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The regulation of neuronal cell fate by the interaction of the Brn-3a transcription factors and the p73 family of proteins.

By

Chantelle Daniella Hudson

A thesis submitted for the degree of Doctor of Philosophy

September 2005

Medical Molecular Biology Unit

Institute of Child Health

University College London

London
Abstract

The Brn-3a and Brn-3b POU transcription factors are expressed in the developing nervous system where Brn-3a is associated with sensory neuronal differentiation and survival. It has been shown that Brn-3a directly interacts with the p53 protein and this interaction resulted in differential regulation of gene targets which affect cell fate i.e. death or differentiation. The p53 related protein, p73, is involved in neuronal development and is expressed as multiple alternatively spliced C-terminal isoforms (TAp73α–ζ), and N-terminally deleted, dominant negative proteins (ΔNp73α–β) that show reciprocal function to the TA forms and p53.

In this study we show that the Brn-3a/Brn-3b proteins also physically interacted with the p73 isoforms via the POU domain of Brn-3 and the region containing the OD domain of p73 and this interaction is modulated by the different C-terminals of the p73 isoforms. The effect of the Brn-3a/p73 interaction was tested on p53 target genes where co-expression of Brn-3a was shown to potentiate the transcriptional effect of TAp73 on the p21^{CIP1/Waf1} promoter whilst antagonizing TAp73/p53 mediated activation of the pro-apoptotic genes bax and noxa. Additionally Brn-3a uses a variety of different mechanisms to modulate the expression of different p53 target genes as well as playing a role in determining the selectivity of individual TAp73 members in controlling the expression of the bax promoter. In agreement with a functional effect, co-expression of Brn-3a and TAp73 increased cell cycle arrest and survival in the ND7 neuronal cell line, whereas co-expression of Brn-3a and ΔNp73 had no effect on cell cycle arrest but increased cell survival.

Similar to p53, p73 (TA and ΔN) co-localised to a subset of Brn-3a positive neural crest cells (NCC) fated for a sensory lineage. Some of these Brn-3a/p73 co-expressing cells suggested a differentiated cell type and co-localization of TAp73 but not ΔNp73 with the differentiation marker NF-160, suggested that TAp73, like p53 is associated with differentiation of Brn-3a positive NCC. Functional effects of Brn-3a-p73/p53 co-expression was analysed in NCC cultured from Brn-3a-/- embryos showed significantly increased apoptosis upon induction of p53/p73 compared with WT cultures, suggesting that Brn-3a is necessary to overcome the p73/p53 apoptotic pathway. Thus, interaction with Brn-3a in sensory neurones may be critical for modulating p73/p53 mediated gene expression and hence cell fate.
Acknowledgments

I would like to thank my supervisors Dr Vishwanie Budhram-Mahadeo and Professor David S. Latchman for all their individual guidance, support and valuable advice throughout my time in their laboratory. I would also like to thank Professor Gerry Melino and his group for reagents, practical advice and their time. I would also like to thank members of MMBU past and present for all their help and support and for making my time in the lab enjoyable and entertaining. Particular thanks goes to Jennifer who passed on the technique of NCC culturing, Duncan for his sound advice and Sonia for her friendship and support.

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## Abbreviations

<table>
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<th>Description</th>
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<tbody>
<tr>
<td>Bax</td>
<td>Bcl-2-associated X protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>Bcl-2 related gene x</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain Derived Neurotrophic Factor</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent kinase</td>
</tr>
<tr>
<td>CKI</td>
<td>Cyclin dependent Inhibitor</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>Cis-Platium(II)diamminedichloride</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulphoxide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetracetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>[ethylene-bis(oxyethylenenitrilo)]tetraacetic acid</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks’s Balanced Salt Solution</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminal kinase</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCC</td>
<td>Neural crest cells</td>
</tr>
<tr>
<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
</tr>
<tr>
<td>Noxa</td>
<td>For damage</td>
</tr>
<tr>
<td>OD</td>
<td>Oligomerization domain</td>
</tr>
<tr>
<td>p21</td>
<td>p21&lt;sup&gt;CIP1/Waf1&lt;/sup&gt;</td>
</tr>
<tr>
<td>PIC</td>
<td>Pre-initiation complex</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>POU</td>
<td>Pit-Oct-Unc</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>RGC</td>
<td>Retinal ganglion cells</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SCG</td>
<td>Sympathetic cervical ganglion</td>
</tr>
<tr>
<td>SDS PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>Synaptosomal-associated protein 25</td>
</tr>
<tr>
<td>TA</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TEMED</td>
<td>N',N',N',N'-Tetramethylethlenediamine</td>
</tr>
<tr>
<td>TFII</td>
<td>General transcription factor II</td>
</tr>
<tr>
<td>TG</td>
<td>Trigeminal ganglia</td>
</tr>
<tr>
<td>Trk</td>
<td>Tyrosine receptor kinase</td>
</tr>
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Publications


CHAPTER 1

Introduction

1.1 Transcriptional regulation in eukaryotes

Multicellular organisms execute complex patterns of gene expression to produce and maintain numerous specialized cell types. In general the development and maintenance of functionally distinct cells is governed by the combination of transcription factors that it contains, which control its pattern of gene expression. This ensures that each cell produces the correct compliment of proteins.

The control of tightly regulated gene expression is achieved at different levels by a number of different processes. These include: chromatin remodeling, that is required to facilitate DNA access; gene transcription which involves the synthesis of mRNA from DNA; post-transcriptional regulation determining the stability of the mRNA transcript and regulation of splicing; translation which involves the synthesis of proteins from a mRNA template and post-translational modifications required for the production of functional proteins or as a mechanism to regulate protein function.

1.2 Pre-transcriptional control of gene expression

The DNA in the eukaryotic nucleus is compacted and organized into chromatin. The fundamental unit of chromatin, the nucleosome core particle, consists of 147 bp of super helical DNA wrapped in 1.75 turns around a histone octamer core composed of two copies of the four core histones, H2A, H2B, H3 and H4. The chromatin structure is repressive for gene transcription as the nucleosomes prevent transcription factors from binding to regulatory elements. Thus in the broadest terms, gene activation in eukaryotic cells takes place in two steps. The first is to penetrate the chromatin barrier; the second is to assemble the pre-initiation complex consisting of RNA pol II and general transcription factors (GTF). Transcriptional activators function at both these steps. As well as enhancing transcription directly by stimulating PIC assembly (see later) transcriptional activators have also been shown to stimulate transcription through interaction with and recruitment of chromatin-
modifying complexes (Narlikar et al., 2002). These complexes can be divided into two classes. The first class includes the ATP dependent remodeling complexes which use energy to noncovently modify chromatin structure. These complexes function by using ATP hydrolysis to slide the DNA with respect to the histone octamer and thereby expose the DNA sequence (Merseman et al., 1992). The second class is composed of histone-modifying complexes which add or remove covalent groups from the amino termini of histones (histone tails) that protrude out of the nucleosome. These modifications include acetylation, methylation, phosphorylation, ubiquitination and ADP-ribosylation (reviewed by Margueron et al., 2005). The best characterized are the histone acetyltransferase complexes (HAT) and the histone deacetylase complexes (HDAC) (Vogelauer et al., 2000) in which hyperacetylation and hypoacetylation correlate with transcriptional activation and repression respectively.

Hence the chromatin structure regulates transcription and provides a highly flexible environment where spatially and temporal coordinated remodeling takes place between transcriptionally repressive and structurally condensed states and transcriptionally active structurally accessible states.

1.3 Eukaryotic transcription

Eukaryotic transcription is the synthesis of RNA from a DNA template by RNA polymerase (RNA pol). There are three classes of RNA pols: I, II and III, each catalyzing the transcription of genes encoding different classes of RNA. RNA pol I is located in the nucleolus and transcribes rRNA except 5S rRNA. RNA pol III functions outside the nucleolus and transcribes 5S rRNA, tRNA and a whole array of small stable RNAs. These latter RNAs include those involved in RNA splicing and 7S RNA of the signal recognition particle involved in the transport of proteins into the endoplasmic reticulum. RNA pol II is involved in the transcription of all protein-coding genes and hence functions in the production of mRNA. RNA pol II also transcribes snRNA genes that take part in RNA splicing. All three polymerases contain two large subunits and 12-15 smaller subunits, some of which are present in two or all three of the polymerases. The carboxyl terminus of the largest subunit in RNA pol II contains a stretch of seven amino acid repeat (consensus
sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser known as the carboxyl terminus domain (CTD). During transcription, the serines and some tyrosines in the CTD are phosphorylated as the polymerase transcribes away from the promoter.

The basal transcription complex includes the RNA polymerase and other general transcription factors that are necessary for the accurate initiation of transcription. Following the formation of the pre-initiation complex (PIC) containing these factors, the RNA pol II then initiates its function by directing 3′-hydroxilation of the RNA chain on the ribonucleotide triphosphate, leading to extension of the chain in a 5′→3′ direction.

**1.4 DNA sequences elements that regulate transcription**

Transcription of eukaryotic genes is regulated by transcription factors that bind to cis-acting DNA response elements. Several types of cis-acting elements have been defined including promoters, enhancers, upstream promoter element (UPEs) and response elements.

Promoter sequences are usually located 5’ end to the coding sequence and analysis of many eukaryotic promoters have revealed short conserved sequences within the proximal promoter that are required for transcription initiation. Many genes, especially those encoding abundantly expressed proteins contain a TATA box element located 25-30 base pairs 5’ to the transcriptional start site and is recognized by the TBP (TATA binding protein). The TATA box functions to position the RNA pol II for transcription initiation. Instead of a TATA box, some house keeping and tissue specific genes contain an initiator (Inr) which is described in the general form Py2CAPy5 and is located between positions -3 and +5. Again, promoters containing an initiator element usually initiate transcription from a single downstream site.

Upstream promoter elements (UPEs) are recognized and bound by specific transcription factors which act to stimulate the activity of the basal promoter. These upstream sequences and the factors that bind them may be common and found in a wide variety of promoters or specific and particular for transcription in a restricted time or place. A variety of UPE common to promoters can contribute to promoter function and some examples of these elements are given in table 1.1. All promoters probably require one or more of these elements
to function efficiently and these elements may be found in different combinations, copy numbers and locations in any given promoter. The simplicity of the elements recognized by upstream factors disguises a complexity of interactions. For instance the ubiquitous CAAT box can be recognized by several transcription factors including the CAAT box transcription factor (CTF) family and poly(C)-binding protein-1 (CP1).

<table>
<thead>
<tr>
<th>Module</th>
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<td>TBP</td>
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<tr>
<td>CAAT box</td>
<td>GGCCATCT</td>
<td>CTF/NF1</td>
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<tr>
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<td>ATF</td>
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Table 1.1: Examples of some common sequence elements found in promoters

Upstream transcription factors bind to sequence elements that are common to mammalian RNA pol II promoters.

Enhancers are DNA sequences that can activate high levels of transcription of a gene or linked genes from distal locations. Enhancers are found in regions that are hypersensitive to nucleases, and are usually located within a few kilo bases 5’ or 3’ of a gene. In some cases they lie much further upstream or downstream or can be found within an exon or intron of a gene. Like promoters, enhancers have a modular structure but tend to contain a greater concentration of transcription factor binding sites. Also similar to promoters, enhancers may contain DNA elements that bind either common or tissue specific activators. However, in contrast to promoters, enhancer function is independent of position and orientation.

Finally, DNA sequences that enhance activation of a promoter in response to exposure of different stimuli are termed response elements. These include the Heat Shock Element (HSE), Glucocorticoid Response Element (GRE), Estrogen Response Element (ERE) and Metal Response Element (MRE). These elements are bound by specific transcription factors induced by a particular stimulus. Furthermore, if these response elements are cloned into
heterologous promoters, these genes become capable of being induced in response to the particular stimulus (reviewed by Latchman, 1997).

1.5 The process of transcription

Transcription factors are proteins that bind to cis-DNA acting elements and play a pivotal role in the regulation of gene expression. Eukaryotic transcription factors can be divided into two main groups, namely general transcription factors that form the PIC and gene specific transcription factors which act to either stimulate or repress the assembly of the PIC.

The general transcription factors (GTF) are sufficient for low levels of accurate transcription initiated by RNA pol II from core promoters, many of which contain the TATA box or Inr sequence (reviewed by Roeder, 1996). On a TATA box containing promoter, TFIID is the first general transcription factor to bind to the promoter. The best characterized subunit of TFIID, TBP (TATA box binding protein) binds the TATA box sequence specifically through minor groove contacts which bends the DNA considerably bringing the sequences upstream and downstream of the TATA element into closer apposition. As the TATA box is a fixed distance from the start site, its recognition by TBP is important for positioning the RNA polymerase. The complex is then greatly stabilized by TFIIA binding, which also acts to inhibit TBP bound negative co-factors that prevent TFIIIB binding to the PIC. TFIIIB binds adjacent to TBP and recruits the pre-formed RNA pol II-TFIIF complex by direct interaction with both TFIIF and RNA-pol II which has an unphosphorylated CTD at this stage. TFIIF is also involved in transcription elongation. TFIIE binds next followed by TFIIH which completes the assembly of the PIC. TFIIH has DNA helicase activity and separates the strands of the duplex template DNA, allowing RNA pol II to initiate transcription. TFIIH also phosphorylates the CTD of the largest subunit of RNA pol II which is thought to be important for releasing the polymerase from the initiation complex, as it initiates transcription (Roeder 1996).

1.6 Regulation of transcription

While general transcription factors are common to all RNA pol II transcribed genes,
independent of cell type, gene specific transcription factors are responsible for activation or repression of specific genes. These ensure tightly controlled temporal and /or tissue specific expression. These transcription factors bound to upstream elements or enhancers influence the initiation of transcription by recruiting chromatin modifiers and/or members of the PIC.

Thus a transcription factor contacts the general transcription factors via its regulatory (activator or repressor) domain to either promote or inhibit assembly of the PIC. For activators this might involve recruitment of the general transcription factors to form the PIC. For example, assembly of TFIID into the complex is a rate limiting step and is stimulated by the presence of transcriptional activators that bind the TFIID complex by contacting TBP or specific TAF (TBP associated factor) subunits. One example is Sp1 which interacts with dTAF110 and facilitates PIC assembly. Other activation domains bind and recruit other general transcription factors to the PIC (Roeder et al., 1996). Alternatively, activators may recruit factors that modulate chromatin structure to make the promoter and enhancer elements more accessible. For example CREB (cyclic AMP response element binding protein) recruits a co-activator such as CBP (Creb Binding Protein). As well as interacting with specific general transcription factors and thereby linking CREB to this complex and allowing gene activation, CBP also possess histone acetyltransferase activity which leads to chromatin remodeling through histone acetylation which allows the DNA to become more assessable. Conversely transcriptional repressors may act by preventing activators binding to their DNA binding sites either by competition or formation of non-DNA binding protein-protein complexes with activators. Inhibitors may also interact with the activator and block the activity of the activation domain, in a phenomenon known as quenching. Direct inhibition may also occur by transcriptional repressors binding to their DNA elements in promoters and making contacts with the general transcription factors that hinders the formation of the PIC (reviewed by Latchman, 1996). An example is the Eve (Drosophila even skipped) protein which functions by preventing the association of TFIID with the TATA box element.

Additionally, some transcription factors bind co-activators or co-repressors that in turn interact with the basal apparatus, highlighting the importance of protein-protein interactions in the control of transcription. These co-activators and co-repressors themselves do not bind DNA but their specificity is conferred by their ability to associate with DNA binding transcription factors. Like the transcription factors themselves, co-activators and co-
repressors may be common regulatory proteins or cell type specific.

As higher eukaryotic promoters and enhancers contain multiple binding sites for transcriptional activators, this posed the question of how multiple activators co-operate with one another to activate transcription. The first model suggests that different activators act at distinct steps—thus one activator could stimulate PIC assembly while others could recruit an essential chromatin modifying activity or promote transcriptional elongation. An alternative model is that the multiple bound activators simultaneously interact with different PIC components, synergistically increasing PIC assembly and transcription. Recently Fishburn et al. 2005 showed that a single DNA bound activator can interact with multiple components of the PIC. A combination of interactions would result in increased stabilization of the factors on the promoter, thus increasing transcription.

Thus, transcription factors function to regulate gene expression by binding to specific elements in promoters and act to control the assembly of the PIC and/or affect chromatin remodeling. Likewise the transcription factors bound to distinct enhancers also interact directly with the PIC, by forming a DNA loop between the enhancer and proximal promoter. It is generally believed that transcription takes place at loci enriched for RNA pol II and transcriptional machinery, known as transcription factories (Osborne et al., 2004). Expressed genes appear to migrate to these regions by looping out of their chromosome territories away from non-expressed genes. By this mechanism the active gene is brought in close proximity to its enhancer sequence (reviewed by West et al., 2005).

1.7 Specific Transcription factors

Although examples of regulation of eukaryotic gene expression at different levels have been discovered, transcription initiation is by far the most widespread mechanism to control gene expression in eukaryotes. The precise temporal and spatial regulation of transcription by RNA pol II is pivotal to the execution of precise and complex gene expression programmes in mammalian cells in response to growth, developmental and homeostatic signals. Coordinated gene expression is brought about by gene specific transcription factors. Transcription factors require the ability to bind DNA and then to influence transcription either positively or negatively. Many transcription factors are modular proteins
consisting of defined functionally independent domains: one or more regulatory domains are connected to a sequence specific DNA binding domain through relatively flexible protein domains. This allows the flexibility required for the protein-protein interactions formed to regulate transcription.

The activity of specific transcription factors can be regulated in several ways. For example they may have restricted distribution being synthesized only in certain tissues or at a specific time which is typical of factors that regulate development. The MyoD family of closely related myogenic proteins (MyoD, Myf5, MRF4 and myogenin), represent a family of transcription factors only expressed in a specific cell type. MyoD is expressed only in skeletal muscle cells and induces muscle specific genes. Over-expression of MyoD in undifferentiated fibroblasts is sufficient to convert them into skeletal muscle cells (Edmondson and Olson., 1993) indicating that MyoD activates transcription of all genes involved in muscle differentiation. The MyoD family is switched on early in myoblasts and some of these members activate their own expression as well as other family members in order to maintain their expression. Under suitable conditions these transcription factors then activate the expression of muscle-specific genes (see below).

Additionally transcription factors may be regulated by tissue specific co-factors. For example the octamer motif is recognized by the POU proteins of which the best characterized are Oct-1 and Oct-2. Oct-1 is ubiquitously expressed and correlates with activation of the histone H2B genes and expression of snRNA genes. In contrast Oct-2 is expressed in B lymphocytes and together with Oct-1 transactivates several lymphoid specific promoters (Wirth et al., 1987). The reason why Oct-1 does not transactivate lymphoid specific promoters in other cell types is because both Oct-1 and Oct2 require the presence of the B-cell specific co-activator OCA-B. By interacting with both Oct-1 and Oct-2, OCA-B is recruited to a subset of octamer sites where it serves as a co-activator for transcription of specific genes. Interestingly Oct-2 is also expressed in a subset of neuronal cells, but rather than activating expression of genes, the Oct-2 transcription factor was found to inhibit expression of many target neuronal genes (Lillycrop et al., 1991; Dawson et al., 1994). This was due to the expression of different Oct-2 isoforms as a result of alternative splicing in different tissues. Thus in B-lymphocytes the predominant Oct-2.1 isoform has activator function. However in neuronal cells the Oct-2.4 and Oct-2.5 isoforms are more abundant. These two isoforms lack the activation
domain which results in the general repressive effects of these isoforms on gene transcription (Wirth et al., 1991; Lillycrop and Latchman., 1992).

Transcription factors may become active under certain conditions by protein modification. For example p53 is activated by DNA damage by a variety of protein modifications which functions to stabilize the protein and enhances its transcriptional activity. The AP-1 transcription factor (a heterodimer between the subunits Jun and Fos) is converted to the active form by phosphorylation of the Jun subunit. Interaction between different transcription factors as dimers may also result in different effects so that a particular binding partner causes it to be inactive, whereas the synthesis of the active partner would displace the inactive partner to allow an active complex to form. Such a network of alternative pairing is seen with the HLH proteins. For instance MyoD, is expressed in myoblasts well before activation of its target genes. Its activity at this stage is tightly regulated by the production of inhibitory binding partners (such as twist, another HLH protein). Twist is down regulated preceding cell specific differentiation and is absent from the myotome, the compartment of the somite where skeletal muscle differentiation takes place. This allows MyoD to form active complexes allowing for expression of muscle specific genes (reviewed by Berkes and Tapscott, 2005). Transcription factors may also be regulated by sequestration upon interaction with inhibitory partners. For example NF-kB is sequestered in the cytoplasm by I-kB. Similarly E2F-1 is held in an inactive complex by pRb. Release of these inhibitory factors via phosphorylation of either I-kB or pRb allows the transcription factors to become active either by moving into the nucleus or by being free to activate transcription of target genes. A factor may also be activated or inactivated by binding a ligand. The steroid receptors are prime examples, in which ligand binding may cause the receptor to translocate to the nucleus thus allowing gene transcription to occur, as well as determining its ability to bind DNA.

Hence in a specific cell type or in response to a stimulus, specific transcription factors are synthesized or become active. The binding of these transcription factors to their response elements in different promoters thus produces highly orchestrated patterns of gene expression.
1.8 Families of transcription factors

Most transcription factors have been grouped into families on the basis of conserved DNA binding domains (DBD). These include the zinc finger motif, the basic leucine zipper motif, beta-scaffold factors with minor groove contacts, the helix-loop-helix motif (HLH) and the related helix-turn-helix motif (HTH).

The zinc finger motif was originally identified in TFIIIA which is required for RNA pol III to transcribe 5S rRNA genes. It has since been identified in several other transcription factors (and presumed transcription factors). There are three classes of Zinc finger proteins. The first is termed C2H2 and consists of the repeating unit (Tyr/Phe)X Cys X2-4 Cys X3 (Phe/Tyr) X5 Lue X2 His X3-4 His, where X is any amino acid. Each repeating unit binds one zinc ion though the two histidine and cysteine side chains. The binding of the zinc ion by the two cysteines and histidines folds the relatively short polypeptide sequence into a compact domain that inserts its α helix into the major groove of the DNA. The second class of the zinc finger motif termed C4 found in the steroid receptors consists of Cys X2 Cys X13 Cys X2 Cys X14 Cys X5 Cys X9 Cys X2 Cys. The two groups of four cysteines each bind a zinc ion but unlike the C2H2 zinc finger proteins the C4 zinc finger proteins bind DNA as homodimers or heterodimers. The DNA binding domain in the yeast Gal4 protein exhibits the third type of zinc finger motif which is known as the C6 (class 3) which has the consensus sequence Cys X2 Cys X6 Cys X5-6 Cys X2 Cys X6 Cys. The six cysteines bind two zinc ions folding the region into a compact globular domain. This class of zinc finger proteins also binds DNA as a dimer.

Leucine zippers consist of a stretch of amino acids with a leucine residue at every seventh position. A leucine zipper in one polypeptide interacts with a zipper in another polypeptide to form a dimer. Adjacent to each zipper is a stretch of positively charged residues that is involved in DNA binding (Fig 1.1). The leucine zipper underlies the interactions between the proto-oncogene products Jun and Fos, which constitutes the heterodimeric transcription factor activating protein-1 (AP-1), a well known downstream target of the mitogenic activated protein kinase (MAP kinase) signaling pathway.
Figure 1.1: A leucine zipper dimer bound to DNA
Two α-helical DNA binding domains (bottom) dimerize through their helical leucine zipper region (top) to form an inverted Y-shaped structure. Each α-helix binds to one half of a symmetric DNA structure in the major of DNA (Adapted from Alberts et al, 1994 Molecular biology of the cell-third edition).

The beta-scaffold factors with minor groove contacts, includes the STAT transcription factors and p53. The three-dimensional structure of the p53 DBD, complexed with DNA has been determined (Fig 1.2) (Cho et al., 1994) and binds to its DNA site as a tetramer. The core domain consists of a large β sheet-sandwich of two anti-parallel sheets of 4 and 5 strands, respectively. This acts as a scaffold supporting a loop-sheet-helix motif (L1), and two large loops (L2 and L3). L2 and L3 are stabilized by zinc coordination (via three cysteines and one histidine) and side-chain interactions. DNA is bound by L1 and L3 in which the helix and loop of L1 slot into the major groove and L3 binds in the minor groove. The L2 loop stabilizes L3 by packing against it. The function of p53 with particular focus on its homologue, p73 is discussed in later sections (1.26-1.39).

Figure 1.2: Structure of the p53 DBD-DNA complex.
Ribbon model of the p53 DBD (light blue) with some of the most frequently mutated residues indicated in yellow. The DNA is coloured dark blue and the zinc atom is represented as a red sphere (Adapted from Alberts et al, 1994 Molecular biology of the cell-third edition).
The amphipathic helix-loop-helix (HLH) motif has been identified in some developmental regulators. Each amphipathic helix presents a face of hydrophobic residues on one side and charged residues on the other side. The length of the connecting loop varies from 12-23 amino acids. The motif enables proteins to dimerize and a basic region near this motif contacts DNA (Fig 1.3a). bHLH heterodimers bind the sequence CANNTG known as the E-box and they function in the co-ordinated regulation of gene expression, cell lineage commitment, and cell differentiation in most mammalian tissues. An example of a bHLH protein is Mash1 which is involved in neuronal differentiation (Nakada et al., 2004).

The helix-turn-helix motif was originally identified as the DNA binding domain of phage repressors. One α-helix lies in the wide groove of DNA (α-helix -3 or the recognition helix), the other lies at an angle across the DNA (α-helix -2) (Fig 1.3b). A related form of the motif is present in the homeodomain, a sequence first characterized in several proteins coded by genes concerned with developmental regulation in Drosophila. Homeodomain proteins are also found in mammalian cells and the 60 amino acid domain termed the homeobox is highly conserved.

**Figure 1.3: Helix-loop-helix and helix-turn helix motifs**

a) Helix loop helix dimer bound to DNA. The two monomers are held together in a four-helix bundle: each monomer contributes two α-helices connected by a flexible loop of protein (red) b) The helix-turn-helix motif is shown where each white circle denotes the central carbon of an amino acid. The carboxyl-terminal α-helix is called the recognition helix because it participates in sequence-specific recognition of DNA. This helix fits into the major groove of DNA (Adapted from Alberts et al, 1994 Molecular biology of the cell-third edition).

Further characterization of transcription factors with homology in the homeodomain gave rise to a sub-classification of these proteins. One such class contained a unique POU domain in addition to the homeodomain, which include the Brn-3 family of transcription factors. The structure and binding properties of the POU domain factors is discussed next followed by a
detailed account of the Brn-3 proteins focusing on the Brn-3a transcription factor.

1.9 The POU domain family of transcription factors

The POU transcription factors were named after identifying high homology in a region common to the original proteins, namely the mammalian pituitary specific Pit1, the ubiquitously expressed Oct-1, the largely B-cell restricted Oct-2 and the nematode gene Unc-86 which were discovered simultaneously in 1988 (Sturm et al., 1988; Bodner et al., 1988; Ingraham et al., 1988; Clerc et al., 1988; Finney et al., 1988). All members of this family have been demonstrated to play important roles in the development of specific cell types, particularly in the nervous system, where they were first identified as powerful transcription factors.

1.10 The structure of the POU domain

Subsequent comparison of the nucleotide and amino acid sequences of the founding members identified a unique bipartite DNA binding domain that is common to all these factors, termed the POU domain (named by using the first letter of the founding members, Pit1, Oct-1/2, and Unc-86). The POU domain comprises two highly conserved sub-domains referred to as the POU specific (POUs) and the POU homeodomain (POUhd) tethered by a more poorly conserved linker region (Herr et al., 1988). The N-terminal POUs domain is 70-80 amino acids long and is unique to this subclass of transcription factors whereas the C-terminal 60 amino acid POUhd is homologous to homeodomains found in the homeobox transcription factors. These two sub-domains are linked by a less conserved linker region of 15-25 amino acids in length (Fig.1.4). Despite the structural independence of the two sub-domains (Botfield et al., 1992), they are always found together and mutational analysis of the POU domain of Pit1 and Oct-1 have shown that both subdomains are required for sequence specific high affinity DNA binding to their appropriate response elements (Sturm and Herr, 1988; Ingraham et al., 1990; Verrijzer et al., 1990a; Aurora and Herr, 1992; Verrijzer et al.,
The POU domain can be subdivided into the highly conserved POU$_S$ domain and the POU$_{hd}$ separated by a linker region that varies in length (14-25 amino acids). The POU$_S$ domain consists of 4 $\alpha$-helices (cylinders) while the POU$_{hd}$ consists of 3 $\alpha$-helices. Conserved regions of basic amino acids are found in both the POU$_S$ and POU$_{hd}$ (indicated by black boxes). (Adapted from Wegner, 1993).

The 3D structure of the POU domain of the Oct-1 protein was the first to be determined and is representative of the general structure of the POU proteins. Initially, the solution structure of the Oct-1 POU$_S$ domain in isolation was determined by nuclear magnetic resonance spectroscopy (NMR) and was shown to consist of four alpha helices connected by short loops surrounding a conserved hydrophobic core. The second and third helix was shown to form a structure similar to the helix-turn-helix (HTH) motif of the bacteriophage lambda and 434 repressors (Assa-Munt et al., 1993; Dekker et al., 1993; Klemm et al., 1994). It is the third helix that makes contact with bases in the octamer recognition site whilst helices 1 and 4 stabilise the HTH motif. Remarkably, the positions of several residues important for DNA binding are maintained in both the POU$_S$ and bacteriophage HTH motif, illustrating evolutionary conservation of this structure due to its effectiveness at binding DNA (Assa-Munt et al., 1993; Dekker et al., 1993).

Resolution of the crystal structure of the Oct-1 POU domain bound to DNA revealed that the POU$_{hd}$ consists of 3 alpha helices that form a compact globular structure. Helix 2 and helix 3 form the HTH motif with helix 3 making several contacts with the octamer binding site (Klemm et al., 1994). As in all other homeodomains, the single helix 1 stabilises the HTH motif. The poorly conserved linker region does not make specific contacts with the DNA binding site itself (Aurora et al., 1992; Botfield et al., 1992), but acts as a flexible arm to facilitate the co-operative binding and orientation of the two sub-domains to an octamer DNA motif with high affinity (Klemm et al., 1994).
1.11 Sub classification of POU family of transcription factors

In contrast to the highly conserved POU domain, the regions outside the POU domain are highly divergent, and the growing numbers of POU domain transcription factors were grouped into 6 classes, POU I-VI (Table 1.2). Originally this division was based on sequence homology in the linker region and the POU domain, although it became apparent that additional features are conserved within these classes. For example, members of the class III POU domain genes are intronless and have various regions of homo- and poly-meric residues at their amino terminal (reviewed by McEvilly and Rosenfeld, 1997). In contrast, members of the class IV POU proteins contain a highly conserved region in the N terminal activation domain that is referred to as the POU-IV box, that is not found in any other POU containing proteins. Interestingly the POU IV box shares homology with the mammalian gene MYC-N, which is frequently amplified in neuroblastoma. The POU IV family includes the three mammalian Bm-3 proteins, Unc-86 of C.elegans and the D.melanogaster I-POU and t1-POU.

1.12 Characterization of the DNA response elements recognized by the POU domain

Consistent with the high degree of conservation in the POU domain, the POU proteins bind similar DNA recognition sites which are A/T rich. However there is a degree of divergence in the DNA binding sites that are recognised by closely related sub classes of POU proteins. For example the class I Pit-1 protein which controls transcription of pituitary-specific genes including the growth hormone and prolactin genes (Rosenfeld, 1991), efficiently binds the consensus sequence 5' ATGNATAWW-'3 (where N= any base and W= A/T). The class II proteins (e.g. Oct-1/2) prefer the octamer sequence ATGCAAT, found in a variety of different promoters (Verrijzer et al., 1990;Aurora and Herr, 1992;Verrijzer et al., 1992). However, both Oct-1 and Oct-2 recognise cis-regulatory sites which bear little homology to the octamer motif such as the (OCTA-)TAATGARAT (where R= A/T) site that is critical for herpes simplex virus gene regulation (Baumrucker et al., 1998;Verrijzer and van der Vliet, 1993). However these non octamer sites bind Oct-1/2 with reduced affinity (Baumrucker et
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<td>Brn-3a</td>
<td>Mouse 14, Human 13</td>
<td>Nervous,</td>
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<td>Nervous,</td>
<td>Loss of inner ear</td>
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<td>Oct3/4</td>
<td>Mouse 17, Human 6</td>
<td>Testis,</td>
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Table 1.2: The six classes of POU transcription factors

The chromosomal location of the different POU proteins in both mouse and humans and expression pattern is indicated. The phenotype of the knock out mouse models, where known, is also briefly discussed. (CNS-central nervous system, ES-embryonic stem cell). (Adapted from Andersen and Rosenfeld, 2001)
al., 1988; Aurora et al., 1992; Verrijzer et al., 1992). The class III and IV POU proteins bind to and activate promoters with the consensus sequence CAT(N)nWAAT (where N=any base, W=A/T) whose class specificity is achieved by accommodating different nucleotide spacing between the bipartite core DNA binding motifs. For example, class III POU proteins bind this sequence where n=0, 2 and 3 whereas class IV proteins bind with high affinity only when the core motifs are spaced by 3 nucleotides (n=3) (Li et al., 1993).

1.13 Bipartite DNA binding by the POU proteins

Sequence specific, high affinity binding of the POU proteins requires both the POU_S and POU_HD sub-domains which both contain HTH motifs (Sturm et al., 1988; Ingraham et al., 1990). However, isolation of either the POU_S or POU_HD of Oct-1 demonstrated that both domains can bind separately to sequence specific DNA but with low affinity. *In vitro* binding site selection and band shift assays, as well as ultraviolet cross linking assays have revealed that each sub-domain makes contact with a subset of residues contacted by the entire POU domain. Specifically it was shown that the POU_S domain recognised the 5’-half of the octamer sequence (ATGC) whilst the POU_HD domain target sequence corresponded to the 3’-half (AT rich region) of the POU domain octamer site (ATGC(A/T)AAT) (Verrijzer et al., 1990b; Verrijzer et al., 1992a).

These results were confirmed by crystallography studies of the Oct-1 POU domain bound to its octamer sequence in the H2B promoter (Klemm et al., 1994). It was shown that the third helix of the POU_S domain binds the ATGC region in the major groove of the DNA, while the third helix (termed recognition helix) of the POU_HD was shown to bind the AAAT sub-site of the octamer sequence in the major groove of the DNA on the opposite side. Only part of the linker that joins the subdomains is visible and this segment is found to track along the minor groove, while the remaining residues of the linker are disordered, indicating unrestrained flexibility, (Fig 1.5a + b). The region of this third helix which makes contact with the AAAT sub-site contains the invariant RVWFCN residues which are present in all members of the POU proteins.

The contacts made by the POU domain of Oct-1 with specific bases on its DNA recognition
Figure 1.5: Crystal structure of the Oct-1 protein bound on the H2B promoter and schematic representation of the contacts made by the POUs and POUHd domains upon binding to the DNA recognition site

a) View of the crystal structure of Oct-1 bound to its site on the H2B promoter looking into the major groove of the DNA double helix, showing the POUs and POUHd bound to their distinct sites on the DNA. The helices are labeled H1-4 and the N and C termini of the protein domains are indicated. b) View down the DNA axis of the Oct-1/H2B crystal structure. c) Schematic view of the DNA contacts made by the POU domain of Oct-1. Hydrogen bonds are shown by unbroken arrows while non-polar interactions are depicted by broken arrows. Interactions with the minor groove are indicated by turquoise arrows while all other arrows represent interactions with the major groove. The octamer binding site is highlighted in pink. (Adapted from Phillips, 2000)
sequence is illustrated in Fig 1.5c. The highly conserved Gln44, Thr45 and Arg49 in helix 3 of the POU\textsubscript{S} domain makes hydrogen bonds with the ATGC subsite while there is one hydrophobic interaction between Leu50 and this subsite. These direct sequence-specific interactions are complemented by a large array of phosphate backbone contacts which are contributed by residues in all four of the POU\textsubscript{S} helices and some in the loops. The details of the POU\textsubscript{HD} interaction are very similar to those observed from other homeodomain/DNA complex structures. In addition, the first nine residues of the POU\textsubscript{HD} form an extended N-terminal arm which comprises part of the flexible linker peptide that fits into the minor groove of the DNA and the POU\textsubscript{HD} contains a set of basic amino acids at both the amino and carboxyl terminals and these regions have been shown to be critical for DNA binding (Li et al., 1993).

Interestingly, when bound to DNA the POU\textsubscript{S} and POU\textsubscript{HD} do not contact each other, so that their only connection is provided by the linker region which acts as a flexible arm allowing for different arrangements of the two sub-domains on the DNA. Thus this linker region provides for flexible DNA sequence recognition by the POU proteins.

1.14 The POU IV subclass of POU proteins

Following the identification of the founder POU domain proteins, He and colleagues (1989) isolated novel POU proteins one of which was Brn-3a (also referred to as Brn-3.0 or POU4F1) form rat brain using a reverse transcriptase/polymerase chain reaction (RT/PCR) approach with degenerate oligonucleotides corresponding to conserved regions of the POU domain (He et al., 1989). The same technique was employed by Lillycrop and colleagues (1992) but using cDNA obtained from the neuronal cell line, ND7 (Lillycrop et al., 1992) which is derived from a fusion of a mouse neuroblastoma cell line and sensory neurones derived from dorsal root ganglia (DRG). These studies resulting in the isolation of a novel Brn-3 protein with high homology to Brn-3a now referred to as Brn-3b (Brn-3.2 or POU4F2). Subsequent studies resulting in the isolation of a third member of this family, Brn-3c from a cDNA library prepared from DRG and spinal cord (Ninkina et al., 1993), showed that there are, in fact, 3 distinct Brn-3 proteins transcribed from separate genes (Theil et al., 1994). Brn-3a, Brn-3b and Brn-3c are found on human chromosomes 13, 4 and 5.
respectively and the murine homologues are located on chromosomes 14, 8 and 18 respectively (Collum et al., 1992; Xia et al., 1993; Xiang et al., 1993; Theil et al., 1994).

The Brn-3 factors, the nematode Unc-86 and the drosophila I-POU and tiPOU proteins are members of the POU-IV sub-family. Proteins in this family share a particularly high homology in the POU domain but additionally some members also share a highly conserved region in the N-terminal domain referred to as the POU-IV box (Theil et al., 1993; Gerrero et al., 1993). The POU-IV box, which is 40 amino acid long, shares homology with the N-terminal region of c-Myc, and in fact the human Brn-3a transcription factor was isolated from human placental DNA on the basis of hybridizing to DNA probes derived from the N-terminus of c-Myc (Collum et al., 1992).

Brn-3a has particularly high homology to Unc-86 which has been shown to be critical for determining specific neuronal cell types, especially sensory lineages in the nematode. It was therefore speculated that the Brn-3 transcription factors may have a similar role in neuronal development and as such most studies have focussed on their expression and function in the nervous system.

1.15 The different isoforms of Brn-3a and Brn-3b

Analysis of the genomic sequence encoding Brn-3a and Brn-3b revealed a relatively simple organization in which two exons are separated by an intron. Both Brn-3a and Brn-3b proteins are expressed as two distinct isoforms which differ in the N terminal region. The longer isoforms are encoded by exons 1 and 2 whereas the shorter proteins are encoded by exon 2 (Theil et al., 1993; Gerrero et al., 1993; Xiang et al., 1993; Theil et al., 1994; Turner et al., 1994).

Analysis of the Brn-3a locus identified two transcriptional start sites located about 500 base pairs apart in the gene sequence, and alternative promoter usage generates two distinct mRNAs that differ at their 5' end which is shown in Fig 1.6 (Theil et al., 1993; Theil et al., 1994) The upstream promoter of Brn-3a gives rise to a pre-mRNA which is spliced to produce the long variant 46 KDa Brn-3a(l) containing an additional N terminal domain encoded by exon 1 which is not present in the cDNA of the short isoform. Similarly,
transcription from the upstream promoter in the Brn-3b gene generates the 42 KDa Brn-3b(1) which also contains the additional N terminal domain (Xiang et al., 1993; Turner et al., 1994). In contrast, transcription from the downstream promoter of both Brn-3a and Brn-3b generates the shorter isoforms of these proteins, 35 KDa Brn-3a(s) and 32 kDa Brn-3b(s) that lacks approximately 80 amino terminal residues and thus both the short forms lack the POU-IV box found in the long isoforms of Brn-3a and Brn-3b.

The functional significance of this region in the Brn-3b protein has yet to be determined but in the case of Brn-3a(1), the N-terminal domain containing the POU-IV box is required for Brn-3a to activate a subset of its target promoters, involved in neuronal survival (see section 1.27.1). Thus the N-terminal domain in Brn-3a may represent an interface for interactions with other co-activators or co-repressors that may modify transcription on specific target genes.

![Figure 1.6: Schematic representation of the Brn-3a and Brn-3b transcription factors and their two isoforms.](image)

The long isoform [Brn-3a(1) and Brn-3b(1)] of both transcription factors contain an extra N-terminal domain that contains a highly conserved region, the POU IV box. The highly conserved POU IV box is only found in the long isoforms of both proteins while the POU domain is found in both long and short isoforms of each protein. (Adapted from a PhD thesis Transcriptional regulation by Brn-3a POU domain containing transcription factors, Author Dr J. Dennis, 1999)

### 1.16 The developing nervous system

The Brn-3 factors are expressed in distinct but overlapping subsets of neurones in the developing and adult nervous systems, further suggesting that they play key roles in the
development of specific neuronal cell types in mammals. In fact Brn-3a has been shown to be necessary for the differentiation and survival of sensory neurones (sections 1.18 and 1.19). These processes will be discussed below before focusing on the expression and function of Brn-3a protein in the developing nervous system which together with its interaction with the p53 family of proteins may be important in modulating neuronal cell fate, and so forms the focus of this study.

The nervous system develops from the embryonic ectoderm (Fig 1.7) in which the neural tube gives rise to the central nervous system whereas the neural crest cells gives rise to most of the neurones and supporting cells of the peripheral nervous system (PNS). Neuronal cells are produced from dividing progenitor cells which at the appropriate time exit the cell cycle and extend axons which innervate their target tissue. The target cell releases a limited amount of specific neurotrophic factor that are required by the neurones innervating that target in order to survive (section 1.16.2). Those neurones that compete successfully go on to differentiate into specific cell type whereas those neurones that do not compete successfully die by programme cell death.

**Figure 1.7: The three primary cell lineages**

During gastrulation, 3 major cell lineages are being established. These are the Ectoderm, Endoderm and Mesoderm. Following gastrulation, various cell lineages are derived from these three primary cell types. For example the ectoderm gives rise to the central nervous system as well to the epidermis and its derivatives such as nails hair and teeth.
1.16.1 Neuronal development and differentiation

Neuronal development and differentiation can be divided into 4 phases; neural induction, patterning of the neural tube, lineage determination and differentiation of adult neuronal phenotypes. Each stage is controlled by several classes of transcription factors that display defined spatial and temporal expression during development.

The formation of the neural tube and crest cells involves the folding of the flat neural plate into the neural tube a process termed neurulation. During this process the dorsal midline of the neural tube forms from the edges of the neural plate that abut the epidermal ectoderm, and this is called the neural fold (Fig 1.8a-c). The interaction between the neural plate and epidermal ectoderm are required for the induction of the neural crest and other cell types of the neural tube. More specifically, the Bmp family of signalling molecules (specifically Bmp-4 and Bmp-7) expressed by the epidermal ectoderm have been shown to induce the production of neural crest cells from the neural plate tissue. Also expression of Noelin-1, a secreted glycoprotein present in the neural plate, is required for neural crest development (Bronner-Fraser, 2002). Once the neural crest cells have been induced, they must further undergo an epithelial to mesenchymal transition to allow for migration, a process called delamination. This process is also under the control of Bmps and includes changes of expression of cell adhesion molecules. Patterning of the neural tube takes place among dividing neuroepithelial cells and lineage determination also takes place in cells that are still dividing, while expression of many differentiation markers occurs in post-mitotic neurones.

The neural tube is divided into distinct anterior/posterior domains; the cephalic, trunk, vagal and sacral crest (Fig 1.8d). The cephalic neural crest migrate into the developing brain to generate bone, cartilage, connective tissue, smooth muscle cells and the cranial ganglia. In contrast, the first structures that the trunk neural crest encounter is the somites. (Somites are paired, segmented masses of mesodermal tissue that form along the length of the neural tube during the early stage of embryonic development. They will eventually give rise to the vertebral column and other tissues including muscle, bone, and the dermal layers of the skin). Trunk neural crest cells follow one of two distinct pathways of migration. The ventral pathway passes through the sclerotome, the region of the somite that forms the axial skeleton,
Figure 1.8: Induction of the neural crest during neurulation and main regions of the neural crest

a-c) The neural plate (light blue) progressively folds to form the neural tube. The ventral midline of the neural plate attaches to the notochord (red). The neural folds (yellow) form at the interface between the neural plate and the epidermal ectoderm (dark blue) and the interaction between these two tissues is required for the induction of the neural crest. The neural folds progressively come together to form the dorsal aspect of the neural tube and the neural crest (yellow). Adapted from Graham, 2003. d) The four main regions of the neural crest (adapted from Duband et al, 1995-Neural crest culture, A Practical Approach)
but only through the anterior half. This gives rise to the dorsal root ganglia, the sympathetic ganglia, adrenal medulla and also makes contributions to the heart. The dorsolateral pathway passes between the epidermis and the somites and gives rise to the melanocytes. Finally cells from the vagal and sacral neural crest contribute cells to the network of enteric ganglia.

Clonal analysis of neural crest cells and \textit{in vivo} cell lineage analysis using a variety of labelling techniques have demonstrated that even at the migratory stage the neural crest is made up of cells at different states of determination. For instance there are multipotent progenitor populations that can give rise to both sensory and sympathetic neurones as well as associated glia. Furthermore both \textit{in vivo} and \textit{in vitro} studies have demonstrated the presence of neural crest stem cells displaying both self-renewal and pluripotent behaviour. In contrast, monopotent cells generating only neurones or only glia cells for example when exposed to a range of environmental conditions, have been demonstrated (Frank and Sanes, 1991; Luo et al., 2003), suggesting there is also some element of autonomous commitment to particular fates.

Taken together the evidence implies that neural crest cells comprises both pluripotent and fate restricted progenitor cells, so that the fate of specific neural crest populations must be controlled though environmental factors during normal development. For example, the sympathetic ganglia settle in the vicinity of the dorsal aorta which secretes Bmp-4 and Bmp-7 both of which trigger the differentiation of sympathetic neurones, whereas the formation of the dorsal root ganglia depends on exposure to brain derived neurotrophic factor (BDNF). Such environmental factors would alter gene expression in the neural crest cells by switching on the expression of transcription factors that are important for controlling the fate of these cells.

Whilst most is known about the role and function of mammalian transcription factors that control pattern formation, recent studies are beginning to unravel the transcription factors involved in the process of lineage determination and differentiation of specific neuronal subtypes. In general, genes controlling pattern formation during development show segmental expression in the neuroepithelium and are generally expressed in diverse cell types within a region. For example the homeobox containing genes of the HOX family have a clear role in hindbrain segmentation. In contrast, transcription factors that play a role in lineage
determination and differentiation of neurones are expressed in cells that share common
features. Several classes of transcription factors have been shown to play a role in lineage
determination and in terminal differentiation and these can be used to identify specific cell
types in these populations such as sensory neurones or autonomic neurones. For instance, the
basic helix-loop-helix (bHLH) factor Mash-1 is required for the development of specific
olfactory and autonomic neural lineages. Mash-1 is expressed in dividing neural crest
precursor cells destined for these neuronal lineages. As expected for a lineage determining
factor it is switched on just before they exit the cell cycle (Greenwood et al., 2000).

In contrast the bHLH family of transcription factors, neurogenin-1 (Ngn-1) and neurogenin-2
(Ngn-2) are required for early development of sensory neurones. Expression of Ngn-2 has
been well documented in migrating neural crest cells. Studies using an inducible Cre
recombinase-based fate mapping system to mark a subpopulation of NCC that expresses
Ngn2 (Zirlinger et al., 2002), showed that Ngn2 expressing cells are 20 times more likely to
generate sensory than autonomic neurones. Thus this data suggests that other determinants of
sensory identity are required in addition to Ngns in order to commit cells to a sensory fate.
The expression of Brn-3a is one such factor that shows specific expression in sensory
neurones and is induced by Ngns. Thus Brn-3a is expressed at a later stage than Ngns but is
found in precursors destined for a sensory lineage as well as differentiating sensory neurones
(Greenwood et al., 1999).

Interestingly, Ngn2 expression was observed in both pre-migratory and migrating neural
crest cells (Sommer et al., 1996;Zirlinger et al., 2002) suggesting that the sensory
developmental bias acquired by this subset of cells occurs at a relatively early stage in their
ontogeny, and supports findings that the neural crest is made up of both pluripotent and fate
restricted neural crest cells. The expression and role of Brn-3a in sensory neurones is
discussed in more detail in later sections.

1.16.2 Neuronal developmental cell death

Cellular apoptosis is crucial for the proper development of the nervous system as both
progenitor cells and post-mitotic neurones are overproduced in the nervous system. Through
a process of elimination, those cells that have made appropriate connections survive and

42
differentiate into neurones whereas others undergo apoptosis. This process of elimination occurs at two distinct phases of neuronal development. The first phase takes place just prior to the onset of neurogenesis when neural stem cells and progenitor cells migrate and populate the brain and spinal cord. This phase serves to eliminate excess progenitor cells and immature neurones to ensure that the correct number of cells is generated in structures such as the cerebral cortex. The existence of this period of developmental cell death has only recently been appreciated and the underlying mechanisms that determine life versus death in these cells are not well understood (reviewed by Kuan et al., 2000; Roth and D'SA et al, 2001).

The second period of developmental cell death in the nervous system takes place after newly differentiated post-mitotic neurones have migrated to their final destinations, extended axons and have attempted to establish appropriate target connections. This period of naturally occurring cell death is crucial to match neuronal numbers to the size of the target tissue and results in elimination of approximately half of the neurones in any given population. The mechanisms controlling this process are best understood in developing sympathetic neurones, which compete for limited amounts of target derived trophic factors such as nerve growth factor (NGF). Those neurones that compete successfully survive and go on to innervate their target tissue in an NGF dependent fashion, whereas those neurones that fail rapidly undergo apoptosis.

NGF promotes survival by binding to neuronal TrkA tyrosine kinase receptors on axon terminals and mediates a retrograde survival signal (Miller and Kaplan, 2001) the most important of which is the Ras-P13-kinase-Akt pathway (see section 1.35) (Kaplan and Miller, 2000). This pathway supports sympathetic neuronal survival by blocking apoptotic signals that initiate from a second neurotrophin receptor, the p75 neurotrophin receptor (p75NTR) (Kaplan and Miller, 2000). Evidence for the functional antagonism between these two neurotrophin receptors is derived from analysis of TrkA-/- and p75NTR-/- animals. Firstly, during the post natal period of naturally occurring death, virtually all sympathetic neurones die in the TrkA-/- animal (Smeyne et al., 1994) whilst naturally occurring sympathetic neurone death is greatly delayed in p75NTR-/- mice (Majdan et al., 2001). Secondly a proportion of the sympathetic neurone apoptosis seen in TrkA-/- animals is rescued by the concomitant loss of p75NTR (Majdan et al., 2001), indicating that sympathetic neurones
are destined to die as a consequence of ongoing p75\textsuperscript{NTR} mediated apoptotic signalling and survive only if they sequester sufficient NGF to robustly activate TrkA.

Whether or not neurotrophins trigger neuronal apoptosis or survival depends on which Trk receptors are expressed in the cell. For instance the p75\textsuperscript{NTR} binds all neurotrophins, whereas TrkA preferentially binds NGF whereas TrkB binds BDNF, NT-3 and NT-4. Thus NGF has the potential to be pro-apoptotic for cells that do not express TrkA, whereas BDNF would be pro-apoptotic for those cells that do not express TrkB. Therefore, upon reaching the appropriate target, a neurone must sequester enough of the appropriate pro-survival factor which reflects its expression of Trk receptors. Therefore a sympathetic neurone arriving to its target destination would sequester NGF leading to robust TrkA activation, over-riding the p75\textsuperscript{NTR} signal. However if the sympathetic neurone arrives late or to the wrong target, TrkA will be weakly activated whereas p75\textsuperscript{NTR} can be robustly activated by non-TrkA-binding neurotrophins encountered at the target environment such as BDNF, leading to apoptosis.

Apoptosis is a highly conserved process to rid of unwanted cells either during development or in damaged cells to halt the propagation of damaged DNA. Two major apoptotic pathways are defined in cells, the intrinsic and extrinsic pathways (Fig 1.9). The intrinsic pathway is engaged when the cells are challenged by stress and it is primarily mediated by the mitochondria, in which the Bcl-2 family members play a key role regulating this pathway (Korsmeyer, 1999). The extrinsic pathway is mediated by the death receptor family of proteins which involves ligand binding, the recruitment of adaptor molecules and resultant activation of Caspase 8 and apoptosis (Korsmeyer, 1999). Apoptosis can also be induced via endoplasmic reticulum (ER) pathways. The ER pathway is triggered by unfolded proteins and other abnormal cytosolic signals. The p53 tumour suppressor plays a key role in all three of these apoptotic pathways (see later).

In the case of neuronal cells, apoptosis occurs due to an imbalance of pro-apoptotic signals over anti-apoptotic which are regulated by the levels of various neurotrophic factors. The central role of the JNK-p53-Bax apoptotic pathway in response to neurotrophic withdrawal or p75\textsuperscript{NTR} activation is discussed in detail in section 1.35. The general mechanisms of p53 mediated apoptosis in the nervous system are reviewed here and shown in Fig 1.9.
Apoptosis is regulated by the interaction of pro- and anti-apoptotic members of the Bcl2 family which function to either promote (Bax, Noxa, Puma, Bid and Bad) or prevent (Bcl-2, Bcl-xL) membrane permeability transition and the release of Cytochrome c (Yang et al., 1997; Narita et al., 1998). There are approximately 15 members of the Bcl-2 family which share one or more Bcl-2 homology domains (BH1-BH4) (Korsmeyer, 1999). The anti-apoptotic members of the Bcl-2 family, all possess BH1 and BH2 domains with some also containing additional BH3 and BH4 domains. The pro-apoptotic members all contain the BH3 domain which is essential for their pro-apoptotic effect and can be subdivided based on the presence or absence of other BH domains.

The pro-apoptotic Bax protein has been shown to interact with Bcl-xL and Bcl-2 to regulate mitochondrial Cytochrome c release. Thus heterodimerization of Bax with either Bcl-2 or Bcl-xL inhibits the pro-apoptotic functions of Bax. However activation of Bax, results in the formation of Bax-Bax homodimers which function by disrupting the mitochondria membrane resulting in the loss of Cytochrome c and apoptosis. The BH3 only proteins (eg Noxa and

Figure 1.9: Downstream targets of p53 mediated apoptosis
The role of p53 and p73 in neuronal developmental apoptosis is discussed in section 1.35. Purple targets represent genes regulated by the p53 transcription factor. Note that some of these targets are also regulated by p73 such as Bax, Noxa, Puma, p53AIP1 and Fas.
Puma) act upstream of Bax where they interact with the anti-apoptotic family members and inhibit their function.

The anti-apoptotic Bcl-2 and Bcl-xL proteins are widely expressed in both the embryonic and adult nervous system and are required for the survival of specific populations of neurones in both the CNS and PNS. (Allsopp et al., 1993; Motoyama et al., 1995; Gonzalez-Garcia et al., 1995). Furthermore, the Bcl-x gene can undergo alternative splicing to produce the anti-apoptotic form Bcl-xL and the pro-apoptotic form Bcl-xs, of which Bcl-xL is the predominant transcript found in the developing and adult nervous system (Gonzalez-Garcia et al., 1994). However, Bcl-2 KO mice showed only a subtle neuronal developmental phenotype affecting primarily the maintenance of specific neuronal subpopulations during postnatal life (Michaelidis et al., 1996). In contrast, disruption of the Bcl-x gene results in massive apoptosis of post-mitotic neurones throughout the developing nervous system but fails to affect apoptosis of neural precursor cells in the ventricular zone. As such these mice die during embryogenesis at E13.5 (Motoyama et al., 1995). Moreover Bcl-x -/- /Bcl-2 -/- double deficient mice shown even more loss of post-mitotic neurones (Shindler et al., 1998) suggesting that Bcl-2 may compliment Bcl-xL in these subpopulations, as well as its main function in the survival of mature neurones.

Of the ten known pro-apoptotic Bcl-2 members, Bax has proven to be the most important in neuronal development. Indeed, Bax -/- animals show dramatically increased neuronal cell numbers as a result of lack of naturally occurring death of post-mitotic neurones. Moreover, Bax-/- neurones survive even in the absence of neurotrophic factors (Deckwerth et al., 1996; White et al., 1998). Furthermore the generation of Bcl-xL/Bax double knock out mice demonstrate that loss of Bax rescues the increased apoptosis seen in the Bcl-xL-/- null mice (Shindler et al., 1997). Thus the relative levels of expression of Bax and Bcl-xL appear to determine neuronal susceptibility to apoptosis during naturally occurring cell death.

