. (Control dishes were mock transfected or transfected with PCDNA control vector and treated as above.)
E) Phase contrast micrographs of cells transfected with PCDNA3.1 lac Z and RafTR-IRES EGFP (i, ii) or PCDNA3.1 lac Z and pPG6 RafTR - IRES EGFP (iii, iv) and treated with (ii, iv) or without (i, iii) dcAMP as described for A-D. Cells have been stained for β-galactosidase activity (blue cells) to determine the transfection efficiency. Scale bar 40μm. F) EGFP positive cells were counted in 120 fields of view using an inverted fluorescent microscope, before staining for β-galactosidase activity (as detailed in Materials and Methods). Samples were normalized for β-galactosidase activity to control for variations in transfection efficiency. The mean values +/- SEM are shown from 2 separate experiments with 2 dishes per condition.
Examination of the RafTR transgenic mice

Generation of potential transgenic mice and screening for the presence of the transgene in gDNA

The pPG6 RafTR IRES-EGFP construct was sent to our collaborators at the Breakthrough Breast Cancer Centre. The final construct containing RafTR IRES-EGFP under the control of the p0 promoter was excised and microinjected into embryos of the B6CBF1 line, which were then implanted into pseudopregnant females and allowed to develop to term.

To determine whether the resulting pups carried the transgene, I analysed genomic DNA from the tail tips of the potential transgenic mice using Southern blotting (Fig. 5.5.A). Animals 957 and 963 were positive for the transgene using this technique, which was confirmed using PCR amplification across the RafTR boundary (Fig. 5.5.B). (PCR was used in all subsequent screening for the transgene.) These results showed that I had two mice carrying the pPG6 RafTR IRES-EGFP transgene. In order to confirm germline transmission the offspring of animals 963 and 957 were screened by PCR for the presence of the transgene (Fig. 5.6.). In both cases, the founder mouse passed on the transgene to a proportion of her offspring, confirming that I had two lines of pG6 RafTR IRES-EGFP transgenic mice.

Investigation of transgene expression in the two lines of transgenic mice

Since both founder mice carried the transgene I then determined whether they expressed RafTR and EGFP protein in myelinating Schwann cells. I was unable to detect RafTR expression by Northern analysis of pooled sciatic nerves, probably as a result of problems with sensitivity due to the relatively few mice available at the time. To overcome this problem I examined the expression of RafTR using RT-PCR, including reverse transcriptase negative controls to confirm that genomic DNA was not responsible for any positive results obtained (Fig. 5.7). RT-PCR analysis of both lines resulted in the
Fig. 5.5. pPG6-RafTR IRES-EGFP is detectable in 957 and 963 transgenic mice.

(A) Genomic DNA was extracted from mouse tail tips and subjected to \textit{XmnI} restriction endonuclease treatment to generate fragments of genomic DNA. The restriction digests were analysed by Southern blotting, using an α\textsuperscript{32}P labelled RafTR IRES-EGFP fragment as a probe. The expected size of fragment generated by \textit{XmnI} treatment of pPG6-RafTR-IRES EGFP was 3kb. Positive mice are shown in bold. (Mouse 962 was only screened by PCR.)
Fig. 5.5 cont. (B) PCR amplification of a 475bp fragment of genomic DNA, spanning the RafTR boundary, was used to confirm the presence of pPG6-RafTR-IRES-EGFP in genomic DNA of 957 and 963 transgenic mice. (See materials and methods for details of primers and conditions used.) Positive mice are shown in bold. Mouse 967 was from the parental strain and thus known to be negative for the transgene. The positive control reaction used RafTR-LXSN3 as a template.
**Fig. 5.6. Germline transmission is observed in both parental lines.**
The offspring of the two transgenic mice identified in Fig. 5.5. were PCR screened, as described before, for the presence of pPG6-RafTR -IRES EGFP DNA. Since numbers 777, 781-4 were positive for presence of the construct, germline transmission had occurred in both parental lines. (Positive mice are shown in bold.)
Fig.5.7. The 963 line expresses RafTR mRNA.
The expression of mRNA by the two transgenic lines was determined using RT-PCR. mRNA was extracted from the sciatic nerves of pools of 957 or 963 line mice and treated with RNase free DNase to remove any contaminating DNA. The mRNA was then converted to cDNA by reverse transcription and the presence of cDNA was confirmed by PCR for GAPDH. Control reactions lacking reverse transcriptase (RT) were also carried out to allow detection of DNA contamination. The cDNA was screened for RafTR in a similar manner to Fig.4.6, but using fewer cycles for amplification. (See Materials and Methods for details.)
detection of RafTR in cDNA derived from 963 line transgene positive mice only, confirming that the 963 line is expressing RafTR mRNA in a peripheral nerve. In contrast the 957 line does not appear to be expressing RafTR mRNA.

To determine whether RafTR and EGFP proteins were produced in the peripheral nerves of my transgenic lines, I decided to analyse sciatic nerves using Western blotting. I was however, unable to detect expression of these proteins in sciatic nerve-derived lysates. Since lysates from control NS cells infected with RafTR or EGFP expressing retroviruses were positive for these proteins, the Western blotting analysis was not at fault. Moreover, the successful extraction of myelinating Schwann cell protein was confirmed by the detection of p0 in the sciatic nerve-derived lysates. However, in future I will examine the expression of a less abundant myelination associated protein, such as periaxin, in the sciatic nerve-derived lysates to confirm that the extraction process is suitably efficient. I will also increase the amount of sciatic nerve-derived protein under analysis to overcome any sensitivity problems.

In summary, I have generated two pPG6 RafTR IRES-EGFP transgenic lines and have shown that the 963 line expresses RafTR mRNA in a peripheral nerve, suggesting that the construct is functioning as required in this transgenic line. In the future I will generate further pPG6 RafTR IRES-EGFP transgenic lines to ensure that any phenotypical changes detected are not due to positional effects caused by transgene insertion. I will also confirm the expression of RafTR in sciatic nerves using a combination of RT-PCR to detect mRNA and Western blotting for protein expression. Subsequently I will determine the expression pattern of RafTR/EGFP in the transgenic lines by RT-PCR and/ or Western blotting to ensure that the proteins are specifically expressed in peripheral nerves and use immunofluorescence of sciatic nerve cryosections to confirm that RafTR expression in the peripheral nerve is confined to Schwann cells.

