TP63 is implicated in apoptotic dysregulation in melanoma

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DECLARATION

I, Rubeta N H Matin declare that the work presented in this thesis is my own, unless stated otherwise, and is in accordance with the University of London's regulations for the degree of Doctor of Philosophy.

Signed

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ABSTRACT

Cutaneous melanoma is an aggressive malignancy accounting for 4% of skin cancers but 80% of all skin-cancer related deaths. Its incidence is rapidly rising and advanced disease is notoriously treatment-resistant. The role of apoptosis in melanoma pathogenesis and chemoresistance is poorly characterised. Mutations in TP53 occur infrequently and are not critical for tumour development, yet the TP53 apoptotic pathway is abrogated; this may alternatively result from TP53 pathway defects or from alterations in other members of the TP53 family, including the TP53 homologue, TP63. The hypothesis of this thesis was that TP63 has an anti-apoptotic role in melanoma and is responsible for mediating chemoresistance. The primary aims were to investigate the biological role of TP63 in melanoma, to explore regulation of p63 expression and to understand its role in apoptosis and dysregulation of the TP53 apoptotic pathway in melanoma.

Although p63 was not expressed in primary melanocytes, upregulation of both p63 mRNA and protein was observed in melanoma cell lines and tissue samples. This is the first report of significant p63 expression in this lineage. Furthermore, aberrant cytoplasmic p63 expression significantly correlated with poor overall outcome in melanoma patients. Multiple possible mechanisms were demonstrated to regulate TP63 expression in melanoma, including epigenetic modulation, microRNA regulation of gene transcription and proteosome-dependent stability of p63 protein. In response to genotoxic stress, endogenous p63 isoforms were stabilised in both nuclear and mitochondrial subcellular compartments. Translocation of p63 to the mitochondria occurred through a co-dependent process with p53 but accumulation of wt-p53 in the nucleus was inhibited by p63. Using RNAi technology, both isoforms of p63 (TA and ΔNp63) were demonstrated to confer chemoresistance in melanoma. In addition, the truncated variant, ΔNp63, was enriched in a subset of melanomas expressing CD133, pointing to an anti-apoptotic role for p63 in putative cancer stem cells in this aggressive tumour.

Taken together, these data suggest that in melanoma, p63 is an oncogene which contributes to dysregulation of wt-p53 function and has an important role in mediating chemoresistance. Ultimately, these observations may provide the rationale for novel approaches aimed at sensitising advanced melanoma to chemotherapeutic agents.

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ABBREVIATIONS

5-Aza	5- Azacytidine
AA	amino acid
ABCB5	ATP-binding Cassette Subfamily B, Member 5
AIP1	ASK-interacting protein
AJCC	American Joint Committee on Cancer
Akt	Protein kinase B
Apaf1	Apoptotic protease activating factor-1
Arg	Arginine
ASIP	agouti signalling protein
ASPP	Apoptosis stimulating protein of p53
ATM	ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	ataxia telangiectasia and RAd3 related
BIDN	benign intradermal naevi
BLT	Bart's and the London Hospitals NHS Trust
bp	base pairs
BSA	bovine serum albumin
BSA-c	acetylated BSA
CDK4	cyclin-dependent kinase-4
CDKN1A	cyclin-dependent kinase inhibitor-1A
cDNA	complementary DNA
Chk1	checkpoint kinase-1
Chk2	checkpoint kinase-2
COREC	Central Office for Research Ethics Committees
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
DBD	DNA binding domain
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanine triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	DL-Dithiothreitol

dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic Acid
EORTC	European Organisation for Research and Treatment of Cancer
EtOH	Ethanol
FACS	Fluorescence activated cell sorting
FCS	Foetal calf serum
FFPE	formalin-fixed paraffin embedded
FLIP	FLICE inhibitor protein
FOXO3	forkhead box 03
FSC	Forward scatter
g	grams
g	gravity acceleration
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSK3β	glycogen synthetase kinase-3 beta
GUS	beta glucuronidase
HAUSP	herpes virus-associated ubiquitin-specific protease
Hcds1	human cds-1 kinase
HEMa	normal human epidermal melanocytes-adult
HRP	horseradish peroxidase conjugated
hrs	hours
hSCF	human stem cell factor
IF	immunofluorescence
IGF-BP3	Insulin-like growth factor binding protein 3
IHC	immunohistochemistry
IRCC	Institute for Cancer Research and Treatment Ethics Committee
J	joules
L	litres
LB	Luria Bertani

m	metres
M	moles
MAPK	mitogen activated protein kinase
MC1R	melanocortin-1 receptor
Mdm2	mouse-double-minute-2
MDM4	mouse-double-minute-4
Met	metastatic
$MgCl_2$	magnesium chloride
MIB	Mitochondrial isolation buffer
mins	minutes
miRNA	microRNA
MITF	microphthalmia-associated transcription factor
mRNA	messenger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Mut	mutant
NaCl	sodium chloride
NCRN	National Cancer Research Network
NFkB	Nuclear factor kappa-B
NHEM	Normal human epidermal melanocytes
NI	Nuclear isolation buffer
OD	oligomerisation domain
PARP	Poly(ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PERP	p53 apoptosis effector related to PMP-22
PFA	paraformaldehyde
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
PIC	protease inhibitor cocktail
PIDD	p53-induced protein with death domain
PIG3	p53-inducible gene-3
Pin1	peptidyl-prolyl cis/trans isomerase -1

Pri-miRNA	primary miRNA
Pro	Proline
PRO	proline rich domain
PS	phosphatidylserine
PTEN	phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
Q-PCR	quantitative-PCR
RACk1	Receptor for activated C kinase 1
RGP	radial growth phase
RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
RT-PCR	reverse-transcriptase PCR
s	seconds
SAM	sterile alpha motif
SBL	scramble
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of mean
ser	serine
shRNA	short hairpin RNA
siRNA	small interfering RNA
SSC	Side scatter
TA	transactivation domain
TBE	Tris borate EDTA
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TID	transactivation inhibitory domain
TKI	tyrosine kinase inhibitors
TMA	tissue microarray
TNF-α	Tumour necrosis factor- alpha
TPA	12-O-tetradecanoyl-phorbol-13-acetate

TRAF2	TNF-receptor associated factor 2
TSA	trichostatin A
TYR	tyrosinase
TYRP1	tyrosinase related protein-1
UV	ultraviolet
UVA	ultraviolet-A
UVB	ultraviolet-B
UVR	ultraviolet radiation
V	volts
VGP	vertical growth phase
Vol	volume
W	watts
WB	Western blotting
WT	wild-type
Wt	weight
WWP1	WW domain containing E3 ubiquitin protein ligase 1

PUBLICATIONS, PRESENTATIONS AND AWARDS

Publications

Brown VL, Matin RN *et al*. Melanomas in renal transplant recipients: the London experience, and invitation to participate in a European study (2007). Br J Dermatol; 156(1):165-7

Matin RN, Mesher D *et al*. Melanoma in organ transplant recipients: clinicopathological features and outcome in 100 cases (2008). Am J Transplant; 8(9):1891-900

Presentations

June 2010	p63 is implicated in apoptotic dysregulation in melanoma (oral). 10th Meeting of THESIS (THESIS/BAD/BSID Research Course)
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Awards

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2007 Dowling Club / BAD travel award (Stiefel)
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2010 BAD Travel Bursary for Dermatology SpRs in Training
To attend BSID meeting, Edinburgh

CHAPTER 1: INTRODUCTION

1.1 MELANOMA SKIN CANCER

Melanoma is an aggressive cutaneous malignancy accounting for just 4% of skin cancers but resulting in 80% of all skin-cancer related deaths (Miller and Mihm 2006). The clinicopathological classification of cutaneous melanoma is primarily based on anatomic location and patterns of growth [Table 1.1; Figure 1.1]. Non-cutaneous melanoma accounts for 5% of melanoma and includes ocular and mucosal malignancies. The aetiology, prognostic features and treatment of these differs from that of cutaneous subtypes.

1.1.1 Epidemiology of cutaneous melanoma

The incidence of cutaneous melanoma is rising faster than for any other major cancer, and rates are set to treble over the next 30 years (Diffey 2004). More than 10,400 cases of melanoma are diagnosed annually in the UK with over 2,000 deaths attributable to melanoma (Statistical information team Cancer Research UK 2009). In 2006, melanoma was more common in females than males with a M:F ratio of 4:5. The incidence of melanoma rises with age but a disproportionately high incidence of melanoma is observed among young people; almost a third of cases (31%) occur in people aged < 50 years and it is the commonest cancer among 15-34 year olds. For females, it is the 6th most common cancer and for males it is the 8th. The current lifetime risk for males in the UK is about 1:91 and 1:77 for females.

1.1.2 Aetiology of melanoma

The aetiology of cutaneous melanoma is multi-factorial and a number of risk factors have been identified [Table 1.2].

Table 1.1: Clinical classification of cutaneous melanoma. Adapted from Chudovsky (2005).

Melanoma subtype	Frequency (%)	Gender M:F	Common site	Other key features
Superficial spreading melanoma (SSM)	50 – 70	F>M	Back (M) Leg (F)	Often arising from pre-existing naevi
Nodular melanoma (NMM)	15 – 35	M>F	Trunk	
Acral lentiginous melanoma (ALM)	10 (Caucasian) 50 (African or Asian origin)	M>F	Feet>hands Subungual	Not related to UV exposure
Lentigo maligna melanoma (LMM)	5 – 15	M=F	Face	Associated with chronic UV exposure

M – males, F – females, UV – ultraviolet radiation

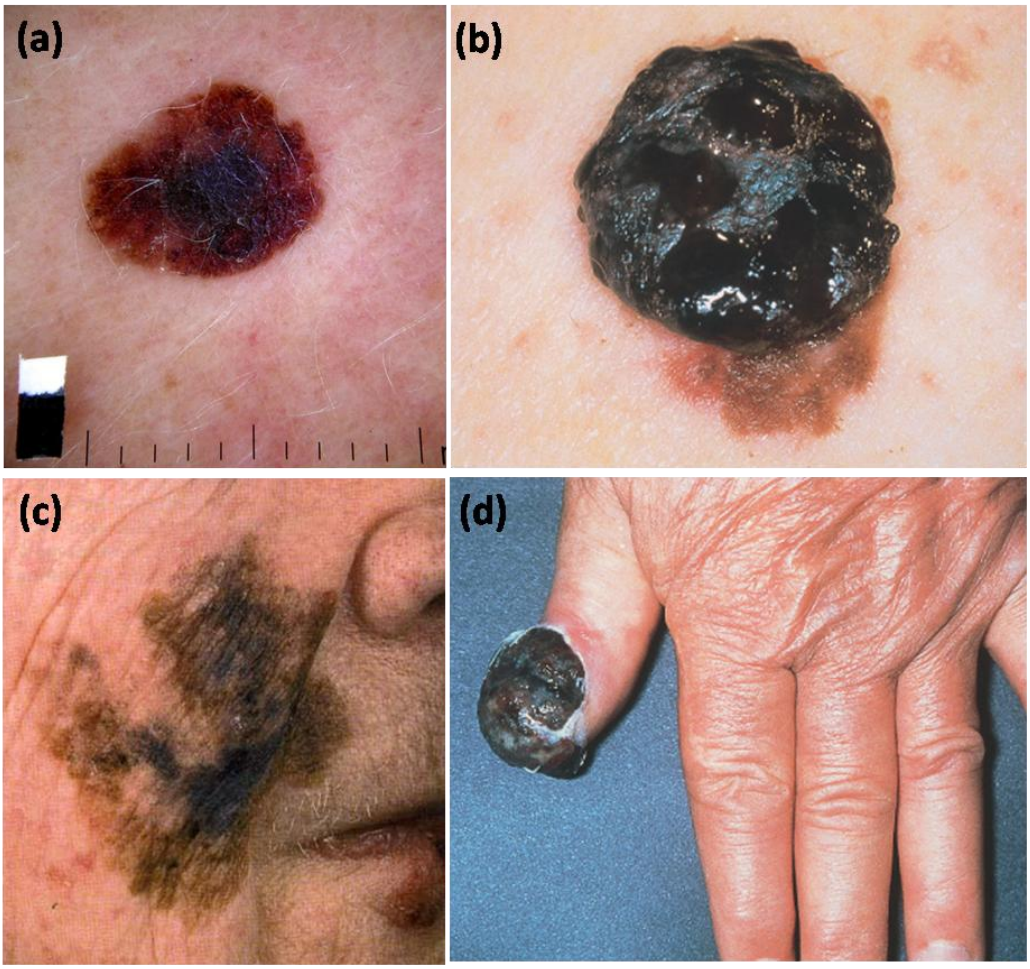


Figure 1.1: Clinical photographs of common histopathological subtypes of primary melanoma. (a) Asymmetrical lesion >1cm in diameter on leg, with variable pigmentation and irregular border – clinical features consistent with a diagnosis of superficial spreading melanoma. (b) Ulcerated, friable, rapidly enlarging necrotic nodular melanoma on back. (c) Lentigo maligna melanoma – poorly circumscribed, variably pigmented patch over the right cheek, enlarging slowly over time. (d) Ulcerated necrotic black pigmented subungual lesion on left thumb, consistent with a diagnosis of acral lentiginous melanoma.

Table 1.2: Risk factors for melanoma predisposition

Risk factor		Relative risk for developing melanoma (OR)
Strong family history of melanoma*		35 – 70 (Goldstein and Tucker 1995; Stehmeier and Muller 2009b)
Previous history of melanoma		8.5 (Tucker et al. 1985)
Low penetrance alleles	MC1R	1.03 – 2.45 (Gudbjartsson et al. 2008; Raimondi et al. 2008)
	ASIP	1.45 – 1.75 (Brown et al. 2008; Gudbjartsson et al. 2008)
	TYR	1.21 (Gudbjartsson et al. 2008)
	TYRP1	1.15 (Gudbjartsson et al. 2008)
UV exposure – history of > 10 blistering sunburns		3.9 (Han et al. 2006)
Fitzpatrick skin type I (burns without tanning)#		1.7 (Bliss et al. 1995; Marrett et al. 1992)
Multiple benign naevi		11 (Berwick and Halpern 1997; Grulich et al. 1996)
Multiple atypical naevi		11 (Newton 1993; Tucker et al. 1997)
Previous history of non-melanoma skin cancer (BCC and/or SCC)		2.9 (Bower et al. 2000)
Immunosuppression Human immunodeficiency virus Post-transplantation		1.5 (Grulich et al. 2007; Patel et al. 2008) 2 – 12 (Baccarani et al. 2009; Birkeland et al. 1995; Bordea et al. 2004; Bouwes Bavinck et al. 1996; Greene et al. 1981; Hollenbeak et al. 2005; Jain et al. 1998; Jensen et al. 2000; Kasiske et al. 2004; Lanoy et al. 2009; Le Mire et al. 2006; Leveque et al. 2000; Lindelof et al. 2000; Moloney et al. 2006; Rigel and Carucci 2000; Sheil 1986; Sheil et al. 1985)

* Defined as ≥3 family members affected

Fitzpatrick skin types developed to classify skin tolerance to ultraviolet radiation using skin colour, susceptibility to burning and ability to tan (Fitzpatrick 1988): I: always burns/never tans; II: usually burns/sometimes tans; III: usually tans/sometimes burns; IV: always tans/rarely burns; V: Asian; VI: Black African and Afro-Caribbean.

BCC – basal cell carcinoma, SCC – squamous cell carcinoma, MC1R – melanocortin-1 receptor, ASIP – agouti stimulating protein, TYR – tyrosinase, TYRP1 – tyrosinase-related protein-1, UV – ultraviolet radiation, OR – odds ratio.