The action of the Bcl-2 family is frequently mediated via activation of caspases whose defined role in neuronal cell death has been well established. Caspases comprise a family of cysteine containing aspartate-specific proteases which contain three domains. These are the N-terminal domain, a large subunit and a small subunit. Caspases are present in the cell as proenzymes and activation of caspases involves proteolytic cleavage and the association of
the large and small subunits to form an active heterodimer or tetramer (Thomberry and Lazebnik, 1998). Once activated caspases cleave other caspases further downstream, an event that leads to cell death. For example, the cleavage of Caspase-3 by either caspase 8 (activated via the death receptor pathway) or Caspase-9 (activated via the mitochondria pathway) is irreversible and commits the cell to undergo cell death. The importance of caspases in neuronal cell death is shown by the phenotypes of mice in which the Caspase-3 or Caspase-9 genes have been deleted (Kuida et al., 1996; Kuida et al., 1998).

Both Caspase-3 and Caspase-9 null mutants are embryonic lethal and in both cases show gross structural abnormalities in the nervous system, including indentations of the cerebrum and periventricular masses as a consequence of a failure of progenitor cells to undergo apoptosis (Kuida et al., 1996; Kuida et al., 1998). The similar neuronal phenotypes of these caspase knock out mice is also common to Apaf1 null animals (Cecconi et al., 1998) and a subset of p53-/- animals (see section 1.32) (Armstrong et al., 1995; Sah et al., 1995) suggesting that these different proteins may function in the same death pathway in neuronal cells. Indeed p53 acts upstream of Apaf1 and induces its expression (Kannan et al., 2001). The Apaf1 protein mediates apoptosis by activating Caspase-9 after mitochondrial damage. Thus, Apaf1 forms a complex with Cytochrome c and Caspase-9 via the caspase recruitment domain (CARD) motif that is present in Caspase-9 but not Caspase-3. The interaction of Apaf1, Caspase-9 and Cytochrome c forms an active apoptosome, which results in the cleavage of Caspase-9 which in turn cleaves Caspase-3, leading to apoptosis (Fig 1.9).

Death receptor mediated activation of caspase-8 has also been shown to function in neuronal cells. In this case Caspase-8 mediated cleavage of pro-apoptotic Bid produces truncated Bid (tBid), which functions by aiding Bax insertion into the mitochondrial membrane resulting in membrane dysfunction and subsequent release of cytochrome c (Fig 1.9).

Therefore, the proper control of neuronal proliferation, differentiation and cell death is essential for the normal function of the nervous system. The role of the Brn-3 transcription factor in neuronal development is discussed in the following sections.
1.17 Expression of Brn-3a and Brn-3b in the nervous system

Most of the studies on expression and function of Brn-3 proteins in the nervous system have been focused on the Brn-3a and Brn-3b transcription factors. Both Brn-3a and Brn-3b show extensive overlap in the CNS and PNS during development, but in the adult, Brn-3b expression is more restricted. Table 1.3 summarises the expression of these two genes in the developing and adult nervous system.

1.17.1 Brn-3a in neuronal cells

Brn-3a is the most widely expressed of the Brn-3 transcription factors in the nervous system. In the peripheral nervous system (PNS), Brn-3a is found in the dorsal root ganglia, trigeminal ganglia and spinal cord whereas in the central nervous system (CNS), Brn-3a is expressed in the hindbrain and midbrain. In the PNS, Brn-3a is first detected in the neuronal precursor cells, immediately before the cells exit the cell cycle, as well as in differentiated sensory neurones. However, in the CNS, Brn-3a expression is observed only in post-mitotic neurones. This suggests that Brn-3a is involved in specification/maintenance of a subset set of neurones in the PNS and in the terminal differentiation/maintenance of a subset of neurones in the CNS (Gerrero et al., 1993; Turner et al., 1994; Fedtsova and Turner, 1995; Xiang et al., 1995).

In the PNS, expression of Brn-3a is first detected at E9.0 in the migrating neural crest cells which are destined for a sensory lineage (Fedtsova et al., 1995; Greenwood et al., 1999). Precursors arising from the neural crest can give rise to both sensory and autonomic cells of the PNS, in which Brn-3a is expressed in cells destined for a sensory lineage but not in those of autonomic lineage (Greenwood et al., 1999). At E9.5, Brn-3a is also detected in the trigeminal ganglia (TG), dorsal root ganglia (DRG) and the spinal cord. In the spinal cord, Brn-3a is detected in the dorsal half and intermediate gray of the cord in post-mitotic cells (i.e. outside the ventricular zone) and by E18, Brn-3a is predominantly detected in the deep dorsal horn (Ninkina et al., 1993; Fedtsova et al., 1995). Brn-3a continues to be expressed in the sensory ganglia throughout development when the cells have become post-mitotic and
<table>
<thead>
<tr>
<th>Brain Structures</th>
<th>Brn-3a E15.5</th>
<th>adult</th>
<th>Brn-3b E15.5</th>
<th>adult</th>
<th>Localization</th>
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<tr>
<td>Thalamus</td>
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<td>Medial habenula</td>
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<td>Lateral habenula</td>
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<td>Superior colliculus</td>
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<td>Inferior colliculus</td>
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<td>-Interpeduncular n.</td>
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<td>+</td>
<td>+++</td>
<td>Periaqueductal gray</td>
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<td>-Mesencephalic n.</td>
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<td>-Red nucleus</td>
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<td>Hindbrain</td>
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<td>Brain stem (motor)</td>
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<td>Nucleus ambiguus</td>
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<td>Inferior olivary n.</td>
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<td>Retina</td>
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<td>Sensory neurones</td>
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<tr>
<td>-Trigeminal</td>
<td>+++</td>
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<td>+</td>
<td>+</td>
<td>Brn-3a in dorsal gray</td>
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<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>Brn-3b in intermediate gray</td>
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<td>Dorsal root ganglia</td>
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<td>Spinal cord</td>
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Table 1.3: Expression patterns of Brn-3a and Brn-3b in the developing and the adult nervous system.

Expression of Brn-3a and Brn-3b in the developing and adult nervous system. Strong expression ++++, moderate expression ++, weak expression +, no detectable expression -. Nucleus is abbreviated to n. This table was reproduced from a PhD thesis, titled Regulation and Function of POU domain transcription factors Brn-3a and Brn-3b, Author Dr. Budhram-Mahadeo
remains high in the TG and DRG postnatally (Turner et al., 1994; Fedtsova et al., 1995; Greenwood et al., 1999).

As expression of Brn-3a in the periphery is restricted to the sensory ganglia, and is not detected in any other neural crest derivative, this suggests that Brn-3a is an early specific marker of migrating neural crest cells fated for a sensory lineage which contribute to the DRG and sensory cranial ganglia (the DRG is derived specifically from the neural crest, whereas the different sensory cranial ganglia receive contributions from the neural crest and/or neural placodes which are discrete patches of ectoderm on the head of the vertebrate embryo). The expression of Brn-3a in the sensory neuronal lineage arising from the mammalian neural crest has been investigated by Greenwood and colleagues (1999). In proliferating sensory precursor cells, BrdU +ve cells expressing Brn-3a were shown to divide for 2 days following migration from the neural tube, before differentiating into sensory neurones. This suggests that expression of Brn-3a in NCC might mark the point of commitment to the sensory lineage. This was supported by studies in which addition of BMP2, which induces autonomic neurogenesis did not prevent sensory neurogeneis in Brn-3a expressing NCC (Greenwood et al., 1999). Interestingly, further characterisation of the sensory neurones generated from such precursors exhibited a proprioceptive and/or mechanosensory but not a nociceptive phenotype under these culture conditions, which correspond to a sublineage of early differentiating precursors of Brn-3a-positive large diameter sensory neurones, identified in vivo (Frank et al., 1991).

Thus the early expression of Brn-3a in precursor cells destined for a sensory lineage suggests that Brn-3a is involved in the specification of these cells. This is analogous to the role of Unc-86, which is also expressed prior to the exit of cells from the cell cycle in the neuronal precursor cells in the nematode and plays a critical role in the determination of sensory neurones (Finney and Ruvkun, 1990).

In the central nervous system, Brn-3a is detected at high levels in the developing midbrain, hindbrain and the spinal cord and continues to be expressed in specific regions of the adult brain. Brn-3a is first detected in occasional cells near the midline of the rostral mesencephalic tectum at E8.5, prior to closure of the anterior neuropore (Fedtsova et al., 1995). In contrast to the Brn-3a expressing cells in the PNS, these cells do not express PCNA
and are thought to have exited the cell cycle. By E9.5 Brn-3a mRNA can clearly be detected in the mesencephalic neurones in the midbrain. Between E12.5 and E15.5 Brn-3a expression is observed in the inferior olivary complex of the hindbrain, the outer layer of post-mitotic cells in the tectum of the midbrain, the developing red nucleus and the medial habenula (Turner et al., 1994). Around this time Brn-3a is also expressed in the inner most layer of cells in the retina (which give rise to the ganglion cell population) at the time when these cells start to differentiate and initiate axon growth, with retinal expression continuing postnatally (Gerrero et al., 1993; Turner et al., 1994). Hence during CNS development, Brn-3a expression is restricted to post-mitotic neurones in specific regions of the brain.

In the adult brain, Brn-3a is detected in similar areas to that observed in the developing brain but in a more restricted manner. Thus, Brn-3a is detected in the medial habenula, superficial gray of the superior colliculus, red nucleus, mesencephalic nucleus of the TG, nucleus ambiguous, inferior olivary nucleus, retina and the peripheral sensory ganglia (Turner et al., 1994). Moreover, even though present in lower levels, Brn-3a is also found in the adult heart, (S.Farooqui 2004) lungs, breast epithelium, ovary and testis (Budhram-Mahadeo et al., 2001). Brn-3a mRNA is not detected in other tissues such as the liver, kidney, spleen and adrenal glands (Gerrero et al., 1993; Ninkina et al., 1993; Turner et al., 1994; Fedtsova et al., 1995).

1.17.1.1 Regulation of Brn-3a expression

As already mentioned the expression of Brn-3a is controlled by two distinct promoters (Frass et al., 2002). The first more distal upstream promoter is TATA-less and contains two bona fida mRNA start sites spanning over 500 base pairs 5' of the ATG translation initiation and directs the expression of the long isoform of Brn-3a [Brn-3a(l)]. The second promoter, also TATA-less, spans the first intron of the Brn-3a gene and controls the expression of the shorter form of Brn-3a [Brn-3a(s)] specifically in neuronal cells but not in epithelial cells. Thus, alternative promoter usage allows these two isoforms to be differentially regulated in different cell types and during development.

Interestingly, two clusters of Brn-3 recognition sites have been identified within the Brn-3a locus, which are located approximately 10 and 5 kb from the start site of transcription. The
distal and proximal sites contain 1 and 4 copies, respectively, of a sequence motif that conforms closely to the optimal Brn-3 recognition site. In transfection assays, the proximal cluster was more effective at transactivating Brn-3a expression compared with the distal region (Trieu et al., 1999). Thus, these regions are likely to mediate autoregulation providing a mechanism by which Brn-3a expression in maintained in differentiated neuronal cell types and in the adult nervous system. Furthermore, this autoregulation may provide the positive feedback mechanism that increases expression of Brn-3a to compensate for loss of one allele in Brn-3a +/- animals which develop normally.

1.17.2 Brn-3b in neuronal cells

Brn-3b expression in both the developing and adult brain shows considerable overlap with Brn-3a, but also shows some distinct expression from Brn-3a which strongly suggests that unique roles for each gene exists in the development of specific neuronal phenotypes (see table 1.3).

Brn-3b expression is first detected in the mouse embryonic body at E10.5 with expression in the head observed by E11.5, which is later than that observed for the expression of Brn-3a which is first detected at E9.5 in similar structures (Turner et al., 1994).

In the hindbrain and midbrain, Brn-3b mRNA is detected by E13.5 and the pattern of expression shows some similarity with Brn-3a. For example, both Brn-3a and Brn-3b are detected in the post-mitotic neuronal cell population of the inferior olivary complex by E15.5. However, Brn-3b also demonstrates some sites of unique expression so that by E17.5 Brn-3b expression is detected in the medullary anterior external migratory stream, a group of cells that do not express Brn-3a. In the midbrain, both Brn-3a and Brn-3b are detected in the tectum of the mesencephalon. However, by E17.5 Brn-3b but not Brn-3a is uniquely detected in the interpeduncular of the midbrain where its expression persists into adulthood (Turner et al., 1994). Analysis of Brn-3b protein expression in serial coronal sections from postnatal day 1 (PI) mouse brain, showed that Brn-3b is expressed in the lateral margin of the cerebellum and in the midbrain. Specifically, Brn-3b expression was found in the superior colliculus (which integrates the sensorimotor inputs to control eye movement) and the adjacent
periaqueductal gray of the midbrain (Xiang et al., 1993).

In the retina, Brn-3b mRNA is first detected in the innermost layer of ganglion precursor cells at E11.5 and by E17.5, this expression is restricted to the post-mitotic layer (Turner et al., 1994). In the retina, Brn-3b expression precedes Brn-3a expression (Brn-3b is detected by E11.5 whilst Brn-3a is not detected until two days later). The relative expression of both factors peak at birth but gradually decreases by P30 (Turner et al., 1994).

In contrast, to its expression in the retina, Brn-3b is first detected in the sensory ganglia, TG and DRG, at E13.5, two days later than the onset of Brn-3a expression in these neurones. By E15.5, Brn-3b is also detected in the cochlear and vestibular parts of the cranial nerve ganglion VIII and the ganglion of glosopharyngeal nerve IX. In the spinal cord, Brn-3b is widely expressed by E12.5 but by E15.5, its expression is restricted to the intermediate gray while Brn-3a mRNA is expressed throughout the dorsal gray region (Turner et al., 1994).

In the adult mouse and rat neuronal tissues, Brn-3b expression is restricted to specific regions of the brain and PNS. In the adult rat CNS, Brn-3b is detected in the optical, intermediate and deep gray areas of the superior colliculus, the dorsal column of the mesencephalic, pontine central gray, and the lateral interpeduncular nucleus. It is also expressed in the adult TG, DRG and retina (Turner et al., 1994). Brn-3b is also expressed in the adult heart and reproductive tract but not in the liver, spleen, kidney, lung and thymus (Turner et al., 1994; Budhram-Mahadeo et al., 2001; S. Farooqui 2004).

1.17.2.1 Regulation of Brn-3b activity

The regulation of Brn-3b expression in different cell types has yet to be fully determined and the characterization of the Brn-3b promoter and identifying factors that regulate its activity are currently being investigated in our laboratory.

The co-expression of Brn-3a and Brn-3b in several sub-populations of neurones led to the idea that they may regulate each others expression. Indeed, Brn-3b was shown to activate the proximal regulation sequence in the Brn-3a locus in transfection assays. This is interesting as it suggests that Brn-3b may activate the expression of Brn-3a in neuronal sub-populations in which Brn-3b expression is switched on first such as in the retina and the
inferior olivary. Such a regulatory mechanism may also explain the severity of the retinal
defects in Brn-3b -/- animals (section 1.20) where the absence of Brn-3b would lead to the
absence of Brn-3a which would otherwise compensate for the lack of Brn-3b in these
animals.

Thus, expression studies in the developing nervous system have shown that both Brn-3a and
Brn-3b are expressed in the CNS coinciding with the terminal phase of neuronal
development in each area of their expression, suggesting a developmental role for each factor
in the terminal differentiation and maintenance of specific neuronal phenotypes. The
expression of Brn-3a in sensory neural crest precursors and differentiated sensory neurones is
interesting as it suggests that expression of Brn-3a plays a significant role in committing
these cells to a sensory neuronal fate as well as being required for their differentiation.

The generation of mice models in which the Brn-3a and Brn-3b gene has been deleted have
proved very useful in elucidating the role of these proteins in neuronal development and
confirmed the critical role that these factors play in determining the cell fate of specific
neuronal cell types. The functions of Brn-3a and Brn-3b in neuronal cells are discussed in the
following sections.

1.18 Role of Brn-3a in neuronal survival

The expression pattern of Brn-3a suggested that this transcription factor might play an
important role in the development of sensory ganglia as well as a subset of neurones in the
CNS in which this transcription factor is expressed. Indeed, analysis of Brn-3a-/- embryos
has revealed significant apoptosis in sensory neurones during late embryogenesis in the TG,
DRG as well as in the red nuclei, and nucleus ambiguus of the hindbrain and the inferior
olivary nucleus and medial habenula (Xiang et al., 1996; McEvilly et al., 1996; Huang et al.,
1999). Thus it appears that the neurones that would normally express Brn-3a are specified
but they fail to survive at these sites in the Brn-3a -/- mice, indicating that Brn-3a is essential
for their survival.

The phenotype of Brn-3a -/- animals show gross phenotypic abnormalities which include
ineffective swallowing and uncoordinated limb and trunk movements resulting in the
inability to right themselves. These animals also show intermittent abnormal movement and posture including rigid outstretched limbs and head. Consequently these animals die within 24 hr of birth possibly as a result of the inability to suckle. Both the red nucleus and the inferior olivary nucleus are involved in co-ordination of movement and the striking loss of neurones in these locations in the Brn-3a -/- mouse would contribute to the abnormal locomotion and posture in these animals. Also the loss of motor neurones in the nucleus ambiguus, which innervate the oesophagus, might contribute to the abnormal peristaltic swallowing reflex. In the PNS there is a significant reduction in the biochemical markers that characterise sensory neurones, which can also contribute to the uncoordinated limb and trunk movement in these animals.

To better understand the changes underlying sensory neurogenesis in mice lacking Brn-3a, Eng and colleagues (2001) generated transgenic mice in which they cloned a Lac Z reporter into the Brn-3a locus. Crossing this strain with mice carrying a Brn-3a null allele generated Lac Z Brn-3a knock out embryos which facilitated the analysis of cells that would normally express Brn-3a and to further examine the abnormalities that accompany the loss of this transcription factor. These studies demonstrated that mice lacking Brn-3a have defects in sensory axon growth. Thus at E11.5 the neurones in the TG and DRG condense as expected and grow towards their peripheral targets. However by E13.5 the Brn-3a null mice demonstrate abnormal axon bundles and migration as well as premature branching in the TG. Furthermore, the TG neurones do not effectively innervate their peripheral targets and this is particularly obvious in the whisker follicles. By E15.5 there is extensive apoptotic death resulting in loss of cells that would normally express Brn-3a (Eng et al., 2001) These results suggest that death in Brn-3a-/- mice results from the failure of sensory axons to correctly innervate their targets and access target derived factors. Hence Brn-3a appears to control survival and normal differentiation/path-finding of specific neurones.

The analysis of target gene expression in the TG of Brn-3a-/- embryos showed a decrease in the number of TrkA and TrkB expressing sensory neurones by E13.5 whereas TrkC could not be detected in the TG of Brn-3a-/- embryos. However the timing of initiation of neuronal cell death Brn-3a-/- TG occurs at E15.5. This suggests that Brn-3a, normally expressed at E9 is critical for the activation of TrkA and TrkB genes or the growth promoting effects of Brn-3a is required to maintain TrkA and TrkB expression neurones during the period of
naturally occurring developmental cell death. Conversely the failure of TrkC expressing neurones to emerge in the TG suggests that Brn-3a is involved in their induction. However, despite the expression of Brn-3a in TG precursor cells, loss of Brn-3a does not result in a reduction in the number of these cells (Huang et al., 1999).

Although Brn-3a is critical for the regulation of expression for Trk receptors, these receptors do not appear to directly affect survival. For instance, the wave of apoptosis seen in the TG of Brn-3a -/- mice occurs at E15.5, two days after loss of TrkA and TrkB expression. Moreover, the generation of double KO Brn-3a/Bax mice resulted in surviving neurones but TrkA expression is eventually lost, suggesting that this receptor is necessary for neuronal differentiation rather than the apoptosis observed in the Brn-3a -/- neurones (section 1.19) (Ma et al., 2003). In addition, Brn-3a was shown to induce survival under conditions of neurotrophin deprivation both in vitro and in vivo (see below) (Smith et al., 2001;Ensor et al., 2001) indicating that it is unlikely that the Brn-3a survival function is mediated just by up-regulating neurotrophic receptors (McEvilly et al., 1996). Interestingly, Brn-3a has been shown to control expression of both anti-apoptotic and pro-apoptotic factors which may represent the main mechanism by which Brn-3a controls survival of neurones in which it is expressed (see below). Additionally, recent studies have shown that Brn-3a can control neuronal cell fate by modifying gene transcription by interaction with other cellular proteins such as p53 (section 1.24) (Perez-Sanchez et al., 2002;Budhram-Mahadeo et al., 2002).

The requirement of Brn-3a in neuronal survival has also been shown using in vitro systems. Initial studies were carried out in the ND7 cell line which was derived by fusing neurones from non-dividing rat DRG with the C1300 mouse neuroblastoma cell line (Woods et al., 1990). One resultant clone could proliferate indefinitely in full growth medium behaving like the parental neuroblastoma cells. However these cells can be induced to differentiate upon serum withdrawal or addition of defined medium supplemented with growth factors and cyclic AMP analogues. Upon differentiation, these cells undergo cell cycle arrest and morphological changes such as neurite outgrowth accompanied by movement of secretory granule component and synaptic vesicle component to the apex of processes, mimicking the events associated with differentiation of sensory neurones. These findings make this cell line ideal to investigate the role of Brn-3a in neuronal survival and differentiation.
Thus, *in vitro* studies have shown that the overexpression of Brn-3a in the ND7 neuronal cell line increases survival even upon removal of neurotrophic factors compared with controls (Smith et al., 1998a). This protective effect of high Brn-3a levels is also observed in cultured DRG and TG neurones upon NGF withdrawal (Smith et al., 1998b; Ensor et al., 2001). Similarly, increasing Brn-3a expression protects DRG cells following sciatic nerve lesion *in vivo*. Conversely, the reduction of Brn-3a expression using an anti-sense approach results in apoptosis in ND7, DRG and TG neurones even in the presence of neurotrophic factors (Smith et al., 2001; Ensor et al., 2001).

Subsequently, anti-apoptotic genes associated with survival in these cells were shown to be regulated by Brn-3a. Firstly Brn-3a was shown to strongly activate the Bcl-2 P2 promoter (which is the predominant promoter that drives Bcl-2 expression in neuronal cells) and to a lesser extent the Bcl-2 P1 promoter in ND7 cells as well as in primary DRG cells. Furthermore, Bcl-2 expression was significantly elevated in ND7 cells over-expressing Brn-3a and in primary cultures of DRG and TG neurones transfected with Brn-3a compared with controls (Smith et al., 1998a; Smith et al., 1998b; Ensor et al., 2001). Similarly, a decrease in the levels of Bcl-2 expression is evident in cells transfected with Brn-3a anti-sense which reduced the expression of this transcription factor (Ensor et al., 2001). More recently, Brn-3a was shown to transactivate another anti-apoptotic gene, Bcl-xL, which shares high homology to Bcl-2. Interestingly the phenotype of the Bcl-xL null mice is similar to the Brn-3a knockout in terms of requirement for neuronal survival (Motoyama et al., 1995; Xiang et al., 1996; McEvilly et al., 1996). Hence these findings support the hypothesis of these factors being in a similar pathway that is required for protection of neurones *in vivo*. Moreover, overexpression of Brn-3a in DRG and TG primary cultures results in clear elevation in the endogenous mRNA and protein levels of Bcl-xL (Smith et al., 2001). Conversely, use of anti-sense to decrease levels of Brn-3a show a concomitant decrease in Bcl-xL levels. Furthermore, Brn-3a activates the Bcl-xL promoter in primary sensory neurones and ND7 cells (Smith et al., 2001). Additionally Brn-3a has been shown to inhibit expression of the pro-apoptotic Bcl-2 family member, Bax either directly, through a mild repression and indirectly through antagonism of p53 (see section 1.24) (Budram-Mahadeo et al., 2002).

Thus the direct up-regulation of Bcl-xL and down-regulation of bax may be more relevant in protecting neurones during development and may explain why significant numbers of
sensory neurones fail to survive the naturally occurring death period in Brn-3a-/- animals, as Brn-3a-/- mice and Bcl-xL-/- mice display similar loss of neurones in terms of time of death and in the population of neurones affected (Motoyama et al., 1995; Xiang et al., 1996; McEvilly et al., 1996; White et al., 1998). In contrast, the Brn-3a up-regulation of Bcl-2 may be more important in the survival of postnatal neurones.

1.19 Role of Brn-3a in neuronal differentiation

Both in vivo and in vitro studies have shown that in addition to the protective effects conferred by Brn-3a in neuronal cells, this transcription factor also plays a critical role in neuronal differentiation.

Brn-3a is expressed only in post-mitotic neurones in the CNS and identifies the first differentiated neurones to appear in the midbrain, hindbrain and spinal cord. Thus the onset of Brn-3a expression in the CNS suggests that it is not required for neurogenesis of the neuroepithelium but is involved in specification of mature neuronal phenotypes. In the PNS, Brn-3a expression is initiated in neural crest cells destined for a sensory lineage, just before neurones exit the cell cycle suggesting that Brn-3a is involved in commitment as well as differentiation in these cells that express it (Fedtsova et al., 1995; Greenwood et al., 1999). In particular, the aberrant axon guidance observed in TG of Brn-3a-/- animals at E13.5 suggests a role for this factor in the development of functional axons, (Eng et al., 2001).

As already mentioned the TG ganglia in Brn-3a-/- animals fail to express TrkC and show a significant secondary loss of TrkA and TrkB expressing neurones by E13.5. This suggests that Brn-3a might be important in regulating the expression of these Trk receptors. However, so far Brn-3a has only been shown to directly regulate TrkA (Huang et al., 1999), which may be involved in Brn-3a mediated differentiation rather than survival (Ma et al., 2003). Indeed, lack of TrkA/NGF signalling results in defective innervation of peripheral targets by sensory neurones (Patel et al., 2000). Moreover, the TG of Brn-3a-/- mice display aberrant axonal growth around the time that TrkA protein levels are minimally reduced (Huang., 1999; Eng et al., 2001). Thus it is possible that the abnormal axon defects in Brn-3a-/- mice are a result of altered expression of Trk receptors. To date, other regulatory targets through which Brn-3a

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might exert its effects on sensory axon formation are still to be identified.

The requirement for Brn-3a in neuronal differentiation and outgrowth of neuronal processes has also been demonstrated in vitro. Amplification of mRNA from ND7 cells using primers specific to Brn-3a, show very low levels are expressed in actively proliferating ND7 cells. However, Brn-3a mRNA levels are greatly increased when ND7 cells switch from the proliferating phenotype to a differentiated state with neurite outgrowth. This pattern of expression was also confirmed by changes in Brn-3a protein levels (Lillycrop et al., 1992; Budhram-Mahadeo et al., 1995a). Therefore Brn-3a appears to be associated with neuronal differentiation in this cell model. A more detailed investigation in ND7 cells and cultured adult rat DRG neurones revealed that the ratio of the long and short forms of Brn-3a is regulated at the mRNA level in response to various differentiation-inducing stimuli. Upon differentiation it was demonstrated that the increase in Brn-3a mRNA was due to increasing levels of the short form of Brn-3a, whereas the mRNA encoding the long form was only marginally elevated (Liu et al., 1996).

In agreement with a role for Brn-3a in neuronal differentiation, over expressing Brn-3a in ND7 cells is sufficient to cause increased neurite outgrowth by up to 60% even when these cells are grown in conditions that would not normally promote neurite formation (i.e. when grown in full serum medium which promotes proliferation) (Smith et al., 1997b). Furthermore, over-expression of Brn-3a in ND7 cells resulted in enhanced neurofilament heavy (NF-H), neurofilament medium (NF-M) and neurofilament light chain (NF-L) expression at both the mRNA and protein level. Moreover, Brn-3a was shown to transactivate all three neurofilament genes in co-transfection assays (Smith et al., 1997c). Similarly, Brn-3a over-expressing ND7 cells exhibited a clear up-regulation of SNAP-25 mRNA which is involved in vesicle exocytosis and axon outgrowth, and Brn-3a was shown to be a direct activator of the SNAP-25 gene (Lakin et al., 1995). Moreover, Brn-3a was shown to directly activate the neuronal intermediate filament, α-internexin gene (Budhram-Mahadeo et al., 1995b). Finally, up-regulation of three other proteins involved in the synaptic vesicle cycle was exhibited in Brn-3a over-expressing ND7 cells. These were synapsin I, synaptophysin and synaptotagmin I which were found to be up-regulated at both the protein and mRNA level in Brn-3a over-expressing ND7 cells compared to controls (Smith et al., 1997b). Conversely reduction of Brn-3a levels using an anti-sense
approach, results in failure of neurite outgrowth even when cells were grown in optimal differentiation conditions (Lakin et al., 1995).

Activation of the neurofilament genes and the α-internexin gene provides the cytoskeletal framework for neurite elongation and for the radial growth of axons, and are essential for maintaining axon diameter and transport. The regulation of different neurofilament genes by one transcription factor is interesting in view of the fact that neurofilament are obligate heteropolymers and any change in the expression of individual subunits results in aberrant axon size and transport. Hence a common regulator of all three neurofilament genes would be critical for their correct functioning, making Brn-3a a master regulator for controlling the expression of these genes in Brn-3a expressing neurones.

Thus the induction of neurite outgrowth upon Brn-3a over-expression or following the rise in Brn-3a expression during ND7 cell differentiation is likely to be dependent on its ability to activate specific target genes whose protein products are required for neurite outgrowth.

1.20 Role of Brn-3b in neuronal development

Detailed analysis of Brn-3b/- animals showed most significant changes in the eye, where there is loss of approximately 70% of their retinal ganglion cells between P17-20, resulting in these animals being blind (Erkman et al., 1996; Gan et al., 1996). The significance of the phenotype of the Brn-3b animals is demonstrated by its high expression in the retinal ganglion cells which precedes that of Brn-3a. Interestingly, retinal ganglion cells of Brn-3b/- animals show disorganised neurites which fail to fasciculate efficiently and thus undergo apoptosis (Gan et al., 1999; Erkman et al., 2000). Therefore Brn-3b is required for normal retinal ganglion cell differentiation. Even though Brn-3b is expressed in other regions of the CNS and PNS there were no apparent defects in these structures suggesting functional redundancy by the presence of other related proteins such as Brn-3a/Brn-3c.

In differentiating ND7 cells, Brn-3b shows reciprocal expression to that of Brn-3a. Thus proliferating ND7 cells grown in full growth media express high levels of Brn-3b which dramatically decreases upon differentiation (Lillycrop et al., 1992; Budhram-Mahadeo et al., 1995a). Interestingly, when grown in differentiation medium, ND7 cells that artificially
over-express Brn-3b showed reduced length of neurites and reduced expression of SNAP-25, synaptophysin and synaptotagmin that are activated during Brn-3a-induced differentiation (Smith et al., 1997a). The association of Brn-3b with proliferation and Brn-3a with differentiation was further shown in cell lines derived from human neuroblastoma tumours (IMR-32, SK-N-SH and SK-N-MC). Additionally, Brn-3b over-expressing cells failed to respond to growth inhibitory stimulus such as retinoic acid (RA) which causes differentiation of wild type neuroblastoma cells (Irshad et al., 2004). Moreover, treatments that induces differentiation results in activation of a test promoter that is normally induced by Brn-3a and repressed by Brn-3b (Morris et al., 1994; Smith and Latchman, 1996). Indeed Brn-3b has been shown to directly antagonise Brn-3a activation of specific target genes (see section 1.22).

Therefore, these findings indicate that the regulation of neuronal cell differentiation is controlled by the enhanced expression of the differentiation inducing factor, Brn-3a and a fall in the level of the proliferation associated factor, Brn-3b. As these factors are expressed in distinct but overlapping patterns in the developing and adult nervous system (Gerrero et al., 1993; Turner et al., 1994; Fedtsova et al., 1995) it is possible that they play a complex role in regulating neurite outgrowth during development. This is particularly evident for Brn-3b which exhibits different roles in different cell types, as it is associated with the survival and terminal differentiation of retinal ganglion cells whereas in ND7 and breast cancer cells it is associated with proliferation and frequently shows antagonistic effects to Brn-3a.

1.21 Expression and function of Brn-3a and Brn-3b in non-neuronal tissue

Even though Brn-3a and Brn-3b were originally detected in the developing and adult nervous system where they play a critical role in development of specific subsets of neurones, subsequent studies have demonstrated expression of both these transcription factors in non-neuronal cells, specifically in tissues of the reproductive tract (Budhram-Mahadeo et al., 2001) and certain cancers including cervical and breast cancer (Budhram-Mahadeo et al., 1998; Budhram-Mahadeo et al., 1999b; Ndisang et al., 2000; Ndisang et al., 2001).

Using Immunocytochemistry, both Brn-3a and Brn-3b were shown to be expressed in the
adult rat testis with the expression of each factor being observed at distinct stages of germ cell development. Thus, while Brn-3a is detected in the spermatogonia, Brn-3b is detected in post-meiotic spermatids. Interestingly the expression of Brn-3a(l) during germ cell development coincides with that of the BRCA-1 anti-oncogene, which Brn-3a has been shown to transactivate in co-transfection assays, while Brn-3b represses this promoter. Thus Brn-3a and Brn-3b may play distinct roles in the regulation of gene transcription during germ cell development as well as during neuronal development.

Brn-3a and Brn-3b have also been detected in breast epithelia cells but the expression of Brn-3b and not Brn-3a is increased in breast tumours compared with normal mammary tissue. Furthermore malignant tumour samples that show increased expression of Brn-3b also had reduced levels of BRCA-1 at both the mRNA and protein level. Interestingly, Brn-3b but not Brn-3a(l) was shown to strongly repress the BRCA-1 promoter in mammary tumour cells. As BRCA-1 halts cell proliferation in normal mammary cells then the negative regulation of its expression by Brn-3b supports a role for Brn-3b in controlling cellular proliferation of mammary cells (Budhram-Mahadeo et al., 1999b). Indeed, stably transfected MCF7 breast cancer cell lines that over-express Brn-3b, exhibited increased growth and proliferation rates compared to control cells containing empty vector, and also shown enhanced growth under anchorage independent conditions (Dennis et al., 2001). Similarly IMR-32 (neuroblastoma cell line) cells over-expressing Brn-3b also showed increased cellular growth and proliferation in monolayer as well as in an anchorage dependent manner compared with controls whereas stably reducing Brn-3b levels reduced the growth rate of these cells. Brn-3b also increased migration of these cells in migration assays compared to controls. Furthermore, mice injected with Brn-3b over-expressing clones showed rapid increase in tumour volume compared to controls (Irshad et al., 2004). Thus Brn-3b contributes to the proliferation of both neuroblastoma cells and breast cancer cells.

Further analysis of the role of Brn-3b in breast cancer progression, demonstrated that Brn-3b altered gene expression associated with growth and cell cycle progression. For instance Brn-3b could strongly activate CDK4 which is required for the G1 to S transition while it repressed the BRCA1 gene promoter (Samady et al., 2004). Plakogobin, which is involved in cell-cell adhesion, cytoskeleton organization and signal transduction, is repressed by Brn-3b which may explain the increased proliferation and migration of breast cells.
which show increased Brn-3b expression (Samady et al. 2005). Furthermore Brn-3b was shown to activate the HSP-27 promoter whose protein product is associated with increased anchorage-independent growth, increased invasiveness, and is a poor prognosis marker (Lee et al., 2005).

Both Brn-3a and Brn-3b have been detected in cervical epithelia cells. However, while expression of Brn-3b is detected in both cancerous and non-diseased cervical epithelium, Brn-3a expression is highly elevated up to 300-fold in high grade cervical intraepithelial neoplasia type 3 (CIN3) whereas the levels of Brn-3b do not change significantly in these cells (Ndisang et al., 2000). Interestingly Brn-3a was shown to activate the upstream regulatory region of the human papilloma viral genome (HPV) (Morris et al., 1994). As it is generally accepted that HPV (particularly types 16 and 18) infection contributes to the development of cervical neoplasia (Burghardt, 1984) it is possible that both HPV and elevated Brn-3a levels may contribute to the progression of cervical cell transformation.

Thus both Brn-3a and Brn-3b are expressed in neuronal and non-neuronal cells and regulate gene expression associated with survival, differentiation and proliferation. The important role of these transcription factors in determining cell fate is reflected in their altered expression being associated with certain cancers. Specifically Brn-3a is up-regulated in cervical cancer whereas Brn-3b is over-expressed in breast cancer as well as neuroblastoma.

**1.22 Gene targets regulated by the Brn-3a and Brn-3b transcription factors**

In general Brn-3a activates genes involved in neuronal differentiation, survival and cell cycle arrest. Studies undertaken in our lab have shown that Brn-3a activates two types of gene promoters, those that are activated by the C-terminal POU domain of the protein and those that require in addition, the N-terminal activation domain. Additional control of transcriptional regulation is provided by the Brn-3b transcription factor which has been shown to repress the basal activity of many promoters activated by Brn-3a and can also inhibit Brn-3a mediated activation of many neuronal promoters which are generally dependent on the Brn-3a POU domain for activation. Thus the opposite expression pattern of Brn-3a and Brn-3b in proliferating versus differentiated neuronal cells is interesting as it
parallels their antagonistic effect on several different promoters. Table 1.4 gives a summary of the various gene targets regulated by Brn-3a and Brn-3b.

Many Brn-3a target genes that have been characterised are involved in the dynamic process of neuronal differentiation, including neuronal outgrowth, synaptogenesis, and early axonal cytoskeleton establishment. These include α-internexin (Budhram-Mahadeo et al., 1995b), the neurofilament genes (Smith et al., 1997c) SNAP-25 (Lakin et al., 1995) and synapsin I (Morris et al., 1996). Brn-3a over expression in ND7 cells also up regulates synaptophysin and synaptotagmin I (Smith et al., 1997b), though a direct effect of Brn-3a on these promoters has not been investigated. Interestingly Brn-3b has been shown to repress Brn-3a target genes involved in neuronal differentiation including α-internexin (Budhram-Mahadeo et al., 1995b), the neurofilament genes (Smith et al., 1997c) and SNAP-25 (Morris et al., 1996). Interestingly, Brn-3b over-expressing ND7 cells also resulted in reduction of synaptophysin and synaptotagmin I at both the mRNA and protein levels (Smith et al., 1997b) but a direct effect on promoter regulation has not been shown.

This antagonistic effect of Brn-3a and Brn-3b on promoters involved in neuronal differentiation may explain their opposite expression patterns during differentiation of ND7 cells and in primary neuronal cells (Lillycrop et al., 1992;Liu et al., 1996). These target genes regulated by Brn-3a and Brn-3b may also help to understand why ND7 cells over-expressing Brn-3a showed increased neurite outgrowth even in the absence of a differentiation stimuli whereas ND7 cells over-expressing Brn-3b actually inhibits neurite outgrowth even in response to stimuli that normally induce differentiation (Smith et al., 1997a;Smith et al., 1997b). However, Brn-3b was found to activate the promoter synapsin I gene in a manner similar to Brn-3a suggesting that Brn-3b can compensate for Brn-3a in terms of activation of some but not all target genes in neuronal cells (Morris et al., 1996).

Interestingly the induction of neurite outgrowth in ND7 cells could be achieved by over-expressing the isolated POU domain of Brn-3a, indicating that this region of the protein is sufficient for this effect. Overexpression of the POU domain of Brn-3b did not alter neurite extension in these cells (Smith et al., 1997b). This effect of the isolated Brn-3a POU parallels the ability of this domain but not the Brn-3b POU domain to activate an artificial test.
## Table 1.4: Promoter regulation by Brn-3a and Brn-3b transcription factors

Activation of the promoter is indicated by +, repression is indicated by -, no effect is indicated by 0, effect not tested is indicated by N.T and * indicated that Brn-3b not only represses the promoter but also negates activation by Brn-3a. The domain involved in the Brn-3 mediated transcription on each promoter is also listed (Adapted from a PhD thesis Transcriptional regulation by Brn-3a POU domain containing transcription factors, Author Dr J. Dennis, 1999)

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Brn-3a</th>
<th>Brn-3b</th>
<th>Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snap-25</td>
<td>+</td>
<td>-*</td>
<td>N-term</td>
</tr>
<tr>
<td>α-internexin</td>
<td>+</td>
<td>-*</td>
<td>POU</td>
</tr>
<tr>
<td>Synaptotagmin 1</td>
<td>+</td>
<td>-</td>
<td>POU</td>
</tr>
<tr>
<td>Synapsin 1</td>
<td>+</td>
<td>+</td>
<td>POU</td>
</tr>
<tr>
<td>NFH</td>
<td>+</td>
<td>-*</td>
<td>POU</td>
</tr>
<tr>
<td>NFM</td>
<td>+</td>
<td>-*</td>
<td>POU</td>
</tr>
<tr>
<td>NFL</td>
<td>+</td>
<td>-*</td>
<td>POU</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>+</td>
<td>0</td>
<td>N-term</td>
</tr>
<tr>
<td>Bcl-x</td>
<td>+</td>
<td>0</td>
<td>Upstream promoter requires N-term, the downstream promoter requires sequences located between the POU and N-term domain</td>
</tr>
<tr>
<td>Bax</td>
<td>-</td>
<td>N.T</td>
<td>POU and sequences located between the POU and N-term domain</td>
</tr>
<tr>
<td>p21 Waf/Cip1</td>
<td>+</td>
<td>N.T</td>
<td>POU</td>
</tr>
<tr>
<td>CDK4</td>
<td>N.T</td>
<td>+</td>
<td>N.T</td>
</tr>
<tr>
<td>Plakoglobin</td>
<td>N.T</td>
<td>-</td>
<td>N.T</td>
</tr>
<tr>
<td>BRCA 1</td>
<td>+</td>
<td>-</td>
<td>N.T</td>
</tr>
<tr>
<td>HSP-27</td>
<td>+</td>
<td>+</td>
<td>N.T</td>
</tr>
<tr>
<td>Vitellogenein</td>
<td>0</td>
<td>+</td>
<td>POU</td>
</tr>
</tbody>
</table>
promoter containing a synthetic binding site for the Brn-3 factors. In contrast the isolated Brn-3b POU domain repressed this promoter (Morris et al., 1994). Interestingly the differential effect exhibited on the artificial test promoter is associated with the single amino acid difference at position 22 in the first helix of the \textit{POU}_{1D} which is a valine residue in Brn-3a and an isoleucine residue in Brn-3b. Thus, substituting the valine at position 22 in Brn-3a with the isoleucine found in Brn-3b converted Brn-3a into a repressor of the promoters that are normally activated by wild type Brn-3a (Morris et al., 1997;Smith et al., 1997c), and inhibits neurite outgrowth (Smith et al., 1997b). Similarly, the mutant form of Brn-3b containing the valine residue converts it into an activator on promoters that are normally repressed by Brn-3b (Dawson et al., 1996;Morris et al., 1997;Smith et al., 1997c) and allows it to stimulate neurite outgrowth (Smith et al., 1997b).

Although the POU domain of the Brn-3 factors directs their binding to DNA, there is no difference in the DNA binding abilities of both the wild type and mutant forms of Brn-3a and Brn-3b (Budhram-Mahadeo et al., 1996). This is in agreement with the location of this residue at position 22 in the C-terminus of the first helix which does not make contacts with the DNA as shown by crystallographic analysis of the POU domain of the related Oct-1 protein. In fact this residue is located on the surface of the POU domain at a position that could be involved in protein-protein interactions (Klemm et al., 1994).

Therefore it is possible that the different effects of Brn-3a and Brn-3b on the same promoters may be the result of interaction with different co-regulatory factors with transactivator or repressor function. Thus the presence of the valine at this position in Brn-3a or the mutant Brn-3b allows the protein to interact with and recruit a co-activator to the promoter, whilst the isoleucine at this position in Brn-3b or the mutant Brn-3a protein allows the recruitment of a co-repressor to the promoter of these neuronal target genes. Thus interaction of the POU domains proteins with other co-activators/repressors which similar to the POU proteins, may also be cell type specific or present at different developmental stages represents a further mechanism to regulate the activity of the POU proteins.

The Brn-3a and Brn-3b transcription factors have also been shown to regulate non-neuronal target genes. For example, CDK4 and HSP-27 gene promoters are transactivated by Brn-3b in breast cancer cells while BRCA1 and plakoglobin are repressed by Brn-3b, providing a
mechanism by which Brn-3b promotes cellular proliferation and invasion in these cells. Brn-3a has also been shown to activate BRCA1 expression in the testis. Additionally the HSP-27 gene can be transactivated by both Brn-3a and Brn-3b in neuronal and breast cancer cells. Thus Brn-3a and Brn-3b can act as either activators or repressors of gene transcription and this effect depend on the target and cell specificity.

1.22.1 Brn-3a has two distinct transactivation domains

As mentioned, Brn-3a activates two types of gene promoters, those which are dependent on the POU domain and those that require the N terminal activation domain, in addition to the POU domain for their activation. The latter promoters are activated only by the long form of Brn-3a [Brn-3a(l)], (Morris et al., 1994). Thus the relative expression of the Brn-3a isoforms also determines which genes will be expressed in specific cell types by alternative promoter usage generating the two different Brn-3a isoforms. Furthermore production of these two isoforms has been shown to be regulated in different neuronal cell types and in response to specific stimuli (Theil et al., 1993; Liu et al., 1996).

Further investigation revealed that the effects of Brn-3a on neuronal survival and differentiation require distinct domains of this transcription factor. Thus differentiation can be induced by overexpression of the isolated Brn-3a POU domain only (Smith et al., 1997b) and this domain is sufficient for the transcriptional activation of a number of genes involved in neuronal differentiation (Lakin et al., 1995; Smith et al., 1997b; Smith et al., 1997c) as described above. Hence this domain can act as an activation domain as well as a DNA binding domain.

In contrast, only Brn-3a(l) but not Brn-3a(s) or the isolated Brn-3a POU domain could protect ND7 cells against apoptosis as a result of serum withdrawal (Smith et al., 1998a). Thus, in the case of Brn-3a, the differential promoter usage to produce the two isoforms has consequences for cell survival, as the long form can promote both neurite outgrowth and enhanced cell survival while the short form promotes neurite outgrowth without increasing survival. The generation of mice lacking the N-terminal domain of Brn-3a or containing the valine to isoleucine mutation in the Brn-3a POU domain has further helped to investigate the functions of the distinct Brn-3a domains in vivo (Faulkes et al., 2004). While the
homozygous valine to isoleucine (V-I) Brn-3a mutants were viable, the N-terminal deletion
mice die shortly after birth, suggesting that the lethal phenotype that occurs in the complete
Brn-3a knock out is due specifically to loss of function of the N-terminal domain. Thus, it
was demonstrated that a similar loss of neurones seen in Brn-3a null mice was also observed
in mice in which the N-terminal was lost but Brn-3a(s) continued to be expressed. In
contrast, no neuronal loss was observed in the homozygous V-I mice. Hence in vivo as well
as in vitro, the N-terminal domain is essential for the Brn-3a effect on neuronal survival.

More specifically, it was demonstrated that the POU domain of Brn-3a was insufficient for
the activation by Brn-3a of genes encoding factors such as Bcl-2 and Bcl-xL which have a
protective effect against cell death (Smith et al., 1998a;Smith et al., 1998b;Sugars et al.,
2001). Thus Bcl-2 activation was only observed by Brn-3a(l) but not Brn-3a(s) or the isolated
Brn-3a POU domain in co-transfection experiments. Additionally this activation was not
affected by altering the valine at position 22 in the POU domain of Brn-3a to isoleucine
which prevents activation of promoters which are dependent on the POU domain of Brn-3a
although it does not prevent DNA binding. Similarly, Brn-3b carrying the converse
isoleucine to valine change was not able to activate this promoter although this change allows
Brn-3b to activate POU domain dependent promoters. Thus these co-transfection
experiments demonstrate that that activation of the Bcl-2 promoters is dependent upon the N-
terminus of Brn-3a being present (Smith et al., 1998a). Furthermore transfecting in the
isolated N-terminal domain together with Brn-3a(l) resulted in inhibition of Bcl-2 activation
suggesting that addition of the N-terminal domain binds to and removes factors required for
activation of this promoter while the POU domain is responsible for DNA binding. Therefore
the N-terminal domain is required for transactivation, possibly by interacting with a neuronal
specific co-activator that may either be bound at another site of the promoter or is recruited
to the promoter by interaction with the N-terminus, thus allowing transactivation of this
promoter (Smith et al., 1998a).

Transactivation of the Bcl-xL gene promoter by Brn-3a was proven to be more complex.
Like, Bcl-2, the Bcl-xL gene has two distinct promoters, both of which are activated by Brn-
3a (Smith et al., 1998b;Sugars et al., 2001). However, whilst activation of the Bcl-2 and the
upstream Bcl-xL promoter depends upon the presence of the N-terminal domain of Brn-3a
(Smith et al., 1998b;Sugars et al., 2001), this is not the case for the downstream Bcl-xL
promoter in which further sequences located downstream of the N-terminal but upstream of the POU domain, as well as the POU domain, are required for activation of this promoter (Sugars et al., 2001). This is interesting as under some conditions, Bcl-xL expression but not Bcl-2 expression can be induced by Brn-3a. This may be of particular significance in view of the key role played by Bcl-xL in regulating the extent of naturally occurring cell death in the development of the nervous system.

Interestingly, another gene that requires the N-terminal of Brn-3a for its activation is the α-internexin gene which encodes a type IV intermediate filament protein (Budhram-Mahadeo et al., 1995b). This is an interesting observation as expression of the neurofilament genes (dependent on the POU domain for activation) and α-internexin show different expression patterns which may be a consequence of the different alternatively spliced Brn-3a isoforms which have shown to be regulated under different conditions (Liu et al., 1996). However, unlike both the Bcl-2 and Bcl-xL genes that also require the N-terminal of Brn-3a for activation, Brn-3a activation of the α-internexin gene can be repressed by Brn-3b (Budhram-Mahadeo et al., 1995b).

Thus, the generation of these novel mice (the N-terminal null and V-I mice) has confirmed in vivo, the results that were obtained from in vitro experiments. That is, the N-terminal domain is critical for neuronal survival and the POU domain, in particular the valine at position 22 is critical for neurite outgrowth. These studies indicate that not only the correct regulation of Brn-3a and Brn-3b but also the correct regulation of the Brn-3a isoforms is critical for the proper development and functioning of the nervous system.

### 1.23 Mechanism of Brn-3a regulation on its target promoter

Other than the requirement of domains outside the POU domain, promoters activated by Brn-3a also differ in the nature of the sequences required for their transcriptional activation. Some promoters contain distinct upstream binding sites for Brn-3a which is essential for their transcriptional activation by Brn-3a. Such promoters include those encoding SNAP-25 and Bcl-2 (Lakin et al., 1995; Smith et al., 1998b). In contrast, in a number of other promoters it is not possible to dissect away the Brn-3a response from the minimal promoter activity. Promoters of this type include those of the genes encoding Bcl-xL, p21Cip1/Waf1, the
neurofilaments and α-internexin (Budhram-Mahadeo et al., 1995b; Smith et al., 1997c; Sugars et al., 2001; Budram-Mahadeo et al., 2002). Thus in some cases Brn-3a induces transcription by binding to upstream sequences whereas in other cases Brn-3a participates in a minimal basal transcriptional complex required for gene transcription. Thus Brn-3a responsive promoters differ in both their requirement for the N terminus of Brn-3a and in the nature of the sequences required for their response.

### 1.24 Interactions of Brn-3a/Brn-3b with other proteins to regulate gene transcription

As a transcription factor, Brn-3a and Brn-3b must associate with other cellular proteins to modify transcription. These include specific co-activators and repressors, the basal transcription apparatus and other cellular proteins that can modulate transcription. Interaction with other transcription factors would effectively increase the range of target genes regulated by the Brn-3 proteins. Therefore studies were undertaken to identify interaction partners that associated with the Brn-3 proteins.

Yeast 2 hybrid studies demonstrated association of Brn-3a with p53 and affinity chromatography showed direct physical interaction between Brn-3a and p53 which occurs via the POU domain of Brn-3a and the DNA binding domain of p53. This interaction resulted in differential regulation of both Brn-3a and p53 mediated gene targets. Indeed in co-transfection experiments in the ND7 neuronal cell line, p53 reduced the Brn-3a mediated activation of the Bcl-2 and Bcl-xL promoters compared with the activity observed in the presence of Brn-3a alone (Budhram-Mahadeo et al., 1999a; Sugars et al., 2001).

The effect of the Brn-3a/p53 interaction on apoptosis was further characterised by investigating the effect of Brn-3a on p53 specific target genes. Therefore the effect of Brn-3a on the bax and p21\(^{Cip1/Waf1}\) promoter was investigated in transient co-transfection experiments carried out in our laboratory. Brn-3a was shown to weakly repress the bax promoter but more importantly it also repressed the p53 mediated activation of the bax promoter to background levels. In addition, Brn-3a alone was able to up-regulate the p21\(^{Cip1/Waf1}\) promoter by 2-fold compared to empty vector control, while upon co-transfection with p53 a strong activation of the promoter was obtained which was significantly greater than that observed with p53 alone.
Functionally this was reflected in decreased expression of endogenous Bax protein and increased expression of endogenous $p21^{Cip/Waf1}$ protein in ND7 cells co-expressing Brn-3a and p53 compared with p53 alone (Budram-Mahadeo et al., 2002).

Thus from these results it follows that in cells co-expressing both these proteins, Brn-3a may increase neuronal survival by antagonising p53 mediated apoptosis and repressing p53-dependent activation of the $bax$ promoter whilst promoting cell cycle arrest that precedes differentiation, via its synergistic activation with p53 on the $p21^{Cip/Waf1}$ promoter. Indeed it was shown that ND7 cells over-expressing Brn-3a and p53 show enhanced growth arrest and reduced apoptosis compared to cells expressing p53 alone, confirming that, at least in ND7 cells, the presence of Brn-3a can increase cell cycle arrest and over-ride p53 mediated apoptosis (Budram-Mahadeo et al., 2002).

Thus it can be hypothesised that during neuronal development Brn-3a can also regulate cell fate by repressing p53 mediated activation of pro-apoptotic genes and co-operating on the $p21^{Cip/Waf1}$ promoter to enhance cell survival and cell cycle arrest preceding differentiation. As Brn-3a increases the expression of neuronal proteins such as neurotrophic receptors (TrkA), neurofilament and α-internexin this would enhance differentiation of surviving neurones into mature neurones (Fig 1.10).

**Figure 1.10: Model for the role of Brn-3a in modulating p53 mediated cell fate**

Expression of Brn-3a would result in activation of anti-apoptotic factors such as $Bcl-2$ and $Bcl-xL$ while repressing the expression of $Bax$. In the presence of p53, Brn-3a represses p53 mediated activation of Bax but co-operates with p53 to increase $p21$ levels with consequent cell survival and cell cycle...
arrest. Brn-3a can then stimulate the expression of genes involved in neuronal differentiation. In the absence of Brn-3a, p53 induction would result in high levels of Bax resulting in apoptosis. Thus in this neuronal cell model, Brn-3a may play an important role in determining the p53 pathway that is activated and thus the fate of these cells. (Adapted from Budram-Mahadeo et al., 2002)

Another factor that associates with Brn-3a and Brn-3b is the proliferation-associated estrogen receptor (ER) which transactivates estrogen responsive element (ERE) containing promoters. In breast cancer cells, Brn-3b/ER interaction results in greater induction of ERE containing promoters compared with Brn-3b alone. Conversely the Brn-3a/ER interaction results in a mild repression of ERE promoters, suggesting a negative role for Brn-3a in cellular proliferation in these cells compared to the enhanced proliferative effect of Brn-3b in breast cancer cells (Budhram-Mahadeo et al., 1998).

1.25 Mechanism of regulation of bax and p21<sup>Cip1/Waf1</sup> promoters

The ability of Brn-3a to differentially modulate expression of p53 target genes has also proven to be complex. For example, the bax promoter contains a p53 response element, that is required for p53 activation of this promoter, and the p53 response element was found to be flanked by two consensus binding sites for Brn-3a. Both of these Brn-3a binding sites are required for its effects on this promoter as mutation of both these sites results in a failure of Brn-3a to repress transcription either alone or via association with p53. Furthermore, the isolated Brn-3a POU domain was not sufficient to repress the bax promoter either directly or indirectly with p53, illustrating that sequences outside the POU domain are required to repress p53 induction of this promoter (Perez-Sanchez et al., 2002).

In contrast, on the p21<sup>Cip1/Waf1</sup> promoter, Brn-3a appeared to mediate its effect by recruitment to the basal transcriptional complex, as Brn-3a activation was lost concomitantly with loss of basal promoter activity. However, the synergistic effect of Brn-3a and p53 on this promoter requires the presence of the upstream p53 response elements (Perez-Sanchez et al., 2002). Furthermore, it was demonstrated that the isolated POU domain of Brn-3a was sufficient to activate both the full length and minimal p21<sup>Cip1/Waf1</sup> promoter as well as synergize with p53 on the full length p21<sup>Cip1/Waf1</sup> promoter, which is in contrast to that shown on the bax promoter. Thus the p21<sup>Cip1/Waf1</sup> promoter is a target of Brn-3a whose activation is dependent on basal promoter sequences and the Brn-3a POU domain whilst repression of bax by Brn-3a
depends on upstream Brn-3a binding sites as well as sequences outside the POU domain.

Therefore the ability of Brn-3a to modulate the expression of p53 target genes is complex and describes an alternative mechanism by which Brn-3a controls the fate of cells. The p53 related protein, p73, has a clear role in the development of the nervous system and is expressed in both pro-apoptotic and anti-apoptotic forms. The work described in this thesis aims to characterize the interaction between Brn-3a and the p73 proteins and to analysis the functional role of this interaction in terms of their effects on neuronal cell fate. The p53 family of proteins is discussed in the following sections.