I will then be able to carry out a number of experiments:
1. To analyse the effects of Raf activation in the transgenic mice I will treat the animals with Tmx in drinking water or by injection into muscle surrounding the sciatic nerve
and look for physiological changes over a relatively short time period. Since activation of the RafER \textit{in vitro} results in Schwann cell dedifferentiation, I expect that activation of the RafTR in the transgenic mice will cause the downregulation of myelination-associated proteins, potentially resulting in demyelination, and may have a gross effect on movements, such as walking, as a result. Raf activation could also result in the disruption of Schwann cell-axon interactions potentially predisposing the Schwann cells to unregulated proliferation. I will analyse any Raf induced changes in the ultrastructure of peripheral nerves by cyrosectioning and EM.

2. \textit{In vitro} Raf activation is insufficient to cause Schwann cell proliferation and instead results in a p53 dependent cell cycle arrest. Therefore activation of Raf in the RafTR transgenic mice may result in Schwann cell dedifferentiation but is not expected to induce proliferation as well. To investigate whether Raf activation in the absence of p53 function is able to induce Schwann cell dedifferentiation and proliferation I will generate \textit{p53} null RafTR transgenic mice. To do this I shall cross the RafTR transgenic with \textit{p53}+/- mice to produce RafTR \textit{p53}+/- mice, which can be intercrossed to generate RafTR \textit{p53} -/- mice as required.
Chapter 6: Discussion
Characterising the Raf induced arrest in primary Schwann cells

Oncogenic Ras signalling in many primary cell types results in a cell cycle arrest associated with the induction of CDKIs, which is mediated by the Raf/MAPK pathway. However, the mechanism responsible for the induction of these molecules appears to differ between cell types. For example, in primary MEFs oncogenic Ras expression results in the induction of p21\textsuperscript{Cip1}, which is thought to be due to p19\textsuperscript{ARF} mediated activation of p53, and increased levels of p16\textsuperscript{INK4a} and p15\textsuperscript{INK4b} (Serrano et al., 1997; Palmero et al., 1998; Malumbres et al., 2000). In contrast in primary HDFs, the oncogenic Ras/Raf induced arrest is associated with the induction of p16\textsuperscript{INK4a} and p21\textsuperscript{Cip1} in a p19\textsuperscript{ARF} independent manner (Serrano et al., 1997; Zhu, 1998; Wei et al., 2001).

In primary Schwann cells activation of Raf results in a p53 dependent cell cycle arrest that is mediated, at least in part, by the induction of p21\textsuperscript{Cip1} (Lloyd et al., 1997). I have shown that Raf activation also induces p15\textsuperscript{INK4b} expression, which may also be involved in mediating the cell cycle arrest in these cells. This is similar to the situation observed with primary MEFs, which arrest in a p15\textsuperscript{INK4b} dependent manner in response to oncogenic Ras (Malumbres et al., 2000). In contrast, I observed that p19\textsuperscript{INK4d} levels decrease in response to Raf activation in primary Schwann cells, whilst p18\textsuperscript{INK4c} levels remain unchanged. Therefore neither p19\textsuperscript{INK4d} nor p18\textsuperscript{INK4c} are involved in mediating the Raf induced cell cycle arrest. This is not especially unexpected as p19\textsuperscript{INK4d} and p18\textsuperscript{INK4c} have been implicated in mediating cell cycle arrests during development and terminal differentiation of a number of cell types, including myoblasts and adipocytes, rather than inducing an arrest following oncogenic stress (Zindy et al., 1997a; Zindy et al., 1997b; Phelps et al., 1998).

Surprisingly I observed that p16\textsuperscript{INK4a} levels also decrease in response to Raf activation and therefore this CDKI is not involved in mediating the cell cycle arrest in primary Schwann cells. This is in contrast to a number of primary cell types where p16\textsuperscript{INK4a} expression is induced in response to oncogenic Ras/Raf signalling (Serrano et al., 1997; Zhu, 1998). Moreover in HDFs, p16\textsuperscript{INK4a} induction is associated with a cell cycle arrest in p53 or p21\textsuperscript{Cip1} null cells and mutation of p16\textsuperscript{INK4a} in HDFs derived from a familial melanoma patient.
appears to abrogate the Ras induced arrest, suggesting that p16\textsuperscript{INK4a} is an important mediator of the oncogenic Ras/Raf induced cell cycle arrest in this cell type (Zhu, 1998; Wei \textit{et al.}, 2001). p16\textsuperscript{INK4a} levels can be regulated by a number of transcription factors (Ohtani \textit{et al.}, 2001; Jacobs \textit{et al.}, 1999), however, the reduction in p16\textsuperscript{INK4a} protein levels following Raf activation was not due to reduced mRNA levels, suggesting that an as yet unidentified post-transcriptional mechanism is responsible for this effect. The reduction in p16\textsuperscript{INK4a} protein levels and the induction of cyclin D1 may form part of the mechanism by which Ras/Raf signalling promotes cell cycle progression in primary Schwann cells under appropriate environmental conditions.