1.1.2.1 Ultraviolet radiation

Melanomagenesis is associated with exposure to solar ultraviolet radiation (UVR). UVR promotes malignancy through direct mutagenic effects of UVB, production of growth factors, reduction of cutaneous immunesurveillance and formation of reactive oxygen species via UVA (Jean et al. 2001). There is compelling evidence for a causative link with sunlight exposure, with intermittent, high intensity exposure in early life correlating best with melanoma risk (Armstrong and Kricker 2001; Tucker and Goldstein 2003). A history of sunburn doubles the risk of developing melanoma (Elwood and Jopson 1997; Gandini et al. 2005), and the use of sunbeds and sunlamps (an artificial source of UVR) increases the risk of melanoma in young adults (Working 2007).

1.1.2.2 Genetics

A family history of melanoma is a significant risk factor [Table 1.2] and an estimated 10% of all melanoma cases report a first- or second- degree relative with melanoma. In families with multiple cases of melanoma, the pattern of susceptibility is consistent with autosomal dominant inheritance of a single gene. However, the majority of familial aggregations show a complex pattern of inheritance suggesting melanoma is a complex trait influenced by the interaction between genetic and environmental factors. The genetic melanoma predisposition factors identified to date are stratified by risk profile into high penetrance genes and low penetrance alleles.

1.1.2.2.1 *High penetrance melanoma susceptibility genes*

In the 1990's, germline mutations in the CDKN2A locus and cyclin-dependent kinase-4 (CDK4) segregated in melanoma-prone kindreds in a dominant pattern (Hussussian et al. 1994; Kamb et al. 1994; Zuo et al. 1996), and to date, these are the only high penetrance melanoma genes identified. The Melanoma Genetics Consortium (GenoMEL; http://www.genomel.org) reported on CDKN2A alterations (single-base changes, small insertions and deletions or large deletions) in 40% of melanoma-prone families (Goldstein et al. 2006). Germline mutations in the CDK4 gene (Arg24His and Arg24Cis) are rare compared to CDKN2A but are found in 2% of the families in GenoMEL (Goldstein et al. 2006), with 14 families harbouring mutations worldwide (Helsing et al. 2008; Molven et al. 2005; Pjanova et al. 2009; Soufir et al. 1998; Zuo et al. 1996). Moreover, penetrance of the CDKN2A mutations is influenced by UV exposure demonstrating an important gene-environment interaction (Meyle and Guldberg 2009).

1.1.2.2.2 *Low penetrance melanoma susceptibility alleles*

Epidemiological studies have established that the risk of developing melanoma is directly related to pigmentation phenotype. Fair skin, poor tanning response, red or blonde hair and freckles are all known melanoma risk factors (Bliss et al. 1995). Genome wide association studies and systematic analyses of candidate genes have identified a large number of genetic determinants of skin, hair and eye colour variation (Sturm 2009). A number of single nucleotide polymorphisms associated with genes involved in the melanin synthesis pathway have emerged as strong candidates for low penetrance melanoma susceptibility factors: melanocortin-1 receptor (MC1R), agouti stimulating protein (ASIP), tyrosinase (TYR) and tyrosinase-related protein-1 (TYRP1) (Brown et al. 2008; Gudbjartsson et al. 2008; Raimondi et al. 2008). A total of nine MC1R variants are associated with increased melanoma risk and this risk persists after adjustment for hair colour and skin type (Kennedy et al. 2001; Landi et al. 2005; Palmer et al. 2000; Raimondi et al. 2008). ASIP, TYR and TYRP1 variants show a small but significant increased melanoma risk [Table 1.2]. In addition, MC1R variants influence penetrance of BRAF mutations with a 5-15-fold increased risk of BRAF-mutant melanomas demonstrated in MC1R variant carriers (Fargnoli et al. 2008; Landi et al. 2006).

1.1.2.3 Immunosuppression

An intact immune system and surveillance are crucial in protecting the body from melanoma; it is therefore expected that immunosuppressed patients would be at increased risk for melanoma (Hollenbeak et al. 2005; Zimmerman and Esch 1978). The possibility that immunosuppression can lead to melanoma is supported by clinical observations that melanoma can spontaneously regress, and in such cases, an immune cell infiltrate is observed within the tumour lesion (Nathanson 1976). Immunological factors are also implicated in both development and progression of melanoma (Greene et al. 1981; Rigel and Carucci 2000).

Significant advances in transplantation have been made over the last several decades benefiting thousands of patients. Long term survival after organ transplantation is increasing and the major cause of late morbidity and mortality in organ transplant recipients (OTR) is post-transplant malignancy (Buell et al. 2005). An increased incidence of skin malignancies following organ transplantation is well established, in particular squamous (SCC) and basal cell carcinomas (BCC) where the excess risk is in the order of 50-100 fold and 10-fold respectively (Ajithkumar et al. 2007; Grulich et al. 2007). For melanoma, an excess relative risk attributable to

transplantation is reported to be up to 12 (Baccarani et al. 2009; Birkeland et al. 1995; Bordea et al. 2004; Bouwes Bavinck et al. 1996; Greene et al. 1981; Hollenbeak et al. 2005; Jain et al. 1998; Jensen et al. 2000; Kasiske et al. 2004; Lanoy et al. 2009; Le Mire et al. 2006; Leveque et al. 2000; Lindelof et al. 2000; Moloney et al. 2006; Rigel and Carucci 2000; Sheil 1986; Sheil et al. 1985).

A recent multi-centre retrospective analysis to assess outcome in 91 post-transplant melanomas was undertaken by stratifying patients according to a number of American Joint Cancer Committee (AJCC) prognostic criteria (Matin et al. 2008). Outcome for post-transplant melanoma was similar to that of a matched non-transplant population (Balch et al. 2001b) for T1 and T2 tumours (≤2mm thickness); but was significantly worse for T3 and T4 tumours (>2mm thickness) ($p<0.0001$) (Matin et al. 2008). Cohort studies of HIV-infected individuals have also demonstrated increased melanoma risk (Grulich et al. 2007; Patel et al. 2008) [Table 1.2], but no analysis of outcome has been undertaken in these individuals.

1.1.3 Melanomagenesis

1.1.3.1 Clark model

Melanomagenesis and disease progression are commonly described as 'de-differentiation' processes of transformed, mature melanocytes enabling the linear stepwise transformation from naevus to melanoma. The Clark model of melanomagenesis represents the proliferation of melanocytes in the process of naevi formation and subsequent development of dysplasia, hyperplasia, invasion, and metastasis (Clark et al. 1984) [Figure 1.2]. The clinicopathological progression from normal melanocyte to benign and dysplastic naevi, horizontal and vertical growth phase primary melanomas and ultimately metastatic melanoma reflects the accumulation of acquired genetic and epigenetic changes in genes with critical functions in the control of cell proliferation, differentiation, motility and apoptosis [Section 1.1.4].

1.1.3.1.1 *Melanocytic naevi*

In the Clark model, the first phenotypic change in melanocytes leads to development of benign naevi, which are composed of nests of naeval melanocytes. Naevi are classified into three main pathological variants – junctional, compound and intradermal [Figure 1.3] based on the location of naeval melanocytes in relation to the dermo-epidermal junction. The control of growth in these cells is disrupted, but growth of a naevus is limited and rarely progresses to cancer (Clark et al. 1984). The

absence of progression is probably due to oncogene-induced cell senescence, in which growth stimulated by oncogenic pathways is limited (Braig and Schmitt 2006).

Melanocytic naevi are thought to originate from the clonal expansion of a single melanocyte stem cell acquiring a BRAF mutation. After an initial phase of proliferation, naevus cells enter senescence. The presence of the $BRAF^{V600}$ mutation alone is not sufficient to transform naevi cells to melanoma since these mutations occur at a similar frequency in benign naevi and in primary and metastatic melanomas (Pollock et al. 2003). Escape from senescence requires one or more additional hit(s), which might collaborate with loss of $p16^{INK4a}$ activity and trigger the naevus cell to proliferate. Immortalisation, however, requires cells to overcome replicative senescence by inactivating the $p16^{INK4a}$/pRB pathway and by maintaining a minimal telomere length, which can be achieved by activation of hTERT. Full oncogenic transformation to melanoma is then likely to require further (epi-) genetic hits (Michaloglou et al. 2008).

Although the Clark model was widely accepted, clinical and pathological evidence suggests that a significant proportion of melanomas do not arise from naevi and that this model of progression is somewhat oversimplified. More recently, evidence of melanoma heterogeneity and plasticity has led to an alternative hypothesis in line with a cancer stem cell concept, proposing mutated melanocyte stem cells or immature progenitor cells present in skin as precursors to melanoma (Fang et al. 2005; Frank et al. 2005; Grichnik et al. 2006; Monzani et al. 2007; Topczewska et al. 2006). These could be involved in initiating cancer and in conjunction with distinct changes in the microenvironment, would regulate the malignant transformation from melanocyte to melanoma [Figure 1.4].

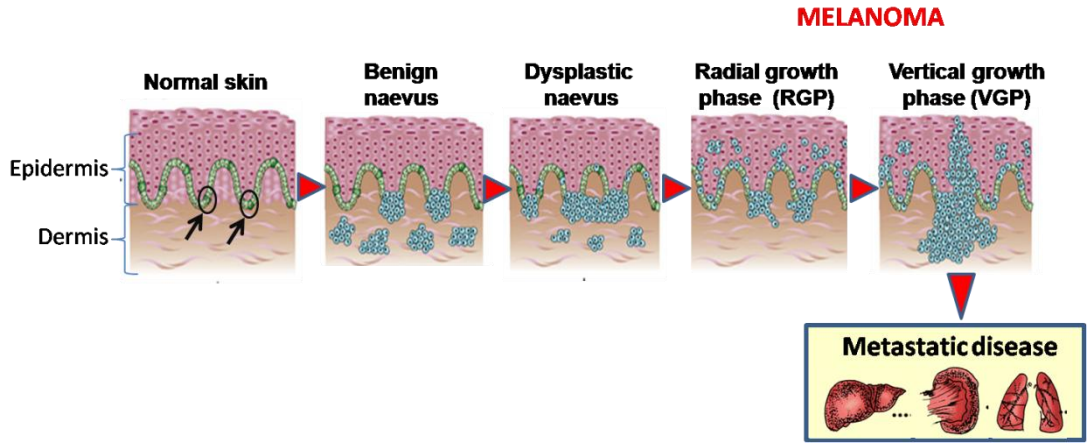


Figure 1.2: Clark Model of Melanomagenesis. Primary melanocytes (black arrows) are interspersed with keratinocytes, in a 1:6 ratio, in the basal layer of the epidermis of normal skin. The Clark model is based on the linear progression of a primary melanocyte to develop nests forming a benign naevus which following genetic aberrations develop into a dysplastic naevus. Further genetic changes result in development of a primary melanoma, initially in a radial growth phase which if it exceeds beyond the basement membrane, grows in a vertical direction (VGP) and through haematogenous and lymphatic spread can metastasise to various organs of the body.

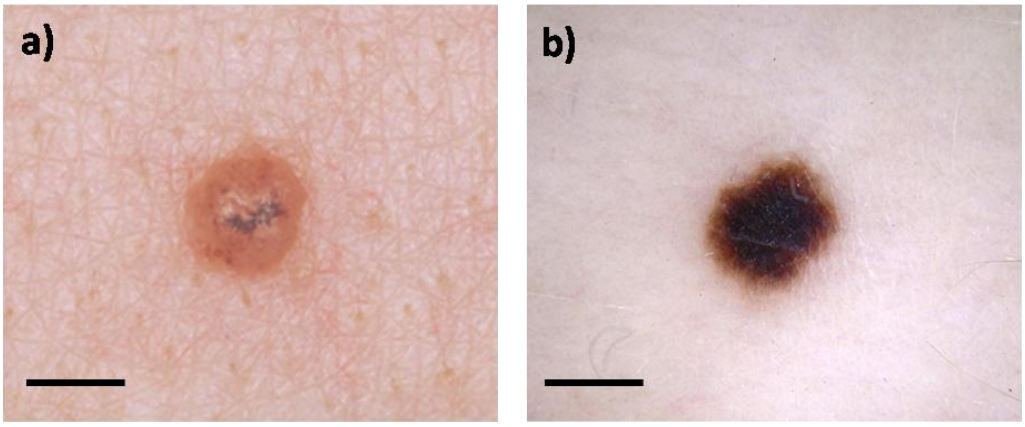


Figure 1.3: Clinical photographs of naevi. a) Well circumscribed pink nodule with linear dermal pigmentation, characteristic of benign intradermal naevus (BIDN). b) Well circumscribed, evenly pigmented macule consistent with a diagnosis of a junctional naevus. Histopathological examination reveals nests of melanocytes in the dermo-epidermal junction. Bar 10mm. Patient consent provided for images.

1.1.3.2 Cancer stem cell model

The hypothesis that cancer stem cells lead to the tumourigenic process has received increasing attention over recent years. It proposes that tumours are initiated and maintained by a subset of cancer cells capable of self-renewal and differentiation into bulk tumour cells (Reya et al. 2001) [Figure 1.5].

There are several features of the stem cell hypothesis in melanocytic neoplasia. Melanocytic naevi and melanoma would both have a common origin, being derived from a genetically defective stem cell. The severity of the defect in growth regulation would determine the extent of the growth; self-limiting defects would be benign (giving rise to naevi), while non-limiting defects would be malignant (resulting in tumour formation). The concept of melanoma 'de-differentiation' from a melanocyte could be explained by the unmasking of a malignant stem/precursor cell with decreased ability to produce competent differentiated daughter cells. Melanomas show phenotypic heterogeneity both *in vivo* and *in vitro*, suggesting an origin from a cell with multi-lineage differentiation abilities. Delayed onset of disease recurrence could be more easily explained where mutated stem cells could exist dormant in the body for many years until the environmental stimulus was triggered. The aggressive nature of melanoma against a heightened immunological response could also be explained whereby daughter cells were destroyed but the malignant stem-like cells might be overlooked by the immune system. Moreover, most chemotherapies achieve a limited response, resulting in no overall survival benefit, and many patients die with relapsing disease that is resistant to further therapy. The recurrence of cancer following a primary response to treatment can be explained by the survival of a subset of cancer stem cells that display an intrinsic resistance to treatment-induced death (Reya et al. 2001). Thus, a number of known biological characteristics of melanomas supports the theory that melanoma arises from a mutated stem cell.

The biological and molecular characterisation of melanoma stem cells is still preliminary. The origin of melanoma stem cells is yet to be determined. Whether melanoma stem cells are derived from melanocyte stem cells, melanocyte progenitors or more mature melanocytes that have de-differentiated, remains unclear [Figure 1.6]. Classification of cell-surface molecules specific to melanoma stem cells could allow for the purification and characterisation of these cells from the bulk tumour population and a number of these have been identified in melanoma [section 3.1.2].