1.26 The p53 tumour suppressor protein

First identified in 1979, p53 (chromosome 17p13.1) was found to act as a tumour suppressor gene that was inactivated in more than 50% of all human cancers. The importance of the p53 tumour suppressor activity stems from its ability to preserve genomic integrity by arresting cell cycle progression or activating apoptosis in response to a wide range of genotoxic and cellular stresses, including DNA damage, oncogene activation, ribonucleotide depletion, mitotic spindle damage and hypoxia (Ko and Prives, 1996).

The p53 protein is highly conserved throughout evolution (Soussi et al., 1996) and like other transcription factors, p53 is a modular protein containing structurally independent transactivation and DNA binding domains. The structure of the p53 protein is divided into four functional domains (Fig 1.11). The N-terminal acidic transactivation domain TAD (amino- acids 1-42) contacts specific components of the PIC, including the TBP and associated factors TAFII40 and TAFII60, which allows p53 to recruit the basal transcription factors required for transcriptional initiation. This region is also involved in the regulation of stability and activity of p53 via interactions with MDM2. Binding of MDM2 to p53 blocks the ability of p53 to transactivate its target genes as well as targeting the p53 protein to the ubiquitin-mediated proteolytic machinery (Haupt et al., 1997; Vousden, 2000). The region between the TAD and DBD contains a proline rich region containing several PXXP motifs. Deletion of this region results in loss of pro-apoptotic function in some experimental systems (Sakamuro et al., 1997).
Figure 1.11: DNA damage induced post-modifications to human p53 and cellular proteins known to interact with and influence p53 function

The transactivation domain (TAD), proline rich region (PXXP), DNA binding (DBD), oligomerization (OD), and a C-terminal basic domain are represented. The positions of known phosphorylations, (S=serine, T=threonine) are shown in pink, acetylations (Ac, K=lysine) are shown in purple and sumoylation (Sumo, K=lysine) is shown in green. Proteins that interact with the p53 protein and modulate p53 transactivation of target genes are represented underneath the p53 domain they were found to interact. Regulatory proteins that are boxed have yet to have their interaction with p53 mapped to specific domains. ASPP, apoptosis stimulating proteins of p53; CTF2, CCAAT binding transcription factor 2; MDM, mouse double minutes; WT1, Wilms tumour protein.

The sequence specific DNA binding domain (DBD) of p53 is localised between amino acids 113 and 292. It is a protease resistant and independently folded domain containing a zinc ion. This domain folds into a four stranded and five stranded antiparallel β sheet that in turn is a scaffold for two α-helical loops that interact directly with the DNA. The majority of missense mutations (>90%) seen in human cancers occur in the DNA binding domain, in which either the critical amino acids involved in contacting DNA are altered or residues that disrupt the whole conformation of the DBD are altered.

The C-terminus contains the OD domain (325-363) which is required for oligomerization of the p53 protein to form a tetramer that is efficient for transactivation (Pietenpol et al., 1994). The structure of the OD domain reveals an α-sheet-turn-β-helix motif which forms a pair of dimers (Jeffrey et al., 1994). Adjacent to the OD domain is a basic region (amino acids 363-393). This region contains a DNA damage recognition domain which can bind to DNA ends and single stranded DNA. The C-terminal interacts with a number of cellular proteins such as the TFIIH dual transcription/ DNA repair factor and other helicase proteins involved in DNA
repair such as CSB (Wang et al., 1995). The p53 C-terminal region also contains NLS and NES that lie within the OD (Strommel et al., 1999).

Activation of the p53 protein in response to stresses is mediated and regulated by protein kinases, histone acetyl-transferases, ubiquitin and sumo ligases. Once activated the p53 protein transactivates downstream targets involved in two major pathways: cell cycle arrest and apoptosis. These include p21\textsuperscript{Cip1/Waf1}, 14-3-3, GADD45, MDM2, Bax, Puma, Noxa and Apaf1. Additionally the activated p53 protein associates with other cellular proteins which selectively modulates p53 transcription of target genes, and thus influence p53 function. The cellular proteins that interact directly with p53 and selectively alter p53 gene transcription are shown in Fig 1.11 and are discussed in more detail in section 1.39.

1.27 Sequence specific binding by p53

The activities of the p53 tumour suppressor protein are mainly due to the ability of p53 to form homotetramers that bind to specific DNA sequences and transactivate a great number of downstream genes (Jeffrey et al., 1995). Promoter recognition by p53 is determined by the presence of p53-response elements (PREs) which share homology with the consensus sequence which consists of two identical repeats of the decamer sequence 5'-(PuPuPuC(A/T)(T/A)GPyPyPy)-3' separated by 0-13 base pairs (El Deiry et al., 1992). However, comparison with functional PREs found in p53 regulated promoters shown that they can differ significantly from this optimised version, and generally consist of multiple decamers. Specifically, only one decamer stringently adheres to the consensus whereas the other decamer(s) deviate from the consensus to a varying extent and in some cases the p53 responsiveness may be mediated by sequences other than the defined PRE (Contente et al., 2002). The explanation for this sequence variation comes from findings that p53 interactions with DNA depend on both sequence specific elements and DNA structure. p53 interaction with linear binding sites was shown to be weak, while presentation of the same target site in a stem loop confirmation strongly promotes specific DNA binding of p53 (Gohler et al., 2002). Thus, the multidecameric composition provide the PREs to adopt different confirmations, allowing individual decamers that deviate from the consensus to be converted from poor binding sites to structurally favourable sites that strongly bind p53.
1.28 Discovery of p53 homologues

The possible existence of p53 homologues was suggested by findings showing that regulation of p53 targets were observed independently of p53 (Weinberg et al., 1995). In 1997, Kaghad and colleagues (1997) discovered the p53 homologue referred to as p73 which shares high sequence homology with p53, particularly in the functional domains. The gene encoding p73 is located on chromosome 1p36, a region frequently deleted in neuroblastoma and other tumours. In 1998, Yang and colleagues (1998) discovered a further homologue with high homology to p53, p63 which is located on chromosome 3q27. Both the p73 and p63 proteins share high homology to p53 in their DNA binding domains and have subsequently been shown to transactivate a wide range of p53 regulated genes (reviewed by De-Laurenzi and Melino, 2000). Since this study deals primarily with p73, the discussion of the p53 family will mainly be focused on the p73 transcription factor.

1.29 Gene organization and protein structure of the p53 family

The human p53 gene is located on chromosome 17p13 and is approximately 20kb and consists of 11 exons while p73 and p63 are both over 65kb with 14 and 15 exons respectively (reviewed by Marin and Kaelin, Jr. 2000). All three genes share a large first intron (10.7kb in p53 and 32kb in p73 and p63) and a non-coding first exon which may influence translation. Interestingly, a 1 kb region in the intronic sequence found upstream of the ATG in the p73 gene functions as a silencer, suggesting that regulatory sequences located in this region may contribute to the tight regulation of p73 expression (Fontemaggi et al., 2001). Essentially, the human p53 gene has a single promoter which encodes a single protein of 393 amino acids. However accumulating data has reported the existence of shorter forms of p53 that arise from various mechanisms such as alternative splicing, autodegradation, proteolytic cleavage and use of an in-frame ATG located at codon 40. However the shorter isoforms of p53 appear to be expressed at very low levels and in specific cell types suggesting they play very narrow and limited roles (Courtois et al., 2004). In contrast, both the p73 and p63 genes consistently produce multiple mRNA transcripts resulting from C-terminal splicing giving rise to six different p73 isoforms (α, β, γ, δ, ε, ζ) (Kaghad et al., 1997; De Laurenzi et al., 1998; Zaika et al., 1999; De Laurenzi et al., 1999; Moll et al., 2001) and three isoforms of p63 (α, β, γ) (Yang 76
et al., 1998). These p73 and p63 proteins are referred to as TAp73 and TAp63 respectively. In addition, truncated isoforms of p63 and p73 that lack the N terminal transactivation domain (ΔNp73 and ΔNp63) have been described in both mice and humans (Yang and McKeon, 2000; Yang et al., 2000; Ishimoto et al., 2002). These isoforms are generated by an alternative promoter spanning exon 3 and exist as several isoforms that contain the different carboxyl-terminal domains seen in the TA isoforms. Furthermore, other p73 splice variants that lack exon2 (Δ2p73) or exon3 (Δ3p73) and hence the transactivation domain have been described in cancer cell lines (Fillippovich et al. 2001). The TA promoter (P1) and ΔN promoter (P2) of the p73 gene are 30-40 kb apart and are differentially regulated. For example E2F-1 sites are present in the TA promoter but not in the ΔN promoter (Irwin et al., 2000), while p53 sites have been characterised in the ΔN promoter but not in the TA promoter (Grob et al., 2001; Kartasheva et al., 2002; Vossio et al., 2002).

The p73 and p63 proteins share high sequence homology to p53 in the regions corresponding to the acidic N-terminal activation domain (TAD), the central DNA binding domain (DBD) and the C-terminal oligomerization domain (OD). Comparison of the similarity in structure of the different domains of the p53 proteins and major isoforms encoded by p73 is shown in Fig 1.12. The p73 N-terminal TAD domain which is missing in the ΔN forms, is the least conserved of the three domains among the family (30% identity between p73 and p53). This domain of all three proteins is acidic in nature and for both TAp73 and p53 the TAD facilitates interaction with the MDM2 protein, which plays a critical role in regulating the stability of the p53/p73 (Vousden, 2000).

Although the DNA binding domains of p53 and p73 show 63% homology, the 3-dimensional structure of the two regions are almost identical (reviewed by Melino et al., 2002). Interestingly, the residues of p53 that directly contact DNA, including those most frequently mutated in cancers, are conserved in the DNA binding domain of p63 and p73 (Kaghad et al., 1997), suggesting that they could activate the same target genes, and indeed, both p73 and p63 have been shown to bind to p53 response elements and transactivate p53 target genes (Kaghad et al., 1997; Jost et al., 1997; De Laurenzi et al., 1998; Yang et al., 1998; Ueda et al., 1999; Lee and La Thangue., 1999; Zheng et al., 1999)
Figure 1.12: Comparative protein structures and functional organization of the p53 family members.

a) The transactivation domain (TAD), proline rich region (PXXP), DNA binding (DBD), oligomerization (OD), sterile α motif (SAM) and post SAM basic domains are represented.

b) Genomic organization of the p73 gene. The proximal promoter (P1) yields TAp73α, β, γ, δ, ε, ζ isoforms while the distal promoter (P2), spanning intron 3, gives rise to the ΔNp73α, β isoforms. (Adapted from Benard et al., 2003; Maisse et al., 2003).

Similar to p53, the p73 OD domain consists of an α-sheet-turn-β-helix motif that forms tetramers (Davidson et al., 1999). Using the yeast two hybrid system, it was demonstrated that the p73 isoforms interacted with each other with various affinities. For example p73α showed a low tendency to form homotypic interactions whereas p73β displayed strong homotypic interactions and binds weakly to p73α (Kaghad et al., 1997; De Laurenzi et al., 1998). In contrast, p73γ demonstrated strong interaction with p73α, p73β, p73δ and itself whereas the p73δ isoform homodimerizes efficiently and binds p73α and p73γ but its interaction with p73β is much weaker (De Laurenzi et al., 1998). The different p73 isoforms were also shown to interact in the cell by immunoprecipitation experiments (Ueda et al., 2001). The high conservation of the OD domain (38% homology) between p53 and p73 suggest that members of this family form heterotetramers as well as homotetramers. Using the yeast two hybrid system, it was shown that both p73α and p73β could very weakly interact with p53 (Kaghad et al., 1997; De Laurenzi et al., 1998;) whereas p73γ and p73δ
showed no interaction with p53, suggesting that p53 prefers to form homotetramers rather than heterotetramers with the p73 proteins (De Laurenzi et al., 1998). This is supported by the findings that wild type p53 failed to co-immunoprecipitation with p73 (Di Como et al., 1999; Marin et al., 2000; Gaiddon et al., 2001; Ishimoto et al., 2002).

Also similar to p53, p73 contains two proline rich regions which contain PXXP motifs. These regions are located between the TAD and DBD and the DBD and OD. The occurrence of PXXP motifs suggests that the p53 family can bind proteins with an SH3 (src homology 3) domain. Additionally, the extended C-terminals of the p63 and p73 proteins may perform specific functions, and only the α isoforms of both p63 and p73 contain a sterile alpha motif domain (SAM) which is implicated in protein-protein interactions. Using NMR spectroscopy the structure of the p73 SAM domain reveals a five helix bundle that resembles the SAM domain present in the Eph receptor tyrosine kinases (Chi et al., 1999). The majority of SAM domain-containing proteins are involved in the regulation of development but unlike other SAM domains, those present in p73 and p63 do not mediate homo- or hetero-dimerization (Chi et al., 1999). So far the p73 SAM domain shows differential binding to different artificial lipids, though the functional significance of this has yet to be investigated (Barrera et al., 2002; Barrera et al., 2003).

1.30 Transcriptional regulation by the TA isoforms

TAp73 has been shown to transactivate p53 responsive genes and when overproduced can induce cell cycle arrest or apoptosis (Kaghad et al., 1997; Jost et al., 1997; Osada et al., 1998; Yang et al., 1998). For example, TAp73α can transactivate several p53 responsive genes implicated in cell cycle arrest, DNA repair and apoptosis such as p21Cip1/Waf1, GADD45, mdm2, Bax, Puma, Noxa, 14-3-3, p53AIP and cyclin G (Kaghad et al., 1997; Jost et al., 1997; De Laurenzi et al., 1998; DiComo et al., 1998; Yang et al., 1998; Lee et al., 1999; Takada et al., 1999; Melino et al., 2003). However the transcriptional activity differs between the splice variants of TAp73. For instance, the isoforms containing the shorter C terminus e.g. p73γ and p73ε are poor transcriptional activators (De Laurenzi et al. 1998). The p73β isoform is generally a more potent transcriptional activator than p73α. This is thought to be due to the suppressive effects of the p73α specific C-terminal as deletion of this region
results in increased transactivation of some gene targets (Ueda et al., 2001). Interestingly, in co-transfection assays both p73a and p73ε (which share the C-terminal 110 amino acids, 527-636) were shown to inhibit p73β transactivation of its target promoters possibly resulting from a direct interaction via the OD domain (Ueda et al., 2001).

It is important to note that these effects were demonstrated using systems for over-expressing of the TA isoforms. Nevertheless, differences in potency of these proteins to induce activation of p53 target genes may exist in the cell and this would impact on the cell's decision to induce cell cycle arrest or apoptosis. Furthermore, the relative abundance of the different p53 family members including the TAp73 and TAp63 isoforms in the cell and the resulting functional/non-functional tetrameric complexes formed between these proteins might play an important role in regulating p73 and p63 functions, depending on the cell type.

More recently, several p73 specific target genes have also been identified. For example using cDNA subtraction assays, aquaporin-3 (AQP3) was found to be a p73 regulated gene target, although p53 was also shown to activate this gene, though to a lesser extent (Zheng and Chen, 2001). AQP3 is a glycerol/water transporter and may mediate the activity of p73 in maintaining the cerebral spinal fluid dynamics which is dysregulated in p73-/- animals (Yang et al., 2000). The characterisation of the human adenosine deaminase (ADA) gene, which encodes an enzyme involved in nucleotide metabolism, revealed a putative p53 response element located in the first intron. The ADA gene was demonstrated to be a p73 target gene, coupling dNTP imbalance and cell cycle arrest. Under physiological conditions, the ADA gene did not appear to be a p53 target, demonstrating that p73 and p53 proteins play different roles in growth and development by activating different sets of genes (Tullo et al., 2003). Studies using cDNA microarray analysis, also identified the Interleukin 4 receptor α (IL-4Rα) to be a p73 target and this gene was demonstrated to be up-regulated by p73 in several cancer cell lines and in response to cisplatin treatment in a p73 dependent manner, demonstrating that IL-4Rα can mediate p73 dependent cell death (Sasaki et al., 2003). The p53 protein was shown not to activate this promoter and the length of the spacer sequences between the decamer motifs were shown to be important for specific binding of the p73 transcription factor (Sasaki et al., 2003). Interestingly, abnormal regulation of IL-4R signalling is associated with allergic, autoimmune diseases and parasitic infections. As the p73-/- animals display chronic infections and inflammation (Yang et al., 2000), the finding
that p73 is a direct transcriptional activator of IL-4Rα may explain, in part, some of this phenotype observed in the p73−/− animals.

1.3.1 The ΔN isoforms

The amino terminal truncated isoforms ΔNp73 and ΔNp63 are naturally occurring members identified for the p53 family and are thought to act in a dominant inhibitory manner on the TA isoforms and p53 (Yang et al., 1998; Liefer et al., 2000; Pozniak et al., 2000; Yang et al., 2000; Grob et al., 2001; Ishimoto et al., 2002). Both ΔNp73 and ΔNp63 isoforms lack the TA domain and do not induce growth arrest or apoptosis. Instead, the ΔN isoforms exert a dominant negative effect on p53 and their corresponding TA isoforms by blocking their transactivation activity on promoters such as Bax, p21cip1/Waf1 or MDM2. (Yang et al., 1998; Grob et al., 2001; Ishimoto et al., 2002; Kartsheva et al., 2002; Stiewe et al., 2002a)

Both the ΔNp73 and ΔNp63 isoforms have been shown to interact with their corresponding TA isoforms and their inhibitory function is exerted by the formation of transactivation deficient hetero-duplexes with their corresponding TA isoforms, as well as by direct competition for DNA binding sites, as the ΔN proteins retain the core DNA binding domain (Yang et al., 1998; Grob et al., 2001; Stiewe et al., 2002a; Ishimoto et al., 2002). In contrast, wild type p53 does not associate with p73 in mammalian cells as shown by co-immunoprecipitation assays (Di Como et al., 1999; Marin et al., 2000; Gaiddon et al., 2001; Ishimoto et al., 2002). Thus, in the case of p53, this inhibitory effect is mediated by the ΔN isoforms by direct competition for promoter binding sites (Yang et al., 1998; Grob et al., 2001; Stiewe et al., 2002a; Kartsheva et al., 2002). Therefore the TA and ΔN isoforms show pro- and anti-apoptotic properties respectively. Interestingly, ΔNp73 is the most abundant isoform in the nervous system, and their functionally antagonistic actions towards p53/TAp73 are known to be of key importance in neuronal survival.

Moreover, expression of ΔNp73 is increased at the mRNA level by p53 and TAp73 acting on this promoter, by binding to two p53 REs located in the P2 promoter (Grob et al., 2001; Kartsheva et al., 2002; Vossio et al., 2002). Therefore both p53 and TAp73 induce ΔNp73, which inhibits their effects, forming a negative regulatory loop (Grob et al., 2001; Kartsheva et al., 2002; Vossio et al., 2002; Nakagawa et al., 2002). Thus, the ΔNp73
mediated negative feedback loop that regulates activity of TAp73 and p53 on the ΔNp73 promoter provides a novel autoregulatory system that serves to modulate cell survival and death. This has been shown specifically in malignant cells and in developing neurones, where the relative ratio of ΔNp73 to p53/TAp73 is important in determining the apoptotic response to different types of stresses such as DNA damage and growth factor withdrawal respectively (Pozniak et al., 2000; Zaika et al., 2002).

1.32 The p53 family knock out mouse models

Results of studies supporting the proposal that different p53 family members have distinct biological roles come from analysis of knock out mice in which the genes encoding for p53, or p73 have been specifically deleted. Analysis of these mice lacking each protein provides a powerful model to reveal the developmental alterations but non-cancerous phenotypes of the p73 -/- animals.

1.32.1 p53 knock-out

The p53-/- animals lack an overt developmental phenotype but are highly tumour prone at an early age, (Donehower et al., 1992). Although loss of the p53 gene results in normal development, a subset of p53-/- animals developed mid-brain exencephaly resulting from the overproduction of neural tissue and failure of neural tube closure as well as craniofacial abnormalities (Armstrong et al., 1995; Sah et al., 1995). Until the discovery of p73 and p63, this partial penetrance of the p53-/- phenotype was difficult to explain. It is possible that p73 and p63, both of which are expressed in the embryonic nervous system can compensate for the lack of p53 resulting in normal neuronal development in the p53-/- mouse. The role of p53 and p73 in neuronal developmental is discussed in section 1.35.

1.32.2 p73 knock out

The p73 deficient mice which are functionally deficient for all of the known p73 isoforms, display varying degrees of hydrocephalus, (the basis of which appears to be associated with excessive cerebral spinal fluid production by cells of the choroid plexus), show defects in
hippocampal formation, primarily due to a loss of a discrete set of neurones that act to guide the organisation of the hippocampus and show enlarged ventricles and decreased cortical tissue. There is also an enhancement of developmental death in sympathetic neurones which may be attributed to loss of ΔNp73 which has been shown to be essential for survival of these neurones (discussed in section 1.35). p73 null mice also display abnormal social behaviour due to abnormalities in the development of the olfactory bulb which is involved in pheromone detection, as well as loss of pheromone receptor expression. In addition to these severe neurological defects, p73 null mice suffer from chronic infections and inflammation showing that p73 is required for the normal fluid dynamics of both the cerebrospinal fluid and the respiratory mucosa (Yang et al., 2000).

The striking difference in function of the p53 family is at first surprising as they share ~60% of amino acid identity in the DNA binding domain and as expected transactivates many of the same target genes with the TA members of the family able to induce cell cycle arrest and apoptosis. However, more specific gene targets must exist for each family member, based on their unique developmental functions. Such data will be forthcoming with advances in techniques such as microarray technologies that would help to elucidate the different roles of specific sub-families such as p73 and p63. However, since both p73 and p63 are expressed as a wide array of spliced variants then these variants may also play a role in controlling the specific activities of these proteins in particular cells. Furthermore both p73 and p63 are expressed as dominant negative isoforms. The complex interaction within the p53 family that result in activation or inhibition of individual family members will impact strongly on the ability of these members to modulate cell fate. Furthermore the interaction of the p53 family with other cellular proteins that act to inhibit, activate, stabilise or specifically direct p53 family members to activate subsets of p53 target genes, will also strongly influence each members biological outputs in controlling the diverse cellular activities such as proliferation, differentiation and regulated cell death

1.33 Role of the p53 family in apoptosis

The p53 protein is activated in response to a variety of cellular stresses such as DNA damage, hypoxia, nucleotide depletion and oncogene activation (Prives and Hall, 1999) and
the nature of the damage is communicated to the p53 protein via post-translational modifications. A number of such modifications that alter its function have been identified and include phosphorylation of multiple N terminal residues (Ser6, 9, 15, 20, 33, 37, 46 and Thr18, 81) and C-terminal residues (Ser315, 392) as well as acetylation (Lys320, 373 and 382) and sumoylation (Lys386) (Appella and Anderson, 2001; Harris and Levine, 2005). Furthermore, the combinations of different modifications may well be specific to the type of stress acting on the cell. For example, γ-radiation activates the ATM kinase and the CHK-2 kinase, both of which phosphorylate the p53 protein while UV-radiation activates ATR, CHK-1 and casein kinase which results in the modification of different amino acid residues on the p53 protein (Chehab et al., 1999; Hirao et al., 2000). These alterations appear to change the p53 protein in two ways. Firstly they may act to stabilize the p53 protein by interfering with MDM2 binding, therefore, preventing the targeted degradation of p53. This can increase the half life of the p53 protein from 6-20 min to hours and results in a 3-10 fold increased concentration of the p53 protein in the cell in response to stress. Secondly, these post-translational modifications act to enhance p53 transcriptional activity.

Once activated, the p53 protein transactivates a network of genes whose products are involved in the initiation of either cell cycle arrest or apoptosis, which represent two major pathways induced by p53. For instance transactivation of the p21Cip1/Waf1 gene promoter by this protein is a major player in p53 mediated cell cycle G1 arrest (El-Deiry et al., 1993). p21Cip1/Waf1 inhibits the cyclin D-CDK4/6 and cyclin E-CDK2 complexes resulting in accumulation of the unphosphorylated form of pRB. In this state, pRB binds E2F-1 and thus prevents the activation of the transcription of genes that are required for cell cycle progression from G1 to S phase in the cell cycle (Sherr, 1998). Other p53 targets involved in controlling the cell cycle include GADD45, cyclin G and 14-3-3. p53 also activates transcription of genes involved in auto-regulation of p53 function, such as MDM2 and ΔNp73.

A large number of p53 regulated genes have been shown to control p53 mediated apoptosis via both the intrinsic and extrinsic pathways. The first evidence suggesting that the mitochondria might be involved in p53-dependent apoptosis was demonstrated by the ability of Bcl-2 to protect cells from p53 mediated apoptosis (Gottlieb et al., 1994). Indeed p53 was shown to activate the pro-apoptotic Bax protein (Miyashita and Reed, 1995) and the
BH3 only proteins Noxa (Oda et al., 2000) and Puma (Yu et al., 2001) whereas the anti-apoptotic genes Bcl-2 and Bcl-xL are repressed by p53 (Vousden and Lu, 2002). Cytochrome c interacts with another p53 transcriptional target Apaf-1 which activates Caspase-9 which in turn activates Caspase-3 resulting in apoptosis (Kannan et al., 2001). In addition, p53 regulates a series of other genes that initiate the extrinsic apoptotic pathway (Fas, DR4 and DR5 death receptors) leading to Caspase-8 activation and Caspase-3 cleavage, resulting in apoptosis (Sheikh et al., 1998). It has been recently shown that p53 can trigger apoptosis via transcription independent mechanism by interacting with the mitochondria. In this report, translocation of p53 to the mitochondria was observed in response to DNA damage and this translocation triggered apoptosis that occurred prior to p53 gene activation. It was shown that p53 could activate BAK and permeabilizes the mitochondrial membranes by physically interacting with the anti-apoptotic Bcl-2 proteins (Erster et al., 2004; Erster and Moll, 2005).

Recent studies, utilising mouse embryo fibroblasts deficient for one or a combination of p53 family members, showed interplay among the p53 family members where p53 is completely inactive on promoters of apoptotic genes such as Noxa, PERP and Bax if p63 and p73 are absent (Flores et al., 2002). However, although p73 and p63 are capable of weak heterotypic interactions, wild type p53 was showed not to interact with p73 or p63 (Davison et al., 1999), suggesting that the interplay of these proteins on apoptotic promoters is likely to be complex. A number of different stress signals and upstream regulators that activate the p53 pathway have been described (reviewed by Appella and Anderson, 2001). The stress signals that activate p73 and regulates it function are discussed in this section.

p73 has been shown to accumulate in response to specific forms of DNA damage resulting from specific stresses such as γ-radiation, cisplatin and taxol. This protein was also identified as a target of the non receptor tyrosine kinase c-Abl, which provided evidence that a p73 repair pathway for DNA damage exists that is p53 independent. Gong and colleagues (1999) showed that the half life of p73 is prolonged by cisplatin treatment and demonstrated that MEF cells deficient in mismatch repair proteins or c-Abl are unable to up-regulate p73 and hence are more resistant to cisplatin treatment (Gong et al. 1999). Furthermore Yuan and colleagues (1999) demonstrated that γ-radiation promotes c-Abl induced tyrosine phosphorylation on residue Tyr99 of p73, which results in the potentiation of the pro-
apoptotic activity of p73 and increased stability (Yuan et al. 1999). These post-translational modifications of p73 occur via physical interaction between the SH3 domain of c-Abl with the PXXP motif present at the C-terminus of p73 (Yuan et al., 1999; Agami et al., 1999). As c-Abl is itself phosphorylated by the ATM gene product this suggests that ATM is involved in the pathway leading to c-Abl dependent activation of p73 in response to DNA damage (Shaul, 2000). Furthermore, over-expression of c-Abl also induces p38 dependent phosphorylation on threonine residues adjacent to prolines in the p73 protein (which was detected by the use of specific antibodies against phosphorylated threonine adjacent to a proline). Although the precise threonines were not identified, it is anticipated that p38 would phosphorylate threonine residues in the proline rich regions found in p73 (Fig 1.12) (Sanchez-Prieto et al., 2002). Both these phosphorylation pathways enhance the stability and transcriptional activity of p73.

In addition to DNA damage induced phosphorylation of p73, which increases its stability, p73 is also regulated by acetylation in response to DNA damage. It has been demonstrated that p300 interacts with the N terminal region of p73 and this interaction enhances its pro-apoptotic activity (Zeng et al., 2000). p300 acetylation of p73 on three conserved lysines 321, 327 and 331 occurs in a c-Abl dependent manner, and this increases p73 transcriptional activity on pro-apoptotic targets such as p53AIP1 and potentiated p73-mediated apoptosis. Conversely, non-acetylated p73 protein is transcriptionally inactive on pro-apoptotic genes but can still transactivate cell cycle related genes such as p21^{Cip1/Waf1} (Costanzo et al., 2002) demonstrating how cellular co-activators can couple activation of p73 to either cell cycle arrest or apoptosis.

Both p53 and p73 can also be activated by oncogenes such as E2F-1, c-myc and E1A (Sherr, 1998; Zaika et al., 2001). Here, the inappropriate signalling for entry into the cell cycle is detected by the p53 checkpoint resulting in apoptosis, and is thus an example of the tumour suppressor function of the p53 protein. A key player in this pathway is E2F-1 whose dysregulation by mitogenic oncogenes is believed to trigger p53 dependent apoptosis and also p53 independent apoptosis that was shown to occur via p73 (Irwin et al., 2000; Lowe and Sherr, 2003). In the first instance, deregulated expression of oncogenes causes E2F-1 mediated induction of p19^{ARF} which inhibits MDM2, thus protecting p53 from MDM2-mediated degradation (Bates et al., 1998). p73 activation by oncongenes was
demonstrated by the findings that the disruption of p73 function inhibits oncogene induced apoptosis in p53 null human tumour cells (Zaika et al., 2001). Furthermore, p73 also mediate E2F-1 induced apoptosis in SAOS2 cells and p53-/- MEFs (Irwin et al., 2000) and thus was shown to mediate E2F-1 apoptosis that is p53 independent. Moreover E2F-1 was shown to directly increase p73 expression by binding to the E2F-1 sites within the TAp73 promoter (Irwin et al., 2000). In addition, unlike p53, activation of p73 by E2F-1 is required for apoptosis after T-cell receptor activation (Lissy et al. 2000). Consistent with this finding, E2F-1 null mice have increased numbers of T-cells and splenomegaly (Field et al., 1996) suggesting that p73 also plays a role in tumour surveillance pathways of lymphoid cells.

Thus, several differences are apparent in the post-translational modifications that occur on the p53 family in response to DNA damage and whether or not they are phosphorylated by the same kinases remains to be established. Firstly, p73 is activated by only a subset of DNA damaging agents and unlike p53, the p73 response to stress is mediated by tyrosine phosphorylation which is not observed with p53 (Yuan et al., 1999;Agami et al., 1999). This suggests that p73 recruits proteins with SH3 domains, and hence can mediate apoptosis via distinct pathways from those engaged by p53 upon DNA damage. Secondly E2F-1 induced apoptosis occurs following regulation of the level of p73 mRNA accumulation whereas E2F-1 induced apoptosis associated with p53, occurs indirectly by altering p53 stabilisation.

1.33.1 Mechanism of p73 mediated apoptosis

Several recent papers have addressed the mechanisms by which p73 triggers cell death. Melino and colleagues (2004) demonstrated that p73 induces the mitochondria death pathway via the up-regulation of Bax and Puma. Puma is a BH3 only protein that mediates the mitochondrial translocation of Bax and changes the conformation of the Bax protein into its active state (Melino et al., 2004). Using Tet-On Saos-2 inducible cell lines, it was demonstrated that while p73 overexpression induced the *bax* promoter, the kinetics of apoptosis did not correlate with increased Bax protein levels (Bax levels were induced only after 72-96 hrs whereas cell death occurred 48 hrs after p73 induction). Instead, p73 mediated up regulation of Puma and Puma-dependent mitochondrial translocation of Bax is kinetically compatible with the induction of cell death as this process takes less than 48 hours (Puma
protein was induced after 12 hours of p73 induction and Puma mediated Bax movement from the cytosol to the mitochondria membrane took place within 24 hrs of transfection of Puma in to cells stably transfected with Bax-GFP). Thus, at least in this experimental system, p73 mediated transactivation of PUMA and the subsequent PUMA mediated mitochondrial translocation of Bax is highly efficient for cell death. Furthermore, ΔNp73 inhibited TAp73 mediated apoptosis and importantly, ΔNp73 inhibited TAp73 mediated PUMA up regulation and in turn blocked Bax translocation to the mitochondria and subsequent Cytochrome c release (Melino et al., 2004).

The p73 protein has also been shown to induce apoptosis via the activation of death receptors (Terrasson et al., 2005). Firstly it was shown that p73 expression was sufficient to trigger apoptosis through death receptors, such as the tumour necrosis factor receptor (TNFR)-related proteins, Fas. Moreover this sensitisation to pro-apoptotic ligands of the Fas receptor such as the TNF-related apoptosis inducing ligand (TRAIL) or 7C11, (an agonistic anti Fas antibody) depended on caspase activation. Furthermore, p73 transcriptional activity is required for this effect and sensitisation of cisplatin treated cells to death receptor apoptosis is due to p73 dependent transactivation of Fas and subsequent increased protein expression at the cell surface. In accordance with this, infection with human cytomegalovirus (HCMV) which is known to increase the levels of the ΔN isoform in infected cells, inhibits p73 dependent Fas mediated apoptosis and transfection with small interfering RNA against the ΔN isoform, restored sensitivity to 7C11 in HCMV infected cells (Terrasson et al., 2005).

The p73 protein has also been demonstrated to induce apoptosis via the endoplasmic reticulum (ER). Specifically it was shown that p73 but not ΔNp73 caused changes in the morphology of the ER including calnexin clumping, reduction of intracellular free calcium and induction of Gadd 153, all of which are associated with ER stress. Furthermore p73 and p53 but not ΔNp73 could up-regulate Scotin, a small transmembrane protein (23kDa) that localizes within the ER and triggers apoptosis from the ER as a consequence of ER stress (Terrinoni et al., 2004). Therefore both p73 and p53 can transactivate Scotin and induce cell death via the ER which is an important sensor of abnormal cytosolic signals such as accumulation of unfolded proteins, calcium depletion, inhibition of glycosylation and viral and hypoxic stress.
1.34 Role of p53 family members in cancer

The p53 tumour suppressor gene is the most frequent target for genetic alterations in human cancers (Hollstein et al. 1997). Most mutations occur within the highly conserved DBD of the protein leading to loss of wild type activity (Levine 1997). In addition, patients with germ line mutations in p53 develop the Li-Fraumeni hereditary syndrome which predisposes affected individuals to the development of multiple types of cancers including sarcomas, brain tumours and breast cancer. The data supporting the role of p73 in cancer is presented below.

p73 maps to chromosome 1p36.33 which is frequently deleted in a variety of human cancers including neuroblastoma, melanoma, breast and colon cancer (Kaghad et al., 1997;Irwin and Kaelin, Jr., 2001). However, extensive studies of p73 status in a variety of primary tumours, including those with 1p deletions, revealed that p73 mutations are exceedingly rare. The analysis of p73 as a tumour suppressor was further complicated by the finding that p73 mRNA and protein levels tended to rise in tumour tissue compared with surrounding normal tissue, suggesting that p73 plays a role in tumourigenesis. To date, p73 over-expression has been found in tumours of the breast, lung, esophagus, stomach, colon, ovaries, liver, CML, AML ependymoma and neuroblastoma (Zaika et al., 1999;Levrero et al., 2000;Moll et al., 2001;Stiewe and Putzer, 2002).

Of note, many early studies measured global p73 levels and the recent use of isoform specific RT-PCR has shown that in many cases, it is the ΔNp73 isoform that is specifically up-regulated in primary tumours such as breast carcinoma, gynaecological cancers, hepatocellular carcinoma and neuroblastoma (Zaika et al., 2002;Douc-Rasy et al., 2002). Furthermore, novel truncated isoforms of TAp73 lacking exon 2 or 3 (Δ2p73, Δ3p73) have also been shown to be expressed in neuroblastoma, hepatocellular, breast and ovarian cancers but not the originating normal tissue (Ng et al., 2000;O'Nions et al., 2001;Fillippovich et al., 2001;Stiewe et al., 2004). Moreover increased levels of ΔNp73 were found to be an independent prognostic marker for reduced survival in neuroblastoma patients (Casciano et al., 2002) and the ΔNp73 isoforms have been shown to exhibit oncogenic properties as a result of its ability to inhibit p53/TAp73 mediated transactivation, apoptosis and growth
suppressing functions (Stiewe et al., 2002b; Petrenko et al., 2003).

Interactions between TAp73 and mutant p53 (but not wild type p53) have also been shown to occur in cancer cells and this interaction resulted in inhibition of the apoptotic function of p73 (Di Como et al., 1999; Strano et al., 2000; Gaiddon et al., 2001). This interaction with p73 was shown to be mediated by the DBD of mutant p53 (Strano et al., 2000;Gaiddon et al., 2001) and the DBD and OD of p73 (Strano et al., 2000). Interestingly, cancer cells that express p53 mutants that do not bind to p73 and therefore do not inhibit the functions of p73 have increased sensitivity to anticancer drug induced apoptosis whereas, those cancers that have p53 mutants that efficiently inhibit p73 function show the most resistance (Bergamaschi et al., 2003). Therefore the inactivation of p73 by mutated p53 may provide a selective advantage in promoting tumourigenesis.

1.35 The p53 family and developmental cell death and differentiation in the nervous system

The up-regulation of p53 in the adult nervous system as a result from traumatic insults ranging from ischemia and excitotoxicity to oxidative stress in multiple neuronal populations has been extensively investigated (Li et al., 1994; Sakhi et al., 1994; Wood and Youle, 1995; Morrison et al., 1996). It is thought that DNA damage may be the major common feature integrating the different stress signalling pathways and initiating p53 mediated apoptosis.

However, emerging evidence also indicate that the p53 family play a critical role in both phases of apoptosis occurring during neuronal developmental, which has been widely investigated. The first phase serves to eliminate those progenitors that do not differentiate appropriately and ensures that the correct number of cells is generated in structures of the nervous system, while the second phase of developmental cell death serves to match neuronal number to the size of the target tissue. Recent evidence has also highlighted a role for p53 as well as p73 in neuronal differentiation which is discussed in the next section. The role of the p53 family in developmental apoptosis in the nervous system is discussed in this section.
1.35.1 The role of p53 in determining fate of neural progenitor cells

Initial analysis of animals with a targeted deletion of the p53 gene provided strong support for the hypothesis that p53 was a key tumour suppressor protein because p53-/- mice exhibited a high rate of spontaneous tumour formation (Donehower et al., 1992). However, upon further examination, it was shown that a large number of female p53-/- embryos died in utero as a result of mid-brain exencephaly, a neural tube malformation resulting from the overproduction of neural tissue and failure of neural tube closure (Armstrong et al., 1995; Sah et al., 1995). Interestingly, this phenotype is shared in animals with targeted deletions in genes encoding for other members that act in the intrinsic mitochondrial death pathway including the pro-apoptotic proteins Apaf-1, caspase-9 and caspase-3. In all these cases, exencephaly resulted from a deficiency of apoptosis in progenitor cell in the neuroepithelium of the developing neural tube, thus indicating the importance of the intrinsic apoptotic pathway for controlling neural progenitor cell fate during neuronal development. Apaf-1-/- animals also exhibited craniofacial abnormalities similar to that observed in the p53-/- animals. However the partial penetrance of the p53-/- phenotype was difficult to explain until the discovery of p73 and p63 proteins, both of which are expressed in the embryonic nervous system and can potentially compensate for the lack of p53 resulting in normal neuronal development in the p53-/- mouse.

Additional support of a role for p53 in neuronal developmental cell death comes from analysis of mice with a targeted deletion of the pRb tumour suppressor gene. These mice suffer massive apoptosis in the embryonic nervous system (Jacks et al., 1992). Interestingly, the concomitant deletion of p53 rescued this apoptotic phenotype (Macleod et al., 1996), suggesting that p53 plays a critical role in eliminating neural progenitors and post-mitotic neurones that do not differentiate appropriately.

1.35.2 An essential role for p53 and p73 during naturally occurring neuronal cell death

The first evidence that p53 might play a role in naturally occurring developmental cell death of post-mitotic neurones came from studies of the peripheral sympathetic neurones of the superior cervical ganglion (SCG). During this second wave of apoptosis, approximately 40%
of sympathetic neurones in the SCG are eliminated. This process starts in late embryogenesis in rodents and occurs primarily during the first two weeks after birth. The survival of any given neurone during this period is determined by its ability to compete for limiting amounts of target-derived NGF. Those neurones that do not receive appropriate trophic support undergo apoptosis due to an imbalance of pro-apoptotic signals over anti-apoptotic signals.

A number of studies indicate that p53 and p73 play a role in regulating the survival of sympathetic neurones during this developmental period. Firstly, it was shown that overexpression of p53 was sufficient to cause the death of sympathetic neurones in the presence of NGF which normally protects these cells from apoptosis (Slack et al., 1996). Secondly, p53-/- sympathetic neurones showed enhanced survival in culture in the absence of NGF, (Vogel and Parada, 1998). Thirdly, p53 levels increased when sympathetic neurones undergo apoptosis in response to either NGF withdrawal or activation of p75NTR and this apoptosis could be inhibited if the increase in p53 levels was prevented by concomitant expression of the adenoviral E1B55K protein which inhibits p53 function (Aloyz et al., 1998). Finally, analysis of p53-/- SCG revealed that the rate of apoptosis and loss of neurones was decreased but not eliminated in vivo during the first two postnatal weeks compared to wild type. This also suggests that other factors are involved in the apoptosis of SCG during this developmental cell death. Furthermore the decrease in apoptosis was similar in p53 +/- and p53-/- animals which suggests that developmental compensation occurs in the p53-/- background e.g. by the presence of p73 (Aloyz et al., 1998). Collectively, these findings strongly supports the idea that p53 plays a pro-apoptotic role in developing sympathetic neurones that is activated following NGF withdrawal or p75NTR activation.

The apoptotic pathways that are activated following NGF withdrawal in peripheral neurones has been well characterised and involves at least two apoptotic pathways which may converge on p53 (Fig 1.13a). One is the JNK-p53-Bax pathway that is also activated by p75NTR (Deckwerth et al., 1996;Aloyz et al., 1998;Kaplan et al., 2000). MEKK and JNK function upstream of p53 in p75NTR mediated apoptosis but cdc42/Rac1, Askl, M KK, JNK, c-jun and p53 have all been shown to act in a signalling pathway regulating NGF withdrawal-induced apoptosis. The second pathway shown to be important for apoptosis after NGF withdrawal involves the activation of the cell cycle regulatory molecules CDK4/6
Figure 1.13: The role of p53 and p73 in neuronal developmental cell death

Naturally occurring cell death is regulated by the balance of signals deriving from the NGF/TrkA pro-survival receptor and the pro-apoptotic p75 neurotrophin receptor (p75NTR). a) Withdrawal of the survival ligand triggers two apoptotic cascades, the JNK pathway that is also triggered by p75NTR activation and the cell cycle deregulation pathway that is independent of p75NTR activation. Both of these pathways are essential for neuronal apoptosis. p53 plays an essential pro-apoptotic role in this process while ΔNp73 plays an anti-apoptotic role by antagonising p53 function (see text for details). b) TrkA mediates neuronal survival during naturally occurring death by blocking p75NTR-mediated apoptotic signals. TrkA overrides the p75NTR-JNK-p53 apoptotic pathway via RAS activation. The RAS-PI3K-AKT survival pathway is the major pathway used by TrkA to suppress apoptosis. The Ras activated MEK/MAPK pathway also contributes to survival but to a lesser extent.
which activate pRb by phosphorylation. This results in the release of E2F-1 and subsequently causes sympathetic neurone apoptosis that is p53 dependent (Park et al., 1997). As inappropriate cell signalling has shown to result in E2F-1 mediated activation of p19ARF which activates p53 (by inhibiting MDM2 function) in non neuronal cells, then it follows that this cell cycle pathway may also converge onto p53, in the embryonic nervous system. Therefore, this suggests that p53 plays a pivotal role in integrating these stimuli and thus activating apoptosis in neurones only when these upstream signals reach a certain critical threshold. This cell cycle pathway may also represent a p75NTR independent pathway that is responsible for the delayed apoptosis of p75NTR independent sympathetic neurones.

Activation of the TrkA receptor by binding of NGF, can silence the JNK-p53-Bax cell death pathway via the RAS signalling pathway (shown in Fig 1.13b) which has been shown to be sufficient to suppress p53 mediated apoptosis in sympathetic neurones (Mazzoni et al., 1999). Ras activates several downstream effector proteins including Raf and phosphatidylinositon 3-kinase (PI3-K). Raf binds to and activates the MAP kinase kinase (MEK) signalling cascade that cumulates in the activation of ERK1/2, which can act in direct opposition to JNK (Xia et al., 1995;Mazzoni et al., 1999) and has been shown to protect against p53 mediated apoptosis in sympathetic neurones. PI3-K activates the serine/threonine kinase Akt and is the major pathway by which Ras mediates its anti-apoptotic effects. (Mazzoni et al., 1999). How Akt mediates survival in response to TrkA activation is not well understood, but may do so via both direct and indirect pathways. For instance it is thought to inactivate Bad thus releasing Bcl-xL to antagonise Bax and/or by the down regulation of Puma, a mediator of the p53 family apoptotic response (Datta et al., 1997;Vousden et al., 2002). Furthermore Akt has been shown to phosphorylate and promote translocation of MDM2 to the nucleus, thus facilitating the degradation of p53 (Mayo and Donner, 2001;Vousden et al., 2002).

The pro-apoptotic Bax protein, a downstream transcriptional target of p53, has a defined role in neuronal apoptosis. Importantly, Bax, has been shown to be essential for apoptotic death of most peripheral neurones (Deckwerth et al., 1996;White et al., 1998;Aloyz et al., 1998) and for p53 dependent apoptosis of cortical and cerebellar granule (Xiang et al., 1998;Cregan et al., 1999). After commitment of neuronal cells to undergo apoptosis the balance of pro-apoptotic and anti-apoptotic factors is shifted and the hypothesis that p53 transcription of
Bax contributes considerably to this shift towards cell death, is supported by several findings. These include experiments showing that exogenous p53 is unable to mediate apoptosis of Bax \(-/-\) cortical neurones and Bax \(-/-\) cerebellar granule neurones (Xiang et al., 1998; Cregan et al., 1999). Moreover, Bax \(-/-\) sympathetic neurones do not undergo apoptosis in response to NGF withdrawal (Deckwerth et al., 1996) and the generation of Bax/Bcl-xL double deficient embryos reduced the excessive apoptosis seen in the developing nervous system of Bcl-xL \(-/-\) animals (Shindler et al., 1997).

Interestingly, the increased numbers of cells in the Bax\(-/-\) embryo and decreased number of cells in the Bcl-xL embryo were the result of elimination/survival of post-mitotic neurones but deletion of Bax or Bcl-xL did not affect the apoptosis of progenitor cells (Motoyama et al., 1995; White et al., 1998; Kuan et al., 2000). In contrast, deletion of Caspase-3 gene effectively eliminates death of progenitor cells, resulting in marked hyperplasia of the embryonic nervous system (Kuida et al., 1996). Thus, Caspase-3 and presumably p53, caspase 9 and Apaf-1, which show similar phenotype to Caspase-3\(-/-\) animals, are regulators of progenitor cell proliferation as well as being involved in post-mitotic apoptosis with the latter requiring both Bax and Bcl-xL/Bcl-2 function.

Analysis of p73 expression in the developing brain and sympathetic ganglia showed that the DSnp73 isoform predominates, yielding protein levels that were 20-fold greater than those of the TAp73 isoform (Pozniak et al., 2000; Yang et al., 2000). Pozniak et al (2000) reported that levels of DSnp73 are highest in sympathetic neurones maintained in NGF but decrease significantly upon NGF withdrawal. After NGF withdrawal these neurones undergo p53 mediated apoptosis (Aloyz et al., 1998), a process that is repressed when DSnp73 is exogenously expressed in these sympathetic neurones. Moreover, in p73 \(-/-\) animals there is a 40% loss of sympathetic neurone number in the SGCs, suggesting that DSnp73 plays an essential anti-apoptotic role in vivo. Further evidence supporting DSnp73 as a determinant of survival in developing neurones by blocking p53-dependent apoptosis comes from genetic studies showing that deletion of even one allele of p53 is sufficient to partially rescue the enhanced sympathetic neurone death seen in p73\(-/-\) animals (Lee et al., 2004). These studies demonstrate that wild type p53 and DSnp73 proteins act in a functionally antagonistic manner during naturally occurring death. Specifically DSnp73 blocked the up-regulation of two direct p53 targets, p21\(^{Cip/Weft}\) and Apaf-1 as well as inhibiting cleavage of caspase-3 (Lee et al., 95)
In addition, ΔNp73 was also shown to prevent neuronal apoptosis in cortical neurones (Pozniak et al., 2002) suggesting that the pro-survival role for ΔNp73 may well generalize to other populations of neurones. It can therefore be postulated that the balance between p53 and the TA and ΔN forms of p73 modulate the levels of apoptosis in neurones during development. Additionally other interacting factors such as Brn-3a may be important in modulating the functions of these proteins in determining neuronal cell fate.

The findings that loss of p53 in p73 -/- animals resulted in only partial rescue of apoptosis, indicates that ΔNp73 must mediate neuronal survival via other targets as well. In this regard, Lee et al (2004) shown that exogenous expression of ΔNp73 in sympathetic neurones prevented the release of mitochondrial Cytochrome c, inhibited JNK activation and suppressed the induction of BimEL, a pro-apoptotic Bcl-2 family member, after NGF withdrawal.

The role of ΔNp73 in neuronal survival is not limited to developing neurones as p73 -/- animals exhibit continued neuronal loss throughout life (Pozniak et al., 2002;Lee et al., 2004). Therefore ΔNp73 is an essential survival protein in both the PNS and CNS as well as having a role in the long term maintenance of at least some populations of adult neurones. In agreement with this, ΔNp73 has been demonstrated to be required as a survival factor in the response of some adult neurones to certain stresses such as DNA damage and trauma. The survival of neurones during embryogenesis is dependent on making appropriate connections but after this event the mature neurones become resistant to apoptotic stimuli, so that they survive the remainder of the animal's life time and this is thought to require ΔNp73. Walsh et al (2004) showed that adult p73 +/- DRG neurones were more vulnerable to apoptotic stimuli then were their p73 +/- counterparts and that invulnerability was restored to the p73 +/- neurones by increasing expression of ΔNp73.

In context of these findings the significant neurological abnormalities seen in p73 -/- mice can be explained by either the absence or decrease of neurones. Therefore it can be speculated that the neurological abnormalities in the p73 -/- mouse is due to the loss of the anti-apoptotic form of p73. As the ΔN forms are generated from the same gene as the TA forms of p73 by alternative promoter usage, this provides a mechanism for rapidly altering the ratios of the pro-apoptotic versus anti-apoptotic isoforms of p73 in the nervous system. This would allow...
p73 to compensate for the absence of p53 in the nervous system and may explain the partial penetrance of the neural phenotype in the p53-/- embryos.

1.36 Role of p53 in differentiation

The role of p53 in neuronal differentiation during development has only become a recent field of investigation. A transient increase in the expression of p53 mRNA and protein was demonstrated in rat hippocampal neurones and in PC12 neuronal cells during differentiation and in these studies p53 expression was mainly nuclear. However, in mature differentiated neurones, p53 levels decreased and the protein was mainly found in the cytoplasm. Exogenous expression of a dominant negative mutant of p53 in PC12 cells reduced the percentage of differentiating neurones demonstrating that p53 is directly involved in the differentiation of these cells (Eizenberg et al. 1996). p53 expression has also been associated with differentiation of Oligodendrocyte precursor cells (OPC) so that transfection of dominant negative forms of p53 into purified OPCs inhibited both thyroid hormone (TH) or retinoic acid (RA) induced OPC differentiation but not platelet derived growth factor (PDGF) withdrawal induced differentiation (Billon et al., 2004). Furthermore there is a decrease in Oligodendrocyte number and an increase in the number of OPCs in the P7 optic nerve of p53 -/- mice compared with wild types, suggesting that p53 plays a role in differentiation of OPCs (Billon et al., 2004).

A role for p53 in kidney development and differentiation has also recently been suggested. The expression of p53 in the developing kidney was shown to coincide spatiotemporally with terminal differentiation, so that p53 stabilisation and enhanced DNA binding are accompanied by p53 phosphorylation and acetylation. Moreover p53 was able to transactivate promoters of several genes associated with differentiation in the kidney including the bradykinin B2-receptor (B2R), AQP-2, and angiotensin type 1. (Saifudeen et al., 2002). Furthermore analysis of p53-/- animals exhibited aberrant terminal differentiation of nephrons (Saifudeen et al., 2002).

These results therefore demonstrate that p53 plays an important role in the differentiation of specific cell types, including neurones, during development as well as inducing apoptosis in
these cells during development and in response to stresses.

1.37 Role of p73 in differentiation

In agreement with the neuronal defects found in the p73 knock out mice, *in vitro* studies demonstrated that TAp73 expression increased during retinoic acid induced differentiation of neuroblastoma cells and TAp73 overexpression alone was capable of inducing both morphological (neurite outgrowth) and biochemical markers (NCAM expression) of neuroblastoma differentiation. Moreover, transfection of dominant negative p73 in neuroblastoma cells abrogates the transactivation of NCAM promoter induced by retinoids, a well known trigger of neuronal differentiation in these cells. (De Laurenzi et al., 2000). Moreover, it has recently been demonstrated that over-expressing p73 explanted purified Oligodendrocyte precursor cells (OPCs) increases the spontaneous differentiation of OPCs in the absence of a differentiation stimulus, but also enhanced the differentiation of these cells induced by TH or RA- treatment or withdrawal of PDGF. Furthermore expression of the ΔNp73 transgene in OPCs inhibits all forms of OPC differentiation, possibly by blocking TAp73 function (Billon et al., 2004).

Additionally the TA73 and ΔNp73 isoforms have also been shown to be developmentally regulated during kidney organogenesis. Thus, TAp73 is predominantly expressed in the differentiation domain of the renal cortex whereas ΔNp73 isoforms are induced early in development and are preferentially expressed in proliferating nephron precursors. Furthermore p73 was shown to activate two renal function genes AQP-2 and B2R promoters independently of p53, while ΔNp73 repressed the B2R promoter. Therefore the spatial switch from ΔNp73 to TAp73 may be an important factor in cell fate determination during terminal nephron differentiation (Saifudeen et al., 2005).

Additionally, up-regulation of p73 expression has also been shown to increase during C2C12 muscle cell line differentiation (Fontemaggi et al., 2001;Li et al., 2005) and during myeloid cell line differentiation (Tschan et al., 2000).

Thus, as well as the response to DNA damage, p73 has also been shown to be up regulated during neuronal, muscle, kidney and hematopoietic muscle differentiation (Tschan et al., 2000).
In order to investigate the possible mechanisms of p73 modulation during differentiation, Fontemaggi and colleagues demonstrated that the transcriptional repressor ZEB binds to the first intron of the p73 gene and silences the p73 promoter. Furthermore ZEB binding is greater in proliferating cells than in differentiating cells thus providing a possible mechanism by which p73 levels increase in differentiating cells. This is supported by findings that overexpression of dominant negative ZEB restores p73 mRNA in proliferating C2C12 myoblasts and P19 neuronal cells (Fontemaggi et al., 2001).

These results demonstrate that as with p53, p73 is also associated with differentiation of specific cell types as well as inducing apoptosis in these cells depending on cell type and conditions. However p73 shows greater molecular complexity compared to p53 as it is expressed as multiple spliced N- terminal and C-terminal isoforms that show differential expression during development, indicating they have different biological properties.

1.38 Regulation of stability and function of the p53 family

The levels of p53 and p73 significantly increase in the cell in response to stress signals such as DNA damage, mainly through stabilisation of the protein and subsequent increase in half life. Both p53 and p73 activation leads to the transactivation of a subset of genes that results in either cell cycle arrest or induction of apoptosis depending on the nature of the stress signal, cell type, the combination of p53/p73 modifications and proteins associated with p53/p73. The activity of the p53 family of proteins is tightly regulated and this is achieved by regulation of protein degradation, post-translational modifications and cellular localisation which is discussed in this section. Furthermore the activation of p53/p73 and its network of genes, sets in motion a process of autoregulatory feedback mechanisms that either permits the completion or the reversal of the p53/p73 programmed response to stress (Harris and Levine, 2005)

The MDM2 protein is a crucial regulator of p53 stability, as it is able to bind to the p53 transactivation domain and inhibit its transcriptional activity while stimulating its ubiquitination and subsequent nuclear export (Haupt et al., 1997). Furthermore, p53 induces the expression of MDM2 which in turn controls p53 activity and stability giving rise
to an auto-regulatory feedback loop (Kubbutat et al., 1997). Studies using p53 and/or MDM2 animals in which expression has been lost showed that the early embryonic death observed in the MDM2 -/- embryos is rescued by further deleting the p53 gene. These findings demonstrate that the MDM2/p53 interaction is crucial for proper development (Jones et al., 1995). According to the current model, ubiquitination of p53 by MDM2 uncovers a nuclear export signal for p53, which is inactive when p53 is in a tetramer, thus permitting nuclear export of p53 and degradation by the proteasome (Geyer et al., 2000; Stommel and Wahl, 2004).

