UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Inhibition of p53 function in tumours that express the *PAX3* proto-oncogene

by

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Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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INHIBITION OF p53 FUNCTION IN TUMOURS THAT EXPRESS THE *PAX3* PROTO-ONCOGENE

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Inactivating mutations in the *TP53* gene are an infrequent event in tumours derived from cells of neural crest origin. These tumours are likely, therefore, to be dependent upon alternative mechanisms for the suppression of p53-dependent cell cycle arrest and pro-apoptotic signalling pathways. The survival of cells from these tumours is often dependent upon their expression of PAX3, an embryologically expressed transcription factor that is essential for the suppression of spontaneous p53-dependent apoptosis in the developing neural crest. I hypothesized that PAX3 causes repression of p53 function in tumours of neural crest origin.

I show that PAX3 is required to prevent spontaneous p53-dependent apoptosis in melanoma cells using siRNA. Knockdown of PAX3 expression causes a loss of cell viability of over 90% and induction of the pro-apoptotic protein BAX. This is in contrast to induction of the cyclin dependent kinase inhibitor $p21^{WAF-1}$ and cell cycle arrest in response to HDM2 knockdown. Furthermore, I confirm the importance of PAX3 in the survival of neuroblastoma.

In a model system PAX3 suppresses p53-dependent transcription from promoters including *BAX* and *HDM2* but, importantly, not *WAF-1*. Mutagenesis studies show that this suppression is dependent upon the integrity of PAX3 as an activating transcription factor. PAX3 expression causes a reduction in p53 protein levels by up to 90% and an increase in p53 turn-over, which is not due to classical proteasomal degradation.

My data clearly demonstrates a critical role for the PAX3 oncoprotein in the inhibition of p53-dependent pro-apoptotic pathways in neural crest derived tumours. Furthermore, I have developed a cell based screening assay to identify compounds that inhibit PAX3 function in melanoma cells. Such compounds will be used as tools to study the mechanism of p53 functional inhibition by PAX3 in future work and may also have therapeutic potential.

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This project has been about more than just experiments in a laboratory and the writing of a thesis. I have had the opportunity to meet many people and make long-lasting friendships. I have learnt the basis of sound science and the art of scientific writing. The conclusion of this project is not the end of a journey but the beginning of a new chapter.

This work is dedicated to my wife Sophie and my daughter Charlotte who was born during my second year of study and has changed my life forever.

Abbreviations

ANOVA	Analysis of variance
ARF	Alternate reading frame
AS-ODN	Antisense oligonucleotide
ASPP	Ankyrin-repeat-, SH3-domain- and proline-rich-region-containing proteins
ATM	Ataxia telangiectasia mutated
BCC	Basal cell carcinoma
BSA	Bovine serum albumin
CDK	Cyclin dependent kinase
CMV	Cytomegalovirus
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence in-situ hybridisation
GFP	Green fluorescent protein
HAUSP	Herpes virus-associated ubiquitin specific protease
HBSS	Hanks' balanced salt solution
HD	Homeodomain
HDM2	Human double minute 2
HLH	Helix-loop-helix
ID	Inhibitory domain
Mdm2	Mouse double minute 2
MITF	Microphthalmia associated transcription factor
NCI	National Cancer Institute
NES	Nuclear export signal
NLS	Nuclear localisation signal
NTD	Neural tube defect
OM	Octapeptide motif

PARC	p53-associated Parkin-like protein
PBS	Phosphate buffered saline
PD	Paired domain
PUMA	p53 up-regulated mediator of apoptosis
qRT-PCR	Quantatative real time polymerase chain reaction
RE	Response element
RLU	Relative luciferase units
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
TA	Transactivation domain
UV	Ultraviolet

1. Introduction

PAX3 and p53 are two proteins that play fundamental roles in the development of normal cells and cancer. Although it is tempting to delve directly into the relationship between PAX3 and p53 it is important to understand the relevance of these two proteins in the context of normal and abnormal tissue homeostasis and their role within the cell. The beginning of this thesis will therefore examine the mechanisms that have evolved over millions of years to control normal cellular growth, proliferation and differentiation. With this background in place I will move on to examine some of the principles of cancer biology followed by a detailed examination of the roles of PAX3 and p53 in normal cells and cancer. I will lay out the aims and objectives of this research project and then move on to describe the experiments that have been conducted to fulfil these aims. I will present a model of PAX3 inhibition of p53 function and finally discuss ongoing work designed to discover new inhibitors of PAX3 function.

1.1 Regulation of cell growth, proliferation, differentiation and apoptosis is fundamental to embryonic development and tissue homeostasis in the adult

1.1.1 The importance of controlling cell populations

Human life begins with a single cell, the product of the fusion of sperm and egg. From this microscopic origin a highly complex, multi-cellular organism develops. In order to achieve complexity cells undergo multiple rounds of division and in some organs go on dividing throughout life. However, a simple increase in cell number is not sufficient to produce the diversity of cell function required in an adult. Cells change both form and function in a variety of tightly regulated processes that are tissue and organ dependent [1-3].

The total number of cells within a specific tissue is tightly controlled. Most tissues maintain populations of stem cells that can repopulate the tissue in response to injury [4], or in the case of the gastrointestinal tract, as part of normal cellular turnover [5].

A number of key processes take place at the cellular level to control cell number and function; these are at play during development and throughout the life of the organism. For the sake of clarity I have defined them below:

Cell growth is the increase in the size of a cell. It is seen during the cell cycle as the cell prepares to divide.

Proliferation refers to an increase in cell number normally due to mitosis. It is seen in a number of physiological processes such as endometrial thickening in response to oestrogen. Proliferation also takes place in a range of pathological settings including cancer.

Differentiation describes the process when a cell changes to carry out a specialised function. Cell differentiation creates the diversity of cell types that arise during development and it is differentiation that gives cells their characteristic appearances in different organs. Cell differentiation also defines the fate of the cell. In some organs, such as the liver, differentiated cells retain the ability to proliferate in response to damage whereas in other cell lineages differentiation begins a non-reversible process that ultimately leads to cell death. Other differentiated cells may enter a permanently quiescent state.

Apoptosis is programmed cell death. This is seen in normal development as the cells between fingers and toes die to give independent digits. Apoptosis may also occur in response to cellular damage. Apoptosis has specific characteristics and depends upon the production and accumulation of specific effecter proteins within the cell.

Much of this thesis deals with the interactions of proteins involved in cancer. Cancer is a genetic disease; the accumulation of genetic mutations over time leads to a pattern of gene expression that gives the cell a proliferation and survival advantage over its normal counterparts [6]. Abnormal gene, and therefore, protein expression, whether

in terms of time, location or amount, leads to changes in the fundamental mechanisms that control the growth, proliferation and differentiation status of the cell [6]. These mechanisms are laid down early in development and are to a greater or lesser extent dependent on cellular context. Higher organisms have evolved complex mechanisms to control embryological development, allowing the formation of a range of specialised tissues [1-3, 7, 8]. Often these programs are completed during early development and the genes responsible may continue to perform important roles in the control of cell proliferation and differentiation [3, 7-12]. The inappropriate expression of these master regulatory genes is potentially tumourigenic and this is a concept that will be visited on a number of occasions in this thesis. In the following sections I will discuss the determination of cell fate in the embryo and how this leads to a complex multi-cellular organism. I will introduce the embryological neural crest as the origin of a variety of adult cell types. This will be followed by a brief account of the regulatory mechanisms governing cellular proliferation and differentiation in the adult. This will form a platform of understanding to allow consideration of the role of embryological gene activity in tumours and specifically, how activation of the *PAX3* proto-oncogene in neural crest derived tumours supports tumour cell viability.

1.1.2 Genetic control of normal development with special reference to skeletal muscle

The ultimate complexity of an organism is tightly regulated in a temporospatial manner [1, 2]. Cells undergo multiple rounds of division producing increasing diversity of cell phenotype until the tissues of the adult organism have been established [1, 2]. Early specification of cell fate during development may be determined by spatial cues that consist of localized maternal regulatory factors that are distributed to particular cells with the egg cytoplasm, and are partitioned during cleavage [1]. Alternatively they may consist of signals produced by other cells, as a consequence of their own prior state of specification. Ultimately, these spatial cues affect the progress of development by causing the activation or repression, in a certain region of the embryo, of particular genes encoding transcription factors [1]. Studies in lower organisms, including nematodes and fruit flies, have identified other genes as regulators of developmental timing (heterochronic genes) that are conserved across

species [2]. Some of these genes encode micro RNA's (miRNA) that act to down-regulate the expression of other heterochronic genes creating complex genetic regulatory pathways [2]. In combination, the expression of specific spatial and timing genes act as developmental switches that program specific cell identities [1, 2]. Thus, the activation or repression of a given heterochronic gene or spatial regulatory gene at a specific developmental time is often a crucial event, such that the corresponding change in activity level modulates the progression of a cell to its next fate [1, 2]. Through this process new regions of cellular specification are created leading to ever more complexity in the embryo. The presence of a specific signal at a certain time or position within the embryo is crucial to determine cell fate but the controlling mechanism for this activity is the presence of *cis*-regulatory regions of the target gene [1]. It is the network of all these *cis*-regulatory interactions that is ultimately responsible for driving the process of development [1].

The development of skeletal muscle is an elegant example of the role of transcription factors in tissue development. In this system a limited number of genes are responsible for the commitment of cells to the muscle cell lineage and the subsequent development of adult skeletal muscle [3, 7, 9, 10, 13, 14]. The developmental program of limb skeletal muscle has been well defined in higher organisms and a number of the genes involved at each stage have been identified (Fig. 1.1) [3, 7]. Skeletal muscle develops from areas adjacent to the neural tube known as somites. In the case of limb muscles, cells from the somite are required to delaminate and migrate into the limb bud [7]. Migration of limb muscle precursor cells is dependent on spatial cues from mesenchymal cells of the limb bud; expression of the tyrosine kinase receptor c-Met under the influence of Pax3 enables the cells to follow a migratory route defined by secretion of the c-Met ligand, Hgf, from non-somitic mesodermal cells [7]. Cells that migrate from the somite are yet to undergo myogenic determination. Limb muscle determination and differentiation requires the expression of a family of basic-helix-loop-helix (bHLH) transcription factors, MyoD, Myf5, Mrf4 and Myogenin (MyoG) [7, 15-18]. Expression of this family of transcription factors is believed to be stimulated by external ligands, such as Wnt signalling, providing more spatial information to the developing muscle cells [3, 7]. As the myogenic determination factors MyoD and Myf5 are expressed the muscle precursor cells undergo extensive proliferation and this proliferative phase is, at least in part,

under the influence of Pax3 [7]. MyoD directly regulates genes throughout the program of muscle gene expression, and temporal patterning is achieved by a combination of promoter-specific regulation of MyoD binding and activity in a feed-forward mechanism [3]. In addition, MyoD regulates chromatin-associated proteins to alter the availability of the *cis*-regulatory regions of muscle defining genes [3]. MyoD (in co-operation with Myf5) acts as a master switch to commit precursor cells to the skeletal muscle lineage by virtue of its ability to recruit histone modifying and chromatin-remodeling complexes to muscle promoters and reset the cellular chromatin structure and transcriptional program [3].

This description of the development of a single tissue type in the embryo serves to illustrate the intricacy of gene activity during embryogenesis and also introduces the role of *Pax3* as an early developmental gene. The detailed nature of Pax3 and the variety of roles that Pax3 plays in embryogenesis will be discussed in detail later but it is important to note that continued expression of Pax3 in the myogenic cell lineage acts as an effective block to differentiation [3, 14]. Although Pax3 is required for MyoD expression, Pax3 levels fall at the induction of differentiation and remain low in adult muscle [7]. The one important exception to this is in the satellite cells of skeletal muscle. These cells retain the ability to proliferate after muscle damage and in so doing retain expression of Pax3 (in certain muscle groups only) or its structural homologue Pax7 [3, 7].

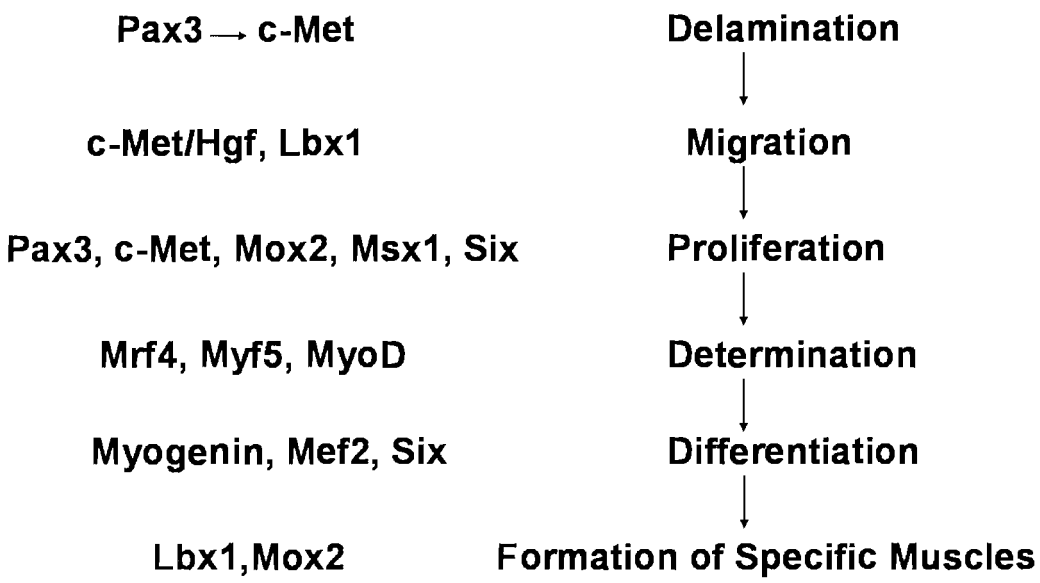

Figure 1.1: The stages of muscle development in the limb and the genes potentially involved at each stage.

The development of individual limb muscles from the somite of the embryo takes place in defined stages under the influence of a range of myogenic transcription factors. These transcription factors must be expressed at the correct time and position within the embryo to enable efficient tissue development. Mutation of one or more of the indicated genes leads to phenotypic abnormalities commensurate with the stage of development. For example, mice with a homozygous *Pax3* deletion develop no skeletal muscle and there is no evidence of precursor migration in the embryo. Prior to expression of the skeletal muscle determination genes *MyoD* and *Myf5* the precursor cells are not committed to the skeletal muscle lineage and in the absence of MyoD and Myf5 no skeletal muscle develops. Absence of Lbx1 or Mox2 leads to loss of specific muscle groups.

1.1.3 The neural crest

The neural crest is a specialised group of cells that arise in the lateral tip of the neural folds during the process of neurulation (Fig. 1.2) [19]. The importance of the neural crest stems from the variety of derivatives that this discrete group of cells yields during development. The majority of the peripheral nervous system, the entirety of the autonomic nervous system, the enteric nervous system and all the

melanocytes of the body, except those of the retina, are derived from neural crest origins [19-24]. In vertebrates the neural crest gives rise to most of the skeleton and connective tissue of the head, face and neck [19]. Neural crest derivatives are absolutely required for vertebrate otongeny and have evolved to take the role of mesoderm to provide a skull cover for the increasing size of the vertebrate brain [19, 25]. Cells destined to populate the neural crest can be identified within the developing neuroepithelium by the expression of specific genes [19]. As the embryonic ectoderm becomes subdivided into the neural plate and epidermis, cells within the boundary region (destined to become neural crest) express a range of transcription factors such as *Slug, Snail, AP-2, Foxd3, PAX3 and Sox9* dependent upon species and their rostrocaudal position within the embryo [19]. These early neural crest markers are under the influence of BMP and Wnt signals from adjacent cells [26, 27]. The early development of the neural crest shows similarities to the development of skeletal muscle described previously, in that an extra-cellular spatial cue is required to drive early specification of cell fate. Specific genetic signals further govern ultimate neural crest cell fate by maintaining a pool of premigratory cells [28], controlling cell cycle progression [29], coordinating neural crest cell delamination and restricting the developmental potential of migratory cells through environmental signals encountered during migration [30]. In summary, the tightly controlled expression of transcriptionally active genes in response to spatial and temporal stimuli give rise to a population of cells that migrate widely throughout the embryo to give rise to a range of essential tissues.

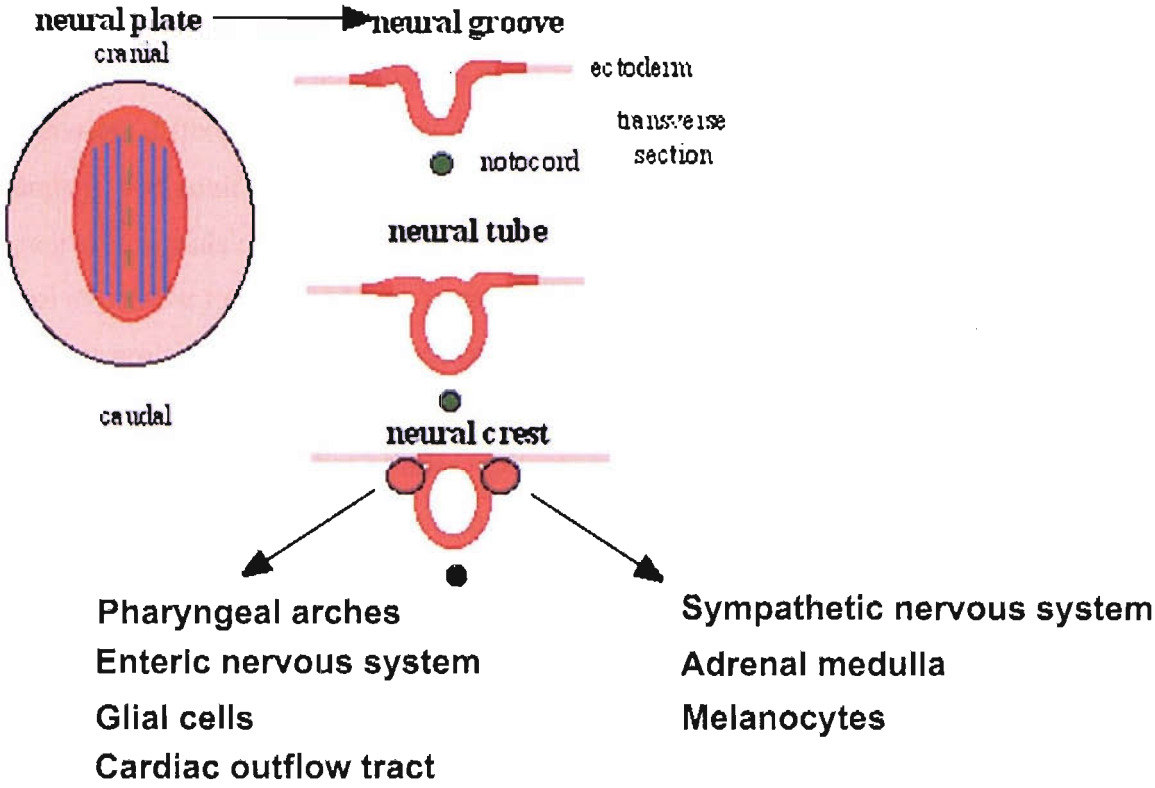

Figure 1.2: Development of the neural crest

The neural crest is a specialised group of cells that arise in the lateral tip of the neural folds during the process of neurulation. After neural tube closure cells of neural crest origin migrate widely to populate diverse areas of the adult organism. Cells of neural crest origin become tissues as varied as melanocytes, glial cells and hormone releasing cells of the adrenal medulla.

Cells of neural crest origin can give rise to a range of solid tumours in the adult [21, 31]. It is possible that the development of the neural crest and malignant cells is related because of the number of cancers arising from neural crest lineages and the phenotypic similarity between neural crest migratory cells and metastasising cells [22, 32]. A comparison of gene expression between migratory neural crest cells and malignant melanoma cells reveals similar patterns of gene expression [22]. This evidence suggests that neural crest derived tumours may rely on the recapitulation of embryonic expression patterns to gain a selective advantage over their normal adult counterparts. Fundamental to tumourigenesis is the ability of the cancer cell to bypass important control mechanisms that exist to restrain undesirable proliferation [33]. Before considering how the expression of embryologically significant genes can have this effect in the adult I will describe some of the mechanisms that exist to prevent uncontrolled cell division.

1.1.4 Cell cycle control in the adult

To maintain tissue homeostasis there is a constant balance between proliferation, differentiation and cell death [34]. These processes can be observed in the embryo and adult organism and are determined, in part, by the balance of positive and negative signals received by the cell [6, 11, 12, 29, 33-41]. This is not the only control of cellular turnover. Many intracellular control mechanisms have developed to regulate proliferation and differentiation; but, once committed to division the final common pathway for all cells is the cell cycle [34, 39, 40]. The cell cycle has evolved as a mechanism to ensure that cellular replication is achieved efficiently and with minimal copying error [40]. The transit of a cell through the cell cycle can be divided into four specific stages as illustrated in Figure 1.3 [40]. Upon receiving the appropriate stimuli to divide a cell enters a preparatory phase G_1. During this phase the cell is preparing for DNA replication. This involves the production of nucleotides and generation of the cellular machinery required for chromosomal duplication. During S phase the DNA replicates and the cell then enters a second gap phase G_2 in preparation for mitosis, M phase. The majority of quiescent cells are in a separate stage named G_0.

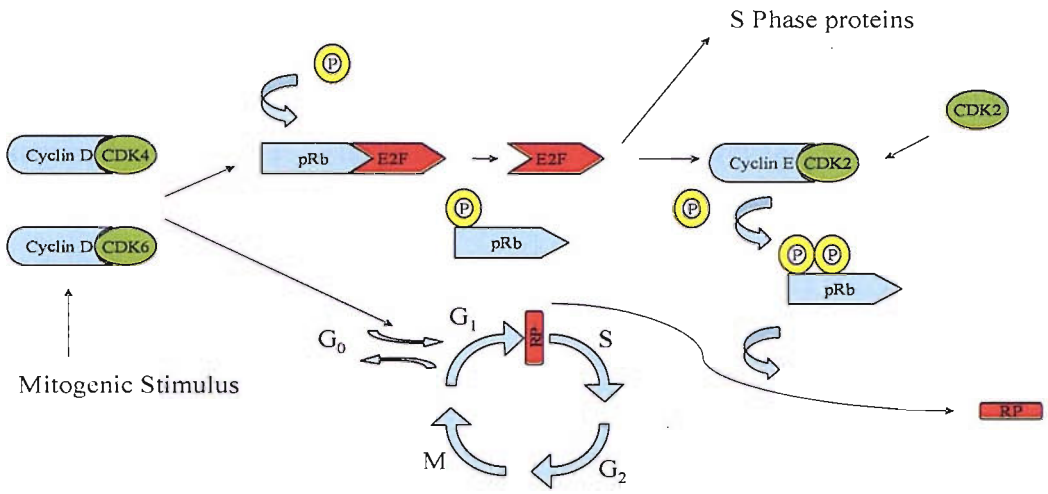

Figure 1.3: A simplified schematic view of some steps in the regulation of the cell cycle

Induction of Cyclin D in response to a mitogenic stimulus leads to phosphorylation of pRb and entry to G_1. Release of the E2F protein stimulates Cyclin E binding of CDK2 and further phosphorylation of pRb. Phosphorylation of pRb allows entry to S phase by overcoming a critical restriction point (represented schematically above) and commits the cell to division.

Control of the cell cycle is intricate and involves the interaction of a number of cellular proteins [34, 39, 40, 42]. Important check points exist as the cell enters G_1 and at the G_1-S boundary. The interplay between the cyclin and cyclin dependent kinase (CDK) family of proteins is key to this regulation [40]. The cellular levels of CDK does not change throughout the cell cycle but their activity as serine/threonine protein kinases is conferred by binding a specific cyclin protein, the level of which changes depending on the phase of the cell cycle [40]. An important restriction point exists at the G_1 to S transition that is governed by phosphorylation of the retinoblastoma protein (pRB). In the absence of mitogenic signals normal cells can not progress past this point [43]. It was believed that if the G_1 to S restriction point is crossed, the cell is committed to divide whatever the extracellular conditions [40], but recent evidence suggests that a second restriction point exists during S phase, also

under mitogenic control [43]. Phosphorylation of pRB may be the final common action that allows cell cycle progression from G_1, but many other proteins influence the G_1 to S transition. One such protein is p53 [34, 41, 42, 44-46]. The actions of p53 on the cell cycle are fundamental to its role as a tumour suppressor gene and will be examined in detail later. The primary role of p53 is to prevent the propagation of damaged DNA and in so doing p53 either acts to prevent cell cycle progression via the activity of the cyclin dependent kinase inhibitor $p21^{WAF1}$ [39], or commit the damaged cell to programmed cell death – apoptosis [39, 41, 44, 47-53].

1.1.5 Apoptosis

Apoptosis exists as a genetically programmed system to dispose of cells [54]. It is a process that is intimately linked with proliferation as a means to control cell number and cell viability [54]. Indeed a number of the proteins indicated in cell cycle control are also involved in apoptotic signalling and the decision of the cell to commit suicide is based on a complex balance of interacting factors [43, 55, 56]. Initiation of apoptosis leads to the activation of caspase proteins. Caspases are a family of cysteine proteases that cleave target proteins at specific aspartate residues [57]. They are produced in cells as catalytically inactive zymogens and can be divided into two classes. The initiator caspases (2, 8, 9 and 10) are subject to autoactivation under apoptotic conditions and lead to the activation of effector caspases (3, 6 and 7) which begin the process of cellular degradation [57]. The caspase cascade can be initiated by two separate molecular pathways. The mitochondrial pathway involves the release of cytochrome c from the mitochondria and the association of APAF-1 with procaspase-9 and formation of the apoptosome [57, 58]. It is the regulation of mitochondrial membrane permeability that governs the signal for apoptosis via this pathway [59]. Proteins such as p53, that respond to DNA damage, influence membrane permeability by increasing expression of the proapoptotic, pore-forming proteins such as BAX [50]. The second major pathway for apoptosis is via death receptor activation by ligand binding [60]. This leads to direct activation of caspase 8. There is also cross talk between the two activation pathways via BID [6]. BID is cleaved by caspase 8 and enhances cytochrome c release from the mitochondria. Once activated (by either pathway) the effector caspases are responsible for the

proteolytic cleavage of a wide spectrum of cellular targets and ultimately cell death [57].

In this section I have described how the diversity of cell phenotypes required in a complex organism, such as a human, is established during embryogenesis and how regulation of ongoing cell proliferation and cell death is fundamental for the prevention of an unwanted expansion in cell number – cancer. I will next describe how the genes implicated in the control of cell number and function in normal tissues can be affected to produce cancer and then describe in detail the activity of PAX3 and p53 and the evidence that suggests that there may be an important interaction between these two proteins in neural crest derived tumours.

1.2 Abnormal proliferation, differentiation and apoptosis - cancer

1.2.1 Tumour suppressor genes and oncogenes

Cancer is a genetic disease [6, 33, 34, 38]. Tumour cells may exhibit a range of abnormalities, from single base mutations, through changes in the epigenetic environment, to changes in chromosome number [33]. Often multiple abnormalities will be apparent in a single tumour and more than one genetic aberration is required for tumourigenesis. The age-dependent incidence of a range of human tumours suggests that between four and seven independent rate-limiting events are necessary for tumour formation [61]. Whatever the biochemical basis the tumour cell will have gained a selective advantage over its normal counterparts. The cancer cell develops immortality, self sufficiency in growth signalling, resistance to antigrowth signals and the ability to promote angiogenesis and metastasize [33]. The cancer cell has established ways to bypass the normal control mechanisms governing proliferation, differentiation and programmed cell death [34].

Mutations occur mainly in two types of genes, (proto-) oncogenes and tumour suppressor genes [6]. Oncogenic products act along the pathways that drive cell proliferation [54]. Mutations lead to over activity often in a dominant fashion. Oncogenes have been described at all levels of the proliferative pathway from cell

surface related proteins such as HER-2 [62], to nuclear phosphoproteins such as C-MYC [63]. In contrast tumour suppressor genes act to inhibit cell proliferation, either by applying brakes or checks to the cell cycle or by stimulating cell death [64]. Usually both alleles will be mutated in a cancer cell (*TP53* being an important exception), for the product of one allele is normally sufficient for protein function [54].

In the majority of solid tumours there is a requirement for cooperation between oncogenes and tumour suppressor genes because a single genetic event is insufficient for tumour development. For example, the development of colonic adenocarcinoma follows a sequence from normal mucosa, through benign polyp to invasive carcinoma [65]. A similar sequence of genetic changes is often observed, involving the mutation of the mismatch repair gene *MSH2* or the *APC* gene [65]. This is followed by changes in methylation status and activation of *K-RAS* [65]. A common final pathway in the majority of colonic tumours is loss of p53 function [66, 67]. Taken together, evidence from human cancers and animal models suggest that tumour development proceeds via a process analogous to Darwinian evolution, in which a succession of genetic changes, each conferring a proliferative advantage, leads to the progressive conversion of normal human cells into cancer cells [33, 68].

1.2.2 Embryonic genes as oncogenes

Oncogenes may have normal functions in the adult cell but in many cancers the activation of genes occurs that would not be normally observed in that tissue or at that stage in development [33]. Genes normally involved in embryological development can have potent oncogenic effects if expressed in the adult. An example is the commonest skin cancer in man, basal cell carcinoma (BCC). BCC is most often sporadic but a rare inherited disorder, Gorlin syndrome, predisposes individuals to BCC and a range of birth defects [69]. Gorlin syndrome is the result of a mutation in the human homologue of a Drosophila gene *patched* [35]. Genetic studies in Drosophila show that *patched* is part of the hedgehog signaling pathway, important in determining embryonic patterning and cell fate in multiple structures of the developing embryo [35]. Human *PATCHED* is mutated in sporadic as well as hereditary BCCs, and inactivation of this gene is probably a necessary, if not

sufficient, step for tumour formation [12, 35]. Another example of the aberrant activity of embryonic genes is observed in the Wnt signaling pathway. The highly regulated Wnt signaling cascade plays a decisive role during embryonic patterning and cell-fate determination [70]. The inappropriate expression of Wnt target genes, resulting from deregulation of this pathway, is also implicated in tumourigenesis [36, 71]. The Wnt canonical pathway regulates, through a core set of evolutionarily highly conserved proteins, the ability of the multi-functional protein and proto-oncogene *β-CATENIN* to activate the transcription of specific target genes [70]. In the absence of Wnt signals β-CATENIN is actively targeted for degradation. Mutations in *APC*, *AXIN* or *β-CATENIN* interfere with this targeted destruction, ultimately leading to cancer development [36, 71]. It is becoming clear that developmental genes are involved in the formation of many tumours, the *Wnt* family and *PATCHED* being two examples.

The PAX family of proteins are vital embryonic gene products that are also implicated in a range of solid tumours including renal cell carcinoma, malignant melanoma and neuroblastoma [10]. The *PAX* gene family is examined in detail in the following section.

1.3 PAX3

1.3.1 The PAX family

PAX proteins are defined by the presence of a 128 amino acid, DNA binding, paired domain (PD) [10, 72]. The PAX family includes 9 known proteins in mammals (Table 1.1) and homologues exist in worms, flies, frogs, fish and birds [10]. PAX family members are expressed throughout embryogenesis and play key regulatory roles in a number of developmental processes during mammalian ontogeny [72]. PAX6 is required for ocular development; heterozygous *PAX6* mutation in humans leads to blindness, aniridia, and cataracts. Homozygous *Pax6* mutation in mice leads to complete failure of ocular development [73, 74]. PAX6 and PAX4 are both involved in pancreatic endocrine cell development and loss of *Pax4* in mice leads to loss of insulin secreting β cells [10]. PAX5 is required for B-cell maturation and is capable of directly regulating transcription of several B-cell-specific genes. *Pax5* knockout mice have a block in B-cell development at the pro-B-cell stage [75].

Pax2 is expressed in the developing mouse kidney and neural structures. Pax2 associates with the Wilms tumour 1 gene and interplay between these two factors determines glomerular development. Loss of Pax2 leads to a variety of defects including absence of kidney, ureter and genital structures [76]. Conversely over expression of PAX2 in humans has been associated with multicystic dysplastic kidney disease, renal cell carcinoma and Wilms tumour [77]. In similar regulatory roles, PAX1 and PAX9 are associated with skeletal development and PAX8 with the thyroid gland [10].

PAX genes are also linked to cancer. Chromosomal translocations involving *PAX* genes have been described in a variety of cancers. A translocation involving *PAX5* results in large-cell lymphoma and a translocation involving *PAX8* and *PPAR gamma 1* causes follicular carcinoma of the thyroid [10].