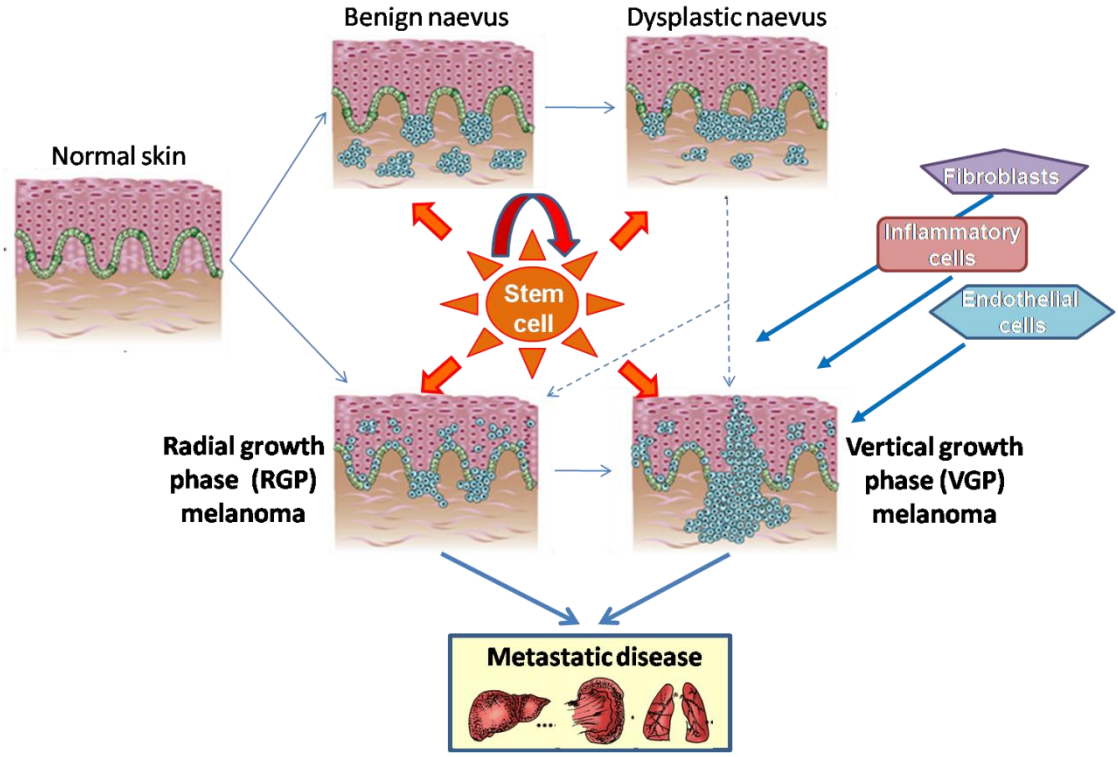


Figure 1.4: Cancer stem cell hypothesis. The melanoma stem cell model proposes that melanocyte stem cell precursors are at the centre of melanomagenesis. It suggests that the stem cell could lead to melanoma development directly or via transition through a naevus. Other cells within the microenvironment (fibroblasts, inflammatory cells and endothelial cells) contribute to regulation of malignant transformation.

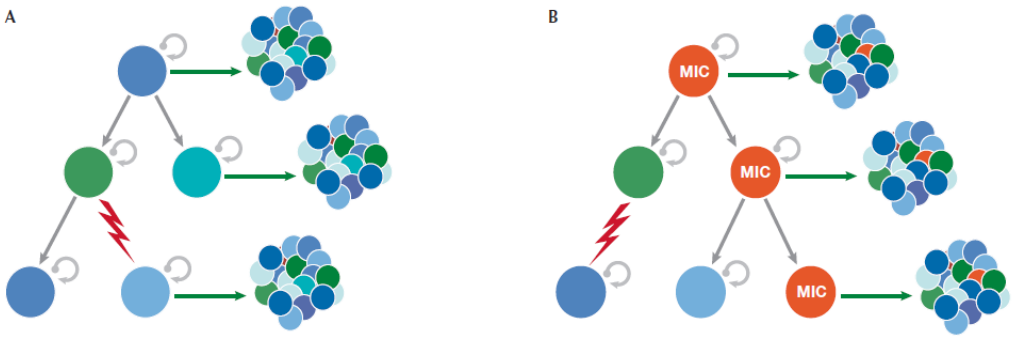


Figure 1.5: Models of cancer growth. (A) The stochastic model predicts that all melanoma cells (coloured circles) derived from a heterogeneous population have the capacity to proliferate, regenerate and maintain tumour growth. (B) The cancer-stem-cell model hypothesises that a subpopulation of melanoma cells within the tumour, the melanoma-initiating cells (MICs – red circles) are able to regenerate, maintaining tumour growth (green arrows). The bulk of the heterogeneous tumour cell population does not share these properties and lacks tumourigenicity. The red zig-zag arrow indicates genetic and/or

epigenetic changes that drive oncogenesis. Circular arrows represent proliferating cells. Figure reproduced from Rafaeli et al. (2009).

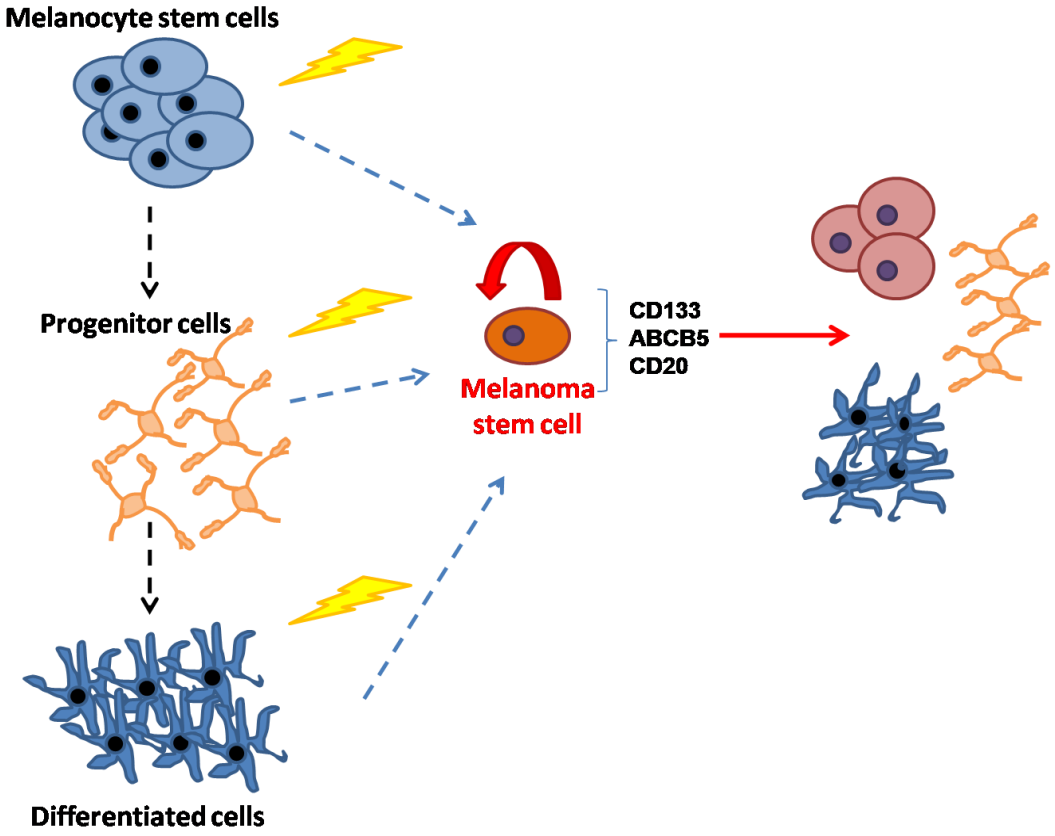


Figure 1.6: Potential origin of melanoma stem cells. Melanoma stem cells may appear after mutations (denoted by yellow zig-zag arrows) occur in melanocyte stem cells or progenitor cells. They may also be the result of transformation in differentiated cells. A number of melanoma stem cell markers have been used to identify these cells from a tumour population, including CD133, ABCB5 and CD20 [detailed in section 3.1.3]. The cancer stem cell hypothesis proposes that these melanoma stem cells are responsible for tumour growth and metastasis. Figure adapted from Sabatino et al. (2009).

1.1.4 Molecular pathogenesis of melanoma

1.1.4.1 Apoptotic pathways

Abnormalities in apoptosis are a common feature of melanoma and occur due to acquired changes in the structure or expression of genes whose products function in various cell death pathways (Soengas and Lowe 2003). The role of apoptosis in melanocyte function and melanomagenesis is not well characterised. However, acquisition of resistance to apoptosis appears important in the transition from normal melanocyte to melanoma (Gilchrest et al. 1999). To support this, apoptosis resistance is demonstrated in naevus cells compared with normal melanocytes (Alanko et al. 1999) and lower levels of spontaneous apoptosis occur in melanoma cells *in vivo* compared with other tumour cell types (Mooney et al. 1995). In addition, occasional overexpression of the anti-apoptotic protein bcl-2 is observed in melanoma (Cerroni et al. 1995; Collins and White 1995; Selzer et al. 1998; Tang et al. 1998).

Most chemotherapeutic agents function by inducing apoptosis in malignant cells (Fisher 1994) and accumulating evidence suggests that melanoma cells exploit their intrinsic resistance to apoptosis to render them resistant to standard chemotherapies (Grossman and Altieri 2001; Satyamoorthy et al. 2001). The expression and activity of apoptotic regulatory proteins maintains a critical balance in cells, with anti-apoptotic and pro-apoptotic regulators exerting influence at multiple control points in apoptotic pathways. Examination of apoptotic dysregulation in melanomas [Figure 1.7; Table 1.3] has revealed:

a) increased expression of apoptotic inhibitors e.g. Livin (Kasof and Gomes 2001); survivin (Bowen et al. 2003; Grossman et al. 1999)
b) loss of pro-apoptotic factors e.g. downregulation of PUMA (Karst et al. 2005); loss of Apaf-1 (Soengas et al. 2001)
c) hyperactivation of survival signalling pathways e.g. Akt is constitutively activated in 45 to 67% melanomas (Robertson 2005a) [section 1.1.4.2]

None of these documented changes in melanoma have thus far, afforded a robust explanation for its characteristic chemoresistance.

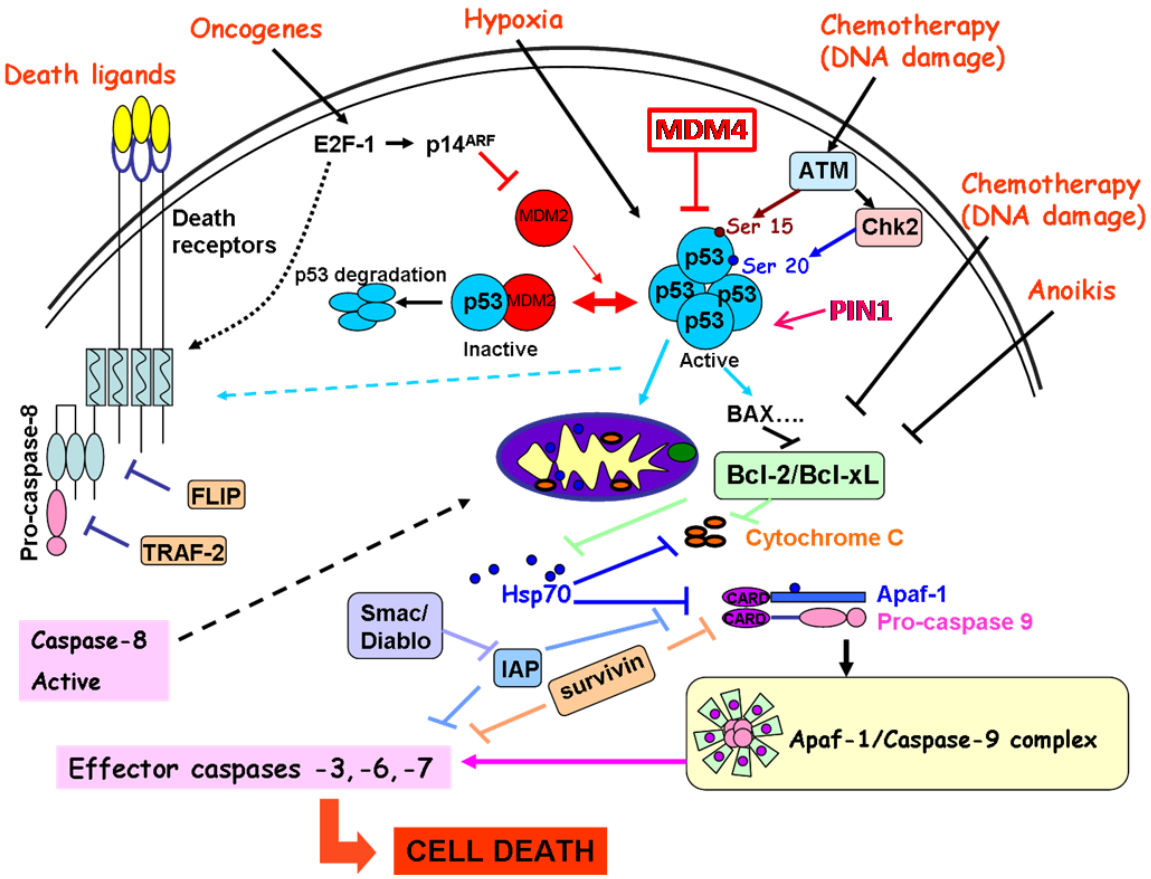


Figure 1.7: Apoptotic dysregulation in melanoma. Simplified schematic of apoptotic pathways in human cells showing multiple levels of regulation, both in extrinsic (death-receptor-mediated) and intrinsic (mitochondrial) apoptosis. A number of these molecules are dysregulated in melanoma [Table 1.3, section 1.2.4]. FLIP – flice inhibitor protein, Hsp70 – heat shock protein 70, IAP – Inhibitor of Apoptosis, TRAIL-R – TNF-related apoptosis inducing ligand, MDM2 – mouse double minute-2, Apaf-1 – Apoptotic protease activating factor-1, ATM – Ataxia telangiectasia mutated, Chk2 – checkpoint kinase-2. Adapted from Soengas and Lowe (2003).

Table 1.3: Dysregulated expression of molecules in melanoma cells [Figure 1.7]

UPREGULATED	DOWNREGULATED
Survivin (Grossman et al. 1999)	Apaf-1 (Soengas et al. 2001)
FLIP (Irmler et al. 1997)	PTEN (Wu et al. 2003b)
MDM2 (Polsky et al. 2002)	$P14^{ARF}$ (Sharpless and Chin 2003)
MDM4 (Ramos et al. 2001)	TRAIL-R (Zhang et al. 1999)
IAPs (Irmler et al. 1997)	PUMA (Karst et al. 2005)
Hsp70 (Dressel et al. 1998; Ricaniadis et al. 2001)	
TRAF2 (Ivanov et al. 2001)	
Bcl-2/Bcl-xL (Tron et al. 1995)	
E2Fs (Halaban et al. 2000)	
ATF-2 (Huang et al. 2008a; Takeda et al. 1991)	

FLIP – flice inhibitor protein, Hsp70 – heat shock protein 70, IAP – Inhibitor of Apoptosis, TRAIL-R – TNF-related apoptosis inducing ligand, MDM2 – mouse double minute-2, MDM4 – mouse double minute-4, Apaf-1 – Apoptotic protease activating factor-1, PUMA – p53-upregulated modulator of apoptosis, PTEN – phosphatase and tensin homologue, ATF-2 – activating transcription factor-2, TRAF-2 – TNF receptor associated factor-2,

1.1.4.2 Survival pathways

There is rigorous evidence for the activation of cell survival pathways in melanoma [Figure 1.8]. Among the main forces limiting cell death are survival signals derived from activation of the Mitogen-Activated Protein Kinase (MAPK), Phosphoinositol-3-kinase (PI3K) / Protein Kinase B (AKT) / Phosphatase and Tensin Homologue (PTEN) and the Nuclear factor-kB (NF-κB) signalling pathways. These signalling cascades are organised in intricate networks and a brief summary of their dysregulation in melanoma is outlined below.