Like p53, p73 levels in the cell are also dependent on the balance between protein synthesis and degradation. The p73 protein also participates in an auto-regulatory feed back loop with MDM2. However, binding of MDM2 to the p73 transactivation domain inhibits p73 mediated transcription and apoptosis but does not target p73 for degradation (Zeng et al., 1999). In fact MDM2 binding promoted p73 stability (Ongkeko et al., 1999). Both 53 and p73 bind the p300 co-activator which is necessary for their transactivation function. Since MDM2 and p73 both bind to the N-terminal domain of the p300 co-activator, it is likely that MDM binding inhibits the function of p73 by competing with it for p300 (Zeng et al., 1999; Zeng et al., 2000). In contrast, MDM2 binding to p53 does not disrupt the p53-p300 complex. Furthermore, the presence of p300 in the p53/MDM2 complex was shown to actually stimulate degradation and that p300 also ubiquitinated p53 (Zeng et al., 1999; Grossman et al., 2003). Additionally, binding of MDM2 to p53 and p73 resulted in completely different subcellular redistributions of these proteins. In contrast to the nuclear export of p53 mediated by MDM2, the p73/MDM2 complex accumulates as nuclear aggregates (Gu et al., 2001). Thus, although the p53/p73-MDM2 negative feedback is preserved, the mechanism of MDM2 regulation of these two proteins is distinct.

Although MDM2 does not target p73 for proteasome dependent degradation, it does appear that p73 stability is dependent on the proteasome as well as on protein modifications (phosphorylation, acetylation). Specifically, it was shown that the steady state levels of p73 increases in the presence of the proteasome inhibitor, LLnL, demonstrating that p73 is degradated by an ubiquitin-dependent proteasome pathway (Lee et al., 1999). Recently it was demonstrated that Itch, a human E3 ligase binds to the PXXP motif found in p73α, p73β and ΔNp73α and targets these proteins for degradation (Rossi et al., 2005).
Furthermore, Itch is downregulated in response to DNA damage and it was therefore anticipated that both TAp73 and ΔNp73 protein levels would rise. However, it was shown that only the TAp73α protein level rises whilst ΔNp73 was rapidly degraded upon DNA damage regardless of the down regulation of Itch (Maisse et al., 2004; Rossi et al., 2005) therefore implying a second pathway that would specifically target ΔNp73α degradation in response to DNA damage. As a consequence of ΔNp73 degradation, both TAp73 and p53 can exert their apoptotic function. The mechanism of this selective degradation of ΔNp73 with respect to TAp73 in response to DNA damage remains to be determined. Additionally, it has been shown that p73α but not p73β can be modified by SUMO-1 (small ubiquitin like molecule) on Lys627 which potentiates p73α’s proteolytic degradation (Minty et al., 2000). Conversely, sumoylation of p53 activates its transcriptional activity (Gostissa et al., 1999). Also, the transcriptional activity of p73 has been shown to be required for its rapid degradation, indicating that direct transcriptional targets of p73 might have the ability to induce its proteolytic degradation (Wu et al., 2004).

p53 is a nuclear phosphoprotein that is phosphorylated by several protein kinases in response to DNA damage and the phosphorylation of specific residues of the p53 protein can lead to increased stabilisation as well as increased activity (Prives et al., 1999). In particular, the phosphorylation of the N-terminus residues Ser15, Ser20, Ser37 and Thr18 has been shown to reduce the interaction of p53 with MDM2 thereby preventing its degradation (Prives et al., 1999). There is also evidence showing that phosphorylation in the C-terminal region of the protein also stabilises p53 (Buschmann et al., 2001) while inhibition of p53 deacetylation which is required for MDM2 mediated degradation increased the half life of p53 (Ito et al., 2002). In contrast, p53 transcription is activated by both C-terminus phosphorylation and p300 mediated acetylation of the p53 C-terminal domain (Lill et al., 1997).

Similar to p53, genotoxic stress prolongs the half life of p73 and enhances its pro-apoptotic activity but in a c-Abl dependent manner (Yuan et al., 1999; Agami et al., 1999; Gong et al., 1999). In response to DNA damage the p73 protein was found to be stabilised by the phosphorylation of Tyr99 by c-Abl and phosphorylation of threonine residues by p38 kinase (Sanchez-Prieto et al., 2002). The transcriptional function of p73 is also regulated by p300 acetylation (Costanzo et al., 2002). As well as activation observed upon p73, phosphorylation can also inhibit function. This was shown by the findings that p73 phosphorylation on
Thr-86 by CDK complexes in a cell dependent manner causes a significant inhibition of p73 transcriptional ability (Gaiddon et al., 2003).

As with all transcription factors, nuclear localisation of the p53 family members is crucial for their transcriptional activity. Thus the MDM2 protein plays an important role in this respect. Cellular proteins that act to retain p53 or p73 in the cytoplasm can also result in inhibition of p53 mediated transcription and apoptosis. Indeed, tumour types with cytoplasmic confinement of p53 are less responsive to genotoxic stress and this is therefore an unfavourable prognostic factor. The p53 protein has been shown to be retained in the cytoplasm via interactions with cytoskeletal filaments, and cytoplasmic proteins such as Parc and mot2 (reviewed by O'Brate, 2003). Recently, the amphiphysin IIb-1 protein was shown to mediate cytoplasmic retention of p73 thus inhibiting p73 function (Kim et al., 2001). Amphiphysin IIb-1 is a member of a super family that play a role in clathrin-mediated endocytosis, particularly of synaptic vesicles.

1.39 Regulation of the transcriptional effects of p53 family members by other cellular proteins

As with p53, it is still not clear what regulates the ability of p73 to activate specific classes of genes e.g. cell cycle genes or apoptotic genes selectivity. However the pattern of post-translational modifications as well as other cellular proteins that interact with the p53 family are thought to play an important role in this decision (reviewed by Melino et al., 2003, and Coutts and La Thangue, 2005). As already stated p300 acetylates p73 upon DNA damage and directs p73 to apoptotic targets (Costanzo et al., 2002). PCAF is also a co-activator of p73 and acetylates the p73 C-terminal, although the significance of this acetylation is not yet known (Zhao et al., 2003). The co-activator p300 also acetylates p53 at K373 and K382 and this modification enhances p53 transcriptional activity (Gu et al., 1997; Gu and Roeder, 1997), and like p73, p300 acetylation of p53 may increase p53 activity on apoptotic targets. Interestingly, PCAF has also been shown to acetylate p53 on K320 which enhances sequence specific DNA binding (Sakaguchi et al., 1998).

A cofactor for p300, junction-mediating and regulatory protein (JMY) has been shown to enhance p53 mediated expression of genes such as Bax whilst not significantly influencing
the induction of p21Cip1/Waf1 (Shikama et al., 1999). Moreover, STAT-1 acts as a co-activator for p53 to induce the expression of Bax, Noxa and Fas (Townsend et al., 2004). The ASPP family members (apoptosis stimulating proteins of p53) bind to all the p53 family of proteins via the highly conserved DNA binding domain and both ASPP1 and ASPP2 specifically stimulate the transactivation function of p53, p73 and p63 on the Bax, PIG3 and Puma promoters but not the mdm2 and p21Cip1/Waf1 promoters. Correspondingly, the ASPP proteins enhances the apoptotic function of all three p53 family members (Samuels-Lev et al., 2001;Bergamaschi et al., 2004). The Wilms tumour 1 (WT1) proteins, which is lost in 15% of wilms tumours, also binds to p53, p73 and p63. However, WT1 was demonstrated to inhibit p73 and p53 mediated transcription of the Bax and mdm2 promoters and reduce p53/p73-induced apoptosis. Additionally, p73 and p53 were also shown to inhibit WT1 transactivation of a reporter containing three copies of the WT1 DNA binding sequence (Scharnhorst et al., 2000). Furthermore binding of WT1 to p53 stabilized the p53 protein whilst WT1 did not stabilize p73, highlighting yet again that different pathways regulate the stabilization of p53 and p73. The Brn-3a transcription factor has been demonstrated to interact with p53 and differentially regulate p53 target genes resulting in protection from apoptosis and increased cell cycle arrest. Conversely p53 was shown to differentially regulate Brn-3a target genes (Budhram-Mahadeo et al., 1999a; Sugars et al., 2001; Budram-Mahadeo et al., 2002; Perez-Sanchez et al., 2002).

The importance of the C-terminal region unique to p73α (but not present in other C-terminal isoforms) in protein-protein interactions has also been widely reported. For example, the receptor for activated C kinase 1, (RACK1) was shown to interact with the unique region of p73α and decreased p73α mediated expression of target genes and its effects on apoptosis whilst having no effect on p73β or p53 (Ozaki et al., 2003). Similarly, the YES associated protein (YAP) was shown to interact with the PPPPY motif present in p73α and p73β but not in the other p73 isoforms and this interaction enhanced p73 mediated transcription of apoptotic targets but not the p21Cip1/Waf1 promoter. It was further demonstrated that YAP requires PML to co-activate p73 and enhance p73 mediated apoptosis in response to DNA damage by potentiating p73 acetylation by p300 (Strano et al., 2001; Strano et al., 2005).

Therefore the cellular proteins that interact with the p53 family members and influence their biological outputs by selectivity activating a subset of p53/p73 target genes play a crucial role.
role in dictating whether cells undergo apoptosis or growth arrest in response to p53/p73 activation.

This study sets out to investigate the possible functional interaction between Brn-3a and p73 in the modulation of cell fate in specific cells. The ability of cellular proteins to interact with and influence the biological output of the p53 family members is important as these regulatory proteins allow for either universal regulation of the p53 family or differential regulation that is cell type specific or dependent on interaction with specific p53 family members. For instance, these regulatory proteins may be widely expressed and function as universal regulators of the p53 family (e.g. the ASPP proteins which regulate the proapoptotic activity of all p53 family members). Alternatively these regulatory proteins may act specifically on a particular p53 sub-family or even on a certain member or restricted members of a sub-family (e.g. RACK1 specifically interacts with p73α and reduces p73α mediated apoptosis). Finally these regulatory molecules may be cell type specific, thus regulating p53 family function spatially (e.g. the ability of Brn-3a to modulate p53 mediated cell fate in sensory neuronal cells). Therefore the possibility of a functional interaction between Brn-3a and p73 is important as it is postulated to regulate p73 mediated cell fate in specific cell types.

1.40 Aims

It has been demonstrated that Brn-3a interacts with the p53 protein via direct protein-protein interaction, between the POU domain of Brn-3a and the DBD of p53. This interaction resulted in differential effects on target gene expression resulting in modulation of p53 mediated cell fate in neuronal cells (Budram-Mahadeo et al., 2002). Because of the high degree of homology between the p53 and p73 DBD, this observation raised the question of a functional interaction between Brn-3a and the p73 proteins, both of which have clear roles in the developing nervous system. The Brn-3a/p73 interaction was assessed as follows:

1. To investigate the interaction between Brn-3a and the different p73 isoforms and effects of these associations in regulating genes such as bax and p21Cl/CIP1/Waf1 in determining cell fate.
2. To investigate the effects of Brn-3a on p53/p73 mediated stimulation of the pro-apoptotic noxa gene

3. To analyse the expression of Brn-3a with p53/p73 proteins in primary cultures of NCC and investigate the effects of this interaction in determining cell fate.
CHAPTER 2
MATERIALS AND METHODS

2.1 materials

2.1.1 Suppliers
Analytical grade laboratory chemicals were obtained from Boehringer Maheim (Lewes, East Sussex, UK), Merck (Poole, Dorset, UK) or Sigma chemical company Ltd (Poole, Dorset, UK). Disposable plasticware was supplied by Greiner (Stonehouse, Gloucester, UK) or Sterilin (Stone, Staffordshire, UK). Additional laboratory materials were obtained from the suppliers mentioned below, unless otherwise stated.

Amersham (Little Chalfont, Bucks, UK)
Glutathione Sepharose 4B, DNase 1, Rainbow™ protein weight marker, dNTPs, Hybond™-C membranes, ECL western blotting detection reagents and analysis system.

BD pharMingen
Annexin V-PE, Annexin Buffer, 7ADD

Bio-Rad (Hemel Hempstead, Herts, UK)
Ammonium persulphate, N,N'-methylene-bis-acrylamide, N’N’N’-tetramethylethylene-diamine (TEMED), GS-250 molecular Imager-densitometer and phosphor imager, SmartSpec 3000 spectrophotometer

Dako (High Wycombe, Bucks, UK)
All peroxidase conjugated secondary antibodies

Difco Laboratories (Basingstoke, Hants, UK)
Bacto-agar, Bacto-tryptone, Yeast extract

Gelman Life Sciences (Ann Arbor, Michigan USA)
Disposable 0.2 and 0.45 μm sterile filters

Gibco-BRL life technologies Ltd (Renfrewshire, Scotland, UK)
1kb DNA ladder, all tissue culture culture solutions, media and supplements
2.1.2 Standard buffers and solutions

The standard buffers and solutions used throughout are given below (all concentrations are
represented at 1X).

**Luria Bertani (LB) media**
- 1% (w/v) Bacto®-tryptone
- 1% (w/v) NaCl
- 0.5% Bacto®-yeast extract

LB media was immediately autoclaved after preparation at 120°C for 20 minutes T 10IB/SQUARE INCH (psi)

**Chloroform / isoamyl alcohol**
- 96% (v/v) chloroform
- 4% (v/v) isoamyl alcohol

**Tris equilibrated phenol**
- Liquefied phenol equilibrated twice with excess 0.1 M Tris-HCL pH 8.0

**TE**
- 10 mM Tris-HCL pH 8.0
- 1 mM EDTA pH 8.0

**Tris-Acetate Buffer (TAE)**
- 400 mM Tris base
- 200 mM Sodium acetate
- 20 mM EDTA pH 8.0

**Tris Borate Buffer (TBE)**
- 90 mM Tris-HCL pH 8.0
- 90 mM Boric acid
- 1 mM EDTA pH 8.0

### 2.1.3 Bacterial strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5-α</td>
<td>E.Coli recA1, endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F'proAB lacIq Z M15 tn 10 (tet))</td>
<td>Stratagene, Cambridge UK</td>
</tr>
<tr>
<td>BL21 (DE3) pLysS</td>
<td>E.Coli B F- ompT hsdS(rB-mB-) dcm+ Tetr gal λ(DE3) endA Hte (pLysS Camr)</td>
<td>Stratagene, Cambridge UK</td>
</tr>
</tbody>
</table>

The BL21(DE3) gold derived expression strains are ultracompetent cells which lack the Lon and Ompt proteases which can degrade recombinant proteins. They can be used in combination with plasmids containing the bacteriophage T7 promoter to control expression of the protein of interest (e.g. pET vectors). The T7 promoter is recognised by the T7 polymerase. To express the gene of interest the BL21(DE3) strain contains the T7 polymerase gene under the control of the lac/UV5 promoter which is induced by isopropyl-1-thio-β-D-galactopyranoside (IPTG). The BL21(DE3)pLysS gold strain carries an
additional plasmid carrying the T7 lysozyme gene derivative to provide tight control of protein expression of toxic proteins. Additionally, the expression of the GST fusion protein can be driven by the host RNA polymerase using a vector utilizing the tac promoter (e.g. pGEX vectors) which is induced by IPTG.

2.1.4 List of Plasmids used

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Resistance</th>
<th>Source/Reference</th>
<th>Genes cloned</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLTR</td>
<td>Eukaryotic expression vector in which the MoMuLV promoter drives the expression of the target gene cloned into it.</td>
<td>Ampicillin</td>
<td>Dr.T.Moroy, Phillips University, Marburg, Germany</td>
<td>pLTR Brn-3a long</td>
<td>Eukaryotic expression vector in which the MoMuLV promoter expressing the long form of Brn-3a. The cDNA of Brn-3a(l) was cloned into the Sall/Nrul site of the pLTR vector.</td>
</tr>
<tr>
<td>pGEX 2T</td>
<td>Prokaryotic expression vector in which the tac promoter drives expression of a GST fusion protein. Appropriate cDNA is cloned downstream of GST domain to express protein of interest with a GST tag at the N-terminal for affinity chromatography purification.</td>
<td>Ampicillin</td>
<td>Amersham Bioscience, Bucks UK</td>
<td>pGEX-Brn-3a(l) and pGEX-Brn-3b(s)</td>
<td>cDNA for either Brn-3a(l) and Brn-3b(s) were cloned into pGEX-2TK to produce the GST-Brn-3a(l) and GST-Brn-3b(s) proteins.</td>
</tr>
<tr>
<td>pDEST15</td>
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</tbody>
</table>
Description: Prokaryotic expression vector in which the T7 promoter drives expression of a GST fusion protein. Appropriate cDNA is cloned downstream of a GST sequence, resulting in expression of a GST-fusion protein that can be used for affinity chromatography purification.

Resistance: Ampicillin

Source/Reference: Invitrogen

Genes cloned: pDEST15-Brn-3b(l), pDEST15-Brn-3a(s), pDEST15-Brn-3aPOU, pDEST15-Brn-3b (POU)

Description: cDNA of either Brn-3b(l) or Brn-3a(s) was cloned into pDEST15 using gateway technology to produce the GST-Brn-3b(l) and GST-Brn-3a(s) proteins. pDEST15-Brn-3a-POU and pDEST15-Brn-3b-POU were cloned using gateway technology and the primers: 3aPOUfoward (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGGATCCCTGGCTTTCGACACACAGCTGGGTTGTCGACTCAGTAAAGTGCGAGAATTTTC-3'), 3aPOUreverse (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGTCGACTCAGTAAAGTGCGAGAATTTTC-3'), 3bPOUfoward (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGGATCCGACGTGGATGCAGACCCG-3'), 3bPOUfoward (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGTCGACTCAGTAAAGTGCGAGAATTTTC-3')

Source/Reference: Dr. D. Falkes ICH, Dr Duncan Gascoyne, ICH (Gascoyne et al., 2004)

Plasmid: pGEX 4T-1

Description: Prokaryotic expression vector in which the tac promoter drives expression of a GST fusion protein. pGEX 4T-1 is derived form pGEX-2T and contains a thrombin recognition site. Appropriate cDNA is cloned downstream of GST domain to express protein of interest with a GST tag at the N-terminal for affinity chromatography purification.

Resistance: Ampicillin

Source/Reference: Amersham bioscience, Bucks UK

Genes cloned: pGEX-Brn-3aN
GST-Brn-3aN was subcloned into the pGEX4T-1 vector from the yeast vector pBD-3aN.

(Gascoyne et al., 2004)

**Plasmid:** pcDNA3.1
**Description:** Eukaryotic expression vector in which the CMV enhancer drives the in vivo expression of the target gene cloned into it and the T7 bacteriophage promoter for in vitro expression.

**Resistance:** Ampicillin

**Source/Reference:** Invitrogen

**Genes cloned:** pcDNA-p53, pcDNA-TAp73α, pcDNA-TAp73β and pcDNA-ΔNp73α

Human p53, p73α, p73β and ΔNp73α was cloned into the *NheI* and *NotI* restriction sites in the pcDNA vector.

(De Laurenzi et al., 1998)

**Genes cloned:** p73 deletions

The deletion constructs p73αN56, p73αN315, p73αN423, p73C505, p73αC424 and p73βOD were subcloned from pcDNA-p73α and pcDNA-p73β using PCR based cloning.

(Takagi et al., 2001)

**Plasmid:** pcDNA3.1-HA
**Description:** Eukaryotic expression vector in which the CMV enhancer drives the in vivo expression of the target gene cloned into it downstream of the HA sequence and the T7 bacteriophage promoter for in vitro expression.

**Resistance:** Ampicillin

**Source/Reference:** Invitrogen

**Genes cloned:** pcDNA-TAp73α, β, γ, δ and ΔNp73α
Description: Human cDNA encoding TAp73α, β, γ and δ and ΔNp73α was cloned into the *NheI* and *NcoI* restriction sites in frame with a HA tag.

**Source/Reference:** Dr Vincent De Laurenzi university Tor Vergata, Rome (De Laurenzi et al., 1998)

**Plasmid:** pGL3-Luciferase

**Description:** Provides a quantitative analysis of factors that may potentially regulate mammalian gene expression. It has a coding region for firefly luciferase, which allows detection of transcriptional activity in transfected eukaryotic cells.

**Resistance:** Ampicillin

**Source/Reference:** Promega, Wisconsin USA

**Genes cloned:** Human bax promoter

**Description:** A 370 bp *EcoR1-sacI* fragment of the human bax promoter was cloned into the pGL3 vector upstream of the luciferase sequence.

**Source/Reference:** (Myashita et al., 1995)

**Genes cloned:** Human p21CIP1/Waf1 promoter

**Description:** A 2326 *HindIII* fragment of the human p21 promoter was cloned into the pGL3 vector upstream of the luciferase sequence.

**Source/Reference:** (EL-Deiry et al., 1993)

**Plasmid:** Picagene

**Description:** Provides a quantitative analysis of factors that may potentially regulate mammalian gene expression. It has a coding region for firefly luciferase, which allows detection of transcriptional activity in transfected eukaryotic cells.

**Resistance:** Ampicillin

**Source/Reference:** Wako, Osaka, Japan

**Genes cloned:** Mouse Noxa promoter and Δp53-Noxa promoter
Description: A 329bp Sac II fragment containing the noxa promoter was inserted into the picagene reporter plasmid. Δp53-Noxa was constructed with a 286bp Xho I to Sac II fragment which lacked the p53 binding site.

Source/Reference: (Oda et al., 2000)

Plasmid: pRL-TK
Description: Renilla lucerase repoter, under the HSV-thymidine kinase promoter. pRL-TK contains cDNA encoding renilla luciferase, acts as a control vector for transactivation efficiency.
Resistance: Ampicillin
Source/Reference: Promega, Wisconsin USA

2.1.5 List of antibodies used in westerns and immunostaining

Actin
Immunogen: human Actin carboxy terminus
Specificity: polyclonal
Dilutions: Western blot 1:2000
Secondary: 1:3000 HRP-conjugated anti-goat Ig
Source: Santa Cruz

Bax (clone 6A7)
Immunogen: amino acids 12-24
Specificity: monoclonal
Dilutions: Western blot 1:500
Secondary: 1:3000 HRP-conjugated anti-mouse
Source: BD PharMingen

Bm-3a mAb
Immunogen: mouse amino acids 186-224
Specificity: monoclonal
Dilutions: Western blot 1:1500, Immunostaining 1:50
Secondary: 1:3000 HRP-conjugated anti-mouse Ig, 1:2000 Alexa Fluor 488 anti-mouse (FITC)
Source: Chemicon International (MAB1585)
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Immunogen</th>
<th>Specificity</th>
<th>Dilutions</th>
<th>Secondary</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brn-3a pAb</td>
<td>Immunogen: synthetic polypeptide amino acids 51-67</td>
<td>Polyclonal</td>
<td>Immunostaining 1:100</td>
<td>1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)</td>
<td>Chemicon International (AB5945)</td>
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<tr>
<td>Cleaved Caspase</td>
<td>Immunogen: large fragment of activated Caspase-3 (17-19kda) resulting from cleavage of adjacent Asp175</td>
<td>Polyclonal</td>
<td>Immunostaining 1:100</td>
<td>1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)</td>
<td>Cell Signalling Technology™</td>
</tr>
<tr>
<td>3 (Asp-175 ab)</td>
<td></td>
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<tr>
<td>HA (805)</td>
<td>Immunogen: YPYDVPDYA (influenza HA epitope)</td>
<td>Polyclonal</td>
<td>Western blot 1:3000</td>
<td>1:2500 HRP-conjugated anti-rabbit Ig</td>
<td>Santa Cruz</td>
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<tr>
<td>Neurofilament-160</td>
<td>Immunogen: Purified neurofilament polypeptides</td>
<td>Monoclonal</td>
<td>Immunostaining 1:100</td>
<td>1:2000 Alexa Fluor 488 anti-mouse (FITC)</td>
<td>Sigma</td>
</tr>
<tr>
<td>(clone NN18)</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>p21^{Cip1/Waf1}</td>
<td>Immunogen: purified mouse p21 protein</td>
<td>Monoclonal</td>
<td>Western blot 1:500</td>
<td>1:3000 HRP-conjugated anti-mouse Ig</td>
<td>BD PharMingen</td>
</tr>
<tr>
<td>p53 (ab26) mAb</td>
<td>Immunogen: mouse amino acids 14-289</td>
<td>Monoclonal</td>
<td>Western blot 1:1000</td>
<td>1:3000 HRP-conjugated anti-mouse Ig</td>
<td>Abcam</td>
</tr>
</tbody>
</table>
p53 (ab-7) pAb

Immunogen: recombinant human p53 protein
Specificity: polyclonal
Dilutions: Western blot 1:1000, Immunostaining 1:100
Secondary: 1:3000 HRP-conjugated anti-mouse Ig, 1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)
Source: Abcam

p73-Total

Immunogen: C-terminus common to all isoforms of p73
Specificity: polyclonal
Dilutions: Immunostaining 1:100
Secondary: 1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)
Source: Emre Sayan, (University of Leicester).

p73-SAM

Immunogen: extreme C-terminal only recognising the α isoforms of TAp73 and ΔNp73
Specificity: polyclonal
Dilutions: Western blot 1:3000, immunostaining 1:100
Secondary: 1:3000 HRP-conjugated anti-mouse Ig, 1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)
Source: Emre Sayan, (University of Leicester).

p73-ΔN

Immunogen: first 16 amino acids of ΔNp73 that is not found in TAp73
Specificity: polyclonal
Dilutions: Western blot 1:3000, Immunostaining 1:100, Co-immunoprecipitation 1 µl.
Secondary: 1:3000 HRP-conjugated anti-mouse Ig, 1:2000 Alexa Fluor 468 anti-Rabbit (Rhodamine)
Source: Emre Sayan, (University of Leicester).

2.1.6 List of oligonucleotides used

All oligonucleotides were manufactured by Thermo Electron Corporation. Primers for Real-time PCR were generated using the primer 3 programme. The p53 response element in the pair of oligonucleotides used for the EMSA assay is highlighted in bold in the sense strand. The primers used in the ChIP assay amplify a 152bp region of the noxa promoter that encompasses the p53 response element.
**Real-time PCR**

- mGAPDH-f: 5'-TTC ACC ACC ATG GAG AAG GC-3'
- mGAPDH-r: 5'-GGC ATG GAC TGT GGT CAT GA-3'
- mBrn-3atotal-f: 5'-TCA CGC TCT CGC ACA ACA AC - 3'
- mBrn-3atotal-r: 5'-TCC GGC TTG TTC ATT TTC TCA- 3'
- mp53-f: 5'-ACC TCA CTG CAT GGA CGA TCT - 3'
- mp53-r: 5'-GAC ACT CGG AGG GCT TCA CTT - 3'
- mANp73-f: 5'-CGA GCC TAC CAT GCT TTA CG-3'
- mANp73-r: 5'-GGA GAC ATG GTG TCG AAG GT-3'
- mTAp73-f: 5'-AGC CAG ACA GCA CCT ACT TTG AC-3'
- mTAp73-r: 5'-GCC TTG CAG GTG GAA GAC AT-3'

**EMSA**

- p53-Noxa-f: 5'-AGG CTT GCC CCG GCA AGT TG-3'
- p53-Noxa-r: 5'-CAA CTT GCC GGG GCA AGC CT-3'

**ChIP assay**

- NoxaChip-f: 5'-CTC GAG ACC TGC TCC ACT TC-3'
- NoxaChip-r: 5'-CGC TGG AAT CCT CTC TGT TC -3'

**Genotyping**

- Bm-3a ko-1: 5-GGC GCG CAG CGT GAG AAA ATG AA-3
- Bm-3a ko2-2: 5-GTC TCA CAC CCT CCT CAG TAA GT-3
- Bm-3a neo-1: 5'-TGA TGC CGC CGT GTT CCG GCT GT - 3'
- Bm-3a neo-2: 5'-TCG CGG CCA AGC TCT TCA GCA AT - 3'
- p53-K01: 5'-ACA CAC CTG TAG CTC CAG CAC - 3'
- p53-K02: 5'-ACG GTC TCA CGA CCT CCG TC - 3'
- OPT-21: 5'-GTG TTC CGG CTG TCA GCG CA - 3'

### 2.1.7 List of cell lines used

**ND7 cell line:** This cell line was derived by fusing non-dividing rat dorsal root ganglion cells with the C1300 mouse neuroblastoma cell line. These cells can be maintained in a
proliferate state and when differentiated exhibit the morphology and biochemical markers of sensory neurones.

Culture medium: 1x L15 with 10% fetal calf serum, 0.3% D-glucose, 0.37% sodium bicarbonate and 0.2mN L-glutamine. Freezing medium: Fetal calf serum + 10% DMSO.

**SAOS-2 cell line:** Human osteogenic sarcoma established from the primary osteogenic sarcoma of an 11-year-old Caucasian female 1973 (American Type Culture Collection (ATCC) HTB 85)

Culture medium: 1x DMSO with 10% fetal calf serum. Freezing medium: Culture medium + 10% DMSO.

**IMR-32 cell line:** Human neuroblastoma cells derived from an abdominal mass removed from a 13-month old Caucasian male. The cell line was submitted to the ATCC in the 36th passage.

Culture medium: 1x DMSO with 10% fetal calf serum, and 1% non-essential amino acids. Freezing medium: Culture medium + 10% DMSO.

### 2.1.8 Primary cell line-Neural crest cells (NCC)

NCC cultures were prepared from neural tube explants removed from E9.5 embryos obtained after timed mating between the CD1 mice strain, Brn-3a heterozygotes or p53 heterozygotes (see section 2.7.4 for detailed method of neural crest dissection and culture).

Culture medium: 1x DMSO with 10% fetal calf serum, basic fibroblast growth factor (bFGF: 50 ng/ml), neurotrophin-3 (NT-3: 40 ng/ml) and antibiotic/antimycotic (Sigma: 10,000 U penicillin 10 mg streptomycin, 25 μg amphotericin per millilitre).

### 2.2 Manipulations in E.coli

#### 2.2.1 Propagation of bacterial strains

Bacteria were grown in either liquid LB media or on plates prepared from LB media
containing 2% Bacto-agar. Liquid cultures were grown overnight at 37° C in an orbital shaker at 200 rpm. Bacterial plates were incubated overnight at 37° C in a standard incubator. Both media contained antibody selection as appropriate. Ampicillin, chloramphenicol and Kanamycin were used as a final concentration of 100 μg/ml, 30 μg/ml and 50 μg/ml respectively.

2.2.2 Long term storage of bacteria

Bacteria were grown overnight as mentioned above with the appropriate antibiotic. The bacteria were distributed in 500 μl aliquots into 1.5 ml autoclaved vials plus 30% (v/v) sterile glycerol. The glycerol cell stocks were slowly cooled to -70 and stored at this temperature until ready to use. The stored strain was revived by streaking onto LB plates containing appropriate antibiotics for a single colony.

2.2.3 Preparation of competent cells

Competent DH5α cells were prepared in transforming buffer (TFB) and stored in frozen storage buffer (FSB). LB plates were streaked from a frozen stock of DH5α cells and incubated at 37° C overnight. A single fresh bacterial colony was used to inoculate 10 mls of LB and grown overnight. In the morning 100 μl of the starter culture was used to inoculate 100 mls of LB and the culture was grown until cell density reached OD₆₀₀ of 0.4-0.8. The culture was then poured into chilled 50 ml falcon tubes and chilled on ice for 5 minutes. The culture was then pelleted in a centrifuge at 3000 rpm for 5 minutes at 4° C, and the supernatant was discarded and the tubes inverted for 1 minute to get rid of any residual LB. The cells were then quickly resuspended in 20 ml of TFB (10mM MES pH5.8, 100mM RbCl₂, 10 mM CaCl₂ and 50 mM MnCl₂) by swirling or gently vortexing. The cells were then incubated on ice for 5 minutes and then pelleted at 3000 rpm for 5 minute at 4° C. The supernatant was discarded and the cell pellet was resuspended in 1ml of FSB (10 mM pipes pH6.5, 75 mM CaCl₂, 10 mM RbCl₂ and 15% (v/v) glycerol) and mixed by swirling or gently vortexing and incubated on ice for 15 minutes. The cells were then stored at -70° C in 100 μl aliquots.
2.2.4 Transformation of Bacteria

Competent cells were transformed using the heat shock protocol. Competent DH5α and Competent BL21 (DE3) cells were placed at room temperature and then transferred to ice just as the cells started to thaw. 50 ng of plasmid was added to 50 μl of competent DH5α or 100 μl BL21 (DE3) cells and incubated on ice for 30 minutes. The samples were heat shocked at 42° C for 90 seconds followed by incubation on ice for 10 minutes. 1ml of LB was then added and the cells incubated in an orbital shaker at 37° C, 200 rpm for 1 hour. The cells were then pelleted, resuspended in 200 μl of LB and plated onto LB agar plates containing the appropriate antibiotic selection. The plates were incubated at 37° C overnight. The plates were then stored at 4° C.

2.3 Nucleic acid isolation

2.3.1 Small scale preparation of plasmid DNA from bacteria cells

Single bacterial colonies were inoculated into 5 ml of LB supplemented with the antibiotic and grown overnight at 37° C in and orbital shaker (200 rpm). Plasmid DNA was extracted using QIAprep Spin Miniprep DNA purification system (Qiagen) according to the manufacturer’s instructions, average yield being approximately 15 μg plasmid DNA from 1.5 ml of overnight culture.

2.3.2 Large scale preparation of plasmid DNA from bacteria cells

A starter culture was set up during the day in 10 mls of LB containing the appropriate antibiotic. The starter culture was then used to inoculate 200mls of LB + antibiotic which was incubated at 37° C overnight. The culture was then pelleted and plasmid DNA was extracted using the Qiagen Maxi-Prep kit according to the manufacturer’s instructions. The concentration of the eluted DNA was measured using a spectrophotometer and subjected to analytical digests and agarose gel electrophoresis before use in either transient transfections or GST affinity pull down chromatography. The average yield of DNA obtained by this method was approximately 500 μg of plasmid DNA.
2.3.3 Genomic DNA isolation from mouse tissue

Approximately 5-10 mm of tail tip from breeding mice or the tail and hind limbs from embryos were removed for genotyping analysis. The mouse tissue was placed in 1.5 ml centrifuge tube containing 700 µl of tail tip buffer (50 mM Tris Ph 8.0, 100 mM EDTA pH 8.0, 100 mM NaCl and 1% SDS). 35 µl of 10 mg/ml proteinase K (Roche) was added and the tubes were incubated at 55° C overnight with occasional agitation. Protein was extracted by mixing with an equal volume of phenol, centrifuging to separate phases and removal of the aqueous phase to a fresh tube. A second phenol extraction was performed followed by a chloroform:iso-amyl alcohol extraction. The DNA was precipitated by adding an equal volume of isopropanol to the final aqueous phase. The DNA was then pelleted by centrifugation, washed in 70% ethanol, dried and resuspended in TE and stored at 4° C until the samples were analysed by PCR.

2.3.4 Spectrophotometric determination of nucleic acids

Nucleic acid concentrations were determined by measuring the UV absorbance of diluted samples at 260 nm using the SmartSpec 3000 spectrophotometer (Bio-Rad). Concentrations were calculated on the basis that 1 absorbance unit at 260 nm corresponds to approximately 50 µg/ml for double stranded DNA and 40 µg/ml for RNA. Absorbance at 280 nm was also measured and the ratio between absorbances at 260 nm and 280 nm were used to provide an estimate of the purity of the nucleic acid sample.

2.3.5 Restriction enzyme digestion

DNA analysis was carried out by restriction digests. These digests were carried out in 20µl reactions with 1 µg of DNA. Restriction enzyme(s) (5 units) were added and the buffer recommended by the manufacturer was used at 1X concentration (Promega). Digests were incubated at the appropriate temperature for 2 hours. The digested DNA was electrophoresed on an agarose gel of appropriate percentage. An undigested sample was run in parallel, as a reference for the enzyme cut samples.
2.3.5 Agarose gel electrophoresis

Depending on the expected sizes of the DNA fragments, appropriate percentage gels in 1 X TAE were cast. For example 1% gel was cast for fragments larger than 1kb, 1.5% for general purpose and 2% to resolve DNA fragments less than 1kb. Ethidium bromide was added to a final concentration of 0.5 μg/ml. Approximately 0.1 volume of 10X loading buffer (1X TAE, 50% v/v glycerol, 0.025% bromophenol blue) was added to the DNA samples prior to loading. For size reference, the 1 kb ladder (Gibco) or 100 bp ladder (Promega) was used. Electrophoresis of the DNA was carried out at 20-90 V for 0.5-2 hours allowing for good resolution of bands. Finally the bands were visualized on a long wave UV transilluminator and photographed using the Syngene doc system.

2.4 polymerase Chain reaction (PCR)

Standard PCR was used to amplify the region of the noxa promoter containing the p53 response element in the ChIP assays (section 2.9) and for genotyping Brn-3a and p53 animals. All PCRs were carried out on an Eppendorf Mastercycler.

2.4.1 Genotyping knock out animals

The Brn-3a-KO 1/2 and Neo-1/2 primers were used to check the genotypes of Brn-3a mice to ensure mating of Brn-3a heterozygous males and females (DNA was isolated from tail tips). The Brn-3a-/-, Brn-3a+/- and Brn-3a+/+ embryos obtained from these matings were used to prepare neural crest cultures and were also genotyped (DNA was isolated from the hind limbs and tail). As the size of the products differed and the conditions of the reaction are the same, it was possible to perform both amplification reactions in the same tube. Together with the template (50 ng of genomic DNA isolated from mouse tails/embryos) each PCR contained 1 unit of Taq polymerase (Promega) in a final concentration of 25 μl [1 X Mg-free buffer, 2.5 mM MgCl2, 1 mM dNTP mix, 100 ng of each primer(Brn-3a-KO 1/2 and Neo 1/2)]. The cycling parameter is shown below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95° C</td>
<td>5 minutes</td>
<td>Denaturation</td>
</tr>
<tr>
<td>2</td>
<td>95° C</td>
<td>45 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>64° C</td>
<td>45 seconds</td>
<td>Annealing</td>
</tr>
</tbody>
</table>

121
4 72° C 45 seconds Extension
Repeat steps 29 times
5 72° C 5 minutes Final extension
6 4° C Until removed

The products were then visualized on a 2% agarose gel. Expected banding pattern: Brn-3a KO = 600bp (neo), Brn-3a WT = 300bp (Brn-3a) and Brn-3a Het = 600bp + 300bp (neo and Brn-3a products)

The p53-KO 1/2 and OPT-21 primers were used to check the genotypes of p53 mice and their progeny to ensure mating of p53 heterozygous males and females to provide p53-/-, p53 +/- and p53+/+ embryos from which neural crest cultures were prepared. As the size of the products differed and the conditions of the reaction are the same, it was possible to perform both amplification reactions in the same tube. Together with the template (50 ng of genomic DNA isolated from mouse tail/embryos) each PCR contained 1 unit of Taq polymerase (Promega) in a final concentration of 25 μl (1 X Mg-free buffer, 2 mM MgCl₂, 0.2 mM dNTP mix, 1 μM of p53-KO-1, 0.5 μM of p53-KO-2 and 0.5 μM of OPT-21). The cycling parameter is shown below:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95° C for 15 minutes</td>
<td>Denaturation</td>
</tr>
<tr>
<td>2</td>
<td>94° C for 45 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>60° C for 1 minute</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72° C for 1 minute</td>
<td>Extension</td>
</tr>
<tr>
<td></td>
<td>Repeat steps 29 times</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72° C for 5 minutes</td>
<td>Final extension</td>
</tr>
<tr>
<td>6</td>
<td>4° C Until removed</td>
<td></td>
</tr>
</tbody>
</table>

The products were then visualized on a 2% agarose gel. Expected banding pattern: p53 KO = 730bp (OPT), p53 WT = 520bp (p53) and p53-HET = 730bp + 520bp (p53 and OPT products)

2.5 RNA methods

2.5.1 Isolation of total RNA

Neural crest cells (NCC) were cultured as described in section 2.7.4 and three separate cultures were harvested at each time point for total RNA (time point: 3, 5, 7, 8, 9 and 12 days
in culture). Total RNA was also extracted from mouse embryo brain. The NCC cultures or mouse embryo tissue was pipetted repetitively in 1ml TRIzol (Gibco) to lyse. If the tissue did not completely homogenize a gauge needle was used so that no pieces of tissue remained. The homogenate was then left to incubate at room temperature for 5 minutes. 200 µl of chloroform was then added and the mixture was shaken vigorously for 15 seconds, followed by 3 min incubation at room temperature before centrifugation at 12000 rpm for 15 minutes at 4° C. The clear upper aqueous phase was removed to a fresh tube and 500 µl of isopropanol was added. The tube was inverted several times to ensure mixing and left at room temperature for 10 minutes to allow of precipitation of RNA. The tube was then centrifuged at 12000 rpm for 10 minutes at 4° C to spin down the RNA which forms a gel-like pellet. The supernatant was carefully removed and the RNA pellet was washed in 70% ethanol and air dried for 5 minutes. The RNA was then dissolved in 15-30 µl Rnase-free H2O by passing the solution through a pipette tip several times and incubating for 10 minutes at 55° C.

Prior to cDNA synthesis the RNA was DNase1 (Amersham) treated to remove genomic contamination. The reaction contained 10 µl of RNA, 1.5 µl of DNase 1, 10 µl of 10 X DNase-1 buffer in a 100 µl reaction made up with DEPC-treated water. The reaction mixture was incubated at 37° C for 30 minutes followed by 10 minutes at 65° C to stop the reaction. 100 µl of DEPC-treated water was added to the reaction mixture so that the total volume was 200 µl, before extraction with 200 µl of phenol saturated with DEPC-water. This was then mixed and centrifuged at 12000 rpm for 10 minutes at 4° C and the aqueous layer was removed to a fresh tube. A further extraction was performed with an equal volume of phenol/chloroform (at a 1:1 ratio) followed by chloroform extraction and the aqueous phase was removed to a new tube. The RNA was the precipitated with 2 X volume of ethanol and 0.1 volume of 3M sodium acetate for 2 hours at -20° C and then pelleted by centrifugation at 12000 rpm for 10 minutes at 4° C. After being washed in 70% ethanol the RNA pellet was then air dried and resuspended in 15-30 µl Rnase-free H2O.

The DNase1 treated RNA was then either run on a 1% agarose gel for 15 minutes at 240 V to assess the quality of RNA and relative quantities (can easily visualise 0.5 µg of RNA) or concentration was determined using a spectrophotometer if RNA concentration was low.
2.5.2 cDNA synthesis

1-5 μg of total RNA isolated from mouse tissue or approximately 0.1 μg of total RNA isolated from NCC cultures was reversed transcribed into first strand complimentary DNA (cDNA) using SUPERSCRIPT™ II enzyme (Gibco) following the manufacturers instructions. In order to amplify total cDNA, 250 ng of random hexamer primers (Roche) were added to the RNA (total volume 11 μl) which was then incubated for 10 minutes at 70° C and was then placed on ice to allow to cool before being spun down for 10 seconds to collect the mixture. During this time the following master mix was made:

<table>
<thead>
<tr>
<th>Component</th>
<th>per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 X first stand buffer</td>
<td>4 μl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2 μl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNAse inhibitor</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

8 μl of the master mix was added to the RNA and random hexamers followed by incubation at 25° C for 2 minutes. 1 μl (200 units) of SUPERSCRIPT II was then added and the mixture was incubated at 25° C for 10 minutes before incubation at 42° C for 90 minutes. The reaction was then terminated by incubation at 70° C for 10 minutes. The cDNA was then stored at -20° C. This protocol results in a final volume of 20 μl. A similar protocol was also carried out to produce a final volume of 50 μl by scaling up the reaction. In parallel, cDNA synthesis was also carried out without the SUPERSCRIPT II enzyme and used in subsequent RT-PCR reactions to check the presence of genomic contamination.

2.5.3 Real time quantitative RT-PCR

Real-time quantitative RT-PCR was carried out to quantify the amount of Brn-3a, p53, TAp73 and ΔNp73 mRNA in neural crest cells kept in culture for 3-12 days. All reactions were carried out on the DNA Engine Opticon (MJ Research) using primers designed with primer express or primer 3. The TA and ΔNp73 primers were designed by targeting regions that are specific to either protein. For TAp73 both forward and reverse primers were targeted to sequences within the TAD which is found in the TA isoforms only. For ΔNp73 the forward primer is specific to the N-terminus only found in ΔNp73 whereas the reverse primer is in a sequence common to both TAp73 and ΔNp73. Real time PCR was carried out utilizing
the Sybr Green technology (QuantiTect /Sybr green- Qiagen) in which the Sybr green fluorescent dye binds to all double stranded DNA molecules and once bound emits a fluorescent signal that can be detected and measured (excitation at 494 nm and emission at 521 nm).

Initially conditions were optimized for each set of primers using cDNA prepared from RNA extracted from mouse embryo brain, as the RNA isolated from NCC cultures were considered too precious for these set up reactions. In general, together with 2 μl of the cDNA template, each PCR contained 1 X QuantiTect /Sybr green PCR Master Mix, 0.3 μM of forward primer and 0.3 μM of reverse primer in a final volume of 20 μl. The reactions were set up in a thin walled microcentrifuge tubes and subjected to the following basic cycling parameters. The annealing temperature, extra conditions and produce size for each primer set are stated (sequences of the primers used are listed in section 2.1.6).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95° C for 15 minutes</td>
<td>Activation of HotStarTaq DNA polymerase</td>
</tr>
<tr>
<td>2</td>
<td>95° C for 30 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>58, 59, 60 or 61° C for 30 seconds</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>72° C for 30 seconds</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Plate read</td>
<td>fluorescence data collection</td>
</tr>
<tr>
<td>6</td>
<td>Repeat 44 times</td>
<td>Cycle number</td>
</tr>
<tr>
<td>7</td>
<td>55-95 ° C, 0.3 ° C, hold 0.00001</td>
<td>Melting curve</td>
</tr>
</tbody>
</table>

For GAPDH reaction, add extra step 80°C for 10 seconds before plate read to melt primer dimers.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Annealing temperature</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>58° C</td>
<td>250bp</td>
</tr>
<tr>
<td>Brn-3a</td>
<td>59° C</td>
<td>100bp</td>
</tr>
<tr>
<td>p53</td>
<td>61 ° C</td>
<td>81bp</td>
</tr>
<tr>
<td>TAp73</td>
<td>60 ° C</td>
<td>100bp</td>
</tr>
<tr>
<td>ΔNp73</td>
<td>60 ° C</td>
<td>195bp</td>
</tr>
</tbody>
</table>

A melt curve is performed at the end of each reaction in order to verify the specificity and identity of the RT-PCR products. The specificity of the RT-PCR products is also assessed by agarose gel electrophoresis. After primer optimization, a set of standards curves were generated using the PCR products of each primer set. Accordingly, the PCR product amplified by each primer set was run out on an agarose gel and the band was excised from
the gel and the DNA purified into 50 µl of double distilled water by use of the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturers instructions. An aliquot of the purified DNA aliquot was then run on an agarose gel next to the Log2 marker (New England Biolabs) to quantify the concentration of each aliquot. A set of standards for each amplicon was then prepared (up to 7 serial dilutions, 1000 fg/µl, 500 fg/µl, 100 fg/µl, 1 fg/µl, 0.1 fg/µl, 0.001 fg/µl and 0.00001 fg/µl to ensure that the unknown amount of target in samples fall within this range), which were used to generate the standard curves in every experiment. The $C_T$ (the cycle at which the aliquot plot crosses the threshold - i.e. the point at which there is a first detectable increase in fluorescence) of the standards is plotted against the log of the template amount resulting in a straight line. The unknown amounts amplified in the samples are then calculated by comparing the $C_T$ values of the samples to the $C_T$ values of the standard curve.

To check for variation in RNA between NCC samples, amplification of the invariant glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was carried out with the Sybr green detection system. The data is produced as a sigmoid amplification plot in which the number of cycles is plotted against fluorescence. The $C_T$ values were determined by using the Opticon software and the standard curve was then used to measure the target expression levels. Once the appropriate amount of cDNA required to equalise for the control mRNA was determined, the Brn-3a, p53, TA73 and ΔNp73 gene targets were amplified under conditions optimised for each set of primers. The control GAPDH was then re-amplified with the same volume of cDNA used to amplify Brn-3a, p53, TA73 and ΔNp73. All values for Brn-3a, p53, TA73 and ΔNp73 expression were normalised relative to GAPDH levels.

RT-PCR reactions were also carried out with each primer sets on cDNA samples prepared in the absence of reverse transcriptase (minus RT controls) to determine if there is any genomic contamination. A no template control was also included in all experiments to control for contamination.
2.6 Protein methods

2.6.1 Extraction of protein from cultured cells

For analysis of endogenous Bax and p21Cip1/Waf1 expression, protein was extracted from ND7 or SAOS-2 cells transfected with the appropriate DNA plasmids in 10 cm plates. Briefly, ND7 cells were transfected with 2 μg of Vector, p53, TAp73α, TAp73β or ΔNp73α in the presence of either 2 μg of Brn-3a or empty vector in 10 cm plates using Fugene reagent (Roche) (see section 2.7.5). After 48 hours, the cells were washed in 1X cold PBS and harvested in 500 μl of standard sample buffer (5% β-mercaptoethanol, 50 mM Tris-HCl pH 8.0, 6% (v/v) glycerol, 2% (w/v) SDS and 0.005% (w/v) bromophenol blue). The samples were kept on ice as much as possible during extraction. The samples were then heated to 95°C for 3 minutes, placed back on ice and either loaded immediately onto a SDS-polyacrylamide gel or stored at -20°C. Protein extraction methods for immunoprecipitation, EMSAs and ChIP assays are described separately.

2.6.2 SDS-polyacrylamide gel electrophoresis

Standard SDS-polyacrylamide gels were prepared with the composition for the stacking and resolving gels as described by Sambrook and colleagues. Protein samples were heated to 95°C for 5 minutes. Approximately 100 μg (total protein) was loaded per lane. Coloured molecular weight protein standards (rainbow marker, Amersham) or broad range prestained protein marker (New England Biolabs) was loaded 3 μl per lane for a small gel, or 7 μl for a large gel. Gels were run at 150 volts for small gels and 200 volts for large gels in 1X running buffer (25mM Tris, 250mM glycine and 0.15 (w/v) SDS). The gels were run until the protein of interest was sufficiently resolved as determined by the migration of the coloured marker.

2.6.3 Equalization of protein loading

In order to establish integrity of protein as well as for variation in total protein concentration, SDS-PAGE mini-gels were run to separate the protein samples. The gel was then placed in coomassie stain solution (2% (w/v) coomassie brilliant blue R250, 50% (w/v) methanol, 10% (v/v) glacial acetic acid) for 30 minutes at room temperature with continual shaking. Any
unbound stain was then removed by repeated replacements of destain (10% (w/v) glacial acetic acid, 30% (v/v) methanol). Any variation in different samples was observed by the intensity of the protein bands in each sample and densitometry was utilized to obtain values for various band intensities. The amount of protein was also calculated by addition of 1 µl of extract to 1 ml of diluted Bradford assay reagent (Biorad) following the manufacturers instructions. Measured absorbance at 595 nm using a spectrophotometer indicated the amount of protein present when compared to a prepared standard.

2.6.4 Transfer of proteins to nitrocellulose membranes (western blotting)

Proteins separated on SDS-PAGE gels were transferred to Hybond C membranes using a wet-transfer method based on that of Sambrook and colleagues (1989). Briefly the SDS-page gel and nitrocellulose membrane were pre-soaked in transfer buffer (192 mM glycine, 25 mM Tris and 20% (v/v) methanol), sandwiched between sheets of pre-soaked Whatman 3MM paper, and a Trans-Blot Cell (BioRad) assembled according to manufacturers instructions. Transfer was carried out overnight at 30 volts and 4°C.

2.6.5 Immunodetection of proteins on Western blots

Following transfer, the membranes were incubated in blocking buffer (1x PBS containing 4% (w/v) skimmed milk powder and 0.1% Tween-20) for 1 hour at room temperature with constant shaking. The membrane was then incubated with primary antibody diluted in blocking buffer for 1-2 hours at room temperature with constant shaking or overnight at 4°C with constant shaking. The unbound antibody was removed by washing the membrane 3 X for 10 minutes in blocking buffer at room temperature with constant shaking. The membrane was then incubated in the appropriate anti-IgG horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking buffer for 1 hour at room temperature with constant shaking. A further 5 X 10 minute washes of the membrane was carried out in 1x PBS containing 4% (w/v) skimmed milk powder and 0.5% Tween-20) at room temperature with constant shaking to remove unbound antibody. The bound HRP was then detected using an enhanced chemiluminescence system (ECL) according to the manufacturer's instructions. The membrane was then exposed to X-ray film to detect the resultant light emissions. The
exposure times were between 1 second and 15 minutes, depending on the strength of the signal.

2.6.6 Expression of labelled proteins by in vitro translation

$^{35}\text{S}$-labelled proteins were expressed by *in vitro* translation using the T7 TNT-coupled transcription-translation system following the conditions as described by the manufacturer (Promega). The reaction mixture (final volume 50 µl) was assembled on ice in a 0.5 ml microcentrifuge tube and incubated at 30° C for 90 minutes. 5 µl of the mixture was then run out on a SDS-PAGE gel and analysed by autoradiography.

2.6.7 Preparation of GST fusion proteins

10 mls of LB containing 100 µg/ml ampicillin and 30 µg/ml chloramphenicol was inoculated with a single colony of BL21 (DE3) pLysS cells containing a recombinant pGEX 2TK or pDEST plasmid and grown overnight at 37° C in an orbital shaker at 200 rpm. The culture was then diluted 1:100 with fresh LB containing 100 µg/ml ampicillin and grown for 2-3 hours until the OD600 reached 0.4. The cells were then induced with 0.3 mM IPTG and incubated for 4-5 hours at 37° C, 200 rpm. The culture was then transferred into 50 ml falcon tubes and the cells were then pelleted by centrifugation and each pellet was resuspended in 1 ml of PBS wash buffer (1x PBS containing 1 M DTT, 0.5 M EDTA, 0.1% igepal and 1x Protease inhibitor solution). The cell suspension was lysed by 3 cycles of freeze/thawing at -70° C/37° C, and the cell debris was then pelleted at 10,000 rpm for 10 minutes at 4° C. The supernatant was recovered and the GST fusion protein purified using glutathione sepharose beads.

2.6.8 Affinity purification of GST fusion proteins using Glutathione sepharose 4B

2 ml of the above supernatant was incubated on a rotating wheel with 150 µl of glutathione sepharose 4B (Sigma) 50% slurry at room temperature for 1 hour. The beads were then pelleted at 3000 rpm for 5 minutes at 4° C and the supernatant was discarded carefully before the beads were washed 3x in PBS wash buffer (1x PBS containing 1 M DTT, 0.5 M EDTA,
0.1% igepl and 1x Protease inhibitor solution) and stored in PBS wash buffer + 15% glycerol at -20. The glutathione sepharose containing the purified fusion protein was then run on a SDS/12.5% polyacrylamide gel to check for size and to visually estimate equal protein concentration for the samples for each pull down experiment.

2.6.9 Glutathione-S-transferase affinity pull-down chromatography

The GST/Bm-3 proteins or GST alone bound to glutathione sepharose beads were resuspended in NENT buffer (100 mM NaCl, 1 mM EDTA, 20 mM Tris pH 8.0, 1% igepl and 0.5% milk powder) and incubated with rotation at room temperature for 30 minutes. The beads were then pelleted at 2000 rpm for 3 minutes and resuspended in 20% milk powder in NENT buffer and incubated with rotation for 15 minutes at room temperature. The beads were then washed in 1 ml of NENT buffer and then once in transcription wash buffer (20 mM HEPES, 60 mM NaCl, 1 mM DDT, 6 mM MgCl, 8.2% glycerol, 0.1 mM EDTA). 5 μl of the in vitro translated proteins were then incubated with the sepharose beads in 100 μl of transcription buffer for 1 hour at room temperature with rotation. The beads were washed 5 X in NENT buffer and the proteins were solubilised in standard sample buffer, heated to 95°C for 5 minutes and resolved on a SDS-10% polyacrylamide gel. The gel was then dried and exposed to radiographic film. The amounts of protein retained following the interaction studies were assessed by comparing the intensity of the bands resulting after the protocol with that resulting when equivalent amounts of proteins (input) were run on a similar gel.

2.6.10 Co-immunoprecipitation

Protein extracts were made from cell lines transfected with the appropriate expression vectors. Adherent cells were washed in ice cold PBS and were then scraped into 800μl of ice cold PBS for 10 cm plates. The cells were pelleted and lysed by putting through a 1 ml syringe, 25 gauge needle in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.5% NP-40 and protein inhibitor at a concentration of 1 X) and left on ice for 20 minutes. 70 μl of protein extract was pre-cleared with 25 μl of protein A/G sepharose on a rotating wheel for 30 minutes at 4°C. The sepharose was then pelleted at 2000 rpm for 5 minutes and the supernatant was collected into a new 1.5 ml microcentrifuge and incubated with the
appropriate antibody overnight at 4°C with rotation. Immunocomplexes were collected by incubation with 30 µl of protein A/G sepharose for 1 hour at 4°C with rotation. The beads were centrifuged at 2000 rpm for 5 minutes and the supernatant was discarded and the beads were washed 5 X in HMKEN buffer (10 mM HEPES ph 7.2, 142 mM KCl, 5 mM MgCl₂ 2 mM EGTA, 0.2% Igepal and protein inhibitor at a concentration of 1X). The beads were then resuspended in 15 µl of standard sample buffer, heated to 95°C for 5 minutes and analysed with SDS/PAGE and Western Immunodetection.