Gene	Structural domains[a]				Target organs/tissues	Chromosome[b]	Associated human disease
	PD	OP	HD1	HD2/3			
PAX1	X	X			Skeleton, thymus	20p11	
PAX9	X	X			Skeleton, cranio-facial, tooth	14q12-q13	Oligodontia
PAX2	X	X	X		CNS, kidney	10q25	Renal-coloboma syndrome
PAX5	X	X	X		CNS, B cells	9p13	Large cell lymphoma
PAX8	X	X	X		CNS, kidney, thyroid	2q12-q14	Thyroid dysplasia, thyroid follicular carcinoma
PAX4	X		X	X	Pancreas	7q	
PAX6	X		X	X	CNS, eye, pancreas	11p13	Aniridia, cataracts
PAX3	X	X	X	X	CNS, neural crest, skeletal muscle	2q35	Waardenburg syndrome, rhabdomyosarcoma
PAX7	X	X	X	X	CNS, cranio-facial, skeletal muscle	1p36.2	Rhabdomyosarcoma

Table 1.1: The PAX gene family in mammals.

[a] Structural domains include the paired domain (PD), octapeptide motif (OP), the first helix of the homeodomain (HD1) and the helix-turn-helix motif of the homeodomain (HD 2/3). X denotes presence of the domain. [b] Human chromosome map position.
(Adapted from Chi and Epstein, Trends in Genetics Vol.18 No1 January 2002 [10])

1.3.2 PAX3 structure and function

PAX3 is a key regulator of mammalian development. In mice Pax3 is expressed in the developing neural tube, neural crest and migrating myoblasts, suggesting a role for this transcription factor in neurogenesis and myogenesis [72]. This has been confirmed with the discovery that the *Pax3* gene is mutated in independent alleles of the mouse *Splotch* mutant [78, 79]. Homozygous *Splotch* mice suffer from a complete lack of limb musculature, severe neural tube defects and cardiac malformations in addition to an absent enteric nervous system that together lead to embryonic lethality by midgestation [10, 20, 80, 81]. Mutations in the human *PAX3* gene are associated with Waardenburg syndrome, a condition characterised by a sensorineuronal (cochlear) deafness and pigment disturbances with occasional limb deformity [82]. Pax3 is down-regulated in later development and its expression is restricted to a small number of highly specialised stem cells such as melanocytes in the bulge region of hair follicles [83]. In this setting Pax3 acts simultaneously to initiate a melanogenic cascade while acting downstream to prevent terminal differentiation, thus maintaining the undifferentiated phenotype of a lineage restricted stem cell [83].

Control of Pax3 expression in the embryo is complex and involves extracellular signaling pathways as well as networks of interacting transcription factors [84]. *In vitro* evidence suggests that Pax3 interacts with members of the Six, Eya and Dach family of transcriptional regulators in early muscle development. In *Drosophila* these genes form a complex regulatory network, including positive feedback regulation of each others' expression [84]. *In vivo* experiments have shown the expression of Pax3 in the somite to be dependent on ectodermal cell contact which can be rescued by ectopic expression of Dach2 [84]. Signaling from the overlying ectoderm in the form of Wnt ligand binding is responsible for Pax3 expression [85]. Extracellular signals are also required for Pax3 expression in the neural crest and recent evidence suggests that this may be mediated by the transcription factor Tead2 [86]. Tead2, and its co-activator Yap65, are co-expressed with Pax3 in the dorsal neural tube and mutation of the Tead2 binding site in the context of Pax3 transgenic constructs abolishes neural expression [86]. Although the number of recognized Pax3 regulatory proteins is limited it is likely that Pax3 transcription is under the influence of a much greater

number of factors, because the five prime Pax3 regulatory region contains multiple positive and negative *cis*-acting elements [87]. Expression of Pax3 in the developing embryo leads to the transcription of a range of target genes dependent on cellular context. In the skeletal muscle lineage Pax3 up-regulates c-Met and members of the MyoD family as previously discussed. In the context of the neural crest Pax3 has been shown to interact physically with Sox10 to transactivate a c-Ret promoter [88]. c-Ret is a protein required for enteric nervous system development, a tissue derived from the neural crest. Pax3 and Sox10 bind independently to a MITF enhancer but cause synergistic activation of MITF reporter constructs in vitro. Mutation of PAX3, SOX10 or MITF is sufficient to cause Waardenburg syndrome [88]. PAX3 activation of MITF is also required to maintain melanocytes stem cells in the adult [83]. Interogation of potential PAX3 target genes expressed during development and in the PAX3 associated paediatric malignancy alveolar rhabdomyosarcoma identified six genes that contain *cis*-regulatory elements bound by PAX3. *TM2A, FATH, FLT1, TGFA, BVES*, and *EN2*, were identified as part of the PAX3 regulatory circuitry during embryogenesis and tumour formation [9]. These genes vary in activity from cell surface receptors (*FLT1*), to other homeobox proteins (*EN2*) [89]. Over expression of PAX3 by stable transfection of a PAX3 expression vector in a medulloblastoma cell line caused a change in expression level of 270 possible target genes when assessed by microarray [90]. Using confirmatory molecular biology techniques STX, a gene required for neural cell adhesion, was identified as a significant biological downstream target of PAX3 [90]. Together these data suggest that the range of PAX3 targets is diverse and dependent on cell lineage and the expression of other transcription factors.

PAX3 consists of 479 amino acids and has a molecular weight of 52967 Da [82]. In addition to the paired domain, PAX3 is one of four PAX proteins (PAX3, 4, 6 and 7) to contain a second DNA-binding domain the paired-type homeodomain (HD) (Fig. 1.4) [91]. The two DNA-binding domains are separated by a linker segment that contains a highly conserved octapeptide motif of unknown function [92-94]. A proline-serine-threonine rich (PST-rich) transcriptional regulatory domain resides at the C-terminus of the protein [93] and together with the octapeptide motif this is responsible for protein-protein interactions to recruit additional transcription factors required for the transcription of target genes [95].

The high resolution crystal structures of the PD and HD have been solved for the closely related *Drosophila* Paired protein [96, 97]. The PD contains two structurally independent subdomains (PAI and RED), each comprising three α-helices that can form a helix-turn-helix motif and can either act independently or cooperatively in the recognition of DNA [93]. A DNA recognition sequence for the PD has been identified as 5'- GTCAC – 3' [98]. The HD also comprises three α-helices that form a helix-turn-helix motif [96]. Interaction of the HD with DNA occurs principally through the N-terminal arm and the third helix and paired type HDs are characterised by their ability to cooperatively dimerise on palindromic sites of the type 5' – $TAAT(N)_{2\text{-}3}ATTA$ – 3' [99]. Both the PD and HD can bind to cognate DNA sequences when expressed individually, but genetic and biochemical data indicates that the two domains are functionally interdependent in the intact PAX3 protein [95]. The *Splotch-delayed* (Sp^d) mouse mutant bears a single mutation in the PD (G42R) which, in addition to abrogating DNA binding by the PD, impairs DNA binding by the HD [100]. Deletion of helix 2 of the PAI subdomain on the background of the Sp^d mutation has been shown to restore DNA binding by the HD [93]. The PD has been shown to modulate DNA binding specificity and the dimerisation potential of the HD [93]. Cysteine scanning mutagenesis studies have shown that modification of a single cysteine within the PD (Cys-82) disables DNA binding by both the PD and HD [101]. Furthermore, modification of a single cysteine in the HD (V263C) abrogates binding by both domains; this led the authors to conclude that the PD and HD do not function as independent DNA binding molecules in PAX3 but seem functionally interdependent [101]. Investigation of point mutations in both the PD and HD observed in Waardenburg syndrome confirm this interdependency. Modelling of these mutations on the three-dimensional structure of the *Drosophila* Paired protein shows that these mutations cluster at the DNA interface, suggesting that a series of DNA contacts are essential for DNA binding by the PD and HD of PAX3 [102].

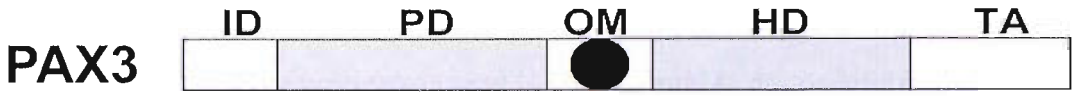

Figure 1.4: Structural organisation of PAX3

PAX3 consists of a number of structural domains as indicated; ID = inhibitiry domain, PD = paired domain, OM = octapeptide motif, HD = homeodomain, TA = Transactivation domain.

PAX3 has oncogenic potential and is able to transform fibroblasts in culture [14]. PAX3, or a PAX3-FKHR fusion protein that is expressed as a consequence of a chromosomal translocation t(2;13) (q35;q14), are involved in the suppression of apoptosis in embryonal rhabdomyosarcoma and paediatric alveolar rhabdomyosarcoma respectively [103]. The translocation identified in alveolar rhabdomyosarcoma leads to the juxtaposition of *PAX3* with a forkhead domain gene *FKHR* [94, 104]. This translocation leads to the expression of a fusion protein containing the N-terminal DNA binding domains of PAX3 and the C-terminal activation domain of FKHR [104]. Treatment of these cells with *PAX3* specific antisense oligonucleotides (AS-ODN) leads to loss of cell viability via an apoptotic pathway [103]. Cell death in this system is also observed after down-regulation of wild type PAX3 or PAX7 implicating PAX proteins in tumour cell survival. *PAX3* mRNA is detectable in 77% of cultured primary malignant melanomas, in which expression increases with advancing tumour stage, and treatment of these cells with *PAX3* AS-ODN leads to apoptotic cell death [105]. A recent report by He *et al.* demonstrated that PAX3 also plays a role in resistance to chemotherapy in malignant melanoma [31]. *PAX3* antisense resulted in a modest increase in the number of p53 immunoreactive cells, though whether p53 was required for the chemosensitisation was not investigated. PAX3 is expressed in neuroblastoma cells and expression is highest in cells that express the oncogene *N-MYC*, as *PAX3* is a direct transcriptional target of N-MYC [106]. Down-regulation of PAX3 in murine neuroblastoma cells leads to rapid morphological differentiation [107].

1.4 p53

The *TP53* tumour suppressor gene is fundamental to our understanding of cancer. The p53 protein is a 393 amino acid protein that acts via transcription dependent and independent mechanisms to induce growth arrest and/or apoptosis in response to a range of cellular stresses [41, 108]. Ionising radiation, ultraviolet light, hypoxia, and aberrant growth signalling lead to rapid accumulation of p53 protein and activation of downstream targets such as BAX and $p21^{WAF1}$ [41, 46, 50]. In this way the p53 protein plays an important role in preventing cancer development by arresting or killing potential tumour cells [45]. During tumour development there is a high selection pressure for p53 network silencing; *TP53* being the most commonly inactivated gene in human cancer [41, 45, 109]. *TP53* is mutated in over 60% of human cancers and the p53 pathway is believed to be abrogated in most, if not all, of the remainder [41]. Suppression of p53 activity appears to be fundamental to tumour development.

The p53 protein has a defined structural organisation (Fig. 1.5) [110]. The amino-terminus contains the acidic transactivation domain and the HDM2 protein binding site. The central region (amino acids 100-300) contains the DNA binding domain. This is the target for 90% of p53 mutations found in human cancers [111]. The tetramerisation domain consists of a beta-strand, followed by an alpha-helix necessary for dimerisation [112]. A nuclear export signal is localized in this oligomerisation domain. The carboxy-terminus of p53 (356-393) contains a non-specific DNA binding domain that binds to damaged DNA [112]. This region is also involved in down-regulation of DNA binding by the central domain [110]. The p53 protein binds DNA as a homotetramer and this oligomerisation is required for the tumour suppressor activity of p53, as oligomerisation-deficient mutants of p53 cannot suppress the growth of tumour cell lines [113]. There is extensive post-translational modification of p53 with at least 24 recognised amino acids targeted for phosphorylation, acetylation, sumoylation or ubiquitination [110]. Distinct signal transduction pathways activate p53-dependent transcription through several common post-translational modifications yet the consequences of p53 activation are both damage dependent and cell-type dependent [110]. Recent studies indicate that several p53 post-translational modifications appear to modulate its transcriptional activity in a

promoter and cell type specific manner [110]. It appears that multiple sites targeted by an integrated network of signaling pathways highly sensitive to genotoxic stresses must be modified to yield a functional p53 [110].

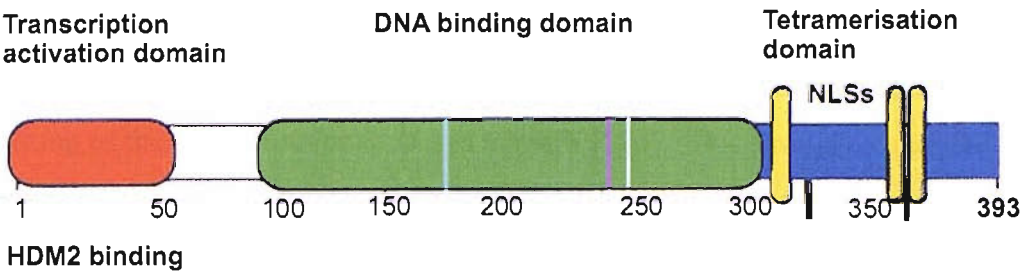

Figure 1.5: Structural organisation of p53

The p53 protein consists of an N-terminal transcription activation domain, a core DNA binding domain and a C-terminal tetramerisatioin domain, within which lie three nuclear localisation signals (NLS) and a nuclear export signal (not shown). HDM2 binds to p53 at the N-terminus, amino acids 18-23 forming the minimal binding site [114].

The p53 protein is a transcription factor that is normally present in very low levels in the cell but is able to respond rapidly to cellular stress [41, 45]. The level of p53 must be tightly regulated in the cell, for on the one hand it has to allow normal growth and proliferation, but on the other hand p53 is responsible for tumour suppression. While most tumour suppressor genes are inactivated by mutations leading to absence of protein synthesis (or production of a truncated product), more than 80% of *TP53* alterations are missence mutations that lead to the synthesis of a stable full-length protein [115]. This selection to maintain mutant p53 in tumour cells is believed to be required for both a dominant negative activity, to inhibit wild-type p53 expressed by the remaining allele, and for gain of function that transforms mutant p53 into a dominant oncogene [116-118]. Although the majority of mutations are localised to the DNA binding domain of p53, mutant p53 proteins exhibit structural, biochemical and biological heterogeneity [111]. Certain p53 mutants are able to transactivate genes involved in proliferation or cell survival and these sites differ from those bound by wild-type p53 [111]. Other p53 mutants are able to interact directly with cellular proteins involved in tumour progression [119]. It is becoming increasing important to

know not only whether, but also how p53 has been disturbed in a cancer. Although p53 is directly altered in the majority of human tumours it remains as wild-type in up to 40% [41]. There is evidence that the p53 network is suppressed in these tumours and this can occur in a variety of ways. For example, it has recently been shown that a single nucleotide polymorphism in the *Mdm2* (the mouse homologue of *HDM2*) promoter leads to higher affinity for the transcription factor Sp1 leading to increased *Mdm2* RNA and protein. This results in both an increase in tumour incidence and lessening of the time to tumour onset in humans [120]. This strongly suggests that elevated levels of HDM2 expression can have a significant impact in dampening the efficiency of tumour suppression by p53. The p53 protein activity can be suppressed in many other ways including; binding to viral proteins (HPV E6 in cervical carcinoma) [121], deletion of genes involved in HDM2 suppression ($p14^{ARF}$ in breast, brain and lung tumours) and mislocalisation of p53 to the cytoplasm (breast cancer and neuroblastoma) [41]. In these circumstances the *TP53* gene is normal.

1.4.1 Activation of p53

Although regulation of p53 at the level of transcription and translation is well documented [45], it is clear that these are not the most important points at which p53 protein levels are determined. The level of p53 in the cell is regulated most closely by control of the rate at which it is degraded, rather than the rate at which it is made [45, 122, 123]. This is mediated by a range of proteins including COP1 [124] and PIRH2 [125], but predominantly by the activity of the p53 transcriptional target HDM2 [126]. The HDM2 protein is a ubiquitin ligase that targets p53 for nuclear export and degradation by the proteasome [45, 127]. Thus a negative feedback loop is created, p53 promoting the transcription of its own negative regulator. Evidence for the importance of *HDM2* as a p53 regulator is suggested from *Mdm2* deficient mice; embryos die in early gestation in a manner that is p53 dependent [128, 129]. Degradation of p53 in response to HDM2 binding is preceded by ubiquitination of specific lysine residues in the C-terminus of the protein [130, 131]; although more recent data suggest that lysine residues within the DNA binding domain are also involved [132]. The ubiquitination of p53 by HDM2 assists in the nuclear export of the p53 protein by activation of the p53 nuclear export signal [133]. HDM2 does not

only negatively regulate p53 by its activity as an ubiquitin ligase enzyme. By virtue of the position of the binding site for HDM2 within the DNA transcription activation domain of p53, HDM2 can directly inhibit p53 dependent transcription [134]. Furthermore a putative inhibitory domain within HDM2 is able to repress basal transcription from p53 responsive promoters [135]. The fundamental interaction between p53 and HDM2; the up-regulation of p53 in response to cellular stress leading to direct transcriptional activation of HDM2 and the eventual destruction of p53 by the proteasome; is a target for the actions of many p53 activating cellular mechanisms.

The activation of p53 in response to cellular stress should be considered on the background of a complex interplay of cell signals and competing pathways. Many protein kinases have been shown to phosphorylate p53 in response to a variety of signals and may have a positive or negative effect on stabilisation depending on the site of phosphorylation [110]. Seven serines and two threonines in the N-terminal domain of p53, specifically Ser 6, 9, 15, 20, 33, 37, 46 and Thr18, and 81, are phosphorylated in response to exposing cells to ionizing radiation or UV light [110]. The N-terminus appears to be a hot spot of p53 modification as it contains the transcription activation domain and the HDM2 binding region of the protein. Although post-translational modifications at most sites occur in response to stress, clear differences in responses at individual sites to different agents have been observed. For Ser15, increased phosphorylation has been observed in response to a range of chemotherapeutic agents [110]. Moreover, in human fibroblasts undergoing replicative senescence or RAS-induced premature senescence, Ser15 phosphorylation was also significantly increased [136], suggesting that phosphorylation at this site may be one of the critical signals through which the p53 response to stress is regulated [110]. A model for stabilisation and activation of p53 via phosphorylation of the N-terminus has been proposed [45]. Phosphorylation in response to cellular stress disrupts HDM2 binding and also prevents p53 nuclear export and degradation. In addition disruption of HDM2 binding to p53 (in particular by Ser 15 phosphorylation) permits enhanced interactions with transcriptional co-activators such as p300/CBP which can acetylate residues in the C-terminus and promote activation of transcription [45]. It is well known that the last 30 amino acids of the C-terminal domain negatively regulate sequence-specific binding by the p53 central core

domain [137]. A range of post-translational modifications of the C-terminus of p53 may, directly or indirectly, relieve DNA binding inhibition by the C-terminus through changes in the binding of interacting proteins that alter p53 structure [110]. Loss of basic charges upon acetylation or sumoylation or addition of negative charges upon phosphorylation could disrupt nonspecific binding to DNA or induce conformational changes that prevent interactions between the C-terminus and the core DNA binding domain [110].

In response to DNA damage caused by ionising radiation the interaction between p53 and HDM2 is inhibited by phosphorylation of amino acids within the HDM2 binding region of p53 by the protein kinases ATM, CHK1 and CHK2 [41, 45, 122]. In this context a single break in DNA can be sufficient to trigger a rise in p53 levels [41]. The activity of ATM, CHK1 and CHK2 is not only confined to phosphorylation of p53. Phosphorylation of serine 20 of p53 by these proteins is important in disruption of HDM2-p53 binding but in addition phosphorylation of HDM2 also occurs [45].

Mechanisms also exist to prevent ubiquitin dependant degradation of p53 in response to cellular stress. The tumour suppressor genes *TSG101* and *c-ABL* both have a negative effect on ubiquitination [122]. Other proteins remove ubiquitin from the p53 protein directly; these include a herpes virus-associated ubiquitin-specific protease (HAUSP) [122]. HAUSP has a dynamic effect on the p53-HDM2 pathway for on the one hand HAUSP is responsible for direct deubiquitination of p53, but on the other it is required for HDM2 stability [138].

p53 is stabilised in response to the deregulated activity of many oncogenes via the nuclear protein ARF ($p19^{ARF}$ in mice/$p14^{ARF}$ in humans). The mechanism of action of the ARF protein is disputed, but there is evidence that ARF acts to sequester HDM2 to the nucleolus thus preventing binding to p53 [41]. This does not seem to be the only mechanism however, as ARF appears able to stabilise p53 without nucleolar sequestration [139]. The importance of the ARF protein has been established in *INK4α/ARF*-deficient mice (the $p19^{ARF}$ protein is transcribed from this locus) that develop a range of tumours [6].

The preceding examples are not the only mechanisms of p53 activation. Down regulation of *HDM2* expression, phosphorylation of HDM2, down-regulation of free ubiquitin and competition for the HDM2 binding site by transcriptional co-activators such as ASPPs [140] are examples of alternate mechanisms of activation. ASPP proteins associate with p53 via the DNA binding domain and influence sequence specific transcriptional regulation of p53 target genes [51, 52]. Other co-activators such as E2F1 and YB1 have been documented to have a similar effect [141, 142].

1.4.3 Localisation of p53

Localisation of p53 to the nucleus is fundamental to its activity as a transcription factor. The C-terminus of p53 contains a number of nuclear localisation signals (NLS) and these contribute to the active nuclear import of the protein [45, 127]. In addition there are two nuclear export signals (NES), one in the N-terminus as already described, and the other in the C-terminus tetramerization domain [112]. It is clear that p53 is exported back to the cytoplasm but the exact mechanism and purpose of this export remain controversial. HDM2 was originally considered necessary for nuclear export via ubiquitination of lysine residues in the C-terminus. These residues are masked when p53 is in its active tetrameric state and may be further concealed by binding of ZBP-89, a protein that inhibits nuclear export [143]. Thus the transcriptionally active p53 is protected from nuclear export. It is now clear that although important, nuclear export is not dependant on HDM2. HDM2 may only be responsible for mono-ubiquitination of p53 and therefore another factor may be necessary for poly-ubiquitination and recognition by the proteasome [45, 122, 127]. A p53-associated Parkin-like protein (PARC) has recently been put forward as a candidate for p53 polyubiquitinisation [144]. In addition, PARC has been implicated as a cytoplasmic anchor for p53 (in some tumours p53 is inactivated by cytoplasmic sequestration).

1.4.4 The cellular response to p53 – repair or apoptosis?

The active p53 protein has a range of biological functions, the best documented of which is the ability to bind specific DNA sequences and initiate gene

transcription. The majority of naturally occurring p53 mutants have mutations within this DNA binding region underlining the significance of this function [41]. The genes that are transcribed in response to p53 have a range of functions including cell cycle arrest, induction of apoptosis, inhibition of angiogenesis and influence on genetic stability [41, 137].

Cell cycle arrest precedes apoptosis and is one of the first effects of induction of p53. The p53 responsive gene $p21^{WAF1}$ is an inhibitor of CDKs [39]. $p21^{WAF1}$ is able to provide a block to the G_1-S and S-M transitions of the cell cycle. Other genetic targets such as *REPRIMO* and *14-3-3σ* help to maintain a block at the G_2 stage [41].

The induction of apoptosis by p53 follows expression of mitochondrial related proteins such as BAX (a pro-apoptotic member of the BCL-2 family), NOXA and P53AIPI [41, 45]. These genes possess direct p53 response elements and may induce apoptosis by altering mitochondrial membrane permeability. Recently the BAX related BH3 only protein PUMA (p53 up-regulated mediator of apoptosis) has been identified as a direct mediator of apoptosis in response to p53 activation [49].

It would seem logical that the cell would be forced directly from cell cycle arrest to apoptosis if all these p53 responsive genes were transcribed at similar rates upon p53 activation. The cell is able to modify these effects to produce an appropriate outcome. This is based in-part upon cell lineage, location and genetic history; however, other transcriptional cofactors modify the p53 response. The p53 protein is believed to cause the transcription of selected targets based on these associations. The ASPP family of cofactors interact with p53 within the DNA binding domain and enhance the affinity of p53 for apoptotic targets [45, 127]. There is direct competition for the ASPP binding site of p53 with the inhibitor of ASPP (iASPP) [141, 145]. They are supported in this role by E2F1 which, in addition to enhancing the affinity of p53 for pro-apoptotic target gene promoters, also leads to the phosphorylation of p53 [141]. Conversely expression of YB1 inhibits the ability of p53 to transactivate pro-death genes [141]. The availability of these cofactors may determine the response of the cell to p53 activation via cell cycle arrest and/or apoptosis.

1.5 PAX3 and p53

The induction of apoptosis in response to *PAX3* specific antisense oligonucleotides in melanoma and rhabdomyosarcoma suggests a link between PAX3 function and inhibition of apoptosis [31, 103, 105]. Further support for a direct link between PAX3 and apoptosis comes from *Splotch* mice; neural tube defects in *Sp/Sp* embryos are associated with neuroepithelial apoptosis [146]. The recent finding that the embryonic lethality seen in *Sp/Sp* embryos can be abrogated by crossing into a *TP53* null background or inhibiting p53 function via chemical inhibition has shown a direct link between PAX3 and p53 in embryology [20]. Indeed the sole function of PAX3 in the neural crest may be to keep p53 down-regulated and prevent apoptosis until a specific developmental program is completed [20].

Tumour cells gain a selective advantage over their normal counterparts as a result of mutations in a number to classes of genes. These genes often affect cell signalling, cell cycle progression and immune recognition [33]. One of the commonest abnormalities in human cancer is loss of p53 function [34, 41, 45, 111, 123, 147]. This is often a late occurrence in the malignant transformation of the cell. A lack of p53 function leads to loss of cell cycle arrest, the accumulation of further mutations and a failure to undergo apoptosis. In the majority of neural crest derived tumours p53 is not mutated [148-150]. In malignant melanoma p53 has been demonstrated to be wild type in 34 of 34 biopsy specimens by PCR [148] and in a study of malignant melanoma cell lines five out of six retained wild type p53 [47, 105]. Expression of wild-type p53 ranges from 35% in primary lesions to 70% in metastatic disease but the mutation rate remains low [149]. In neuroblastoma two separate studies have reported p53 mutations in 0 of 29 [151] and 0 of 38 [152] primary tumour specimens and a further study has shown significant p53 mutation at tumour relapse after combined chemo-radiotherapy [153]. Furthermore, perturbation of the p53 response has been observed in *N-MYC* amplified neuroblastoma cell lines [153]. In the absence of p53 inactivating mutations other mechanisms are likely to be involved in p53 suppression [41, 109]. The low frequency of *TP53* mutation in melanoma and neuroblastoma tumours, which are derived from cells with a common origin in the neural crest [148, 152], is a striking example of the influence that cell type-specific

characteristics play in defining the selection pressure for *TP53* mutation during cancer development [109].

Other PAX proteins have been shown to inhibit p53 expression via transcriptional repression. PAX2, PAX5 and PAX8 inhibit p53 by binding to the *TP53* promoter [154]. In human astrocytoma the level of p53 correlates inversely with PAX5 expression via this mechanism. Transcriptional repression would not seem to be the mechanism of p53 down-regulation by PAX3. There is no identifiable PAX3 binding site in a 530 base pair (b.p.) upstream region of the murine *tp53* [20]. *Splotch* mice are observed to have high levels of p53 protein compared to their normal counterparts suggesting that PAX3 is able to repress p53 protein levels in the embryo [20]. Together, the experimental data from murine development and cancer cell lines raise the possibility that PAX3 may be promoting the survival of a defined subset of human tumours through the suppression of the p53 pathway. It remains unknown if PAX3 expression in neural crest derived tumours causes repression of p53 function.

2. Aims and objectives

This study is based on four key observations:

1. PAX3 inhibits p53 function in the embryo
2. PAX3 is a survival factor for neural crest derived tumours
3. Neural crest derived tumours undergo apoptosis when PAX3 is down-regulated
4. Neural crest derived tumours have low rates of p53 mutation.

These findings lead to the formulation of a working hypothesis:

The PAX3 proto-oncogene inhibits p53 function in neural crest derived tumors

At the outset of this project little was known about the relationship between PAX3 and p53. This meant that the initial aims of the project were broad but could be broken down into manageable sections for study. The aims were:

1. To determine whether PAX3 inhibits p53 function in an *in vitro* cancer cell model.
2. To define the regions of each protein required for this inhibition
3. To define the mechanisms of inhibition of p53 function by PAX3
4. To determine the biological significance of p53 repression by PAX3 in neural crest derived tumours

3. Methods

3.1 Cell lines, culture conditions and cell passage

Cells were cultured at 37 °C in a humidified atmosphere containing 5-10% CO_2 depending on the cell line. H1299 and UACC-62 cells were grown in RPMI 1640 medium supplemented with 10% v/v foetal bovine serum and 1% v/v penicillin /streptomycin /glutamine. All other cells were grown in DMEM supplemented with 10% v/v foetal bovine serum and 1% v/v penicillin /streptomycin /glutamine. Medium was prewarmed to 37 °C before use. Culture medium was renewed every 48 hours.

Cell passage was performed by removing the existing medium and washing the cells with a calcium and magnesium free Hanks Balanced Salt Solution (HBSS). A solution of Trypsin / EDTA was applied and the cells were incubated at 37 °C for 3-5 minutes or until all the cells had become detached. Cells were typically replated in growth medium at 1:10 dilution.

Greiner UK supplied all tissue culture plastic ware

From Invitrogen life technologies UK:

Hank's Balanced Salt Solution (HBSS)	Cat. No. 14170088
Dulbecco's Modified Eagle Medium (DMEM)	Cat. No. 41966029
RPMI 1640 Medium	Cat. No. 21875-034
Trypsin-EDTA	Cat. No. 25300054
Penicillin-Streptomycin-Glutamine	Cat. No. 10378016

-(100X) Liquid with 10000 U Penicillin, 10000 µg Strepromycin, 29.2 g L-Glutamine/ml

From Autogen Bioclear UK:

Foetal Bovine Serum	Cat. No. 12230687

Freezing mixture:

20% v/v Dimethyl Sulphoxide (DMSO) in Foetal Bovine Serum

Name	ECACC No.	ATCC No.	Tissue	Reference
IMR 32		CCL-127	Human neuroblastoma	[155]
Kelly	92110411		Human neuroblastoma	[156]
NIH3T3		CRL-1658	Mouse embryonic fibroblast	[157]
H1299		CRL-5803	Human non-small cell lung cancer	[158]
UACC-62	Available from the DCTDC repository of the National Cancer Institute U.S.A		Human malignant melanoma	[159]

Table 3.1: Cell lines used in this study
ECACC = European Collection of Cell Cultures
ATCC = American Type Culture Collection

3.2 Mutagenesis

PAX3 deletion mutants were constructed by inserting stop codons in the coding sequence of the PAX3 bacterial expression vector pJ7PAX3. Nucleotide mutations were inserted into the same vector to produce PAX3 protein mutants corresponding to the known amino acid changes observed in Splotch delayed mice (SD42) and Waardenberg syndrome in humans (WS265 and N47H). Mutagenesis was conducted using the QuikChange XL Site-Directed mutagenesis Kit (Stratagene, UK) according to the manufacturers' instructions using the primers shown in Table 3.2.

PAX3 mutant name	Primer sequence
PAX3 Stop34	CCACCCCTCTTGGCtagGGCCGAGTCAACC
PAX3 Stop222	CAGCGCAGGAGCtgaACCACCTTCACGGC
PAX3 Stop281	CTGGAGCCAATtagCTGATGGCTTTCAACC
PAX3 SD42	CGAGTCAACCAGCTCcGAGGAGTATTTATC
PAX3 WS265	GAGGCCCGAGTGCAGtTCTGGTTTAGCAAC
PAX3 N47H	GGAGGAGTATTTATCcACGGCAGGCCTCTGCC

Table 3.2: Oligonucleotide primers designed to generate specific PAX3 mutations

A series of oligonucleotide primers were designed from the published PAX3 nucleotide sequence to generate specific PAX3 mutations as shown. Nucleotides shown in lower case indicate stop codons for Stop 34, Stop 222 and Stop 281 and single nucleotide substitutions for SD42 and WS265. Only the forward primer is shown. The other primer used was reverse complement to that shown.

3.3 Small-scale preparation of plasmid DNA (minipreps)

Selected bacterial colonies were picked in sterile conditions and inoculated into 5 ml of LB medium containing the appropriate antibiotic. The cultures were incubated at 37 °C overnight with vigorous shaking (220r.p.m). The QIAprep Miniprep Kit (Qiagen, UK) was used for purification of plasmid DNA as per manufacturers' instructions.

Luria-Bertani (LB) broth

25 g/L LB-Broth powder (Merck)

LB plate media

37 g/L LB-plate powder (Merck)

3.4 Large-scale preparation of plasmid DNA (maxipreps)

The QIAGEN Plasmid Maxi kit (Qiagen, UK) was used for large-scale purification of plasmid DNA from bacterial cultures (100 ml-500 ml). The concentration of DNA was determined by standard UV spectrophotometry.