1.1.4.2.1 *RAS/RAF/MAPK Pathway*

The MAPK pathway is primarily involved in regulation of cell growth. Stimulation of the pathway occurs after interaction of growth factors with their respective cell surface receptors e.g. receptor tyrosine kinases, stem cell factor / c-KIT, and the transmission of these signals through the small GTPase, RAS (Robinson and Cobb 1997). Upon activation, RAS in turn, activates the RAF family of serine/threonine kinases which stimulates the MAPK cascade [Figure 1.8]. Hyperactivation of the MAPK pathway is an early event in melanoma progression (Cohen et al. 2002). Mutations in NRAS and BRAF, which occur exclusively of each other, cause constitutive activation of the serine–threonine kinases in the ERK–MAPK pathway (Albino et al. 1989; Davies et al. 2002; Omholt et al. 2003); somatic mutations of N-RAS occur in 15-20% of melanomas (Brose et al. 2002; Davies et al. 2002); more commonly mutations in BRAF (downstream of NRAS) at position V600 are responsible and occur in up to 70% of melanomas (Davies et al. 2002; Forbes et al. 2008; Garnett and Marais 2004; Smalley and Flaherty 2009) [Figure 1.8]. Activated RAS/MAP kinase signalling strongly upregulates expression of the POU domain transcription factor, Brn-2 (Goodall et al. 2004b). High levels of Brn-2 are expressed in melanoma compared with primary melanocytes (Eisen et al. 1995; Sturm et al. 1994; Thomson et al. 1995) and it was demonstrated to be an effector of BRAF downstream of the MAP kinase cascade linking BRAF signalling to proliferation (Goodall et al. 2004a).

Recently an inhibitor of activated BRAF V600E, plexxikon (PLX4032), has shown promising results in phase I/II clinical trials (Flaherty and Smalley 2009) and currently recruitment is taking place for the BRIM3 trial, an international phase III trial for this drug for use in metastatic melanoma (Plexxikon, Inc). Other selective BRAF inhibitors are also being tested in pre-clinical phase trials e.g. PLX4720, 885-A and GDC-0879,

providing new possibilities for therapeutic success in melanoma (Hatzivassilliou et al. 2010, Heidorn et al. 2010).

1.1.4.2.2 *KIT*

Possibly the greatest therapeutic potential in melanoma comes from evidence confirming that specific molecular events are associated with particular clinicopathological subtypes of melanoma. Curtin *et al.* (2006) demonstrated frequently occurring genetic aberrations affecting the *KIT* oncogene in mucosal and acral melanomas and in melanomas from chronically sun-exposed areas. No *KIT* mutations were found in melanomas from intermittently sun-exposed skin. Improved understanding of such genotype-phenotype correlations is impacting on the development of novel therapies as major progress has been achieved in the targeted treatment with tyrosine kinase inhibitors (TKI) of gastrointestinal stromal tumours (GISTs), where *KIT* mutations are present in up to 80% of cases.

To date, most of the *KIT* mutations reported in melanoma are of the type predicted to be sensitive to TKI; major responses to TKI, such as imatinib, have been seen in patients with melanoma harbouring *KIT* mutations (Jiang et al. 2008; Wyman et al. 2006). Furthermore, a phase II clinical trial of imatinib reported clinical response in an acral melanoma which expressed high levels of c-KIT receptor but no c-KIT mutation (Kim et al. 2008). There is also evidence that targeting c-KIT mutated melanomas with imatinib simultaneously inhibits the MAPK and PI3K/AKT pathways, suggesting an interconnected network of these pathways exists to perpetuate melanoma (Jiang et al. 2008).

1.1.4.2.3 *PI3K/AKT/PTEN Pathway*

Activation of the MAPK pathway does not account for all aspects of melanoma progression and there is evidence that the PI3K/AKT/PTEN signalling pathway may be equally important [Figure 1.8]. PTEN expression is reduced in up to 30% of primary melanomas as a result of mutation or transcriptional silencing suggesting inactivation of PTEN is a late but frequent event in melanomagenesis (Birck et al. 2000; Lin et al. 2008; Whiteman et al. 2002; Zhou et al. 2000). PTEN can also function as a haploinsufficient tumour suppressor with allelic loss in up to 50% melanoma metastases (Birck et al. 2000) and reports of PTEN methylation have demonstrated it to be an independent predictor of poor survival in melanoma (Lahtz et al. 2010).

Up to 50% of melanomas have selective constitutive activity of AKT3 (Stahl et al. 2004) and activating mutations, although rare, have also been reported in this gene (Davies et al. 2008) [section 1.2.4.4.2]. This pathway is therefore a suitable candidate for therapeutic intervention and the clinical development of small molecule inhibitors of PI3K and AKT are currently undergoing evaluation.

1.1.4.2.4 *Microphthalmia-associated transcription factor (MITF)*

MITF encodes basic helix-loop-helix-leucine-zipper transcription factors, among which the M-isoform is specifically expressed in melanocyte (Hodgkinson et al. 1993). It is the earliest known marker for the melanocyte lineage and is essential for melanocyte development. It is considered the master gene of melanocyte differentiation and induces the transcription of genes involved in melanin production including TYR and TYRP1 [section 1.1.2.2.2] (Steingrimsson et al. 2004). Although conflicting data exist regarding the role of MITF in melanoma development, evidence supporting a specific tumourigenic role is mounting (Fecher et al. 2007). MITF increases the transcription of bcl-2 (McGill et al. 2002), CDK2 (Du et al. 2004), INK4A (Loercher et al. 2005), and p21 (Carreira et al. 2005). Further, MITF resides downstream of the ERK and the PI3-kinase survival pathways, suggesting that MITF integrates extracellular pro-survival signals (Goding 2000). These observations directly implicate MITF in melanoma survival (Larribere et al. 2005). The role of MITF is still under intense investigation and clinical agents are not yet available to target this gene.

1.1.4.2.5 *NF-κB Pathway*

In melanoma cells, the NF-κB pathway is altered by upregulation of the NF-κB subunits, p50 and Rel-A (McNulty et al. 2001; Meyskens et al. 1999) and by downregulation of the NF-κB inhibitor, IκB (Dhawan and Richmond 2002; Yang and Richmond 2001). Gene transfer approaches have been used in melanoma to therapeutically inactivate Rel-A and overexpress IκB with pre-clinical success (Bakker et al. 1999; Huang et al. 2000; McNulty et al. 2001).

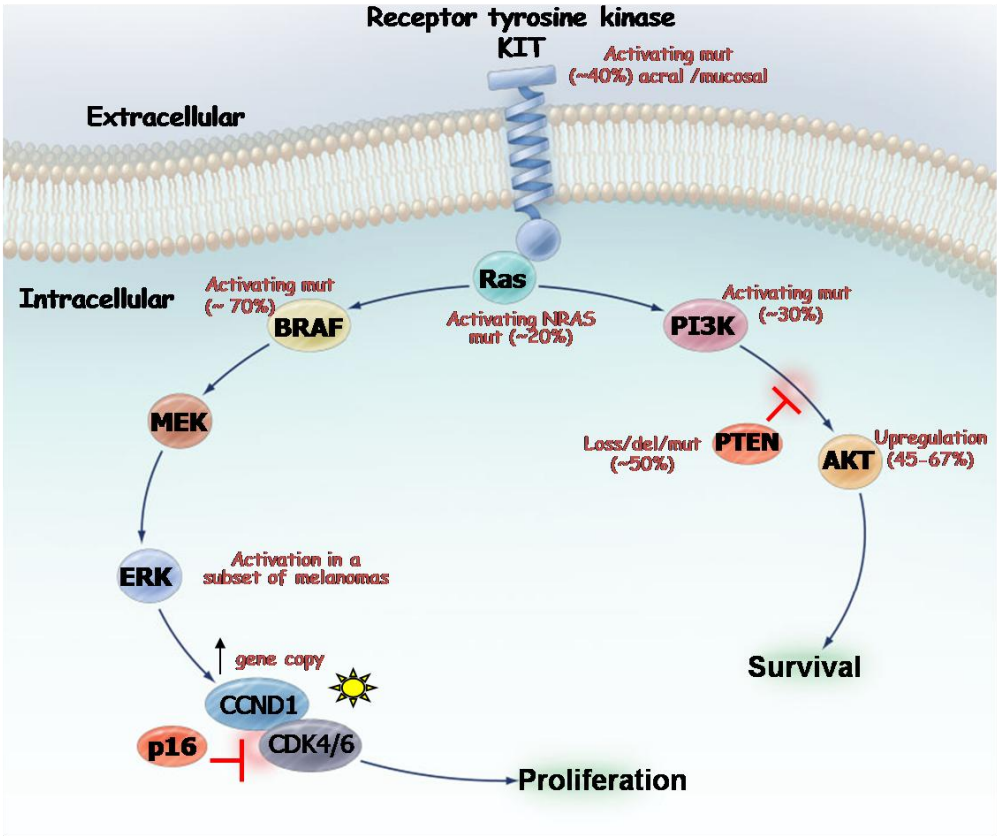


Figure 1.8: Dysregulation of survival pathways in melanoma. Signals from receptor tyrosine kinases can promote proliferation through the mitogen-activated protein (MAP) kinase (left branch) and survival through the phosphatidylinositol 3' kinase (PI3K) pathway (right branch). Frequency of mutations is shown in brackets. mut – mutation, del – deletion. Figure adapted from Curtin et al. (2005).

1.1.5 Role of microRNAs in melanoma progression

MicroRNAs (miRNA) are a class of small non-coding RNAs, first described in *Caenorhabditis elegans* in 1993 (Lee et al 1993, Wightman et al 1993). They are ~22-nucleotide long, double-stranded, non-coding RNAs, excised from longer (60-110 nucleotides) stem-loop containing RNA precursor structures. They constitute an important layer of post-transcriptional regulation of gene expression via degradation of target mRNAs and/or impairment of mRNA translation, mediated by the RNA-induced silencing complex (RISC) which is a cytosolic ribonucleoprotein complex [Figure 1.9]. Many miRNAs are well conserved, especially in vertebrates, and display distinct temporal and spatial expression patterns (Farh et al. 2005; Landgraf et al. 2007). The roughly 500 mammalian miRNAs regulate up to 30% of protein-coding genes and are involved in both physiological and pathological processes including development and cancer. As part of known signalling pathways and regulatory circuits, miRNAs can exert oncogenic or tumour suppressor functions and miRNA expression profiles are emerging as viable markers for diagnosis and treatment of cancer.

Initially, miRNA expression profiling was performed in melanoma tissue samples (Lu et al. 2005) but more recently was extended to include melanoma cell lines (Gaur et al. 2007; Zhang et al. 2006); Lu *et al.* demonstrated that miRNA expression profiles reflect the developmental lineage and differentiation state of the melanoma; Zhang *et al*. demonstrated that 85.9% of genomic loci harbouring one or more of the 283 examined miRNA genes exhibited DNA copy number alterations in melanoma and Gaur *et al*. identified 15 miRNAs which were significantly dysregulated in melanoma cell lines. However, none of these studies compared the differential expression of miRNAs in melanoma to the normal biological correlate, normal human melanocytes. The first study to address this issue demonstrated a number of novel miRNAs dysregulated in melanoma when compared with melanocytes (Mueller et al. 2009). Their relevance was confirmed *in vivo* and the functional characterisation of the effects of each miRNA is detailed in table 1.4.

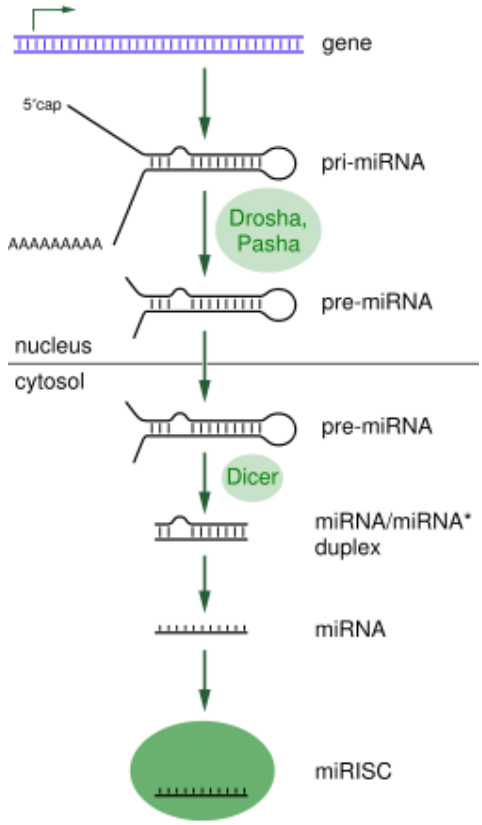


Figure 1.9: Biogenesis of microRNAs. MicroRNAs are ~22-nucleotide double stranded non-coding RNAs endogenously encoded by the genome. They are transcribed by RNA polymerase II into the primary miRNAs (pri-mRNA) which have 5' caps and poly-A tails. The pri-miRNAs are processed by RNAse II, Drosha with its partner Pasha, into the precursor miRNAs (pre-miRNAs). These are exported into the cytoplasm and further processed by another RNAse II, Dicer, into mature miRNAs. The mature miRNA is incorporated into the RISC complex which negatively regulates target mRNA via degradation of target mRNAs and/or impairment of translation. RISC - RNA-induced silencing complex miRNA – microRNA.

Table 1.4: Functional effects of single miRNAs dysregulated in melanoma

Effect of melanoma progression on miRNA	miRNA	Functional effect in melanoma	Reference
Upregulated	miR137	Negative regulator of MITF*	(Bemis et al. 2008)
	miR182	Overexpression results in increased survival and invasive potential by repression of MITF & FOXO3	(Segura et al. 2009)
	miR 221/222	Reduced expression of c-KIT receptor during melanoma progression with increased invasive chemotactic capabilities in mouse model	(Felicetti et al. 2008a; Felicetti et al. 2008b)
Downregulated	Let-7a	Mechanism for regulation of integrin-β3. Depletion results in increased migration and invasion	(Muller and Bosserhoff 2008)
	Let 7b	Directly & indirectly targets cell cycle regulators (e.g. cyclin D1) and interferes with anchorage-independent growth	(Schultz et al. 2008)

*MITF – microphthalmia-associated transcription factor - master regulator of melanocyte growth, maturation, apoptosis and pigmentation,

FOXO3 – forkhead box 03

1.1.6 Prognostic factors

Overall, males diagnosed with melanoma have a 5-year survival of 78%, for females this is 91% (Cancer Research UK) but advanced disease has a dismal prognosis with a 5-year survival reaching only 11% (Barth et al. 1995). The AJCC predicts 5-year survival for individuals with early (stage I/II) melanoma to be 85% (Balch et al. 2001a; Balch et al. 2009) which falls to approximately 6% in cases with visceral metastases where the median survival is 7.5 mths (Barth et al. 1995) [Figure 1.10]. Increases in Breslow thickness (the vertical distance measured in millimetres from the granular cell layer to the deepest part of the tumour), dermal mitotic rate and microscopic ulceration are the most important histological determinants of prognosis, all inversely correlated with survival [Table 1.5]. However, nodal status as determined by sentinel lymph node biopsy has emerged as the most powerful predictor of recurrence and survival and in patients with stage III regional nodal disease the 5-year survival ranges from 24% to 69.5% depending on the number of lymph nodes affected and the tumour burden (Balch et al. 2001a; Balch et al. 2009).