The importance of p21\textsuperscript{Cip1} in mediating the Raf induced arrest in primary Schwann cells has been clearly demonstrated using p21\textsuperscript{Cip1} antisense RNA, which partially abrogates the cell cycle arrest (Lloyd \textit{et al.}, 1997). The mechanism of p21\textsuperscript{Cip1} induction by oncogenic Ras/Raf in these cells is unclear, however, I found that p21\textsuperscript{Cip1} induction is associated with elevated p21\textsuperscript{Cip1} RNA levels, which is consistent with increased transcription. Since the Raf induced arrest in primary Schwann cells is p53 dependent (Lloyd \textit{et al.}, 1997), an increase in p53 transcriptional activity may be responsible for the increased p21\textsuperscript{Cip1} levels. However, although p21\textsuperscript{Cip1} is a transcriptional target of p53 in response to stimuli such as DNA damage and oncogenic stress, p21\textsuperscript{Cip1} can also be induced independently of p53 in response to oncogenic Ras/Raf by the transcription factors E2F1 and Sp1/2 in other cell types (Serrano \textit{et al.}, 1997; Woods \textit{et al.}, 1997; Kivinen \textit{et al.}, 1999; Gartel \textit{et al.}, 2000). Moreover, in other circumstances p21\textsuperscript{Cip1} induction can be mediated by transcription factors such as IRF-1 that require the presence of basal levels of p53 (Tanaka \textit{et al.}, 1996). Alternatively the elevated levels of p21\textsuperscript{Cip1} RNA could indicate an increase in RNA stability.

Since the increase in p21\textsuperscript{Cip1} RNA is relatively small compared to the fold induction of protein, it is possible that other mechanisms are also involved in increasing p21\textsuperscript{Cip1} protein levels. It has been demonstrated that p21\textsuperscript{Cip1} levels can be regulated post-transcriptionally. For example C/EBP\textalpha{} (CCAAT/ enhancer –binding protein \textalpha{}) induces elevated p21\textsuperscript{Cip1} protein levels via a combination of increased p21\textsuperscript{Cip1} RNA and post-translational
stabilisation of the p21\textsuperscript{CIP}\textsubscript{1} protein (Timchenko \textit{et al.}, 1996), whilst conditional expression of oncogenic Ras in NIH3T3 cells resulted in increased protein synthesis in the absence of enhanced transcription or protein stability, suggesting that increased translation was occurring (Kivinen \textit{et al.}, 1999). However, I found that Raf activation in primary Schwann cells does not increase p21\textsuperscript{CIP}\textsubscript{1} protein stability.

In primary MEFs the oncogenic Ras induced cell cycle arrest is associated with increased p53 levels (Serrano \textit{et al.}, 1997). In contrast, I showed that p53 levels do not increase in response to Raf activation in primary Schwann cells. Since p53 activity is normally associated with increased stability this may suggest that p53 is not actively involved in transcription of p21\textsuperscript{CIP}\textsubscript{1} in response to Raf activation in these cells. A similar situation exists in HDFs where activation of Raf results in the induction of p21\textsuperscript{CIP}\textsubscript{1} in a p53 dependent manner, but is not associated with a detectable increase in p53 levels (Zhu 1998). However, p53 stabilisation is not essential for activation of p53 in NIH3T3 cells and MEFs (Korgaonkar \textit{et al.}, 2002), suggesting that p53 activity could be increased in primary Schwann cells in the absence of elevated p53 levels. In HDFs and MEFs p53 activity can also be enhanced by interaction with PML in response to oncogenic Ras/Raf and it is possible a similar interaction may occur in primary Schwann cells, however this interaction might also be expected to result in increased p53 levels (Ferbeyre, 2000) Pearson \textit{et al.}, 2000).

Mutant p53 proteins, which are used as dominant negative inhibitors of wild-type p53 activity, have also been observed to inhibit the functions of the p53 homologues p63 and p73, raising the possibility that the Raf induced arrest in primary Schwann cells is dependent on the functions of either or both molecules instead of p53 ([Yang, \textit{et al.}, 2002; Di Como \textit{et al.}, 1999; Strano \textit{et al.}, 2002). However, p63 and p73 are rarely mutated in human cancers, p73 null mice are not predisposed to tumorigenesis and p73 inactivation is not required for viral transformation of cells, suggesting that p63 and p73 may not be tumour suppressor proteins (Marin \textit{et al.}, 1998; Yang \textit{et al.}, 2002). However, p63 may be involved in the p53 response to UV, and p73 is stabilised by γ-irradiation and can be induced by oncogenes in p53 deficient cells resulting in apoptosis or the transcription of p53 targets.
(Zaika et al., 2001; Yang et al., 2002). Thus the importance of p63 and p73 in response to p53 activating stimuli remains unclear. It will be interesting to examine whether Raf activation in primary Schwann cells is associated with increased levels and activity of p63 or p73.

p19ARF has been implicated in the regulation of p53 stability and activity in response to oncogenic stress in primary MEFs (Palmero et al., 1998; Damalas et al., 2001). In contrast, in HDFs the oncogenic Ras induced increase in p53 levels appears to be p19ARF independent (Wei et al., 2001). In primary Schwann cells, I observed that Raf activation results in the induction of p19ARF RNA, which is associated with increased p19ARF protein levels. Increased p19ARF RNA levels may reflect increased p19ARF transcription, which may be mediated by the transcription factors E2F (Bates et al., 1998) or DMP1 (Inoue et al., 1999). Alternatively Raf activation may result in the reduction in levels or activity of the transcriptional repressors bmi-1, Twist or TBX2 (Jacobs et al., 1999; Maestro et al., 1999; Jacobs et al., 2000) or may induce increased p19ARF RNA by another mechanism independently of increased transcription. In primary MEFs the oncogenic Ras/Raf induced arrest is p19ARF dependent (Kamijo et al., 1997). However, the importance of p19ARF in mediating the Raf induced arrest in Schwann cells and whether Raf activation increases p53 transcriptional activity via p19ARF induction has yet to be determined. A model for the mechanism of the oncogenic Ras/Raf induced cell cycle arrest based on these results and the previous findings of Lloyd et al., is detailed in Fig. 6.1 (Lloyd et al., 1997).
Fig. 6.1. Model of the mechanism of the oncogenic Ras/Raf induced cell cycle arrest in primary Schwann cells.