3.5 Transfection method

The cationic lipid Lipofectamine 2000 (Invitrogen) was used for both transient and stable transfections. Cells were plated the day before transfection or grown to 90% confluency in the appropriate sized dish. Growth medium was changed the day before transfection in all cases. A ratio of 1:2.5, DNA (in μg) to Lipofectamine (in μl), was used for all transfections. On the day of transfection the appropriate amount of DNA was suspended in Opti-MEM reduced serum medium. In a separate tube the appropriate amount of Lipofectamine 2000 was also suspended in Opti-MEM. After 5 minutes the DNA and Lipofectamine 2000 mixtures were combined and incubated for a further 20 minutes at room temperature. The mixture was then added to the cells.

3.6 Reporter gene assay

Reporter gene assays were based on the transfection of the firefly luciferase gene under the control of a test promoter. Changes in transcription of the firefly luciferase gene were measured as changes in light production compared with an internal control plasmid. Cells were plated the day before transfection at a density appropriate to achieve 90% confluency on the day of transfection in 96 well plates. Cells were transfected using Lipofectamine 2000 (Invitrogen). A constant amount of reporter gene (100 ng per well for most experiments) was transfected into the cells with varying amounts of p53 and PAX3 expression plasmids. The total amount of plasmid transfected was normalised with an empty vector construct such as pJ7. A constant amount (20 ng per well) of the renilla control plasmid pRLSV40 was included in each transfection to control for transfection efficiency and non-specific effects on transcription. 48 hours post transfection luciferase and renilla activity were determined using the Dual-Glo system (Promega) as per manufacturers instructions and a Topcount microplate luminometer (Packard). Figure 3.1 demonstrates validation of the use of the renilla internal control plasmid under experimental conditions. There is no significant repression of renilla activity in the presence of PAX3 plasmid up to 100ng (the highest amount used in any reporter assay).

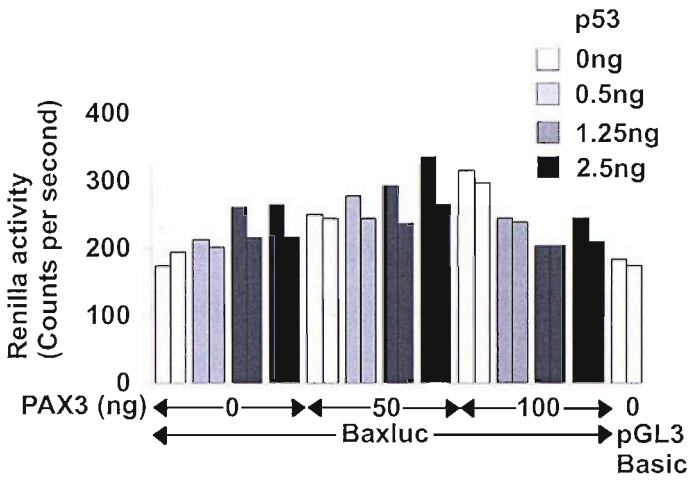

Figure 3.1: Renilla activity is not altered by addition of up to 100ng PAX3 plasmid under test conditions

Human neuroblastoma IMR 32 cells were transfected with 100 ng of Baxluc or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection. Increasing amounts of pJ7 PAX3 was added as shown. Total amounts of transfected plasmid were equalized using empty pJ7 vector.

3.7 Preparation of cell pellets for protein and RNA extraction

Plates of cells were removed from the incubator and placed on ice. The growth medium was removed and the cells were washed with ice cold PBS. The PBS wash was removed and 1ml per plate ice cold PBS placed on the cells. The cells were scraped into suspension using a disposable cell scraper or the plunger from a sterile syringe of the appropriate size. The suspension was removed to a 1ml eppendorf and centrifuged at 1000 r.p.m at 4 °C for 4 minutes to pellet the cells. The supernatant was then removed and the cell pellets either snap frozen or immediately subjected to the appropriate assay. Pellets were placed at -70 °C for long term storage

Phosphate Buffered Saline (PBS) 30X concentrate (2.5 L)

600 g NaCl

15 g KCl

108 g Na_2HPO_4

18 g KH_2PO_4

2.5 l H_2O

3.8 Urea whole cell lysis

Urea lysis was used to extract total cellular protein from mammalian cells for western blotting. Cell pellets were prepared as previously described. The cell pellets were resuspended in two volumes of urea lysis buffer and allowed to lyse for 15 minutes on ice. The mixture was then centrifuged at 11000 r.p.m at 4 °C for 10 minutes. The supernatant (containing cellular protein) was transferred to a 0.5 ml eppendorf and snap frozen and stored at -70 °C or analysed immediately.

3.9 Protein quantitation

Total protein quantitation was estimated by comparison of sample protein light absorbance at 595 nm using Bio-Rad protein quantitation reagent (Bio-Rad, UK) against known BSA standards. A 1 in 5 dilution of Bio-Rad reagent was made and aliquoted into 1 ml plastic cuvettes (BDH Laboratory supplies). 1 µl of the protein sample was then added to each cuvette and mixed thoroughly. Each sample was quantified in duplicate. Absorbance was measured in a spectrophotometer and protein concentration calculated against a BSA standard curve.

3.10 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein separation was conducted by SDS-PAGE prior to further analysis. Pre-cast 4-15% polyacrylamide gels were used throughout (Bio-Rad, UK). Protein concentration was estimated using Bio-Rad's protein assay reagent as described. SDS-PAGE sample buffer was then added to each sample to standardised concentration between samples. Samples were heated to 95 °C for 5 minutes and equal volumes loaded into individual wells. Electrophoresis was conducted at 175 V for 50 minutes or until the dye front had reached the bottom of the gel.

4X SDS-PAGE sample buffer	Final concentration
EDTA	10 mM
SDS	4% w/v
Glycerol	20% v/v

Tris pH 6.0	20 mM
Bromophenol Blue	to colour
DTT	200 mM

Dithiothreitol (DTT)

1 M stock made in 0.01 M Sodium Acetate pH 5.2

10X Running Buffer

Tris Base	30 g
Glycine	144 g
SDS	10 g
H_2O	to 1 L

3.11 Western blotting

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Hybond-C, Amersham) using a mini-protean II transfer unit at 100 V for 1 hour according to the manufacturers instructions. The membrane was then rinsed with Tween 20 0.1% v/v/PBS and transfer efficiency checked by Indian ink staining. The membrane was rinsed again in Tween 20 0.1% v/v/PBS and incubated for 1 hour at room temperature (or overnight at 4 °C) with gentle rolling in 5% non-fat milk (Marvel) in Tween 20 0.1% v/v/PBS to block any subsequent non-specific antibody binding. The blocking mixture was discarded and the membrane incubated with primary antibody at the appropriate concentration in 3% w/v milk/Tween 20 0.1%v/v/PBS at room temperature (or 37 °C for anti PAX3 antibody) for 1 hour with gentle rolling. The membrane was then washed in Tween 20 0.1% v/v/PBS 3 times for 5 minutes. The membrane was then incubated with the appropriate secondary horse radish peroxidase conjugated antibody in 3% w/v milk/ Tween 20 0.1% v/v/PBS for 1 hour at room temperature with gentle rolling. The membrane was then washed once for 15 minutes and then twice for 10 minutes with Tween 20 0.1% v/v/PBS. The membrane was then incubated with SuperSignal West Pico Substrate Chemiluminescent working solution (Pierce) for 10 minutes. The membrane was blotted dry and wrapped in parafilm. Chemiluminescence was detected using a Bio-Rad Flour-S Multimager and Quantity One software.

Transfer Buffer

10X Tris-Glycine(TG)	100 ml

H_2O	700 ml
Methanol	200 ml

10XTG

Tris Base	29 g
Glycine	145 g
H_2O	to 1 L

Antibody	Antigen	Antibody type	Dilution	Source
PAX3	PAX3	Rabbit polyclonal	1:2000	Active Motif
DO-1	P53	Mouse monoclonal	1:1000	Serotec
Ab290	GFP	Rabbit polyclonal	1:2500	Clontech
PAb-421	P53	Mouse monoclonal	1:250	T.R. Hupp
2A9	HDM2	Mouse monoclonal	1:100	A.J. Levine
Bax	BAX	Mouse monoclonal	1:1000	Santa Cruz
P21	$P21^{WAF1}$	Mouse monoclonal	1:1000	Calbiochem
A5060	ACTIN	Rabbit polyclonal	1:2000	Sigma
AM30	PARP	Rabbit polyclonal	1:100	Calbiochem
P0448	Rabbit IgG	Goat polyclonal	1:2000	DAKO
SAM	Mouse IgG	Sheep polyclonal	1:2000	Amersham Biosciences

Table 3.3: Antibodies used in this project

3.12 RNA extraction

Total RNA was extracted from cell pellets using RNeasy Mini Columns (Qiagen) with on-column DNase treatment using the RNase-Free DNase Set (Qiagen) as per manufacturers instructions. RNA was quantified by spectrophotometry and stored at -80 °C for future use.

3.13 Synthesis of cDNA

First Strand cDNA synthesis was conducted using Superscript II RT (Invitrogen) as per manufacturers' instructions. A typical reaction would contain the following:

Oligo (dT)12-18 (500 μg/ml)	1 μl
10 mM dNTP mix	1 μl
500 ng RNA	
Sterile water	to 12 μl

This was heated to 65 °C for 5 min and quick chilled on ice. The contents of the tube were collected by centrifugation and the following added:

5X First-Strand buffer	4 μl
0.1 M DTT	2 μl
RNaseOUT	1 μl
Superscript II RT	1 μl

The mixture was incubated for 50 min at 42 °C and the reaction inactivated by heating at 70 °C for 15 min.

3.14 Quantitative real-time polymerase chain reaction (qRT-PCR) – Taqman

Changes in gene expression were detected by measuring the relative abundance of mRNA for specific genes by quantitative RT-PCR. cDNA produced using Superscript II RT was used for all experiments. Reactions were conducted in 96 well plates using an ABI PRISM 7500 Real-Time PCR system and software (PerkinElmer Life Sciences). Primers and probe for *gapdh*, which was used as an endogenous control gene, were purchased as a Pre-Developed TaqMan® reagent

assay (PerkinElmer Life Sciences). Primer and probe for all other genes were purchased as a pre-designed, pre-optimised TaqMan® gene expression assay (Applied Biosystems), except PAX3 and HDM2 which were designed using Primer Express, Version 2.0 (PerkinElmer Life Sciences) or Primer3 (MIT) and were synthesized by MWG Biotech: *HDM2*-coding sequence F (TCTACAGGGACGCCATCGA) and *HDM2*-coding sequence R (CTGATCCAACCAATCACCTGAA) and *HDM2*-coding sequence probe (FAM-TTCACTTACACCAGCATCAAGATCCGGA-TAMRA). *PAX3* F (GAGCGAGCGAGCCTCAGCAC) and *PAX3* R (AGGTGGTTCGGCTTCTGCG) and *PAX3* probe (FAM-AGGCTCTGATATTGACTCTGAACCAGATTTACC-TAMRA). qRT-PCR was performed in 20 µl reaction volumes containing 1x qPCR™; Mastermix (Eurogentec), cDNA (from 5 ng of RNA), and the appropriate sets of primers and probes. Threshold cycle values were converted to relative transcript levels using a standard curve.

3.15 In vivo ubiquitination assay

Cell pellets were prepared as described with 20% separated for direct western analysis. The remaining pellet was lysed on ice for 10 mins in 40 µl lysis buffer and passed through an 8 gauge needle 7 times before being transferred to a falcon tube containing a further 4 ml of lysis buffer. 75 µl Ni^{2+} - agarose beads (Qiagen) were added to each tube and placed on a rotating table at 4 °C overnight. The next day beads were collected by centrifugation at 2000 rpm for 3 mins and the supernatant aspirated off. The beads were then washed for 10 mins in 750 µl of buffers 1-5 at room temperature. 75 µl of elution buffer was added and incubated for 30 mins at room temperature. Eluates were collected by centrifugation at 11000 rpm for 5 mins at 4 °C before being mixed with an equal volume of sample buffer and analysed by SDS-PAGE.

In vivo ubiquitination assay reagents

Buffer 1

Guanidinium-HCl	114.6 g
0.2 M Na_2HPO_4	94.7 ml
0.2 M NaH_2PO_4	5.3 ml
1 M Tris-HCl, pH 8.0	2 ml

14.3 M β-mercaptoethanol	140 µl

Adjusted to pH 8.0 and made up to 200 ml with H_2O

Lysis Buffer

100 ml Buffer 1 with 500 µl 1 M Imidazole (fresh)

Buffer 2

Urea	48 g
0.2 M Na_2HPO_4	47.35 ml
0.2 M NaH_2PO_4	2.65 ml
1 M Tris-HCl, pH 8.0	1 ml
14.3 M β-mercaptoethanol	70 µl

Adjusted to pH 8.0 and made up to 100 ml with H_2O

Buffer 3

Urea	96 g
0.2 M Na_2HPO_4	22.5 ml
0.2 M NaH_2PO_4	77.5 ml
1 M Tris-HCl, pH 6.3	2 ml
14.3 M β-mercaptoethanol	140 µl

Adjusted to pH 6.3 and made up to 200 ml with H_2O

Buffer 4

10 ml of buffer 3 with 200 µl of 10% v/v Triton X-100

Buffer 5

10 ml of buffer 3 with 100 µl of 10% v/v Triton X-100

Elution Buffer

1 M Imidazole	200 µl
10% w/v SDS	500 µl
1 M Tris-HCl, pH 6.7	150 µl
Glycerol	100 µl
14.3 M β-mercaptoethanol	50 µl

3.16 Preparation of cells for Fluorescence In-Situ Hybridisation (FISH)

1% v/v colcemid was added to cells in culture and incubated at 37 °C for 30 minutes to 2 hours. Cells were lifted into suspension using a sterile cell scraper and collected by centrifugation for 3 minutes at 1100 rpm. The supernatant was discarded and 10ml potassium chloride added before incubation at 37 °C for 10 minutes. The cells were collected by centrifugation at 1000 rpm for 3 minutes, the supernatant was discarded and the pellet vortexed. 1 ml of fixative (3:1 methanol: acetic acid) was added 1 drop at a time whilst vortexing, then topped up to 5 ml. The sample was centrifuged at 1,100 rpm for 3 minutes, the supernatant was discarded and the sample was topped up with fresh fixative. Fixed cells were then processed by The Wessex Regional Cytogenetics Laboratory.

3.17 Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's HSD post-hoc testing using SPSS for Windows version 12 was used for all experiments except cell viability assays in chapter 7 where paired T-tests were conducted. Statistical significance is represented for each experiment using the following: * = $p<0.05$, ** = $p<0.001$ and is measured against the appropriate untreated control. For example; 2.5 ng p53, 100 ng PAX3 has been compared against 2.5 ng p53, 0 ng PAX3 in figure 4.2.

4. The role of PAX3 in repression of p53 dependent transcription

4.1 Introduction

The critical role of p53 in mediating stress-induced cell cycle arrest or apoptosis results in a strong selection pressure for tumour cells with an attenuated p53 pathway [34]. The documented low frequency of *TP53* mutation in neural crest derived tumours suggests that these cells are able to by-pass p53 tumour suppression [148, 152, 160]. In addition, malignant melanoma cells have been shown to undergo apoptosis in response to p53 [47]. In humans, *PAX3* is frequently expressed in cancers derived from cells of neural crest origin, notably neuroblastoma and melanoma [8]. *PAX3* mRNA is detectable in 77% of cultured primary malignant melanomas in which expression increases with advancing tumour stage [105]. In neuroblastoma cell lines it is expressed due to transcriptional up regulation by N-MYC [106]. *PAX3* is also expressed in myogenic cell lineages as PAX3, or a PAX3-FKHR fusion protein in embryonal rhabdomyosarcoma and paediatric alveolar rhabdomyosarcoma respectively [103]. Treatment of neural crest and myogenic derived tumour cell lines with PAX3 antisense leads to apoptosis [103, 105]. One possible explanation for these findings is that p53 function is inhibited by PAX3. Support for this interaction comes from work on *Splotch* embryos. Homozygous *Splotch* embryos develop open neural tube defects by day ten and show high levels of neuroepithelial apoptosis [20, 81]. *Splotch* embryos exhibit increased levels of p53 protein, but not mRNA compared to their normal litter-mates [20]. When crossed into a *TP53* null background neural tube defects in *Splotch* embryos are abolished and the *Splotch* embryos are indistinguishable from their litter mates. A partial rescue from NTD is seen when the mothers of *Splotch* embryos are treated with the chemical p53 inhibitor pifithrin-α [20]. PAX proteins have been documented to inhibit p53 transcription directly [154]. No such evidence exists for PAX3.

The collected experimental data from murine development and cancer cell lines has raised the possibility that PAX3 may be promoting the survival of neural crest derived

tumours and myogenic tumours through the suppression of the p53 pathway [8-10, 20, 31, 80, 94, 103-105, 149, 161, 162]. This chapter describes the investigation of this hypothesis. I initially describe the creation of a model system to study p53 activity and the effects of PAX3 in this system. Later the creation of a series of mutant PAX3 proteins is documented and the ability of these proteins to repress p53 function is tested.

4.2 Results

4.2.1 Generation of a model system to study p53 dependent transcription

The tumour suppressor protein p53 acts on a range of cellular effectors to produce cell cycle arrest and/or apoptosis [41]. p53 is a transcription factor; the ability of p53 to influence cell fate relies on its ability to modulate target gene transcription [41, 45, 46, 50, 110, 112, 137]. To study the biological activity of p53 it is therefore necessary to examine the ability of p53 to act as a transcription factor. To do this a number of specific p53 targets were selected. The ability of p53 to induce transcription from the promoter region of each target was then measured using a reporter gene assay. A reporter gene consists of the promoter region from the gene of interest fused to the coding region of a gene that when transcribed can be measured in an *in vitro* system e.g. the firefly luciferase protein. In the case of luciferase, stimulation of the promoter is detected as emitted light in the presence of the appropriate luciferase substrate. Prior to my arrival, our laboratory had been using β galactosidase as an internal control in a 60mm dish format but this had the drawback of being relatively time consuming and offering no real way of comparing results between experiments. I conducted an initial optimisation of this system and moved to a 96 well format. This enabled high throughput of samples with relatively few cells (as little as 2500 per well) for each experiment. I also decided to use the renilla internal control plasmid pRLSV40 and the Dual-Glo^{TM} reporter assay system (Promega). The pRLSV40 plasmid contains the constitutively active renilla luciferase protein under the control of the SV40 promoter. The inclusion of pRLSV40 controls for variations in transfection efficiency and the possibility of general transcriptional repression by PAX3. This form of internal control also makes it possible to compare results between separate experiments. For each experiment duplicate wells were transfected with the appropriate reporter gene and pRLSV40. Firefly luciferase activity relative to renilla luciferase activity was calculated for each well and a mean relative luciferase unit (RLU) figure was generated for each duplicate set. For comparison between experiments the sum of the means for duplicate wells was calculated for all experiments. These were then used to calculate a conversion factor between plates using the plate with the highest overall mean total as a reference.

Initial experiments were conducted using the promoter region from the BCL-2 family protein BAX. The *BAX* gene is a direct transcriptional target of p53 and contains a direct p53 response element [41, 45]. Increased transcription of *BAX* is one of the first steps in the p53 induced apoptosis pathway, changing the balance between pro- and anti-apoptotic BCL-2 protein family members. BAX is therefore a biologically relevant p53 target for study. Baxluc contains a 369 b.p. fragment of the *BAX* promoter region, retaining the p53 response element, in pGL3Basic [50].

Figure 4.1 shows the optimisation of p53 induced *BAX* transcription in three separate cell lines. For each cell line the experiment was conducted at least twice. In Figure 4.1A the human neuroblastoma cell line IMR 32 was used. This is a cell line known to express PAX3 and have wild type p53 [106, 163]. The relative luciferase activity at all amounts of p53 is high, possibly reflecting endogenous p53 activity. Sequential induction of *BAX* is observed as the quantity of p53 expression vector is increased. At 2.5 ng of p53 expression vector, *BAX* reporter induction is seventeen times that of baseline. Inclusion of the pGL3Basic plasmid controlled for any non-specific induction of reporter activity. Figure 4.1B shows the results of similar experiments in the mouse embryonic fibroblast cell line NIH3T3. This cell line is not known to express PAX3 and has wild type p53. Relative luciferase activity in this system was somewhat lower than in IMR 32 cells. It is possible that the endogenous p53 was not as active in this cell line, or that the endogenous mouse p53 protein was unable to bind the human *BAX* promoter. Once again sequential induction of the Bax reporter was observed with increasing levels of p53. Induction eleven times that of baseline was observed at 5 ng of p53. In Figure 4.1C the human non-small cell lung cancer cell line H1299 was used. H1299 cells have a homozygous partial deletion of the p53 gene and lack expression of p53 but retain intact downstream p53 pathways [164, 165]. In this cell system it is possible to study the relationship between PAX3 and p53 without the potential influence of endogenous proteins. The lack of endogenous p53 activity in H1299 cells has meant that the relative luciferase activity observed was much less than in IMR 32 or NIH3T3 cells. Titration of reporter activity was still observed in the H1299 system with induction of *BAX* reporter activity at 5 ng of p53 expression plasmid twelve times that of base line levels.

These experiments established the reporter gene assay as a reliable and reproducible system in which to study the effects of PAX3 on p53 dependent transcription.

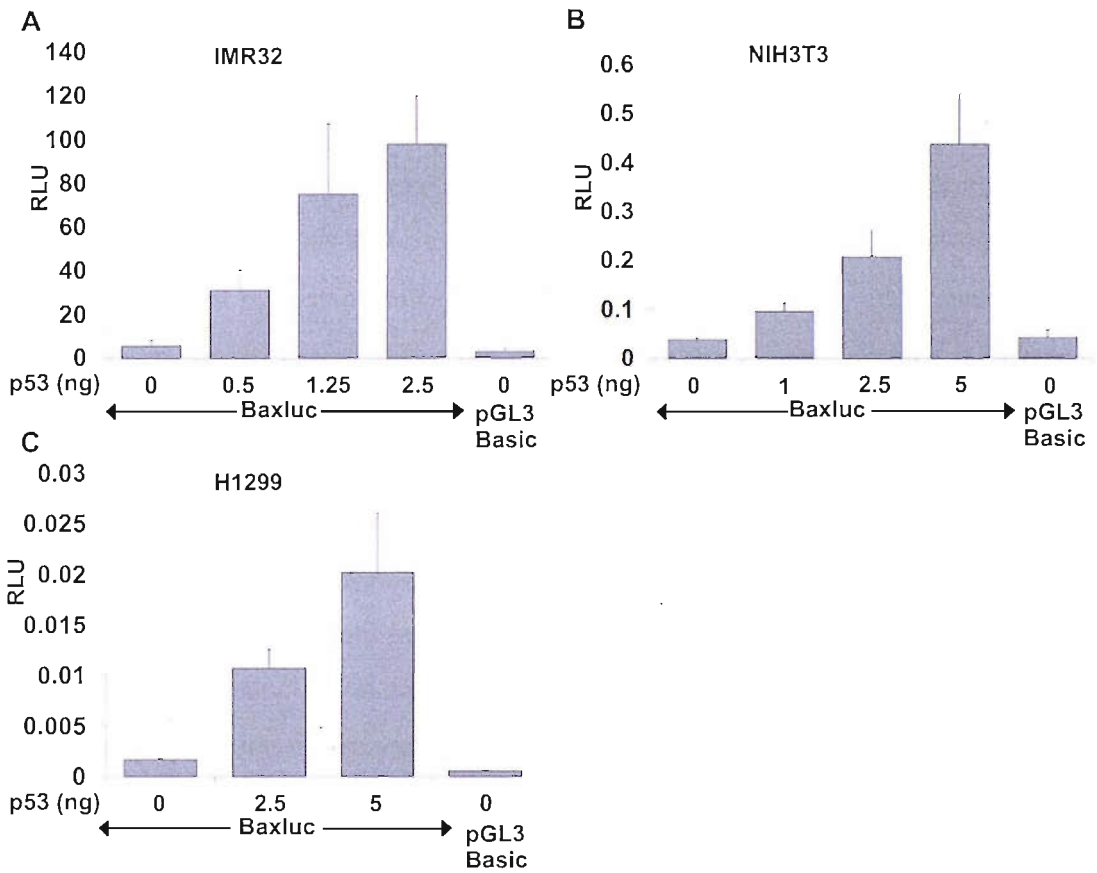

Figure 4.1: p53 expression induces transcription of Baxluc in three different cell lines in a dose dependent manner

Human neuroblastoma IMR 32 cells (A), mouse fibroblast NIH3T3 cells (B) and human non-small cell lung cancer H1299 cells (p53 null) (C) were transfected with 100 ng of Baxluc or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=4). All data are mean RLU ± S.E.M.

4.2.2 PAX3 inhibits p53 dependent transcription of a Bax reporter gene

Having established that p53 caused a reproducible induction of Baxluc in the cell lines of interest the ability of PAX3 to inhibit this induction was tested. Figure 4.2 shows the effects of varying concentrations of PAX3 expression vector in the Baxluc reporter system. Figure 4.2A shows the effect of PAX3 expression in IMR 32 cells. There was clear induction of Bax reporter transcription in the presence of p53 (seventeen times relative luciferase activity at 2.5 ng p53 compared to baseline). Upon the addition of PAX3 to the system two observations can be made. Firstly, there is an apparent repression of reporter gene induction caused by endogenous p53 (open bars). However when the raw data for this experiment was assessed it became clear that this was due to effects of PAX3 on the renilla control plasmid (data not shown). The second and more striking observation is the repression of p53 mediated reporter induction observed with increasing levels of exogenous p53 in the presence of PAX3. At 2.5 ng of p53 mean relative *BAX* reporter activity is 97.67; this falls to 26.22 with 50 ng of PAX3 and falls further to 20.46 in the presence of 100 ng of PAX3. There is a reduction in activity of 79% ($p<0.01$). At these higher levels of *BAX* reporter induction the non-specific effects of PAX3 on renilla transcription were not observed. Figure 4.2B shows the results of the same experiment conducted in NIH3T3 cells. There is marked repression of p53 dependent reporter activity with both 50 ng and 100 ng of PAX3. Maximal inhibition of reporter activity is seen with the higher dose of PAX3 and amounts to a repression of 77% (comparison between 2.5 ng p53, no PAX3 and 2.5 ng p53, 100 ng PAX3) ($p<0.001$). In Figure 2.2C the observations have been extended to the H1299 cell line. This system was chosen because there is no endogenous p53 activity. The low level baseline activity of the *BAX* reporter gene is not affected by the addition of PAX3 to this system (open bars). This suggests that PAX3 has a specific effect on p53 dependent transcription and is not simply acting to shut down transcription in general. This possibility is further controlled for by the inclusion of the renilla plasmid. Repression of p53 dependent *BAX* reporter activity by PAX3 is seen with all doses of PAX3 but is best observed by comparing the levels of activity observed at 5 ng of p53 expression vector (black bars). Maximal repression of p53 dependent transcription is 74% at this dose of p53

with 100 ng of PAX3 ($p<0.001$).

Taken together the results from all three cell lines show that PAX3 specifically represses p53 dependent transcription from a *BAX* reporter construct. The repression is PAX3 dose-dependent and not due to a general shut down of transcription. *BAX* is only one target of p53 and to confirm these initial observations it was important to test other p53 targets in the same assay. To do this I used the H1299 cell line. This cell line enabled the study of the p53/PAX3 interaction without the confliction of endogenous protein activity and allowed investigation of protein levels and p53 activity in subsequent experiments (described later).

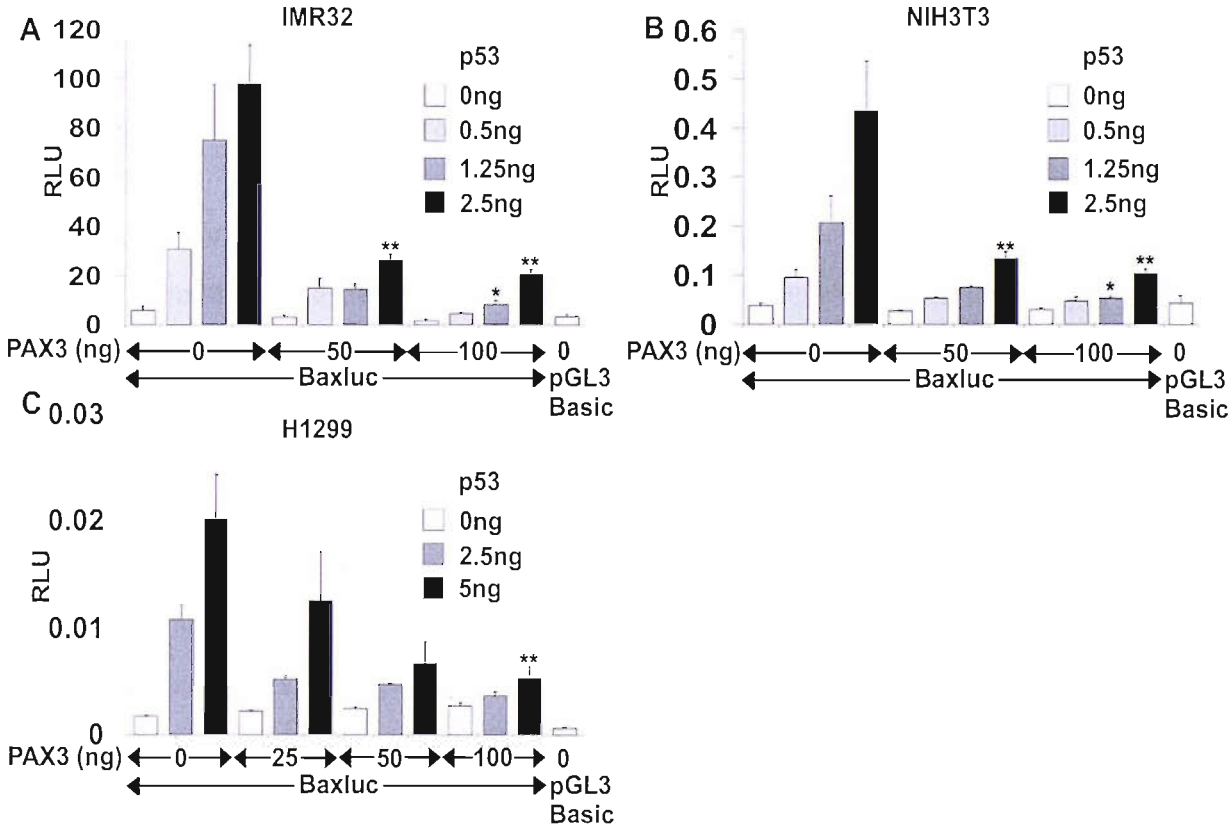

Figure 4.2: PAX3 inhibits p53 dependent transcription of a Baxluc reporter gene in three different cell lines

Human neuroblastoma IMR 32 cells (A), mouse fibroblast NIH3T3 cells (B) and human non-small cell lung cancer H1299 cells (p53 null) (C) were transfected with 100 ng of Baxluc or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=4). Increasing amounts of pJ7 PAX3 was added as shown. Total amounts of transfected

plasmid were equalized using empty pJ7 vector. All data are mean RLU ± S.E.M.

4.2.3 PAX3 inhibits p53 dependent transcription of an HDM2 reporter gene

One of the best described targets of p53 is the p53 ubiquitin ligase HDM2 [120, 126, 130, 137, 147, 166-168]. The major cellular mechanism for p53 protein regulation is via HDM2 mediated ubiquitination, leading to nuclear export and proteasomal degradation. The rate of HDM2 protein synthesis in response to rising p53 levels is rapid leading to tight control of p53 activity [45, 147, 169]. HDM2 expression is regulated by transcription from two distinct promoters, P1 and P2 [170]. Transcription from P1 is constitutive at low levels in most cells, whereas P2 is highly induced by p53 because of the presence of two conserved p53-REs in both the murine *Mdm2-P2* [126, 168] and human *HDM2-P2* promoters [170]. Studies dissecting the mechanisms that control transcription of *Mdm2* and *HDM2* have focused on the P2 promoter, primarily because the human P1 promoter-dependent transcript is poorly translated [171]. The *HDM2-P2* promoter is therefore an excellent target for use in a reporter assay because it requires minimally toxic, low levels of p53 to be activated. The Hdm2luc03 reporter construct used throughout this study contains 165 b.p. (-132 to +33) of the P2 promoter region including both p53-RE's. Before considering the effects of PAX3 on the *HDM2* reporter it was necessary to titrate the level of induction of *HDM2* by p53 to a level comparable with the *BAX* construct. Figure 4.3 shows the results of titrating p53 to achieve induction of the *HDM2* reporter gene. As expected the relative activity of the *HDM2* reporter is much higher than the *BAX* reporter gene as evidenced by the small quantities of p53 required to produce a similar level of luciferase activity. Using the *BAX* reporter 5 ng of p53 is required to achieve a relative luciferase activity of 0.02 (Fig. 4.2). In the same cell line only 0.015 ng of p53 is required to achieve the same level of activity when using the *HDM2* reporter gene (Fig. 4.3). This is likely to be due to the additional p53 binding site present in the *HDM2* promoter. To enable comparison between p53 reporter targets p53 at 0.015 ng and 0.0625 ng was used for further experiments with *HDM2* in H1299 cells.