1.1.7 Management of melanoma

Early detection of melanoma renders the disease curable in patients undergoing surgical excision. For a vast majority of patients with metastatic disease, there are no effective treatments and despite testing of numerous novel therapeutic strategies over the past three decades, there has been little improvement in survival rates from advanced disease (Atkins et al. 2002; Chapman et al. 1999; Gause et al. 1998; Gogas et al. 2004a; Grossman and Altieri 2001; Helmbach et al. 2001; Hochster et al. 1999; Hwu et al. 2003; Marsoni et al. 1987; Samuel et al. 1994). Advanced melanoma is notoriously chemo- and radioresistant. Most available therapies provide a short-lived palliative benefit (Middleton et al. 2000). These studies have demonstrated that very few of the drugs in our chemotherapeutic armamentarium have been successful in treating melanoma thus the development of effective therapies for this cancer remains a priority [Table 1.6]. Melanoma cells have acquired the ability to evade the diverse range of actions employed by cytotoxic agents e.g. DNA damage (e.g. by alkylation, methylation or crosslinking), microtubule destabilisation or topoisomerase inhibition. Complete responses to chemotherapeutic regimens rarely benefit more than 20% of patients with remission in melanoma rarely reported. It is therefore unlikely that drug resistance is due primarily to acquired genetic alterations selected during or after therapy, but rather inherent to the malignant behaviour of cells at diagnosis.

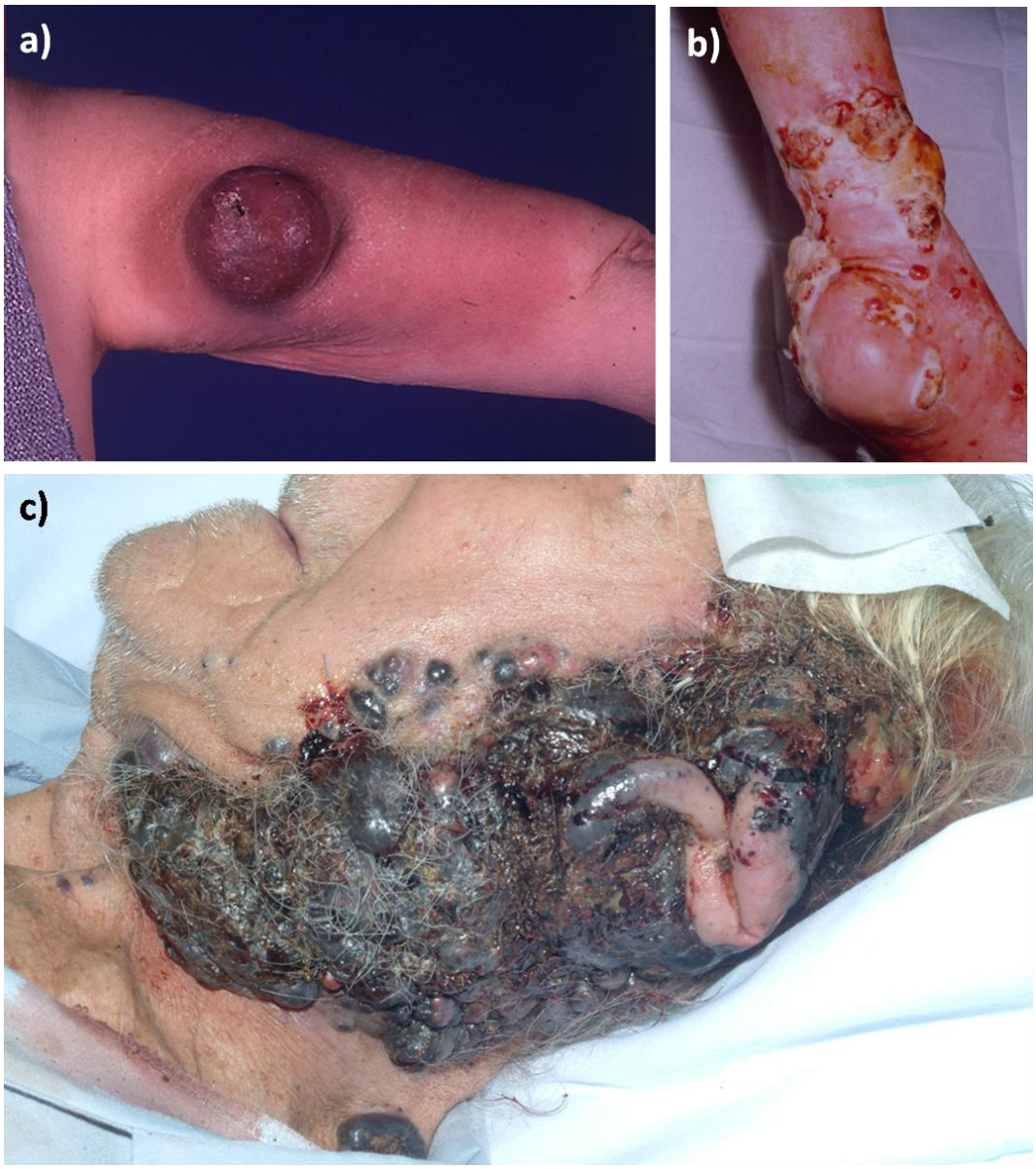


Figure 1.10: Clinical photographs of metastatic melanoma. a) Solitary large cutaneous nodule of metastatic melanoma on right upper arm. (b) Multiple haemorrhagic, macerated locoregional skin metastases affecting the left lower limb. (c) Multiple haemorrhagic and necrotic nodules of cutaneous metastatic melanoma with cervical, parotid and widespread lymphatic involvement (courtesy of A. Sahota, Whipps Cross Hospital). Patient consent obtained for images.

Table 1.5: AJCC classification for staging of cutaneous melanoma (Balch et al. 2001a; Balch et al. 2009)

Stage	Description	5-year survival (%)
0	Melanoma *in situ*	100
IA	Tumour thickness ≤1.0mm (T1)	95.3
IB	Tumour thickness ≤1.0mm with ulceration* OR tumour thickness 1.01 – 2.0mm (T2) without ulceration	89 – 90.9
IIA	Tumour thickness 1.01 – 2.0mm with ulceration OR tumour thickness 2.01 – 4.0mm (T3) without ulceration	77.4 – 78.7
IIB	Tumour thickness 2.01 – 4.0mm with ulceration OR tumour thickness >4.0mm (T4) without ulceration	63 – 67.4
IIC	Tumour thickness >4.0mm with ulceration	45.1
IIIA	Any tumour thickness, no ulceration, 1-3 microscopic nodes involved	63.3 – 69.5
IIIB	Any tumour thickness with ulceration and 1-3 microscopic nodes OR any tumour thickness without ulceration and 1-3 macroscopic nodes OR any tumour with satellite(s) / in-transit metastases	46.3 – 52.8
IIIC	Any tumour thickness with ulceration and 1-3 macroscopic nodes / satellite(s) / in-transit metastases OR any tumour with >4 metastatic nodes	26.7 – 29
IV	Any tumour thickness, any number of nodes, any distant skin, subcutaneous, nodal or visceral metastases	6.7 – 18.8

*Melanoma ulceration is the absence of an intact epidermis overlying a major portion of the primary melanoma based on microscopic examination of histological sections (Balch et al. 1978; Balch et al. 1979; Balch et al. 1980; McGovern et al. 1982).

Table 1.6: Selection of chemotherapeutic drugs used in the treatment of melanoma

Chemotherapy	Mode of action	Use in melanoma
Platinum Cisplatin Carboplatin	DNA and protein crosslinking Induces single (ss) & double-stranded (ds) DNA breaks activating signal-transduction pathways e.g. DNA-damage repair, cell-cycle arrest, apoptosis (Siddik 2003) Induces changes in DNA structure and inhibits DNA and RNA synthesis	Definite (though minor) degree of activity in melanoma (Casper and Bajorin 1990; Chang et al. 1993; Evans et al. 1987; Karakousis et al. 1979)
Taxanes Taxol Paclitaxel Docetaxel	Microtubule disruption (prevent depolymerisation altering cell division and motility)	Minor clinical activity in clinical trials (Aamdal et al. 1994; Bedikian et al. 1995; Einzig et al. 1991; Legha et al. 1990)
Epipodophyllotoxin Etoposide	Inhibits topoisomerase II Induces dsDNA breaks (Hande 1998)	Chemoresistant (Atkins 2002)
Anthracyclines Doxorubicin	DNA intercalating agents Induces ssDNA breaks, DNA crosslinking, inhibits DNA and RNA replication	Chemoresistant (Atkins 2002)
Alkylating agents Dacarbazine (DTIC) Temozolomide	DNA alkylation & methylation Inhibits nucleic acid and protein synthesis DTIC derivative	Only FDA approved drug used in melanoma (Lee et al. 1995; Serrone et al. 2000) Used in patients with brain metastasis (Quirbt et al. 2007)

1.2 TP53

1.2.1 Background

TP53 was first discovered 30 years ago as a cellular partner of simian virus 40 large T-antigen, the oncoprotein of this tumour virus (Lane and Crawford 1979). It is among the most extensively investigated of all genes and proteins. The first decade of research confirmed p53 to be a tumour suppressor with a major role in the cellular response to a diverse range of stress signals including DNA damage, genomic instability, hypoxia or oncogene activation (Vogelstein et al. 2000) [Figure 1.11]. TP53 is described as the 'guardian of the genome' because its activation induces a range of responses from cell cycle arrest or DNA repair to apoptosis or senescence. These responses contribute to tumour suppression either by preventing or repairing genomic damage or through elimination of potentially oncogenic cells from the proliferating population (Vousden 2006). Although the best studied functions of p53 are the control of cell cycle arrest and apoptosis, more recently it has been implicated in other cellular processes, including regulation of glucose metabolism and oxidative stress (Bensaad and Vousden 2007).

The p53 protein is a transcription factor and cellular response to p53 activation is determined by the differential activation of p53 target genes (Liu and Chen 2006). Based on several studies, a model has begun to emerge in which p53 drives two broad categories of response (Bensaad and Vousden 2005; Sablina et al. 2005) [Figure 1.11]. The first of these, elicited under mild or transient stress, induces p53-dependent cell cycle arrest and DNA repair before the cell re-enters a normal proliferative state upon resolution of the stress. The second induces permanent inhibition of cell proliferation, through apoptosis or senescence, in response to severe, irreparable stress.

p53 activity depends on its quantity within a cell and on qualitative changes in its structure, intracellular localisation, DNA binding activity and interaction with other proteins. A multitude of parameters govern expression, modification, accumulation and localisation of p53 proteins, which may explain how a single gene can display such an extensive repertoire of activities.

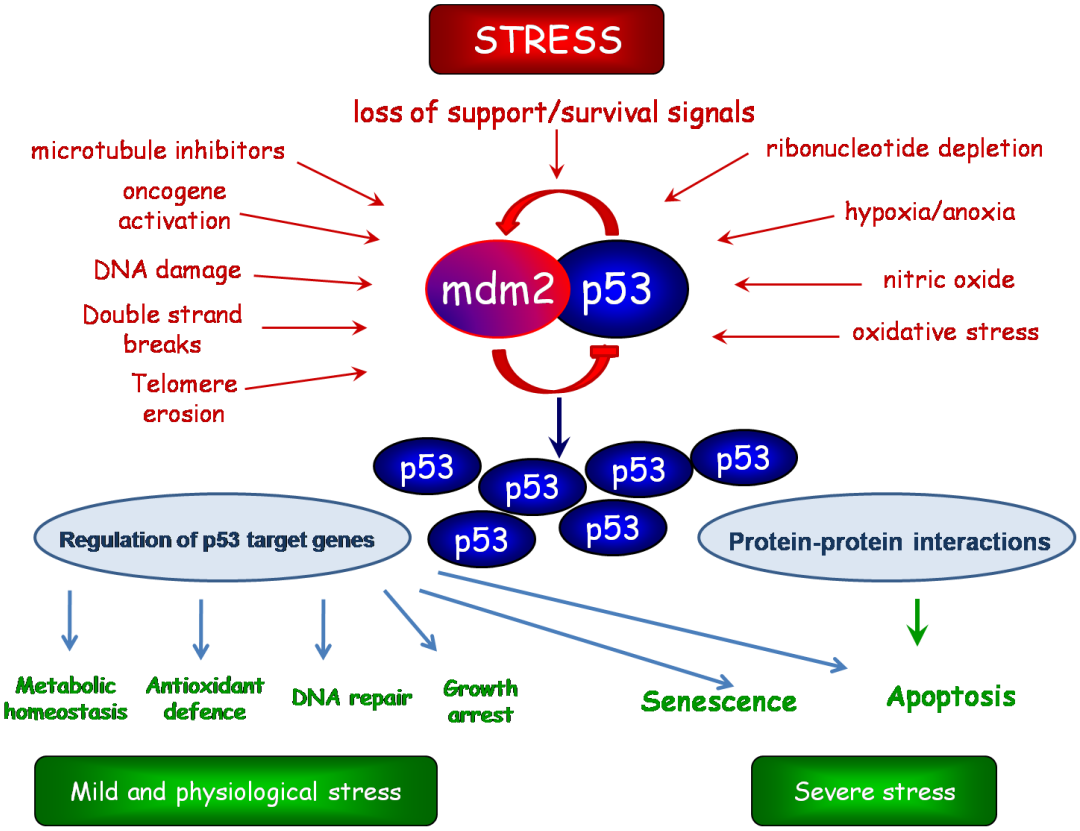


Figure 1.11: Simplified scheme showing activation of the p53 pathway. The p53–MDM2 feedback loop is central to the p53 pathway. Under normal conditions, it maintains low steady-state levels of p53. Various stress signals activating p53, act on this central loop to release p53 from MDM2-mediated inhibition. This increases p53 protein levels and activity, inducing various phenotypic changes. The downstream effects of p53 are a result of its ability to transactivate and repress various subsets of target genes. The phenotypic response to p53 activation is, at least in part, proportionate to the amplitude, duration and nature of the activating signal. Severe stress induces apoptosis and senescence, whereas milder stress leads to a transient growth arrest and repair of stress-induced damage. Recent evidence indicates that p53 has an additional role to adjust cell metabolism in response to mild physiological fluctuations. Adapted from Levine and Oren (2009).

1.2.2 Structure of TP53

The recent discovery that all p53 family members possess a dual gene structure, has added complexity to our understanding of the role of p53 pathway abnormalities in cancer. Bourdon et al. (2005) showed that the p53 gene encodes at least nine protein isoforms as a result of alternative splicing, alternative promoter usage and alternative translation initiation. Most of these isoforms retain their DNA binding domains (DBD) but differ in their N' and C' terminal regions (Bourdon et al. 2005; Courtois et al. 2004). p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ isoforms result from alternative splicing of intron 9 and usage of an alternative promoter in intron 4. Isoforms Δ40p53, Δ40p53β and Δ40p53γ are due to alternative splicing of intron 9 and alternative initiation of translation or alternative splicing of intron 2 (Ghosh et al. 2004) [Figure 1.12]. Furthermore p53 variant mRNA is expressed in several normal human tissues in a tissue-dependent manner, indicating that the internal promoter and the alternative splicing of p53 can be regulated. Tissue-specific expression of the p53 isoforms may explain the tissue-specific regulation of p53 transcriptional activity in response to stressors such as ionising radiation, UVR, pH and hypoxia (Bourdon et al. 2005).

1.2.3 Regulation of p53

As p53 is such a potent inhibitor of cell growth, its function must be tightly controlled to allow normal growth and development. This is achieved through several mechanisms including regulation of protein stability, subcellular localisation and activity.