2.6.11 Immunofluorescence

Immunostaining of NCC were carried out to detect expression of different proteins. After 7 days of culture, NCC cultures were gently washed 3 X in 1 X TBS (50 mM Tis HCL pH 7.5 and 150 mM NaCl) and fixed in 4% paraformaldehyde (PFA) for 15 minutes. Cells were washed 3 X in 1 X TBS followed by incubation in 1 X TBST (TBS + 0.1% triton X-100) for 5 minutes to permeabilise the cells. The cells were then blocked in 1 X TBS containing 10% goat serum for 20 minutes followed by incubation with first primary antibody in 1 X TBST + 1% serum. If double immunostaining was to be carried out, the other primary antibody was added after 3 X washes in TBS, and incubation was as described above. After incubation with primary antibodies, the cells were washed 3 X in 1 X TBS and the appropriate Alexa Fluor secondary antibodies (Molecular Probes) were added to the cells diluted in 1 X TBST + 1% serum for 30 minutes. The cells were then washed 5 X in 1 X TBS and mounted in fluorescent mounting medium (Dako). Images were obtained using the Zeiss Axioscop 2 fluorescent microscope fitted with an Axiophoto camera and analyzed with Axiovision software.

2.7 Cell culture

2.7.1 Culture conditions

For long term storage all cell lines were stored in liquid nitrogen at -180°C. During culture the cells were maintained at 37°C in a 5% CO₂ incubator. All manipulations of cells were carried out under aseptic techniques.
2.7.2 Freezing and recovery of cell stocks

Cell stocks for storage were prepared by resuspending the cell pellet from one 175 cm$^3$ flask in 1.5ml of 10% (v/v) dimethysulphoxide (DMSO), 90% foetal calf serum (FCS). The temperature of the vials was slowly decreased to -70° C before being immersed in liquid nitrogen. For growth from frozen stocks, cells were recovered by rapidly thawing the contents of one vial and transferring the cell suspension in to a 25 cm$^3$ flask containing 8ml of pre-warmed growth media. The medium was changed or the cells passaged the following day.

2.7.3 Routine cell passage

Cells were grown in the appropriate growth medium until the flasks were ~80% confluent. They were passaged as follows. The monolayer of ND7 cells were washed with Hanks Balanced Salt Solution (HBSS) free of calcium and magnesium ions. Fresh medium was added to the cells and the cells were detached by tapping the side of the flask. Fresh flasks were seeded at a ratio of 1:10. SAOS-2 cells were washed with Hanks Balanced Salt Solution (HBSS) free of calcium and magnesium ions. Trypsin/versene (1:10) were added to the flask for 30 seconds before being removed and the flask was left for 2 minutes to allow for the cells to detach. Fresh medium was added to the flask and the cells were seeded at a ratio of 1:3 into a new flask containing fresh medium.

2.7.4 NCC cultures

2.7.4.1 Preparation of coverslips

NCC cultures were grown on 13 mm diameter coverslips that were acid washed and baked, before being coated with poly-l-lysine (Sigma) and fibronectin (Sigma). Briefly, 13 mm coverslips were placed in a glass container and rinsed in concentrated nitric acid for 30 minutes at room temperature with constant shaking followed by a rinsing for 3 X 10 minute in distilled water. The coverslips were then rinsed for 30 minutes in methanol at room temperature with constant shaking. The methanol was removed and the coverslips were air dried. The coverslips were then baked in a sealed glass container in an oven at 150° C for 4
hours. Coating of coverslips took place in a sterile fume hood and was carried out on the morning of dissection. The acid/baked coverslips were placed into 24 well plates and 150 μl of 0.5 mg/ml of poly-l-lysine (Sigma) was added on top of each coverslip and left to incubate for 20 minutes. The poly-l-lysine was then removed and the coverslips were air dried for 20 minutes before adding 150 μl of 40 μg/ml fibronectin (Sigma) to each coverslip and allowed to incubate for 20 minutes. The fibronectin was then removed and the coverslips were air dried for 20 minutes, at which point they are ready to use.

2.7.4.2 Dissection of neural crest

NCC cultures were prepared from E9.5 embryos obtained after timed mating, with midday on the day of finding the copulation plug considered as E0.5. Dissections were carried out in a fume hood with the aid of a microscope. The pregnant mouse was first sacrificed and the embryo sack was removed to a dish containing HBSS. Primary neural crest explants were prepared from embryos by removing the portion of neural tube corresponding to the trunk level (somites 7-18). After separation of fetal membranes, the explants were enzymatically treated with 0.5 mg/ml collagenase in HBSS for 5 minutes, to further aid separation of the neural tube from the ectoderm and somites. After enzyme treatment, 1 ml of DMEM was added to stop the enzyme reaction. Further dissection was carried out to remove excess tissue, which was made easier following enzyme treatment. The neural tube explant was then transferred onto poly-l-lysine and fibronectin coated coverslips in a droplet of DMEM supplemented with 10% fetal calf serum (FCS). Explants were maintained in culture in a humidified incubator with 5% CO₂ at 37°C. The neural tube explant was removed and discarded the following day and the NCC cultures were maintained for the required period, in 0.5 ml growth medium consisting of DMEM with 10% FCS, 50 ng/ml of basic fibroblast growth factor (bFGF,R and D systems) 40 ng/ml of neurotrophin-3 (NT-3, PeproTech EC Ltd) and antibiotic/antimycotic (Sigma; 10,000 U penicillin, 10 mg streptomycin, 25 μg amphotericin per millilitre). The medium was replaced every 2-3 days. At the appropriate times cells were either harvested for extraction of total RNA (section 2.5.1) or fixed in 4% PFA and processed for immunostaining (section 2.6.11).
2.7.4.3 Analysis of apoptosis in NCC from Brn-3a-/− mutants

Timed mating of Brn-3a heterozygous males and females were used to provide Brn-3a-/− mutants as well as littermates [wild type (WT+/+) and heterozygous (-/+)] which were used as controls. Neural crest cultures were prepared from embryos as described, whereas DNA was extracted from the hind legs and tail of the embryo for genotyping experiments which were carried out as described in section 2.4.1. After 7 days, matched Brn-3a-/− NCC cultures and wild-type (+/+) and heterozygous (-/+) controls were treated with 20 μM cisplatin for 6 hours to induce apoptosis. Cells were then fixed and immunostained for activated caspase-3 (detected with FITC-conjugated secondary Ab). To quantify the apoptotic cells in NCC cultures derived from WT, KO or Het embryos, counts were made of cells that were positive for activated Caspase-3 in at least six KO embryos and matching WT and HET controls.

2.7.4.4 Analysis of p73 expression in NCC from p53-/− mutants

Timed mating of p53 heterozygous males and females were used to provide p53-/− mutants as well as littermates [wild type (WT+/+) and heterozygous (-/+)] which were used as controls. Neural crest cultures were prepared from embryos as described whereas DNA was extracted from the hind legs and tail of the embryo for genotyping experiments which were carried out as described in section 2.4.1. After 7 days, matched p53 -/- NCC cultures and wild-type (+/+) and heterozygous (-/+)) controls were fixed and immunostained for p73 (detected with rhodamine-conjugated secondary Ab) and Brn-3a expression (detected with FITC-conjugated secondary Ab).

2.7.5 Transient transfection

ND7 were transfected using the calcium phosphate method or Fugene (Roche). Note that most of the transfections in ND7 cells were carried out with Fugene.

Calcium phosphate method - One day before transfection cells were plated out at 5×10^5 in 10cm plates. The next morning the medium was replaced with 5 mls of pre-warmed DMEM plus 10% FCS and was incubated for 2 hours. The DNA plasmids, reporter plasmid and TK renila plasmid (at 1/10 the concentration of reporter) and HS DNA were combined to a final
concentration of 30 μg per transfection in a sterile microcentrifuge tube. 31 μl of 2 M CaCl₂ was added to each plasmid DNA solution and the final volume was brought up to 250 μl with sterile dd-H₂O. The DNA mix was then added to 250 μl of 2 X HBS (1.64% (w/v) NaCl, 1.19% (w/v) HEPES, 0.04% (w/v) Na₂HPO₄ adjusted to pH 7.12) dropwise whilst vortexing. The precipitate was allowed to form for 30 minutes at room temperature before a final vortex and addition of the 500μl of the precipitate to the cells. The cells were then returned to the incubator for 5 hours. After 5 hours the cells were washed in pre-warmed HBSS hanks buffer and replaced in pre-warmed growth medium and incubated for 48 hours.

Fugene- ND7 cells were plated out at 1x10⁵ in 6 well plates the day before transfection. The next morning the growth medium was replaced with fresh pre-warmed DMEM plus 10% FCS. 3 μg of total DNA was then diluted in 100 μl of DMEM without FCS. 6 μl of Fugene was added and gently mixed before being left to incubate for 30 minutes at room temperature to allow the DNA-fugene complex to form. The DNA-fugene complex was then added to the cells drop wise and the cells were incubated for 5 hours. After this period the cells are washed with HBSS buffer and replaced with 2 mls of full growth medium and incubated for 48 hours. Transfections were also carried out in 10 cm plates when extracting protein for co-immunoprecipitation experiments or detecting expression of endogenous protein. Transfections were carried out as before except ND7 were plated out one day before transfection at 5x10⁵ in 10cm plates. DNA:Fugene ratio was 4 μg :9 μl.

Lipofectimine (Invitrogen) was used to transfect SAOS-2 cells plated out at 0.5x10⁵ in 6 well plates the day before transfection. The next morning the growth medium was replaced with fresh pre-warmed DMEM without FCS. Briefly 3 μg of total DNA was diluted in 100 μl of DMEM without FCS. 6 μl of Lipofectimine was diluted in 100 μl of DMEM without FCS and left to incubate for 5 minutes before being combined with the diluted DNA. The combined diluted DNA and diluted Lipofectimine were incubated for 20 minutes at room temperature to allow the DNA-liposome complex to form. The DNA-liposome complex was then added to the cells drop wise and the cells were incubated for 5 hours. After this period the cells are washed with HBSS buffer and replaced with 2 mls of growth medium and incubated for 48 hours.
2.7.6 Luciferase assays

After 48 hours, transfected cells were washed in ice cold PBS and lysed in 1 x passive lysis buffer (Promega), 100 μl were used for 6 wells, 500 μl for 10 cm plates. The plates were incubated on a shaker for 15 minutes and the lysate were transferred to 1.5ml micro centrifuge tubes and centrifuged at 13 000 rpm for 1 minute to pellet cell debris and supernatant was collected into a fresh eppendorfs. 30 μl from each lysate was used to measure firefly and Renilla luciferase activities. Both luciferase assays were quantified using a commercially available kit (Promega) and a TD-20e Luminometer. Values for firefly luciferase were divided by their corresponding Renilla luciferase values to obtain relative luciferase units (RLU).

2.7.7 FACS analysis

2.7.7.1 Propidium Iodide (PI) staining for cell cycle analysis

ND7 cells were transfected with the appropriate DNA plasmids together with 100 ng of GFP-spectrin expression vector to mark transfected cells. Cells were then cultured for 30 hours, scraped into the media, pelleted and washed in PBS. The pellet was resuspended in 100 μl PBS and 1ml of ice cold ethanol was added to the samples dropwise whilst vortexing and stored at -20 until the day of analysis. At that time cells were pelleted and resuspended in 300 μl of staining solution (50 μl/ml of PI (Roche), 100 μl/ml RNaseA in PI lysis buffer (250 mg sodium citrate and 0.1% triton x100) and incubated for 15 minutes at 37° C prior to analysis for DNA content. Flow cytometric analysis for GFP and PI fluorescence was performed using a 4-colour Epics XL flow cyclemeter (Beckman) equipped with an argon laser. For each analysis at least 30,000 events were collected to permit cell cycle analysis of both GFP (+) and GFP(-) cell populations. A 525 nm long pass filter was used to collect GFP emission and 675 nm long pass filter was used to detect PI. Data analysis was preformed using Expo32 software (Beckman, High Wycombe) in the first instance. The cell cycle of the GFP/PI stained cells was further analysed by the Multicycle software (Phoenix Flow Systems, San Diego) which is more accurate as it mathematically models cell cycle data by deconstructing linear histograms obtained by the Expo32 software into normal distribution. The set up
conditions to collect the cell cycle data was carried out by Jo Buddie (UCL, London)

2.7.7.2 Annexin V-PE staining for determination of apoptosis

ND7 cells were transfected with the appropriate DNA plasmids together with 100 ng of GFP-spectrin expression vector to mark transfected cells. Cells were then cultured for 48 hours, scraped into the media, pelleted and washed in PBS. The pellet was resuspended in 100 µl of 1 X Annexin buffer (BD Pharmingen; 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂), 3 µl of Annexin V-PE (BD Pharmingen) and 5 µl of 7-Amino-actinomycin (7-AAD) (BD Pharmingen). The solution was incubated for 15 minutes in the dark at room temperature. 300 µl of annexin buffer was then added to each sample and the cells were immediately analysed using a 4-colour Epics XL flow cytemeter (Beckman) equipped with an argon laser. For each analysis at least 30,000 events were collected to permit cell cycle analysis of both GFP (+) and GFP(-) cell populations. A 525 nm long pass filter was used to collect GFP emission and a 675 nm and 650 nm long pass filter was used to detect annexin V-PE and 7-AAD respectively. The set up conditions to collect the cell cycle data was carried out by Jo Buddie (UCL, London). Annexin V-PE negative and 7-AAD negative identified live cells, Annexin V-PE positive and 7-AAD negative identified early apoptotic cells and annexin V-PE positive and 7-AAD positive identified necrotic cells. Finally annexin V-PE negative, 7-AAD positive represented nuclear debris.

2.8 Electrophoretic Mobility Shift Assay (EMSA)

EMSA is a technique used to study protein-DNA interactions to determine if a particular protein binds to a specific DNA regulatory sequence. This assay is performed by incubating cellular extracts or in vitro translated protein with a Radiolabelled oligonucleotide that contains the putative binding site. The complex is then analyzed on a non-denaturing PAGE gel. Proteins that are bound to the labeled oligonucleotide will be visible by autoradiography. To detect for specificity, addition of an antibody that recognizes the protein in the DNA complex will result in a supershifted band.
2.8.1 Nuclei extract from cells

Nuclear protein was extracted from IMR-32 cells over-expressing Brn-3a and expressing wild type p53. IMR-32 cells were scraped from 10 cm plates in ice cold PBS. After centrifugation the cell pellet was resuspended in cold buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and cells were allowed to swell on ice for 10 minutes before vortexing and centrifugation at 700 rpm for 5 minutes at 4° C to pellet nuclei. Nuclei were resuspended in cold buffer C (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF), lysed for 10 minutes on ice and centrifuged at 13,000 rpm for 10 minutes at 4° C to pellet nuclear debris. Protein concentration of the extract was determined by Bradford assay. 5 µg of the extract was used in the bandshift reactions.

2.8.2 Labeling of the oligonucleotide probe

Single stranded oligonucleotides corresponding to the Noxa promoter containing the p53 response element (section 2.1.6) were reconstituted in dd-H$_2$O at 160 pmoles. Equimolar amounts of complementary oligonucleotides were annealed by adding commercially available buffer C (Promega), heating to 90° C for 3 minutes and leaving at room temperature for 1 hour to allow annealing to take place. The double stranded oligonucleotide was diluted to the working concentration of 16 pmoles. End labelling of the double stranded probe was carried out using T4 kinase (Promega) which labels the 5'-OH ends. Typically 16 pmoles of the double stranded probe was incubated with 20 µCi [$^{32}$P] -γATP, 1 X T4 buffer (70 mM Tris-Hcl (pH 7.6), 10 mM MgCl$_2$, 5 mM DTT) and 1 µl (10 units) of T4 polynucleotide kinase and incubated at 37° C for 30 minutes. The 50 µl reaction was increased to 100 µl by addition of dd-H$_2$O and excess unlabelled nucleotides removed by passing the sample through a micro-spin™ G-25 column (Amersham). The labelling efficiency was measured using a Geiger counter, comparing the elute reading value to the radioactivity left in the column. The final volume should be approximately 100 µl containing 160 fmoles of the labelled probe. The labelled probe was stored at -20.
2.8.3 Gel retardation assay

Analysis of protein binding to the labeled oligonucleotide corresponding to the \textit{noxa} promoter containing the p53 binding site was preformed using a modified method described by Gasgoyne and colleagues 2003. 5 μg (~ 1 μl) of IMR-32 cellular extract was added to 10 μl of 2 X EMSA buffer (20 mM HEPES pH 7.9, 5 mM MgCl₂, 50 mM KCl, 0.1 mM EDTA, 15 % glycerol, 1 mM DTT and 5 ng/μl Poly(dl-dC)-Amersham). For competition experiments, 16 pmoles or 32 pmoles of cold oligonucleotide was added to the appropriate tubes resulting in 50 or 100 fold molar excess of specific competitor. 32 pmoles (100 X excess) of unlabelled oligonucleotide corresponding to the estrogen receptor element (ERE) was used as a non-specific competitor. An oligonucleotide known to bind the Brn-3a protein was used to test whether this protein was in the complex and whether binding to its site could compete with binding to the probe. 1 μl of p53 PAb (Oncogene), actin PAb (Santa Cruz Biotechnology) or Brn-3a Mab (Chemicon) were also used to test whether the p53 protein bound to the p53 element in the noxa promoter and whether Brn-3a was in this complex. In all cases dd-H₂O was added to give a final volume of 18 μl for each reaction mixture before adding 2 μl of the labelled probe (320 fmoles) per tube. The reaction mixture was then incubated on ice for 1 hour. Protein-DNA complexes were separated from free probe by electrophoresis carried out on a pre-run 7 % non-denaturing polyacrylamide gel run in 0.5 % TBE, at 220 volts for 2 hours at 4° C. Gels were carefully removed from the running apparatus by the removal of one glass plate and attaching 2 sheets of 3MM paper to the gel and carefully peeling off, and covering with cling film. The gels were dried at 80° C for 1 hour and exposed to X-ray film overnight at 80° C.

2.9 Chromatin Immunoprecipitation (ChIP) assay

This assay was performed to test whether Brn-3a could be immunoprecipitated when bound to p53 on the wild type \textit{noxa} promoter but not on the \textit{noxa} promoter in which the p53 site has been deleted in intact cells. ND7 cells were co-transfected in 10 cm plates with the wild type or mutant promoter together with p53 and Brn-3a. One set of transfections were repeated 4 times so that 4 X 10 cm plates were harvested into one tube. 48 hours after transfection cross linking was performed by adding 270 μl of 37% formaldehyde in 10 ml of medium to the 10 cm plates and incubating the cells for 15 minutes at 37° C wrapped in parafilm. Cells
were then washed 2 X in ice cold PBS before scraping the cells into 1ml of PBS which was transferred into a 50 ml falcon (4 X 10 cm plates were harvested per 50 ml falcon tube). The cells were centrifuged at 700 rpm for 5 minutes and the supernatant was removed. Each cell pellet was lysed in 400 μl of lysis buffer (1 % SDS, 10 mM EDTA, 50 mM Tris HCL pH 8.0 and 1 X protease inhibitor) and incubated on ice for 10 minutes. The lysates were then sonicated for 3 X 10 seconds at 7 micron to shear the DNA into 500 to 1 kb fragments. The supernatant was transferred to 1.5 microcentrifuge tubes and centrifuged at 13,000 for 10 minutes at 4° C and the supernatant was removed into a fresh cold Eppendorf. 40 μl of the supernatant was removed and added to 160 μl of IP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM ETDA, 150 mM NaCl, 20 mM Tris HCL pH 8.0 and 1 X protease inhibitor). This is used as the input sample for subsequent PCR analysis. The remaining 360 μl lysate was then divided into two eppendorfs and 720 μl of IP dilution buffer was added to each 180 μl of lysate. 5 μg of secondary anti-mouse IgG was added to one tube while 5 μg of Bm-3a MAb (Chemicon) was added to the other tube, and the lysates were incubated over night at 4° C with rotation. Incubation with 20 μl of protein G-Sepharose bead for 1 hour at 4° C allowed immobilization of the protein-antibody complex. The beads were pelleted at 1000 rpm for 1 minute and washed in buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM ETDA, 10 mM Tris HCL pH 8.0, 150 mM NaCl), pelleted as above, and followed by a wash in 1 ml of buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM ETDA, 10 mM Tris HCL pH 8.0, 0.5 M NaCl), followed by a final wash in buffer 3 (2.5 M LiCl, 1% Igepal, 200 mg Deoxycholic acid, 1 mM ETDA, 2 mM Tris HCL pH 8.0) and centrifugation at 1000 rpm for 1 minute to pellet the beads. The supernatant was discarded and the beads were then vortexed briefly in 250 μl of freshly prepared elution buffer (1% SDS in 0.1 M NaCO₃), and incubated for 15 minutes at room temperature with rotation. The beads were then pelleted and the supernatant was transferred to a fresh tube. A further 250 μl of elution buffer was added to the beads and incubated for 15 minutes with rotation. The beads were centrifuged and the 250 μl elute was combined with the first elute. 20 μl of 5 M NaCl was added to each elute and the samples, together with the input were incubated at 65° C for 4 hours to reverse cross link between the DNA and protein. 10 μl of 0.5 M EDTA + 20 μl of 1 M Tris pH 6.8 and 20 μl of 1 mg/ml proteinase K was added to the sample elutes and 20 μl of 1mg/ml proteinase K was added to the input samples followed by incubation at 45° C for 1 hour to digest away the protein. The volume of the input was made up to 500 μl with dd-H₂O to match the volume of the
sample elutes. All samples were extracted with 250 μl phenol/250 μl chloroform followed by extraction with 500 μl of chloroform to remove any remaining traces of protein. To visualise pellet of DNA upon precipitation, 20 μg of glycogen was added to each sample before addition of 1 ml of ice cold ethanol and incubation at -20 for 1 hour. The samples were then centrifuged at 13,000 for 10 minutes at 4 °C. The supernatant was removed and the pellet was air dried. The input samples were resuspended in 75 μl of TE and the test samples were resuspended in 30 μl of TE. 0.5 μl and 1.5 μl of the input and test sample DNA were used for the PCR reactions respectively.

The 152 bp product of the noxa promoter containing the p53 site was amplified with NoxaChip-f and NoxaChip-r primers shown in section 2.1.6. Together with the template each PCR contained 1 unit of Taq polymerase (Promega) in a final concentration of 25 μl (1 X Mg-free buffer, 2.5 mM MgCl₂, 1 mM dNTP mix, 100 ng of each primer) and subjected to the following cycling parameter: After 1 cycle at 94° C for 3 minutes, the samples underwent 25 cycles of denaturation at 94° C for 30 seconds, annealing at 60° C for 30 seconds and amplification at 72° C for 30 seconds followed by 1 cycle of 72° C for 5 minutes. The PCR products were resolved on a 2.5% agarose gel.

2.10 Statistical analysis

Data points were compared using the Students T-test analysis and the level of significance of the test is given as a p-value in the text. Statistical help was provided by Dr. V. Budhram-Mahadeo.
The Brn-3a transcription factor physically interacts with the different p73 isoforms and differentially regulates p73 mediated transcription to determine cell fate.

3.1 Introduction

The Brn-3a transcription factor is required for the survival and differentiation of sensory neurones during development. In particular, Brn-3a knock out mice demonstrate significant loss of specific sensory neurones during development, and these mutants die soon after birth (McEvilly et al., 1996). Interestingly, cells that would normally express Brn-3a are specified but failed to survive because of a wave of apoptosis in later development. This suggests that the progenitor cells are present, but once they are committed to their developmental fate, Brn-3a is necessary for survival and differentiation.

Brn-3a can directly transactivate genes associated with neuronal survival such as Bcl-2 and Bcl-xL and neuronal differentiation such as Trk receptors, the neurofilament proteins, α-internexin and Snap-25 (Budhram-Mahadeo et al., 1995b; Smith et al., 1997b; Smith et al., 1998; Huang et al., 1999; Sugars et al., 2001) as well as regulating gene expression associated with cell fate, indirectly via the interaction with p53 (Budhram-Mahadeo et al., 1999; Sugars et al., 2001; Perez-Sanchez et al., 2002; Budram-Mahadeo et al., 2002). The ability of Brn-3a to alter p53 mediated cell fate is interesting as it is the first protein shown to decrease apoptosis but increase cell cycle arrest function of the p53 protein.

p73 is a recently discovered member of the p53 family which can induce cell cycle arrest and apoptosis in cells irrespective of their p53 status (Kaghad et al., 1997; Jost et al., 1997; De, V et al., 2000). Despite the structural and functional similarities between p53 and p73, p73 knock out mice do not develop spontaneous tumours but show defects in neuronal development (Yang et al., 2000). The striking difference in function between p73 and p53 is underscored by the fact that p73 shows much greater molecular complexity than p53 as it is expressed as multiple alternatively spliced C-terminal and N-terminal isoforms (TAp73α-ζ and ΔNp73α-β) (Kaghad et al., 1997; De, V et al., 1998a; Ueda et al., 1999) (Please refer to
introduction sections 1.29, 1.30 and 1.31). Although the physiological functions of these different p73 isoforms are still unknown, they have different transcriptional and biological properties (Kaghad et al., 1997; Jost et al., 1997; Zhu et al., 1998; De, V et al., 1998b; Ueda et al., 1999). Furthermore, the ΔNp73 isoforms which lack the N-terminal transactivation domain are dominant negative proteins that show reciprocal function to the TA isoforms and p53 (Pozniak et al., 2000).

The ability of Brn-3a to modulate the activity of p53 on promoters associated with cell fate may be an important mechanism by which this transcription factor can help to determine survival and differentiation of neuronal cells that co-express both these proteins. The importance of p73 in neuronal development as shown by the phenotype of the p73 null mice, raised the possibility of a functional interaction between the Brn-3a and p73 proteins in specific cells that result in the modulation of cell fate. Thus the interaction and functional effects of this interaction between Brn-3a and the p73 proteins were investigated.

3.2 Brn-3a and Brn-3b specifically binds to the different p73 isoforms

In order to determine if Brn-3a and Brn-3b proteins interact physically with the p73 proteins, \textit{in vitro} interaction experiments ("GST pull-down" chromatography) were performed. Initial studies were carried out using the p73\textalpha{} and p73\textbeta{} isoforms. Fusion proteins were prepared for Brn-3a(l), Brn-3a(s), Brn-3b(l) and Brn-3b(s) using constructs in which the cDNA encoding each protein was cloned into the pGEX or pDEST15 vector downstream of a GST sequence. GST alone was also prepared and used as a negative control for all the interaction studies. The fusion proteins were expressed as described in materials and methods, immobilised on sepharose beads and incubated with \textit{in vitro} translated (IVT) \textsuperscript{35}S-labeled p73\textalpha{} and p73\textbeta{} proteins. \textit{In vitro} translated p53 was used as a positive control. Following washes to remove unbound proteins, the bound complexes were then resolved on a 10\% SDS polyacrylamide gel (see methods). Radiolabelled p73 proteins retained by the Brn-3-GST proteins were detected after the gel was vacuum dried and exposed to film. The amounts of protein retained following the interaction studies were assessed by comparing the intensity of the bands with 10\% input on the same gel using scanning densitometry. The results show the relative
affinity of binding of the Bm-3 family of proteins and the p53 family of proteins

As shown in Fig 3.1, IVT p73α and p73β proteins readily interacted with Bm-3a(l) (Fig 3.1a) and Bm-3a(s) (Fig 3.1b). Similarly these proteins also readily interacted with the Bm-3b proteins, Bm-3b(l) (Fig 3.1c) and Bm-3b(s) (Fig 3.1d). No interaction was observed between IVT p73α and p73β proteins with GST alone confirming that the interaction with the Bm-3 proteins was specific and significant. p53 was included in these experiments as a positive control and as expected this protein did interact with both Bm-3a and Bm-3b. In contrast the labelled luciferase control protein failed to interact with any of the Bm-3-GST fusion proteins or with GST alone, once again confirming the specificity of the interaction. Each of the “pull-down” experiments were repeated in three independent experiments and the scanned results are represented in graphical form in Fig 3.1e. These results show that while the Bm-3 proteins interacted with the p73 proteins, the strength of the interaction differed between the Bm-3 proteins and the different p73 proteins. The longer isoforms of Bm-3a and Bm-3b interacted with higher relative affinity with all of the p73 isoforms when compared with the shorter isoforms of Bm-3a and Bm-3b (for example, Bm-3a(l) and Bm-3b(l) retained 23% and 31% of p73β respectively, while Bm-3a(s) and Bm-3b(s) retained only 9% and 3% of p73β respectively). Further analysis also showed that the p73 proteins appear to interact with Bm-3a and Bm-3b more strongly than p53, with p73β consistently showing stronger association with the Bm-3 proteins. This suggests that p73β binds the Bm-3 proteins with the greatest affinity. The difference between the p73α and p73β isoforms lies in the C-terminal sequence. The p73β isoform arises from the alternative splicing of exon 13 in the mRNA which results in a shorter protein. Apart from the last 5 amino acids which are unique to p73β, the rest of the protein is identical to the corresponding regions in p73α. This suggests that the longer C-terminal sequence specific to p73α may reduce p73 binding to the Bm-3 proteins (please see later).

As p53 and p73 proteins can bind to DNA, it was necessary to identify if contaminating nucleic acid in these assays might mediate interaction with the Bm-3 proteins. In order to assess this, the interaction studies were repeated in the absence or presence of ethidium bromide (EtBr). The EtBr was added in excess to the samples to sequester contaminating nucleic acid. Importantly the interaction between Brn-3 and p53/p73 was maintained in the
Figure 3.1: Affinity chromatography “pull-down” assay used to test for interaction of *in vitro* translated $^{35}$S-labeled p53, p73α and p73β with Brn3a(l)-GST, Brn3a(s)-GST and GST alone.

a) lanes 1-4 shows 1/10 of the input protein used in the “pull-down” assays, lanes 5-8 shows proteins retained following incubation with Brn3a(l)/GST fusion proteins. While the IVT control protein, luciferase was not retained in a complex with Brn-3a(l), the positive control p53 as well as p73α and p73β proteins were associated with Brn-3a(l). This effect is specific to Brm-3a proteins as the GST moiety on its own failed to bind to p53, p73α or p73β (lanes 9-12) b) Similar to Brn-3a(l), Brn-3a(s) could also interact specifically with p53 (lane 6) and p73α and p73β (lanes 7,8) but not the negative control (lane 5).
Figure 3.1 continued: Affinity chromatography "pull-down" assay used to test for interaction of in vitro translated 35S-labeled p53, p73α and p73β with Brn3b(l)-GST, Brn3b(s)-GST and GST alone.

c) lanes 5-8 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-4 shows proteins retained following incubation with Brn3b(l)/GST fusion proteins. While the IVT control protein, luciferase was not retained in a complex with Brn-3b(l), the positive control p53 as well as p73α and p73β proteins were associated with Brn-3b(l). This effect is specific to Brn-3b proteins as the GST moiety on its own failed to bind to p53, p73α or p73β (lanes 9-12). b) Similar to Brn-3b(l), Brn-3b(s) could also interact specifically with p53 (lane 2) and p73α and p73β (lanes 3,4) but not the negative control (lane 1).
Figure 3.1 continued: e) Interaction of Brn-3a and p73 proteins as measured by percentage of \(^{35}\)S labeled in vitro translated p53, p73α and p73β retained by Brn3-GST proteins. Densitometer scans of "pull-down" assays expressed as a percentage of the input of \(^{35}\)S labeled IVT p53 and p73 proteins retained by Brn-3a(l), Brn-3b(l), Brn-3a(s) and Brn-3b(s) in the same experiment. Data is the average of 3 independent experiments with standard error shown. f) GST pull-down assays demonstrating interaction of p53, p73α and p73β with Brn-3a(l) in the absence/presence of EtBr. Assays were preformed as before but in the absence (-) or presence (+) of 50 μg ethidium bromide per sample. Interactions were observed in the presence of ethidium bromide although interaction of p53, p73α and p73β with Brn-3a(l) were reduced in the presence of ethidium bromide.
presence of EtBr but at slightly reduced levels (Fig 3.1f), indicating that binding of these proteins to Brn-3a may be strengthened by nucleic acid.

3.3 ΔNp73 which lacks the N-terminal TA domain also interacts with Brn-3

In addition to the alternatively spliced C-terminal isomeric forms of p73 that contain the TA domain, the p73 gene can also be transcribed from a second downstream promoter, spanning exon 3, producing the ΔNp73 isoforms that lack the transactivation domain and starts with an alternative exon (exon 3′) (Yang et al., 2000; Ishimoto et al., 2002) (please refer to sections 1.29 and 1.31). To assess whether ΔNp73α could also interact with Brn-3a to the same extent as the TA isoforms already tested (p73α and p73β), similar pull-down assays were performed using Brn-3a(l)-GST and Brn-3a(s)-GST fusion proteins with IVT ΔNp73α. IVT p73α and p73β were included as positive controls. As shown in Fig 3.2 ΔNp73α readily interacts with the Brn-3a proteins, with 10-20% of protein being retained in this assay. Therefore, it appears that ΔNp73α also interacts with the long and short forms of Brn-3a in a manner similar to that seen with the TA isoforms. Additionally, interaction of ΔNp73α with Brn-3a confirms that the interaction is not mediated via the TA domain of p73.

3.4 p73 interacts with the POU domain of Brn-3a

In order to determine the domain(s) of Brn-3a that are required for interaction with p73, similar experiments were carried out using constructs containing the Brn-3a POU domain only and the N-terminal activation domain only illustrated in Fig 3.3c. The interactions were compared with Brn-3a(l)-GST and Brn-3a(s)-GST proteins. As expected, full length Brn-3a associates more strongly with the p73 isoforms (p73α, p73β and ΔNp73α) than Brn-3a(s) (Fig 3.3a and Fig 3.3b) and ΔNp73β is retained the most by full length Brn-3a. All the p73 isoforms bound very weakly to the N-terminus of Brn-3a suggesting that this region on its own is not sufficient for the strong interaction with p73. In contrast, the isolated POU domain interacted with all the p73 isoforms with similar affinity to that observed with Brn-3a(l). This suggests that the interaction between Brn-3a and p73 is primarily via the Brn-3a POU
Figure 3.2: Affinity chromatography "pull-down" assays showing ΔNp73α isoform interacts with the Brn3a-GST proteins with similar affinity to TAp73.

a) lanes 10-12 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-2, 4-5, 7-8 shows p73α, p73β and ΔNp73α proteins retained by Brn3a(l)/GST and Brn3a(s)/GST fusion proteins. This effect is specific to Brn-3a proteins as the GST moiety on its own failed to bind to p73α, p73β or ΔNp73α (lanes 3, 6, 9). In vitro translation of ΔNp73α shows a double band due to use of two different ATGs.

b) Densitometer scans of "pull-down" assays expressed as a percentage of the input of 35S labelled IVT p73 proteins retained by Brn-3a(l), and Brn-3a(s) in the same experiment. Data is the average of 3 independent experiments with standard error shown.
Interaction between the p73 proteins and Brn-3a domains

Figure 3.3: The isolated Brn-3a POU domain but not the isolated N-terminal domain is sufficient for interaction with the p73 proteins.

a) Lanes 16-18 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-4, 6-9 and 11-14 shows p73α, p73β and ΔNp73α proteins respectively retained following incubation with the indicated Brn-3a-GST fusion proteins. The Brn-3a isolated POU domain interacts with the p73 proteins with similar affinity to Brn-3α(i) (compare lanes 1 and 3, for p73α, lanes 6 and 8 for p73β and lanes 11 and 13 for interaction with ΔNp73α). The isolated N-terminal domain binds weakly to p73α (lane 4), p73β (lane 9) and ΔNp73α (lane 14). This effect is specific to Brn-3a proteins as the GST moiety on its own failed to bind to p73α, p73β or ΔNp73α (lanes 5, 10, 15). b) Densitometer scans of "pull-down" assays expressed as a percentage of the input of 35S labeled IVT p73 proteins retained by Brn-3α(i), Brn-3α(s), and the isolated Brn-3a POUS and N-terminal GST fusion proteins. Data is the average of 3 independent experiments with standard error shown. c) GST fusion proteins containing Brn-3a domains were purified from Escherichia coli using glutathione beads. Domains are indicated I-IV, homologous N-terminal activation domain is shaded, C-terminal activation domain are shown striped.
domain but the N-terminal domain must contribute as Brn-3a(l) is better than Brn-3a(s) in retaining the p73 proteins.

3.5 The p73 oligomerization domain (OD) is critical for the Brn-3/p73 interaction and this interaction is modulated by the p73 extended C-terminal

To precisely define the region(s) of the p73 protein that are required for the interaction with the Brn-3 proteins, GST pull-down assays were carried out using in vitro translated proteins (shown in Fig 3.4d), transcribed from constructs in which specific regions of p73α were deleted and cloned into the pCDNA vector (constructs kindly provided by K. Shimotohno, Institute of Viral Research, Kyoto University). The deletion constructs either have N-terminal or C-terminal deletions of p73α. The p73N56 construct lacks the first 56 amino acids resulting in a protein that lacks the entire N terminal transactivation domain. The p73N315 polypeptide lacks the first 315 amino acids resulting in a protein lacking the TA and DBD domains. p73N423 lacks the sequences encoding the first 423 amino acid sequence resulting in a C-terminal protein that lacks the TA, DBD and the OD domain. The C-terminal deletion constructs, p73C505 and p73C424, lacking either the last 132 or 213 amino acid sequences respectively, result in proteins lacking the extreme C-terminal region but both retaining the OD domain. In contrast p73βdelOD is a derivative of p73β in which the sequences for the OD domain was deleted resulting in a protein that lacks the OD domain only.

In vitro translated 35S-labeled truncated p73 proteins with appropriate deletions were incubated with either Brn-3a(l) or the Brn-3a isolated POU domain linked to GST using the methods described. The isolated Brn-3a POU domain was included as it has been previously shown to be sufficient to interact with the p73 proteins. As shown in Fig 3.4a and Fig 3.4b and graphically represented in Fig 3.4c, full length p73α interacts with Brn-3(l) and the Brn-3a POU domain with the same affinity as seen previously. The deletion protein lacking the amino terminal transactivation domain p73N56 also binds Brn-3a(l) and the isolated POU domain to a similar extent shown with full length p73α. This deletion protein is similar to the truncated isoform ΔNp73α which has been previously shown to bind to both Brn-3a(l) and
Fig 3.4c

Interaction of Brn-3a(l)/POU with p73 deletion proteins

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Brn-3a(l) | Brn-3aPOU

Figure 3.4: The p73 region containing the oligomerization domain (OD) is critical for the Brn-3a/p73 interaction.

a) Affinity chromatography "pull-down" assays showing interaction between in vitro translated p73 deletion proteins with Brn3a(l)-GST proteins and GST alone. Lanes 9-16 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-8 shows proteins retained following incubation with Brn-3a(l)/GST fusion proteins. While the luciferase control protein was not retained in a complex with Brn-3a(l) (lane 1), the positive control p73a (lane 2) and to varying extents the deletion proteins (lanes 3-8) were associated with Brn-3a(l). Deletion proteins p73N423 and p73pdel OD both of which lack the OD domain fail to bind significantly to Brn-3a(l) whilst the deletion proteins p73C505 and p73C424 show increased binding to Brn-3a(l) compared to p73a. This effect is specific to Brn-3a proteins as the GST moiety on its own failed to bind to p73a or the deletion proteins (lanes 17-24) b) Similar to Brn-3a(l), Brm-3a isolated POU domain could also interact specifically with p73a (lane 2) and to varying extents the deletion proteins (lanes 3-8) but not the negative control (lane 1). Again, the deletion proteins p73N423 and p73pdel OD both of which lack the OD domain fail to bind significantly to the isolated Brn-3a POU domain whilst the deletion proteins p73C505 and p73C424 show increased binding to the Brn-3a POU domain compared to p73a. c) Graphical representation of densitometer scans of "pull-down" assays expressed as a percentage of the input of 35S labeled IVT p73 proteins retained by Brn-3a(l) and the Brn-3a isolated POU domain. Data is the average of 3 independent experiments with standard error shown. d) A schematic representation of the p73 deletion constructs of p73a and Brm-3aPOU domain to map the region required for interaction with the Brn-3 proteins. The indicated TAD, DBD and OD refer to the transactivation domain, DNA-binding domain and oligomerization domains respectively. The numbers above the boxes denotes the amino acid positions in the wild-type p73a and Brm-3aPOU domain.
the isolated POU domain.

Interaction of the polypeptide, p73N315 (which lacks the DBD) with Brn-3a(l) appears to be similar to that of p73α, whilst interaction of p73N315 and the isolated Brn-3a POU domain is compromised compared to that observed with full length p73α. This suggests that full length Brn-3a, in its natural conformation can continue to interact with the p73N315 truncated protein. However the isolated POU domain requires binding to the DBD (as seen with p53). These results imply that full length Brn-3a might make some additional contacts with the p73 protein lacking the DNA binding domain compared with the isolated POU domain. This may be accomplished by a) binding of full length Brn-3a might affect the conformation of the p73 protein in order to increase affinity for p73 binding or b) that other domains in Brn-3a might make contact with the p73 protein lacking the DBD to retain binding.

Interestingly, the p73N423 C-terminal protein fails to bind significantly to either Brn-3a(l) or the isolated POU domain. The only difference between p73N423 which fails to bind Brn-3a and p73N315 which still binds to Brn-3a, is that p73N423 lacks the OD domain. Furthermore the p73β protein that lacks the OD domain only, (p73βdelOD) also fails to interact with Brn-3a(l) or the isolated POU domain. Since it was previously shown that p73β can interact very strongly with Brn-3a(l) and the isolated POU domain, these results suggest that the region containing the OD domain is critical for the p73/Brn-3a interaction. Since p73 polypeptides lacking the OD domain can no longer form tetramers, it can be argued that Brn-3a can either directly contact points within the OD domain or is incapable of binding to monomers of p73.

The deletion proteins p73C505 and p73C424, both of which contain the OD domain but lack 132 or 213 amino acids respectively from the C-terminal, continue to bind to the Brn-3a(l) and the isolated POU domain, but with greater affinity than wild-type p73α. The binding affinity of these two deletion constructs to Brn-3a is similar to that found with p73β, which lacks the 216 amino acid C-terminal region encoded by codons 421-636 found in p73α due to splicing out of exon 13 in the p73β mRNA. This further suggests that sequences within the C-terminal region specific to p73α may act to reduce its association with both Brn-3a(l) and the isolated POU domain.

Similarly the p73 deletion proteins showed a similar binding pattern with Brn-3a(s) as they
did with Brn-3a(l) (data not shown) in which p73N56 and p73N315 bound Brn-3a(s) with similar affinity to p73a. The truncated proteins p73N423 and p73βdelOD failed to bind Brn-3a(s) whilst the p73 C-terminal deleted polypeptides p73C505 and p73C424 interacted with Brn-3a(a) with stronger affinity than p73a. In addition Brn-3a(s) interacted with the p73 polypeptides with weaker affinity compared to the interaction of these polypeptides with Brn-3a(l).

These results demonstrate that Brn-3a interacts with p73 and this interaction is mediated mainly via the POU domain of Brn-3a and the OD domain of p73. The DBD of p73 also contributes by contacting the POU domain of Brn-3a, in a manner similar to that seen with p53. Furthermore, this interaction is modulated by the C-terminal region of p73. This is interesting, as it is possible that Brn-3a may differentially regulate the functions of the different C-terminal isomeric forms of p73, as a direct effect of different affinities of interaction provided by the variation in the p73 C-terminal region.

### 3.5.1 Interaction of Brn-3b with the p73 truncated proteins

Since Brn-3b was also shown to interact with the p73 proteins, a similar study was carried out to look at which regions of p73 are required to interact with Brn-3b. Using the methods described above, Brn-3b(l) and the isolated Brn-3b POU domain linked to GST were incubated with the 35S labeled p73 deletion proteins (Fig 3.5a and Fig 3.5b and graphically represented in Fig 3.5c). As expected p73a and p73N56 bound strongly to both Brn-3b(l) and the isolated Brn-3b POU domain, suggesting that p73 interaction with Brn-3b is also mediated via the Brn-3b POU domain. As was the case with Brn-3a, the p73N315 polypeptide, appears to interact with Brn-3b(l) to a similar extent to that observed with p73a, whilst interaction of p73N315 and the isolated Brn-3b POU domain is compromised compared to that observed with full length p73a. Again, this suggests that the full length Brn-3b protein in its natural conformation can continue to bind p73N315 (which lacks the DBD) but the isolated Brn-3b POU domain further requires the DBD for strong interaction. Thus, as with Brn-3a, the correct folding of full length Brn-3b is required for strong interaction with p73 lacking the DBD or other domains in Brn-3a might make contact with the p73 protein lacking the DBD to retain binding.
Fig 3.5a

Bm-3b(l)-GST

GST only

Fig 3.5b

Bm-3bPOU-GST

GST only

1/10 input
Interaction of Brn-3b(l) and Brn-3b POU domain with p73 deletion proteins

Figure 3.5: The p73 region containing the oligomerization domain (OD) is critical for the Brn-3b/p73 interaction.

a) Affinity chromatography "pull-down" assays showing interaction between in vitro translated p73 deletion proteins with Brn-3b(l)-GST proteins and GST alone. Lanes 17-24 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-8 shows proteins retained following incubation with Brn-3b(l)/GST fusion proteins. While the luciferase control protein was not retained in a complex with Brn-3b(l) (lane 1), the positive control p73a (lane 2) and to varying extents the deletion proteins (lanes 3-8) were associated with Brn-3b(s). Deletion proteins p73N423 and p73del OD both of which lack the OD domain fail to bind significantly to Brn-3b(l) whilst the deletion proteins p73C505 and p73C424 show increased binding to Brn-3b(l) compared to p73a. This effect is specific to Brn-3b proteins as the GST moiety on its own failed to bind to p73a or the deletion proteins (lanes 9-16) b) Similar to Brn-3b(l), the isolated POU domain from Brn-3b proteins could also interact specifically with p73a (lane 2) and to varying extends the deletion proteins (lanes 3-8) but not the negative control (lane 1). Again, the deletion proteins p73N423 and p73delOD both of which lack the OD domain fail to bind significantly to the isolated Brn-3b POU domain whilst the deletion proteins p73C505 and p73C424 show increased binding to the Brn-3b POU domain compared to p73a. c) Graphical representation of densitometer scans of "pull-down" assays expressed as a percentage of the input of 35S labeled IVT p73 proteins retained by Brn-3b(l) and the isolated Brn-3b POU domain. Data is the average of 3 independent experiments with standard error shown.
As seen with Brn-3a, the p73N423 polypeptide that lacks the amino terminus and the OD domain and p73βdelOD which just lacks the OD domain failed to bind significantly to Brn-3b(l) or the isolated Brn-3b POU domain. This suggests that Brn-3b also interacts with the p73 OD domain. In addition, the polypeptides p73C505 and p73C424 which lack the C-terminal of p73α also show increased binding to Brn-3b(l) and the Brn-3b isolated POU domain compared to that obtained with full length p73α, suggesting that as with Brn-3a, sequences within the p73α specific C-terminal region may inhibit association with the Brn-3b protein. As was the case with the Brn-3a/p73 interaction, it is possible that the different interactions between Brn-3b and the C-terminal isomers of p73 may be a determinant of precise biological outputs.

Similarly the p73 deletion proteins showed a similar binding pattern with Brn-3b(s) as they did with Brn-3b(l) (data not shown) in which p73N56 and p73N315 bound Brn-3b(s) with similar affinity to p73α. The truncated proteins p73N423 and p73βdelOD failed to bind Brn-3b(s) whilst the p73 C-terminal deleted polypeptides p73C505 and p73C424 interacted with Brn-3b(s) with stronger affinity than p73α. In addition Brn-3b(s) interacted with the p73 polypeptides with much weaker affinity compared to Brn-3b(l)

Therefore both isoforms of Brn-3a and Brn-3b interact with p73 via the Brn-3 POU domain and the OD of p73 which is critical for this interaction. Additionally, the DBD of p73 also makes contributions to this association via the POU domain. This interaction is further modulated by the p73 extended C-terminal region.

3.6 Brn-3a interacts with p73 in vivo

In order to examine whether these interactions occur in vivo, in the context of intact cells, co-immunoprecipitation experiments were carried out in ND7 cells transfected with Brn-3a and p73α.

These experiments were carried out with the ΔNp73α isoform as this protein is more stable than TAp73α when transfected into ND7 cells. Also the p73-SAM Ab (which recognizes TAp73α and ΔNp73α only) does not appear to be as good as the p73-ΔN Ab (recognises the ΔNp73 isoform only) for co-immunoprecipitation experiments (these antibodies were a kind
gift from Emre Sayan, University of Leicester). Importantly, since the Brn-3a/p73 interaction is not dependent on the p73 N-terminal but on the common OD domain, it was desirable to use the p73-ΔN Ab to assess in vivo interactions between p73 and Brn-3a.

Therefore, ΔNp73α-containing protein complexes were immunoprecipitated from ND7 cells transfected with Brn-3a and ΔNp73a and analyzed for the presence of Brn-3a by immunoblot analysis. The transfected ND7 cells were also immunoprecipitated with actin pAb antibody control or rabbit IgG. In these experiments, Brn-3a protein was detected in immunoprecipitates pulled down with p73-ΔN pAb antibody but not in immunoprecipitates pulled down with actin pAb antibody or rabbit IgG (Fig 3.6), confirming that p73 interacts with Brn-3a in vivo.

**Figure 3.6: Brn-3a and ΔNp73α interact in vivo**
ND7 cells were transfected with Brn-3a and ΔNp73a and cultured for 48 h. Cell lysates were prepared, and immunoprecipitated with rabbit IgG, anti-Actin antibody or anti-p73 antibody. 10% of the lysate was retained for input sample. After SDS-PAGE expression and efficient purification of ΔNp73α was confirmed by immunoblot with p73-ΔN antibody with detects the ΔNp73 isoform, and presence of Brn-3a in the immunocomplexes were identified by immunoblot for Brn-3a. No Brn-3a was observed in the immunoprecipitate with Rabbit IgG. Similarly no Brn-3a was observed when immunoprecipitation was carried out with actin protein which does not interact with Brn-3a, but is present in immunoprecipitates pulled down with p73 antibody.
3.7 Brn-3a co-operates with TA\textit{p}73\textalpha and TA\textit{p}73\textbeta on activating the \textit{p21\textsuperscript{CIP1/Waf1}} promoter in the ND7 cell line

Brn-3a can induce neuronal differentiation and is associated with growth arrest and increased activity of the promoter that drives expression of the cyclin-dependent kinase inhibitor \textit{p21\textsuperscript{CIP1/Waf1}} gene (Perez-Sanchez et al., 2002; Budram-Mahadeo et al., 2002). p53 exerts its protective effects by inducing cell cycle arrest (with possible DNA repair) or apoptosis which is largely caused by differential transcriptional effects on target genes that control cell cycle (e.g. \textit{p21\textsuperscript{CIP1/Waf1}}) or apoptosis (e.g. \textit{bax, puma, apafl-1, fas}). It has been previously shown that Brn-3a interacts with and enhances p53 mediated activation of the \textit{p21\textsuperscript{CIP1/Waf1}} promoter, suggesting that Brn-3a is a determinant in the decision of p53 mediated cell cycle arrest rather than apoptosis (Perez-Sanchez et al., 2002; Budram-Mahadeo et al., 2002). Similar to p53, p73 can also activate the \textit{p21\textsuperscript{CIP1/Waf1}} promoter and induce cell cycle arrest when exogenously expressed (Kaghad et al., 1997; Levrero et al., 2000). To examine if a similar functional effect is observed upon interaction of Brn-3a with p73, transient co-transfections were carried out using the reporter construct containing the promoter that drives the expression of \textit{p21\textsuperscript{CIP1/Waf1}} cloned upstream of a luciferase reporter gene.

Characterization of Brn-3a as a transcription factor has been mostly investigated in neuronal cells with many studies carried out in the neuronal derived ND7 cell line. This cell line was derived from rat dorsal root ganglion neurons immortalized by fusion with C1300 mouse neuroblastoma cells (Woods et al., 1990). When ND7 cells are induced to differentiate (by serum withdrawal or by addition of defined medium supplemented with growth factors or cyclic AMP analogues) they undergo morphological changes and express biochemical markers mimicking the events associated with differentiation of sensory neurones (Suburo et al., 1992). Brn-3a mRNA and protein levels are low in actively proliferating ND7 cells, but upon differentiation, Brn-3a levels are significantly elevated (Lillycrop et al., 1992; Budhram-Mahadeo et al., 1995a). In contrast expression of Brn-3b is higher in proliferating ND7 cells, whereas Brn-3b levels are dramatically decreased upon differentiation of these cells (Lillycrop et al., 1992; Budhram-Mahadeo et al., 1995a). Thus ND7 cells are ideal to investigate the role of Brn-3a and Brn-3b in neuronal survival and differentiation.

To test whether Brn-3a could modulate p73 mediated activation of the \textit{p21\textsuperscript{CIP1/Waf1}},
increasing concentrations of expression vectors encoding either p73α or p73β (2-20 μg) were co-transfected with the p21^{CIP1/Waf1} reporter plasmid (5 μg) in ND7 cells either in the presence of 5 μg of Brn-3a expression vector or the LTR empty vector control. These transfections were carried out in 10 cm plates using the calcium phosphate method (see methods). Transfection of p53 either alone or with Brn-3a was included as positive controls as earlier studies have shown that Brn-3a could enhance p53 mediated activation of the p21^{CIP1/Waf1} promoter. The transfections resulted in the changes of expression of Brn-3a, p53 and the p73 proteins in the transfected cells as shown by western blot (Fig 3.7d). Unfortunately the p73-Full Ab which detects all p73 isoforms did not work well in western blots. Therefore, to assess for transfected p73 expression, the p73-SAM Ab was used which detects the α isoforms of both TAp73 and ΔNp73. Thus expression of introduced p73β could not be confirmed in these experiments. However, since transfection of p73α and ΔNp73α were shown to be efficiently expressed and the p73β construct were cloned in the same expression vector, this suggests that high levels of p73β would also be expressed in cells transfected with the p73β expression vector (and this was confirmed by the observed effects on target gene expression).

As expected, increasing concentrations of either p73α or p73β results in increased activation of the p21^{CIP1/Waf1} promoter, with p73β being the stronger activator on this promoter (Fig 3.7a and Fig 3.7b). For instance, p73α could activate the p21^{CIP1/Waf1} promoter up to 30 fold, p73β activated this promoter up to 60 fold. However, 20 μg of p73α resulted in reduced activation of the p21^{CIP1/Waf1} promoter suggesting a squelching effect, in which such high concentrations of this protein lead to depletion of essential co-activators, thus resulting in transcriptional repression.

Whilst Brn-3a on its own can mildly activate the p21^{CIP1/Waf1} promoter as previously seen, co-expression of Brn-3a and p73 resulted in increased activation of the p21^{CIP1/Waf1} promoter compared to that observed with either Brn-3a or p73 alone at their specified concentrations. For example, whereas 2 μg of p73α alone resulted in 10 fold activation, co-expression of Brn-3a with 2 μg of p73α resulted in 25 fold activation of the p21^{CIP1/Waf1} promoter (p<0.05). In particular the effect was marked at 20μg of p73α which resulted in 22 fold activation of the p21^{CIP1/Waf1} whilst addition of Brn-3a resulted in 44 fold activation (p<0.005),
Figure 3.7: Brn-3a enhances p73α and p73β activation of the p21\textsuperscript{CIP1/WAF1} promoter in ND7 cells.

Brn-3a alone activated the p21\textsuperscript{CIP1/WAF1} promoter while p73α or p73β alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with a) p73α or b) p73β significantly enhances p73α/p73β mediated activation of the p21\textsuperscript{CIP1/WAF1} promoter. ND7 cells were transfected with 5 μg of the p21\textsuperscript{CIP1/WAF1} promoter luciferase reporter construct with increasing amounts of p73α or β (1-20μg) in the presence of 5 μg of either Brn-3a expression vector or empty vector using the calcium phosphate transfection method. c) ΔNp73α has no effect on p21 promoter activity compared to vector alone. Co-transfection of Brn-3a with ΔNp73 resulted in loss of Brn-3a mediated transactivation of the p21\textsuperscript{CIP1/WAF1} promoter compared with Brn-3a alone. ND7 cells were transfected with 1 μg of p21\textsuperscript{CIP1/WAF1} promoter luciferase reporter construct with increasing amounts of ΔNp73 (50-200ng) in the presence of 1 μg of either Brn-3a expression vector or empty vector using Fugene. All luciferase values were equalized to the activity of a co-transfected renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. d) Brn-3a and/or p53, p73α, p73β, ΔNp73α expression in transiently transfected ND7 cells were analyzed by Western blot after whole cell lysis.
highlighting the importance of this co-factor in stimulating increased expression of this promoter. Similarly, whereas 2 μg of p73β alone resulted in 12 fold activation of the p21\textsuperscript{CIP1/WAF1} promoter, co-expression of Brn-3a with 2 μg of p73β resulted in 26 fold activation of the p21\textsuperscript{CIP1/WAF1} promoter (\(p<0.005\)). However, whereas 20 μg of p73β resulted in a 60 fold activation of the p21\textsuperscript{CIP1/WAF1} promoter, co-expression of Brn-3a with 20 μg of p73β did not further enhance p21\textsuperscript{CIP1/WAF1} promoter activity. This is probably because p73β at this concentration of 20 μg, there is maximal stimulation of this promoter so that co-expression of Brn-3a can not further enhance expression above the level observed with 20 μg of p73β alone. The observation that Brn-3a can still co-operate with p73α and significantly enhance p73α mediated activation of the p21\textsuperscript{CIP1/WAF1} promoter when 20 μg of p73α is transfected is most likely due to the lower activation of p73α alone on this promoter which can still be enhanced upon co-expression of Brn-3a.