Activation of the Raf/MAPK pathway results in a cell cycle arrest associated with elevated levels of the CDKIs p21$^{Cip1}$ and p15$^{INK4b}$ in a p53 dependent manner. However, p16$^{INK4a}$ and p19$^{INK4d}$ levels decrease and so these factors are not involved in inducing the arrest. Regulation of the levels of p15$^{INK4b}$, p16$^{INK4a}$ and p19$^{INK4d}$ by Raf may be mediated by the MAPK pathway, but this has yet to be confirmed. Raf activation also induces p19$^{ARF}$ expression, which may result in p53 activation in the absence of stabilisation of p53 protein levels. Transcriptionally active p53 may directly induce p21Cip1 expression or other transcription factors may be required instead of or as well as p53. The mechanism of Raf/MAPK induction of p15$^{INK4b}$ has yet to be investigated in these cells. Dashed arrows indicate potential interactions. See text for more details.
Examining the effects of Raf activation on Schwann cell differentiation

Ras/Raf/MAPK signalling has been implicated in promoting differentiation of a number of cell types in vitro, including PC12 cells and thymocytes (Marshall, 1995; Bain et al., 2001). Furthermore, in vivo Ras/Raf/MAPK signalling is involved in regulating differentiation in the Drosophila eye and vulval development in C. elegans (Freeman, 1998; Sternberg and Han, 1998). In contrast, I have shown that activation of Raf/MAPK signalling blocks the differentiation of primary Schwann cells and results in Schwann cell dedifferentiation. These experiments were carried out with Schwann cells under non-proliferative conditions, facilitating the examination of the effects of Raf activation on differentiation independently of effects on the cell cycle.

Examining the implications of Raf activation on Schwann cell behaviour during Wallerian degeneration

Following nerve injury it has yet to be determined whether loss of a signal that maintains Schwann cell differentiation, production of a new signal or the release of a signalling molecule from the myelin debris initiates Schwann cell dedifferentiation (Scherer and Salzer, 2001). However, recent work by a number of groups has implicated NRG-1 signalling as a potential regulator of this process. Expression of GGF, a NRG-1 isoform, and the receptor heterodimer proteins ErbB2 and ErbB3 are elevated during Wallerian degeneration (Carroll et al., 1997). Addition of GGF to differentiated Schwann cell-DRG co-cultures has been shown to cause demyelination (Zanazzi et al., 2001). Moreover, ErbB2 phosphorylation has been detected in Schwann cells distal to the nerve damage at a site that couples the receptor to the Ras/Raf/MAPK pathway (Kwon et al., 1997). Thus, in view of these results and my findings on the effects of Raf activation on Schwann cell differentiation, increased GGF signalling following nerve injury could activate the Ras/Raf/MAPK pathway to downregulate expression of myelin genes, leading to Schwann cell dedifferentiation and demyelination. We could examine this possibility in vivo by crushing rat sciatic nerves and determining the effect on Raf/MAPK activity.

I have shown that Raf induced dedifferentiation or inhibition of Schwann cell differentiation is associated with the downregulation of the mRNA levels of a number of...
myelination markers. The expression of these proteins appears to be regulated at the mRNA level during development and Wallerian degeneration by a number of transcription factors including Pax-3 Oct-6, Krox-20 and Sox-10 (Topilko and Meier, 2001). Raf activation reduces the expression of the transcription factors Oct-6 and Krox-20, suggesting a possible mechanism by which the expression of the myelination- associated genes is reduced. Oct-6 and Krox-20 are thought to promote differentiation, with Oct-6 potentially transcriptionally activating expression of Krox-20 which may then induce the transcription of myelin associated genes including P0, MBP, PMP22 and periaxin. Thus the reduction in levels of Oct-6 and Krox-20 by Raf activation may directly result in reduced expression of myelin genes. It will be interesting to examine this possibility further in the future.

Another possible mechanism of decreasing myelin gene expression during Wallerian degeneration or in response to Raf activation may involve the transcriptional repressors Id1/3, which antagonise basic helix-loop-helix (bHLH) transcription factors. Thatikunta and colleagues have shown that Id1/3 can repress myelin gene expression, probably by sequestering bHLH containing transcription factors that promote expression of myelin sheath proteins such as P0 (Thatikunta et al., 1999). Moreover, since Ras/Raf/MAPK activity can induce Id3 expression in thymocytes (Bain et al., 2001). Raf activation in primary Schwann cells may also induce Id3 expression. However, in contrast to Schwann cells, Ras/Raf/MAPK signalling and induction of Id3 is associated with thymocyte differentiation. We will look at the effect of Ras/MAPK signalling on Id1 and Id3 expression in our system in future experiments.

Raf/MAPK activity in Schwann cells differentiated by elevated cAMP also results in the induction of Krox-24, a transcription factor that is expressed during development in precursors and immature Schwann cells and upregulated in dedifferentiating Schwann cells during Wallerian degeneration (Topilko et al., 1997). Interestingly, I have shown that Krox-24 induction appears to require activation of both the Raf/MAPK and cAMP signalling pathways, suggesting that these pathways synergise to induce Krox-24 expression. In contrast, Raf or cAMP signalling alone is insufficient for Krox-24 induction. Moreover, control Schwann cells do not express detectable levels of Krox-24 in vitro under these
culture conditions. The downregulation of Oct-6 and Krox-20 levels concomitant with Krox-24 re-expression suggests that activation of Raf in differentiated Schwann cells (or cells under differentiating conditions) results in dedifferentiation to the immature stage of development, similar to during Wallerian degeneration. This raises the possibility that Raf/MAPK activation may provide the signal that induces Schwann cell dedifferentiation following nerve injury (Fig. 6.2.B.).

I have also shown that Raf/MAPK activation causes the induction of cyclin D1, which is required for Schwann cell proliferation during Wallerian degeneration (Kim et al., 2000). Thus Raf/MAPK signalling may facilitate Schwann cell proliferation, in addition to dedifferentiation, in response to nerve damage. GGF is also able to induce proliferation of dedifferentiated Schwann cells in co-cultures containing demyelinated axons, although proliferation only appears to occur following the clearance of myelin debris (Zanazzi et al., 2001). It is unclear which pathway(s) are responsible for mediating Schwann cell proliferation during Wallerian degeneration or following GGF induced demyelination and it will be interesting to investigate this further using inhibitors of the PI3K and MAPK pathways.