1.2.3.1 Post-translational modifications of TP53

Active p53 is subject to a complex and diverse array of post-translational modifications, which markedly influence the expression of p53 target genes. Post-translational modification of p53 involves the covalent addition of a functional group to the p53 protein after its translation. The most commonly reported post-translational modifications of p53 include phosphorylation of serines and/or threonines and acetylation, ubiquitylation and sumoylation of lysine residues [Figure 1.13]. Phosphorylation and acetylation have been associated with stabilisation and transcriptional activation of p53 (Meek 1998). Neddylation of p53 has also been recently reported involving covalent addition of the C-terminal glycine residue of the ubiquitin-like protein NEDD8 to Lys370, Lys372 and/or Lys373 of p53 (Xirodimas et al. 2004). Sumoylation is catalysed by small ubiquitin modifier–1 (SUMO-1) which targets a C-terminal lysine in human p53 (K386), blocking acetylation by p300, and

impairing DNA binding capabilities (Stehmeier and Muller 2009a; Wu and Chiang 2009). Other reported modifications of p53 include glycosylation (Shaw et al. 1996) and ribosylation (Wesierska-Gadek et al. 1996), but the significance of these modifications in carcinogenesis is unclear.

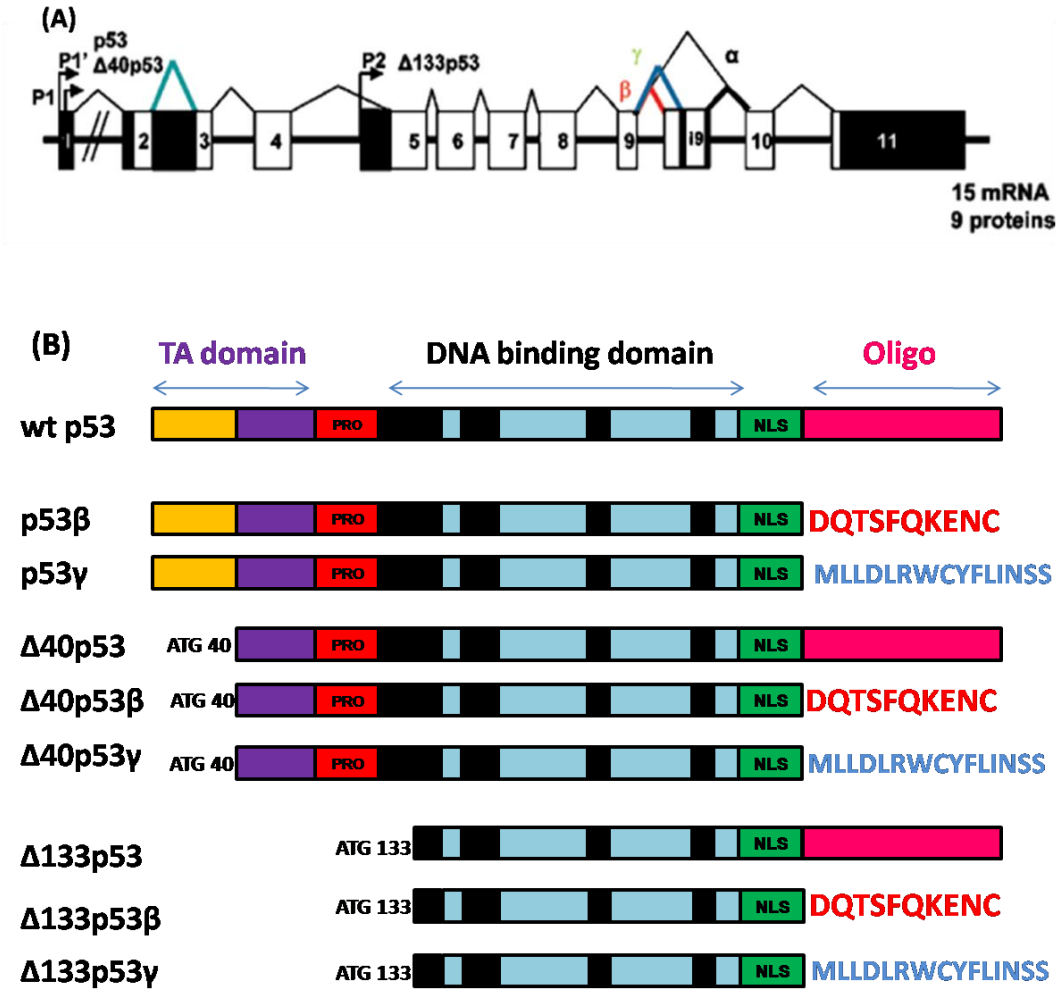


Figure 1.12: Schematics for human p53 gene structure and p53 protein isoforms. (A) Schematic of the human p53 gene structure. (B) The p53 gene encodes at least nine protein isoforms. p53, p53β, p53γ, Δ133p53, Δ133p53β and Δ133p53γ isoforms result from alternative splicing of intron 9 and usage of an alternative promoter in intron 4. Isoforms Δ40p53, Δ40p53β and Δ40p53γ are due to alternative splicing of intron 9 and alternative initiation of translation or alternative splicing of intron 2. TA – transactivation, Oligo – oligomerisation domain. Adapted from Bourdon et al. (2005).

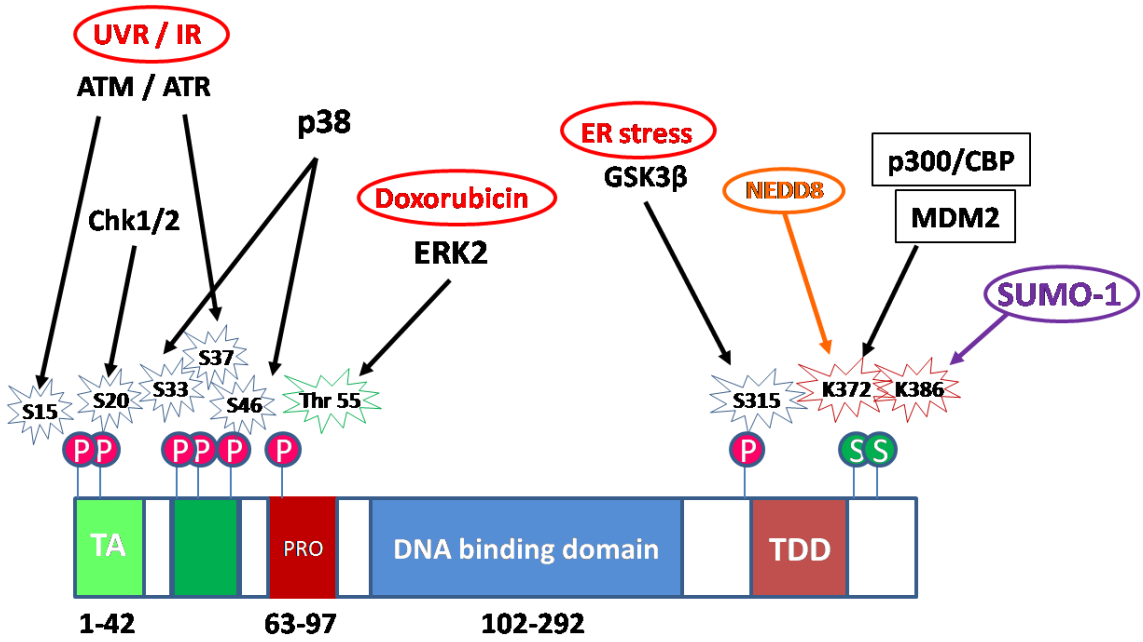


Figure 1.13: Post-translational modification sites on p53. The p53 protein consists of 393 amino acids. The TA domain is required for transactivation activity and interacts with various transcription factors, acetyltransferases and MDM2. Examples of stressors (denoted by red panels) which are reported to activate upstream pathways in cancers to result in phosphorylation (pink P circles) of specific serine (S) and threonine (Thr) residues are shown. Neddylation involving the covalent addition of NEDD8 to Lysine 372 (K372) is shown (Xirodimas et al. 2004). Sumoylation catalysed by small ubiquitin modifier–1 (SUMO-1) targets a C-terminal Lysine 386 (K386), blocking acetylation (lysine residues green S circles) by p300, and impairing DNA binding capabilities (Stehmeier and Muller 2009a; Wu and Chiang 2009). UVR – ultraviolet radiation, IR – ionising radiation, Chk1/2 – checkpoint kinase 1/2, ATM – ataxia telangiectasia mutated, ATR – ataxia telangiectasia and Rad3 related, ERK2 – extracellular signal-related kinase 2, GSK3β – glycogen synthase kinase 3β, CBP – CREB-binding protein, MDM2 – mouse-double-minute 2.

1.2.3.2 Cellular localisation of p53

p53 is best characterised as a nuclear transcription factor that binds to specific DNA sequences and transactivates a number of genes with a variety of functions (Riley et al. 2008). In addition, p53 possesses biological activities that are cytosolic and transcription-independent. p53 mutants lacking a transactivation domain can induce apoptosis (Kakudo et al. 2005) and activation of p53 in the absence of a nucleus also triggers apoptosis (Chipuk et al. 2003), supporting the notion that cytoplasmic p53 can induce apoptosis through a transactivation-independent mechanism.

The transcription-independent p53-death pathway couples the nuclear and extra-nuclear actions of p53. In unstressed cells, cytosolic p53 is sequestered into an inactive complex by soluble cytosolic bcl-XL. In response to stress, nuclear p53 first transactivates its target gene PUMA, which liberates p53 from its cytosolic bcl-XL inhibition by forming a PUMA/bcl-XL complex instead. p53 is then free to activate monomeric Bax in the cytosol (Chipuk et al. 2005).

Upon exposure to apoptotic stimuli, total cellular levels of p53 quickly stabilise and a fraction accumulates at the mitochondria, where it induces an apoptotic programme. Activated p53 rapidly translocates to the outer membrane of mitochondria, where it engages in inhibitory and activating complexes with the anti- and pro-apoptotic members of the Bcl-2 family of mitochondrial permeability regulators (bcl-XL/bcl-2 and BAK respectively). This translocation precedes changes of mitochondrial membrane potential, cytochrome c release and caspase activation. This induces outer membrane permeabilisation and the release of apoptotic activators (Arima et al. 2005; Mihara and Moll 2003; Moll et al. 2006; Nemajerova et al. 2005; Sansome et al. 2001). Mitochondrial translocation of endogenous wt-p53 occurs both *in vitro* and *in vivo,* in response to a wide spectrum of p53-activating cellular stresses in different cell types (Arima et al. 2005; Mihara and Moll 2003; Moll et al. 2006; Sansome et al. 2001).

The targeting of p53 to the mitochondria has received considerable interest. There is no reported mitochondrial translocation motif within the p53 polypeptide sequence and N- and C-terminal phosphorylation / acetylation modifications play no major role in mitochondrial targeting of p53 (Nemajerova et al. 2005). Recent evidence supports a process whereby monoubiquitylation of p53 provides a trafficking signal that redirects it from MDM2-mediated degradation in unstressed cells, to mitochondrial translocation and activation, early during the stress response (Marchenko et al.

2007). Nuclear export of p53 is not necessary for mitochondrial translocation upon DNA damage and instead distinct nuclear and cytoplasmic p53 pools become simultaneously and rapidly stabilised after genotoxic stress.

1.2.3.3 p53-Binding Proteins

There are at least 80 p53-binding proteins, of which two or more independent *in vivo* observations of complex formation have been reported (Braithwaite et al. 2006). These interactions frequently dictate how p53 behaves and a few key proteins linking p53 to its apoptotic function have been selected for review.

1.2.3.3.1 *p53 family members*

Identification of p53 homologues, p63 and p73, both of which exist in multiply spliced isoforms, has added considerable complexity to delineating the role of individual p53 family members in cell death (Kaghad et al. 1997; Yang et al. 1998). Proteins encoded by these two genes are more structurally similar to each other than to p53; however all three family members possess several conserved protein domains [Figures 1.12, 1.14, 1.15]. These include an N-terminal transactivation (TA) domain, a central DNA binding domain (DBD) and an oligomerisation domain (OD) (Yang and McKeon 2000). Both homologues share approximately 80% amino acid identity within the DBD with each other and >60% amino acid identity with p53, thus regulating an overlapping set of target genes (Kaghad et al. 1997; Yang et al. 1998). Conservation of all essential DNA contact residues (De Laurenzi et al. 2000) allows p63 and p73 to share functions of p53 such as induction of cell cycle arrest and apoptosis (Melino et al. 2003; Moll and Slade 2004; Osada et al. 1998; Vousden and Woude 2000). Nevertheless, a number of functional differences exist between the three family members making them distinct proteins.

p63 and p73 each have two promoters, resulting in two proteins with opposing functions: p53-like proteins containing the TA domain, and inhibitory proteins (called ΔN) lacking the TA domain. The full-length TA isoforms of these proteins are pro-apoptotic, whereas naturally occurring N-terminal truncated ΔN variants act as pro-survival factors, at least in part by antagonising activities of the full-length family members (Melino et al. 2002; Melino et al. 2003; Moll and Slade 2004; Yang et al. 1999a; Zaika et al. 2002). In addition both of these genes undergo alternative splicing at the COOH- terminus [Figures 1.14, 1.15].

1.2.3.3.2 *p53 family co-operation*

Interactions among p53 family members appear to be supported by evolutionary, genetic and biochemical evidence (Yang et al. 2002) and there is intense debate as to whether, and how, p63 and p73 interact with p53 in apoptosis and tumour suppression (Benchimol 2004). Although initial evidence suggests that induction of death by p53 requires the partnership of p63 and p73 in E1a-expressing mouse embryo fibroblasts and in primary neuronal cultures (Flores et al. 2002), this has been challenged in thymocytes where p53-dependent apoptosis occurred independently of p63 and p73 (Senoo et al. 2004). In neuronal cells, whilst p63 alone is sufficient to promote neuronal apoptosis, it is also an obligate pro-apoptotic partner for p53 and essential for p53-induced apoptotic cell death (Jacobs et al. 2005). In hepatocellular carcinoma all three p53 family members were involved in the DNA damage response to genotoxic agents and revealed a central role for the p53/p63/p73 network in treatment response and prognosis of this cancer (Gressner et al. 2005; Muller et al. 2006; Seitz et al. 2009).