Hence, as with p53, Brn-3a can co-operate with the TAp73 isoforms, p73α and p73β to stimulate the activity of the p21\textsuperscript{CIP1/WAF1} promoter.

The effect of ΔNp73α with or without Brn-3a was also tested on the p21\textsuperscript{CIP1/WAF1} promoter. Increasing concentrations of expression vectors encoding ΔNp73α (50-200 ng) were co-transfected with the p21\textsuperscript{CIP1/WAF1} reporter plasmid (1 μg) in ND7 cells either in the presence of 1 μg of empty vector or Brn-3a, in 6 well plates using Fugene (Roche) method of transfection (see methods). This set of transfections and subsequent transfections were carried out with the Fugene reagent as it is more efficient than the calcium phosphate method resulting in an 80% transfection rate rather than 30-40% transfection rate achieved with the calcium phosphate method. Thus lower concentrations of plasmid DNA were used and transfections were carried out in 6 well plates when using the Fugene reagent.

Fig 3.7d shows the expression of ΔNp73α and Brn-3a in transfected cells. As shown in Fig 3.7c ΔNp73α failed to activate the p21\textsuperscript{CIP1/WAF1} promoter, which is expected as ΔNp73α does not contain the N-terminal TA domain required for transactivation. Again Brn-3a was shown to activate the p21\textsuperscript{CIP1/WAF1} promoter by 2.5 fold which is consistent with published data. Furthermore, co-expression of Brn-3a and ΔNp73α appeared to reduce Brn-3a activation of the p21\textsuperscript{CIP1/WAF1} promoter when compared to Brn-3a alone (\(p>0.005\)). Thus while Brn-3a co-
operates with the TA isoforms p73α and p73β to increase $p21^{CIP1/Waf1}$ activation, ΔNp73α acts to decrease Brn-3a mediated activation of this promoter in reporter assays.

3.8 Brn-3a co-operates with TAp73α and TAp73β on activating the $p21^{CIP1/Waf1}$ promoter in the SAOS-2 cell line

In order to determine if the co-operative effect of Brn-3a and TAp73 on the $p21^{CIP1/Waf1}$ is dependent on other neuronal factors, or endogenous p53, the above transfections were repeated in the SAOS-2 cell line which is derived from an osteogenic sarcoma harboring a homozygous deletion of the p53 gene. Additionally this cell line does not express detectable endogenous p73 protein (Kaghad et al., 1997) or Brn-3a (personal communication with C Perez-Sanchez and Fig 3.8b), hence providing a suitable model to look at target gene expression of the p53 family and the functional effect of this expression. In similar co-transfection studies described before, the $p21^{CIP1/Waf1}$ luciferase reporter construct was co-transfected into SAOS-2 cells with increasing amounts of Brn-3α with or without p73α or p73β (Brn-3α:p73 ratios were 1:1, 2:1, 3:1 and 4:1) using Lipofectimine (Invitrogen). As this cell line has no Brn-3α expression, a titration was carried out to ascertain how much Brn-3α was required to affect the promoter activity in the absence or presence of the p73 proteins. Again p53 was included as a positive control. Fig 3.8b shows the expression of the Brn-3α and p53/p73 proteins in the transiently transfected SAOS-2 cells. As expected, both p53 and the p73 proteins strongly activated the $p21^{CIP1/Waf1}$ promoter, whilst Brn-3α alone could also induce the $p21^{CIP1/Waf1}$ promoter, being more effective at higher concentrations (Fig 3.8a). As was the case in the ND7 cells, co-transfection of Brn-3α with either p53 or the p73 proteins resulted in significant enhancement of $p21^{CIP1/Waf1}$ promoter activity compared with either p53 or p73 alone (Fig 3.8a). It is noteworthy that the maximal activation of the p21 promoter upon co-expression of Brn-3α and p73 was when the ratio between Brn-3α and p73α was 1:1 or 2:1 and a ratio of 3:1 between Brn-3α and p73β.

Hence Brn-3α can enhance p53/p73 mediated activation of the $p21^{CIP1/Waf1}$ promoter in both non neuronal (SAOS-2) and neuronal (ND7) cell lines suggesting that the effect of Brn-3α on p53/p73 mediated transcription of the $p21^{CIP1/Waf1}$ promoter does not depend on other neuronal specific co-factors. Also the co-operative effects of Brn-3α and TAp73 on the
a) Effect of Brn-3a and p73α/p73β on p21 promoter activity in SAOS-2 cells

![Graph showing the effect of Brn-3a and p73α/p73β on p21 promoter activity in SAOS-2 cells.]

b) Brn-3a and/or p53, p73α expression in transiently transfected SAOS2 cells were analysed by Western blot after whole cell lysis

**Figure 3.8: Brn-3a enhances p73α and p73β activation of the p21cip1/waf1 promoter in SAOS2 cells**

Brn-3a alone activated the p21cip1/waf1 promoter while p53, p73α or p73β alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with a) p73α or p73β significantly enhances p73α/p73β mediated activation of the p21cip1/waf1 promoter. SAOS-2 cells were transfected with increasing amounts of Brn-3a (1:1, 1:2, 1:3, 1:4 ratio with p73) with 1.5μg of p21cip1/waf1 vector in the presence or absence of 1.5μg of p73α or p73β. All luciferase values were equalised on the basis of the activity observed upon co transfection with a control renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. b) Brn-3a and/or p53, p73α expression in transiently transfected SAOS2 cells were analysed by Western blot after whole cell lysis.
The $p2^1_{\text{CIP1/Waf1}}$ promoter is observed in both a p53 wild type cell line (ND7) and a p53 null cell line (SOAS-2 cells) suggesting that p73 activation of the promoter and co-operative effects of Brm-3a and TAp73 on the $p2^1_{\text{CIP1/Waf1}}$ promoter is independent of p53 expression.

### 3.9 Brm-3a represses TAp73α and TAp73β mediated activation of the pro-apoptotic Bax promoter in the ND7 cell line

Brm-3a also promotes sensory neuronal survival by activating transcription of the anti-apoptotic Bcl-2 and Bcl-xl proteins and inhibiting the basal transcription of the pro-apoptotic $bax$ promoter (Smith et al., 1998; Smith et al., 2001; Ensor et al., 2001; Budram-Mahadeo et al., 2002). The p53 family of proteins induce apoptosis by transactivating pro-apoptotic gene targets such as $bax$. Moreover it has been shown that Brm-3a interacts with wild-type p53 and antagonizes p53 mediated activation of the $bax$ promoter, thus contributing to the survival of sensory neurones conferred by Brm-3a (Budram-Mahadeo et al., 2002).

Thus, in order to test the effect of Brm-3a on the p73 mediated activation of the $bax$ promoter, co-transfection experiments were carried out similar to those undertaken with the $p2^1_{\text{CIP1/Waf1}}$ promoter in the ND7 neuronal cell line. The $bax$ reporter gene was co-transfected with increasing concentrations of expression vectors encoding either p73α or p73β (50-200 ng) in the presence of either 1 μg of Brm-3a expression vector or empty vector control, using Fugene. As before p53 alone or together with Brm-3a was included as a positive control.

Western blots carried out using protein extracts prepared from transfected cells show that the changes observed on the activity of the $bax$ promoter is due to the changes in expression of Brm-3a, p53 and the p73 proteins in the transfected ND7 cells (see section 3.7, Fig 3.7d). As expected, p73α and p73β could strongly activate the $bax$ promoter (Fig 3.9a and 3.9b) compared with empty vector control. Interestingly, both p73 isoforms appeared to activate the $bax$ promoter more strongly than p53, but in contrast to the $p2^1_{\text{CIP1/Waf1}}$ promoter, where p73β was the stronger activator, the p73α isoform was better at transactivating the $bax$ promoter. Thus whilst p73α could transactivate the $bax$ promoter up to 115 fold, p73β induced this promoter by 58 fold. Brm-3a on its own resulted in slight repression of the $bax$ promoter as previously shown while co-expression of Brm-3a with either p73α or p73β
Brn-3a alone slightly repressed bax promoter activity while p73α or p73β alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with a) p73α or b) p73β significantly repressed p73α or p73β mediated activation of the bax promoter. c) ΔNp73α alone failed to activate the bax promoter and co-transfection of Brn-3a with ΔNp73α failed to significantly alter bax activity. All luciferase values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. ND7 cells were transfected with increasing amounts of p73α, p73β or ΔNp73α (50-200ng) with 1μg of bax promoter in the presence or absence of 1μg of Brn-3a using Fugene reagent.
resulted in strong repression of the transactivation observed with either p73α or p73β alone respectively on this promoter. For example, whereas 50 ng of p73α alone resulted in 93 fold activation of the bax promoter, co-expression of Brn-3a with 50 ng of p73α resulted in a 75.8% decrease in p73α mediated activation of this promoter (p<0.005). Similarly, co-expression of Brn-3a with 100 ng or 200ng of p73α resulted in a 65.3% (p<0.005) and 43.6% (p<0.05) decreased activation of the bax promoter compared to the activation observed with 100 ng or 200 ng of p73α alone, respectively. As with p73α, co-expression of Brn-3a with 50 ng, 100 ng or 200 ng of p73β resulted in a 63% (p<0.05), 40.7% (p<0.05) and 37.2% reduction in bax promoter activity compared with the transactivation observed with 50 ng, 100 ng and 200 ng of p73β alone respectively. Moreover, the Brn-3a mediated repression on p73-mediated activation of the bax promoter is reduced as the concentration of p73 is increased.

The effect of ΔNp73α with or without co-expression of Brn-3a was also tested on the bax promoter. As expected, ΔNp73α failed to activate the bax promoter (Fig 3.9c) compared to the activation observed with p73α or p73β. Furthermore co-expression of Brn-3a did not significantly alter bax promoter activation compared with ΔNp73α alone.

Hence Brn-3a can strongly inhibit the stimulatory effect of the TAp73 isoforms p73α and p73β on the bax promoter, and co-operate with TAp73α and TAp73β on the p21CIP1/Waf1 promoter in a manner previously shown with p53. In contrast ΔNp73α fails to activate either of these promoters, though it does appear to reduce Brn-3a mediated activation of the p21CIP1/Waf1 promoter.

3.10 Brn-3a represses TAp73α and TAp73β mediated activation of the pro-apoptotic bax promoter in the SAOS-2 cell line

Recent findings have demonstrated that the p53 family members require each other to function as transcription factors on apoptotic gene targets (Flores et al. 2002). Therefore the above transfections were repeated in the SAOS-2 cell line. As before SAOS-2 cells were co-transfected with the bax reporter gene and increasing amounts of Brn-3a with or without p73α or p73β using Lipofectimine. Fig 3.8b shows the expression of the Brn-3a and p53/p73
proteins in the transiently transfected SAOS-2 cells

As expected, p53, p73α and p73β strongly activated the \textit{bax} promoter, with the p73 isoforms being the stronger activators of the \textit{bax} promoter (Fig 3.10.) For example, whereas p73α activated the \textit{bax} promoter by 76 fold, p73β activated the \textit{bax} promoter by 55 fold. Increasing concentrations of Brn-3a on its own resulted in slight repression of the \textit{bax} promoter as previously shown in ND7 transfections. Moreover the addition of increasing concentrations of Brn-3a with p53 or p73 resulted in repression of the p53 or p73 mediated activation of the \textit{bax} promoter similar to that seen with the ND7 cell line. Hence coin­expression of Brn-3a with either p53, p73α or p73β at a 1:1 ratio resulted in 72.2%, 54.1% and 45.2% decreased activation of the \textit{bax} promoter compared with p53, p73α or p73β alone respectively (p<0.05). Maximal repression is seen when the Brn-3a/p53 or Brn-3a/p73 ratio is 1:1 where maximum saturation is reached as increasing the levels of Brn-3a to p53 or the p73 isoforms does not reduce the \textit{bax} promoter activity further.

Hence Brn-3a represses both TAp73α and TAp73β mediated activation of the \textit{bax} promoter in both ND7 and SAOS-2 lines, suggesting that other neuronal factors and the expression of p53 is not required for this effect.

\textbf{3.11 Brn-3a reduces the levels of endogenous Bax while increasing the levels of p21 protein when co-transfected with p73.}

It has been previously shown that the co-operative effects of Brn-3a on p53 mediated activation of the \textit{p21^{CIP1/Waf1}} promoter as well as the Brn-3a antagonism on p53 mediated activation of the \textit{bax} promoter is reflected in increased endogenous \textit{p21^{CIP1/Waf1}} protein levels and reduced expression of endogenous Bax protein in ND7 cells transfected with both Brn-3a and p53 compared with levels in cells transfected with either Brn-3a or p53 alone (Budram-Mahadeo et al., 2002). It was therefore investigated whether the ability of Brn-3a to co­operate with p73 on the \textit{p21^{CIP1/Waf1}} promoter or repress p73-mediated activation of the \textit{bax} promoter could be reflected in changes of \textit{p21^{CIP1/Waf1}} and Bax protein expression. Since TAp73 is a strong transactivator of the \textit{p21^{CIP1/Waf1}} and \textit{bax} promoters and the effects of Brn-3a on these targets were so significant, changes in \textit{p21^{CIP1/Waf1}} and Bax expression was
Effect of Brn-3a and p73 and on Bax promoter activity in SAOS-2 cells

Figure 3.10: Brn-3a represses p73α and p73β mediated activation of the bax promoter in SAOS2 cells

Brn-3a alone slightly repressed bax promoter activity while p53, p73α or p73β alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with p53, p73α or p73β significantly repressed p53/p73α/p73β mediated activation of the bax promoter. All luciferase values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector and expressed as a percentage of the empty LTR control vector. The results represent the mean and standard error of three independent experiments. SAOS-2 cells were transfected with increasing amounts of Brn-3a (in a 1:1, 1:2, 1:3 and 1:4 ratio with p73) in the presence or absence of 1.5μg of p73α or p73β.
assessed by western blot analysis. Therefore, ND7 cells were transfected with p73α, p73β or ΔNp73α alone or in combination with Brn-3a in 10cm plates. p53 alone or together with Brn-3a was included as a positive control. The lysates obtained from the transfected cells were then used to carry out western blot analysis for endogenous p21^{CIP1/Waf1} and Bax proteins in the different transfected cells. Quantification of p21^{CIP1/Waf1} and Bax levels were carried out by densitometry and values were equalized for actin. Fold changes were obtained by comparing the levels of p21^{CIP1/Waf1} and Bax protein in cells transfected with p53 or the appropriate p73 constructs alone (value set as 1) to that obtained upon co-expression of p53 or the appropriate p73 constructs with Brn-3a.

As shown in Fig 3.11a, p21^{CIP1/Waf1} levels were significantly elevated in cells expressing p53, p73α, p73β but not ΔNp73α compared with the control (p<0.05). This reflects the strong activation of the bax promoter by p53, p73α and p73β in transfection experiments, whilst ΔNp73α fails to activate this promoter. Moreover, quantification of p21^{CIP1/Waf1} protein obtained from 3 independent experiments shown that, as predicated from the co-operative effect of Brn-3a and p73α or p73β on this promoter, there were significantly higher levels of the p21^{CIP1/Waf1} protein expressed in cells co-transfected with Brn-3a and p73α compared with p73α alone (1.7 fold increase, p<0.05) and with Brn-3a and p73β compared with p73β only expressing cells (2.9 fold increase, p<0.05) (Fig 311c). Thus, whereas Brn-3a alone only mildly activated the p21^{CIP1/Waf1} promoter, co-expression of Brn-3a with p73α or p73β can enhance the activity of the p21^{CIP1/Waf1} promoter and this is reproduced at the protein level. p53 was also included, and in accordance with published data, increased levels of p21^{CIP1/Waf1} protein is observed when Brn-3a is co-transfected with p53 compared to p53 expressing cells only (2.4 fold increase, p<0.05) (Budram-Mahadeo et al., 2002). In contrast the levels of p21^{CIP1/Waf1} protein in cells transfected with ΔNp73α alone compared to cells co-transfected with Brn-3a and ΔNp73α did not differ significantly.

Thus Brn-3a co-operates with both p73α and p73β on the p21^{CIP1/Waf1} promoter and this results in significantly enhanced p21^{CIP1/Waf1} protein levels in cells that co-express these proteins. In contrast, co-expression of Brn-3a and ΔNp73α had no effect on p21^{CIP1/Waf1} levels compared with ΔNp73α alone.
Figure 3.11: Brn-3a represses TAp73 mediated expression of endogenous Bax protein and increases TAp73 mediated expression of endogenous p21(CIP1/WAF1) protein

Representative western blot showing a) Levels of p21(CIP1/WAF1) and b) Bax protein in ND7 cells transfected with vector alone, p53, p73α, p73β or ΔNp73α with or without Brn-3a. The levels of Brn-3a and of the control actin protein are shown in each transfected cell population for comparison. c) Fold increase of p21 and d) Decrease of Bax protein levels upon co-expression of Brn-3a compared with p53, p73α, p73β or ΔNp73α alone. Quantification of p21 and Bax levels were carried out by densitometry and values were equalized for actin. Fold changes were obtained by comparing the levels of protein in cells transfected with p53 and appropriate p73 constructs alone to that obtained upon co-expression of Brn-3α with p53 or the appropriate p73 constructs. Values are expressed as a proportion of cells transfected with p53 and the appropriate p73 constructs alone which were taken as 1. Data is from 3 independent experiments.
As expected, Bax levels were significantly elevated in cells expressing p53 p73α or p73β compared with control transfected with empty vector only (p<0.05) (Fig 3.11b), reflecting their ability to strongly transactivate this promote in transfection assays. However, quantification of Bax protein obtained from 3 independent experiments shown that the induction of Bax expression by p73α or p73β is significantly reduced upon co-expression of Brn-3a compared with p73α or p73β alone (32% and 46% decrease in Bax levels respectively, p<0.05), (Fig 3.11d) which supports the antagonistic effects of Brn-3a on p73α and p73β mediated activation of the bax promoter. In addition, significantly lower levels of Bax protein is observed when Brn-3a is co-transfected with p53, compared to p53 expressing cells only (46% decrease in Bax levels, p<0.05) which is consistent with published data (Budram-Mahadeo et al., 2002). As expected, the levels of Bax protein in cells transfected with ΔNp73α, which fails to activate this promoter in ND7 cells, remained similar to that observed in the control cells. Furthermore, co-expression of Brn-3a with ΔNp73α did not significantly alter the levels of Bax expression compared with ΔNp73α alone.

Thus Brn-3a antagonizes TAfp73α and TAfp73β mediated activation of the bax promoter which results in significantly reduced Bax protein in cells that co-express these proteins. In contrast, co-expression of Brn-3a and ΔNp73α had no effect on Bax protein levels.

3.12 Brn-3a differentially regulates p73 mediated cell cycle arrest and apoptosis

3.12.1 Brn-3a co-operates with TAfp73α and TAfp73β to induce cell cycle arrest

It has been previously demonstrated that in the ND7 cell line, co-expression of Brn-3a and p53 is accompanied by increased survival and a higher proportion of cells in the G0/G1 phase of the cell cycle, compared to cells transfected with either factor alone (Budram-Mahadeo et al., 2002). The co-operative effect of Brn-3a and p73α or p73β on the p21^{Clp1/Waf1} promoter and increased p21^{Clp1/Waf1} protein in cells co-transfected with both factors compared with either alone, also suggest that induced cell growth arrest associated with differentiation may be enhanced when Brn-3a is co-expressed with the p73α or p73β isoforms. Additionally, the ability of Brn-3a to inhibit p73α or p73β mediated activation of the bax gene and decrease in endogenous Bax protein levels when both factors are
expressed suggests that Brn-3a might antagonize the pro-apoptotic effect of p73α or p73β in neuronal cells. Thus the effect of Brn-3a on p73 mediated cell cycle arrest and apoptosis was investigated, in the ND7 cell line.

Flow cytometry and fluorescence-activated cell sorting (FACS) analysis was carried out on ND7 cells transfected with either Brn-3a or the p73 isoforms (p73α, p73β and ΔNp73α) alone, as well as co-expressing Brn-3a with either p73α or p73β or ΔNp73α. p53 was included as a positive control, whereas the empty vector was used as a control to compare the effects of Brn-3a and/or p73. p73βdelOD which fails to interact with Brn-3a and is expected not to be functional due to its inability to form tetramers was included as a negative control. GFP-spectrin expression vector was included in all experiments to mark transfected cells. From previous titration experiments the maximal activation of the p21CIP1/WAF1 promoter is achieved when ND7 cells are transfected with the p73/Brn-3a ratio of 1:10 so this ratio was used in these studies. Transfected cells were maintained in culture for 30 hours, fixed in ethanol and stored at -20 till day of analysis. Cells were then stained with propidium iodide (PI) which binds to dsDNA and can therefore assess the DNA content in cells. PI staining is monitored in the FL2 channel, and the ability of the cytometer to exclude doublets (distinguishing between 2 cells in G1 passing through the beam together from a single G2 cell) allows for the estimation of the percentage of cells in G1, S phase and G2M using software analysis.

As well as using PI staining for cell cycle analysis of the total cell population, the cell cycle of GFP positive cells only can also be analysed, which is useful when assessing the cell cycle of transfected cells only (as marked by the expression of transfected GFP). The GFP plasmid makes up a small fraction of the total DNA transfected in the cells so that cells transfected with GFP are likely to express other transfected plasmids.

The total cell population as well as just the GFP positive cell population were analysed for DNA content to obtain their cell cycle profile by using Expo32 software in the first instance. The cell cycle of the GFP positive cells stained with PI was further analysed by the Multicycle software (Phoenix Flow Systems, San Diego) which is a more accurate analysis as it mathematically models cell cycle data by deconstructing linear histograms obtained by the Expo32 software into a normal distribution. Even though values from the Multicycle
software were used in the analysis showed herein, they were similar to the cell cycle data obtained from the Expo32 software. Fig 3.12a demonstrates a typical cell cycle profile of GFP positive cells transfected with vector only using the Expo32 analytical software, compared with the same data analysed by the Multicycle software that gives a more accurate measurement of the percentage of cells in each stage of the cell cycle. Fig 3.12b shows a typical cell cycle profile obtained in a set of experiments when analysed by the Multicycle software.

![Figure 3.12a: Comparison of the two different software's used for cell cycle analysis in these experiments](image)

At least 30,000 events were collected using a Beckman epicsXL flow cytometer equipped with a 488nm argon laser and 525nm band filter to collect GFP emission and 675nm band filter for PI detection. When set up to exclude cell doublets, the percentage of cells in G1, S phase and G2M can be estimated using i) Expo32 software or by using a programme such as ii) Multicycle which will mathematically deconvolute the DNA histogram thus giving a more accurate measurement of the cells in each phase.
Figure 3.12b: Brn-3a increases TAp73 mediated cell cycle arrest

Cell cycle analysis of GFP positive ND7 cells transfected with Brn-3a (b), p53 alone (c) or together with Brn-3a (d) p73α alone (e) or together with Brn-3a (f) or p73β alone (g) or together with Brn-3a (h) ΔNp73α alone (i) or together with Brn-3a (j), compared with empty control vector (a). At least 30,000 events were collected using a Beckman epicsXL flow cytometer equipped with a 488nm argon laser and 525nm band filter to collect GFP emission and 675nm band filter for PI detection. Data analyzed by Multicycle software.
Fig 3.12ci shows a graphical representation of the percentage of cells in G0/G1 obtained from at least three independent experiments derived from data generated by the Multicycle software. Brn-3a alone resulted in an increase in G0/G1 arrest compared to control as expected from results of previous studies (control = 35% of cells in G0/G1, Brn-3a = 40% of cells in G0/G1, p<0.005). As expected p53 alone increased cell cycle arrest compared with control and co-expression of Brn-3a enhanced p53 cell cycle arrest as shown previously (Budram-Mahadeo et al., 2002).

Also as expected, a high proportion of cells transfected with p73α or p73β alone are in the G0/G1 phase of the cell cycle compared with control (p<0.0005). Cells transfected with p73α alone resulted in 45% of cells in G0/G1 whilst co-expression of Brn-3a with p73α resulted in a significant increase in the proportion of cells in G0/G1 phase (52%) compared with p73α alone (p<0.005). Similarly, p73β alone resulted in 45% of cells in G0/G1 whilst co-expression of Brn-3a with p73β resulted in a significant increase in the proportion of cells in G0/G1 phase (55%) compared with p73β alone (p<0.0005). Thus the increase in cell cycle arrest appears to be more pronounced upon co-expression of Brn-3a with p73β, compared with p73β alone. Also the G0/G1:S phase ratio was highest in cells co-expressing Brn-3a/p53, Brn-3a/p73α and Brn-3a/p73β indicating that fewer cells are transiting the G0/G1 boundary into S phase compared with either protein alone or vector control (Fig 3.12cii). Hence, as observed with Brn-3a and p53, Brn-3a also increases both p73α and p73β mediated cell cycle arrest.

In contrast transfection with ΔNp73α did not significantly alter the proportion of cells in G0/G1 compared with cells transfected with empty vector. Furthermore co-expression of Brn-3a with ΔNp73α did not significantly alter the proportion of cells in G0/G1 compared with Brn-3a alone, demonstrating that ΔNp73α fails to induce cell cycle arrest and does not significantly affect the ability of Brn-3a to induce cell cycle arrest. As expected transfection of p73βdelOD did not alter the proportion of cells in G0/G1 compared with the empty vector control and co-expression of Brn-3a with p73βdelOD did not increase cell cycle arrest above that seen with Brn-3a alone. Thus Brn-3a can co-operate with p53 and the TA isoforms p73α and p73β but not with ΔNp73α in inducing cell cycle arrest in ND7 cells. This would suggest that the ability of Brn-3a to enhance cell cycle arrest would depend on which p73 isoform
i) **Effect of Brn-3a and p73 on cell cycle arrest in G0/G1 phase of cycle in ND7 cells**

![Bar chart showing percentage of cells in G0/G1 phase](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>G0/G1</th>
<th>S phase</th>
<th>G2/M</th>
<th>G0/G1:S phase ratio</th>
</tr>
</thead>
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<td>43.3</td>
<td>22.3</td>
<td>1.1</td>
</tr>
<tr>
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<td>36.6</td>
<td>22.0</td>
<td>1.5</td>
</tr>
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<td>18.8</td>
<td>1.2</td>
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<tr>
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</tr>
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<td>30.6</td>
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</tr>
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<td>19.4</td>
<td>0.9</td>
</tr>
<tr>
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<td>20.7</td>
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<td>p73βΔOD</td>
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<td>21.7</td>
<td>0.9</td>
</tr>
<tr>
<td>p73βΔOD + Brn-3a</td>
<td>40.7</td>
<td>40.6</td>
<td>22.1</td>
<td>1.0</td>
</tr>
</tbody>
</table>

**Figure 3.12c: Brn-3a increase p73α and p73β mediated G1 arrest**

i) Percentage of cells in G0/G1 transfected with Brn-3a, p53, p73α, p73β or ΔNp73α alone or together with Brn-3a compared to empty vector. Brn-3a increases p73α and p73β mediated cell cycle arrest compared to p73α or p73β alone (* p<0.005, ** p<0.0005). The results represent the mean and standard error of at least three independent experiments. ND7 cells were co-transfected with 100ng of the p53/p73 isoforms or 1μg of Brn-3a alone or together with 100ng of GFP-spectrin to facilitate the sorting of transfected cells from untransfected cells. Cells were harvested after 30 hours, fixed in ethanol and stained with PI. The DNA content was then measured by FACS as previously described.

ii) Percentage of ND7 cells in the different phases (G0/G1, S or G2/M). The G0/G1:S phase ratio represents the change in proportion of cells progressing through the cell cycle. The higher the number indicates that fewer cells are transiting the G0/G1 boundary into S phase. These values represent the mean of 3 independent experiments.
predominates in the cell (i.e. TA versus ΔN).

3.12.2 Brn-3a antagonizes TAp73α and TAp73β mediated apoptosis and co-operates with ΔNp73 to increase cell survival

The ability of Brn-3a to inhibit TAp73α or TAp73β mediated activation of the bax gene and decrease in endogenous Bax protein levels when both factors are expressed suggests that Brn-3a might antagonize the pro-apoptotic effect of p73α or p73β in neuronal cells. Thus the effect of Brn-3a on p73 mediated apoptosis was investigated, in the ND7 cell line.

To assess whether Brn-3a can over-ride p73 mediated apoptosis, in a manner similar to that seen with p53, the percentage of cells undergoing apoptosis was assessed in cells transfected with the p73 expression vectors, with or without Brn-3a. As before, ND7 cells were transfected with Brn-3a or the p73 isoforms (p73α, p73β and ΔNp73α) alone as well as Brn-3a with either p73α or p73β or ΔNp73α (p73/Brn-3a ratio 1:10). p53 was included as a positive control and empty vector was used as a negative control. GFP-spectrin expression vector was again included in all experiments to mark transfected cells. Annexin V labelling was used to assess for cells undergoing early apoptosis, which recognizes modified phosphatidylserine on the surface of apoptotic cells. After 30 hours following transfection, cells were harvested in 1x PBS and stained with Annexin V-PE and the percentage of early apoptotic cells was measured by FACS analysis on the GFP positive population.

Fig 3.13a shows the number of surviving cells (annexin V negative) from a representative experiment. Increased protection is obtained following transfection of Brn-3a alone compared with controls (Brn-3a= 65% survival, control= 46% survival p<0.0005) which is in accordance with previous studies. Transfection of ΔNp73α also shows slight protection compared to control while transfection of p53, p73α or p73β alone resulted in an increase in apoptosis compared with control which was significant for p73α and p73β (40.1% and 34.4% survival respectively p<0.05). The findings that transfection of p53 or p73 failed to induce significantly more death is perhaps due to the high level of cell death already observed in the vector control as a result of the transfection. This procedure stresses the cells and might induce endogenous p53/p73 protein and hence cell death in the absence of Brn-3a.
**Effect of Brn-3a and p73 on cell survival in ND7 cells**

![Figure 3.13: Brn-3a protects cells from p73α or p73β mediated apoptosis and co-operates with ΔNp73α to increase survival](image)

Percentage of surviving ND7 cells transfected with Brn-3a and/or p53, p73α, p73β, ΔNp73α expression vectors measured by lack of annexin V-PE staining in GFP positive cells. ND7 cells were co-transfected with 100ng of the p53/p73 isoforms or 1μg of Brn-3a alone or together with 100ng of GFP-spectrin to facilitate the sorting of transfected cells from untransfected cells. Cells were harvested in 1x PBS after 30 hours and labeled with Annexin V-PE according to manufacturers protocol and then measured by flow cytometry and FACS analysis.

Thus it is difficult to induce increased cell death much over the basal level seen in the control transfected with vector only. However, upon co-transfection of Brn-3a with p53, there is a 2 fold increase in survival compared with p53 alone (p<0.05). Furthermore the increased survival observed upon Brn-3a and p53 co-expression was also above the levels observed with Brn-3a alone (p<0.05), demonstrating that Brn-3a overrides the p53 mediated apoptotic pathway, but also co-operates with p53 to enhance survival. Co-transfection of Brn-3a with either p73α or p73β resulted in a 1.5 fold and 1.3 fold increase respectively compared with p73α or p73β alone (p<0.05), but the survival observed was less than that obtained with Brn-3a alone. This result may be due to the increased apoptosis of these cells conferred by p73α or p73β compared to p53, resulting in Brn-3a only being able to rescue a smaller fraction of these cells. However, it is clear that Brn-3a reduces p73α and p73β pro-apoptotic effects in a significant percentage of these cells. In contrast, co-transfection of Brn-3a with ΔNp73α resulted in a 2 fold increased survival compared with ΔNp73α alone (p<0.05). Furthermore this increase in survival is above the levels observed with Brn-3a alone (p<0.05). Thus Brn-3a co-operates with ΔNp73α to further increase cell survival.

Thus while Brn-3a confers some protection over p73α and p73β induced cell death, Brn-3a is more effective at inhibiting the pro-apoptotic effect of p53 and synergizing with ΔNp73α to
3.13 Specific regions in the p73 C-terminus is required for Brn-3a to repress p73 mediated activation of the \textit{bax} promoter but not for Brn-3a to enhance the p73 mediated activation of the \textit{p21} promoter

In this study it has been shown that Brn-3a can enhance p73 mediated activation of the \textit{p21} promoter while repressing p73 mediated activation of the \textit{bax} promoter in a similar manner to p53. However there is a marked increase in complexity of p73 compared to p53, largely owing to their extended C-terminal sequence, in which p73 has up to 6 alternatively spliced C-terminal isomeric forms, which may be a determinant of unique biological functions. Furthermore, these C-terminal isomers can also be transcribed from the second downstream promoter to produce the N-terminally truncated isoforms (ΔN) that lack the TA domain (DeLaurenzi and Melino, 2000). It was therefore necessary to establish whether the extended C-terminal region found in p73α and the N-terminal domains of p73 could alter the ability of Brn-3a to enhance p73-mediated activation of the \textit{p21} promoter or repress p73-mediated activation on the \textit{bax} promoter. To investigate this, the transcriptional proficiency of the truncated p73 polypeptides (used previously for the interaction studies, see section 3.5) on these promoters was tested in the absence or presence of Brn-3a.

Therefore similar co-transfection experiments were carried out as described previously in the ND7 neuronal cell line. 1 μg of the \textit{bax} reporter gene was co-transfected with 100 ng of expression vector encoding the appropriate p73 deletion protein in the presence of either 1 μg of Brn-3a expression vector or empty vector. The wild type p73α and p73β with or without Brn-3a were included as positive controls. As shown in Fig 3.14a, wild type p73α and p73β transactivated the promoter and this was inhibited by Brn-3a. In contrast, p73N56 or p73N315, both of which lack the TA domain, failed to activate the \textit{bax} promoter. Similarly co-transfection of p73N423 (which is incapable of forming tetramers, and lacks the TA domain) and p73βdelOD, (which fails to oligomerize) also failed to activate the \textit{bax} promoter activity. Furthermore, co-transfection of Brn-3a with either of these polypeptides did not result in significant changes in the activity of the \textit{bax} promoter, compared to either of the p73
Effect of Brn-3a and p73 deletion proteins on bax promoter activity in ND7 cells

![Graph showing bax promoter activity with and without Brn-3a](image)

Effect of Brn-3a and p73 deletion proteins on p21 promoter activity in ND7 cells

![Graph showing p21 promoter activity with and without Brn-3a](image)

Figure 3.14: Deletion of the p73a C-terminal inhibits the ability of Brn-3a to repress p73 mediated activation of the bax promoter but not for Brn-3a to enhance p73 mediated activation of the p21/Waf1/Cip1 promoter.

a) Brn-3a alone slightly repressed bax promoter activity while p73α, p73β, p73αC505 and p73αC424 alone strongly activated this promoter compared with vector only. Constructs that lack the N-terminal activation domain (p73αN56, N315, N423) or constructs that lack the OD domain (p73βdelOD) failed to activate the bax promoter. Co-transfection of Brn-3a with p73α or p73β significantly repressed p73α or p73β mediated activation of the bax promoter as seen previously. In contrast, Co-transfection of Brn-3a with either of the p73α C-terminal deleted proteins, p73αC505 or p73αC424 failed to repress bax promoter activity.

b) Constructs that lack the N-terminal activation domain (p73αN56, N315, N423) or constructs that lack the OD domain (p73βdelOD) failed to activate the p21 promoter, whilst p73α, p73β and both the C-terminal deleted proteins alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with p73α, p73β, p73αC505 or p73αC424 significantly increased p73 mediated p21 activation showing that loss of the extended C-terminal does not affect the ability of Brn-3a to increase p73 activation of the p21 promoter. All luciferase values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. ND7 cells were transfected with either 1 μg of bax or p21/Waf1/Cip1 promoter with 100ng of expression vector encoding either p73α, p73β or the appropriate p73 deletion protein in the presence of either 1 μg of Brn-3a expression vector or empty vector using Fugene reagent.
polypeptides alone. Hence these results further confirm that the TA domain is essential for gene activation and also show that the OD is required for activation presumably because the active form of p73 is a tetramer (Davison et al., 1999).

However, co-transfection of the C-terminal deleted polypeptides, p73C505 and p73C424, both of which contain the TA domain and OD domain but lacks 132 and 213 amino acids at the C-terminus respectively, resulted in strong activation of the \( bax \) promoter. The p73C505 polypeptide is a stronger activator than p73\( \beta \) but less than p73\( \alpha \) which has been consistently shown to be the strongest activator of the \( bax \) promoter in the ND7 cell line. P73C424 lacking the C-terminal 213 amino acid was able to transactivate the \( bax \) promoter, though less efficiently. Interestingly, co-transfection of Brn-3a with either p73C505 or p73C424 failed to repress \( bax \) promoter activity, in contrast to the significant repression of Brn-3a on p73\( \alpha \) or p73\( \beta \) mediated activation of the \( bax \) promoter as shown previously. As Brn-3a and the p73C505/p73C424 polypeptides have been shown to interact (see Fig 3.4), the lack of repression observed by addition of Brn-3a must be due to the missing C-terminal 132 or 213 amino acids not found in the p73C505 and p73C424 polypeptides respectively.

Hence specific region(s) of the C-terminal sequence of p73 is essential for Brn-3a to repress p73 mediated activation of the \( bax \) promoter. However as repression by Brn-3a is not lost on p73\( \beta \) mediated \( bax \) activation, this would suggest that the sequence encoded by exon 13 which is lost by splicing in the p73\( \beta \) mRNA is not important for Brn-3a to repress p73 mediated activation of the \( bax \) promoter.

Since Brn-3a differentially regulates p73 mediated effects on the \( p21^{CIP1/Waf1} \) promoter with co-operation instead of repression, it was tested whether this effect was also dependent on the C-terminus region of p73. In experiments similar to those described above, the transcriptional proficiency of the different truncated p73 proteins on the \( p21^{CIP1/Waf1} \) promoter and the Brn-3a effect on this activation was investigated. As before, ND7 cells were co-transfected with 1 \( \mu \)g of the \( p21^{CIP1/Waf1} \) reporter gene with 100 ng of expression vector encoding the different p73 deletion proteins in the presence of either 1 \( \mu \)g of Brn-3a expression vector or empty vector. p73\( \alpha \) and p73\( \beta \), with or without Brn-3a were included as positive controls. As expected, co-transfection of p73N56, p73N315, p73N423 and p73\( \beta \)delOD which lack the TA domain or OD domain or both, were unable to transactivate the \( p21^{CIP1/Waf1} \) promoter.
Furthermore, co-transfection of Brn-3a with any of these p73 polypeptides resulted in no significant changes in the activity of the \( p21^{cip1/waf1} \) promoter, compared to either of the p73 polypeptides alone or Brn-3a alone which as shown previously can activate the \( p21^{cip1/waf1} \) promoter. In contrast, co-transfection of the C-terminal deleted polypeptides, p73C505 and p73C424, resulted in strong activation of the \( p21^{cip1/waf1} \) promoter, with p73C424 activating the \( p21^{cip1/waf1} \) promoter the least, whilst p73C505 activation was comparable to that obtained with p73a and p73β. Co-transfection of Brn-3a with either p73C505 or p73C424 resulted in a significant increase in \( p21^{cip1/waf1} \) promoter activity, compared to p73C505 or p73C424 alone. Therefore, loss of 131 amino acids in p73C505 or 213 amino acids in p73C424 of the p73a C-terminus does not affect the ability of Brn-3a to significantly enhance p73 mediated \( p21^{cip1/waf1} \) promoter activity.

Thus, the requirement of the p73 C-terminal for Brn-3a mediated effects on p73 transcription is specific to the \( bax \) promoter as the Brn-3a/p73 co-operation on the \( p21^{cip1/waf1} \) promoter is not lost upon deletion of the p73 C-terminal. These results suggest that the mechanisms by which Brn-3a regulates the p73 proteins on the \( bax \) and \( p21^{cip1/waf1} \) promoters are distinct. This parallels the findings of the distinct mechanism used by Brn-3a to regulate p53 mediated transcription on these promoters. Hence, whereas Brn-3a binding sites are required for it to repress p53 mediated transcription on the \( bax \) promoter (Budram-Mahadeo et al., 2002), Brn-3a mediated enhancement of p53 transcription on the \( p21^{cip1/waf1} \) promoter, was achieved by recruitment to the basal transcriptional complex (Perez-Sanchez et al., 2002).

### 3.13.1 Brn-3a differentially regulates the transcriptional effect of the different TAp73 isoforms on the \( bax \) promoter but not on the \( p21^{cip1/waf1} \) promoter

The findings that Brn-3a could repress p73α (636 aa) and p73β (resulting from splicing of exon 13) mediated activation of the \( bax \) promoter but this repression was lost upon deletion of 132 or 213 amino acids from the p73α C-terminal, suggested that Brn-3a could differentially regulate the transcriptional effect of the different p73 C-terminal isoforms on \( bax \) promoter activity. Therefore the effects of the truncated naturally occurring p73γ and p73δ mediated activation of the \( bax \) promoter was investigated in the absence and presence of Brn-3a. Both p73γ and p73δ isoforms possess much shorter C-terminals than either p73α or p73β. The splicing of exon 11 in p73γ results in a frame shift from the original
reading frame leading to a translation of 76 amino acids that is different from the sequence in p73α. The splicing of exons 11, 12 and 13 in p73δ results in a p73 isoform that is 403 amino acids long and bears the most striking resemblance to p53, as it completely lacks the COOH terminus extension that are found in the other forms of p73. As already mentioned p73β is produced by splicing of exon 13 and is identical to the corresponding regions of p73α with an additional 5 residues at the end which is specific to p73β.

As before, the bax reporter gene was co-transfected with 100 ng of expression vectors encoding either p73γ or p73δ in the presence of either 1 μg of Brn-3α expression vector or empty vector. As both p73γ and p73δ proteins are HA tagged then HA tagged p73α and p73β were included as positive controls (all HA-tagged constructs were a kind gift from Professor Jerry Melino). Western blot analysis shows that each of the transfected p73 isoforms are expressed in the ND7 cells (Fig 3.15c).

As shown in Fig 3.15a, p73α is the strongest activator of the bax promoter resulting in a 92 fold induction, whilst p73β activates this promoter 47 fold which is consistent with previous results demonstrating that addition of the HA tag does not effect p73 transcriptional activity on this promoter. As seen previously, co-expression of Brn-3a represses both p73α or p73β mediated activation of the bax promoter, further showing that the HA tag does not affect the repressive effects of Brn-3a on p73 mediated bax activation. Moreover, these results also show that both p73γ and p73δ can activate the bax promoter to a similar extent as p73β. However, co- transfection of Brn-3a with either p73γ or p73δ failed to repress either the p73γ or p73δ mediated activation of the bax promoter.

To confirm that these differences were not due to lack of interaction between the shorter isoforms of p73 and Brn-3a, GST pull down assays were performed as before using Brn-3α(l)-GST fusion proteins with IVT p73γ, p73δ and p73α as a control. As shown in Fig 3.15d both p73γ and p73δ readily interact with Brn-3a(l) and appear to interact more strongly than p73α, being retained 22.5% and 33.2% respectively by Brn-3a(l), compared with 11.3% of p73α. Therefore similar to p73β, it appears that the p73 isoforms with shorter C-terminals interact more strongly with Brn-3α (Brn-3α(l) retained 23.7% of p73β- see section 3.2). Together, these results further provide evidence that the p73 C-terminal influences the ability
a) **Effect of Brn-3a and p73 isoforms on bax promoter activity in ND7 cells**

![Bar chart showing the effect of Brn-3a and p73 isoforms on bax promoter activity](image)

- Brn-3a alone slightly repressed bax promoter activity while p73α, p73β, p73γ and p73δ alone strongly activated this promoter compared with vector only. Co-transfection of Brn-3a with p73α or p73β significantly repressed p73α or p73β mediated activation of the bax promoter as seen previously. In contrast, Co-transfection of Brn-3a with either p73γ or p73δ failed to repress bax promoter activity.

b) **Effect of Brn-3a and p73 isoforms on p21 promoter activity in ND7 cells**

![Bar chart showing the effect of Brn-3a and p73 isoforms on p21 promoter activity](image)

- The p73 C-terminal isoforms p73α, p73β, p73γ and p73δ alone strongly activated the p21 promoter compared with vector only. Co-transfection of Brn-3a with either C-terminal isoform significantly enhanced p73 mediated p21 activation demonstrating that Brn-3a enhancement of p73 mediated p21CIP/Waf1 promoter activation is independent of the p73 C-terminal sequence.

![Western blot analysis of the transfected ND7 lysates](image)

Western blot analysis of the transfected ND7 lysates confirms expression of the transfected p73 proteins.

Figure 3.15: Brn-3a interacts with the shorter isoforms of p73, p73γ and p73δ but fails to inhibit p73γ or p73δ mediated activation of the bax promoter but continues to co-operate with these isoforms on the p21CIP/Waf1 promoter.

ND7 cells were transfected with 1 μg of the p21CIP/Waf1 promoter with 100ng of expression vector encoding the appropriate p73 isoform in the presence of either 1 μg of Brn-3a expression or vector empty vector using Fugene reagent. Western blot analysis of the transfected ND7 lysates confirms expression of the transfected p73 proteins.
**Interaction of Brn-3a(l) with the p73 C-terminal isoforms, p73y and δ**

Figure 3.15 continued: d) GST pull down assay demonstrating interaction between Brn-3a(l) and the shorter isoforms of p73. Lanes 5-8 shows 1/10 of the input protein used in the "pull-down" assays, lanes 1-4 shows proteins retained following incubation with Brn-3a(l)/GST fusion proteins. While the luciferase control protein was not retained in a complex with Brn-3a(l), the positive control p73a as well as p73y and p73δ were associated with Brn-3a(l). This effect is specific to Brn-3a(l) proteins as the GST moiety on its own failed to bind to p73a, p73y or p73δ (lanes 9-12). The percentage interaction is also represented graphically. These results strengthens the importance of the requirement of specific C-terminal sequence not included in the C-terminal sequence of either p73y or p73δ but is present in the C-terminus of both p73a and p73δ, in the ability of Brn-3a to repress p73 mediated bax activation.
of Brn-3a to modulate p73 mediated activation of the \textit{bax} promoter.

Hence the failure of Brn-3a to inhibit p73 mediated activation of the \textit{bax} promoter by the shorter isoforms of p73 strengthens the requirement of specific C-terminal sequence not found in the C-terminal sequence of either p73\textgamma{} or p73\textdelta{} but present in the C-terminus of both p73\textalpha{} and p73\textbeta{}, for Brn-3a to repress p73 mediated \textit{bax} activation. Interestingly as both p73\textgamma{} and p73\textdelta{} lack the sequences encoded by exon 11 and 12 whilst p73\textalpha{} and p73\textbeta{} isoforms retain these exons and original reading frame, this would suggest that the sequence encoded by exon 11 and/or 12 may be required for the repressive effects of Brn-3a on p73 mediated \textit{bax} transactivation.

The non requirement of the C-terminal sequence in the ability of Brn-3a to enhance p73 mediated \textit{p21CIP1/Waf1} promoter activity suggests that Brn-3a would enhance p73 mediated activation of the \textit{p21CIP1/Waf1} promoter by all p73 C-terminal isoforms. Therefore, to determine the effect of Brn-3a on the transcriptional activity of the shorter p73 isoforms, p73\textgamma{} and p73\textdelta{} on \textit{p21CIP1/Waf1} promoter activity, transfections were carried out as before. The \textit{p21CIP1/Waf1} reporter gene was co-transfected with 100 ng of expression vectors encoding either p73\textgamma{} or p73\textdelta{} in the presence of either 1 \textmu{}g of Brn-3a expression vector or empty vector. As before, p73\textalpha{} and p73\textbeta{} with and without Brn-3a were included as positive controls. The results shown in fig 3.15b, demonstrate that p73\textgamma{} and p73\textdelta{} activate the \textit{p21CIP1/Waf1} promoter to a similar extend as p73\textbeta{} which is slightly higher than that observed with p73\textalpha{}. Co-transfection of Brn-3a with either p73\textgamma{} or p73\textdelta{} resulted in co-operation on \textit{p21CIP1/Waf1} promoter activity, as seen with p73\textalpha{} and p73\textbeta{} isoforms. Therefore, unlike the \textit{bax} promoter, the enhancement of p73 mediated \textit{p21CIP1/Waf1} promoter activation by Brn-3a is independent of the p73 C-terminal sequence.

3.13.2 Brn-3a may repress p73 mediated activation of the \textit{bax} promoter by preventing the binding of a co-activator to the p73 C-terminal region

It is possible that the dependence of Brn-3a on the C-terminus of p73 to repress p73 mediated activation of the \textit{bax} promoter occurs because of either 1) the interaction of Brn-3a with p73 on the \textit{bax} promoter may prevent p73 from interacting with cellular protein(s) that have co-activator function and interact with the C terminus of p73 or 2) the interaction of Brn-3a with
p73 on the \textit{bax} promoter may facilitate the binding of a second repressor co-factor to the p73 C-terminus, and this is required for Brn-3a to repress p73 mediated activation of the \textit{bax} promoter or 3) post transcriptional modifications of the p73 C-terminus is required for Brn-3a to repress p73 mediated activation of the \textit{bax} promoter.

In order to address 1 and 2, further transfections were carried out in ND7 cells to determine whether cellular proteins binding to the p73 C-terminus might affect p73 transactivation of the \textit{bax} promoter and alter Brn-3a mediated repression. This was achieved by introducing the p73N423 construct (that encodes the p73a C-terminal domain but lacks the TA, DBD and the OD domain) with specified p73 constructs and/or Brn-3a. Therefore, ND7 cells were co-transfected with 1 \mu g of \textit{bax} reporter plasmid with 100 ng of constructs encoding either p73a or the C-terminal deleted polypeptides p73C505 or p73C424 with increasing concentrations of p73N423 (100 ng or 500 ng) in the absence or presence of 1 \mu g of the Brn-3a construct or empty vector.

As shown in Fig 3.16a, co-expression of the C-terminal polypeptide p73N423 (100 ng) with p73a, represses p73a mediated activation of the \textit{bax} promoter by 50% which is similar to the repression observed when Brn-3a is co-expressed with p73a. Furthermore increasing the concentration of co-expressed p73N424 with p73a further repressed \textit{bax} activity, and in fact makes TAp73a behave like p73C505 on this promoter (Fig 3.16b). This suggests that the excess C-terminal domain is competing for the binding of a cellular protein that might act as a co-activator which is required for maximal activation of the \textit{bax} promoter by p73a. In addition, co-expression of Brn-3a with p73a in the presence of the C-terminal polypeptide, does not further repress \textit{bax} promoter activity, suggesting that Brn-3a represses p73 activation on this promoter in a similar manner achieved by addition of excess C-terminal protein, by interfering with a functional interaction between the p73 C-terminal and a cellular co-activator.

Interestingly, co-expression of the C-terminal polypeptide p73N423 with the C-terminal deleted protein p73C505 significantly increased p73C505 mediated activation of the \textit{bax} promoter in a dose dependent manner so that addition of 100 ng of p73N423 leads to a 31 fold increase in activity whereas addition of 500 ng of p73N423 leads to an 38 fold increased
Figure 3.16: Brn-3a mediates its repressive effects on p73 mediated bax activation by preventing the binding of a co-activator to the p73 C-terminal.

Brn-3a alone slightly repressed bax promoter activity while p73a, p73aC505 and p73aC424 alone strongly activated this promoter compared with vector only. a) Co-expression of the C-terminal polypeptide p73aN423 with p73a, at the concentrations stated, represses p73a mediated activation of the bax promoter to a similar extend to that observed when Brn-3a is co-expressed with p73a. This suggests that Brn-3a represses p73 activation on this promoter in a similar manner achieved by addition of excess C-terminal protein. b) Co-expression of the C-terminal protein p73aN423 with the C-terminal deleted protein p73aC505 significantly increased p73aC505 mediated activation of the bax promoter. This suggests that addition of the C-terminal polypeptide is interacting with and recruiting a co-activator back to the p73C505 complex on the bax promoter. Co-expression of Brn-3a with p73aC505 fails to repress bax activity as seen previously. However, co-expression of Brn-3a with p73aC505 and the C-terminal polypeptide restores the Brn-3a ability to significantly repress the p73aC505 mediated activation of the bax promoter. These results support the idea that Brn-3a mediates its repressive effects on p73 mediated activation by preventing the binding of a co-activator to the p73 C-terminal. Co-expression of the C-terminal protein p73aC424 with the C-terminal deleted protein p73aC424, also increases p73aC424 mediated activation of the bax promoter in a dose dependent manner. However co-expression of Brn-3a with p73aC424 and the C-terminal polypeptide p73aN424 failed to alter the activation of the bax promoter suggesting that this protein does not bind to the co-activator. All luciferase values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. ND7 cells were transfected with 1 μg of bax with 100ng of expression vector encoding the appropriate p73 isoform in the presence of either 1 μg of Brn-3a expression vector or empty vector. 100ng or 500ng of p73aN423 or empty vector was also transfected where indicated, using Fugene reagent.
activity on the \( bax \) promoter compared to the 19 fold induction of the \( bax \) promoter observed with p73C505 alone (\( p<0.005 \)) (Fig3.16b). This enhanced activity observed suggests that addition of the C-terminal polypeptide might allow recruitment of a co-activator back to the p73aC505 complex on the \( bax \) promoter, thus increasing promoter activity. Additionally, co-expression of Brn-3a with p73C505 and the C-terminal polypeptide restores the Brn-3a ability to significantly repress the p73C505 mediated activation of the \( bax \) promoter (\( p<0.005 \)), supporting the idea that Brn-3a mediates its repressive effects on p73 mediated activation by preventing the binding of a co-activator to a region in the p73 C-terminal domain. This is further supported by the observation that co-expression of Brn-3a with p73C505 alone does not repress \( bax \) promoter activity as seen previously (section 3.13, Fig 3.14a). This may be because the p73 co-activator that binds to the p73 C-terminus can not be recruited by the p73aC505 polypeptide to the \( bax \) promoter, therefore resulting in the inability of Brn-3a to further repress \( bax \) activity when co-expressed.

Although co-expression of the C-terminal protein p73N423 with the C-terminal deleted protein p73C424, increased p73C424 mediated activation of the \( bax \) promoter in a dose dependent manner, this effect was less significant than p73C505. Additionally, co-expression of Brn-3a with p73C424 and the C-terminal polypeptide p73N424 fails to repress the activation of the \( bax \) promoter (Fig 3.16b). This suggests that the region of the p73 protein required for binding to a cellular co-activator and thus allowing \( bax \) transactivation is between 423-505 amino acids, which is retained in p73C505 but lost in p73C424.

Collectively, these results suggest that the p73 C-terminal region (from 400 to 494 amino acids) encoded by exons 11 and 12 found in p73\( \alpha \) and p73\( \beta \) but not in p73\( \gamma \) and p73\( \delta \) is required for Brn-3a to repress p73 mediated activation of the \( bax \) promoter. Moreover, the results above suggest that the p73 C-terminal is responsible for binding of a co-activator which is required for maximal activation of the \( bax \) promoter by p73, and that this co-activator interacts with the region spanning 424-505 amino acids of the p73a C-terminus. Thus, when the Brn-3a/p73 interaction occurs, the presence of Brn-3a on the \( bax \) promoter may interfere or prevent the binding of this co-activator to the p73 C-terminal, resulting in repression of \( bax \) promoter activity by disrupting gene expression. Thus the region of the p73 C-terminus that is required for binding of a possible co-activator and repression by Brn-3a can be further refined to 424-494 amino acids that are encoded by exons 11 and 12.
and is thus only found in the p73α and p73β isoforms. Hence, the array of p73 transcripts expressed in the cell and which isoforms are dominant will determine the extent to which Brn-3a can repress p73 mediated bax activation and thus modulate p73 mediated cell death.

As the Brn-3a/p73 co-operation on the p21CIP1/Waf1 promoter was shown to be independent of the p73 C-terminal region, it would therefore be anticipated that adding back the C-terminal peptide in transfections would not effect Brn-3a stimulation of p73 mediated activation of the p21CIP1/Waf1 promoter. Hence to test this, transfections were carried out in ND7 cells in which 1 µg of the p21CIP1/Waf1 reporter plasmid was co-transfected with 100 ng of constructs encoding either p73α or the C-terminal deleted polypeptides p73C505 or p73C424 with increasing concentrations (100 ng or 500 ng) of the p73N423 construct (that encodes the p73α C-terminal domain but lacks the TA, DBD and the OD domain). These were carried out in the absence or presence of 1 µg of the Brn-3a construct or empty vector.

The results show that co-expression of the C-terminal polypeptide p73N423 at the two different concentrations stated with p73α (Fig 3.17a), p73C505 and p73C424 (Fig 3.17b) did not significantly alter p21CIP1/Waf1 activity compared with p73α, p73C505 and p73C424 alone. Moreover, the addition of the C-terminal peptide did not effect the Brn-3a mediated enhancement of either p73α, p73C505 or p73C424 mediated activation of the p21 promoter, confirming that the extended p73 C-terminal domain does not significantly alter the ability of Brn-3a to enhance p73 mediated activation of the p21CIP1/Waf1 promoter.