I have suggested that Raf/MAPK signalling may mediate demyelination in response to GGF signalling during Wallerian degeneration. However, Zanazzi and colleagues report that GGF induced demyelination in Schwann cell-DRG co-cultures is associated with the re-expression of Oct-6, which is expressed during the promyelinating stage of Schwann cell differentiation (Zanazzi et al., 2001). In contrast, I have shown that Oct-6 protein and mRNA levels are downregulated by Raf/MAPK activation in association with Krox-24 re-induction, suggesting that these Schwann cells have dedifferentiated to the immature stage of development. The differences in these findings could be due to differences between the assays used. DRG-Schwann cell co-cultures are capable of undergoing myelination in response to neuron derived signals, whereas Schwann cells differentiated by elevated cAMP levels still have elevated levels of Oct-6 with Krox-20 and P0 expression, suggesting that they remain at the promyelinating stage of differentiation. A similar result has been observed in the case of TGFβ treatment of myelinated DRG-Schwann cell co-
cultures, which induces Oct-6 expression, whilst addition of TGFB to cells with elevated cAMP levels results in reduced Oct-6 transcription (Einheber et al., 1995; Guenard et al., 1995; Awatramani et al., 2002). Alternatively the differences in results may reflect the fact that Ras has additional effector pathways and that GGF signalling can also be mediated by other pathways. In the future, completion of the DRG-Schwann cell co-culture experiments will allow me to determine whether activation of Raf/MAPK signalling induces similar changes in neuronally induced myelin gene and transcription factor expression to those found using the cAMP elevation assay.

My results suggest that Raf/MAPK activation result in Schwann cell dedifferentiation to the immature stage of development. However, it remains to be confirmed that Schwann cell trans-differentiation is not occurring since Krox-24 is also expressed in non-myelinating Schwann cells (Topilko et al., 1997). Confirmation of an immature state is complicated by the maintenance of expression of the majority of immature stage markers in non-myelinating cells. In the future examination of the expression of the lipids GalC/ sGalC, which are expressed in myelinating and non-myelinating cells, but not immature Schwann cells, should allow further investigation of Raf induced dedifferentiation to the immature stage.

**Examining the implications of Raf activation on Schwann cell behaviour during development and axon regeneration following injury**

The apparent ability of the Raf/MAPK pathway to block Schwann cell differentiation raises the possibility that this pathway is involved in preventing premature myelin gene expression during development and re-differentiation of Schwann cells following Wallerian degeneration (Fig.6.2.A). Interestingly, Zanazzi and colleagues have shown that GGF addition is able to prevent myelination in DRG-Schwann cell co-cultures, as well as induce demyelination (Zanazzi et al., 2001), and both effects could potentially be mediated by activation of the Ras/Raf/MAPK pathway.

Paradoxically NRG-1 signalling has also been implicated in promoting myelination. Activation of the PI3K pathway is required for Schwann cell proliferation and survival in
response to axon derived stimuli, and for the initial events of myelination in DRG-Schwann cell co-cultures (Maurel and Salzer, 2000). In this context PI3K signalling is largely activated by axon bound NRG-1 (Zanazzi et al., 2001) and is not required for myelin sheath maintenance (Maurel and Salzer, 2000). Moreover, a mouse model with conditional ErbB2 knockout in Schwann cells has hypomyelinated nerves, supporting a role for NRG-1 signalling in myelination (Garratt et al., 2000). Thus it appears that NRG-1 signalling via the PI3K pathway is required for the initial events of myelination, but is dispensable after a few days under myelinating conditions (Maurel and Salzer, 2000). In contrast, MAPK activity is not required for the development of myelinated axon segments in co-cultures. In agreement with this finding, I have shown that inhibition of MAPK signalling does not prevent P0 expression under myelinating conditions. However, GGF is able to inhibit myelination and cause demyelination of axons in vitro (Zanazzi et al., 2001). From my results the negative effects of GGF on myelination could be mediated by the Ras/Raf/MAPK pathway, suggesting that inhibition of MAPK signalling may be required for Schwann cell differentiation and the maintenance of a myelinating phenotype. This idea is also supported by my observation that Schwann cell differentiation in vitro is associated with a reduction in MAPK activity. However, I found that inhibition of MAPK activation was insufficient to cause Schwann cell differentiation as measured by P0 induction and does not result in increased expression of P0 under differentiating conditions. These results do not exclude the possibility that reduced MAPK activity may be required during a specific time period for differentiation to succeed. In support of this idea, inhibition of the MAPK pathway after 3 days under differentiating conditions was associated with elevated P0 levels, suggesting that inhibition of the MAPK pathway, after the initial events of myelination, may promote myelin gene expression. The use of NSRaFER cells with MEK and PI3K pathway inhibitors will enable me to investigate the requirements for PI3K signalling and inhibition of MAPK activity during myelination.
Fig. 6.2. Models for the role of Raf/MAPK signalling during the development of myelinated peripheral nerves (A) and Wallerian degeneration (B).

(A) Axonally produced NRG-1 may activate the Raf/MAPK pathway to inhibit premature myelin gene expression and myelination. PI3K signalling is required for the initial events of myelination and may be activated by NRG-1 produced by axons. As myelination begins PI3K signalling via AKT may inhibit Raf/MAPK signalling, relieving the inhibition of myelin gene expression. However, since inhibition of MAPK activity is insufficient to induce expression of these genes, additional signals are required for successful myelination. NRG-1 signalling is downregulated in the adult nerve.