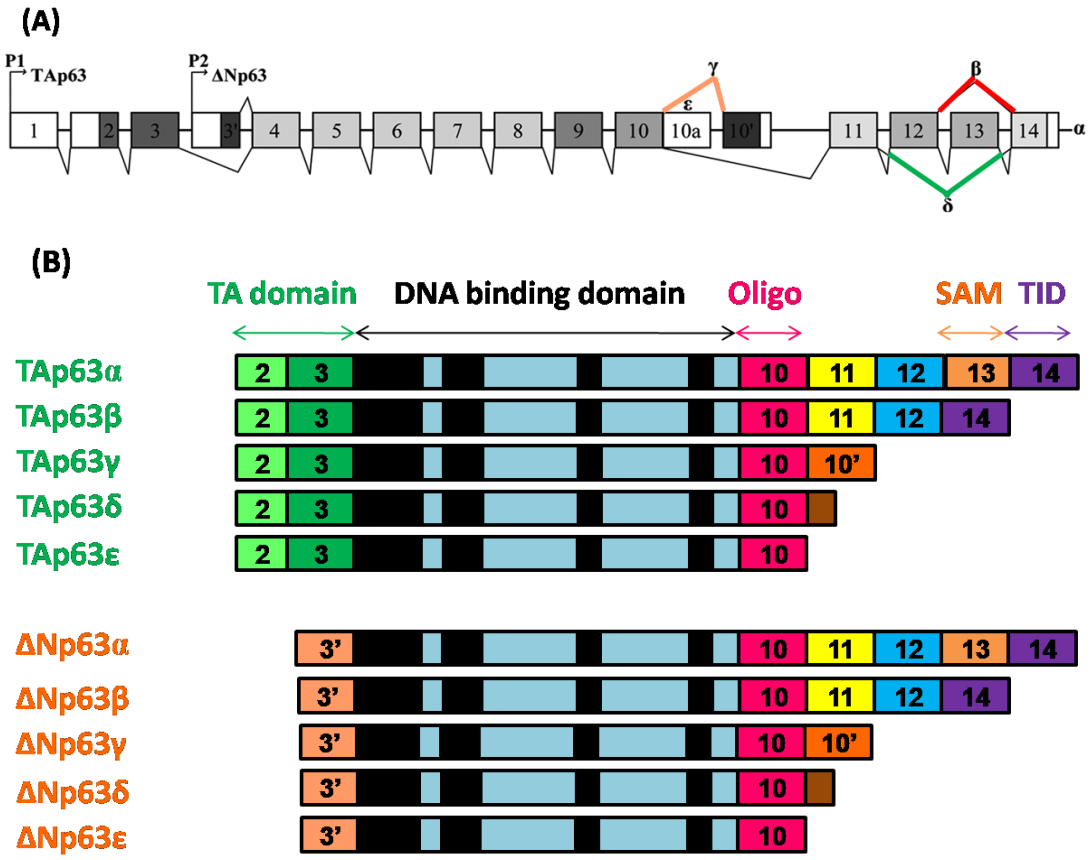


Figure 1.14: Schematic of human p63 gene and protein isoforms. (A) Schematic of human p63 gene structure demonstrating alternative promoters indicated (P1 and P2) giving rise to TA and ΔN isoforms and alternative splicing sites giving rise to α, β and γ, C-terminal splice variants. (B) p63 protein isoforms: TAp63 proteins encoded from promoter P1 contain the transactivation (TA) domains. ΔNp63 proteins encoded from promoter P2 are amino-truncated variants containing a different N-terminal domain to the TAp63 proteins. The numbers indicate the exons encoding p63 protein isoforms. Recent discovery of two new C-terminal splice variants are also shown – δ variant derived from exclusion of exon 12 and 13 and ε variant generated by a premature transcriptional termination in intron 10, retaining the 5' portion of intron 10 which immediately presents a stop codon. Oligo – oligomerisation domain, SAM – sterile alpha motif, TID – transactivation inhibitory domain. Adapted from Murray-Zmijewski et al. (2006) and Mangiulli et al. (2009).

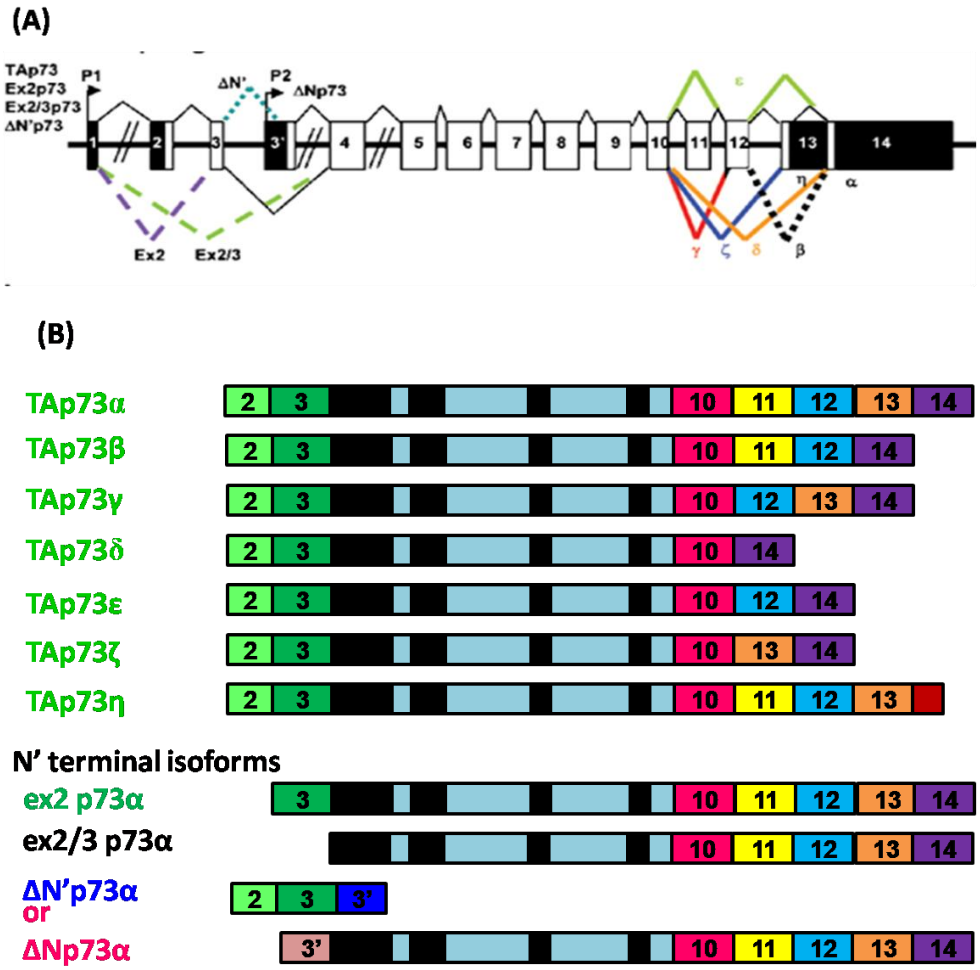


Figure 1.15: Schematic showing human p73 gene and protein structure. (A) Schematic of the human p73 gene structure. Alternative promoters (P1 and P2) are indicated and alternative splicing results in seven C-terminal splice variants. (B) p73 protein isoforms: TAp73 proteins are encoded from promoter P1 and contain a conserved N-terminal TA domain. Ex2p73 proteins are due to alternative splicing of exon 2. They have lost the conventional TA domain but retain part of it (exon 3). Ex2/3p73 proteins arise due to alternative splicing of exons 2 and 3. They have entirely lost the TA domain and are initiated from exon 4. The protein encoded by ΔN'p73 mRNA has not been described yet. ΔN'p73 variant is often overexpressed at the mRNA level in tumours. It is due to alternative splicing of exon 30 contained in intron 3. Theoretically ΔN'p73 mRNA would encode for either a short p73 protein or p73 protein isoforms identical to ΔNp73. ΔN'p73 mRNA contains the normal initiation site of translation in exon 2 (ATG) and a stop codon in exon 30 and could therefore encode for a short p73 protein composed only of the TA domain. It is possible that translation of ΔN'p73 mRNA is initiated from the third ATG present in exon 30, leading to a p73 protein identical to ΔNp73 isoforms. ΔNp73 proteins encoded from promoter P2 are amino-truncated proteins containing a different N-terminal domain to that of TAp73 proteins. Numbers indicate the exons encoding p73 protein isoforms. For brevity, all C-terminal splice variants are only shown for the TA isoforms. Adapted from Murray-Zmijewski et al. (2006).

1.2.3.3.3 *Mouse double minute 2 (MDM2)*

The main regulator of p53 levels is the human homologue of MDM2. MDM2 is a RING-finger E3 ubiquitin ligase which is a nuclear protein which binds to, promotes the nuclear export of, and subsequently degrades p53 through its intrinsic E3 ubiquitin ligase activity (Honda et al. 1997). In addition to its role in proteosomal degradation, the mdm2 binding site domain of p53 overlaps with its transactivation domain enabling mdm2 to inhibit the transcriptional activity of p53 through specific binding (Momand et al. 1992). In unstressed cells, mdm2 and p53 are kept at low levels and mutually regulate their levels through a negative feedback loop [Figures 1.7, 1.11].

1.2.3.3.4 *MDM4*

MDM4 (formerly named MDMX) is historically considered to be a negative regulator of p53 function under basal or sub-lethal conditions. It binds to and inhibits transactivation of p53. In contrast to MDM2, it does not possess intrinsic ubiquitin ligase function but does co-operate with MDM2 to potentiate its activity towards p53 ubiquitination and degradation (Kawai et al. 2007; Linares et al. 2003; Linke et al. 2008; Okamoto et al. 2009) [Figure 1.7]. More recently, under severe genotoxic stress, MDM4 has been shown to anchor at the mitochondria, facilitate localisation of phosphorylated p53 (at Serine 46) and promote its binding to bcl-2 resulting in release of cytochrome c and mitochondrial apoptosis (Mancini et al. 2009). This highlights a novel role for MDM4 as a positive regulator of p53-intrinsic apoptosis and suggests that p53 regulators have an important function in the p53-mediated decision between cell life and death.

1.2.3.3.5 *ASPP Family of Proteins*

The ASPP (Apoptotic-Stimulating Proteins of p53 or ankyrin-repeat-, SH3-domain- and proline-rich-region-containing protein) family are transcriptional co-activators which enhance the DNA binding and transcriptional activity of p53 (Samuels-Lev et al. 2001). Members of the ASPP family have been identified as specific regulators of the p53 family, selectively regulating the apoptotic function of p53. The family comprises three members of which two, ASPP 1 and 2, are pro-apoptotic and the third, inhibitory ASPP (iASPP), is anti-apoptotic [Figure 1.16].

1.2.3.3.6 *ASPP and p53 family members*

All members of the ASPP family interact with all three p53 family members (Bergamaschi et al. 2004; Robinson et al. 2008; Samuels-Lev et al. 2001). ASPP1

and 2 enhance the apoptotic function of p53 by selectively stimulating binding and transactivation of p53, p63 and p73 on specific downstream apoptotic gene promoters (e.g. BAX and PIG3) without acting on cell cycle arrest promoters (e.g. CDKN1A, MDM2 and cyclin G1) (Samuels-Lev et al. 2001) [Figure 1.17].

Unlike ASPP1/2, evidence indicates that iASPP is predominantly an anti-apoptotic gene and this function is evolutionarily conserved (Bergamaschi et al. 2003b). The importance of the ASPP family in regulating p53 function is supported by genetic evidence in *C.elegans* (in which ASPP1/2 do not exist), *C.elegans* iASPP can inhibit the function of human p53 in human cells as effectively as human iASPP (Bergamaschi et al. 2003b). iASPP selectively inhibits p53 apoptotic function by inhibiting transcriptional activity of p53 bound to BAX and PIG3 promoters, but not p53 that is bound to the MDM2 and p21 promoters (Bergamaschi et al. 2003b).

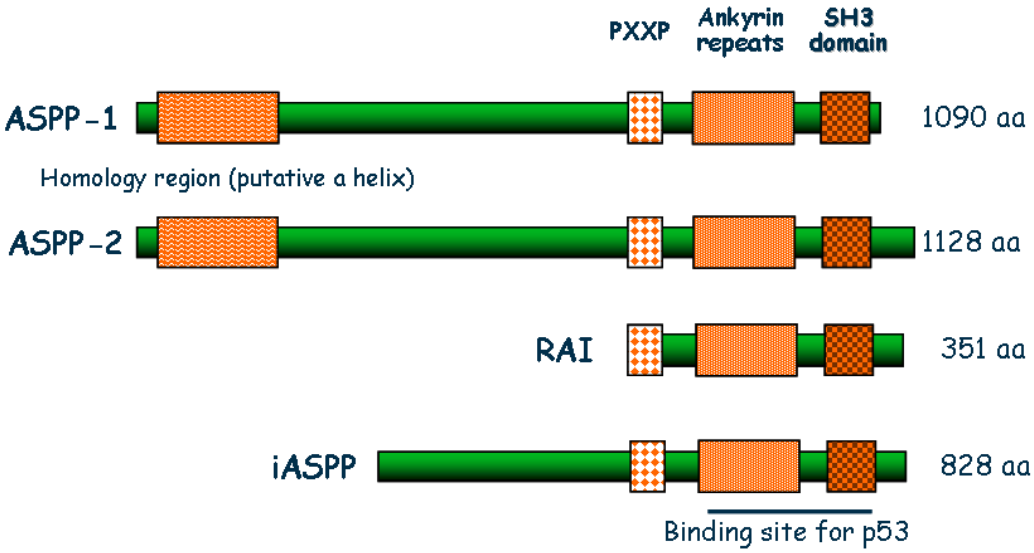


Figure 1.16: Structure of ASPP family of proteins. The members of the ASPP (ankyrin-repeat-, SH3-domain- and proline-rich-region containing protein) family are specific regulators of p53-, p63- and p73-mediated apoptosis. The family comprises three members; two pro-apoptotic proteins, ASPP1 and ASPP2 and a third, iASPP, originally reported as a REL-A/p65-associated inhibitor (RAI) that inhibits the transcriptional activity of REL-A/p65 (Yang et al. 1999b). Subsequent studies showed that the full-length iASPP protein is 828 amino acids in length (Herron et al. 2005; Slee et al. 2004) and its homologue in *Caenorhabditis elegans* is a protein of 769 amino acids which functions as an inhibitor of p53 (Bergamaschi et al. 2003b). aa – amino acids, PXXP – proline repeats.

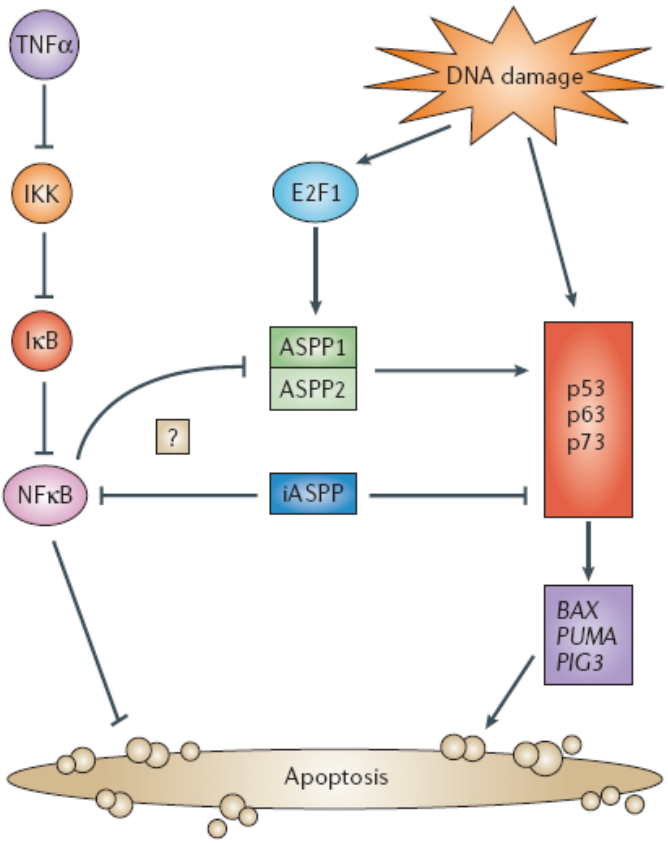


Figure 1.17: ASPP function in cellular pathways. Pro-apoptotic ASPP1 and ASPP2 are induced by E2F1 transcription factor and cooperate with the p53 family in transactivating pro-apoptotic genes such as the BCl2-family member BAX, the BCl2-binding component PUMA and PIG3. The inhibitory family member iASPP, functions as a transrepressor of the same genes. It also affects the NFκB pathway by inhibiting its subunit REL-A/p65. NFκB might also inhibit ASPP1 and ASPP2. IκB – inhibitor of NFκB; IKK – IκB kinase, TNFα – Tumour-necrosis factor-α, PIG3 – p53-induced gene 3, PUMA – p53-upregulated mediator of apoptosis. Image reproduced from Trigiante and Lu (2006).