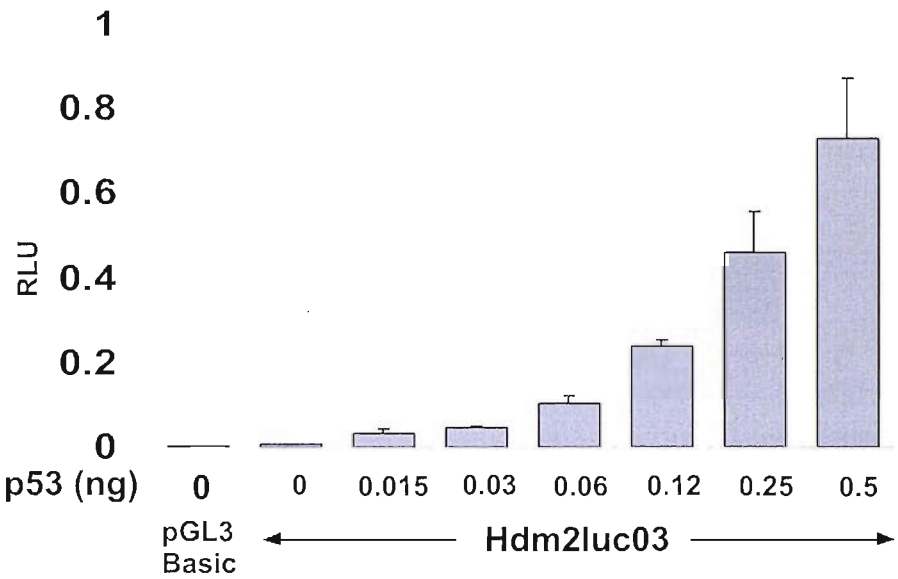

Figure 4.3: Titration of p53 dependent induction of an Hdm2 reporter gene in H1299 cells

H1299 cells were transfected with 100 ng of the p53 reporter construct Hdm2luc03, 20 ng of control renilla plasmid pRLSV40 and increasing amounts of p53 wild type expression vector as indicated. pGL3Basic at 10 ng was transfected as a negative control in a separate well. Dual-Glo™ reporter assays were performed 48 h after transfection (n=2). All data are mean RLU ± S.D.

Having titrated p53 to achieve appropriate levels of induction with the *HDM2* reporter I next tested the ability of PAX3 to inhibit p53 dependent transcription from the *HDM2-P2* promoter. Figure 2.4 represents the data from four separate experiments (n=8). There is a significant (p=0.001) inhibition of Hdm2luc03 reporter activity with the addition of PAX3 at 100 ng at the highest concentration of p53 tested (black bars). This inhibition represents a fall in HDM2 promoter activity of 57%. There is a more modest, but significant, inhibition of p53 dependent Hdm2luc03 activity at 0.015 ng of p53 ($p<0.05$). PAX3 is observed not to affect basal transcription (open bars).

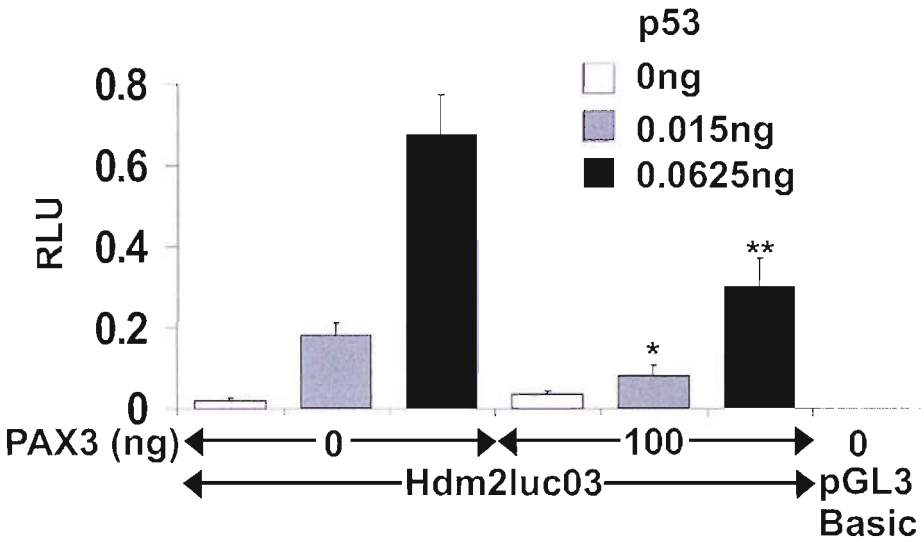

Figure 4.4: PAX3 inhibits p53 dependent transcription of a Hdm2luc03 reporter gene

H1299 cells were transfected with 100 ng of Hdm2luc03 or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=8). Increasing amounts of pJ7 PAX3 was added as shown. Total amounts of transfected plasmid were equalized using empty pJ7 vector. All data are mean RLU ± S.E.M.

I extended this finding by examining the role of PAX3 in suppression of p53 dependent transcription from an endogenous promoter by performing a quantitative RT-PCR for the level of *HDM2-P2* transcript in H1299 cells in the presence or absence of PAX3. PAX3 also suppressed p53-dependent expression of the endogenous *HDM2-P2* transcript (Fig. 4.5), suggesting that the suppression of p53-dependent transcription by PAX3 is not restricted to synthetic reporter gene assays.

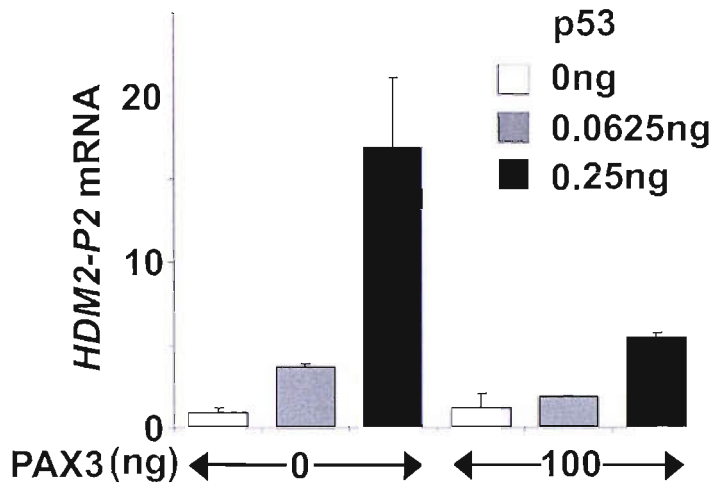

Figure 4.5: PAX3 reduces the relative abundance of p53 dependent HDM2 transcripts

The abundance of endogenous *HDM2-P2* mRNA transcripts was determined by qRT-PCR in H1299 cells transfected with increasing amounts of p53 expression vector and pJ7PAX3 as indicated. Total plasmid concentrations were normalized with pJ7. Data is normalised to *GAPDH*, is mean $\pm$ SD for duplicate qRT-PCR assays, and is representative of two independent experiments (Transfections were in 60 mm dishes. For ease of comparison the amounts of plasmids shown are the equivalents by surface area of a 96 well plate transfection).

To prove that the effects observed on the Hdm2luc03 reporter gene were due to p53-dependent transcription a further control experiment was conducted (Fig. 4.6). The reporter construct Hdm2luc13 contains 616 b.p. (-583 to +33) of the *HDM2-P2* promoter region, with two recognised p53 response elements [172]. Hdm2luc17 was derived from Hdm2luc13 by site directed mutagenesis to generate two independent 2 b.p. substitutions in the two p53-response elements. PAX3 is clearly observed to inhibit transcription from Hdm2luc13 that is p53 dose dependent ($p<0.05$), whereas Hdm2luc17 has no activity.

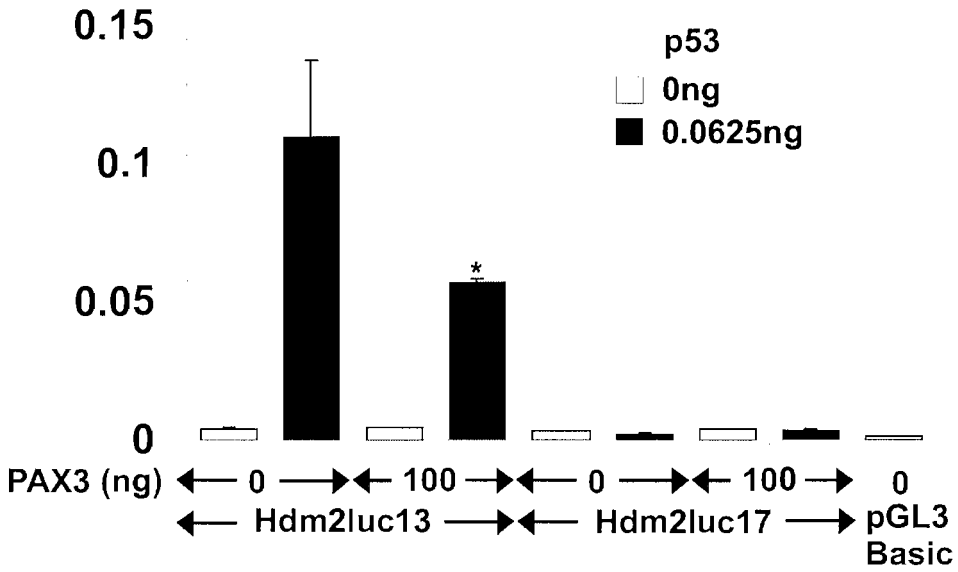

Figure 4.6: PAX3 inhibition of Hdm2 reporter gene transcription is p53 dependent

H1299 cells were transfected with 100 ng of Hdm2luc13, Hdm2luc17 or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=2). Increasing amounts of pJ7 PAX3 was added as shown. Total amounts of transfected plasmid were equalized using empty pJ7 vector. All data are mean RLU ± S.E.M.

4.2.4 PAX3 promotes transcription of *WAF1* and *PUMA* reporter genes independently of p53

PAX3 has been shown to inhibit the p53 dependent induction of two p53 target genes using the reporter assay system, *BAX* and *HDM2*. Both BAX and HDM2 are important p53 targets responsible for significant cellular events but they are not the only targets of p53. BAX is a pro-apoptotic protein that acts in the mitochondrial death pathway [45]. Another effector of p53 stimulated apoptosis is PUMA (p53 up-regulated mediator of apoptosis). PUMA is a BH3 only protein that may act as a direct p53 mediator of apoptosis [49]. PUMA was therefore considered as a possible target for PAX3 mediated transcriptional repression. To consider the range of effectors involved after p53 stimulation one must consider cell cycle arrest as well as apoptosis. One of the critical p53 responsive cell cycle arrest proteins is the cyclin dependent kinase inhibitor $p21^{WAF1}$ [46]. I tested a *PUMA* and a *WAF1* reporter alongside the Hdm2luc03 reporter to determine if PAX3 inhibited p53 dependent transcription from these promoters (Fig. 4.7). In the absence of PAX3, reporter gene

induction is observed with increasing amounts of p53. This finding replicates the observations for both *BAX* and *HDM2*. Interestingly for both *WAF1* and *PUMA* it appears that PAX3 is able to induce transcription independently of p53. At the highest levels of p53 used (0.3125 ng, black bars) there is an increase in reporter activity of 70% for *WAF1* and 60% for *PUMA* in the presence of 100 ng of PAX3 compared to no PAX3. I was confident that the induction of transcription from these promoters was not p53 related as an increase in luciferase activity is seen with increasing doses of PAX3 in the absence of p53 (open bars). This finding made it impossible to use the *PUMA* and *WAF1* reporter genes to further study the inhibition of p53 function by PAX3, but is an interesting finding in its own right. It suggests the possibility that PAX3 has promoter specific effects on p53 targets.

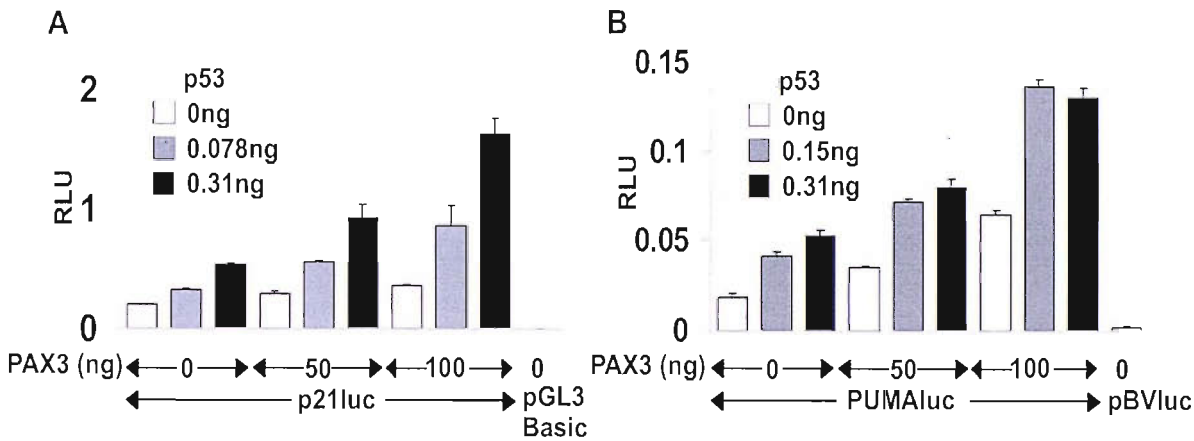

Figure 4.7: PAX3 induces transcription of p21 Luc and PUMA Luc independently from p53

H1299 cells were transfected with 100ng of p21luc or PUMAluc or the appropriate empty vector as indicated, 20ng pRLSV40 and the indicated amount of p53 plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=4). Increasing amounts of pJ7 PAX3 was added as shown. Total amounts of transfected plasmid were equalized using empty pJ7 vector. All data are mean RLU ± S.E.M.

4.2.5 Generation of mutant PAX3 proteins

PAX3 is a transcription factor. PAX family proteins bind to specific DNA sequences and may cooperate with other DNA binding proteins to regulate transcription [173]. The protein structure of PAX3 has been resolved [21]. The full length PAX3 protein consists of 479 amino acids and contains a number of specialised domains that are required for its transcriptional activity (Fig. 4.8). Using the published nucleotide sequence for *PAX3,* and with the help of 4th year medical student Mr. Jay Amin, I generated a series of mutations in the *PAX3* coding sequence to create proteins with altered structure. This was performed by site directed mutagenesis. Initially a series of truncations were generated by inserting stop codons into the *PAX3* coding sequence (Fig. 4.8). The position of each stop was carefully designed to remove each active domain of PAX3 in turn from the C-terminus. The largest mutant PAX3 protein Stop281 contains 281 amino acids of the full length protein but lacks the C-terminal transactivation domain. Both the DNA binding paired domain and homeodomain are intact. The next largest mutant PAX3 protein, Stop 222 consists of 222 amino acids and lacks both the transactivation domain and the homeodomain but retains the paired domain and octapeptide motif. Stop 34 is the shortest mutant PAX3 protein generated and only retains a part of the N-terminal inhibitory domain.

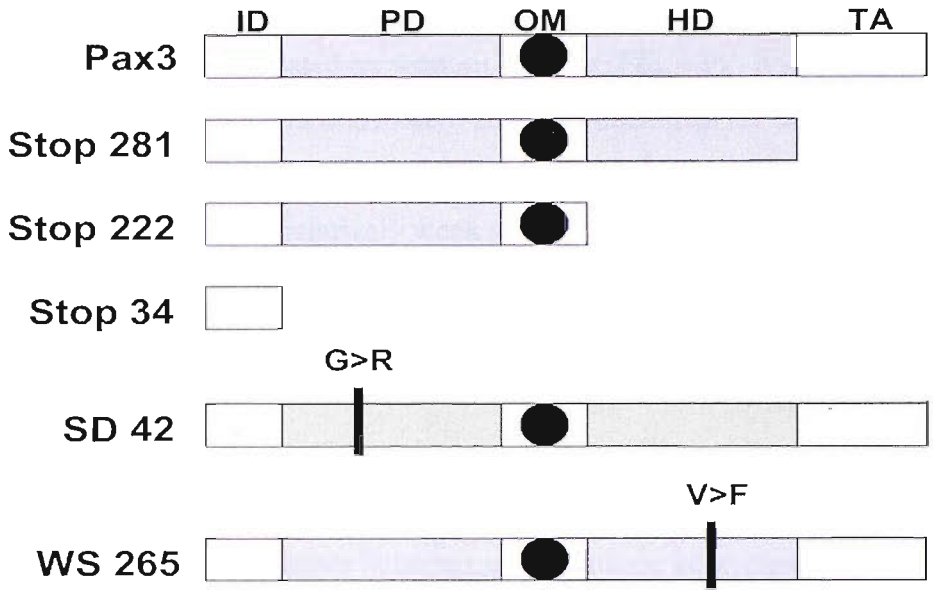

Figure 4.8: Mutant PAX3 proteins

PAX3 protein structure, and a description of some of the mutant proteins used in this study. ID = inhibitory domain, PD = paired domain, OM = octapeptide motif, HD = homeodomain, TA = transactivation domain. Three truncations of PAX3 were generated using site directed mutagenesis. Each one in turn removes a domain implicated in transcriptional activity. The point mutants SD42 and WS265 mimic disease specific mutations in mouse and human respectively.

In addition to the truncation mutants generated we also constructed two disease specific point mutants. The mutant PAX3 protein SD42 corresponds to a documented mutation leading to the *Splotch delayed* (SpD) phenotype in mouse [79, 100, 174]. A single base substitution, glycine to arginine substitution at amino acid 42 within the DNA binding paired domain, leads to a 17-fold reduction in paired domain binding to a paired domain specific oligonucleotide [100]. The SpD mutation also decreases homeodomain binding by 21-fold *in vitro* [100]. The mutant PAX3 protein WS265 is taken from the human PAX3 related disease Waardenburg syndrome. A single nucleotide substitution leads to a valine to phenylalanine substitution at amino acid 265 within the homeodomain [93, 95]. The generation of single point mutants allowed both of the DNA binding domains to be altered at sites documented to affect DNA binding function without changing the conformation of the entire protein.

Each of the mutants generated was validated by DNA sequencing. Plasmids containing the correct mutation and with no other mutations in the PAX3 coding sequence were further validated by western blotting (Fig. 4.9). It is clear that the mutants are well expressed in this system and the truncations are at a size corresponding to their expected size based on the DNA sequence. The shortest mutant Stop 34 produces a relatively weak signal. This may be because an important antibody epitope is contained in the deleted C-terminal region (the PAX3 antibody used is polyclonal and was raised against a synthetic peptide representing amino acids 1-347) or because the mRNA or protein is less stable. Differences in expression are unlikely given the similarity of the expression vector. The point mutants SD42 and WS265 run at the same size as the full length PAX3 protein and their expression has not been affected by the changes in amino acid sequence generated.

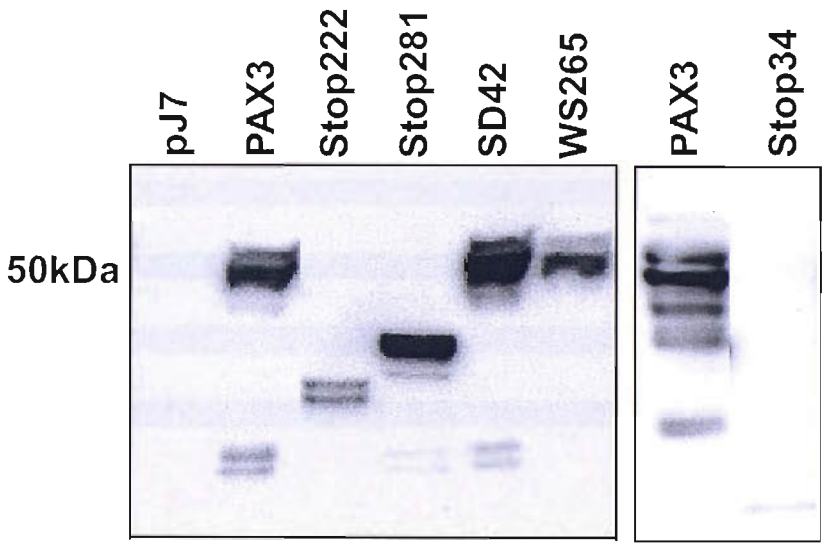

Figure 4.9: Validation of mutant PAX3 protein expression by western blot

Representative western blots showing a number of the PAX3 mutant proteins generated. Equal amounts of each PAX3 mutant was transfected into H1299 cells in 60mm dishes. pJ7 = empty vector.

4.2.6 PAX3 must retain transcriptional integrity to repress p53 dependent transcription

The panel of mutant PAX3 proteins generated by site directed mutagenesis were tested for their ability to inhibit p53 dependent transcription in H1299 cells.

Experiments were conducted in the same way as described in the previous sections with the effect of mutant PAX3 proteins being tested against full length PAX3. Initially the Hdm2luc03 reporter construct was used. The *HDM2* reporter was chosen because it requires the least p53 for activation and therefore any subtle changes in the ability of the mutant PAX3 proteins to inhibit transcription would be most easily detected. As previously demonstrated, PAX3 significantly represses p53 dependent transcription from the Hdm2luc03 construct by approximately 50% ($p<0.001$) (0.0625 ng p53 vector) (Fig. 4.4 and 4.9). None of the mutant PAX3 proteins have this effect (Fig.4.9).

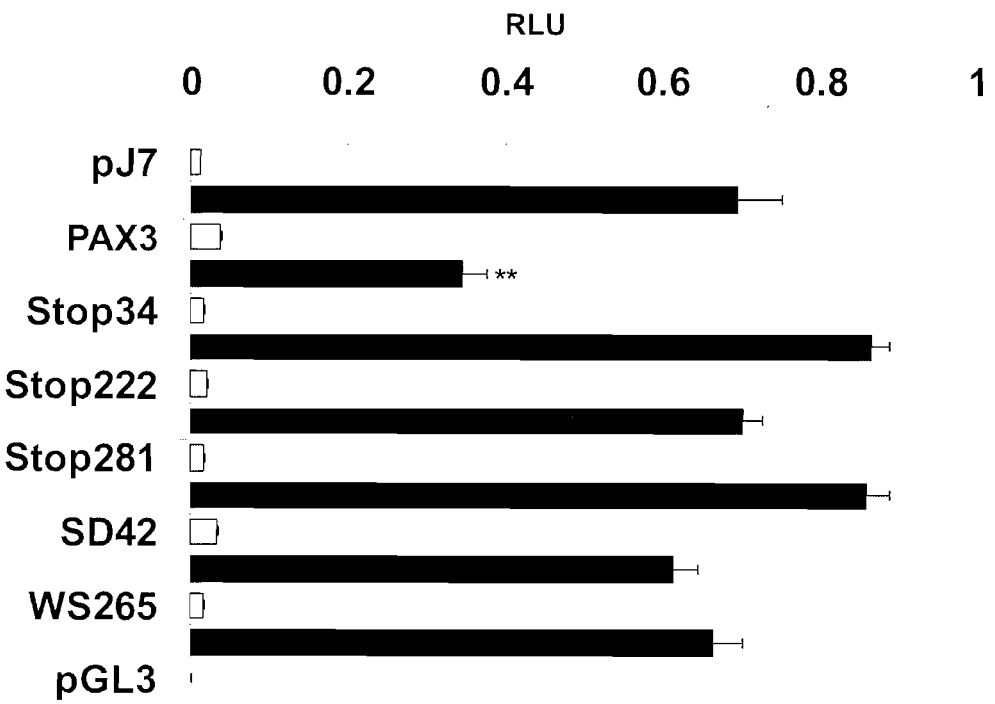

Figure 4.10: PAX3 must retain transcriptional integrity to inhibit p53 dependent transcription from an *HDM2* promoter

Mutant PAX3 proteins were tested for repression of p53 dependent transcription in a reporter assay in H1299 cells in a 96 well format. Cells were transfected with 20 ng pRLSV40, 100 ng pJ7 empty vector or the indicated PAX3 mutant and 100 ng Hdm2luc03 or pGL3Basic. Open bars = 0 ng p53 plasmid, closed bars = 0.0625 ng p53 plasmid. All data are mean RLU $\pm$ S.E.M (n=8).

The mutant PAX3 proteins were unable to repress p53-dependent transcription from the *HDM2* promoter. The mutant PAX3 proteins were next tested against the *BAX* reporter construct. PAX3 represses p53 dependent transcription from the *BAX* promoter by over 45% in all experiments at 2.5 ng of p53 ($p<0.05$) (Fig. 4.2 and Fig.

4.11). Figure 4.11 shows that only the transcriptionally competent PAX3 protein represses p53 dependent transcription and none of the mutant PAX3 proteins generated have this effect.

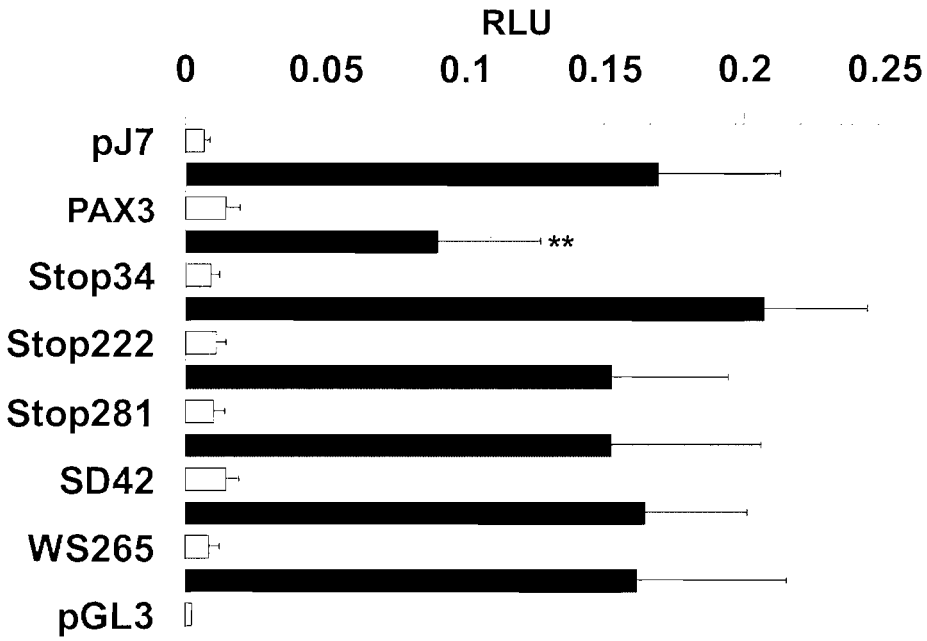

Figure 4.11: PAX3 must retain transcriptional integrity to inhibit p53 dependent transcription from a *BAX* promoter

Mutant PAX3 proteins were tested for repression of p53 dependent transcription in a reporter assay in H1299 cells in a 96 well format. Cells were transfected with 20 ng pRLSV40, 100 ng pJ7 empty vector or the indicated PAX3 mutant and 100 ng Baxluc or pGL3Basic. Open bars = 0 ng p53 plasmid, closed bars = 2.5 ng p53 plasmid. All data are mean RLU ± S.E.M (n=8).

4.2.7 DNA binding by both the paired domain and homeodomain of PAX3 is required for inhibition of p53 dependent transcription

The mutant PAX3 proteins SD42 and WS26 were generated due their disease specificity in mice and humans respectively. This strategy enabled the results obtained with these mutants to be interpreted with a degree of biological significance. These two mutants did not, however, provide information that would allow one to attribute the specific domains of PAX3 responsible for its effect on p53 transcription. More specifically, the G>R mutation in the PD of SD42 causes a 17-fold decrease in

its ability to bind to a PD consensus sequence as well as a 21-fold decrease in the ability of the unmutated HD to bind onto a P2 site [100]. This means that the SD42 mutant is unable to efficiently bind DNA by either DNA binding motif. The possibility remained that DNA binding by either the paired domain or homeodomain in isolation (but within the context of the full length protein) was sufficient to generate inhibition of p53 function by PAX3. To test this possibility I generated a further PAX3 mutant N47H (Fig.4.11), that has been shown to abrogate DNA binding by the PD but increase HD binding activity [102]. I confirmed the expression of N47H in H1299 cells and it was tested for repression of p53 dependent activity of the *HDM2* promoter. Once again wild-type PAX3 was observed to repress p53 dependent transcription ($p<0.05$) but the N47H mutant did not have this effect (Fig. 4.12B).

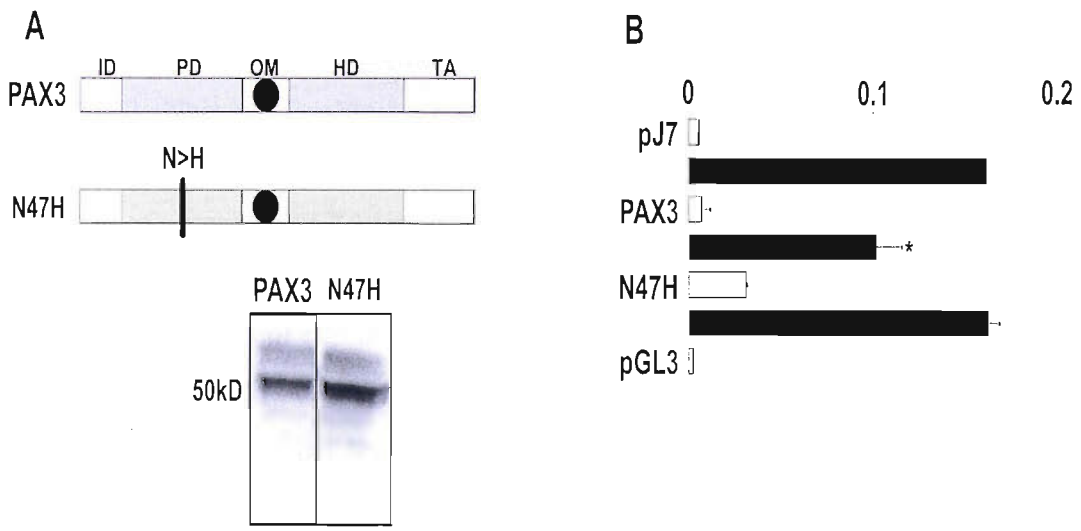

Figure 4.12: The mutant PAX3 protein N47H is expressed in H1299 cells and is unable to repress p53 dependent transcription from an *HDM2* promoter

(A) The PAX3 mutant protein N47H was generated by site directed mutagenesis and expressed in H1299 cells. N47H mutant was tested for repression of p53 dependent transcription in a reporter assay in H1299 cells in a 96 well format. Cells were transfected with 20 ng pRLSV40, 100 ng pJ7 empty vector or the N47H mutant and 100 ng Hdm2luc03 or pGL3Basic. Open bars = 0 ng p53 plasmid, closed bars = 0.0625 ng p53 plasmid. All data are mean RLU $\pm$ S.D (n=2).

4.3 Discussion

A growing body of evidence suggests a link between PAX3 expression and inhibition of p53 function [20, 44, 47, 103]. Much of this evidence relates down-regulation of PAX3 with apoptosis, although only recently has a direct link with p53 been suggested [20]. The actions of p53 in the cell are predominantly mediated via its transcriptional activity. I have optimised a model system to study the ability of p53 to function as a transcription factor, making use of well characterised p53 target genes. The reporter assay system created allows rapid analysis of p53 transcriptional behaviour using a small number of cells. Reproducible induction of the p53 targets *BAX* and *HDM2* has been demonstrated in the cell lines IMR 32, NIH3T3 and H1299 in response to p53. Induction is dependent on the amount of p53 expression vector transfected. The three cell lines used differ in their species of origin and p53 status. IMR 32 cells are derived from a neuroblastoma cell line demonstrated to express PAX3 [106]. This cell line retains the cellular machinery necessary for PAX3 expression and would be expected to be a cellular environment conducive to the expression of potential PAX3 target genes. The mouse fibroblast cell line NIH3T3 is not known to express PAX3 and has intact p53 [157]. H1299, a non-small cell lung cancer cell line does not express p53 by virtue of a homozygous partial deletion and has not been demonstrated to express PAX3 [158]. H1299 cells therefore represent a "cellular test-tube" to allow the study of two completely exogenous proteins.

I have shown for the first time in an *in vitro* model that PAX3 inhibits the p53 dependent induction of reporter constructs containing elements of the *HDM2* and *BAX* gene promoter regions. This finding is reproducible between cell lines. Results from the p53 null H1299 cells show that PAX3 represses p53 function in the absence of endogenous PAX3 or p53 expression. PAX3 has been documented to possess a transcriptional repressive function and can alter the epigenetic environment through association with the co-repressor KAP1 [175, 176]. To control for the possibility of overall transcriptional inhibition masking a specific effect on p53 dependent transcription the renilla internal control plasmid was use in all transfections. While there was some evidence of transcriptional shut-down with very high amounts of PAX3 expression vector this was not observed within the experimental range

presented in this study. The possibility that the observed effects of PAX3 on p53 targets were independent of p53 was examined by experiments using the Hdm2luc13 and Hdm2luc17 constructs. These experiments confirm that PAX3 acts on transcription driven from the recognised p53 response elements in the *HDM2* promoter.