(B) Following nerve injury NRG-1 may be released from the damaged nerves, activating the Raf/MAPK pathway and resulting in the downregulation of myelin gene expression. This may cause demyelination and induce Schwann cell proliferation or additional signals may also be required. Once successful axonal regeneration has been achieved Raf/MAPK signalling may be reduced to facilitate remyelination in response to other axonal signals. NF1 patients appear to have abnormal responses to wounding. Neurofibromin loss, resulting in constitutively elevated Ras signalling, may disrupt (NF1-/-) Schwann cell re-differentiation and axonal remyelination via activation of the Raf/MAPK pathway. A subpopulation of Schwann cells may retain an immature phenotype and continue proliferating in the cytokine rich environment provided by NF1+/- mast cells initiating neurofibroma formation. Dashed arrows or symbols indicate potential effects or interactions.
NRG-1

ErbB2/3

Ras

PI3K

AKT ?

Myelin gene expression

Additional signals

Peripheral nerve damage

NRG-1

ErbB2/3

Ras

Raf/MAPK

↓Oct-6

↓Krox-20

↓Myelin gene

De-myelination

Schwann cell proliferation

Axonal regeneration and remyelination

Additional signals

Axonal contact and signals

Reduced Raf/MAPK signalling?
To accommodate the different effects of NRG-1 signalling during myelination, Zanazzi and colleagues proposed that NRG-1 signalling via PI3K is required to establish Schwann cell-axon association and the initial events of myelination (Zanazzi et al., 2001). However, after these events are complete NRG-1 signalling becomes inhibitory to myelination. This switch in effect on myelination may coincide with a change to activation of the Ras/Raf/MAPK pathway, which may be used to prevent premature completion of myelination during development and nerve regeneration. This idea is consistent with the downregulation of NRG-1 signalling during development in adult animals. For example CD44, which enhances NRG-1 signalling, and the NRG-1 receptor proteins ErbB2/3 are downregulated during postnatal development (Grinspan et al., 1996; Sherman et al., 2000b) However, elevated GGF levels persist during nerve repair and ErbB2/3 level are not reduced (Carroll et al., 1997), suggesting that re-differentiating Schwann cells may downregulate the effects of NRG-1 signalling by alternative means.

The regulation of Schwann cell differentiation may have similarities to skeletal myoblast differentiation where Ras/Raf/MAPK signalling, during early differentiation, inhibits myoblast fusion and the expression of muscle specific genes (Olson et al., 1987; Bennett and Tonks, 199); Coolican et al., 1997; Dorman and Johnson, 1999; Wu 2000). In addition, similar to Schwann cell differentiation, myoblast fusion can also be inhibited by TGFβ and FGF, and mitogen induced expression of Id factors has been found to prevent the expression of muscle specific genes by antagonising the activity of myogenic regulatory factors (Kitzmann and Fernandez, 2001). Myoblast differentiation is promoted by PI3K/AKT signalling (Jiang et al., 1999; Jiang et al., 1998). Moreover, AKT has been shown to inhibit Raf signalling in differentiated myotubes, but not their myoblast precursors, and this interaction does not appear to be confined to cells of the skeletal muscle lineage (Rommel et al., 1999; Zimmermann and Moelling, 1999). This raises the possibility that in Schwann cells GGF activation of PI3K/AKT could inhibit GGF induced Raf signalling during early myelination, and this may prevent the inhibition of myelin gene expression by the Raf/MAPK pathway. However, since inhibition of the MAPK pathway is insufficient to induce Schwann cell differentiation, PI3K activation must promote the initial events of myelination by other means.
I have shown that activation of the Raf/MAPK pathway in differentiated Schwann cells causes Schwann cell dedifferentiation in association with downregulation of PO expression; whilst in cells under differentiating conditions Raf activation blocks PO expression and differentiation. However, Rosenbaum and colleagues have reported that oncogenic Ras induces PO expression (Rosenbaum, 1999). It is unclear how these opposing results can be reconciled, but Ras has other effectors including PI3K, which is required for the initial events of myelination and thus may promote myelin gene expression (Maurel and Salzer, 2000). Alternatively differences in the intensity of Raf/ MAPK activation may result in different effects, similar to the situation in PC12 cells. The oncogenic Ras expressing cells used by the Ratner group (Kim et al., 1997; Rosenbaum, 1999) are still able to proliferate at a reduced rate in response to mitogens, whereas other Schwann cells expressing oncogenic Ras or with Raf activation via the RafER are cell cycle arrested (Ridley et al., 1988; Lloyd et al., 1997). This suggests that the oncogenic Ras expressing cells used by the Ratner group have been selected for lower levels of Ras activity.