These results demonstrate that the p73 C-terminal domain is required for Brn-3a to inhibit p73 mediated activation of the bax promoter, but is not required for Brn-3a to enhance p73 mediated activation of the p21CIP1/Waf1 promoter. This suggests the existence of different regulatory mechanisms used by Brn-3a to modulate the transactivation function of the p73 family members on the two different promoters tested in this study. Thus, while Brn-3a is able to co-operate with all the different C-terminal p73 isoforms tested (p73α, p73β, p73γ and p73δ) to enhance p73-mediated activation of the p21CIP1/Waf1 promoter, Brn-3a can only repress p73α and p73β mediated activation of the bax promoter whilst having no effect on p73γ and p73δ mediated activation of this promoter. Hence Brn-3a may play an important role in determining the selectivity of individual p73 members in controlling the expression of
3) Effect of Brn-3a and the p73 C-terminal on p73 activation of p21 promoter activity in ND7 cells

![Graph showing effect of Brn-3a and p73 C-terminal on p73 activation of p21 promoter activity in ND7 cells.]

b) Effect of Brn-3a and the p73 C-terminal on p73aC505/C424 activation of p21 promoter activity in ND7 cells

![Graph showing effect of Brn-3a and p73 C-terminal on p73aC505/C424 activation of p21 promoter activity in ND7 cells.]

Figure 3.17: The extended p73 C-terminal sequence is not required for Brn-3a to enhance p73 mediated activation of the p21CIP1/WAF1 promoter.

Co-expression of the C-terminal polypeptide p73aN423 at the two different concentrations stated with a) p73a or the C-terminal deleted proteins b) p73aC505 and p73aC424 did not significantly alter p21CIP1/WAF1 activity compared with p73a, p73aC505 and p73aC424 alone. Additionally, co-transfection of the C-terminal peptide did not effect the Brn-3a mediated enhancement of either p73a, p73aC505 or p73aC424 mediated activation of the p21CIP1/WAF1 promoter, again confirming that the extended p73 C-terminal is not a factor in the ability of Brn-3a to enhance p73 mediated activation of the p21CIP1/WAF1 promoter. All luciferase values were equalized on the basis of the activity observed upon co-transfection with a control renilla expression vector and expressed as a percentage of the empty control vector. The results represent the mean and standard error of three independent experiments. ND7 cells were transfected with 1 µg of p21CIP1/WAF1 with 100ng of expression vector encoding the appropriate p73 isoform in the presence of either 1 µg of Brn-3a expression vector or empty vector. 100ng or 500ng of p73aN423 or empty vector was also transfected where indicated, using Fugene reagent.
their target genes.

Discussion

Since Brn-3a was shown to interact with p53 and these two factors were found to modulate the effects of each other on their target gene expression (Sugars et al., 2001; Perez-Sanchez et al., 2002; Budram-Mahadeo et al., 2002), part of this study set out to examine the physical interaction and the functional effect of the association between the Brn-3a and the p73 proteins. Critically the interaction of Brn-3a and p53 resulted in the enhancement of p53 mediated transcription of $p21^{CIP1/Waf1}$ gene associated with cell cycle arrest and antagonism of p53 mediated $bax$ activation. p53 plays an essential role in regulating apoptosis in the nervous system in response to injury or stress (for review see Hughes et al. 1997), as well as in developmental cell death as regulated by the TrkA and p75 neurotrophin receptors (Aloyz et al., 1998) and has also been shown to be up-regulated and activated in some neurones as they migrate to their target field and differentiate (Eizenberg et al., 1996). Thus it was anticipated that neuronal cells that co-express Brn-3a and p53 are more likely to survive and undergo cell cycle arrest that precedes differentiation. In the absence of Brn-3a, p53 would induce high levels of Bax resulting in an increase in cell death (Budram-Mahadeo et al., 2002). Hence Brn-3a may be an important determinant in p53 mediated cell fate.

Employing the use of “GST pull-down” chromatography this study has shown that the TA isoforms of p73, p73a and p73b also specifically interact with both isoforms of Brn-3a and Brn-3b. In addition the $\Delta N p73$ isoform that lacks the N-terminal TA domain, has also been shown to interact with Brn-3a. Co-immunoprecipitation studies also confirm that Brn-3a and $\Delta N p73$ interact in vivo. Studies to map the domains of Brn-3a and Brn-3b required for association with the p73 proteins showed that the isolated POU domain of the Brn-3 proteins is sufficient for interaction with the p73 proteins, whilst the isolated N-terminal domain of Brn-3a shows relatively weak association with the p73 proteins. Additionally, the longer isoforms Brn-3a(l) and Brn-3b(l) interacted with higher affinity with the p73 proteins than the shorter Brn-3a(s) and Brn-3b(s) isoforms with Brn3b(s) interacting with the least affinity with the p73 proteins. The stronger interaction of the p73 proteins with the longer forms of Brn-3a and Brn-3b proteins could be due to sequences in the N terminal region found in the
longer isoforms that may enhance binding to the p73 proteins. However, since the Brn-3a N-terminal alone did not interact strongly with p73 it is likely that the N-terminal contributes to binding of the p73 proteins by altering the conformation of the longer Brn-3 isoforms.

This differential interaction that was observed between the long and short forms of Brn-3a with the p73 proteins, are supported by the functional differences between the long and short forms of the Brn-3a transcription factors. For instance Brn-3a(l) has been shown to be required not only for neuronal differentiation but is also essential for neuronal survival, whilst Brn-3a(s) promotes neuronal differentiation without enhancing cell survival (refer to section 1.22.1). As TAp73 has been shown to be involved in neuronal differentiation and apoptosis, then a possible functional interaction between TAp73 and Brn-3a(l) proteins may have consequences for the survival as well as the differentiation of neuronal cells that express both these proteins in the developing nervous system.

Similar studies to map the region of the p73 protein required for this interaction was undertaken using p73 deletion proteins and confirmed that the region of p73 containing the OD domain is critical for this interaction, although the DBD does contribute to the interaction, which was particularly evident with the interaction studies carried out with the isolated POU domain which required the DBD of p73 for efficient binding. In this respect the Brn-3a/p73 interaction is similar to the Brn-3a/p53 interaction which is mediated via the POU domain of Brn-3a and the DBD of p53. However, the Brn-3a/p73 interaction also requires the OD of p73 suggesting that Brn-3a only binds to tetrameric p73 or Brn-3a contacts specific regions in the OD of p73.

Interestingly, the p73α C-terminal deletion mutants showed increased binding to both Brn-3a and Brn-3b compared to wild type p73α. Additionally, the shorter p73β isoform which lacks codons 421-636 found in p73α appears to associate with higher affinity with the Brn-3a and Brn-3b proteins compared to p73α. This may be a consequence of the structural differences in the C-terminus of p73α and p73β which appears to affect interaction with the Brn-3a/Brn-3b proteins, with the extended C-terminus specific to p73α possibly interfering with association with the Brn-3a/Brn-3b proteins. This is further supported by the findings that p73γ and p73δ, both of which have shorter C-terminals also binds Brn-3a with higher affinity than p73α. Interestingly, the differential binding of various isoforms of p73 and Brn-3a/Brn-
3b that is conferred by the p73 C-terminus may be a determinant of precise biological outputs. Thus, it appears that Brn-3a interacts with p73 via the POU domain of the Brn-3 proteins and the OD domain of p73 with contributions from the DBD of p73. This interaction is further modulated by the C-terminal of the p73 proteins.

The effects of Brn-3a on p73 mediated activation of the \( p21^{CIP1/Waf1} \) and \( bax \) promoters were tested in the ND7 neuronal cell line. As seen previously with p53, Brn-3a antagonized p73\( \alpha \) and p73\( \beta \) mediated activation of the \( bax \) promoter in reporter assays and this is reflected in a decrease of endogenous Bax protein upon co-expression of Brn-3a with p73\( \alpha \) or p73\( \beta \) compared with levels seen with p73\( \alpha \) or p73\( \beta \) respectively. Similarly, Brn-3a co-operates with p73\( \alpha \) or p73\( \beta \) on the \( p21^{CIP1/Waf1} \) promoter in reporter assays and this is reflected in increased endogenous \( p21^{CIP1/Waf1} \) protein levels in cells co-expressing Brn-3a with p73\( \alpha \) or p73\( \beta \) compared with either p73\( \alpha \) or p73\( \beta \) expressing cells only. Additionally, Brn-3a co-operation on the \( p21^{CIP1/Waf1} \) promoter and repression of p73 mediated activation of the \( bax \) promoter was reproduced in the osteogenic SAOS-2 cell line demonstrating that the Brn-3a modulation of p73 mediated activation observed was not neuronal specific or dependent on the endogenous expression of p53.

Thus Brn-3a antagonizes TAp73\( \alpha \) and TAp73\( \beta \) mediated activation of the \( bax \) promoter whilst co-operating on the \( p21^{CIP1/Waf1} \) promoter, in a manner similar to that seen with p53.

In contrast, \( \Delta Np73\alpha \) alone failed to activate either of the \( p21^{CIP1/Waf1} \) or \( bax \) promoters in the ND7 cell line. Furthermore, co-expression of Brn-3a with \( \Delta Np73\alpha \) did not significantly alter the activity of the \( bax \) promoter compared with \( \Delta Np73\alpha \) alone and this is represented in the lack of change of Bax protein levels upon co-expression of Brn-3a and \( \Delta Np73\alpha \) compared with \( \Delta Np73\alpha \) alone or control vector. However, co-expression of Brn-3a with \( \Delta Np73\alpha \) resulted in repression of Brn-3a mediated activation of the \( p21^{CIP1/Waf1} \) promoter in reporter assays but this is not reflected in changes of \( p21^{CIP1/Waf1} \) protein levels in cells co-expressing Brn-3a and \( \Delta Np73\alpha \) compared to Brn-3a only expressing cells. However, the fold changes are probably too small to be detected at the protein level, thus such small changes would be better investigated by RT-PCR to look at the changes in \( p21^{CIP1/Waf1} \) mRNA level expression.

Importantly, the interaction of Brn-3a and the different p73 isoforms had distinct effects on
regulation of cell cycle arrest and cell survival in the neuronal cell line ND7. Thus, co-expression of Brn-3a with p73α or p73β resulted in enhanced cell cycle arrest compared with p73α or p73β alone. Furthermore, the increase in percentage of cells arrested in G0/G1 phase of the cell cycle observed appeared to be more pronounced upon co-expression of Brn-3a with p73β than with p73α. This suggests that the extent to which Brn-3a can enhance TAp73 mediated induced cell cycle arrest in cells co-expressing these proteins depends largely on which TAp73 C-terminal isomers are present in the cell. As expected ΔNp73α did not increase cell cycle arrest compared with control vector, and co-expression of Brn-3a with ΔNp73α did not increase cell cycle arrest above that seen with Brn-3a alone. Thus Brn-3a can co-operate with p73α and p73β but not with ΔNp73α in inducing cell cycle arrest.

The strong repression of p73 mediated bax activation conferred by Brn-3a suggest that less apoptotic cells will be observed in cells co-expressing Brn-3a and p73 than cells transfected with p73 alone, and this was indeed the case. However even though co-expressing of Brn-3a with p73α or p73β increased cell survival compared to p73α or p73β alone, the level of protection was less that that seen with Brn-3a alone. This is in contrast to the effect of Brn-3a on p53 induced cell death in which co-expression of Brn-3a with p53 resulted in increased survival that was higher than that observed with Brn-3a alone. Intriguingly co-expression of Brn-3a and ΔNp73α also resulted in increased survival above the levels observed with either Brn-3a or ΔNp73α alone. Thus the anti-apoptotic effects of Brn-3a co-operate with ΔNp73α to further increase cell survival as well as overriding the pro-apoptotic effects of p53 and to a lesser extend the pro-apoptotic effects of p73α and p73β.

The ΔNp73 isoforms are highly potent survival proteins, inhibiting death of cultured sympathetic and cortical neurones treated with a variety of stimuli including NGF withdrawal and DNA damage (Pozniak et al., 2000; Pozniak et al., 2002). One of the ways ΔNp73α mediates these effects is by acting as a naturally occurring dominant inhibitory antagonist of p53 and TAp73. More recently ΔNp73α has also been shown to mediate survival in neurones via p53 independent mechanisms by inhibiting multiple points in the mitochondrial apoptotic pathway and inhibiting JNK activation after NGF withdrawal (Lee et al., 2004). Even though the molecular mechanism by which the Brn-3a/ΔNp73α interaction co-operates to increase cell survival remains to be characterized, it can be hypothesized that they may act co-operatively on other anti-apoptotic targets such as the Bcl-2 and Bcl-xL, both of which
are known targets of Brn-3a, or on as yet unknown targets of ΔNp73α.

Thus while Brn-3a co-operates with the TAp73 isoforms in enhancing cycle arrest when co-expressed, Brn-3a modulates both TAp73 and ΔNp73 mediated cell fate leading to increased cell survival when co-expressed.

As demonstrated with the data generated so far, Brn-3a can physically interact in vitro with the different isoforms of p73 and has been shown to interact with ΔNp73 in intact cells. Furthermore Brn-3a can modulate p73 transcriptional activity and functional effects on cell cycle and apoptosis in a similar manner shown previously with p53. As the p73 proteins are expressed as multiple alternatively spliced C-terminal isoforms and as N-terminally truncated isoforms, it was interesting to see if Brn-3a could differentially modulate the individual p73 members in controlling the transactivation of the p21\(^{CIP1/Waf1}\) and bax promoters. It has already been shown that the ΔNp73 does not activate either of these promoters, but does co-operate with Brn-3a in increasing cell survival of ND7 cells. Therefore the different p73 deletion proteins lacking either the N or C terminal sequences were tested for transactivation of either the p21\(^{CIP1/Waf1}\) or bax promoters and the effect of Brn-3a on this transactivation was tested.

As expected none of the p73 deleted proteins which lacked the N-terminal transactivation domain could activate transcription from either the p21\(^{CIP1/Waf1}\) or bax promoters, though the two C-terminal deleted polypeptides p73C505 and p73C424 could transactivate both promoters. Interestingly, the deletion of either 131 or 213 amino acids from the extreme C-terminus of p73, (in p73C505 and p73C424 respectively) prevented Brn-3a from repressing p73 mediated bax activation, demonstrating that regions in the C-terminal domain of p73 are essential for Brn-3a to repress p73 transactivation of this promoter. Upon further analysis, this study demonstrated that specific regions in the p73 C-terminus found in p73α and p73β but not in p73γ and p73δ are required for Brn-3a to repress p73 mediated activation of the bax promoter. The C-terminal regions of p73α, p73β, p73γ and p73δ are shown below (Fig 3.18). The fact that both p73γ and p73δ lack the sequences encoded by exon 11 and 12 (due to the alternative reading frame of p73γ and splicing out of these exons in the mRNA of p73δ) whilst p73α and p73β isoforms retain these exons and original reading frame, this would suggest that sequences encoded by exon 11 and/or 12 may be required for the
repressive effects of Brn-3a on p73 mediated bax transactivation. Exon 11 and 12 encodes the region 400-494 that is between the OD (345-390) and SAM (526-549) domain of p73α and is shared by p73β. Interestingly this region contains the PPPPY (residues 482-488, underlined in Fig 3.18) motif that has been shown to interact with other cellular proteins (see later). It is possible that co-factors bound to this region of p73α or p73β are affected when Brn-3a binds to these proteins on the bax promoter.

Figure 3.18 Amino acid alignment of the C-terminal region of the four splice variants

The human amino acid sequences of the C-terminal regions of the p73 splice variants are shown. The corresponding exons are shown above the p73α amino acid sequence.

The idea of a co-factor bound to these proteins was strengthened by results of co-transfection studies in which the isolated C-terminal polypeptide (p73N423) repressed p73α mediated bax activation by 50-60%. This suggested that transfecting in excess isolated p73 C-terminal polypeptide is competing for the binding of cellular proteins with co-activator function which is required for maximal activation of the bax promoter by p73. In contrast, co-expression of the C-terminal protein p73N423 with the C-terminal deleted protein p73C505 which lacks 131 amino acids from the extreme C-terminus of p73α, significantly increased p73C505 mediated activation of the bax promoter in a dose dependent manner by up to 19 fold, compared to the activation of this promoter by p73C505 alone. This enhanced activity observed suggests that addition of the C-terminal polypeptide allows recruitment of a co-activator back to the p73C505 complex on the bax promoter. In contrast, addition of
p73N423 to p73C424 did increase p73C424 activity on the bax promoter but not as significantly as p73C505. The struggle to recapitulate full activation of this p73 construct which lacks the C-terminal 213 amino acids, suggests that the region 424-505 that is lacking in the p73C424 protein is required for binding of a co-activator protein by p73 that allows maximal activation of this promoter.

Furthermore, the observation that co-transfection of Brn-3a with full length p73α in the presence of the C-terminal polypeptide did not further repress bax promoter activity, suggested that both the C-terminus and Brn-3a were repressing p73 mediated bax activation by a similar mechanism, probably by interfering with the binding of a co-activator to the p73α C-terminus. Moreover, since co-expression of the C-terminal polypeptide with p73C505 restored the ability of Brn-3a to significantly repress the p73C505 mediated activation of the bax promoter, it can be concluded that Brn-3a acts by interfering with the binding of a co-activator which is being added back to the complex by the C-terminal polypeptide. Additionally, co-expression of Brn-3a with p73C424 and the C-terminal polypeptide failed to repress further the p73C424 activation of the bax promoter suggesting that p73C424 lacked the sequences that bind the co-activator. Therefore, it is clear that Brn-3a can repress the p73 mediated activation of the bax promoter whilst co-operating with p73 to activate the p21CIP1/Waf1 promoter. Furthermore, specific regions of the p73 C-terminus is required for the ability of Brn-3a to repress p73 mediated activation of the bax promoter.

Although the specific co-activator which is affected by Brn-3a to represses p73 mediated bax activation requires further investigation, these results collectively, suggest that sequences within the region 400-494 that is encoded by exons 11 and 12 and the region 424-505 that is lacking from the C-terminal of the p73C424 is required for binding of a co-activator by p73, which is possibly disrupted by the presence of Brn-3a. This further narrows the region to 425-494. The identification of this co-activator would be the next step in elucidating the mechanism by which Brn-3a represses p73 mediated activation on the bax promoter.

Transcriptional co-activators play a major role in enhancing both p53 and p73 in stimulating the expression of their target genes. For example the apoptosis stimulating protein of p53 (ASPP) proteins, which bind to all p53 family members are required to potentiate p53 family mediated activation of pro-apoptotic promoters such as pig and bax and is an important
determinant in the apoptotic pathway (Samuels-Lev et al., 2001; Bergamaschi et al., 2004). The co-activator PCAF, specifically stimulates p73 mediated transactivation on promoters such as PG13 (an artificial reporter construct containing several p53 binding sites) and p21^{CIP1/Waf1} and binding of PCAF to p73 is necessary for p73 transactivation activity (Zhao et al., 2003). Additionally, PCAF specific siRNA reduces p73 mediated activation of the PG13 promoter by 50%, and co-transfection of PCAF and p73 leads to increased apoptosis. However, although these two co-activators are clearly involved in increasing p73 activation of apoptotic targets, neither of these co-activators bind to the extreme p73 C-terminus.

Interestingly, it has been recently demonstrated that the WW domain adaptor phosphoprotein YAP (Yes-associated protein) has been shown to interact with the C-terminal PPPPY proline rich region (482-488 amino acids encoded by exon 12) found in the p73α and p73β C-terminal but not in p73γ, p73δ or p53. Furthermore it was demonstrated that YAP stimulated p73α mediated activation of the bax and mdm promoters in H1299 cells and binding of YAP to p73 is necessary for the co-activation of p73 (Strano et al., 2001). Therefore it can be speculated that when bound to p73, Brn-3a may disrupt the interaction between p73 and a co-activator such as YAP resulting in repression of p73 mediated bax activation. This can be addressed in future studies by titrating in YAP in cells co-transfected with Brn-3a and p73α with the bax reporter gene, to investigate whether increasing concentrations of YAP can antagonize Brn-3a mediated inhibition of p73α transactivation of the bax promoter. Further analysis would then require the use of siRNA-mediated inactivation of each of the endogenous proteins to test the effect on survival/apoptosis.

Also, to further identify other cellular proteins that interact with the p73 C-terminus, a strategy that can be employed would be to use the yeast based 2 hybrid screen, in which the cDNA encoding specific regions of the p73 C-terminus (particularly the cDNA spanning exons 11 and/or 12 of p73α) are used as bait. As both Brn-3a and p73 are highly expressed in the brain, and as most of the transient transfections were performed in the ND7 neuronal cell line, it would be appropriate to carry out the screening of the cDNA libraries derived from human fatal brain or a neuronal cell line. Once identified the effects of these cellular proteins that interact with the p73 C-terminus on p73 mediated bax transactivation and on the repression of this activation by Brn-3a can be investigated, in a similar manner mentioned
As well as interaction with other cellular proteins, the activities of p73 are also regulated by phosphorylation and acetylation. Thus modifications specific to the C-terminal of p73 may also affect the ability of Brn-3a to repress p73 mediated activation of the \( bax \) promoter. In this respect p73 is a substrate of p300 and PCAF acetylation of lysine residuals in the p73 DBD and C-terminus of p73\( \alpha \) respectively. Although the functional significance of the acetylation of the C-terminus by PCAF is not known, acetylation by p300, which is markedly increased in cells treated with DNA damage agents, enhances the transcriptional activity on pro-apoptotic targets such as \( p53AIP1 \) (Costanzo et al., 2002; Zhao et al., 2003). To test whether these modifications on the C-terminus of p73 change the ability of Brn-3a to repress p73 mediated activation of the \( bax \) promoter, studies can be undertaken to assess the effects of mutating these sites to either express constitutively acetylated proteins or to prevent acetylation. The effects of these mutations in the absence or presence of Brn-3a can then be tested.

Hence, while Brn-3a can significantly repress both p53 and p73 mediated activation of the \( bax \) promoter, this study also demonstrates a further level of specificity in the ability of Brn-3a to repress p73 mediated \( bax \) activation which exists among the different C-terminal isomers of p73. Thus, whereas Brn-3a can repress p73\( \alpha \) and p73\( \beta \) mediated activation of the \( bax \) promoter, and increase cell survival when co-expressed with either p73\( \alpha \) or p73\( \beta \) in the ND7 cell line, Brn-3a has no effect on p73\( \gamma \) and p73\( \delta \) mediated activation of this promoter. Like p73\( \alpha \) and p73\( \beta \), both p73\( \gamma \) and p73\( \delta \) have been shown to be expressed in normal tissue (De Laurenzi et al., 1998; Ueda et al., 1999). However, these splice variants showed different expression patterns in different tissues as well as at different stages of differentiation suggesting that they perform different functions. Thus the role of Brn-3a in determining the selectivity of individual p73 members in controlling the expression of the \( bax \) promoter may have consequences on cell death/survival under different conditions or in different cell types.

Furthermore each of the splice variants exhibited different transactivation properties on a p53 consensus site in transfection assays (De Laurenzi et al., 1998; Ueda et al., 1999). Moreover yeast-2 hybrid system experiments demonstrated that each isoform can form both homodimeric and heterodimeric interactions to varying extents (De-Laurenzi et al., 1998)
and these interactions between different variants can further modulate the transcriptional proficiency of the p73 complex on a p53 consensus site (Ueda et al., 2001). Hence it can be postulated that the array of p73 transcripts expressed in the cell at any given time and also which isoforms are dominant will determine the extent by which Brn-3a can repress bax activation and hence the ability of Brn-3a to modulate p73 mediated cell fate.

In summary, although the complete functional implications of this differential repression on p73 mediated bax transactivation conferred by Brn-3a requires further analysis, these observations provide an understanding in the fine tuning of the biological outputs of the p73 family, as a result from interaction with other proteins such as Brn-3a.

In contrast the C-terminal was shown not to be of such critical importance in the ability of Brn-3a to enhance p73 mediated activation of the p21CIP1/Waf1 promoter. Therefore, Brn-3a was able to enhance p21CIP1/Waf1 activation of all four p73 C-terminal isomers tested in this study. However, the ability of Brn-3a to enhance cell cycle arrest differed between the isoforms tested. Thus Brn-3a may differentially enhance cell cycle arrest depending on the p73 C-terminal isomers present in the cell.

The use of different regulatory mechanisms by Brn-3a to modify p73 transactivation of its target genes is reflected in the different mechanisms employed by Brn-3a to modulate p53 target genes. Thus, on the p21CIP1/Waf1 promoter, Brn-3a appeared to mediate its effects by recruitment to the basal transcription complex, whilst intact Brn-3a binding sites are required for it to repress p53 mediated activation of the bax promoter. These findings demonstrate the complexity of regulation of expression of genes associated with cell death/survival /differentiation by Brn-3a

Brn-3a and p73 are both implicated in the differentiation of neuronal cells by up-regulating genes involved in neuronal differentiation (Budhram-Mahadeo et al., 1995; Smith et al., 1997a; Smith et al., 1997b; Huang et al., 1999; De Laurenzi et al., 2000). Similarly p53 has been associated with differentiation of sympathetic and sensory neurones (Eizenberg et al., 1996; Hudson et al., 2004) (also see chapter 5). Clearly the effects of p53 or p73 on cells (apoptosis or differentiation) are dependent on the presence of other specific or common members of the p53 family of regulatory proteins that result in subtle modification of their
biological effects. Brn-3a is one such protein, which has been shown to interact with and differentially regulate p53 or p73 mediated transcription. The ability of Brn-3a to synergize with p53 or p73 on the p21^{CIP1/Waf1} promoter but strongly repress p73 or p53 mediated apoptotic gene expression (bax promoter) suggest that Brn-3a plays a critical role in determining the cellular status and survival of neuronal cells which co-express these proteins. In particular Brn-3a may be important in determining the fate of neuronal cells where it has been shown that p53 and p73 is also up-regulated during neuronal differentiation, during developmental death, or when cells are subject to stress or injury resulting in activation of the p53/p73 gene. Hence similar to the Brn-3a/p53 effect, it can be postulated that in developing neuronal cells that express both p73 and Brn-3a, the pro-apoptotic effect of p73 will be antagonized by Brn-3a whilst both proteins co-operate to induce cell cycle arrest. Because Brn-3a increases expression of neuronal proteins such as neurotrophic receptors (trkA), neurofilament and α-internexin, this would enhance differentiation of surviving cells into mature neurones.

In this respect it was demonstrated that p53 co-localizes to a subset of Brn-3a positive neural crest derived cells fated for a sensory neuronal linage. Furthermore the distinct morphology of the Brn-3a/p53 co-expressing cells suggested a differentiated neuronal cell type which was confirmed by co-localizing p53 with the differentiation marker NF-160. The functional effects of the Brn-3a/p53 co-expression and the localization of TAp73 and ΔNp73 with respect to Brn-3a in the neural crest derived cells is investigated in chapter 5.
CHAPTER 4

Mechanism by which the Brn-3a transcription factor blocks p53 and TA73 mediated expression of pro-apoptotic target gene, Noxa.

4.1 Introduction

The ability of Brn-3a to enhance neuronal survival and differentiation is mediated by direct regulation of target genes or indirectly upon interaction with proteins such as p53 or p73. The ability of Brn-3a to directly repress the bax promoter and antagonise p53 and p73 activity on this promoter is interesting as brn-3a/- embryos show significant loss of specific neurones as a consequence of increased apoptosis. Bax plays a role in naturally occurring apoptosis in many neurones during development including sensory neurones in the DRG and trigeminal ganglia which are known to express Brn-3a. In support of this, neuronal cells from bax/- mice demonstrated decreased apoptosis (DRG, TG, spinal ganglia, brainstem nuclear complex and cerebellum). Moreover, neurones cultured from bax/- mice survive even in the absence of neurotrophic factors (Deckwerth et al., 1996; White et al., 1998). However apoptosis is still observed in some sensory neurones in bax/- mice which is independent of Bax. This suggests that while Bax can contribute to some apoptosis in sensory neurones, other pro-apoptotic factors are likely to contribute to the fate of these cells.

Noxa is another known pro-apoptotic p53 target gene that is induced by stresses which can activate p53 expression (Oda et al., 2000). Noxa encodes a Bcl-2 homology 3 (BH3)-only member of the Bcl-2 family of proteins. When expressed, Noxa becomes localized to the mitochondria where it interacts with the anti-apoptotic Bcl-2 family members via its BH3 domain and inhibits their pro-survival function (Oda et al., 2000; Chen et al., 2005). This in turn leads to bax activation and subsequent Cytochrome c release, though the mechanism is as yet unclear (Green et al., 2004). Both Noxa and Bax expression are elevated upon induction of p53 in neuronal cells (Yang et al., 2004) while p73 has been shown to activate noxa directly (Flores et al., 2002; Flinterman et al., 2004) or by co-operation with adenovirus E1A (Flinterman et al., 2004). Therefore it is possible that like Bax, Noxa may also contribute to apoptosis during neuronal development and their regulation by p53 and/or p73

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may be important in controlling cell fate. The antagonistic effect of Brn-3a on p53 and TAp73 mediated activation of the bax promoter suggests that Brn-3a may be important in controlling expression of pro-apoptotic factors and hence determine cellular status and survival of neuronal cells that co-express Brn-3a and p53/p73 during development. The effect of the Brn-3a/p53 and Brn-3a/p73 interaction on the noxa promoter and functional effects of this interaction were investigated in this study.

4.2 The p53 and TAp73 mediated activation of the noxa promoter is repressed by Brn-3a

Since Brn-3a could block p53 or TAp73 mediated activation of the bax promoter but enhance expression of the cell cycle arrest associated gene, \( p21^{CIP1/Waf1} \), the possibility that Brn-3a could also modulate the effect of p53 on the promoter of another pro-apoptotic target gene, noxa was investigated. As the effect of p73 on the noxa promoter in ND7 cells had not been investigated, experiments were also carried out to test if the noxa promoter is responsive to p73 in these cells and whether Brn-3a could modify p73 mediated effect on this promoter.

Similar to co-transfection experiments carried out previously in the ND7 neuronal cell line, a reporter construct in which the regulatory region of the noxa gene (-183 to +158) (Oda et al., 2000) drives expression of the luciferase reporter gene was co-transfected with increasing concentrations of expression vectors encoding either p53 (2-20 ng) or p73\( \alpha \) (10-50 ng) in the presence of either 1 \( \mu \)g of Brn-3a expression vector or empty vector. As expected, co-transfection with p53 expression vector resulted in strong transactivation of the noxa promoter (Fig 4.1a) with up to 8 fold increased activity compared with the empty vector control (\( p<0.0005 \)). Similarly co-transfection with p73\( \alpha \) could also strongly activate the noxa promoter with up to 13 fold increased activity compared with the empty vector control (\( p<0.0005 \)), suggesting that like other p53 target promoters, noxa is also responsive to p73. As was the case on the bax promoter, p73 appeared to activate the noxa promoter more strongly than p53, with increasing transactivation seen with increasing p73 concentration. Whilst Brn-3a alone did not significantly alter noxa promoter activity, co-transfection of Brn-3a with either p53 or p73\( \alpha \) resulted in a significant decrease of the transactivation observed with either p53 or p73\( \alpha \) alone (\( p<0.005 \)), respectively on this promoter. These effects resulted
Figure 4.1: Brn-3a antagonizes p53/p73α mediated activation of the noxa promoter and this effect is lost upon mutation of the p53 site in the noxa promoter

a) Brn-3a alone had a minimal effect on noxa promoter activity compared with vector only control. Co-expression of p53 or p73α alone resulted in significant activation of the promoter. Co-transfection of Brn-3a with p53 or p73α resulted in significant loss of p53 or p73α mediated transactivation of the noxa promoter. b) Whereas Brn-3a alone had a minimal effect on the noxa promoter lacking the p53 site, the stimulation of the promoter by p53 or p73 is lost. Co-transfection of Brn-3a with p53 or p73 did not significantly modify the promoter activity. The ND7 cells were transfected with 1 μg of the noxa promoter luciferase reporter construct (wild type or Δp53-Noxa) and increasing amounts of p53 or p73α (2-50ng) in the presence of 1 μg of either empty vector or Brn-3a. All luciferase values were equalized to the activity of a co-transfected renilla expression vector and expressed as a percentage of the empty LTR control vector. The results represent the mean and standard error of three independent experiments. c) Brn-3a and/or p53, p73α, ΔNp73α expression in transiently transfected ND7 cells were analyzed by Western blot after whole cell lysis.
from the changes in the expression of Brn-3a, p53 and p73 proteins in the transfected cells (fig 4.1c). Therefore, similar to the \textit{bax} promoter, Brn-3a can strongly inhibit the stimulatory effect of p53 and p73\(\alpha\) on the \textit{noxa} promoter, highlighting a trend in which Brn-3a down-regulates the expression of apoptotic genes, which could contribute to its ability to enhance survival. However, while Brn-3a alone reduced the endogenous activity of the \textit{bax} promoter (Budram-Mahadeo et al., 2002), no such direct repressive effect was observed on the \textit{noxa} promoter.

Whereas the \textit{bax} promoter contains p53 sites flanked by two Brn-3 binding sites, both of which are required for Brn-3a to repress the promoter directly or indirectly via p53 (Perez-Sanchez et al., 2002), no such Brn-3 consensus sites were observed in the sequence of the \textit{noxa} promoter used in these studies. However since Brn-3a could still efficiently block p53 or p73 mediated transactivation of this promoter, it was investigated whether this effect resulted from the direct interaction of Brn-3a with p53 or p73 and hence was dependent on the p53 consensus site present in the promoter.

Co-transfection studies were carried out using a reporter construct containing the \textit{noxa} promoter in which just the p53 site was deleted (\(\Delta p53\)-\textit{noxa}). While the basal promoter activity was still present and similar to the wild type \textit{noxa} promoter (basal activity of \(\Delta p53\)-\textit{noxa} \(=7.46\) RLU \(\pm 0.18\) compared with wild type \textit{noxa} \(= 6.31\) RLU \(\pm 0.77\)), p53 and p73\(\alpha\) mediated activation of the \textit{noxa} promoter is greatly reduced upon loss of the p53 binding site (Fig 4.1b). Brn-3a on its own had little effect on promoter activity while co-expression of Brn-3a and p53 or Brn-3a and p73\(\alpha\) had no effect compared with p53 or p73\(\alpha\) only, respectively. The expression of Brn-3a, p53 and p73\(\alpha\) proteins are clearly increased in the transfected cells as shown by Western blot (Fig 4.1c). Therefore loss of transcription by p53/p73 on the \(\Delta p53\)-\textit{noxa} promoter occurs as a result of loss of the p53 consensus site which prevented binding of the p53 or p73 proteins. Therefore it appears that the association of Brn-3a with p53 or p73\(\alpha\) antagonizes their activity on the \textit{noxa} promoter and this effect is dependent on the p53 cognate DNA site being present on the promoter.

\(\Delta Np73\alpha\) was shown to have no direct effect on \textit{bax} or \textit{p2\textsuperscript{CIP1/Wf4}} promoter activity in ND7 cells, but its effect on the \textit{noxa} promoter is not known. In order to investigate the effect of \(\Delta Np73\alpha\) on the \textit{noxa} promoter in ND7 cells, and effect of the \(\Delta Np73\alpha\)/Brn-3a interaction on
the possible regulation of this promoter, co-transfection experiments were carried out as previously described in the ND7 neuronal cell line. The reporter construct containing the wild type noxa promoter (-183 to +158) (Oda et al., 2000) was co-transfected with increasing concentrations of expression vector encoding ΔNp73α (10-50 ng) in the presence of 1 μg of either the empty vector or Brn-3a expression vector. Co-transfection with ΔNp73α expression vector resulted in a slight repression of the basal activity of the noxa promoter, but this was not significant (Fig 4.2). As previously shown, Brn-3a alone did not significantly alter noxa promoter activity and similarly, co-expression of Brn-3a with ΔNp73α did not affect noxa promoter activity compared with Brn-3a alone. Levels of expression of transfected constructs were assessed by western blotting, showing that both transfected Brn-3a and ΔNp73 are expressed in these cells (Fig 4.1c). Hence lack of transactivation by both Brn-3a and ΔNp73 of this promoter was not due to their lack of expression, but reflect different functional effects on this promoter.

**Effect of Brn-3a and ΔNp73 on Noxa promoter activity**

![Figure 4.2: ΔNp73α does not significantly alter noxa promoter activity](image)

Brn-3a alone had a minimal effect on noxa promoter activity compared with vector only control. Co-expression of ΔNp73α alone did not significantly alter promoter activity. Co-transfection of Brn-3a with ΔNp73α did not significantly modify the promoter activity compared to control. The ND7 cells were transfected with 1 μg of wild type noxa promoter luciferase reporter construct and increasing amounts of ΔNp73α (10-50ng) in the presence of 1 μg of either empty vector or Brn-3a(l). All luciferase values were equalized to the activity of a co-transfected renilla expression vector and expressed as a percentage of the empty LTR control vector. The results represent the mean and standard error of three independent experiments.
4.3 Brn-3a does not prevent p53 binding to its cognate sequence in the noxa promoter

In order to investigate whether Brn-3a acted by blocking p53 binding to its consensus site in the noxa promoter or whether its association with p53 prevented the interaction with other co-factors which may be required for the activation of the noxa promoter, electrophoretic mobility shift assays (EMSA) were carried out using cellular extracts obtained from neuroblastoma cells (IMR-32 cells stably over-expressing Brn-3a made by Shazia Irshad, ICH). These cells, which express high levels of Brn-3a, are known to express wild type p53 which is also shown by Western blot analysis (Fig 4.3a). As shown in figure Fig 4.3b, incubation of IMR-32 cell extract with $^{32}$P labelled oligonucleotide probe corresponding to the noxa p53 binding site resulted in specific complexes (lane 1) which were competed by addition of x50 and x100 excess of unlabelled p53 binding site (lanes 2+3) but not the non-specific oligonucleotide (lane 4). The presence of two main retarded complexes would suggest that p53 associates with either itself or other cellular proteins to form complexes of different sizes. Furthermore, addition of p53 pAb resulted in further retardation of the complexes, indicated by asterix (lanes 5+6) but this was not seen upon addition of the pAb against the unrelated actin protein (lane 7), confirming that p53 protein is present in these complexes. Interestingly, addition of an excess of the oligonucleotide that corresponds to the Brn-3a site resulted in a loss of the complex binding to the probe (lanes 8+9). This suggests that Brn-3a is associated with p53 in this complex and the excess Brn-3a site is competing away the Brn-3a/p53 complex from the labelled probe. Moreover, addition of Brn-3a mAb resulted in a supershift of the complex with one larger complex observed, further showing that Brn-3a interacts with p53 when p53 is bound to the noxa promoter (lane 9). However there also appears to be some competition when Brn-3a antibody is added suggesting that binding of this antibody to Brn-3a within the complex may prevent p53 binding to the labelled DNA, perhaps by steric hindrance.

The results described above confirm that Brn-3a is bound to p53 on the p53 consensus site found in the noxa promoter. Moreover, it appears that Brn-3a does not prevent p53 binding to this sequence but may act by preventing the recruitment of co-factors such as ASPP.
Figure 4.3: Brn-3a does not prevent p53 binding to its cognate sequence in the noxa promoter

a) Western blot using IMR-32 cell extract (used in EMSA assay) showing expression of Brn-3a and wild type p53. b) Electrophoretic mobility shift assays (EMSA) of protein binding on the p53 DNA sequence element taken from the noxa promoter. Cellular extracts were made from IMR-32 cells. $^{32}$P-labelled oligonucleotide comprising the p53 DNA binding site in the noxa promoter were incubated with the cellular lysate and subjected to non denaturing gel electrophoresis. Specific competition was 50 and 100x excess of the same oligonucleotide, unlabelled, non-specific competition was 100x excess CREB oligonucleotide. Addition of p53 pAb or Brn-3a mAb resulted in supershift of the complexes (indicated by asterices). Addition of a Brn-3a consensus site resulted in competition of this complex.
(apoptosis-stimulating protein of p53 family members) proteins required for stimulation of pro-apoptotic proteins such as Bax (Bergamaschi et al., 2004).

Next, the association of Brn-3a with the noxa promoter was investigated further in intact cells by carrying out Chromatin Immunoprecipitation (ChIP) assays in order to determine whether Brn-3a is associated with the wild type but not mutant noxa promoter. ND7 cells were transfected with the wild type noxa promoter with the intact p53 site or the Δp53-noxa promoter which does not contain the p53 binding site. Expression vectors encoding Brn-3a and p53 were also co-transfected as endogenous levels of both proteins are relatively low in normal proliferating ND7 cells. Primers were designed to amplify 152 bp-fragment of the promoter encompassing the p53 binding site. As can be seen in fig 4.4, lane 2 and 5 indicates the input sample whilst lanes 3 and 6 shows amplification following immuno-precipitation of cells transfected with the wild type and Δp53-noxa promoters respectively, with Brn-3a mouse mAb. Lanes 4 and 7 indicate the amplification from cells that were immuno-precipitated with the control anti-mouse secondary antibody only. Significantly higher amplification of the noxa promoter region was observed in cells transfected with the wild type promoter compared with those with the promoter lacking the p53 site from which low levels of the promoter sequence were amplified. Lane 1 indicates the 100bp ladder.

These results demonstrate that in the intact cell, Brn-3a is associated with the region of the noxa promoter that contains the p53 binding site. The failure of the mutant promoter to be immuno-precipitated with Brn-3a suggests that Brn-3a is bound to p53 that is bound to this site so that loss of p53 binding to this site prevents the promoter from being immuno-precipitated with Brn-3a in the ChIP assay in these cells.

The finding that TAp73 activated the wild type noxa promoter but not the Δp53-noxa promoter suggests that TAp73 activates this promoter by binding to the p53 RE, though evidence of direct binding would need to be tested (e.g. EMSAs, whereas ChIP assays will determine if TAp73 binds this site in the intact cell). Investigation of the mechanism by which Brn-3a inhibits TAp73-mediated activation of the noxa promoter can then be carried out by performing similar EMSA/ChIP assays described above. Due to time constraints this work was not undertaken.
Chromatin immunoprecipitation assay were carried out in ND7 cells transfected with p53 and Brn-3a with either wild type or p53 site deleted noxa promoters. Following immunoprecipitation with Brn-3a antibody, PCR amplification was carried out using specific primers flanking the p53 site in the noxa promoter. PCR amplification of the positive control input (total chromatin extract before immunoprecipitation) is shown in lane 2. The wild type noxa promoter immuno-precipitated with Brn-3a is shown on lane 3 whilst the negative control in which secondary antibody was used to immuno-precipitate the wild type noxa promoter is shown in lane 4. Lane 5 shows the input from cells transfected with the p53 site deleted noxa promoter whilst lane 6 shows immunoprecipitation with the Brn-3a antibody which is similar to the background level seen with the control secondary antibody only in both wild type and control experiments.

Figure 4.4: Immunoprecipitation of Brn-3a on wild type noxa promoter but not on the mutant noxa promoter
4.4 Levels of pro-apoptotic proteins, Bax and Noxa in Brn-3a -/- mice

Knock out mice lacking Brn-3a demonstrate significant loss of specific neuronal cells as a result of apoptosis that peaks at E15.5. In order to address the question of whether loss of Brn-3a resulted in increased levels of pro-apoptotic p53/p73 target genes in vivo, Bax and Noxa protein levels were analyzed in pooled neuronal tissue known to express Brn-3a (midbrain, hindbrain, trigeminal ganglia, spinal cord and DRG) dissected from Brn-3a -/- embryos compared with similar tissue taken from wild type litter mates (Fig 4.5-this work was done by Peter Morris and Budram-Mahadeo, ICH). Quantification for protein levels obtained from a number of litters showed significant increased levels of both Bax and Noxa protein in E14.5 Brn-3a -/- embryos compared with levels in wild type litter mates. However, by E16.5 the levels of both Bax and Noxa proteins were similar in the wild type and knock out litter mates with no significant differences evident upon quantification (Fig 4.5) (Hudson et al., 2005)

Therefore loss of Brn-3a in mice with wild type p53/p73 expression, results in higher levels of the pro-apoptotic proteins, Bax and Noxa, at a time preceding maximal apoptosis in the Brn-3a -/- embryos. This suggests that Brn-3a may be important in regulating the expression of these pro-apoptotic target proteins during development.

4.5 Discussion

These results discussed in this chapter has demonstrated that Brn-3a represses the transcriptional activity of p53 on another pro-apoptotic p53 target gene, noxa, in a manner similar to that seen on the bax promoter (Budram-Mahadeo et al., 2002). Similar to p53, p73α was also shown to strongly activate the noxa promoter in ND7 cells, while co-expression of Brn-3a with p73α resulted in a strong repression of the p73 mediated activation of the noxa promoter. Hence Brn-3a antagonizes p53 and p73α mediated activation of the noxa gene promoter in reporter assays.

ΔNp73α acts as a potent transdominant inhibitor of gene expression by p53 or TAp73,
Figure 4.5: Elevated levels of Bax and Noxa protein in Brn-3a-/- embryos at E14.5 but not at E16.5

a) Representative western blot analysis showing the expression of Bax and Noxa proteins in tissues taken from either Brn-3a-/- embryos or from wild type embryos at E14.5. Actin levels are shown for the same samples. b) Representative western blot analysis showing the expression of Bax and Noxa proteins in tissues taken from either Brn-3a-/- embryos or from wild type embryos at E16.5. Actin levels are shown for the same samples.
Grob et al., 2001; Kartasheva et al., 2002; Stiewe et al., 2002; Zaika et al., 2002; Vossio et al., 2002) as well as regulating the expression of a variety of genes independently of p53 (Kartasheva et al., 2003). Thus the effect of ANp73α on the noxa promoter was tested. As was the case on the p21CIP1/Waf1 and bax promoters, ANp73α did not significantly alter the activity of the noxa promoter.

It is interesting that while Brn-3α alone could mildly repress bax promoter activity this was not observed on the noxa promoter used in this study. However whereas the bax promoter contains two consensus binding sites for Brn-3α that flanks the p53 sites, no such Brn-3α sites were observed on the noxa promoter tested in this study. In the bax promoter both Brn-3α binding sites are required for its effects on this promoter as mutation of these sites resulted in a failure of Brn-3α to repress transcription either alone or via association with p53 (Perez-Sanchez et al., 2002). The lack of Brn-3α consensus binding sites on the noxa promoter may explain why Brn-3α did not directly repress promoter activity in these experiments. However, since Brn-3α could significantly antagonize p53 or p73 mediated stimulation of this promoter, this effect must arise from its ability to physically interact with the p53 and p73 proteins. Hence, Brn-3α could block p53/p73 mediated activation either by 1) the association of Brn-3α with p53/p73 prevents the p53/p73 protein from binding to its consensus site in the noxa promoter, or 2) interaction of Brn-3α with p53/p73 when it is bound to the promoter prevents the association of other proteins that are required for full p53/p73 transactivation of this promoter. Results of electrophoretic mobility shift assay shows that Brn-3α is, in fact, associated with p53 when it is bound to its DNA binding site in the noxa promoter. This suggest that the interaction of Brn-3α with p53 may prevent the transactivation of the noxa promoter possibly by excluding binding of co-activators required for the stimulation of pro-apoptotic gene promoters by p53. This was strengthened by the finding that in ChIP assays, immunoprecipitation of Brn-3α in ND7 cells containing the wild type p53 site in the noxa promoter resulted in amplification of the promoter DNA but this was not observed on the same promoter lacking the p53 site.

In response to a stress signal, activated p53 regulates genes involved in many cellular functions, the most important being cell cycle arrest and apoptosis. It is clear that cellular proteins co-expressed with the p53 family of proteins can alter the effect of this protein on determining cell fate by modifying target genes regulated by the p53 family. The co-
expression of ASPP proteins (ASPP1/2) which interacts with p53, p73 and p63 increases expression of pro-apoptotic genes such as \textit{bax}, \textit{pig3} and \textit{puma} and are clearly important for determining the apoptotic pathway (Samuels-Lev et al., 2001; Bergamaschi et al., 2004). The apoptotic function of p73 is also potentiated by c-abl kinase activity and the co-activators p300 and PCAF by increasing its ability to activate the transcription of pro-apoptotic gene targets (Gong et al., 1999; Costanzo et al., 2002; Zhao et al., 2003). In contrast co-expression of Brn-3a with p53 or p73 increases expression of \textit{p21CIP1/Waf1} but antagonizes the expression of the p53/p73 apoptotic target \textit{bax} (Budram-Mahadeo et al., 2002), as well as \textit{noxa} and so contribute to increased survival and cell cycle arrest. Whether Brn-3a has similar biological impacts on p63 remains to be established.

The ability of Brn-3a to differentially modulate expression of p53 target genes appears to be achieved by different mechanisms. Whilst Brn-3a binding sites are required for it to repress p53 mediated transcription on the \textit{bax} promoter (Budram-Mahadeo et al., 2002), it can repress activity on the \textit{noxa} promoter by direct protein-protein interaction with p53. In contrast, on the \textit{p21CIP1/Waf1} promoter, Brn-3a appeared to mediate its effect by recruitment to the basal transcriptional complex (Perez-Sanchez et al., 2002). These findings demonstrate the complexity of regulation of expression of genes associated with cell death/survival by Brn-3a.

In this regard, it is therefore possible that during neuronal development, cells which co-express Brn-3a and p53 and/or p73 will survive because of the repression of pro-apoptotic factors whereas co-operation on \textit{p21CIP1/Waf1} promoter would enhance cell cycle arrest. Additionally, Brn-3a increases the expression of genes involved in neuronal differentiation (the neurofilament genes, $\alpha$-internexin, Snap-25) and neurotrophic receptors (TrkA) which would enhance differentiation of surviving cells into mature neurones. In agreement with this hypothesis it has been demonstrated that in neuronal tissue taken from Brn-3a $\sim$/- mice compared with wild type, loss of Brn-3a resulted in significant increases in levels of both Bax and Noxa at specific times during development. Both Bax and Noxa proteins are elevated by E14.5 in Brn-3a $\sim$/- embryos compared with wild type litter mates at a time that coincides with increased apoptosis in these mutants (Eng et al., 2001). By E16.5 when most neurones which would normally express Brn-3a are lost in \textit{Brn-3a} $\sim$/- embryos, the levels of Bax and Noxa proteins remained similar in the wild type embryos compared with the
mutants. Therefore in Brn-3a expressing cells such as sensory neurones, it is likely that the expression of bax and noxa are controlled by the ability of this transcription factor to alter p53/Tap73 mediated gene transcription.

Thus, the increase in pro-apoptotic Bax and Noxa, in the absence of Brn-3a, may result from lack of antagonism of p53/TAp73 mediated transcription and contribute to the increased death of sensory neurones which would normally express Brn-3a. As well as having a clear role in naturally occurring apoptosis in many sensory and sympathetic neurones during development (Deckwerth et al., 1996; White et al., 1998; Cregan et al., 1999), Bax mediated apoptosis is also clearly important for loss of sensory neurones in Brn-3a -/- embryos since crosses of Brn-3a KO with Bax KO resulted in rescue of many sensory neurones that would normally express Brn-3a (Ma et al., 2003). However, there is still apoptosis in some sensory neurones which is independent of Bax and it is likely that other pro-apoptotic factors contribute to the fate of these cells. The finding that Brn-3a antagonizes p53/TAp73 activity on the pro-apoptotic noxa promoter, as well as the bax promoter, suggests that during normal development, the interaction of Brn-3a with p53/TAp73 will be important in determining the fate of neuronal cells which co-express these proteins by the ability of Brn-3a to repress p53/p73 target genes involved in apoptosis. Thus the transcriptional regulation of p53 and TAp73 function by Brn-3a describes an alternative mechanism by which this transcription factor can control the fate of cells which express it and supports previous findings showing that Brn-3a is a critical regulator of neuronal cell fate (Smith et al., 1997; Ensor et al., 2001; Budram-Mahadeo et al., 2002; Faulkes et al., 2004).
CHAPTER 5
Co-expression of Brn-3a with p53 and p73 proteins in Neural Crest cell cultures

5.1 Introduction

Sensory neurones in the peripheral nervous system (PNS) arise from neural crest cells (NCC). These are pluripotent neuroepithelial cells that migrate from the neural tube during early development and colonize target tissues, where they differentiate into specific cell types. The cell types formed depend both on their anterior/posterior origin in the neural tube, the environment they encounter as they migrate and on their ultimate destination in the periphery (Dupin et al., 1993; Sieber-Blum, 2000). The factors that influence the survival and fate of these cells are still poorly understood, but transcription factors expressed in these cells appear to play a critical role in their specification and subsequent differentiation into distinct phenotypes (Sieber-Blum, 2000) (refer to sections 1.16.2).

Brn-3a is detected in the migrating neuronal precursors arising from the neural tube as early as E9.0. This transcription factor is expressed in a sub population of neuronal precursor cells destined for a sensory lineage (Fedtsova and Turner, 1995; Greenwood et al., 1999) and is considered a marker of sensory neuronal precursor and differentiated sensory neuronal cells. Brn-3a plays a critical role in the survival and differentiation of sensory neurones by regulating expression of target genes associated with survival and differentiation either directly or indirectly e.g. via its interaction with p53 or p73. In the ND7 neuronal cell line model, co-expression of Brn-3a with p53 or TAp73 isoforms resulted in increased survival and cell cycle arrest, whilst co-expression of Brn-3a and ΔNp73α resulted in increased survival but did not increase cell cycle arrest. However the effects of the Brn-3a/p73 interaction in progenitor cells, such as those that arise from the neural crest are not known.

Extensive studies have shown that p53 and TAp73 play critical roles in the pathways that determine cell death in the developing and mature nervous system (Aloyz et al., 1998; Pozniak et al., 2000), but their role in neuronal differentiation though less well studied, is now shown to be important in cell fate determination. Eizenberg et al (1996) demonstrated
transient up-regulation of p53 in sympathetic neurones undergoing differentiation. A role for p73 in neuronal differentiation is suggested by the findings that RA-induced differentiation of a mouse neuroblastoma line depends on p73, and overexpression of p73 in these cells induces their differentiation in the absence of RA (De, Laurenzi et al., 2000).

In this regard a role for p53 with Brn-3a in sensory neuronal differentiation has been demonstrated. Nuclear p53 expression was shown in a population of Brn-3a positive neural crest-derived cells displaying distinct morphology of differentiated neurones, implicating p53 in the differentiation of sensory neurones. p53 expression in these differentiated neurones was further confirmed by co-localization of p53 with the differentiation marker NF-160. Furthermore induction of p53 by cisplatin treatment of NCC resulted in p53 co-expression with activated caspase-3, which is a marker of irreversible apoptosis, but also there were several p53 expressing populations which were negative for activated caspase-3 staining and had intact flattened morphology that suggested they were differentiated cells (Hudson et al., 2004). This observation further supports the hypothesis that expression of p53 in cells arising from the neural crest is not just associated with apoptosis. Thus p53 is associated with differentiation of some NCC and the co-expression of other factors such as Brn-3a can enhance p53 mediated differentiation by modulating its apoptotic effects.

In order to investigate the functional effect of the interaction between Brn-3a and p73 during sensory neuronal development, analysis of the expression of p73 in Brn-3a positive neural crest-derived cells destined for sensory neuronal lineage were carried out in a similar manner to p53. The importance of the Brn-3a/p53 and Brn-3a/p73 co-expression in determining cell fate was further analyzed using NCC cultured from both wild type and Brn-3a/-/- embryos.

5.2 Expression of p53, TAp73, ΔNp73 and Brn-3a in NCC.

Brn-3a expressing cells derived from trunk neural crest cultures have previously been shown to be destined for a sensory lineage (Greenwood et al., 1999), but the expression of p53 and the pro-apoptotic and anti-apoptotic isoforms of p73 (TAp73 and ΔNp73, respectively) had not been studied in these NCC. Therefore to determine the expression levels of p53, TAp73 and ΔNp73 in NCC, primary neural tube explants were prepared from E9.5 mouse embryos by dissecting out the portion of neural tube corresponding to the trunk level (somites 7
to 18). After separation of the fetal membranes, explants were enzymatically treated to separate the neural tube from the ectoderm and somites and then transferred onto poly-lysine and fibronectin coated coverslips in DMEM supplemented with 10% fetal calf serum (FCS), basic fibroblast growth factor (bFGF), neurotrophin-3 (NT-3) and antibiotic/antimycotic to prevent infections. Explants were maintained in a humidified incubator with 5% CO$_2$ at 37°C. After 24 hrs, the neural tube explant was removed and discarded, and the NCC cultures were maintained for the required period, replacing the media every 2-3 days (see Materials and Methods). Cells were then harvested for extraction of total RNA after 3, 5, 7, 8, 9 and 12 days. Brn-3a expression was also included to determine the time of maximal expression, in order to facilitate subsequent studies to analyze whether p53 and the p73 proteins are localized in Brn-3a positive cells. Because of the limited amount of RNA obtained from each neural crest culture, changes in the levels of Brn-3a, p53 and p73 mRNA were quantified by using real-time (quantitative) reverse transcriptase-polymerase chain reaction (qRT-PCR). RNA obtained from the cultured NCC was digested with DNase 1 (to remove genomic DNA) and cDNA was synthesized from approximately 0.1 μg total RNA by using Superscript enzyme (Invitrogen). qRT-PCR for specific mRNA was carried out on the MJ Opticon by using primers designed for use with the Sybr green detection system. Conditions were optimised for each set of primers used to amplify Brn-3a, p53, TAp73 and ΔNp73 to ensure that a single product was seen. A set of standards was generated by serial dilution of known amounts of each aplicon, and these standards were used to generate standard curves in all subsequent experiments. The transcript encoding the invariant GAPD was amplified and used to correct for variation in RNA prepared from different samples (see Materials and Methods).