Interestingly PAX3 is shown to induce transcription from the promoters for *WAF1* and *PUMA* independently of p53. This is likely to be due to promoter specific effects of PAX3 on p53 target genes as these experiments were conducted in the H1299 cell line that does not express p53. A recent report supporting this possibility has shown that $p21^{WAF1}$ is up-regulated by the PAX3 transcriptional target MITF [177]. To date there have been no studies to examine the relative levels of specific p53 targets in tumours that express PAX3 and this is an area of study that should be explored.

These experiments were conducted in a transient transfection setting in relatively artificial cellular systems. There are significant drawbacks of using transient expression systems that make it hard to extrapolate these results to endogenous genes. For example, the regulatory context of endogenous protein interactions may differ in terms of gene dosage, chromatin formation, local gene effects and the presence of gene regulatory elements not on the reporter gene construct. However, I have demonstrated that PAX3 suppressed p53-dependent expression of the endogenous *HDM2-P2* transcript, demonstrating that the suppression of p53-dependent transcription by PAX3 is not restricted to synthetic reporter gene assays and may be equally relevant to *in vivo* regulation of p53 function in neural crest derived tumours.

The establishment of a robust model that clearly shows PAX3 inhibition of p53 dependent transcription was the first step in understanding the mechanisms involved in the PAX3/p53 interaction. To investigate the structural elements of PAX3 required for inhibition of p53 dependent transcription a panel of PAX3 mutants were generated with the assistance of Mr. Jay Amin. Each mutant protein was designed to specifically alter a region of the PAX3 protein known to play a role in PAX3 transcriptional control. None of the mutant PAX3 proteins are able to inhibit p53 dependent transcription in H1299 cells. This finding suggests that PAX3 must retain transcriptional integrity in order to inhibit p53 function. By introducing disease

specific point mutations into the paired domain and homeodomain I have shown that these DNA binding motifs must be fully intact for p53 functional repression to occur. Moreover, it is only necessary to disrupt one of the DNA binding domains to completely inhibit PAX3 repression of p53 dependent transcription. There is evidence that binding by one domain causes a conformational change in the other, altering its ability to bind DNA [95, 178]. The reporter assay data presented here lends support to this observation. The loss of p53 inhibition observed with the truncation mutant Stop281 suggests that PAX3 must not only be able to bind DNA, but also the recruitment of other transcription factors is required to affect p53 function. The C-terminal deletion of the transactivation domain is enough to abrogate PAX3 inhibition of p53 function. Together these findings suggest that PAX3 may drive the expression of another, as yet undefined, protein (or proteins) in order to inhibit p53 transcriptional function. In neural crest derived lineages PAX3 has been shown to be not only required for tumour cell survival [31, 105], but also to prevent terminal differentiation and this is likely to require the regulation of a number of target genes [83].

The generation of the H1299 cell model system described in this chapter has been used to further study the mechanisms of PAX3 inhibition of p53 function. The findings from these experiments are described in the next chapter.

5. The effect of PAX3 on p53 protein abundance

5.1 Introduction

The primary cellular mechanism for regulation of p53 protein level is via degradation of p53 [41, 45, 122, 128, 129, 139, 147, 169]. HDM2 is the key regulator of p53 in the majority of cellular systems; p53 is targeted for nuclear export and proteasomal degradation by HDM2 binding leading to C-terminal ubiquitination. In response to cellular stress p53 can be stabilised and degradation prevented. Stabilisation of p53 can occur via phosphorylation of key amino acids within the HDM2 binding domain [110] or by the inhibition of HDM2 by stress response proteins such as ARF [41]. The balance between p53 and its direct transcriptional target HDM2 is the key to p53 activity. Any rise in the cellular level of p53 protein will lead to the appropriate p53 response for that cell.

I have established in the previous section that p53 is able to induce promoter activity of selected target genes in a model system and that this induction is repressed by the addition of PAX3. The mechanism of PAX3 repression of p53 dependent transcription is unknown but may be due to a direct effect of PAX3 on p53 protein levels. This theory is supported by evidence from the mouse embryo [20]. No studies have directly examined the effect of PAX3 on p53 protein level or stability. This chapter outlines the findings from a series of experiments designed to investigate the effects of PAX3 on p53 protein levels.

5.2 Results

5.2.1 Adaptation of the H1299 reporter assay system for p53 protein analysis

The H1299 cell line was used for all work involving p53 protein levels because it does not express endogenous p53. This meant that small changes in the levels of observed p53 would be easily detectable. The levels of p53 and PAX3 required for significant repression of p53 dependent transcription had been defined using the reporter assay system. The quantities of reagents used in the 96 well format were scaled up for use in 60mm dishes based on relative surface area, to a maximum of 4μg of transfected DNA per plate. The use of 60mm dishes was required to enable enough cells to be transfected for protein analysis by SDS-PAGE and western blotting. The first step in producing a meaningful protein assay was detection of p53 at the appropriate levels. Figure 5.1A shows a western blot for p53 protein using the DO-1 mouse monoclonal antibody. The amount of p53 transfected in each lane in Figure 5.1A corresponds to the level used to achieve induction of the *BAX* reporter gene in the 96 well format. At these levels of p53 there is no increase in expressed p53 quantity despite an increase in p53 plasmid. It is possible that the transcriptional machinery is saturated at this level of p53. It was therefore important to titrate the level of p53 downwards toward the range used for HDM2 induction in the reporter assays. Figure 5.1B shows the results of such a titration experiment. It can be seen that p53 is detectable using the DO-1 antibody when transfected at amounts known to induce HDM2 reporter activity (1.13 to 18 ng of p53 plasmid).

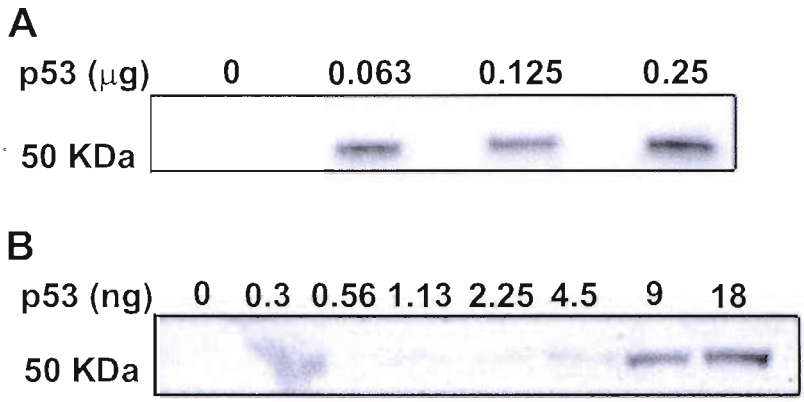

Figure 5.1: Titration of p53 in H1299 cells for detection with DO-1
H1299 cells were transfected in 60mm dishes with p53 at varying amounts as indicated. Transfected cells were analysed by western blotting at 48 h.

5.2.2 PAX3 decreases p53 protein abundance in H1299 cells

p53 was transfected into H1299 cells in 60mm dishes in the presence or absence of PAX3 (Fig. 5.2). Care was taken to ensure that the ratio of p53 to PAX3 corresponded to that used in the reporter assay experiments. In all experiments a small amount (5ng per plate) of the green fluorescent protein (GFP) plasmid EGFPN1 was added to control for differences in transfection efficiency between plates and to control for the possibility that PAX3 would act as a transcriptional repressor on the CMV promoter of the p53 expression vector. If this were the case the level of GFP would be expected to be reduced by a similar amount, as it is driven by the same promoter. The quantity of expressed p53 was estimated based on the band intensity observed in each lane. A Bio-Rad Fluor-S Multimager and Quantity One software was used for all experiments to estimate band intensity and protein abundance relative to GFP. Figure 5.2 shows that PAX3 represses the expression of p53 in H1299 cells at all levels of p53 tested. The western blot in Figure 5.2A clearly shows repression of p53 expression in the lanes where PAX3 is also present. This observation is most marked at the 0.3ng p53 level. Figure 5.2B shows the relative level of p53 repression by PAX3 as a percentage of the p53 alone band. PAX3 represses p53 protein level by 90% at 0.3ng of transfected p53.

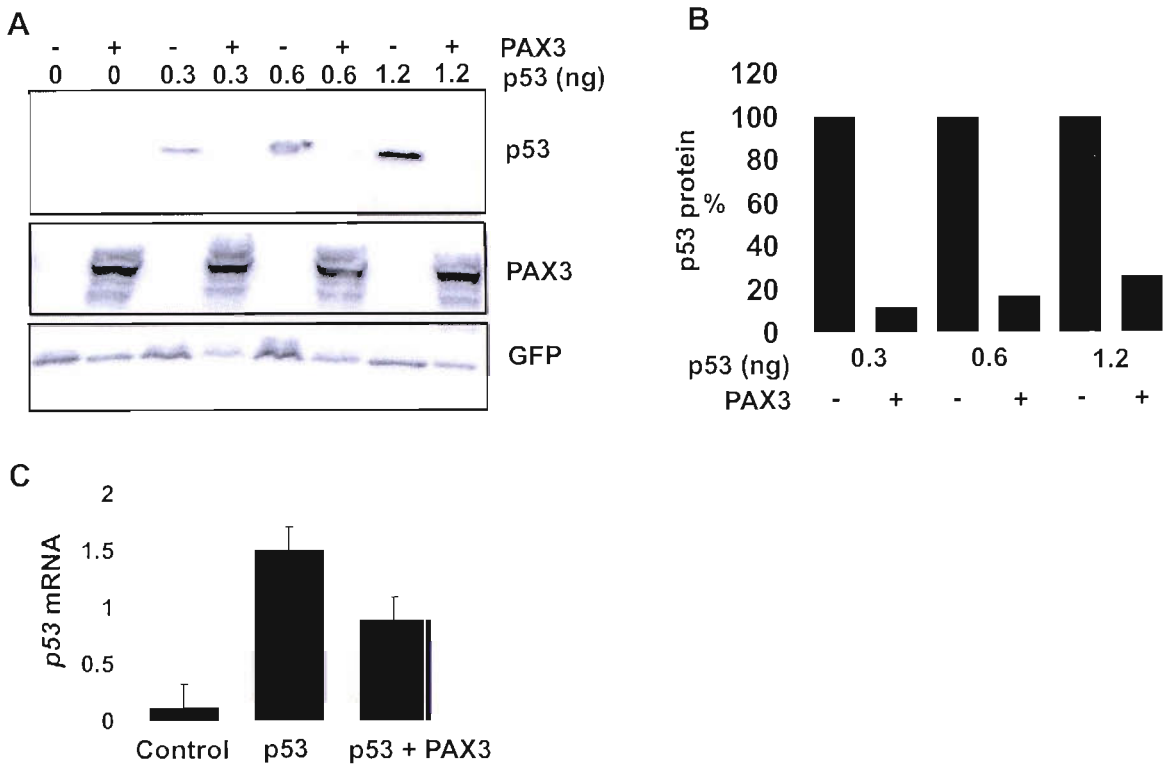

Figure 5.2: PAX3 represses p53 protein abundance in H1299 cells

(A) H1299 cells in 60mm dishes were transfected with EGFPN1 at 5ng per plate, pGL3 Basic at 1.8µg per plate and the indicated amount of p53. PAX3 or empty vector was added at 1.8 µg per plate as indicated. Total transfected plasmid was equalized with pJ7. Cells were harvested at 48 h. (B) Quantification of (A) showing relative abundance of p53 protein. (C) Cells were transfected as in (A). Quantitative RT-PCR was performed on duplicate samples transfected with the indicated expression plasmid (control = not transfected) and harvested at 48 h. *TP53* mRNA levels were normalised to *GAPDH* housekeeping gene expression and are expressed as a mean relative abundance ± S.D. of duplicate assays.

In an attempt to confirm that PAX3 was suppressing p53 protein expression and not inhibiting *TP53* gene transcription I performed a quantitative RT-PCR on samples prepared in an identical manner to those assessed for p53 protein. Figure 5.2C indicates a modest fall (~30%) in *TP53* mRNA in the presence of PAX3, although the effect that this would have on p53 protein expression is unclear. This modest fall in *TP53* mRNA in the presence of PAX3 should be considered in conjunction with the observed fall in EGFP protein expression used as a control for p53 protein expression. Although the level of EGFP mRNA in the presence of PAX3 has not been measured directly the fall in EGFP protein in the presence of PAX3 suggested that the observed

changes in p53 protein abundance in the presence of PAX3 were due to post-transcriptional repression of p53.

5.2.3 PAX3 increases the turnover of p53 in H1299 cells

The major degradation pathway for p53 is via the proteasome. It is possible that the repression of p53 protein level observed in the presence of PAX3 is mediated through increased p53 breakdown. To test this hypothesis I decided to block new protein synthesis and observe the time taken for p53 to be degraded in the presence or absence of PAX3. From this an estimate of the half-life of p53 could be made. Cycloheximide inhibits peptidyl transferase at the ribosome and effectively blocks all new protein synthesis [179]. Figure 5.3 shows the results of an initial experiment using cycloheximide. The half-life of p53 in most systems is short, between 15 and 45 minutes [180]. The initial time span for the experiment shown in Figure 4.3 extends beyond this period but it is clear that levels of p53 protein have not begun to reduce at 90 minutes. In addition, there is no change in the relative levels of p53 in the presence or absence of PAX3.

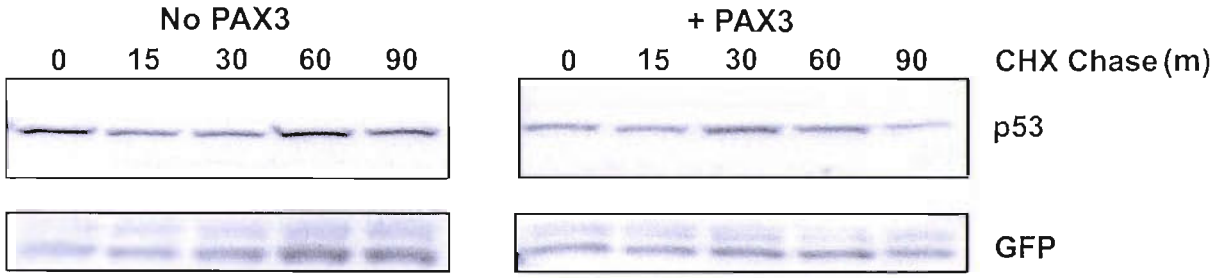

Figure 5.3: Relative p53 protein levels do not fall up to 90 minutes after cycloheximide treatment in H1299 cells

H1299 cells were transfected in 60mm dishes with 1.125ng p53, 1.8μg pGL3 Basic, 5ng EGFPN1 and 1.8 μg PAX3 or empty vector as indicated. At 48Hrs cycloheximide 100 μg/ml was added and the cells harvested for western blot at the times indicated.

The p53 protein appeared to be stable in H1299 cells for longer than expected. There is some support for this finding in the literature [181]. Yamauchi et al have found that p53 is stable for up to six times longer in H1299 cells than normal fibroblasts. The authors found no abnormality in phosphorylation or ubiquitination of p53 expressed in H1299 cells compared to normal human cells, but did observe a defect in

proteasomal degradation of p53 [181]. Having made this finding the time course of the cycloheximide chase experiment was extended overnight. Figure 5.4 shows a representative result of three separate experiments. In the absence of PAX3 (left hand panel) relative p53 levels decline to 50% of their staring point at eight hours. The decay kinetics of p53 in this experiment are not constant and there appears to be a rise in relative p53 abundance between 2 to 4 hours after the addition of cycloheximide. In the right hand panel, with the addition of PAX3, p53 abundance has fallen to 50% of staring levels by 2 hours. In this experiment the level of PAX3 added to each plate was critical; any increase in PAX3 led to total repression of p53 expression. Equally the amount of p53 had to be tightly controlled. A number of attempts were made to try and shorten the p53 half life by using less p53 but in each case p53 expression was totally abolished in the presence of PAX3. These experiments confirm that PAX3 increases the turnover of p53 in H1299 cells.

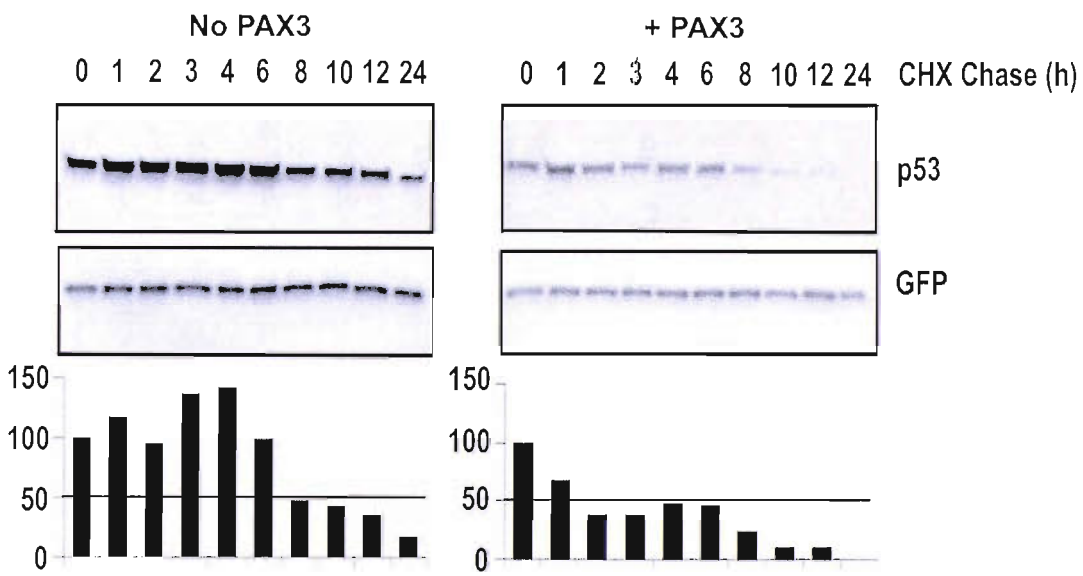

Figure 5.4: PAX3 increases p53 turnover in H1299 cells

H1299 cells were transfected in 60 mm dishes with 1.125 ng p53 plasmid, 1.8 µg pGL3 Basic, 5 ng EGFPN1 and 0.9 µg pJ7PAX3 or empty vector as indicated. At 48 h post transfection, 100 µg/ml cycloheximide (CHX) was added to block new protein synthesis and cells harvested for western blotting at the times indicated. The bar charts show the abundance of p53 protein relative to EGFP as a percentage of time zero (solid line = 50%). Representative of three independent experiments.

5.2.4 The effects of mutant PAX3 proteins on p53 protein levels

The series of experiments described so far in this chapter have shown that PAX3 represses p53 protein levels in H1299 cells by increasing p53 turnover. To define the elements of PAX3 required for these effects the panel of mutant PAX3 proteins described in the previous chapter were tested for their effect on p53 protein levels. Figure 5.5 shows the composite results for two separate experiments. The blots on the left hand site are representative of both experiments. There is a clear reduction in p53 protein abundance in the presence of PAX3 compared to the empty vector control (over 75% comparing pJ7 and PAX3). Repression is not observed with the mutant Stop 281 and there is partial recovery of p53 abundance with the other mutant PAX3 proteins. One would expect complete recovery of expression with the shortest truncation mutant Stop34 but this is not observed; repression of 37% is still seen. The point mutants SD42 and WS265 appear to be able to repress p53 protein levels but not to the extent of full length PAX3 (56% for SD42 and 52% for WS265).

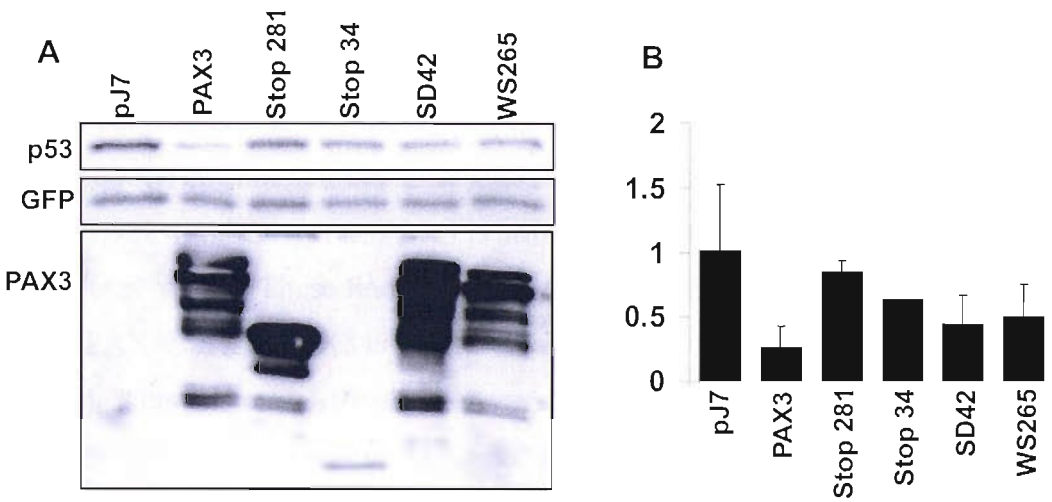

Figure 5.5: Mutant PAX3 proteins have varying affects on p53 protein abundance

(A) H1299 cells were transfected in 60mm dishes with 1.125ng p53, 1.8μg pGL3 Basic, 5ng EGFPN1 and 0.9 μg PAX3, empty vector or PAX3 mutant protein as indicated. Blots were quantified and relative p53 level calculated. (B) Mean relative levels of p53 protein from two separate experiments ± S.D.

5.3 Discussion

Protein function can be regulated at a variety of levels from transcription through translation and post-transational modification to degradation. The regulation of p53 takes place to some extent at every level and factors have been found to influence p53 regulation at every step [41]. One of the most important determinants of p53 function is regulation of p53 degradation. In this chapter I have addressed the regulation of p53 protein levels by PAX3. Initially a system based on the reporter assay experiments of the previous chapter was optimised for western blotting p53 and PAX3 proteins. p53 could be detected using the DO-1 mouse monoclonal antibody at levels comparable with those used in the *HDM2* reporter assay experiments. This was important as low levels of p53 protein are less likely to cause cellular toxicity. It also meant that any small changes in p53 protein abundance could be detected.

p53 was found to be very stable in H1299 cells, another group has shown this to be despite normal expression of HDM2 [181]. A possible explanation of this finding is that a mediator exists between p53 ubiquitination and proteasomal degradation and this is defective in H1299 cells [181]. Full length PAX3 was observed to repress p53 protein levels in H1299 cells. PAX3 is able to increase the turnover of p53. It is possible that this increased turnover is observed in the presence of PAX3 simply because the starting amount of p53 at time zero is much less than in the absence of PAX3 (Figure 4.4). However, PAX3 is also responsible for the reduction in the starting level of p53. These findings are dependent on exact concentrations of both p53 and PAX3. When PAX3 is expressed at higher levels p53 expression is totally repressed. This makes it difficult to interpret these findings in terms of endogenous function as the relative levels of PAX3 and p53 in neural crest derived tumours is unknown.

The experiments in this chapter that raise a number of potential problems that could provide alternative explanations for the finding of decreased p53 protein abundance and increased p53 turnover in the presence of PAX3. It is clear from figure 5.2 that the EGFP control protein abundance is repressed by PAX3 and this is presumably through an inhibitory effect on the CMV promoter. This is a reproducible effect in these assays and validated the use of EGFP as the internal control as the p53

expression plasmids is driven by a similar CMV promoter. PAX3 probably causes a small reduction in p53 transcription but the relative reduction in p53 protein levels in the presence of PAX3 is far greater than EGFP. To further define this phenomenon I conducted an analysis of p53 transcript levels in the presence of PAX3 and there was a modest fall in p53 mRNA. However, this is much less than the effects on p53 protein levels suggesting that the predominant effect of PAX3 on p53 is post-transcriptional. The experiments concerning p53 turnover did not provide evidence of classical protein degradation kinetics. There appears to be a small increase in p53 protein abundance up to four hours after new protein synthesis is blocked by cycloheximide. This implies a lag in the termination of new protein synthesis or that there is differential destruction kinetics for p53 and EGFP (used as a control for gene expression and transfection efficiency). Furthermore, the documented deficit of p53 degradation in H1299 cells could also have a significant effect on degradation dynamics for p53 [181].

Having established not only that PAX3 represses p53 function, but represses p53 protein levels as well, the panel of mutant PAX3 proteins generated in Chapter 3 were tested for their ability to repress p53 protein abundance. It is clear that full length PAX3 is required for maximal repression of p53 protein abundance. Loss of the transactivation domain of PAX3 (Stop 281) leads to total loss of p53 protein repression. This suggests that PAX3 is stimulating the transcription of other, as yet unidentified, proteins to enable p53 repression. Although disruption of either the paired domain or homeodomain is sufficient to rescue from PAX3 inhibition of p53 dependent transcription this is not the case for repression of p53 protein levels. Both SD42 and WS265 exhibit some ability to repress p53 protein levels, although not to the same extent as PAX3. It is possible that some variation in repressive activity is caused by variable expression of the PAX3 mutants. I am unable to be confident about this because the only effective PAX3 antibody available at the time these experiments were conducted was raised as a polyclonal against a very large polypeptide. Therefore it is likely that the individual clones within the PAX3 antiserum recognise diverse epitopes on the PAX3 protein.

The findings in this chapter show that PAX3 is able to repress p53 protein levels and increase p53 protein turnover and in the next chapter I have considered the role of HDM2 in this process.

6. The role of HDM2 and ubiquitination in repression of p53 function by PAX3

6.1 Introduction

The importance of the HDM2/p53 interaction to both normal proliferating cells and tumours has been documented extensively and described in previous chapters[45, 120, 123, 127-130, 147, 166, 167, 169, 182, 183]. A crucial step in the targeting of p53 for nuclear export and eventual degradation is binding of p53 by HDM2 [167, 184]. Indeed inhibition of HDM2 binding to p53 by a mini-protein [185] or a chemical inhibitor of HDM2 [183] leads to p53 activation. Binding of HDM2 to p53 is dependent upon the integrity of amino acids F19, W23 and L26 of p53 and L66, Y67 and E69 of HDM2 [184]. The phenylalanine residue at position 19 of the amino terminus of p53 is critical for HDM2 binding and substitution by alanine (p53 F19A) prevents HDM2 dependent ubiquitination of p53 despite adequate induction of HDM2 transcription [186, 187]. N-terminal binding of p53 by HDM2 promotes ubiquitination of specific lysine residues at the C-terminus (370, 372, 373, 381, 382, and 386) [169, 188]. Mutation of these residues to arginine (6KR) or alanine (6KA) has been shown to significantly reduced ubiquitination in comparison to wild-type p53 [133]. Furthermore the 6KR mutant is unable to be exported from the nucleus [133]. In this chapter I have made use of the p53 F19A and p53 6KR mutant proteins to dissect the mechanism of p53 protein turnover in the presence of PAX3. I also show the results of a series of experiments investigating the ubiquitination of p53 in the presence of PAX3. I have attempted to answer two broad questions; firstly, is HDM2 required for inhibition of p53 function by PAX3, and secondly, does PAX3 expression lead to increased ubiquitination of p53?

6.2 Results

6.2.1 Titration of p53 mutant proteins F19A and 6KR in a 96 well format

Initial experiments using the F19A and 6KR mutant proteins were designed to take place in the 96 well reporter assay format to assess the ability of PAX3 to repress p53 dependent transcription. Plasmids expressing the mutant proteins were first titrated into H1299 cells to assess their relative transcriptional activity on the Hdm2luc03 construct. Scaling up for direct assessment of p53 protein could then be calculated from the levels required to generate similar reporter induction as with wild type p53. Figure 6.1 shows the titration results for both mutant proteins. For all future reporter experiments F19A was used at 0.5 ng per well and 6KR at 0.25 ng per well.

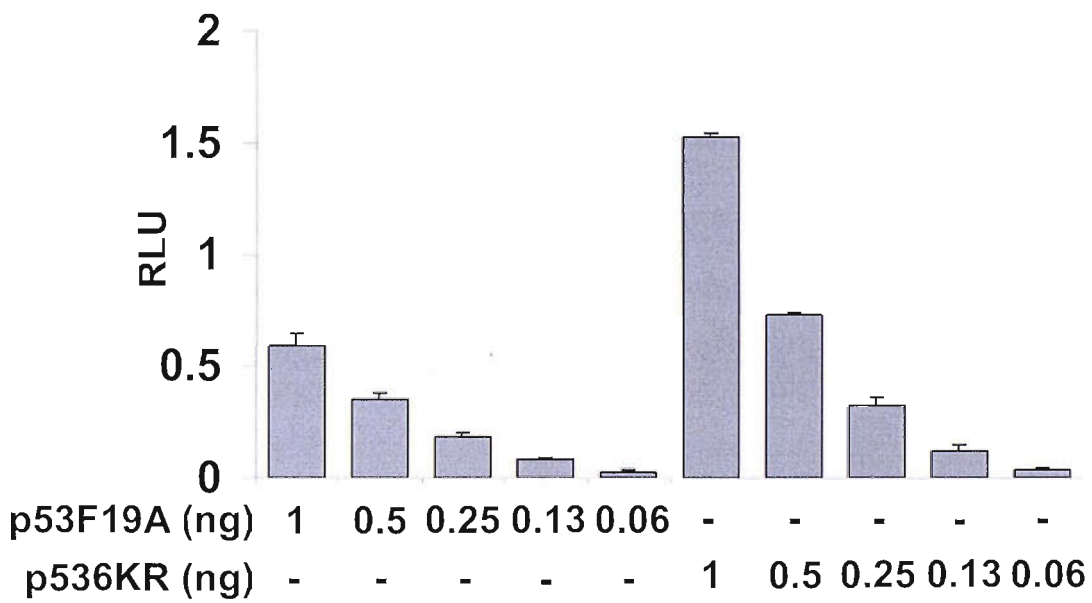

Figure 6.1: Induction of an *HDM2* reporter gene in H1299 cells by F19A and 6KR

H1299 cells were transfected with 100 ng of the p53 reporter construct Hdm2luc03, 20 ng of control renilla plasmid pRLSV40 and increasing amounts of p53 mutant expression vectors as indicated. Dual-Glo™ reporter assays were performed 48 h after transfection (n=2). All data are mean RLU ± S.D.

6.2.2 HDM2 binding is not required for inhibition of p53 dependent transcription by PAX3

Using p53 F19A the requirement for HDM2 activity in PAX3 inhibition of p53 transcriptional function was tested (Fig. 6.2). Figure 6.2A shows that PAX3 caused a 6 fold reduction in activation of an *HDM2* reporter construct by 0.5 ng of p53 F19A ($p<0.001$). While an interesting result in its own right further confirmation of the integrity of the F19A mutant was required. In figure 6.2B the ability of p53 F19A to transactivate the same *HDM2* reporter construct has been tested in the presence of a ten-fold excess of HDM2. In this experiment HDM2 was unable to down-regulate p53 F19A activity. The same ten-fold excess of HDM2 expression vector was observed to significantly inhibit the activity of wild type p53 ($p<0.05$ at 0.0625 ng p53) (Fig. 6.2C). Further confirmation that HDM2 was not required for PAX3 inhibition of p53 transcriptional activity was obtained using the HDM2 chemical inhibitor Nutlin-3 [183] (Fig. 6.2D). There is a >40% ($p<0.05$) decrease in p53 transcriptional activity in the presence of PAX3 despite Nutlin-3 at a dose known to cause HDM2 inhibition [183] (comparison between 0.0625 ng p53, 0 ng PAX3 and 0.0625 ng p53, 100 ng PAX3).