Potential relevance of Raf activation in Schwann cells for NF1

NF1 sufferers are predisposed to the development of benign neurofibromas and malignant peripheral nerve sheath tumours (MPNSTs). LOH in human neurofibroma–derived Schwann cells has recently been demonstrated at the NF1 locus (Serra et al., 2000; Wallace et al., 2000) and in a mouse model this is sufficient to initiate neurofibroma formation in a NF1+/- background (Zhu et al., 2002). NF1/- mouse and human neurofibroma derived Schwann cells have elevated Ras-GTP levels, similar to oncogenic Ras expressing cells, suggesting that Neurofibromin is an important RasGAP in these cells (Kim et al., 1997; Sherman et al., 2000a). In addition, NF1 -/- Schwann cells behave in an abnormal manner in a number of assays and appear morphologically different to wild-type cells, but are similar to oncogenic Ras expressing Schwann cells and cells with activated Raf signalling (Ridley et al., 1988, Kim et al., 1997; Kim et al., 1995; Lloyd et al., 1997). Thus, although Neurofibromin has non-Ras related effects and Ras has other effector pathways than Raf/MAPK, investigation of the effects of Raf signalling in Schwann cells may still provide information on Schwann cell differentiation that is relevant to NF1.
Since activation of Raf in primary Schwann cells in vitro is able to cause Schwann cell dedifferentiation, elevated Raf/MAPK signalling in NF1 may disrupt Schwann cell differentiation, potentially facilitating cell cycle re-entry and unregulated proliferation. However, in the Ratner lab NF1-/- mouse Schwann cells appear to have constitutive expression of P0 in vitro (Rosenbaum, 1999), similar to oncogenic Ras expressing cells experiments in their hands (see above). Surprisingly given this result, co-cultures of NF1-/- Schwann cells with NF1-/- DRG undergo reduced myelination compared to wild-type cultures. The Schwann cells appeared to be blocked at the promyelinating state of development, suggesting that Neurofibromin activity is required for successful myelination. In addition, Schwann cells appear to require Neurofibromin to express S100, since plexiform neurofibromas in NF1-/- chimaeric mice do not express S100 (Cichowski et al., 1999). Since loss of Neurofibromin is known to result in elevated Ras-GTP levels in Schwann cells, this suggests that a reduction in Ras signalling is required for myelination, which would be compatible with my observation that signalling by the Ras effector Raf can inhibit myelin gene expression and that expression of P0 in vitro is associated with reduced Raf/MAPK activity. It is unclear why Rosenbaum and colleagues have such different results (Rosenbaum, 1999). However, Neurofibromin may be required independently of an effect on P0 levels for successful myelination or reduced levels of NF1 activity in the surrounding cell types may result in altered Schwann cell behaviour (Zhu et al., 2002). For example, it has been observed that NF1+/- mice and plexiform tumour from NF1 patients have enlarged peripheral nerves in association with an apparent increase in Schwann cell proliferation (Zhu et al., 2002). Alternatively, the elevated levels of P0 induced by loss of Neurofibromin may disrupt myelin sheath formation, which is supported by the finding that P0 overexpression in a mouse model causes hypomyelination (Wrabetz et al., 2000). The differences in the effects on P0 expression in Schwann cell with activated Raf compared to NF1-/- cells, could potentially be due to differences in the intensity of MAPK signalling. The NF1-/- Schwann cells used by Kim and colleagues exhibit reduced proliferation in vitro, in contrast to the cell cycle arrest induced by RafER activation in Schwann cells in our hands, suggesting that NF1-/- cells have lower levels of Ras/Raf/MAPK activity (Lloyd et al., 1997). This may be a result of adaptation to defects in proliferation occurring during
the development of NF1-/- mice. Examination of the effects of Raf activation during DRG myelination and in myelinated co-cultures will enable confirmation of the results I have obtained using the cAMP elevation assay.

Neurofibromas consist of a number of cell types including an abundant population of Schwann cells and fibroblasts in a collagen-rich matrix (Poirier et al., 1968; Waggener, 1966; Stefansson et al., 1982). Interestingly, although Schwann cells are frequently observed in the absence of contact with axons in neurofibromas in vitro NF1-/- Schwann cells can apparently associate normally with axons in vitro (Kim et al., 1995) and human neurofibroma derived Schwann cells have been reported to associate with host axons on injection into nude mice (Sheela et al., 1990). To examine the effects of Raf activation on Schwann cell–axon interactions I used the DRG-Schwann cell co-culture system with NSRafER cells, which enabled investigation of the effects of Raf activation firstly on the recognition of axons, establishment of Schwann cell-axon interactions and myelination, and secondly on the maintenance of these interactions. Schwann cells with activated Raf were still able to rapidly recognise and associate with axons. Thus Raf/MAPK signalling was not sufficient to overcome the signals responsible for recognition of axons by Schwann cells, despite Raf activation in vitro being associated with Schwann cells migrating over each other due to loss of contact inhibition of movement (Lloyd et al., 1997). Moreover, the majority of these Schwann cells remained associated with the axon network over time. Similar results were obtained with Schwann cells that were allowed to associate with the axon network prior to Raf activation. These results are comparable to those of Kim and colleagues who reported that oncogenic Ras expressing cells were able to associate normally with axons in co-cultures (Kim et al., 1995). However, I have examined the interactions between Schwann cells with activated Raf and axons in detail and have observed some subtle defects. Although the vast majority of Schwann cells were able to stably associate with axons or remained associated, a small but significant number of cells with activated Raf were found to be poorly associated with the axons. Moreover, this effect was more pronounced in Schwann cells with Raf activated prior to addition to the DRG culture, suggesting that the strength of the interaction between Schwann cells and axons increases over time reducing the effect of Raf activation.
This finding was investigated further using time-lapse microscopy, which revealed that a number of Schwann cells with activated Raf prior to association with axons or following stable association were able to completely dissociate from the axon network and move across the culture dish independently of axon contact at any one time. Thus activation of Raf/MAPK signalling in Schwann cells is sufficient to induce dissociation of a small number of cells from axons. I also observed that some Schwann cells with Raf activation following stable association with axons were also stimulated to move at a significantly increased rate, which may further contribute to a reduction in stable axon association. Thus elevated Ras/Raf signalling as a result of the loss of Neurofibromin activity may increase the chance of Schwann cell dissociation, leading to a few cells losing contact with axons, which may then be stimulated to proliferate by other mutations or an altered mitogenic or cellular environment.

The events surrounding the initiation of neurofibroma formation in humans are unclear. LOH at the \textit{NF1} locus in Schwann cells in a \textit{NF1+/-} background has been shown to be sufficient for the development of neurofibromas in a mouse model and is associated with an increased population of infiltrating \textit{NF1+/-} mast cells, which may secrete cytokines that promote Schwann cell proliferation (Zhu \textit{et al.}, 2002). It has been suggested that neurofibroma formation in \textit{NF1} patients may be triggered by an abnormal response to trauma (Riccardi 1990; Ratner and Daston, 2001). In support of this idea, wounding of \textit{NF1+/-} mice results in abnormal fibroblast proliferation, which is associated with the accumulation of cytokines and increased collagen deposition (Atit \textit{et al.}, 1999). The proliferative cytokines may be produced by mast cells that have been stimulated to infiltrate peripheral nerves by injury and are observed in neurofibromas (Riccardi, 1990). In addition, \textit{NF1+/-} sciatic nerve injury has been reported to result in the formation of rare tumours and hyperpigmentation near the site of the wound (Ratner and Daston, 2001). Moreover \textit{NF1-/-} Schwann cells have been shown to behave abnormally under certain culture conditions, undergoing hyperproliferation in response to elevated cAMP levels (Kim 1997). Thus peripheral nerve injury in \textit{NF1} patients may result in abnormal Schwann cell proliferation, facilitated by an aberrant cellular and mitogenic environment and by the
constitutive elevation of cyclin D1 levels associated with Neurofibromin loss (Kim et al., 2001). However, following nerve repair NF1-/- Schwann cells may have defects in re-differentiation, with elevated Ras/Raf signalling potentially promoting maintenance of a proliferative, immature phenotype and thus facilitating the generation of an abundant Schwann cell population in the absence of axon contact. Moreover, if even a single Schwann cell did not re-associate and re-differentiate, but remained proliferative it could generate a large population of cells without axon contact, similar to that observed in neurofibromas.