The standards preparation for quantification, for Brn-3a, p53, TAp73 and ΔNp73 are shown in Fig 5.1a + b. Aliquots of the same standard curves were used in all experiments to allow for comparison between experiments. Fig 5.1c shows an example of the quantification of a set of samples (cDNA from NCC harvested for RNA at the relevant time points) for each aplicon. Fig 5.1d shows the melting curve for each aplicon to confirm the specificity of amplification. Initially the melting curve for GAPD showed a smaller peak at 76°C which represents primer dimers in addition to the major peak at 85°C that represents the GAPD product. The smaller peak was eliminated by the addition of an extra step, at 80°C, for 10
i) Quantification of Brn-3a mRNA by Real-Time PCR

- a) standards
- b) standard curve
- c) standard curve + samples
- d) melt curve

ii) Quantification of p53 mRNA by Real-Time PCR

- a) standards
- b) standard curve
- c) standard curve + samples
- d) melt curve
iii  Quantification of TAp73 mRNA by Real-Time PCR

a) standards  b) standard curve  c) standard curve + samples

iv  Quantification of ΔNp73 mRNA by Real-Time PCR

a) standards  b) standard curve  c) standard curve + samples
Quantification of GAPD mRNA by Real-Time PCR

- **a) standards**
- **b) Standard curve**
- **c) Standard curve + samples**
- **d) melt curve**
- **e) melt curve**

Figure 5.1: Measurement of Brn-3a, p53, TAp73 and ΔNp73 mRNA in neural crest-derived cells maintained for different times in culture by qRT-PCR using primers designed specifically to amplify Brn-3a, p53, TAp73 or ΔNp73.

For each aplicon a) shows the standards generated following serial dilution of known amount of aplicon. Red, 1000 fg/μg green 500 fg/μg, purple 100 fg/μg, yellow 1 fg/μg, pink 0.1 fg/μg, blue 0.001 fg/μg, brown 0.00001 fg/μg b) Plot of the standards gives rise to a linear curve. c) Plot showing standards together with samples from one experiment. The samples fall within the range of the standards validating the use of the standard curve for quantification of unknown samples. d) The melting curve, for each aplicon, showing that conditions were optimized so that one product is detected. Whereas conditions for Brn-3a(i), p53(ii), TAp73(iii) and ΔNp73(iv) gave rise to a single peak, Initial conditions for GAPDH(v) resulted in two peaks, with the lower melting peak representing primer dimers in addition to the major peak at 85°C that represents the GAPD product. e) The smaller peak was eliminated by the addition of an extra incubation step at 80°C for 10 second just before fluorescence reading.
seconds just before florescence reading (the plate read step), which resulted in melting of primer dimers. This was used in all subsequent PCR reactions when amplifying GAPD, to ensure only the fluorescence emitted from the GAPD product was detected.

Analysis of mRNA prepared from NCC at different times in culture demonstrated low levels of Brn-3a at day 3, which increased over time with a peak observed between 7 and 8 days in culture, followed by a decrease so that by day 9 the level of Brn-3a expression correspond to those observed at day 5 (Fig 5.2a). Since Greenwood et al. (1999) showed that Brn-3a expression is restricted to cells of sensory neuronal lineage in NCC, the change in Brn-3a expression may reflect changes in the relative ratio of this subset of cells in relation to the total cell population as well as possible changes in the levels of mRNA encoding Brn-3a in specific cells. In contrast, relatively low levels of p53 mRNA were detected. There is a modest increase from day 3 to day 5 after which the levels of p53 mRNA remained unchanged over the rest of the period of study. This may result from the expression of p53 in NCC destined for other lineages, such as sympathetic neurones, which also express p53 (Eizenberg et al., 1996).

The levels of p73 mRNA are shown in Fig 5.2b. Low levels of both TAp73 and ΔNp73 isoforms were found to be expressed in NCC. A modest increase in ΔNp73 mRNA is detected between day 3 and day 5 after which the levels of ΔNp73 mRNA remain unchanged over the rest of the period of study. TAp73 mRNA levels is detected at a slightly higher level than ΔNp73 at day 3. However between days 3 and 8 the level of TAp73 mRNA decreases, after which there is a significant increase in the levels of TAp73 mRNA (p< 0.05) which continues until day 12 which was the last time point collected in these studies. As TAp73 has been shown to be up-regulated during neuronal differentiation (De Laurenzi et al., 2000), the increase in TAp73 expression after day 8 may reflect an increase in the number of neuronal cells undergoing differentiation in the cultured NCC at this time. The initial modest increase in mRNA levels of the anti-apoptotic ΔNp73 isoform between day 3 and 5 may reflect survival of the neuronal precursor cells in the neural crest cultures, prior to differentiation.
Expression of Brn-3a and p53 in neural crest derived cells maintained in culture over 3-12 days

Figure 5.2: Expression of Brn-3a, p53, full length TAp73 and N-terminal truncated ANp73 in neural crest-derived cells maintained in culture for different times as established by qRT-PCR using primers designed specifically to amplify either Brn-3a, p53, TAp73 or ANp73

a) Whereas Brn-3a levels peak between day 7 and 8 in culture, there was a modest increase in p53 mRNA between day 3 and 5, followed by a relatively steady level over the time course studied.

b) Low levels of both TAp73 and ANp73 isoforms were expressed in NCC. Initially ANp73 mRNA levels increase above the levels of TAp73. After the initial modest increase in ANp73 levels between day 3 and 5, the levels of ANp73 mRNA remains constant. In contrast at day 8, TAp73 levels significantly increase. As TAp73 expression is not restricted to Brn-3a expressing cells, the increase at this time can be contributed by many cell types that express TAp73 in this culture. Amplification of the invariant GAPD was used to equalize for variation in mRNA from different samples. The results represent the data from three independent cultures.
5.3 Localization of both ΔNp73 and TAp73 isoforms in a subset of Brn-3a positive NCC

In order to investigate the co-expression of these factors in NCC, immunostaining experiments were carried out in NCC grown in DMEM supplemented with 10% FCS and neurotrophic factors NT-3 and FGF. After 7 days, when Brn-3a levels are high, the NCC was fixed in 4% paraformaldehyde (PFA) and processed for immunostaining (see methods). Initial studies were carried out to assess the expression of Brn-3a in the NCC cultures. As shown in figure 5.3, two morphologically distinct populations of Brn-3a positive cells were observed within the neural crest cultures which is in accordance with previously published data (Greenwood et al., 1999). The typically smaller, more rounded Brn-3a expressing cells (figure 5.3a) that were more commonly found nearer the site of migration, were clearly distinct from the larger, more flattened Brn-3a positive cells, many of which display neurite outgrowth (figure 5.3b). Greenwood et al (1999) previously showed that the smaller Brn-3a-positive population of cells were neuronal precursor cells fated for the sensory lineage that were still undergoing cell division as demonstrated by bromodeoxyuridine (BrdU) incorporation. These cells were also negative for the differentiation marker NF-160, indicating an undifferentiated, dividing phenotype. However the larger Brn-3a positive cells were differentiated sensory neurones that also expressed DRG11 which marks differentiated sensory neurones and NF-160. The observation that Brn-3a is expressed in precursor cells that are still dividing, but clearly destined for a sensory lineage as well as in differentiated sensory neurones is interesting, suggesting that Brn-3a expression may commit neuronal progenitor cells to a specific cell fate and underscores its importance in determining the fate of these cells.

Co-localization studies were undertaken to look at p53 expression in Brn-3a expressing cells. The results shown in Fig 5.4 show that nuclear p53 is only observed in the more flattened Brn-3a positive cells with neurite extensions but not in the smaller, more rounded Brn-3a positive cells (Conditions for Brn-3a/p53 co-expression were set up and carried out by Jennifer Podesta, ICH).

To investigate whether p73 co-localized with Brn-3a in cells derived from neural crest, co-immunostaining experiments were carried out in NCC grown in culture for 7 days as
Figure 5.3: Brn-3a is expressed in two morphologically distinct populations in Neural crest-derived cell cultures.

Brn-3a protein was observed in two subsets of neural crest-derived cells with distinct morphology characteristics. The smaller more rounded cells are shown in (a) which are typically nearer the site of migration, whereas the larger, more flattened cells with neurite processes are shown in (b). Neural crest cells were fixed in PFA after 7 days in culture and immunostained for Brn-3a expression (Brn-3a mouse Mab, Chemicon). Magnification x40
Figure 5.4: Nuclear localization of p53 with Brn-3a in larger neural crest derived cells

Larger more flattened cells express both Brn-3a (a) and p53 (b) and the proteins are co-expressed in the nucleus (c). In contrast, smaller Brn-3a positive cells (asterisks) are negative for p53 expression. Neural crest cells were fixed in PFA after 7 days in culture and immunostained for Brn-3a expression (Brn-3a mouse Mab, Chemicon) and p53 expression (p53 sheep PAb, PC35, Oncogene). Magnification x60
described previously. Three different antibodies were used to detect the p73 proteins. These include the anti-p73-ΔN (p73-ΔN) which was raised against the first 16 amino acids of ΔNp73 and thus specifically recognises the ΔNp73 isoform only, the anti-p73-SAM (p73-SAM) which was raised against the SAM domain and so recognizes TAp73α and ΔNp73α only (the only two p73 isoforms that contain the SAM domain) and anti-p73-Total (p73-Total) that recognizes all isoforms, i.e. ΔNp73 isoforms α, β and C-terminal isoforms TAp73α, β, γ, ε, δ and ζ. (All three p73 antibodies were a kind gift from Emre Sayan, University of Leicester).

As shown in figure 5.5i, the p73-SAM antibody could positively stain Brn-3a expressing cells with the more flattened phenotype. This antibody recognises both TAp73α and ΔNp73α. Within this population it can be seen that some of these cells that co-express Brn-3a and p73, have neurite outgrowth indicative of differentiated neurones (fig5.5i a-d). In contrast, there was no apparent co-localization in the smaller, more rounded Brn-3a positive cells that were commonly found near the site of migration (fig5.5ii a-c). Co-immunostaining experiments using p73-ΔN or p73-Total antibodies with Brn-3a also gave the same pattern of co-expression observed with p73-SAM, in that no p73 protein was observed in the small rounded Brn-3a positive population, but co-expression was seen in the larger Brn-3a positive population (data not shown). Furthermore, all 3 antibodies appeared to stain equally well. Thus, as was the case with p53, the cells that co-expressed Brn-3a and p73 were morphological distinct from those expressing Brn-3a alone.

Upon closer inspection at higher magnification, cells co-expressing Brn-3a and p73 recognised by p73-SAM (detects both ΔNp73α and TAp73α) and p73-Total, shows localization of p73 protein being cytoplasmic as well as nuclear (Fig 5.6i p73-SAM antibody and Fig 5.6iii +iv p73-Total antibody). However the ΔN specific antibody showed mostly cytoplasmic localisation in Brn-3a positive cells (Fig 5.6ii p73-ΔN antibody). This suggests that the cytoplasmic staining seen with the p73-SAM and p73-Total antibodies is likely to be the ΔNp73 form whereas the nuclear staining detected by p73-SAM and p73-Total in Brn-3a positive cells is likely to be the TA forms. This suggests parallel roles between Brn-3a/TAp73 and Brn-3a/p53 in which p53 is also found in the nucleus when co-expressed with
Figure 5.5: p73 co-localizes in a subset Brn-3a positive cells displaying distinct morphology of differentiated neurones.

**Fig 5.5i** Expression of p73 (b, c) is clearly observed in the larger Brn-3a expressing cells (a, c) which display a flattened morphology and neurite outgrowth (d) but not in the more rounded Brn-3a positive cells that were more commonly found near the site of migration (**fig 5.5ii a-c**). Neural crest cells were fixed in PFA after 7 days in culture and immunostained for Brn-3a expression (Brn-3a mouse Mab, Chemicon) and p73 expression (p73-SAM, rabbit PAb). Magnification X20
Figure 5.6: Examples of localization of p73 protein in NCC derived cells.

i). Staining with p73-SAM antibody that detects both TAp73α and ΔNp73α expression shows p73 expression in the large flattened Brn-3a positive cells. In these cells p73 can either be cytoplasmic (two bottom cells) as well as nuclear (asterisk). Hoechst staining is shown insert of phase, with the nucleus of the cells co-expressing Brn-3a and p73 are marked (asterisk). Neural crest cells were fixed in PFA after 7 days in culture and immunostained for Brn-3a expression (Brn-3a mouse MAb, Chemicon) and p73 expression (p73-SAM, rabbit PAb). Magnification X40
Figure 5.6 continued: ii) Staining with p73-ΔN antibody to detect ΔNp73α expression only, shows that ΔNp73α is also expressed in the large flattened Brn-3a positive cells and that staining is mostly cytoplasmic. Neural crest cells were processed for immunostaining with Brn-3a and p73-ΔN antibodies. Magnification X40
Figure 5.6 continued: iii) Staining with p73-Total antibody which detects all isoforms of TAp73 and ΔNp73 expression shows that expression of p73 in the large flattened Brn-3a positive neural crest cells can be evenly distributed in both the nucleus and cytoplasm or iv) shows predominantly nuclear staining. Hoechst staining is shown, insert of overlay. Cells were processed for immunostaining with Brn-3a and p73-Total antibodies. Magnification X40.
Bm-3a. Thus, similar to p53, TAp73 may also have a role in sensory neuronal differentiation. The availability of good TA specific antibodies will help clarify the location of ΔN versus TA proteins in cells co-expressing Bm-3a.

Both p73-SAM and p73-Total antibodies also stained other NCC that were not Bm-3a positive. Some of these cells appeared to be undergoing apoptosis as assessed by morphological changes in the NCC. Therefore in NCC, TAp73 is co-expressed in Bm-3a positive cells that are more differentiated but is also seen in Bm-3a negative cells that have the morphology indicative of cells undergoing apoptosis e.g. nuclear condensation and DNA fragmentation (Fig 5.7).

This is an interesting observation as it supports a crucial role for Bm-3a in determining the pathway taken by p73 (by antagonizing p73 mediated activation of pro-apoptotic genes such as bax and noxa, whilst co-operating on the p21^{CIP1/WD40} promoter) when co-expressed during development and thus in controlling the fate of these cells. Hence, under the conditions used in these experiments, p73 is detected in Bm-3a expressing neurones that display flattened morphology with some displaying neurite extensions suggestive of differentiated neurones, whereas its expression in cells not expressing Bm-3a demonstrate morphology that is indicative of apoptosis. However this is assessed on morphology alone. Co-immunostaining with p73 and a marker of apoptosis (i.e. Caspase-3 or Bax) would need to be carried out to confirm the presence of p73 proteins in cells undergoing apoptosis.

It is clear that as was found with p53, the p73 proteins are also co-expressed with Bm-3a in a subpopulation of NCC. Under the growth conditions used in these experiments, p73 shows cytoplasmic as well as nuclear expression in cells co-expressing Bm-3a. Similar to p53, p73 function is regulated by cellular location, with sequestration in the cytoplasm being a mechanism by which its activity is regulated. As nuclear p73 expression is seen in Bm-3a positive cells with neurite outgrowth, it suggests that p73 proteins are associated with differentiated sensory neurones and may have a role in this process.
Figure 5.7: p73 is expressed in Brn-3a positive and Brn-3a negative cells

Co-immunostaining studies show that p73 (as detected by p73-SAM) is expressed in cells not expressing Brn-3a that showed distinct morphological changes indicative of apoptotic cells (asterisk), as well as being co-expressed with Brn-3a in intact cells with flattened morphology that would suggest that these cells are differentiated. Magnification X40.
5.4 Co-expression of Brn-3a and TAp73 but not ΔNp73 in differentiated sensory neurones

The co-expression of Brn-3a and p73 in NCC with flattened morphology, many of which showed extensive neurite outgrowth, suggested that these cells may be undergoing differentiation or were already differentiated. Previous studies have shown that nuclear p53 is co-expressed with NF-160 (which marks differentiated neurones), in cells that had similar morphology to those that co-expressed p53 and Brn-3a. Similarly studies to look at co-localization of Brn-3a and NF-160 showed that the larger Brn-3a positive cells with neurite extensions also expressed the neurofilament protein, but co-expression was not observed in the smaller Brn-3a positive cells (see figure 5.8iii). Whilst triple immunostaining was not possible in these experiments, given the similar morphology of cells that co-expressed Brn-3a/p53 and NF-160/p53, it is likely that the NF-160/p53 co-expressing cells will also be Brn-3a positive, supporting the hypothesis that p53 is involved in differentiation of sensory neurones (Hudson et al., 2004).

Given the results obtained for co-localisation of Brn-3a and p73 in the NCC cultures, the next step was to investigate whether the large flattened cells expressing p73, as seen when co-expressed with Brn-3a, were differentiated neurones. Similar co-immunostaining experiments were carried out using an antibody to NF-160 and either p73-SAM to detect both TAp73α and ΔNp73α or p73-AN to detect the truncated form of p73 only. As shown in figure 5.8i, the p73-SAM antibody stained a number of cells expressing p73 proteins that were also positive for neurofilament expression. In contrast figure 5.8ii shows that NF expression is not co-localised with ΔNp73 although other cells expressing NF were seen in this culture. These co-immunostaining results suggest that there are 2 populations of p73 positive cells, one identified by p73-SAM Ab which will include TAp73α and ΔNp73α and the other by p73-AN that only detects the ΔNp73 proteins. Thus the co-expression of p73 with NF-160 observed with the p73-SAM antibody is likely to be the TA isoform, p73α but not the ΔNp73α isoform. As with the p53 studies, TAp73/NF-160 and Brn-3a/NF-160 were observed in similar cell types so it is likely that NF-160/TAp73 co-expressing cells will also be positive for Brn-3a.
Figure 5.8i

P73-SAM pAB-Rh  NF-160 mAB-FITC  NF-160/p73-SAM overlay  Phase

Figure 5.8ii

P73-ΔN pAB-Rh  NF-160 mAB-FITC  NF-160/ΔN overlay  Phase

Figure 5.8iii

Brn-3a pAB-Rh  NF-160 mAB-FITC  Brn-3a/NF-160 overlay

Figure 5.8: Co-expression of NF-160, a marker of differentiated neurones with TAp73 but not with ΔNp73

5.8i) demonstrates that some p73 expressing cells (TAp73α and ΔNp73α as detected by p73-SAM) were also positive for NF-160. These large, flattened cells had intact morphology and neurite extensions characteristic of cells either undergoing differentiation or differentiated. ii) Cells expressing ΔNp73 (stained for by ΔN specific antibody) were negative for NF-160 expression suggesting that the co-localization of p73 with NF-160 shown in figure 4.8i is due to the TA isoform that is detected by p73-SAM. This suggests that not all large, flattened Brn-3a positive cells are differentiated and that these cells co-express ΔNp73 while differentiated Brn-3a cells co-express TAp73. Insert shows that differentiated neurones are stained with NF-160 in this culture iii) co-localization of NF-160 with Brn-3a protein in the larger more flattened Brn-3a positive cells (*), but not in the smaller Brn-3a positive cells confirms that a subset of Brn-3a positive neural crest cells are differentiated.
in utero as a result of hindbrain exencephaly. This indicated that p53 may play a role in normal development of the embryonic nervous system (Armstrong et al., 1995). This partial penetrance observed in the p53 -/- phenotype was difficult to interpret due to the identification of p73 and p63, both of which are expressed in the embryonic nervous system and which could potentially play a compensatory role in a p53-/- background. In order to assess whether p73 compensates for p53 function in sensory neuronal development, the expression of p73 in relation to Brn-3a was analyzed in NCC cultures prepared from normal and p53 KO embryos.

Figure 5.9 shows the location of staining of NCC using p73-SAM that recognizes proteins including TAp73 and ANp73, in NCC cultured from p53 +/-, +/- or -/- embryos. As before, co-localization of p73 with Brn-3a was observed in the larger, more Brn-3a positive cells in NCC cultures prepared from p53 wild type (fig5.9i), heterozygous (fig5.9ii) and KO (fig5.9iii) embryos, while no co-localization was found in the smaller Brn-3a positive cells (data not shown). Similar results were obtained when the experiments were repeated with p73- AN and p73-Full antibodies to detect just the ANp73 isoforms (data not shown). In these co-localization experiments, there were no gross differences in the expression of p73 in relation to Brn-3a observed in the NCC cultures prepared from the different p53 genotypes. Lack of gross differences in p73 expression across NCC cultured from p53 +/-, +/- and -/- made it difficult to ascertain if p73 compensates for loss of p53 in sensory neuronal development. To better answer this question, it would be necessary to assess the levels of p73 mRNA in NCC cultured from p53 -/- embryos compared to the wild type. If p73 compensates for loss of p53, then it would be expected that the expression of p73 would be increased in NCC taken from p53 -/- embryos. But due to time constraints, this level of investigation was not pursued.
Figure 5.9: p73 expression in relation to Brn-3a is not significantly altered in NCC grown from p53-/- or p53-/+ embryos.

NCC cultures prepared from i) wild type, ii) p53-/+ or iii) p53-/- embryos were immunostained for Brn-3a and p73 expression (p73-SAM which recognizes both TA and ΔNp73 isoforms). As before, co-expression was found in the more flattened Brn-3a positive population while no co-localization was found in the smaller, more rounded Brn-3a population. No changes in p73 expression or location were detected in the NCC cultured from the different p53 genotypes, in relation to Brn-3a.
embryos were grown in culture for 7 days, before treatment with 20 \( \mu \)M of \( \alpha \)-tubulin for 24 hr to induce p53/p73 expression. The NCC were then immunostained for activated caspase-3 which is used as a marker of cells undergoing apoptosis. Positive cells were counted in matched NCC cultures from Brn-3a -/-, +/- and +/+ and used as a measure of cells undergoing apoptosis.

As shown in figure 5.10, a significantly greater number of caspase-3-positive cells were found in cultures prepared from Brn-3a -/- embryos compared with wild type (students t-test, \( p<0.0005 \)). This suggests that, in the absence of Brn-3a protein, p53/p73 expression results in an increase in the number of cells undergoing apoptosis compared with cultures prepared from wild type littermates.

Cultures prepared from heterozygous (+/-) mice demonstrated a response similar to that with wild type. This is as expected since Brn-3a +/- embryos develop normally as a result of a positive feedback mechanism (Trieu et al., 1999; Trieu et al., 2000). The expression of Brn-3a to compensate for loss of one allele. Thus, induction of p53/p73 in the absence of Brn-3a leads to increased apoptosis in NCC, supporting the hypothesis that Brn-3a is required to modulate the effects of p53/p73 in cells that express these proteins.
Levels of apoptotic death observed in cis-platinum treated NCC prepared from Brn-3a wild type, heterozygous and Knock Out littermates

![Graph showing % Caspase-3 positive NCC for Brn-3a WT, Brn-3a Het, and Brn-3a KO.]

- Brm-3a WT: X=24±/-.10.5
- Brm-3a Het: X=27±/-.11.6
- Brm-3a KO: X=54±/-.11.9

**Figure 5.10:** NCC cultured from Brn-3a +/- embryos demonstrated significant apoptosis upon stimulation of p53/TAp73 expression with cis-platinum

Measurement of apoptotic cells immunostained for activated caspase-3 showed a significant increase in apoptotic cells in NCC cultures prepared from Brn-3a +/- embryos compared with cultures prepared from wild-type littermates. The level of apoptotic cells in cultures prepared from Brn-3a +/- littermates were similar to wild type, which is expected as the levels of p53/TAp73 increases in the Brn-3a heterozygote due to a positive feedback mechanism. The data is based on counts from cultures taken from at least six independent embryos.
neural crest cultures in which Brn-3a is expressed in progenitor cells that are sensory lineage, as well as in differentiated sensory neurones, allowed for the mechanism by which Brn-3a mediates its effect in these cells, with specific association with p73.

The levels of mRNA encoding p53, TAp73 and ΔNp73 were assessed in the over a time period of 3-12 days in culture, using qRT-PCR and these were Brn-3a mRNA expression over the same time period. The levels of p53 expressed at relatively low but steady levels over the time course studied, which with findings that p53 is not restricted to Brn-3a expressing cells in this culture p73 isoforms showed that ΔNp73 mRNA levels modestly increase between days at which the levels of ΔNp73 mRNA remained constant. TAp73 mRNA is detected a higher level than ΔNp73 at day 3 which is followed by a slight decrease in this until day 8. This is consistent with findings that TAp73 induces expression of ΔNp73 (Grob et al., 2001;Kartasheva et al., 2002;Vossio et al., 2002;Nakagawa et al., 2003). At day 8, there is a significant rise in TAp73 mRNA. been shown to be up-regulated during neuronal differentiation (De, Laurenzi increase in TAp73 expression may reflect an increase in the number of cells undergoing differentiation in the cultured NCC at this time. The initial small mRNA levels of the anti-apoptotic ΔNp73 between day 3 and 5 may be a survival preceding differentiation of the neuronal cells in the neural crest cult of ΔNp73 in neuronal survival has been well characterised in developing neuron (et al., 2000;Lee et al., 2004). Furthermore recent evidence suggests that Δ are required to maintain the survival of mature sensory neurones (Walsh et al., 2000).

Brn-3a mRNA levels showed a significant increase in cells that were kept in days, followed by a decrease to basal levels. The striking changes in Brn-3a-
3a expressing cells in the cultures may contribute to the apparent decrease of Brn-3a total RNA. It is interesting that TAp73 levels increases at day 8 while Brn-3a tends to decrease at this time. However since TAp73 expression is not restricted to Brn-3a expressing cells, the increase at this time may be contributed by many cell types expressing TAp73 in this culture. Hence these results provide an indication of changes in the mRNA encoding Brn-3a, p53, TAp73 and ΔNp73 in the total cell population used to determine the best time for subsequent co-localization studies of Brn-3a destined for a sensory neuronal lineage.

The data so far generated has shown that Brn-3a can physically interact with isoforms of p73 and can modulate p73 transcriptional activity and functional cycle and apoptosis in the ND7 cell line in a similar manner shown previously. The ability of Brn-3a to synergize with p53 or TAp73 on the p21^CIP1/Waf1 promoter repress p53 or TAp73 mediated activation of the bax and noxa promoters suggests plays a critical role in determining the cellular status and survival of neural crest cells which co-express these proteins. Thus co-immunostaining experiments were carried out in neural crest derived cells which provides a dynamic model to investigate protein interactions. Furthermore Brn-3a expression has been well characterized in NCC cell populations. In agreement with published data (Greenwood) Brn-3a was detected in two distinct populations of NCC, a smaller more rounded Brn-3a positive population that were common to the site of migration and a Brn-3a positive population that displayed a larger cell morphology with many showing neurite extensions indicative of differentiated cells. In previous studies, p53 was shown to co-localise in the more flattened Brn-3a positive cells with neurite extensions but not in the smaller, more rounded Brn-3a positive cells.

In this study co-immunostaining experiments also demonstrated that p73 co-expressed with Brn-3a in a subset of neural crest cells. Thus, whereas Brn-3a was detected in all populations of NCC, co-expression with p73 was observed only in Brn-3a positive cells.
deduced by comparing the staining for p73 expression with the p73-SAM antibody with that obtained with the AN-specific antibody.

Interestingly, when co-expressed with Brn-3a, localisation of p73 protein with p73-ΔN Ab was mostly cytoplasmic, whereas p73-SAM and p73-Total antibodies showed both cytoplasmic and nuclear staining. This suggests that ΔNp73α is mostly cytoplasmic in these cells and the nuclear staining detected by the p73-SAM and p73-Total antibodies is likely to be the TA isoforms. This is an interesting observation, as like p53, these proteins are synthesized in the cytoplasm and must transport into the nucleus to exert its effect on downstream targets. Thus, subcellular sequestration of p73 or p53 in the cytoplasm inactivate their activity. Thus, the findings that p53 was always nuclear and TA isoforms of p73 also appear to be nuclear in Brn-3a positive cells, suggests that p53 is in an active state when co-expressed with Brn-3a.

Interestingly, under these growth conditions, the expression of TAp73 and ΔNp73 in NCC were observed in Brn-3a expressing cells with flattened morphology and membranes, while TAp73 was also detected in some cells not expressing Brn-3a but undergoing apoptosis as shown by morphological changes. These results support the hypothesis that Brn-3a can modulate TAp73 as well as p53 mediated cell failure, in which co-express Brn-3a and TAp73 and p53 are more likely to survive and proliferate because of the repression of pro-apoptotic bax and noxa and co-operation on the p73 promoter to promote cell cycle arrest.

To characterize the phenotype of p73 expressing cells further, co-immunostaining with NF-160 Ab and NF-160 Ab was carried out and this showed that TAp73 but not ΔNp73α co-localised with NF-160 in cells with flattened morphology. The differences in expression length and truncated forms of p73 with NF-160 suggests that the large fl
these cells may precede differentiation since both ΔNp73 and Brn-3a have been shown to protect neuronal cells from apoptosis (Smith et al., 1998; Pozniak et al., 2000; 2001; Ensor et al., 2001; Nakagawa et al., 2003). However the co-expression of Brn-3a in the large flattened cells and co-expression of TAp73/NF-160 and Brn-3a in the same cell types suggest that TAp73, as with p53, is associated with differentiation of neurones.

Hence, given these results it is possible that there is a switch in expression of isoforms during sensory neuronal differentiation, so that ΔNp73 is the major isoform expressed with Brn-3a in these cells preceding differentiation, whilst upon differentiation of these cells, TAp73 is the major isoform co-expressed with Brn-3a. Indeed, this supports the hypothesis that the co-operative expression of Brn-3a/ΔNp73 in one cell type may be an important factor in cell fate determination during sensory neuronal development.

The finding that TAp73 as well as p53 is associated with differentiation of sensory neurones is interesting as it is possible that TAp73 and p53 may co-operate or compete with each other during sensory neuronal differentiation. These proteins may also act as transcription factors on targets involved in neuronal differentiation. For example p73 but not p53 can activate the neuronal differentiation marker N-CAM during neuroblastoma cell line differentiation (De Laurenzi et al., 2000). Furthermore, TAp73 isoforms have been shown to be required to induce neuronal differentiation in a mouse neuroblastoma cell line, suggesting that TAp73 can directly affect differentiation of neuronal cells (De Laurenzzi et al., 2000). The observation that ΔNp73 is not co-expressed with NF-160 in sensory neurones would support the hypothesis that expression of this isoform in the Brn-3a positive cells, occurs preceding differentiation which may reflect its role as a pro-survival protein in the nervous system (Pozniak et al., 2000; Nakagawa et al., 2003). In addition the Brn-3a/ΔNp73 interaction may act to further enhance survival of these cells compared to the levels seen when either factor was expressed alone.
clear that triple co-immunostaining techniques are required to further confirm
of Brn-3a/TAp73/NF-160 and Brn-3a/ΔNp73 but not NF-160 in the same
experiments can also be done by combining in-situ for one factor with co-i
for other proteins. For example, analysing for p73 mRNA expression can
using either TA or ΔN-specific ribo-probes, followed by immunostaining for NF-
160 expression. This approach would also help to analyze ΔN versus TA
separately in these cells in the absence of good TA specific antibodies.

The results of the studies discussed above implicate TAp73 in sens
differentiation, which has already been demonstrated for p53. It is likely that during
development both p53 and p73 might contribute to both differentiation and
eurones depending on which other proteins are co-expressed with it. To further
the requirement for co-expression of p53/TAp73 with Brn-3a in determining

cultures were prepared from Brn-3a-/- embryos and induced for p53/TAp73

cis-platinum treatment. Induction of wild type p53/TAp73 proteins in these
loss of Brn-3a gave rise to significantly increased apoptosis compared to wi
grown in similar conditions. In support of this finding, neuronal progenitor
null embryos are formed but die by apoptosis at later stages of developme
(2001), so the increased cell death observed in Brn-3a-/- NCC must be associ

cells that would normally express Brn-3a. These findings suggest that Brn-3a
overcome the apoptotic pathway by repressing the expression of pro-apoptotic
bax and noxa that are stimulated by both p53 and TAp73 in specifi
development. Since p53 and p73 expression is not restricted to Brn-3a pos
conceivable that other factors in cells of other lineages may also interact with
survival and p53/p73 mediated cell cycle arrest, in a manner similar to that
observed in sensory neurones.

The co-expression of TAp73 with Brn-3a (and similarly the co-expressio
mediated transcription of pro-apoptotic factors such as \textit{bax} and \textit{noxa}, but TAp73/p53 mediated \textit{p21}\textsubscript{Clp1/Waf1} expression, would increase cell survival arrest. At the same time, direct activation by Brn-3a of neuronal target genes such as internexin, neurofilament, synaptophysin, Trk receptors and Snap-25 would promote differentiation of these cells. Also direct activation of neuronal target genes like N-CAM would further enhance differentiation.

The co-expression of \(\Delta\text{N}\text{p73}\) in a subset of Brn-3a positive cells which are differentiated but not in the small, rounded proliferating Brn-3a positive population suggests that \(\Delta\text{N}\text{p73}\) has a role in sensory neuronal survival just preceding differentiation, though both factors alone can directly affect the survival of cells that express them (Smith et al., 1998; Smith et al., 2001; Ensor et al., 2001; Zaika et al., 2002; Nakagawa et al., 2003) the promoter targets that are possibly modulated by the Brn-3a/\(\Delta\text{N}\text{p73}\) interaction that would result in increased survival of cells that co-express proteins are not yet known. However, it is possible that \(\Delta\text{N}\text{p73}\) may have co-ordinated interaction on some Brn-3a gene targets such as Bcl-2 and Bcl-\text{x}\textsubscript{L}.
preferentially switch on particular subsets of genes. The molecular basis for activation of particular sets of genes by the p53 family is not fully understood; molecular mechanisms are thought to contribute to target gene selectivity.

Covalent modifications have been shown to play a critical role in target protein selection. For instance, phosphorylation of serine 46 is required for p53 to efficiently transactivate the apoptotic p53AIP1 gene (Oda et al., 2000) and p73 acetylation by p300 has been shown to direct p73 to pro-apoptotic targets (Costanzo et al., 2002). Cellular proteins that interact with the p53 family members have also been demonstrated to dictate the choice of target genes that are activated by the p53 family members. Such proteins include the protein JMY which favors the transactivation of pro-apoptotic genes by p53 and the p53 response towards apoptosis (Shikama et al., 1999). The ASPP family members p53, p73 and p63 to pro-apoptotic targets (Samuels-Lev et al., 2001; Berg, 2004). In contrast, WT1 inhibits the pro-apoptotic effect of p53 and p73 (Scholz, 2000). The Brn-3a transcription factor has also been demonstrated to inhibit the apoptotic effects of p53 whilst enhancing p53 mediated cell cycle arrest (Buettner et al., 2002). (see below). Similarly this study shows that Brn-3a also interacts with the p53 family members and inhibits p73 mediated apoptosis but enhances p73 mediated cell cycle arrest.

The POU proteins are a family of gene regulators found in organisms as diverse as Drosophila, Xenopus, zebrafish and humans (reviewed by Anderson and Roelink). All members of this family have been demonstrated to play important roles in the development of specific cell types, particularly in the nervous system, where they are identified as powerful transcription factors. The Brn-3a and Brn-3b transcription factors belong to the class IV subgroup of the POU family transcription factors and have particularly high homology to Unc-86 which has been shown to be critical for the establishment of specific neuronal cell types, especially sensory lineages in C.elegans. It has been speculated that the Brn-3 transcription factors may have a similar role.
inferior olivary nucleus and medial habenula (Xiang et al., 1996; McEvilly et al., 1999). Thus it appears that the neurones that would normally express Bm-3a are necessary for neuronal survival and differentiation. In vivo and in vitro studies also showed that the N-terminal domain of Bm-3a is required for its survival effects whereas the POU domain of Bm-3a is sufficient for neuronal differentiation of cells that express Bm-3a (Lakin et al., 1995; Smith et al., 1998a; Smith et al., 1998b; Smith et al., 2001; Enser et al., 2001; Faulkes et al., 2001).

The ability of Bm-3a to enhance neuronal survival and differentiation was further elucidated by direct regulation of target genes associated with protection against apoptosis (Bcl-2 and Bcl-xL) and differentiation (the neurofilament genes, \( \alpha \)-internexin, synaptophysin and TrkA) (Budhram-Mahadeo et al., 1995b; Lakin et al., 1997c; Smith et al., 1998a; Smith et al., 1998b; Smith et al., 2001; Enser et al., 2001). In order to further elucidate the mechanisms by which the Brn-3a transcription factor promotes neuronal survival and differentiation, studies were undertaken to determine the physical interactions that occur between Brn-3a and other cellular proteins. It was demonstrated that interaction with other transcription factors would effectively increase the range of genes regulated by the Brn-3 proteins and provide further insight into the molecular mechanisms by which Brn-3a controls cell fate.

Initially, yeast 2 hybrid studies demonstrated association of Brn-3a with the p53 tumor suppressor protein and affinity chromatography showed a direct physical interaction between the POU domain of Brn-3a and the DNA binding domain of p53. Further studies using immunoprecipitation experiments showed that this interaction occurred \( \text{in vivo} \) (Budhram-Mahadeo et al., 1999). Critically this interaction resulted in differentially regulated expression of both Brn-3a and p53 mediated gene targets. Thus Brn-3a was shown to promote p53 mediated transcription of the \( p21^{\text{Clp1/Waf1}} \) gene associated with cell cycle arrest.
The p53 related gene, p73 shares high homology to p53 in the TAD, DBD and NTD (reviewed by Melino et al., 2003). The high homology shared between these genes particularly in the DBD lead to the idea that Brn-3a may also interact with p73 in p73 mediated cell fate. Moreover, p73-/- knock out mice exhibit severe neuronal defects highlighting the importance of the p73 protein in neuronal development, (Yan et al., 2002) further suggesting that a functional Brn-3a/p73 interaction may be important in the fate of particular neuronal cell types that co-express these proteins.

In contrast to p53, the p73 gene encodes multiple alternatively spliced C-terminal as well as N-terminally truncated isoforms (TAp73α–ζ and ΔNp73α-β) (Kaghad et al., 1997; Laurenzi et al., 1998;Ueda et al., 1999). The TA isoforms, which similar to p53, mediate cell cycle arrest or apoptosis, have been shown to be differentially regulated in various tissues and at different stages of differentiation, suggesting that they play distinct roles (Kaghad et al., 1997;Jost et al., 1997;Zhu et al., 1998;De, V et al., 1998b;Ueda et al., 1999). Furthermore, the ΔN isoforms act as dominant inhibitory proteins towards the TA and p53. Thus the TA and ΔN isoforms of p73 show pro-apoptotic and anti-apoptotic functions respectively (reviewed by Melino et al., 2002). This increased complexity could explain the distinct biological roles shown by the p53 and p73 proteins that have been identified (Zheng and Chen, 2001;Sasaki et al., 2003;Tullo et al., 2003).

In chapter 3, the physical interaction between Brn-3a and p73 and the functional significance of this association was investigated. Using affinity chromatography it was shown that Brn-3a could physically interact with the p73 isoforms tested (the TA isoforms p73
of p73 which was critical for this interaction, with contributions from the DBD and OD termini. The contribution of the DBD for this interaction was highlighted by findings that the POU domain of Brn-3a required the DBD for interaction but the OD domain interfered with and blocked the strong interaction with the intact Brn-3a protein. The essential requirement of the OD terminus for this interaction suggests that either Brn-3a contacts specific regions in the OD domain or the OD domain can only bind to tetrameric p73. The possibility that Brn-3a specifically binds tetrameric p73 is interesting as tetramerisation of p73 is required for it to transactivate gene expression.

Furthermore, the Brn-3a/p73 interaction was shown to be modulated by the C-terminal domain present in the various p73 isoforms so that the extended C-terminus specifically interacts with Brn-3a whereas the truncated variant of p73(a) retained 10.5% of IVT p73a whereas Brn-3a(l) retained 22.8% and 33.2% of IVT p73β, p73γ and p73δ respectively. Indeed deletion of either termini of the p73 C-terminus resulted in increased binding to both Brn-3a and Brn-3b compared to that observed with wild type p73. Such differential binding confirms that the C-terminus could be a determinant of precise biological outputs. Indeed Brn-3a(p73) can differentially regulate the activity of the different p73 isoforms on the bax promoter (see later).

The observation that the p73 protein interacts with both Brn-3a and Brn-3b is significant as the interaction occurs via the POU domain, which is highly conserved across the p73 family of proteins. Additionally, the longer isoforms Brn-3a(l) and Brn-3b(l) interacted with the p73 proteins with higher affinity than the shorter Brn-3a(s) and Brn-3b(s) isoforms. The isolated Brn-3a N-terminal alone did not interact strongly with p73, this suggests that the N-terminal domain might contribute to stronger binding of the p73 proteins, possibly due to the conformation of the longer Brn-3 isoforms that results in tighter binding. The interaction observed between the long and short forms of Brn-3a with the p73 proteins...
be involved in neuronal differentiation and apoptosis, then a possible function between TA$p73$ and Brn-3a(l) proteins may have consequences for the survival and differentiation of neuronal cells that express both these proteins in the developing system. The importance of the Brn-3b N-terminal domain found in Brn-3b(l) and Brn-3b(s) in carrying out specific functions remains to be determined.

The effect of the Brn-3a/p73 interaction was tested on the p53/p73 target genes and $bax$ as Brn-3a was shown to differentially regulate p53 transcription of. Furthermore the greater molecular complexity exhibited by p73 suggested that it might mediate differential regulation of the different p73 members on these target genes demonstrated to be the case on the $bax$ promoter.

As seen previously with p53, results in chapter 3 show that in the ND7 cells antagonized p73α and p73β mediated activation of the $bax$ promoter in reporter assays. This is reflected in a decrease of endogenous Bax protein upon co-expression of p73α or p73β compared with levels seen with p73α or p73β respectively. Similarly, ANp73α co-operates with p73α or p73β on the $p21^{CIP1/Waf1}$ promoter in reporter assays reflected in increased endogenous p21$^{CIP1/Waf1}$ protein levels in cells co-expressing with p73α or p73β compared with either p73α or p73β expressing cells. The antagonistic effects of Brn-3a on p73α or p73β mediated activation of the $bax$ promoter were also reproduced in the SAC demonstrating that these differential effects of Brn-3a on p73 mediated transcription were neuronal specific or dependent on the presence of p53 in these cells.

Importantly, the interaction of Brn-3a and the different p73 isoforms had disordered regulation of cell cycle arrest and cell survival in the ND7 neuronal cell lines. Brn-3a can co-operate with p73α and p73β but not with ΔNp73α in inducing cell death in contrast the anti-apoptotic effects of Brn-3a co-operate with ΔNp73α to further...
survival by both p53-dependent and independent mechanisms, with the latter involving inhibiting JNK which is activated by NGF withdrawal (Lee et al., 2004). This suggests that ΔNp73 can also enhance survival independently of p53 by interacting with as yet unknown transcription factor. Though the molecular mechanism remains to be determined, it is hypothesized that the Brn-3a/ΔNp73α interaction may act co-operatively on apoptotic targets such as the Bcl-2 and Bcl-xL, both of which are known targets of p53.

The ability of Brn-3a to directly repress the bax promoter and antagonise p53 activity on this promoter is interesting as Brn-3a-/- embryos show significant neuronal deficits as a consequence of increased apoptosis. Bax plays a critical role in many cells undergoing apoptosis in many neurones during development including sensory neurones which are known to express Brn-3a (Deckwerth et al., 1996; White et al., 1998). Genetic crosses of Brn-3a-/- with Bax-/- resulted in rescue of many sensory neurones which normally express Brn-3a (Ma et al., 2003). However there is still apoptosis in some of these neurones which is independent of Bax and it is likely that other pro-apoptotic genes contribute to the fate of these cells.

It was therefore tested whether Brn-3a could repress other p53/p73 target genes involved in apoptosis. Results in chapter 4 demonstrated that Brn-3a also inhibits p53 mediated activation of the noxa gene which encodes a BH3 only, pro-apoptotic protein of the Bcl-2 family (Oda et al., 2000). The mechanism by which Brn-3a inhibits activation of the noxa gene was further investigated. Unlike the bax promoter which contains two Brn-3a sites required for Brn-3a mediated repression, either directly or in concert with p53 (Perez-Sanchez et al., 2002), the noxa promoter contains no Brn-3a sites. Since Brn-3a could significantly antagonize p53 or p73 mediated stimulation of this promoter, this effect must arise from its ability to physically interact...
immunoprecipitation of Brn-3a in ND7 cells containing the wild type p53 site + promoter resulted in amplification of the promoter DNA but this was not obtained with the same promoter lacking the p53 site.

Therefore, the antagonistic effect of Brn-3a on p53/p73 mediated activation of noxa genes is interesting as it suggests that one mechanism by which Bm-3a promotes neuronal survival is by repressing p53/p73 target genes involved in apoptosis. In agreement with this hypothesis it has been demonstrated that in neuronal tissue taken from Brn-3a -/- embryos compared with wild type, loss of Brn-3a resulted in significant increases in the expression of Bax and Noxa at specific times during development. Both Bax and Noxa are elevated by E14.5 in Brn-3a -/- embryos compared with wild type litter mates and this coincides with increased apoptosis in these mutants (Eng et al., 2001). By E15.5, neurones which would normally express Brn-3a are lost in Brn-3a -/- embryos whilst Bax and Noxa proteins remained similar in the wild type embryos compared with the mutants. Therefore, in Brn-3a expressing cells such as sensory neurones, it is likely that expression of Bax and Noxa are controlled by the ability of this transcript to repress p53/TAp73 mediated gene transcription.

Thus Brn-3a was shown to repress two p53/p73 pro-apoptotic targets involved in the mitochondrial death pathway. It would be of interest to determine if Brn-3a can also repress p53/p73 targets that mediate cell death, such as Puma, which is shown to be an important mediator of p53/p73 induced apoptosis (Yu et al., 2001; Melino et al., 2004). And the ability of Brn-3a to repress p53/p73 target genes involved in the extrinsic death pathway of neuronal cell death.

The ability of Brn-3a to repress p73α and p73β mediated activation of the p21cip1/waf1 promoter whilst enhancing p73α and p73β mediated activation of the p21cip1/waf1 promoter...
by exon 11 and/or 12 may be required for the repressive effects of Brn-3a on bax transactivation. Furthermore, it was demonstrated that the region 424-501 from the C-terminal of p73C424 is required for binding of a co-activator by possibly disrupted by the presence of Brn-3a on the bax promoter. In contrast, the C-terminal was shown not to be important for Brn-3a mediated co-operation with p21^{CIP1/Waf1} promoter, thus this differential regulation of the p73 splice variant specific to the bax promoter.

Interestingly this region contains the PPPPY motif (residues 482-488, encoded underlined in Fig 3.18) that has been shown to interact with cellular proteins mediated transcription. The WW adaptor, Yes-associated protein (YAP), demonstrated to interact with the p73 PPPPY motif (Strano et al., 2001). YAP stimulate p73α mediated activation of the bax and mdm promoters in H1299 cells. The binding of YAP to p73 is necessary for the co-activation of p73 (Strano et al., 2001). Therefore it can be speculated that when bound to p73 on the bax promoter, disrupt the interaction between p73 and a co-activator such as YAP resulting p73 mediated bax activation. The identification of this co-activator would be discussed in chapter 3.

Therefore Brn-3a can differentially regulate transcriptional activation by the C-terminal isomers on the bax promoter. This is interesting as these TA splice different abilities to transactivate target genes and increase cell cycle arrest (De-Laurenzi et al., 1998; Ueda et al., 1999; Ueda et al., 2001). One mechanism these isoforms may achieve this is by interacting with cellular proteins which the selectivity of individual p73 members in controlling the expression of targets. The effect of Brn-3a on p73γ and p73δ mediated apoptosis was not study but it is anticipated that addition of Brn-3a would not increase survi.
The ability of Brn-3a to synergize with p53 or p73 on the p21CIP1/Waf1 promoter repress p73 or p53 mediated apoptotic gene expression (bax and noxa promoters) shows that Brn-3a plays a critical role in determining the cellular status and survival of cells which co-express these proteins. In particular Brn-3a may be important in the fate of neuronal cells where it has been shown that p53 and p73 are all activated during neuronal differentiation, during developmental cell death or when cells are under stress or injury resulting in activation of the p53/p73 gene.

Therefore, the co-expression of Brn-3a and p73 was analyzed in neuronal progenitor cells during development and the effects of this interaction were investigated by studying the fate of cells destined for a sensory neuronal lineage, under different conditions (conditioning). Utilization of neural crest cultures in which Brn-3a is expressed in progenitors committed to a sensory lineage (Greenwood et al., 1999) provides a dynamic model for the mechanism by which Brn-3a mediates its effect on these cells, with specific association with p53 and p73.

In accordance with published data, analysis of Brn-3a expression in neural crest cultures showed that Brn-3a was detected in two distinct populations of NCC, a smaller proliferative Brn-3a-positive population that were commonly found near the site ...
It was therefore hypothesized that TAp73 may also be involved in senescence development, particularly in the view of findings that TAp73 is itself sufficient for the differentiation of certain neuronal cell lines (De Laurenzi et al., 2000). To detect the p73 protein were used in this study. These were p73-Total which detects the alpha isoforms of both TAp73 and ANp73, p73-AN that detects the only and p73-Total which detects all p73 isoforms. Similar to p53, co-immunostaining experiments also demonstrated that p73 co-localized in the larger Brn-3a expressing cells displayed flattened morphology with many showing neurite extensions, whereas smaller, more rounded Brn-3a positive cells. Interestingly both p73-Total and p73-SAM showed both nuclear and cytoplasmic staining whereas staining with p73-AN only exhibited cytoplasmic localisation. It can therefore be inferred that the cytoplasmic staining by both p73-Total and p73-SAM is likely to be due to the ANp73 isoform in most cases whereas nuclear staining detected by these two antibodies is likely to be due to other isoforms. This suggests parallel roles between TAp73 and p53, both of which are involved in the differentiation of these cells. Obviously the availability of isoform specific antibody will help to clarify the sub cellular locations of the TA and NCC co-expressing Brn-3a.

To characterize the phenotype of p73 expressing cells further, co-immunostaining with NF-160 showed that TAp73 but not ANp73 co-localizes with NF-160 in cells displaying flattened morphology. As TAp73/NF-160, Brn-3a/NF1-60 and Brn-3a/p73 were observed to be expressed in different cell types it is likely that Brn-3a/TAp73 positive cells arising from the neural crest are differentiated neurones. In contrast the co-expression of ANp73/Brn-3a...
2. The large flattened ANp73/Brn-3a positive population that has not begun differentiation (lacking NF-160 expression).

3. The large flattened TAp73/Brn-3a positive population which are undergoing differentiation or are differentiated (co-expressing NF-160)

Hence, these results suggest that there is a switch in expression of the AN and TAp73 isoforms during sensory neuronal differentiation and that TAp73 is associated with differentiating sensory neurones. p73 has been shown to be up-regulated during musculoskeletal and hematopoietic differentiation (Tschan et al., 2000; De, V et al., 2000; Fontema et al., 2001) suggesting a general role in the differentiation of a variety of cell types. In addition, ANp73 plays an essential anti-apoptotic role in developing neurones as well as contributing to sensory neuronal survival just preceding differentiation. This will need to be confirmed in sensory neuronal survival just preceding differentiation. This will need to be confirmed for the survival of adult DRGs (Pozniak et al., 2000; Walsh et al., 2004). Interestingly, isoforms have been shown to be required to induce neurite outgrowth in a neuroblastoma cell line, suggesting that TAp73 can directly affect differentiation of these cells (De, Laurenzi et al., 2000).

Additionally, the TAp73 and ANp73 forms have also been shown to be developmentally regulated during kidney organogenesis. Thus, TAp73 is predominantly expressed in the differentiation domain of the renal cortex whereas ANp73 isoforms are in the proliferation domain and are preferentially expressed in proliferating nephron precursors (De, Laurenzi et al., 2005). In this study TAp73 is co-expressed with Brn-3a in differentiating neurones, suggesting a role for TAp73 in this process. In contrast, the co-expression of ANp73 in a subset of Brn-3a positive cells which have not yet differentiated suggests that ANp73 might play a role in sensory neuronal survival just preceding differentiation. This will need to be confirmed.
activate genes involved in neuronal differentiation. However, TAp73 was shown to activate the neuronal differentiation marker N-CAM during neuroblastoma differentiation (Laurenzi et al., 2000). It would be interesting to see if Brn-3a and TAp73 can also induce other effects on genes involved in neuronal differentiation. Indeed, p53 can enhance Brn-3a mediated activation of the α-internexin promoter (Budhram-1999). The observation that ΔNp73 is not co-expressed with NF-160 may reflect a role for ΔNp73 to act as a pro-survival factor rather than a differentiation factor (Pozniak et al., 2003). In addition the Brn-3a/ΔNp73 interaction may act to further enhance neuronal differentiation, as seen in the ND7 neuronal cell line model, in which co-expression of Brn-3a/ΔNp73 enhanced survival of these cells compared to the levels seen when either was expressed alone.

Due to the limitations of double staining techniques used in this study, the actual fact that Brn-3a, TAp73 and NF-160 are expressed in the same cell and NF-160 is not co-expressed with the cells that co-express Brn-3a/ΔNp73 is based on similarity in the distinct populations of subsets of cells. Clearly, if triple co-immunostaining techniques can be optimised (dependent on suitable antibodies becoming available), this will further confirm the expression of Brn-3a /TAp73 /NF-160 and Brn-3a/ΔNp73 but not NF-160 in a neuronal cell type. However, in the absence of suitable antibodies, such experiments can be combined in-situ for one factor and co-immunostaining for other proteins. ANp73, TA or ΔN-specific ribo-probes, would help in deciphering TA verses ΔN is expressed in these cells in the absence of good TA-specific antibodies.

However, on the available evidence it is likely that during neuronal development, p53 and p73 contribute to both differentiation and apoptosis of neurones depending on other proteins are co-expressed with it. To further demonstrate the requirement of expression of p53/TAp73 with Brn-3a in determining cell fate, NCC cultures...
must be associated with loss of cells that would normally express Brn-3a. Further support the hypothesis that Brn-3a is necessary to overcome the apoptotic repressing the expression of pro-apoptotic factors such as \textit{bax} and \textit{noxa} that are both p53 and TAp73 in specific cells during development.

Therefore similar to the Brn-3a/p53 effect, the results of studies carried out in this thesis support the hypothesis that during neuronal development Brn-3a represses p73 mediated activation of pro-apoptotic genes such as \textit{bax} and \textit{noxa} operating on the \textit{p21CIP1/Waf1} promoter to enhance cell survival and cell cycle arrest during differentiation. Thus, interaction with Brn-3a in sensory neurones may be in modulating p73/p53 mediated gene expression and hence cell fate of neurons express these proteins.

**Future direction**

In this study, Brn-3a was shown to differentially regulate p73 mediated activation of \textit{p21CIP1/Waf1}, \textit{bax} and \textit{noxa} promoters, and modulate p73 mediated cell fate. By assessing whether p73 modulates Brn-3a expression of its target promoters associated with neuronal development. Also many genes specifically regulated by p73 are being identified and would be interesting to assess whether Brn-3a can modulate p73 mediated expression of these genes, particularly gene targets involved in neuronal development such as \textit{p21CIP1/Waf1}.

It will be interesting to determine whether or not the Brn-3a enhancement of p73 mediated activation of the \textit{p21CIP1/Waf1} promoter would result in an additive gene expression in sensory neurones expressing all three proteins. This can be assessed by carrying out triple transfections in which Brn-3a and p53 are co-expressed with the \textit{p21CIP1/Waf1} reporter construct in the presence or absence of the different p73 isoforms. Similarly the effect
Given the data obtained in these studies, it appears that there may be possible roles for p53 and TAp73 in sensory neuronal differentiation, and that the TAp73 isoforms may compensate for loss of p53 in these neurones during development. Therefore, it would be interesting to test whether the levels of TAp73 mRNA in NCC taken from p53-/- mice are elevated compared to NCC prepared from p53 wild type embryos. This might help to explain the low penetrance of the neural phenotype in the p53-/- mice. Similarly, to determine whether p53 compensates for loss of p73 in sensory neuronal development, the levels of TAp73 mRNA in NCC can be assessed and compared in NCC cultures prepared from wild type embryos.

Double crosses between Brn-3a and p73 knock out mice to generate Brn-3a/p73 and/or Brn-3a/p73/p53 modified by Brn-3a/p73 and/or Brn-3a/p73/p53 compared with Brn-3a, p73 and p53 knock out mice would greatly help to decipher the roles of these proteins in sensory neuronal fate. It would be anticipated that in NCC cultures prepared from these double knockouts, there would be a reduced number of sensory neurones present and less differentiation markers compared to controls (Brn-3a +/− p73+/−, or wild type). The ability of Brn-3a to modulate p53/p73 mediated cell fate can also be investigated in cells cultured from either p53−/−, p73−/− or Brn-3a−/− animals.

The promoters that produce the TA and ΔN forms of p73 (P1 and P2 regions) are differentially regulated. For instance, the TA isoforms and p53 were shown to bind to the ΔN promoter but not the TA promoter, leading to increased ΔNp73 expression and inhibition of TAp73/p53 transactivation (Grob et al., 2001; Kartasheva et al., 2002; Nakagawa et al., 2002). The interaction of the Brn-3a and p73 proteins in the modulation of p73 mediated cell fate suggests that these proteins may regulate ΔNp73 expression. Indeed it has been shown that Brn-3a transactivates the p53 gene...
TPAp73a and Brn-3a enhanced the activity of the ΔNp73 promoter above that seen for the factor alone (data not shown). Similarly p53/p73 may regulate Brn-3a expression, which can be assessed by carrying out co-transfection assays.

If p53 and/or p73 are shown to regulate Brn-3a expression, it can be assessed whether p53 or p73 affects Brn-3a expression in NCC. Therefore the levels of Brn-3a mRNA taken from p53-/− and p73-/− embryos can be measured and compared to the controls (NCC cultures prepared from wild type littermates).
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