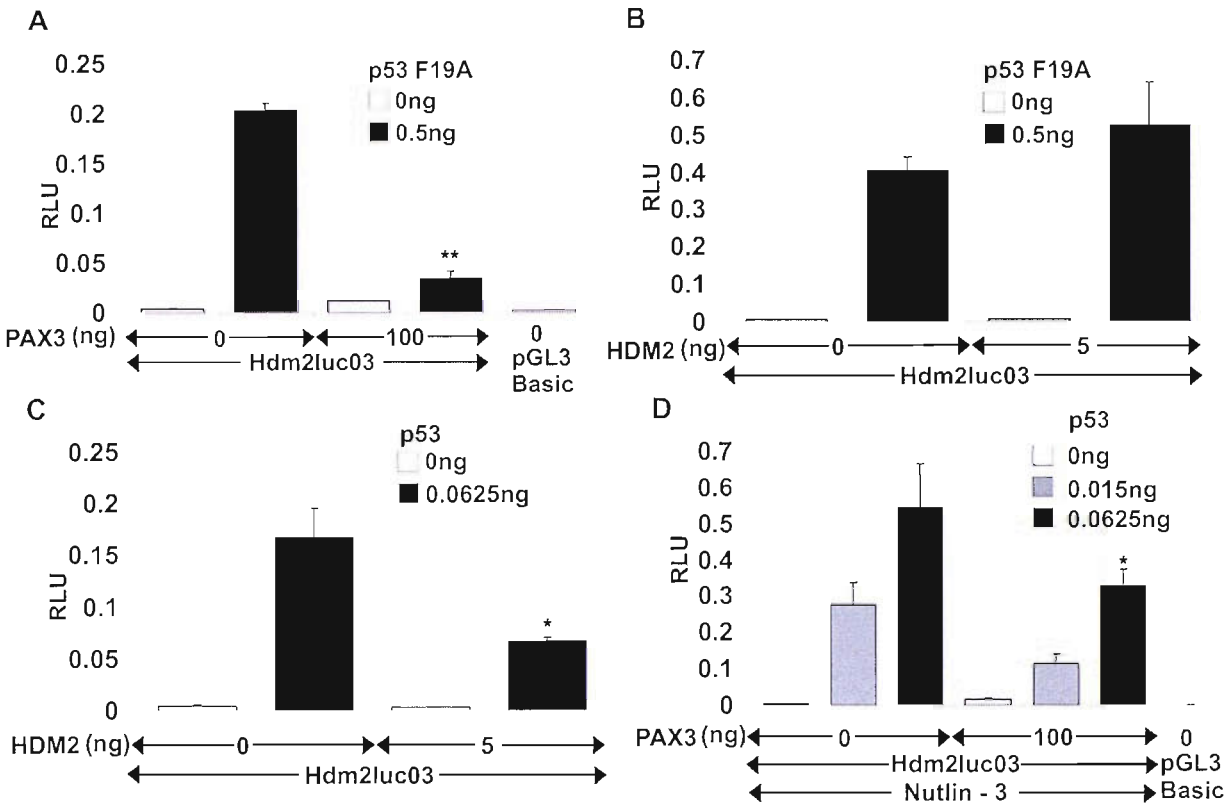

Figure 6.2: Analysis of the requirement for HDM2 in repression of p53 function by PAX3.

H1299 cells were transfected with 100 ng of Hdm2luc03 or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid or p53 F19A plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (A-C, n=4; D n=2). Increasing amounts of pJ7 PAX3 or Hdm2 plasmid was added as indicated. Total amounts of transfected plasmid were equalized using empty pJ7 vector. (D) Nutlin-3 5μM was added at 3 hr after transfection. Data are mean RLU + S.E.M.

6.2.3 PAX3 represses p53 F19A protein abundance in H1299 cells

PAX3 has been shown to lower the protein levels of wild type p53 in Chapter 5. Having shown that PAX3 was able to suppress transcriptional activation by p53 F19A it was interesting to consider whether or not PAX3 was able to suppress the level of F19A protein. A direct scale-up from the 96 well plate format to 60mm dishes allowed analysis of total cellular protein from H1299 cells transfected with p53 F19A with or without PAX3. Figure 6.3 shows that despite the documented resistance to HDM2 mediated degradation of p53 F19A the observed protein levels

are lowered by PAX3. In this experiment expression of the EGFP control protein is unaffected.

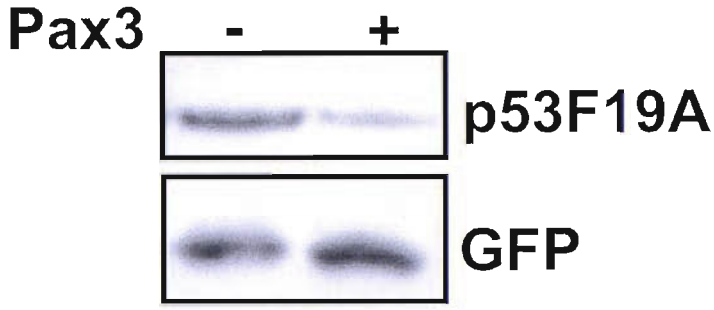

Figure 6.3: PAX3 represses p53 F19A protein levels in H1299 cells
H1299 cells in 60mm dishes were transfected with EGFPN1 at 5ng per plate, pGL3 Basic at 1.8μg per plate and 1.125 ng of p53 F19A. PAX3 or empty vector was added at 1.8 μg per plate as indicated. Cells were harvested at 48 h and western blotting performed with PAb-421.

Careful consideration of the anti-p53 antibody used in this experiment had to be made. Initial experiments indicated that DO-1 (used in all previous experiments) could not be used to accurately quantify p53 F19A expression. The C-terminal binding antibody PAb-421 (epitope, a.a 372-382) was therefore used.

6.2.4 PAX3 expression does not promote ubiquitination of p53

Having established that HDM2 was not required for PAX3 to inhibit p53 transcriptional activity and protein expression, I considered whether or not ubiquitination of p53 was taking place in this process. I first made use of the C-terminal lysine mutant p53 6KR. Figure 6.4A shows that PAX3 is able to repress p53 6KR dependent transcription from an *HDM2* reporter ($p<0.001$ at 0.25 ng p53 6KR). Transfection of a ten fold excess of HDM2 expression vector sufficient to significantly inhibit the activity of wild type p53 (Fig. 6.3C $p<0.05$) had no effect on the p53 6KR mutant (Fig. 6.4B).

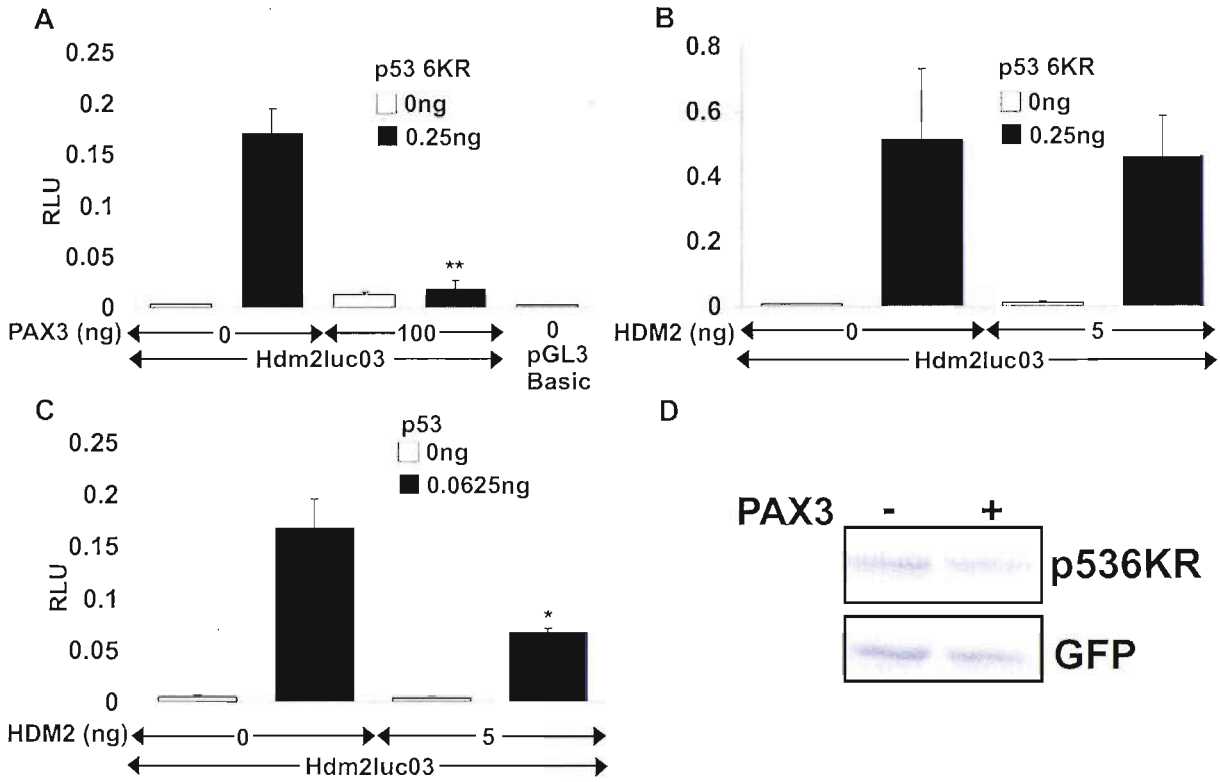

Figure 6.4: Analysis of the role of C-terminal ubiquitination of p53 in repression of p53 by PAX3.

(A, B & C) H1299 cells were transfected with 100 ng of Hdm2luc03 or pGL3Basic as indicated, 20 ng pRLSV40 and the indicated amount of p53 plasmid or p53 6KR plasmid in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=4). Increasing amounts of pJ7 PAX3 or Hdm2 plasmid was added as indicated. Total amounts of transfected plasmid were equalized using empty pJ7 vector. Data are mean RLU + S.E.M. (D) H1299 cells in 60mm dishes were transfected with EGFPN1 at 5ng per plate, pGL3 Basic at 1.8μg per plate and 1.125 ng of p53 F19A. PAX3 or empty vector was added at 1.8 μg per plate as indicated. Cells were harvested at 48 h and western blotting performed with DO-1.

Panel D shows that expression of p53 6KR is suppressed in the presence of PAX3 in H1299 cells to a similar extent to wild type p53 (Fig. 5.2).

The preceding experiments confirmed that the 6 C-terminal lysine residues of p53 established as targets for ubiquitination by HDM2 are not required for PAX3 suppression of p53 function or expression. These are not the only possible targets for ubiquitination of p53 by HDM2 or other E3-ligases. Recent evidence has suggested that the C-terminal lysines may not be absolutely required for p53 ubiquitination and residues within the DNA binding domain fulfil this role [132]. To investigate the

overall requirement for ubiquitination of p53 in PAX3 mediated repression of p53 function a direct measure of p53 ubiquitination was used. The addition of ubiquitin to p53 can be detected by expressing His-6 tagged ubiquitin and p53 in H1299 cells. His-6 tagged ubiquitin is subsequently bound to Ni^{2+} agarose beads and associated proteins defined by western blotting. Using this protocol Xirodimas et al. have shown that the tumour suppressor $p14^{ARF}$ inhibits the ability of HDM2 to promote p53 ubiquitination [189]. Figure 6.5 shows the results of a similar experiment to test the ability of PAX3 to promote p53 ubiquitination. In H1299 cells there is little evidence of ubiquitination of p53 without the expression of exogenous HDM2 (lane 1). Upon the addition of an HDM2 expression vector to the system ubiquitinated p53 is observed (lane 2 upper panel) that is also evident in the western blot for total p53 (lower panel). Use of the proteasome inhibitor MG132 further enhances ubiquitinated p53 accumulation (lane 3). PAX3 is observed to have no effect on p53 ubiquitination above baseline (lane 5).

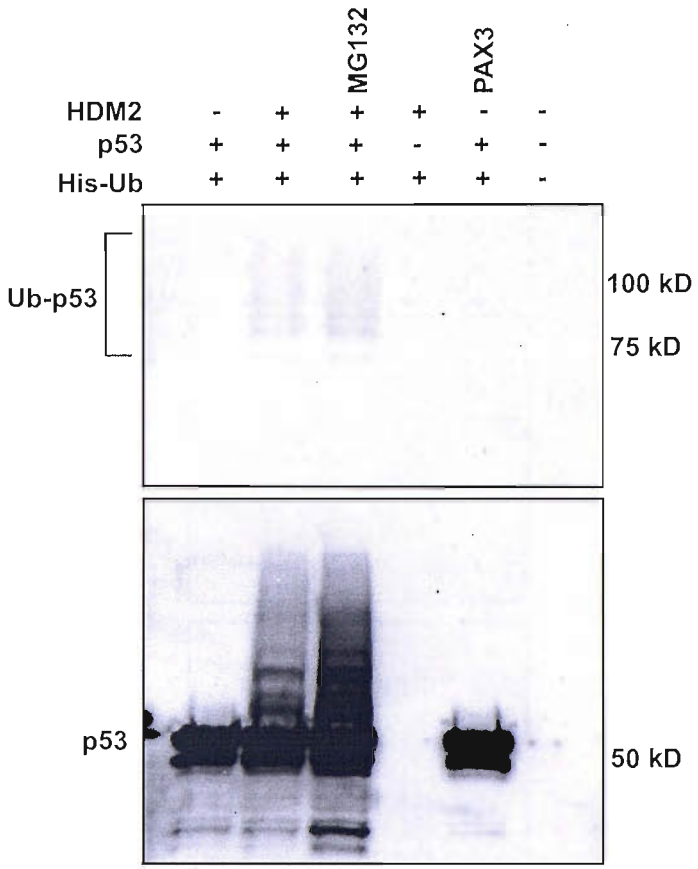

Figure 6.5: An *in vivo* assay for p53 ubiquitination

H1299 cells were transfected with 1µg p53 expression plasmid, 2 µg His-tagged ubiquitin, 2µg Hdm2 (Mdm2) expression plasmid and 2 µg PAX3PJ7 as indicated. 24 h after transfection 25 µM MG132 was added for 3 h where indicated. Ubiquitinated products were separated and analyzed with the DO-1 antibody against p53 (upper panel). Total p53 levels are shown in the lower panel.

The apparent lack of endogenous ubiquitination of p53 by HDM2 in this experiment could have been due to lack of sensitivity in the assay and not a failure of p53 ubiquitination. To test this possibility a similar experiment was conducted with all samples exposed to MG132, a proteasome inhibitor. By blocking proteasomal degradation of p53 I expected any ubiquitinated p53 to accumulate. This, in theory, would lead to any low level activity of either HDM2 or PAX3 as p53 ubiquitin ligases being detected. Figure 6.6 clearly shows that PAX3 does not stimulate p53 ubiquitination in this system. Low level ubiquitination of p53 is observed in the absence of exogenous HDM2 or PAX3 (lane 1 lower panel – arrows). This is unchanged with the addition of PAX3 (lanes 2 lower panel - arrows). The addition of

exogenous HDM2 promotes enhanced ubiquitination of p53 (lane 3 upper and lower panels).

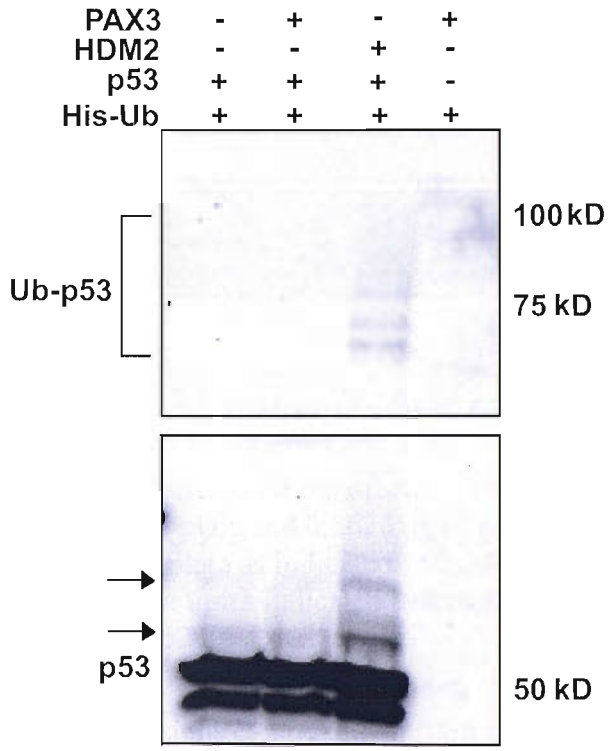

Figure 6.6: PAX3 does not stimulate p53 ubiquitination

H1299 cells were transfected with 1μg p53 expression plasmid, 2 μg His-tagged ubiquitin, 2μg Hdm2 (Mdm2) expression plasmid and 2 μg PAX3PJ7 as indicated. 24 h after transfection 25 μM MG132 was added for 3 h. Ubiquitinated products were separated and analyzed with the DO-1 antibody against p53 (upper panel). Total p53 levels are shown in the lower panel.

6.2.5 PAX3 does not stimulate degradation of p53 by the proteasome

The findings of Chapter 5 showed that degradation of p53 is enhanced in the presence of PAX3. It has been demonstrated in this chapter that the major p53 negative regulator HDM2 is not required for this process and furthermore, that ubiquitination of p53 is not a requirement for PAX3 inhibition of p53 protein levels. Figure 6.7 extends this finding to suggest that the proteasome may not be required at all for p53 degradation in the presence of PAX3.

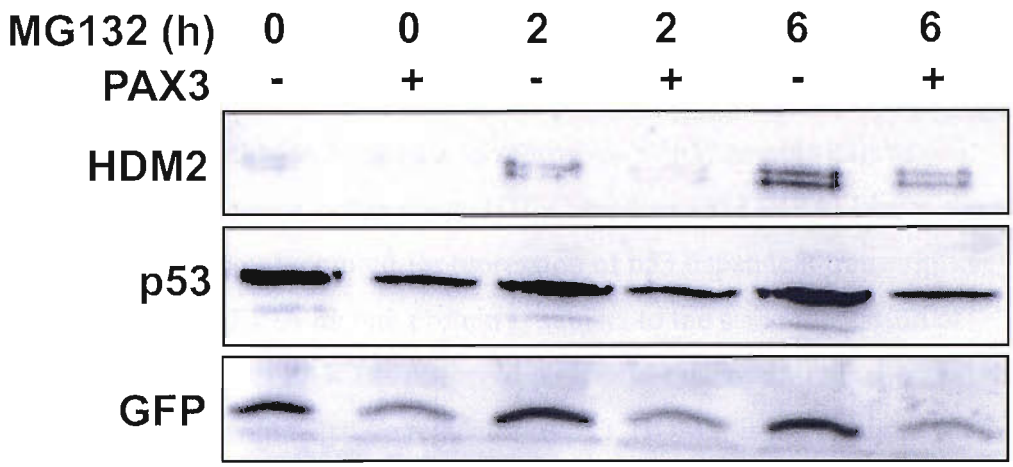

Figure 6.7: Analysis of the requirement of the proteasome in PAX3 inhibition of p53 protein levels

H1299 cells in 60mm dishes were transfected with EGFPN1 at 5ng per plate, pGL3 Basic at 1.8μg per plate and 0.5625 ng of p53. PAX3 or empty vector was added at 0.9 μg per plate as indicated. 50μM MG132 was added at 48 h and cells harvested at the times indicated before analysis by western blotting with DO-1, 2A-9 and Ab290.

A number of interesting conclusions can be drawn from this experiment. Firstly, endogenous HDM2 protein levels (in H1299 cells) are subject to repression by PAX3. At time zero the relative abundance of HDM2 falls by 84% in the presence of PAX3. Secondly, inhibition of proteasomal degradation with MG132 leads to HDM2 accumulation (the relative level of HDM2 rises to 83% of control in the presence of PAX3) indicating that this is the primary route of HDM2 degradation in this assay. Finally, and most importantly, inhibition of p53 protein expression by PAX3 is not rescued by MG132, suggesting that PAX3 is stimulating degradation of p53 via an alternative pathway.

6.3 Discussion

In this chapter I have addressed the requirement for HDM2 and ubiquitin-proteasomal degradation of p53 in the PAX3 mediated repression of p53 protein expression. Using a p53 mutant protein that prevents HDM2 binding (p53 F19A) I have shown that HDM2 binding is not required for repression of p53 dependent transcription. Furthermore, the p53 F19A mutant protein is subject to the same repression of expression in response to PAX3 as observed with wild-type p53. This suggests that the accepted predominant negative regulator of p53, HDM2, is not required for PAX3 stimulated turnover of p53 protein. A number of other proteins have been identified as negative regulators of p53 including PIRH2 [125], COP-1 [124], and CHIP [190]. These proteins share E3 ligase activity and mediate ubiquitination of p53. This meant that before investigating the contribution of PIRH2, COP-1 and CHIP to PAX3 mediated p53 turnover it was prudent to assess the role of ubiquitination and proteasomal degradation in general. Using the 6 C-terminal lysine residue p53 mutant p53 6KR I have demonstrated that ubiquitination of these residues is not required for PAX3 inhibition of p53 expression. This finding implicates a novel mechanism for PAX3 stimulated p53 degradation but does not rule out proteasomal degradation of p53 altogether. Although the 6 C-terminal lysine residues mutated in p53 6KR have been shown to be important for p53 turnover, p53 transcriptional activity [131] and p53 sub-cellular localization [133], more recent reports have suggested other amino acids within the DNA binding domain as targets for ubiquitination by p53 negative regulators [132]. Confirmation that ubiquitination is not required for PAX3 mediated p53 degradation was obtained by performing direct assessment of p53 ubiquitination. The direct assessment of ubiquitination demonstrated in figures 6.5 and 6.6 were based on the published methods of Xirodimas et al. [189]. In their paper the authors demonstrated strong ubiquitination of endogenous p53 that was clearly enhanced with the addition of an HDM2 expression construct. Despite several attempts I was unable to demonstrate clear p53 ubiquitination by endogenous proteins and the possibility remains that PAX3 is able to promote the ubiquitination of p53 in H1299 cells but I am unable to detect this low level activity in my assays. However, the lack of ubiquitination of p53 in the presence of PAX3 after addition of the proteasome inhibitor MG132 (Fig. 6.6) argues against this possibility. In this assay a low level of

p53 ubiquitination by endogenous pathways is observed in the western blot of total cellular p53 that is not enhanced by PAX3.

Proteasomal degradation of p53 does not necessarily require ubiquitination; a ubiquitin-independent mechanism of proteasomal degradation has been suggested regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) [191]. PAX3 recruitment of NQO1 for p53 degradation is made less likely by the finding that proteasomal inhibition using MG132 failed to rescue p53 protein levels from PAX3 induced down-regulation. Together these findings provide evidence that p53 is not being down-regulated by the conventional ubiquitin-proteasome system of protein degradation in the presence of PAX3. This statement must be considered on the background of work that has shown that H1299 cells exhibit a deficiency in p53 turnover despite normal levels of HDM2 and adequate p53 ubiquitination [181]. The apparent lack of proteasomal degradation of p53 in H1299 cells has also been demonstrated in some neuroblastoma cell lines. Over-expression of HDM2 in neuroblastoma cell lines fails to decrease the high steady state levels of endogenous p53. Moreover, exogenous p53, when introduced into these cells, is also resistant to HDM2-directed degradation. This resistance is believed not due to a lack of HDM2 expression in NB cells or a lack of p53-HDM2 interaction, nor is it due to a deficiency in the ubiquitination state of p53 or proteasome dysfunction [192]. Instead, HDM2-resistant p53 from neuroblastoma cells is associated with covalent modification of p53 and masking of the modification-sensitive PAb-421 epitope [192]. The covalent modification of p53 in H1299 cells is a possible mechanism for the observed resistance to proteasomal degradation of p53 and should be considered with future experiments.

If the proteasome, the final common pathway for the majority of protein degradation within the cell, is not being utilized to promote p53 turnover in the presence of p53, what is? Another potential mechanism for p53 negative regulation has been documented in the literature and relies on the activity of the nonlysosomal calcium-activated neutral protease, or calpain [193-195]. Calpain belongs to the family of cysteine proteases and calpains are part of a regulatory proteolytic system [194]. Calpain cleaves p53 within the DO-1 antibody epitope into a 46 kDa form in vitro [194]. The cleaved fragment of p53 is believed to be unstable *in vivo* and cleavage fragments are not detectable in cell based assays [194, 195], however inhibition of

calpain activity with calpastatin *in vivo* provides good evidence for calpain activity. The DO-1 antibody used throughout this project fails to recognize calpain cleaved p53 and only loss of the full-length protein can be detected. To determine the role of calpain cleavage in PAX3 mediated degradation of p53 an experiment to block calpain activity using calpastatin should be conducted.

7. The biological effects of PAX3 repression in neural crest derived tumours

7.1 Introduction

The ability of PAX3 to repress p53 expression and function in an artificial system has been clearly demonstrated in the previous chapters. PAX3 is expressed in the neural crest where the primary role of PAX3 may be to keep p53 down-regulated and prevent apoptosis until a specific developmental programme is completed [20]. PAX3 is down-regulated in later development and its expression is restricted to a small number of highly specialised stem cells such as melanocytes in the bulge region of hair follicles [83]. In this setting Pax3 acts simultaneously to initiate a melanogenic cascade while acting downstream to prevent terminal differentiation, thus maintaining the undifferentiated phenotype of a lineage restricted stem cell [83]. PAX3 is frequently re-expressed in cancer cells of neural crest origin, *PAX3* mRNA is detectable in 77% of cultured primary malignant melanomas, expression increasing with tumour stage [105]. PAX3 is also expressed in neuroblastoma cell lines due to transcriptional up-regulation by N-MYC [106]. Evidence from the myogenic cell lineage supports a role for PAX3 in tumourigenesis. PAX3 is over expressed in embryonal rhabdomyosarcoma and a PAX3:FKHR fusion protein resulting from chromosomal translocation occurs in a high proportion of alveolar rhabdomyosarcomas [103]. This combination of evidence suggests that PAX3 is promoting survival of tumours of neural crest and myogenic origin. Indeed, antisense depletion of PAX3 in melanoma and rhabdomyosarcoma cells leads to loss of cell viability by an apoptotic pathway [103, 105]. More recently it has been demonstrated that PAX3 may play a role in resistance to chemotherapy in malignant melanoma cells [31]. A modest increase in p53 immunoreactive cells was demonstrated after PAX3 antisense treatment, though whether p53 was required for the chemosensitisation was not examined [31].

In this chapter I describe experiments conducted to further examine the role of PAX3 in p53 functional inhibition in cell lines of neural crest origin. Initially I attempted to repeat the published antisense down-regulation of PAX3 and extend these

experiments to the neuroblastoma cell line Kelly. This was followed by targeted down-regulation of PAX3 to define the cellular response to PAX3 suppression in two biologically relevant cell lines, the malignant melanoma cell line UACC-62 and the neuroblastoma cell line IMR 32.

7.2 Results

7.2.1 Down-regulation of PAX3 in Kelly cells leads to loss of cell viability

A report by Bernasconi et al. [103] had shown that treatment of rhabdomyosarcoma with specific PAX3 antisense oligonucleotides (AS-ODN) leads to loss of cell viability. Using commercially generated oligonucleotides of identical sequence to those used by Bernasconi et al. [103] (Table 7.1) the requirement for PAX3 expression in the survival of the neuroblastoma cell line Kelly was tested. Kelly was chosen as the initial target cell line as it has previously been shown to express high levels of PAX3 as a direct target of amplified N-Myc [106].

Oligonucleotide	Sequence
Antisense	5'-gcgtggtcatcctgggggc-3'
Missense	5'-gcgaggacaacgagggggc-3'

Table 7.1: Oligonucleotides used to test PAX3 knock-down in Kelly cells

Initial experiments indicated that a dose of 0.5 μM PAX3 AS-ODN was optimal for suppression of cell viability in Kelly cells. Subsequent experiments were therefore conducted at this dose. Figure 7.1 is representative of three separate experiments and shows that the findings made by Bernasconi et al. [103] can be extended to a neuroblastoma cell line. Background cell death of 20% is observed with transfection reagent alone (lipofectamine 0.5 μM); there is a non-statistically significant rise in cell death to 30% with transfection of 0.5 μM missense ODN. A significant increase in the number of non-viable cells as a percentage of the total cell population is observed with PAX3 specific AS-ODN transfection (>3 fold increase between

lipofectamine alone and antisense; $p<0.001$ and >2 fold increase between missense and antisense; $p<0.001$). Addition of the chemical inhibitor of p53 pifithrin-α leads to a fall in the number of non-viable cells compared to antisense treatment alone from 67% to 46.8% ($p=0.008$).

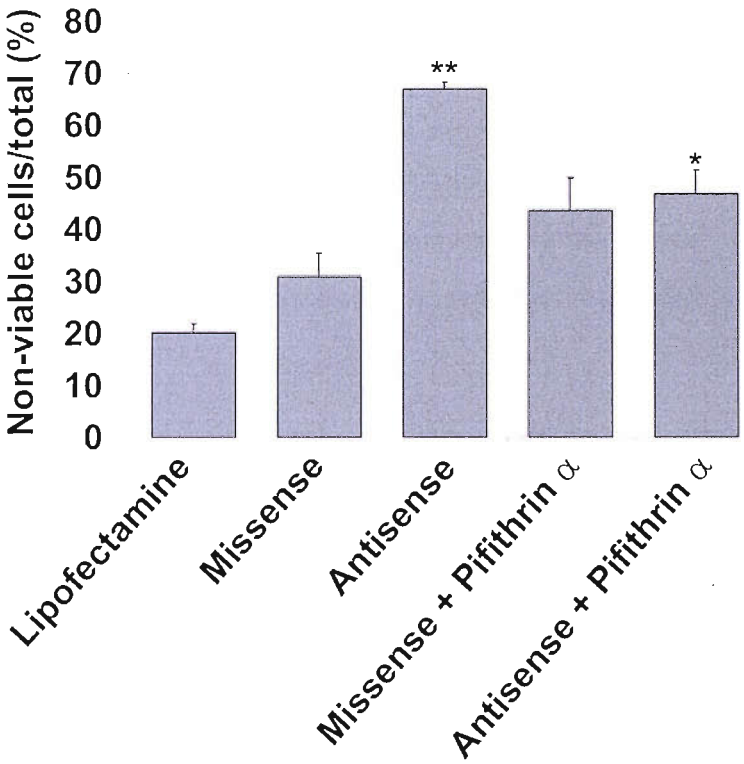

Figure 7.1: PAX3 AS-ODN treatment of Kelly cells leads to loss of cell viability that is rescued by pifithrin-*α*

Kelly cells in 60 mm dishes were transfected in anti-biotic free medium with 0.5 μM missense or antisense oligonucleotides or lipofectamine alone as indicated. Every 24 h Pifithrin-α (20 μM) or DMSO was added as indicated and the number of non-viable cells counted at 72 h using trypan blue exclusion (n=3). All data are non-viable cells as percentage of total cell population ± S.E.M.

7.2.2 Characterisation of the neural crest derived tumour cells lines IMR 32 and UACC- 62

Although Kelly cells seemed a good initial target for study of PAX3 down-regulation there was considerable concern about the high background levels of cell death with transfection and their slow growth in cell culture. A more robust cell line was required that grew quickly in culture and could be relied upon for reproducibility

between assays. Two biologically relevant, neural crest derived cell lines, IMR 32 (neuroblastoma) and UACC-62 (melanoma) were therefore considered. The cell lines IMR 32 and UACC-62 are known to express PAX3 and possess wild-type p53 protein [106, 196, 197]. In the case of IMR 32, expression of PAX3 has been shown to be under the direct control of N-MYC [106]. MYC transactivation of PAX3 promoter activity is dependent upon a non-canonical E box site in the 5' promoter region of PAX3 [106]. No such regulatory mechanism has been documented for UACC-62. Further characterisation of the genetic basis of expression of PAX3 and N-MYC in IMR 32 and UACC-62 was conducted using fluorescence in-situ hybridisation (FISH). Cells were arrested in metaphase by exposure to the spindle disturbing agent colcimid before being fixed and processed by Lisa Russell of the Wessex Regional Cytogenetics Laboratory.

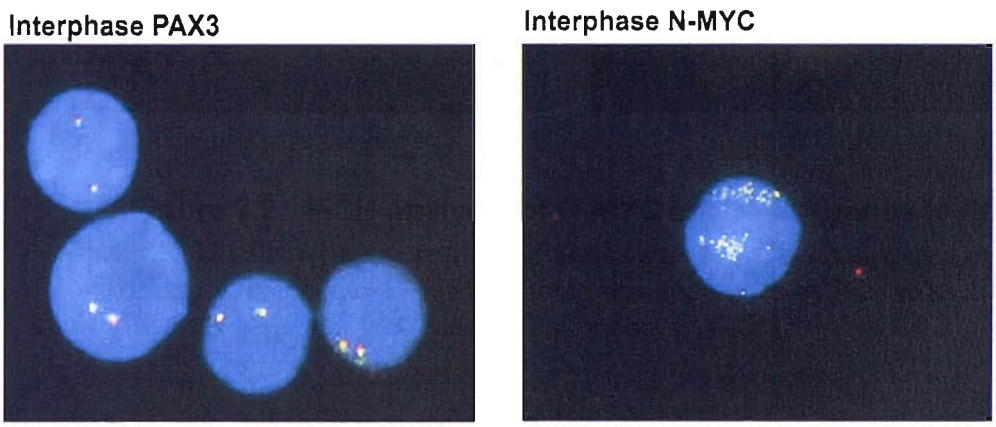

Figure 7.2: FISH analysis of N-MYC and PAX3 genes in IMR-32
IMR32 cells were grown in T125 flasks and treated with colcimid prior to fixing with methanol. FISH staining was conducted by Lisa Russell of the Wessex Regional Cytogenetics Laboratory.

Figure 7.2 shows that there is amplification of *N-MYC* gene copy number in IMR 32 cells. This corresponds with data showing that *N-MYC* is amplified in IMR 32 and N-MYC protein is up-regulated in this cell line leading to up-regulation of PAX3 protein expression [106, 156]. *PAX3* gene copy number is normal (i.e. 2 copies per cell). While these findings are not novel they confirm that the IMR 32 cells in the laboratory have not differed significantly from those in the literature.