Neurofibromin expression is elevated in Schwann cells distal to the site of injury following sciatic nerve damage, which suggested that reduced Ras signalling might be associated with Schwann cell dedifferentiation (Wrabetz et al., 1995). However, an alternative possibility is that reduced Ras signalling is required to facilitate expression of myelination-associated genes prior to Schwann cell re-association and axon re-myelination. This view could be supported by the detection of Neurofibromin upregulation from 4 days to at least 12 days post injury (Wrabetz et al., 1995), which coincides at later time points with the initial re-expression of MBP and P0 mRNA (LeBlanc and Poduslo, 1990). Neurofibromin upregulation during Wallerian degeneration was also reported to be associated with increased Schwann cell proliferation (Wrabetz et al., 1995). However, later studies have shown that loss of Neurofibromin in Schwann cells results in elevated cyclin D1 levels, suggesting that Neurofibromin antagonises cyclin D1 expression (Kim et al., 2001). Since Ras/Raf activation in Schwann cells results in cyclin D1 expression and cyclin D1 is required for Schwann cell proliferation during Wallerian degeneration (Kim et al., 2000), loss of Neurofibromin activity may facilitate Schwann cell proliferation by allowing increased cyclin D1 expression.

Neurofibromin has other non- Ras related functions, which may contribute to tumorigenesis. For example NF1-/- Schwann cell invasiveness in vitro is not dependent on Ras activation (Kim et al., 1997) and could potentially be mediated by elevated cAMP signalling as NF1-/- cells have increased cAMP levels (Kim et al., 2001). However, both NF1-/- mouse neurons in vitro and NF1-/- Drosophila appear to have reduced cAMP
Raf activation in primary Schwann cells induces a cell cycle arrest associated with elevated levels of CDKIs (Lloyd et al., 1997). I have shown that Raf activation in Schwann cells under differentiating conditions, or in differentiated cells, results in reduced levels of myelination-associated proteins in addition to the induction of CDKIs. These experiments used pools of RafER cells selected for their ability to induce a cell cycle arrest with Raf activation due to high levels of MAPK activity. However, Raf/MAPK signalling has been shown to have different effects depending on the level of MAPK activity (Sewing et al., 1997; Woods et al., 1997; Zhu et al., 1998). For example in mouse fibroblasts high levels of Raf/ MAPK activation can induce a cell cycle arrest, whilst lower levels are associated with continued cell cycle proliferation due to the absence of CDKI induction. Thus it is possible that lower levels of MAPK activity could still have effects on Schwann cell differentiation and Krox-24 expression without inducing CDKI expression. Alternatively the effects of Raf activation on Schwann cell differentiation may be dependent on high levels of MAPK activity. Titration of the levels of RafER activation in NSRafER cells, resulting in different levels of MAPK signalling, will allow me to investigate whether the effects of Raf activation on differentiation can be separated from effects on the cell cycle. Moreover, using appropriate culture conditions, I can examine whether lower levels of MAPK activity in differentiated cells can also induce cell cycle re-entry in addition to Schwann cell dedifferentiation, similar to the situation in occurring vivo following peripheral nerve damage. Finally, since I have been using pools of RafER expressing Schwann cells it is likely that there is some variation in the levels of RafER expression between cells. In the DRG-Schwann cell co-culture experiments I have shown that Raf activation results in the dissociation of a number of cells from the axon network and that a subpopulation of Schwann cells show an increased rate of movement. This variation in response to Raf activation could depend on the levels of RafER expression in the NSRafER cells, with the most active cells potentially having the highest levels of MAPK activity. This possibility will be investigated in the future.

The RafTR transgenic was designed to allow investigation of the effects of Raf activation on Schwann cell differentiation in vivo. Expression of the RafTR is under the control of the P0
signalling (The et al., 1997; Töng et al., 2002), thus it is unclear whether Neurofibromin regulates cAMP signalling in a positive or negative manner in mammalian cells. In addition, Ras has other effectors besides Raf that may responsible for some of the phenotypic abnormalities associated with NFI-/ cells. Interpretation of studies examining Neurofibromin expression are also complicated by the existence of a number of isoforms of Neurofibromin, some of which have differing RasGAP capabilities (Viskochil, 1999).

In conclusion, I have shown that activation of Raf/MAPK signalling is able to induce Schwann cell de-differentiation in vitro. Following nerve injury in vivo Schwann cells dedifferentiate and proliferate in a process called Wallerian degeneration, however, the pathways regulating the regenerative capacity of Schwann cells are unclear. My results suggest that the Raf/MAPK pathway may be a good candidate for the signal that induces Schwann cell dedifferentiation following nerve injury. To examine this possibility further in the future we could use sciatic nerve injury to determine whether Raf/MAPK signalling is activated in vivo during Wallerian degeneration. The observed disruption of Schwann cell-axon interactions associated with Raf activation in vitro could also be envisaged to contribute to development of neurofibromas. Investigation of the effects of Raf activation in Schwann cells differentiated in response to axon derived signals, using myelinating DRG-Schwann cells co-cultures and the RafTR transgenic mouse, will allow assessment of these results under more physiological conditions and may enable us to extend our understanding of the pathways regulating the regenerative potential of Schwann cells following nerve damage.
Chapter 7: References

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201


205


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