A similar experiment was then conducted with the melanoma cell line UACC-62. Figure 7.3 shows that although UACC-62 cells are tetraploid, there is no further amplification of either *N-MYC* or *PAX3* genes.

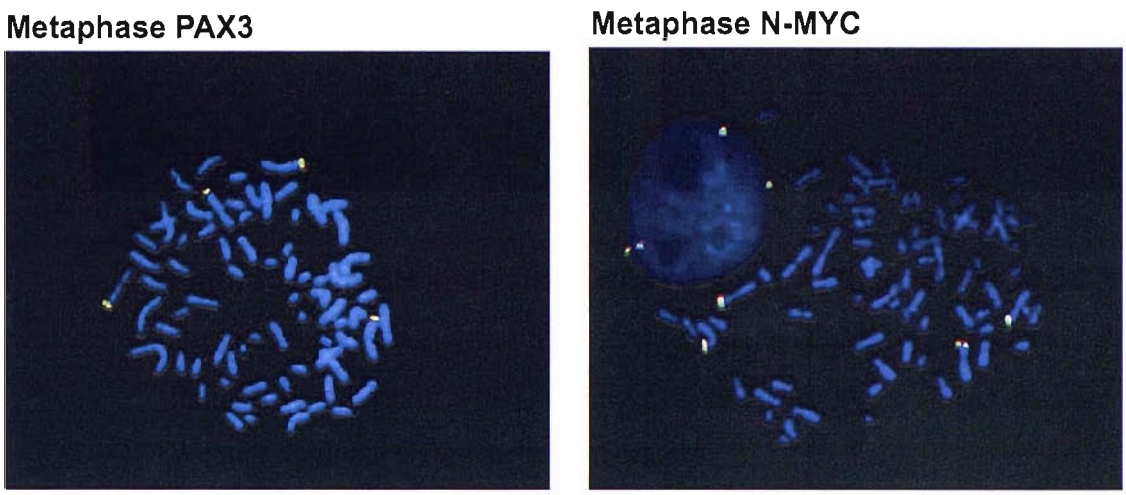

Figure 7.3: FISH analysis of N-MYC and PAX3 genes in UACC-62

UACC-62 cells were grown in T125 flasks and treated with colcimid prior to fixing with methanol. FISH staining was conducted by Lisa Russell of the Wessex Regional Cytogenetics Laboratory.

7.2.3 Down-regulation of PAX3 in IMR 32 leads to decreased cell viability

A high level of background cell death was observed using AS-ODN in Kelly cells. All previous reports of PAX3 down-regulation have used AS-ODN [31, 103, 105, 197]. To extend and complement these findings commercially available short interfering RNA (siRNA) was used to target *PAX3* mRNA for destruction. The use of siRNA offers a number of benefits over AS-ODN mediated mRNA suppression in the cytoplasm. Gene silencing with siRNA is achieved at concentrations lower than those required for other agents [198, 199] and siRNA is believed to produce fewer off-target effects [200]. RNA interference can be delivered by multiple vectors and has potential for *in vivo* use as the interferon pathway induced by AS-ODN is bypassed [200]. To measure the effects of siRNA mediated gene silencing a cell counting assay was developed for use in 24 well and 6 well plate formats. Cells were grown to near

confluency in 24 well plates prior to transfection with gene specific siRNA or control non-silencing siRNA (scrambled) at day 0. Optimisation had shown that IMR 32 cells could not be transfected at lower densities due to non-specific siRNA toxicity. This meant that the cells had to be transferred to 6 well plates at 24 hours before the growth suppressive effects of over-confluency were observed. Cells were counted at days 0, 1, 3 and 5 by trypsinisation and resuspension in medium before being mixed 50:50 with trypan blue. This enabled a count of the total cell population of the well to be made. Down-regulation of PAX3 using specific siRNA led to a decrease in cell viability in IMR 32 cells of over 50% compared to scrambled at day 5 (Fig. 7.4). This loss of cell viability was equivalent to that observed by down-regulating HDM2.

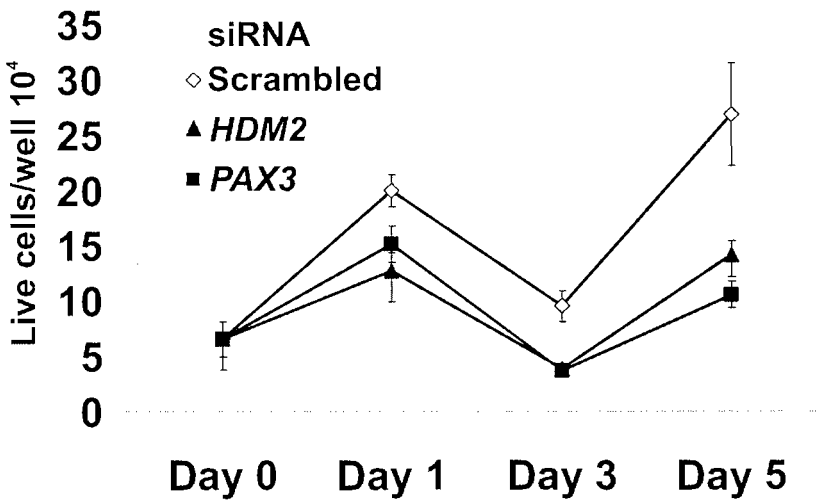

Figure 7.4: Down-regulation of PAX3 leads to decreased cell viability equivalent to down-regulation of HDM2 in IMR 32 cells

IMR 32 cells were transfected at c. 90% (to minimize the non-specific toxicity of siRNA in this cell line) in 24 well plates at day 0 with 20 pmol per well gene specific siRNA as indicated. Cells were transferred to 6 well plates at 24 h. Viable cells were counted at the times indicated using trypan blue exclusion. Triplicate wells were counted for each data point and represent mean cell count $\pm$ S.E.M. Data is representative of three separate experiments.

Despite showing that PAX3 was required for maintained cell viability in IMR 32 cells, further experiments with this cell line proved problematic. IMR 32 cells adhered poorly to plastic and were slow growing. They also required to be maintained at high density for transfection in order to minimise non-specific toxicity of siRNA. This meant that they had to be transferred to 6 well plates at 24 h adding a

further insult to already stressed cells. Therefore, subsequent attempts to rescue the effects of PAX3 down-regulation with p53 knock-down were unsuccessful. I was also unable to demonstrate PAX3 knock-down using either western blotting for protein or qRT-PCR for PAX3 message. This is likely to be because PAX3 expression under the influence of N-MYC is cell cycle time specific. In addition, cells at near confluency are less likely to be actively dividing and therefore will not express PAX3. Further experiments using siRNA were therefore conducted in the melanoma derived cell line UACC-62 and are described in the following sections.

7.2.4 Down-regulation of PAX3 in UACC-62 leads to decreased cell viability

The faster and more reliable growth characteristics of UACC-62 cells meant that they could be cultured at less density and therefore removed the requirement for replating at 24 h. As a positive control for p53 activation PAX3 siRNA was compared to the down-regulation of the major p53 negative regulator HDM2. Figure 7.5A shows that the growth of UACC-62 cells transfected with scrambled siRNA is normal after day 2. There is significant loss of cell viability observed at day 4 that persists up to day 8 for cells transfected with *HDM2* specific ($p<0.001$ at day 6) and *PAX3* specific ($p<0.001$ at day 6) siRNA. Confirmation that the siRNA is having on-target effects is observed in Figure 7.5B. In the presence of scambled siRNA HDM2 and PAX3 protein are expressed and can be detected by western blotting. *HDM2* specific siRNA caused a marked reduction in HDM2 protein expression and PAX3 protein was below the levels of detection after PAX3 specific siRNA down-regulation.

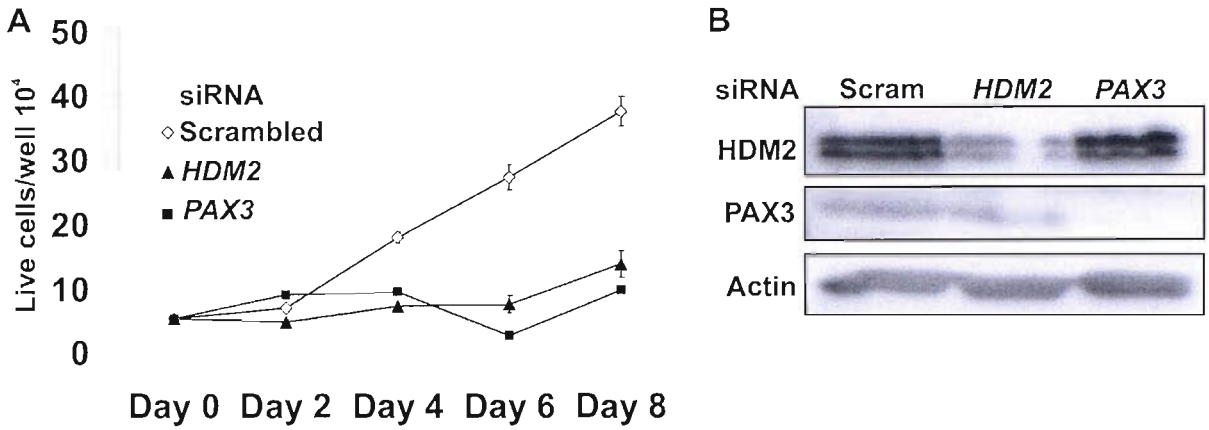

Figure 7.5: Effects of gene specific siRNA in UACC-62 cells

(A) UACC-62 cells were transfected in 24 well plates with 20 pmol siRNA per well as indicated. Medium was changed every 48 h and viable cells were counted at the times indicated using trypan blue exclusion. Triplicate wells were counted for each data point and represent mean cell count ± S.E.M. Data is representative of three separate experiments. (B) UACC-62 cells in 60 mm dishes were transfected with 200 pmol siRNA as indicated and target proteins analysed at 48 h by western blotting.

7.2.5 Down-regulation of PAX3 causes cell death and not cell cycle arrest in UACC-62 cells.

Having established that PAX3 knock-down in UACC-62 caused loss of cell viability it was important to define if this was due to cytostasis or cell death. In Figure 7.6A the number of viable cells per well as a percentage of the total cell population is shown at day 6 after transfection with siRNA. There is a significant loss of cell viability between scrambled and *PAX3* siRNA ($p<0.05$), indicating cell death. Furthermore, *PAX3* siRNA did not cause cell cycle arrest when the cells were analysed for DNA content by propidium iodide staining and flow cytometry (Fig. 7.6B).

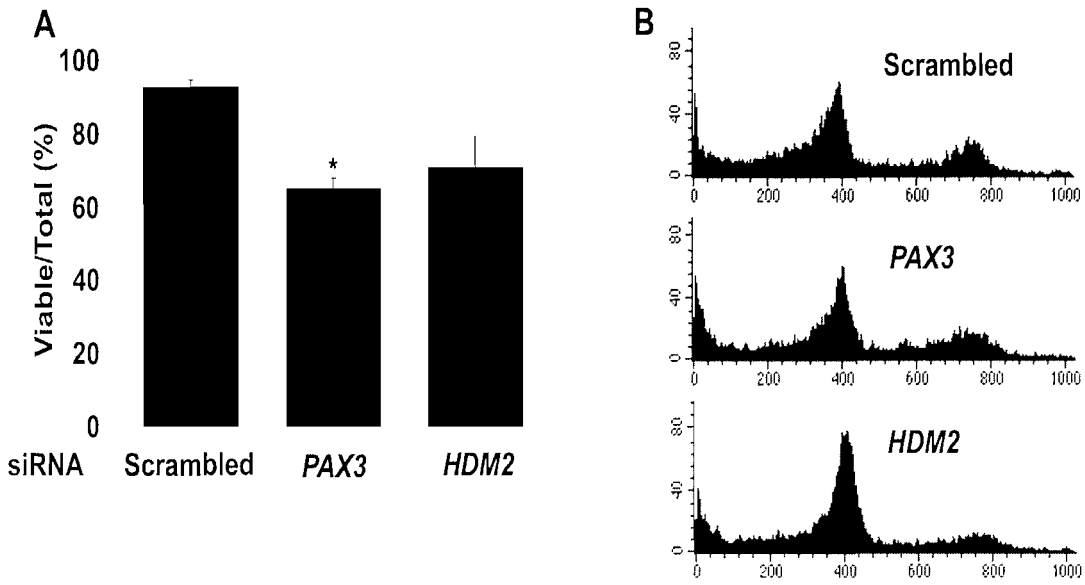

Figure 7.6: Differential effects of gene specific siRNA on UACC-62 cell viability

(A) UACC-62 cells were transfected in 24 well plates with 20 pmol siRNA per well as indicated. Medium was changed every 48 h and viable cells as a percentage of the total cell population were counted at day 6 using trypan blue exclusion. Triplicate wells were counted for each data point and represent mean cell count ± S.E.M. Data is representative of three separate experiments. (B) UACC-62 cells were transfected in 60mm dishes with 200 pmol siRNA as indicated. 3 days post-transfection, cells were fixed in ethanol, stained with propidium iodide, and DNA content was determined by flow cytometry.

In contrast, transfection with *HDM2* specific siRNA caused an increase in the number of cells in G_1 from 61.9% to 77.8% (Fig. 7.6B), but *HDM2* siRNA did not cause a significant decrease in the number of viable cells (Fig. 7.6A). In this assay PAX3 specific siRNA would be expected to show an increase in the number of cells in sub-G1. This was not observed.

To investigate the mechanistic basis for the difference in cellular response to p53 activation by HDM2 or PAX3 down-regulation the major transcriptional targets of p53 were examined. Figure 7.7 shows that in response to HDM2 knock-down there is accumulation of p53 protein (relative levels increase by over 100%). This accumulation of p53 does not lead to apoptosis as evidenced by lack of PARP cleavage. PARP (Poly(ADP-Ribose)Polymerase) is a nuclear protein involved in DNA repair and is one of the earliest targets for caspase cleavage to a signature 89 kDa fragment during apoptosis [201]. Interestingly these cells do not undergo

apoptosis, despite accumulation of the pro-apoptotic p53 target protein BAX. The cyclin dependent kinase inhibitor $p21^{WAF1}$ is seen to accumulate after HDM2 down-regulation. In contrast, suppression of PAX3 expression causes PARP cleavage despite lesser accumulation of p53 (on average 25% over 3 experiments). Accumulation of BAX is clearly evident but there is no evidence of $p21^{WAF1}$ induction.

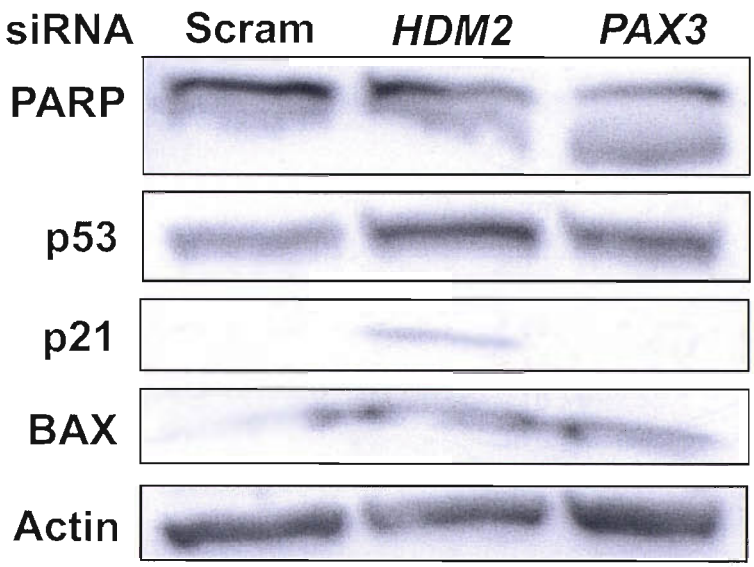

Figure 7.7: Analysis of p53 pathway proteins in response to Hdm2 and PAX3 down-regulation in UACC-62 cells

UACC-62 cells in 60 mm dishes were transfected with 200 pmol siRNA as indicated and p53 pathway proteins analysed at 48 h by western blotting.

7.2.6 The cell death observed after PAX3 knock-down in UACC-62 cells is p53 dependent

Previous reports have shown that UACC-62 cells show significant loss of viability and undergo apoptosis following the transfection of *PAX3* specific antisense oligonucleotides [31, 197]. The findings presented so far in this chapter support these observations and extend them to include PAX3 knock-down with siRNA. Comparison with down-regulation of the p53 major negative regulator HDM2 has indicated a differential response in terms of cell death or cell cycle arrest. Given this difference in response it was important to define whether the cell death observed after

PAX3 siRNA was p53 dependent. To test this hypothesis UACC-62 cells were exposed to *PAX3* or *HDM2* siRNA in the presence or absence of *TP53* siRNA. As expected the decrease in cell viability after HDM2 knock-down is rescued by *TP53* siRNA (Fig. 7.7A). The growth inhibitory effects of *HDM2* siRNA are observed at day 2 and persist up to day 6. Addition of *TP53* specific siRNA leads to complete rescue of growth inhibition at every time point. More strikingly the growth inhibitory effects of *PAX3* siRNA are completely rescued by the addition of *TP53* siRNA at every time point to day 6 (Fig. 7.7B) (p=0.001 between *PAX3* and Scrambled and between *PAX3* and *PAX3* + *TP53* siRNA at day 4).

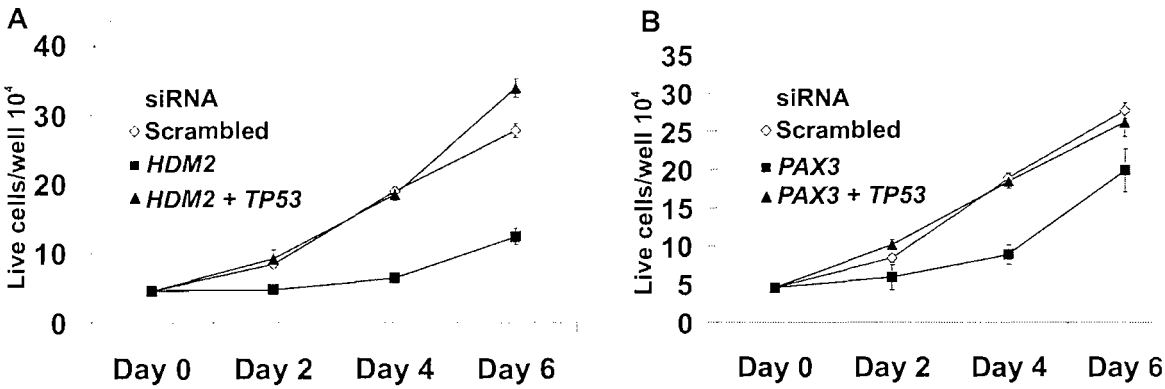

Figure 7.8: Down-regulation of p53 rescues UACC-62 cells from Hdm2 or PAX3 siRNA mediated loss of cell viability

UACC-62 cells were transfected in 24 well plates with 20 pmol gene specific siRNA as indicated. Total siRNA was equalised to 40 pmol with scrambled siRNA. Medium was changed every 48 h and viable cells were counted at the times indicated using trypan blue exclusion. Triplicate wells were counted for each data point and represent mean cell count ± S.E.M. Data is representative of three separate experiments.

A possible explanation for the apparent rescue of *PAX3* siRNA mediated cell death by p53 down-regulation was an interaction between siRNA oligonucleotides, leading to incomplete PAX3 suppression. To investigate this possibility the relative levels of *PAX3* mRNA were measured using quantitative RT-PCR. Transfection of *PAX3* specific siRNA caused a 3 fold reduction in relative *PAX3* mRNA abundance (p=0.001), this was not significantly affected by addition of *TP53* siRNA (Fig. 7.8).

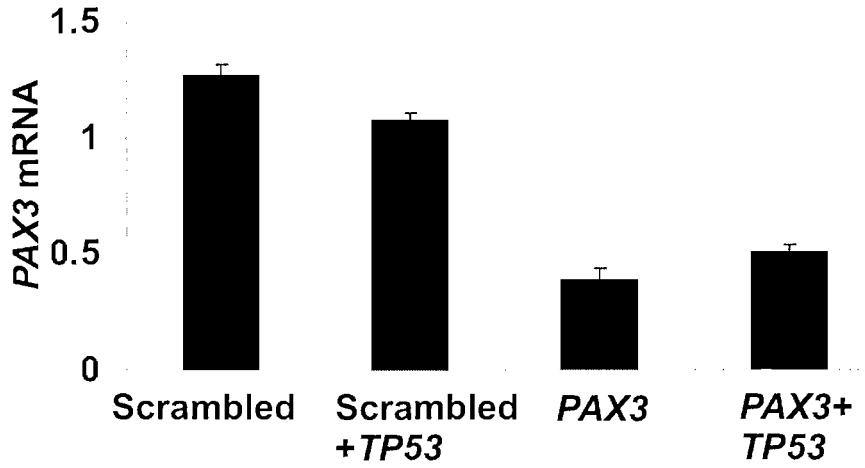

Figure 7.9: Down-regulation of PAX3 mRNA is not affected by p53 siRNA

To confirm *PAX3* mRNA knockdown quantitative RT-PCR was performed on duplicate samples transfected with the indicated siRNA and harvested at day 3. Total siRNA was equalized with Scrambled siRNA. *PAX3* mRNA levels were normalised to *GAPDH* housekeeping gene expression and are expressed as a mean relative abundance ± S.D. of duplicate assays.

7.2.7 Down-regulation of PAX3 in UACC-62 induces apoptosis

I have demonstrated in this chapter that suppression of PAX3 expression in a melanoma cell line leads to p53 activation and cell death. The tumour suppressor function of p53 is in part due to induction of apoptosis in response to ongoing cellular stress [41, 45]. To establish that an apoptotic programme was occurring after PAX3 suppression in UACC-62 cells, PARP cleavage, a marker of active apoptosis was used. At 48 h after transfection of *PAX3* siRNA the amount of cleaved PARP as a percentage of the total cellular PARP is approaching 20% (Fig. 7.10). This is reduced to less than 10% (and below the level observed with control non-silencing siRNA) with the co-transfection of *TP53* specific siRNA. Transfection of Hdm2 specific siRNA does not cause PARP cleavage. This supports the finding shown in figure 7.6, that p53 activation in response to HDM2 down-regulation induces cell cycle arrest.

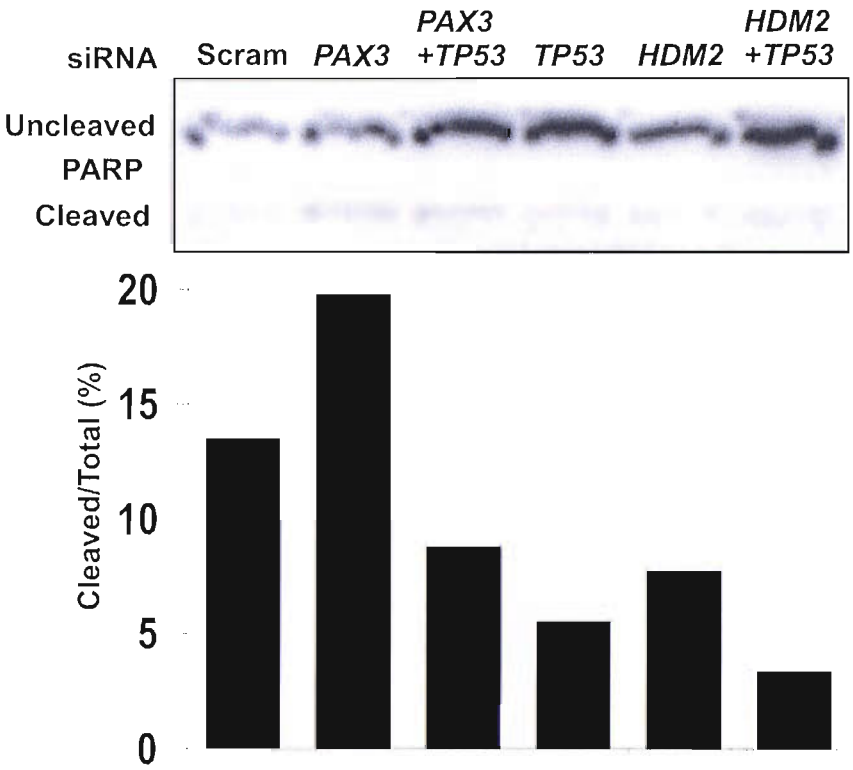

Figure 7.9: PAX3 siRNA induces p53 dependent apoptosis in UACC-62 cells

UACC-62 cells were transfected in 60 mm dishes with 200 pmol gene specific siRNA as indicated. Total transfected siRNA was equalized to 400 pmol with scrambled siRNA. At 48 h PARP cleavage was analysed by western blotting. Representative of 3 independent experiments.

7.3 Discussion

In previous chapters I have made use of artificial cellular systems to study the relationship between over-expressed exogenous proteins. While this has led to many novel and important findings they provide little insight into the biological significance of PAX3 expression in neural crest derived tumours. To address this question I have used targeted down-regulation of endogenously expressed PAX3 in biologically relevant cell lines to further examine PAX3 function. Characterisation of IMR 32 and UACC-62 using FISH has confirmed the many-fold amplification of *N-MYC* in IMR 32 cells, previously shown to influence PAX3 expression [106]. *N-MYC* is not amplified in UACC-62 cells, a finding unsurprising as UACC-62 cells are of melanocyte origin. An alternative mechanism driving aberrant PAX3 expression in these cells remains to be elucidated. PAX3 has been shown to be required for the survival of two cell lines derived from primary neuroblastoma tissue (Kelly and IMR 32) and one malignant melanoma cell line (UACC-62). Further investigation of the mechanistic basis of PAX3 dependent cell survival in the neuroblastoma cell lines was limited by their poor growth characteristics. In addition I was unable to demonstrate PAX3 expression in these cells by either western blotting or qRT-PCR (data not shown). This may be because PAX3 is expressed at levels below the threshold of detection in my assays. PAX3 expression in neuroblastoma cell lines is cell cycle stage dependent [106], but I was unable to successfully transfect either Kelly or IMR 32 cells at low density, due to siRNA toxicity, when they would exhibit the greatest proliferative capacity.

I have shown for the first time that the apoptosis observed after knockdown of PAX3 expression in melanoma cells is p53-dependent. I have demonstrated clear on-target effects of gene specific siRNA leading to loss of cell viability in UACC-62 cells (Fig. 7.5). The loss of cell viability observed after knock-down of both HDM2 and PAX3 is rescued by co-transfection of *TP53* specific siRNA (Fig. 7.7). It is clear in this experiment that the effects of both HDM2 and PAX3 siRNA on cell viability are not as dramatic or long-lasting as that observed in figure 7.5. This is likely to be because the amount of siRNA used in figure 7.7 (40 pmol) is double that used in figure 7.5 (20

pmol), the optimum dose of siRNA in this assay. The 40 pmol concentration is sub-optimal when effects on proliferation and PARP cleavage are assessed.

PAX3 knockdown in melanoma cells leads to the induction of the pro-apoptotic p53 target gene *BAX*. The cyclin-dependent kinase inhibitor $p21^{WAF1}$ is not induced by PAX3 knockdown. This is in contrast to the induction of $p21^{WAF1}$ protein observed after HDM2 knockdown, which appears to lead to cell cycle arrest rather than apoptosis. Using propidium iodide staining and analysis of cellular DNA content by FACS, I was able to show that *HDM2* specific siRNA caused a G_1 arrest in UACC-62 cells. In the same experiment I was unable to see evidence of apoptosis in response to *PAX3* siRNA. This is at odds with the PARP cleavage experiments that clearly show caspase activation in response to PAX3 down-regulation. It is possible that an increase in the sub-G_1 cellular population is being masked by the high background rate of cell fragmentation in control-transfected cells. The literature clearly supports apoptosis in response to PAX3 down-regulation in melanoma cells [31, 105, 197].

Evidence to support the induction of cell cycle arrest in response to HDM2 suppression comes from recent reports using Nutlins, chemical inhibitors of HDM2 [182, 183]. Nutlins have been observed to specifically promote wild-type p53 dependent cell cycle arrest via *WAF1* [182, 183]. It appears that induction of the $p21^{WAF1}$ cell cycle check point is critical in determining the response of UACC-62 cells to p53 activation. PAX3 down-regulation activates p53 but promotes apoptosis rather than cell cycle arrest. There are two possible explanations for this phenomenon. Firstly, PAX3 down-regulation activates p53 in a target specific manner, causing the preferential induction of pro-apoptotic genes. PAX3 may affect the cellular activity of apoptosis regulatory p53 co-factors such as ASPP [145]. The ASPP family consists of two pro-apoptotic members ASPP1 and ASPP2 and one anti-apoptosis member iASPP [51]. ASPP1 and ASPP2 bind to the DNA binding domain of p53 and specifically stimulate the transactivation function of p53 on pro-apoptotic genes [52]. iASPP competes directly with the other ASPP family members for the DNA binding domain of p53 [202]. Studies have confirmed the biological relevance of these proteins by showing down-regulation of ASPP1 and ASPP2 and up-regulation of iASPP in a range of tumours [52, 140]. Further complexity in control of the p53 response is provided by proteins such as E2F1 [142] and YB1 [141], both of

which are potential targets for PAX3 activity. E2F1 interacts directly with p53 to enhance the apoptotic activity of p53 in response to DNA damage [203]; in addition E2F1 can phosphorylate p53 to stimulate apoptosis [53]. YB1 inhibits the ability of p53 to cause cell death and transactivate pro-apoptotic targets but does not interfere with transactivation of p21WAF1 [141]. PAX3 could be stimulating YB1 expression in neural crest derived tumours and withdrawal of the protective effects of YB1 could lead to apoptosis after PAX3 knock-down. Secondly, p53-independent activation of the *WAF1* promoter by PAX3 or PAX3-regulated genes such as microphthalmia-associated transcription factor (*MITF*), a known regulator of $p21^{WAF1}$ expression may be involved [177]. This possibility is supported by the evidence that PAX3 induces activity from a *p21* reporter gene (Fig. 4.6). The mechanisms underlying this differential effect on p53 target genes remain to be identified. Defining the molecular basis for this will be important, as an ability to promote apoptosis rather than cell cycle arrest in response to p53 activation has potential therapeutic implications [42, 145]. The findings presented in this and previous chapters support the growing body of evidence that PAX3 provides a promising target for the therapy of malignant melanoma, and potentially also neuroblastoma and rhabdomyosarcoma in which *PAX3* expression and retention of wild-type p53 are common events [31, 103, 105]. Previous studies have shown not only that ablation of PAX3 expression in cancer cells potently activates pro-apoptotic pathways [31, 103, 105], but also that the expression of PAX3 is essentially restricted to tumour tissue in the adult [105], providing the potential for a high degree of tumour specificity. PAX3 has been suggested as a target for the development of novel anti-cancer therapies [8]. I have identified the transcription factor activity of PAX3 as critical for its role in repression of p53-dependent pro-apoptotic pathways, information that will assist in the development of therapeutic strategies for the treatment of melanoma and other PAX3-dependent tumours. I have begun this process and in the next chapter I detail preliminary experiments to develop a cell-based screen to identify compounds that inhibit endogenous PAX3 transcription factor activity in melanoma cells.

8. Development of a cell based screening assay to identify compounds that inhibit PAX3 function in melanoma cells

8.1 Introduction

Skin cancer is common with over 73000 new cases recorded each year in the UK [204]. Malignant melanoma is the least common skin cancer but the most serious, responsible for over 1800 deaths each year in the UK [204]. The number of melanoma cases worldwide is increasing faster than any other cancer; rates of increase in the order of 3-7% per year have been recorded [205]. Estimates suggest a doubling of melanoma incidence every 10-20 years [206]. Survival is strongly associated with thickness of tumour at the time of diagnosis. Surgery for tumours less than 1.5 mm thick offers a greater than 90% survival at five years, but for tumours thicker than 3.5 mm five year survival is only 47% for men and 55% for women [204]. Melanoma cells exhibit an intrinsic resistance to chemotherapy and the five year survival rate for stage IV disease treated with dacarbazine is only 6% [207]. In addition only high does interferon has been shown to confer a modest long term survival advantage when used as systemic adjuvant therapy for patients at high risk of recurrent malignant melanoma [208]. Malignant melanoma cells may exhibit a range of genetic lesions including activating mutations of NRAS and BRAF leading to self-sufficiency in growh factor signalling, activation of AKT by PTEN deletion causing cell survival signalling and deregulation of cell cycle control proteins [209]. Notably amplification of the PAX3 target MITF occurs most frequently in tumours that have a poor prognosis and is associated with resistance to chemotherapy [210]. MITF may act as a melanoma oncogene and interference with MITF function increases chemosensitivity of a melanoma cell line [210]. The role of PAX3 has not been extensively documented in melanoma but studies that have been performed confirm PAX3 as critical to the survival and chemo-resistance of these tumours [31, 105]. I have extended these findings to show that PAX3 inhibits p53 function in melanoma cells and that the transcriptional integrity of PAX3 is an absolute requirement for this effect *in vitro*. Furthermore the tumour specific expression of PAX3 makes it an

excellent target for therapy. In this chapter I set out the design and initial optimisation of an assay to identify compounds that inhibit PAX3 transcriptional function. I also describe how this assay could be used in future work to dissect further the mechanistic basis of p53 functional inhibition by PAX3.

8.2 Results

8.2.1 Designing an assay to interrogate PAX3 transcriptional function

The detailed description of the design of a cell based high throughput screen is out-with the scope of this thesis and has been reviewed in detail by Johnston and Johnston [211]. In principle a cell based screening assay offers a number of benefits over other assays for lead generation, including removal of the requirement for protein isolation and purification and the ability to assay the function of transcriptionally active proteins in the presence of native co-activators and co-repressors [211]. I have established that the transcriptional activity of PAX3 is fundamental to its ability to repress p53 function in melanoma cells. This finding, coupled with the relatively low expression of PAX3 make a cell based screen the most likely to generate potentially useful chemical agents. In the long term I aim to interrogate large chemical collections to accurately identify active compounds able to repress PAX3 transcriptional function. To do this I needed to develop an assay that provides a robust, reproducible signal with adequate throughput to screen large compound libraries [212]. In addition the assay signal window must provide adequate separation between the maximum and minimum responses to allow confident identification of potential "hits" [212]. The initial considerations to generate such an assay were assay target design, capture of assay signal, optimisation of an appropriate cell model and production of enough cells for screening. Given my previous experience in generating a small scale assay for p53 transcriptional function using a dual luciferase reporter I decided to use this format to generate a PAX3 responsive screen. The use of a dual reporter system controls for the generation of false positive hits due to loss of cellular proliferation or chemical cytotoxicity.

PAX3 has been shown to directly transactivate the MITF promoter and the specific binding sites have been demonstrated [213]. Furthermore a MITF reporter construct has been shown to be efficiently activated in melanoma cells by endogenous PAX3 [213]. Watanabe et al. also demonstrated that disease causing mutations of PAX3 prevented MITF promoter activation by completely inhibiting PAX3 binding to the

consensus sequence ATTAATACTACTGGAAC [213]. The MITF promoter was therefore chosen as the PAX3 responsive target for assay optimisation.

8.2.2 Development of a transient transfection reporter assay to assess PAX3 transactivation of the MITF promoter

The MITF promoter contains a PAX3 response element responsible for over 80% of promoter activity in 624-mel melanoma cells [213]. This activity has been confirmed to reside within a PAX3 consensus binding sequence between positions -263 and -238 of the MITF promoter P1 [213]. In addition a second PAX3 response element, P2, has been identified between positions -40 and -26 [214]. The relative role of these binding sites in PAX3 dependent MITF activity is cell line dependent [214]. I have therefore used a minimal MITF promoter construct consisting of bases -382 to + 96 of the MITF promoter in pGL3Basic for optimisation of PAX3 responsiveness. This construct contains the two PAX3 response elements and also contains binding sites for the transcription factor SOX10, shown to act synergistically with PAX3 to promote MITF transactivation [214].

The ability of PAX3 to transactivate MITF has been reported to be cell line dependent [213, 214], therefore the melanoma cell line UACC-62, which I have shown to express PAX3 was tested for MITF reporter activation. Watanabe et al., in the report first describing the relationship between PAX3 and the MITF promoter, had used relatively large amounts of reporter construct to produce increased relative luciferase activity from the MITF reporter gene [213]. With this in mind a complex titration experiment was conducted to find the conditions best suited to maximal MITF reporter activation. This was important because to produce a robust assay for high throughput screening a large difference between the minimum and maximum response is required.

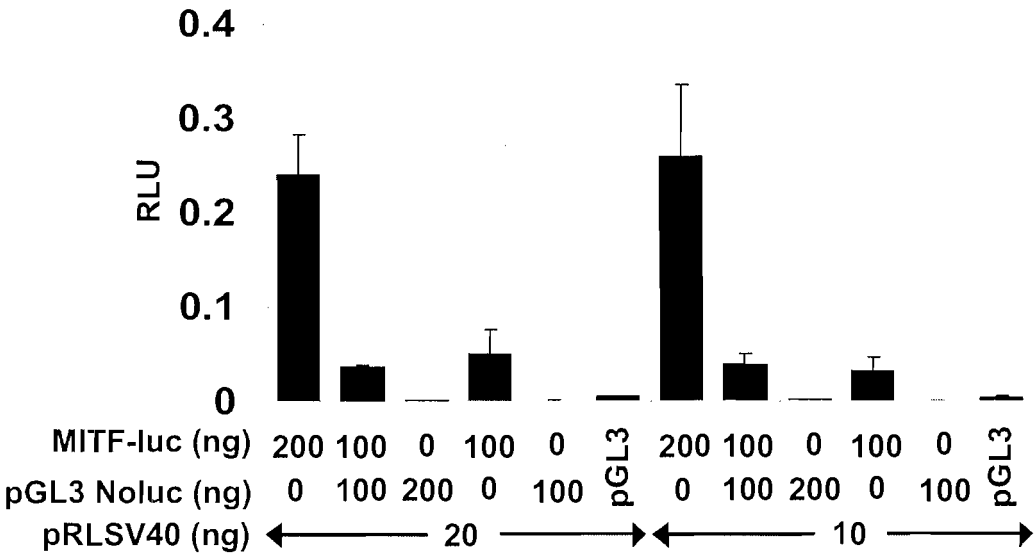

Figure 8.1: Optimization of MITF reporter induction in UACC-62 cells

UACC-62 cells were transfected with the indicated amounts of MITF-luc, pGL3 Noluc or pGL3 Basic as indicated in the presence of varying amounts of pRLSV40 as indicated in 96 well plates. Dual-Glo™ reporter assays were performed 48 h after transfection (n=2). All data are mean RLU ± S.D.

In figure 8.1 the relative amount of MITF reporter has been varied on the background of different total plasmid transfection and different amounts of renilla plasmid. It can be observed that variation between 20 ng and 10 ng renilla plasmid has little effect on MITF activity. In both cases the optimum conditions are 200 ng of MITF reporter with no carrier plasmid. This gives a >50 fold induction of reporter activity compared to pGL3 Basic. By doubling the amount of reporter construct transfected the response has been increased by over 6 times (comparison between MITF-luc 200 ng and MITF-luc 100 ng with 20 ng pRLSV40). This experiment defined the optimum conditions for MITF induction in UACC-62 cells to be carried forward into the next stage of assay optimization.

8.2.3 Transactivation of the MITF promoter can be repressed by PAX3 inhibitors

The development of a high throughput screen to test possible inhibitors of PAX3 transcriptional function is dependent on hit compounds producing significant

down-regulation of the MITF reporter signal. Before trial compounds could be assessed evidence was required that demonstrated that transactivation of MITF in UACC-62 cells was PAX3 dependent and that inhibition of PAX3 transactivation of MITF was possible. In order to demonstrate these effects I used two strategies. Firstly, I have shown that PAX3 specific siRNA has on-target effects in UACC-62 cells. Therefore PAX3 siRNA was used to repress PAX3 expression and any effects on MITF reporter activity observed. Secondly a presentation at the American Association for Cancer Research 97th Annual Meeting had suggested that the transcriptional activity of PAX3 is in part regulated by phosphorylation [215]. Furthermore, use of a kinase inhibitor PKC412, a staurosporine derivative originally developed against protein kinase C (PKC), was demonstrated to produce caspase-3 dependent apoptosis in alveolar rhabdomyosarcoma cells via PAX3/FKHR inhibition [216]. I therefore tested the effect of two kinase inhibitors on PAX3 activation of MITF; UO126 a potent mitogen-activated protein kinase kinase (MEK) inhibitor [217, 218] and LY294002 a specific phosphatidylinositol 3-kinase (PI3K) inhibitor [219]. Figure 8.2 shows the result of an experiment to test both strategies for suppression of PAX3 transactivation of the MITF reporter. There is clear transactivation of the MITF reporter with an over 48 fold increase in relative luciferase activity (comparison between pGL3Basic and control ($p<0.001$)). Addition of the chemical carrier DMSO does not cause any diminution of the MITF reporter activity. UO126 is demonstrated to significantly inhibit MITF reporter induction when compared to control ($p=0.001$). The PI3K inhibitor LY294002 has been excluded from analysis as it inhibited expression of the control renilla luciferase plasmid (fig. 8.2B). Increasing doses of PAX3 specific siRNA are also shown to inhibit MITF transactivation when compared to control ($p<0.004$ for all doses). Optimum inhibition is observed with 0.1 μl siRNA per well (2 pmol). This demonstrates that activity of the MITF reporter construct is repressed by PAX3 inhibitors.

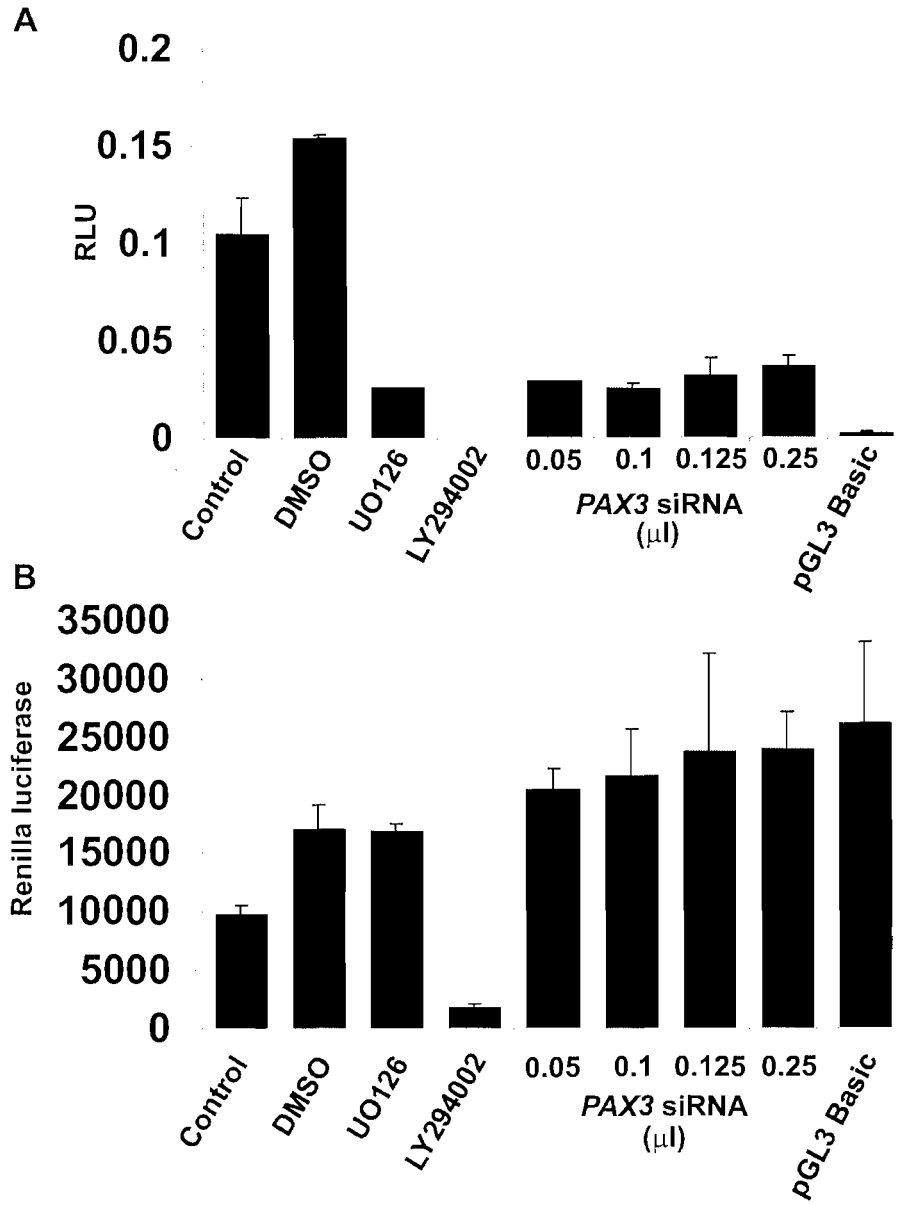

Figure 8.2: PAX3 inhibition represses MITF transactivation in UACC-62 cells

UACC-62 cells were transfected with 200 ng MITF-luc (bars 1-8), or pGL3 Basic (bar 9), 20 ng pRLSV40 and PAX3 siRNA as indicated in 96 well plates. DMSO, or kinase inhibitors UO126 (25µM) or LY294002 (10µM) were added at 24 h where indicated. Dual-Glo™ reporter assays were performed 48 h after transfection (n=2). (A) Data are mean RLU ± S.E.M. (B) Data are renilla luciferase ± S.E.M.

8.3 Discussion

PAX3 has been identified as a potential target for therapy in a range of tumours including malignant melanoma [8]. I have demonstrated that the transcriptional integrity of PAX3 must be retained for it to have this effect. The design and optimisation of a cell based screening assay using UACC-62 cells will allow further investigation of PAX3 transcriptional function and may identify potential lead compounds for therapeutic use. I have demonstrated that the MITF promoter is a valid target for PAX3 transcriptional activity and can be used in a dual reporter assay system. The use of a dual luciferase transient reporter system has a number of advantages including: avoidance of the problems associated with stable reporter cell lines (development time, integration effects and promoter silencing), a reduction in assay variability due to internal normalisation and reduced false positive hits due to non-specific effects of transcription and cell viability.

The activity of PAX3 on the MITF promoter appears to be cell line dependent and this may in part be due to the presence or absence of other transcription factors such as SOX10 [214]. The interaction between PAX3 and SOX10 is not straightforward; the proteins are required to interact physically to transactivate a c-RET enhancer but must bind DNA independently on the MITF promoter to achieve synergy [88, 214]. The expression of SOX10 is limited in the adult [214]. MITF promoter activity is clearly demonstrable in UACC-62 cells and this has previously been shown to be dependent on PAX3 in another melanoma cell line [213]. Optimisation of the assay yielded a low background reporter activity with over 50 times activity of the MITF reporter in the presence of PAX3. This should give a wide enough response window to enable testing of chemical compounds for their effects on PAX3 transcription. Use of genetic and chemical inhibitors of PAX3 has further demonstrated the validity of the assay for interrogating PAX3 function. The kinase inhibitor UO126 repressed MITF reporter activity in this assay, however it has been shown previously that UO126 can also inhibit p53 dependent transcription [220] and will not be taken further into lead generation.

The findings presented in this chapter have shown that the MITF promoter offers a good target to study PAX3 transcriptional activity. The assay will require further optimisation before it can be used for high throughout compound screening. I have shown that PAX3 is required for survival of UACC-62 cells and this occurs by inhibiting p53. Therefore potentially active compounds may be discarded because they decrease overall cell viability and therefore renilla luciferase. To overcome this problem of false negatives the screen will be optimised by co-expression of a dominant negative fragment of p53. It may also be possible to enhance the specificity of MITF reporter activity by using a minimal promoter linked to multiple PAX3 response elements [213]. The final assay will be scaled-up for use in multiple plates and will have to fulfil the criteria of all high throughput screens including optimum Z' score and minimal cross plate and plate to plate variability [212]. This will generate a primary screen for identification of potentially active compounds (Fig. 8.5). The compound libraries available for screening are the NCI challenge set (57 compounds), the NCI mechanistic and diversity sets (~3000 compounds) and a ~1000 compound kinase target-directed library (Biofocus). Positive hits (producing >50% reduction in MITF reporter activity) will be subjected to further analysis to establish PAX3 dependent transcription specificity. Functional analysis of PAX3 inhibition in the form of cell viability and apoptosis assays will be conducted in UACC-62 cells, the PAX3 null melanoma cell line A375 and telomerase-immortalised fibroblasts at IC_{50} concentrations for PAX3 transcriptional repression. This will lead to identification of confirmed hits for further analysis of structure activity relationships and medicinal chemistry (Fig. 8.5).

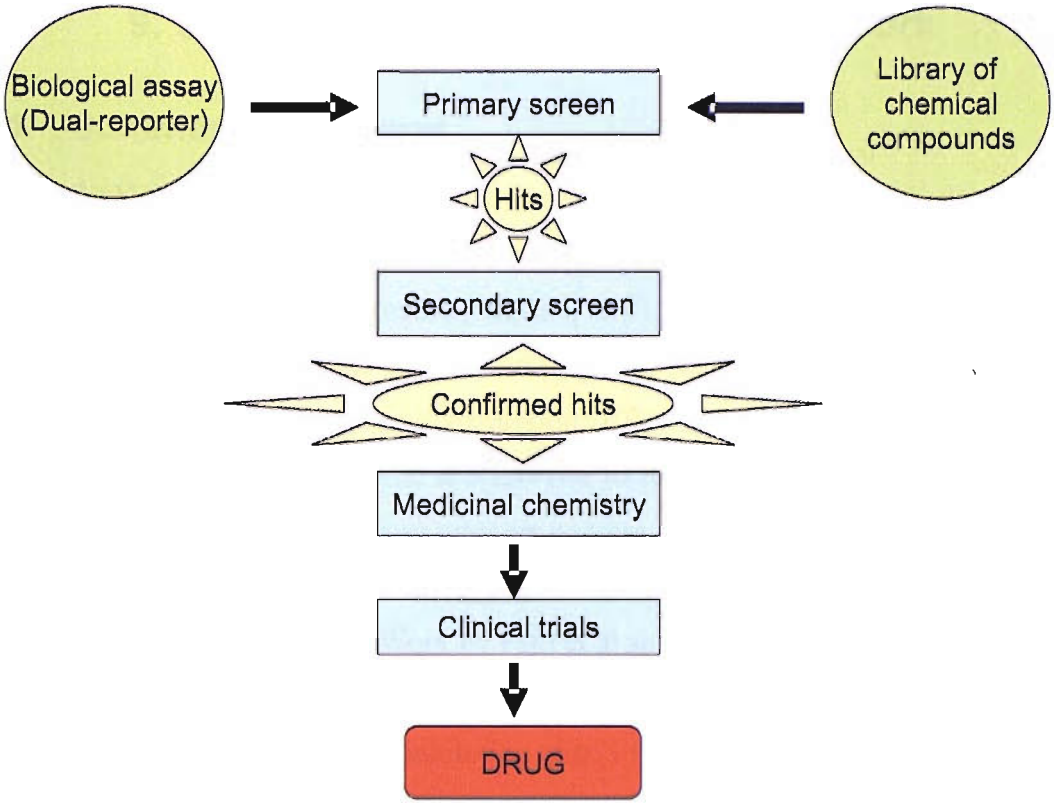

Figure 8.5: High throughput screening to drug discovery
(Adapted From Malo et al. Nature Biotechnology, Volume 24, 167-175, 2006 [221])

The use of screening methods to identify small molecules with a desired biological activity from compound libraries is an approach with many advantages [222]. The regulatory pathways that control PAX3 and p53 activity are not completely understood so a purely rational approach to drug design is limited by current biological understanding [223]. A cell based screening approach, as described here, pre-selects compounds with the desired activity in a cellular context. Such compounds can be used as tools to study the mechanism of p53 functional inhibition by PAX3 and may also have therapeutic potential as lead compounds for medicinal chemistry.

9. Summary of findings and future work

9.1 Summary of findings

PAX proteins are conserved between species and act as master regulators of vital embryological programmes [10, 224]. Aberrant expression of embryological genes is well documented in tumours [70, 224] and many are required for tumour cell survival [8, 10, 35]. A number of PAX family proteins have been documented to inhibit p53 via direct transcriptional repression [10]. This has not been shown for PAX3. PAX3 has been implicated as a survival factor for a range of neural crest derived tumours [103, 105]. These tumours possess wild type p53 but do not undergo spontaneous apoptosis. Inhibition of PAX3 in these tumours leads to cell death via an apoptotic pathway [103, 197]. Work by Pani et al showed that PAX3 inhibited p53 function in the neural crest of mouse embryos [20]. This combination of evidence suggested a role for PAX3 in the inhibition of p53 function in neural crest derived tumours. This became a working hypothesis for study in this project.

The initial objective of this study was to develop a robust and reproducible system in which to investigate p53 function. I made an initial attempt to generate a stable, inducible PAX3 expressing cell line, based on IMR32, using the TET-off system. This system would have been used to assess the effect of PAX3 expression on endogenous p53. Although I was able to achieve stable integration of the TET responsive plasmid, despite many attempts, I was unable to achieve integration of the PAX3 antisense expressing secondary vector. A transient transfection assay was therefore developed to study p53 function in the presence of absence of PAX3. This was achieved by optimisation of a reporter assay system that uses a small number of cells in a 96 well format. I have optimised this system for use in three separate cell lines, using a number of p53 target genes. I have shown for the first time that PAX3 represses p53 transcriptional function. PAX3 was also shown to have direct effects on the promoters of two p53 targets, *PUMA* and *WAF1*.

To further dissect the mechanism of p53 repression by PAX3 a number of mutant PAX3 proteins were generated using site directed mutagenesis. These proteins were carefully designed to determine the role of each active domain of PAX3 in p53

inhibition. The mutant PAX3 proteins became vital tools, not only to study activity of PAX3 in the reporter assay setting, but also later when the effect of PAX3 on p53 protein level was considered. Data from reporter assay experiments shows that PAX3 must retain full transcriptional activity in order to inhibit p53 function. This data also confirms previous findings of interdependence between the DNA binding domains of PAX3 [93, 95, 178].

To study the effects of PAX3 on p53 at the protein level the reporter assay system was scaled up for use in 60mm dishes. Initial observations showed that p53 could be detected at very low levels when expressed in H1299 cells. This meant that small changes to p53 expression in the presence of PAX3 could be detected. PAX3 was observed to repress expression of p53 and furthermore PAX3 was seen to shorten the half life of p53 significantly. Co-expression of PAX3 mutant proteins and p53 showed that PAX3 must retain full transcriptional integrity to have this effect. Indeed, loss of the transactivation domain of PAX3 abolished its ability to repress p53 protein expression, suggesting the involvement of other proteins in PAX3 repression of p53. Further examination of the mechanistic basis of increased p53 turn-over in the presence of PAX3 produced surprising results. Not only was HDM2 not required for increased p53 breakdown, but PAX3 was observed not to induce ubiquitination or proteasomal degradation of p53 protein. This leads to the consideration of other potential mechanisms for p53 degradation in the presence of PAX3 including calpain cleavage [194].

I used a panel of phosphospecific antibodies to interrogate the post-translational modifications of p53 in the presence or absence of p53 (data not shown). These results were inconclusive and this may in part be due to the reduction in p53 levels observed in the presence of PAX3.

The majority of the reporter assay and p53 protein regulation work was conducted in a p53-null, human non small cell lung cancer cell line H1299. This cell line proved to be a reliable vehicle to study the interaction between over-expressed plasmid derived proteins which achieved substantial results. To complement these findings I made use of physiologically relevant cell lines to study the effects of PAX3 down-regulation. I have shown that PAX3 can be targeted efficiently by siRNA technology and that

knock-down of PAX3 causes loss of cell viability in both neuroblastoma and malignant melanoma cells. Furthermore I have shown for the first time that the apoptosis observed in melanoma cells after PAX3 down-regulation is p53 dependent. This is in striking contrast to the effects of down-regulating HDM2 when cell cycle arrest is observed. This finding is supported by data from small molecule inhibitors of HDM2 [183]. The differential activation of the p53 response in UACC-62 cells between PAX3 and HDM2 knock-down is likely to be dependent on the differential expression of the p53 target $p21^{WAF1}$.

The restricted expression of PAX3 in adult tissues [83] and the requirement for expression of PAX3 in tumour survival [31, 103, 197] has led to suggestions for therapeutic down-regulation of PAX3 using antisense or siRNA technology [8]. In vivo delivery of siRNA has so far proved most efficacious as a local rather than systemic therapy to sites such as the eye and lungs [225]. Therefore, in an attempt to develop a therapeutic PAX3 inhibitor I have designed and tested a cell-based screening assay for PAX3 transcriptional function. The promoter of the PAX3 target gene *MITF* has been shown to be significantly transactivated in UACC-62 cells and this is repressed by *PAX3* specific siRNA. The selective MEK inhibitor UO126 was also demonstrated to down-regulate PAX3 induced activity of the *MITF* reporter; although UO126 will not be examined further due to its documented ability to inhibit p53 dependent transcription [220]. It has been suggested that PAX3 transcriptional function is dependent on kinase activity and a number of potential phosphorylation sites have been identified [215]. Screening compound libraries for PAX3 inhibitors will not only lead to new potential therapies for neural crest derived tumours but will yield useful tools to further investigate the mechanism of PAX3 repression of p53 function.

9.2 A model for PAX3 inhibition of p53 function

The exact mechanistic basis of PAX3 repression of p53 function remains to be elucidated. Findings from this project have shed considerable light on the role of PAX3 in neural crest derived tumours and have defined the transcriptional integrity of PAX3 as fundamental to its p53 repressive activity. PAX3 clearly causes increased p53 turn-over; this is supported by data from Pani et al showing that the level of p53

protein expressed in the presence of PAX3 is significantly decreased when compared with embryos with the homozygous Splotch deletion [20]. In a melanoma model the number of p53 immunoreactive cells is increased by down-regulation of p53 [31], suggesting that PAX3 does indeed suppress p53 protein levels in these tumours. Increased p53 turn-over is by a novel mechanism but this is unlikely to be the only activity of PAX3 on the p53 network. For example, I have presented evidence that PAX3 may have direct effects on down-stream p53 targets and this is supported by data showing that $p21^{WAF1}$ is a direct transcriptional target of MITF [177]. It is likely that one or many intermediary proteins are responsible for the overall effect of PAX3 on p53 activity. Direct evidence for this comes from my work with PAX3 mutant proteins showing that PAX3 must be transcriptionally active to inhibit p53. A range of genes have been shown to be up regulated in response to PAX3 expression in micro-array experiments but they appear disparate in function and a clear candidate for repression of p53 function does not stand out [90]. Analysis of the genes regulated by endogenous PAX3 has not been reported.

In addition to its direct effect on p53 protein, it is possible that PAX3 competes with p53 for p53 specific binding sites, causing promoter selective activation or repression of p53 target genes (Fig 9.1A). This mechanism is supported by my data showing PAX3 stimulation of $p21^{WAF1}$ and inhibition of p53 dependent BAX and HDM2 activity. Up-regulation of $p21^{WAF1}$ is potentially protective to tumour cells and may provide a mechanism to bypass stress induced apoptotic pathways [42]. If this is the case in neural crest derived tumours down-regulation of PAX3 will not only lead to accumulation of p53 but will also cause decreased expression of $p21^{WAF1}$, leading to apoptosis (Fig. 9.1B). It is this effect that I have observed in UACC-62 cells upon down-regulation of PAX3 and strengthens the case for targeted suppression of PAX3 in neural crest derived tumours. In melanoma PAX3 has been shown to be required for tumour cell survival and in the melanocyte lineage PAX3 is responsible for preventing terminal differentiation [83]. PAX3 is therefore likely to be responsible for providing two essential functions required by a tumour; prevention of apoptosis by inhibition of p53 function and maintenance of a dedifferentiated phenotype.

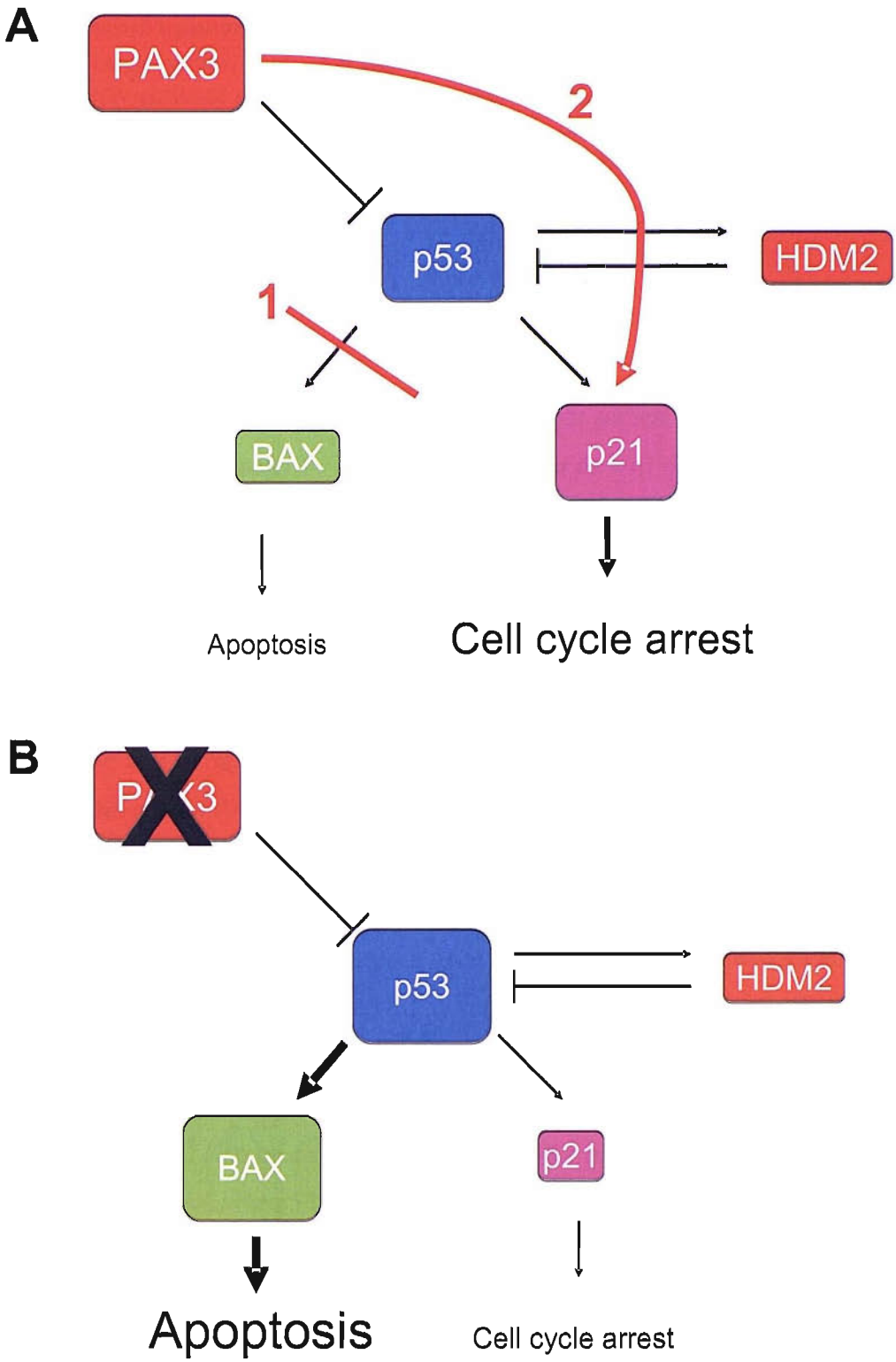

Figure 9.1: A model for PAX3 suppression of p53 function in neural crest derived tumours

(A) In a resting cell p53 is maintained at low levels by the activity of its major negative regulator HDM2. In neural crest derived tumours aberrant

expression of PAX3 causes direct suppression of p53 protein levels via increased turnover. In addition PAX3 may have promoter selective effects on p53 target genes, such as *BAX*, to inhibit apoptosis (1). In addition PAX3 may directly activate the p21WAF1 promoter leading to protection from stress induced apoptosis (2). (B) Down-regulation of PAX3 causes increased p53 protein and withdrawal of $p21^{WAF1}$ activation. The pro-apoptotic p53 target BAX is up-regulated leading to cell death.

9.3 Future work

The further optimisation of the high throughput screen for PAX3 inhibitors has been discussed in chapter 8. Any active compounds will be characterised for their effect on PAX3 and p53 dependent transcriptional control. This work will form part of a PhD studentship. At the time of writing a grant application is being considered for funding by Cancer Research UK. The proposal was drafted in collaboration with my PhD supervisors Dr Jeremy Blaydes and Dr Karen Lillycrop and Cancer Research UK Professor of Medical Oncology Peter Johnson. I am a co-investigator in this proposal. We aim to build on the findings presented in this study to further investigate the role of PAX3 in neural crest derived tumours. Specifically we aim to probe the molecular basis for the suppression of p53-dependent apoptosis by PAX3. We plan to analyse mRNA from UACC-62 cells 48 h post-siRNA transfection (control vs. PAX3) for global changes in gene expression using micro-arrays. Following q RT-PCR validation we will identify candidate genes involved in the up-stream regulation of p53 activity. This will define the molecular pathways regulated by PAX3 that are responsible for p53 attenuation. Changes in expression of known p53 target genes will also be considered and tested for p53 dependency. We also hope to define the basis for p53 promoter specific effects of PAX3 observed in this study. We will examine the sequence specific DNA binding of p53, the regulation of p53 co-factors that confer promoter specificity and p53 independent effects of PAX3 on p53 dependent promoters. This can be achieved using the optimised experimental systems described in this thesis.

There is no doubt that PAX3 inhibits p53 function in neural crest derived tumours, whether or not manipulation of PAX3 expression in these cancers leads to therapeutic success remains to be seen.

10. References

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