1.2.3.3.7 *ASPP and cancer*

ASPP expression correlates with apoptotic sensitivity (ASPP1 and 2 increase sensitivity and iASPP increases resistance) and, given that disruption of the apoptotic pathway is a hallmark of cancer, it is reasonable that the ASPP family has an important function in tumourigenesis. Deregulation of ASPP1/2 has been demonstrated in several cancers including lung cancer and breast cancer (Bergamaschi et al. 2004; Liu et al. 2005; Mori et al. 2004; Mori et al. 2000; Samuels-Lev et al. 2001). Lowered ASPP2 levels correlate with poor prognosis and metastasis in diffuse large B cell lymphoma and breast cancer (Cobleigh et al. 2005; Lossos et al. 2002) and ASPP2 expression is associated with breast cancer progression (Sgroi et al. 1999). More recently, ASPP2 was described as a haploinsufficient tumour suppressor in mice developing lymphomas and sarcomas (Vives et al. 2006).

iASPP, a proto-oncogene is upregulated in a number of cancers including leukaemia, and breast cancer (Bergamaschi et al. 2003a; Liu et al. 2009a; Zhang et al. 2005) and in some cases occurs with a concomitant downregulation of ASPP expression thus providing a further option for targeting the p53 family in the treatment of cancers (Bell and Ryan 2008).

1.2.3.3.8 *Prolyl isomerase Pin1*

The prolyl isomerase Pin1 catalyses phosphorylation-directed prolyl isomerisation. Upon phosphorylation, Pin1 regulates p53 activation by rendering p53 suitable for subsequent modifications and modulating its interaction with DNA and co-factors. Genotoxic stress increases the interaction between p53 and Pin1 by recruiting Pin1 to Ser33, Thr81 and Ser315 (and under certain circumstances Ser46) of p53 (Wulf et al. 2002; Zacchi et al. 2002). Isomerisation of specific proline residues of p53 by Pin1 changes the conformation of p53 thus affecting its interaction with proteins that modulate p53 abundance or function. It is required for the dissociation of Mdm2 and full stabilisation of p53 following UVR (Zacchi et al. 2002). Cells lacking Pin1 fail to efficiently stabilise p53 upon genotoxic stress and consequently, cell cycle arrest and apoptotic responses are compromised (Mantovani et al. 2004a; Zacchi et al. 2002).

Pin1 activity can also restore the occupancy of p53 pro-apoptotic promoters. Pin1 was deemed essential for the apoptotic function of p73, by modifying p73 conformation and promoting its acetylation by p300/CBP (CREB binding protein) in a c-abl dependent fashion under genotoxic stress (Mantovani et al. 2004b). The presence of Pin1 at the p53 and p73-target promoters suggests that it contributes to

promoter selection by stabilising transcriptional complexes involving the p53 family and co-activators, including ASPP and p300. In support of this, Pin1 has recently been demonstrated to orchestrate p53 acetylation at Ser46, by p300 upon genotoxic stress, further mediating dissociation of p53 from iASPP and promoting cell death (Mantovani et al. 2007). These data highlight the complex interactions between p53 family members and p53 co-activator proteins in regulating the transcriptional activity of p53.

1.2.3.3.9 *p300/CBP*

One group of p53 family regulators is p300/CBP. Although both p300 and CBP are distinct proteins encoded by two individual genes (Chrivia et al. 1993; Eckner et al. 1994), both proteins often mediate transcription by binding to similar sets of transcriptional activators and are therefore often referred to as p300/CBP (Kawasaki et al. 1998; Kwok et al. 1994; Lee et al. 1996). p300/CBP acetylates p53 and contributes to its stability and transcriptional activity (Barlev et al. 2001; Gu and Roeder 1997; Gu et al. 1997; Lill et al. 1997). Consequently these effects mediate activation of downstream apoptotic pathways (Mantovani et al. 2007).

1.2.4 TP53 and melanoma

1.2.4.1 TP53 and UVR

TP53 has an important role in protecting against skin cancer induced by UVR (Cleaver and Crowley 2002). UVB is considered to be the most carcinogenic wavelength (280-320nm) inducing erythema, sunburn and is associated with cancer risk. To facilitate survival, melanocytes have developed an attenuated p53-dependent apoptotic response after UV exposure. In keratinocytes, p53 functions as a master regulator of skin cytokine signalling. In normal skin, p53 is a transducer of the tanning signal, thus an essential component of a keratinocyte-melanocyte signalling cycle that regulates skin pigmentation optimally in skin of dark pigmentation (Box and Terzian 2008). In lightly pigmented Caucasian skin, there is diminished pigment synthesis and diminished photoprotection. In response to UVR, p53 is highly induced leading to expression of target genes including paracrine factors which are responsible for upregulating melanogenesis and melanocyte proliferation. These paracrine signalling molecules are highly mitogenic for melanocytes and capable of acting as tumour promoters (Berking et al. 2004; Yamaguchi et al. 2006).

Absorption of UVB by DNA results in the generation of 6-4 photoproducts or pyrimidine dimers leading to genetic mutations such as C→T or CC→TT transitions;

the latter mutation representing the hallmark of UV-induced mutagenesis (Jhappan et al. 2003). These UVB-signature mutations in p53 are common in situations of defective DNA repair (Spatz, Giglia-Mari et al. 2001). Despite the high frequency (>90%) of UV-signature p53 mutations in non-melanoma skin cancer (McGregor et al. 1997), such mutations occur with a low frequency (<10%) in melanoma (Hocker and Tsao 2007; Weiss et al. 1995a; Zerp et al. 1999).

The p53 gene itself may be a melanoma risk gene. The Arg72Pro allele of p53 is associated with melanoma risk, particularly in individuals with dark skin phototypes who do not carry the MC1R variants (Stefanaki et al. 2007). While a clear picture is still emerging as to the functional consequences of p53 activation in melanocytes, studies suggest p53 plays a tumour-suppressive role in melanocytes (Box and Terzian 2008).

1.2.4.2 p53 mutations in melanoma

Mutational inactivation of p53 is rare in melanoma but can occur anywhere in the gene, and is usually a result of a point mutation (Albino et al. 1994; Avery-Kiejda et al. 2008; Gwosdz et al. 2006; Li et al. 2006; Montano et al. 1994; Papp et al. 1996; Soto et al. 2005; Sparrow et al. 1995b; Weiss et al. 1995a; Zerp et al. 1999). Functional studies of p53 mutant protein activity have revealed that over 70% of melanoma-associated p53 mutations retain >50% of wild-type (wt) p53 protein function (Soussi and Lozano 2005). Moreover these mutations tend to occur at later stages of disease suggesting that they are not essential for melanoma progression (Kanoko et al. 1996; Lassam et al. 1993; Stretch et al. 1991; Zerp et al. 1999). Ectopic expression of wt-p53 in melanoma cells harbouring an endogenous mutation of p53 results in apoptosis whereas the same does not occur with ectopic expression of wt-p53 in cells without mutations, suggesting that inactivation of p53 in melanoma is due to inhibition of the function of wt-p53 itself (Kichina et al. 2003).

1.2.4.3 p53 expression in melanoma

High levels of p53 mRNA and protein expression has been reported in melanoma cells and tissue samples (Akslen and Morkve 1992; Bartek et al. 1991; Hussein et al. 2003; McGregor et al. 1993; Sparrow et al. 1995b; Stretch et al. 1991; Weiss et al. 1995b; Yamamoto et al. 1995) often in the absence of point mutations in the gene (Albino et al. 1994; McGregor et al. 1993; Sparrow et al. 1995b). TP53 detection was associated with better prognosis and longer survival for patients with superficial spreading melanomas (Essner et al. 1998; Florenes et al. 1994) but in other studies

p53 immunoreactivity correlates with advanced melanoma and adverse prognostic factors (McGregor et al. 1993; Sparrow et al. 1995a; Yamamoto et al. 1995). Isoforms of p53 have also been demonstrated in melanoma cell lines and fresh isolates of melanoma. In some cases variants were expressed at higher levels than the full length wt-p53 suggesting that they might play an inhibitory role for p53 transactivation (Avery-Kiejda et al. 2008).

1.2.4.4 p53 pathway dysregulation

The ubiquitous loss of the p53 pathway in melanoma occurs rarely by p53 gene mutation and more commonly in tumours expressing the wt-p53 gene, through presumed dysregulated upstream or downstream cell signalling. Understanding p53 tumour suppressor activity has provided insight into melanomagenesis. Evidence from a transgenic zebrafish model expressing mutant BRAF (V600E) revealed expression of mutant, but not wild-type, BRAF led to dramatic patches of ectopic melanocytes, termed fish naevi. In p53-deficient fish, activated BRAF induced formation of melanocytic lesions that rapidly developed into invasive melanomas, resembling human melanomas (Patton et al. 2005). In support of this, an increased incidence of melanoma was observed in BRAF mutant mice with p53 deficiency (Goel et al. 2009) and p53 inactivation promotes malignant transformation in BRAF mutant human melanocytes demonstrating a dysfunctional p53 pathway *in vivo* (Yu et al. 2009). These data confirm that BRAF activation is among the primary events in melanoma development, but more importantly provide direct evidence that the p53 and BRAF pathways interact genetically to produce melanoma.

1.2.4.4.1 *Defective activation of p53*

Activating pathways upstream of p53 are likely to be disrupted in melanomas expressing wt-p53 protein and there is evidence supporting this hypothesis. DNA damage leads to an increase in p53 protein levels (Avery-Kiejda et al. 2008; Bae et al. 1996; Maltzman and Czyzyk 1984) and an increase in the functional activity of the protein (Chernov and Stark 1997; Haapajarvi et al. 1997). The molecular mechanism for increased p53 levels in response to ionizing radiation (IR) involves a signalling pathway including ATM (ataxia telangiectasia mutated) and Chk (checkpoint) kinases; ATM activates Chk1 and Chk2/hcds1, which in turn phosphorylates p53 on ser-20 (Chehab et al. 2000; Hirao et al. 2000; Matsuoka et al. 1998; Shieh et al. 2000) [Figure 1.13]. The latter phosphorylation leads to dissociation of p53 from mdm2 (Chehab et al. 2000) whose normal function is to target p53 for degradation [Figure 1.7]. Dephosphorylation of ser-376 of p53 in response to IR, increases

binding with 14-3-3 proteins enabling transactivation of p53 target genes, inducing cell cycle arrest or apoptosis (Kastan et al. 1991; Kubbutat et al. 1997).

The inhibition of wt-p53 function by upstream signalling pathways that activate p53 in response to DNA damage is supported by mutations in chk2/hcds1 (Chehab et al. 2000; Hirao et al. 2000; Matsuoka et al. 1998; Shieh et al. 2000), which have been identified in a subset of families with the hereditary Li-Fraumeni cancer predisposition syndrome (Bell et al. 1999). An overall increase in phosphorylation of p53 is observed in melanoma cells (Minamoto et al. 2001) and decreased p53 transcriptional activity is demonstrated in melanoma cells exposed to γ-radiation (Bae et al. 1996). In addition, defective activation of p53 occurs in response to DNA damage providing possible explanations for the radioresistance commonly observed in melanoma (Satyamoorthy et al. 2000).

1.2.4.4.2 *Dysregulation of p53 co-activators*

The most commonly cited explanation for p53 inactivation in melanoma is through increased expression of the negative regulator, mdm2 [section 1.2.3.3.3]. However INK4a or CDK4 loss or mutation occurs in only 25-40% of sporadic human melanomas (Ghiorzo and Scarra 2003) resulting in increased mdm2 protein which degrades p53 (Bardeesy et al. 2001). Overexpression of mdm2 protein contributes to the functional inactivation of p53 (Polsky et al. 2002) and in the setting of amplification of 12q14 in a subset of melanomas, may substitute for loss of $p14^{ARF}$ expression (Muthusamy et al. 2006). One possible explanation for MDM2 overexpression is AKT3 which is commonly amplified in melanoma (Stahl et al. 2004) and has been demonstrated to upregulate MDM2 (Moumen et al. 2007). MDM2 may also be elevated because of elevated p53 levels as they are both involved in an autoregulatory loop where mdm2 is a transcriptional target of p53. Other studies have reported that melanoma cells, with few exceptions, do not express high levels of mdm2 (Gelsleichter et al. 1995) implicating alternative methods of attenuation of wt-p53 activity in melanoma.

Although MDM4 is overexpressed in melanoma cell lines (Ramos et al. 2001), the exact relationship between p53 function and mdm2/mdm4 expression in melanoma is yet to be defined. A role for MDM4 in regulating p53 activity was demonstrated when potent activation of wt-p53 in chemoresistant melanoma cells induced by an organometallic glycogen synthase kinase 3β (GSK3β) compound, downregulated both MDM2 and MDM4, subsequently leading to cell death (Smalley et al. 2007).

Dysregulation of the p53 family members, TP63 and TP73 in melanoma has not been robustly investigated, although there is evidence to suggest involvement of TP73 (Tuve et al. 2006), and examination of the co-ordination of activities of each of the members has not been undertaken.

1.2.4.4.3 *Dysregulation of p53 downstream pathways*

Disruption of apoptosis downstream of p53 may alleviate pressure to mutate p53 and simultaneously reduce chemosensitivity in cancers (Schmitt et al. 2002; Schmitt and Lowe 2002). The failure of IR to induce either cell cycle arrest or apoptosis in melanoma cells expressing wt-p53 (ectopically or endogenously), suggests defective signalling pathways downstream of p53 (Bae et al. 1996; Satyamoorthy et al. 2000). In support of this, one of the key mediators of p53-dependent apoptosis, Apoptosis protease-activating factor-1 (Apaf-1), is frequently lost or downregulated in melanoma cell lines and tissue samples, an effect which is associated with melanoma progression and an example of how melanoma cells evade apoptosis (Campioni et al. 2005). Moreover, restoration of physiological levels enhances chemosensitivity by re-establishing the p53 pathway (Soengas et al. 2001).

p53 upregulated modulator of apoptosis (PUMA) is a p53-inducible target gene (Nakano and Vousden 2001; Yu et al. 2001) which shows decreased expression during melanoma progression correlating with lower survival rates (Karst et al. 2005). Anti-senescence factors Tbx2 and Tbx3 are also upregulated in melanoma (Vance et al. 2005) and provide another example of dysregulation of the p53 pathway through suppression of p53-mediated activation of its downstream target, p21 (Hoek et al. 2004; Prince et al. 2004; Vance et al. 2005). Thus, dysregulation of the p53 pathway in melanoma occurs at all levels and is likely to contribute to both pathogenesis and chemoresistance of the disease.

1.3 TP63

1.3.1 Background

TP63 was the third member of the p53 family to be discovered in 1998 by McKeon and Caput (Yang et al. 1998). Phylogenetically, it is the oldest evolutionarily conserved member of the p53 family (Blandino and Dobbelstein 2004).

1.3.2 Structure of p63

The gene is tissue-specifically transcribed into different isoforms whereby two alternative promoters give rise to TA and ΔN isoforms [Figure 1.14] (Osada et al. 1998; Yang et al. 1998). The distal promoter generates TAp63 isoforms with a transactivation domain (TA domain) in their NH_2-terminus capable of transactivating various p53-responsive promoters. The use of the proximal promoter generates ΔNp63 isoforms with a short peptide substituting for the TA domain, which exerts dominant-negative activity against TA isoforms and p53 (Hibi et al. 2000; Patturajan et al. 2002; Ratovitski et al. 2001; Stiewe et al. 2002; Wu et al. 2003a; Yang et al. 1998). Whilst initial studies showed that the TAp63 isoform was a transcriptional activator and ΔNp63 was a dominant negative inhibitor (Yang et al. 1998) later studies confirmed that the ΔNp63 isoform is capable of inducing cell cycle arrest and apoptosis (Dohn et al. 2001) through a second transcriptionally active domain (TA2) between residues 410 and 512 (Duijf et al. 2002; Ghioni et al. 2002; Ponassi et al. 2006; Wu et al. 2003a).

In addition, both isoforms undergo three alternative splicing events at the COOH-terminus generating six different isoforms (Ikawa et al. 1999; Mills 2006; Yang and McKeon 2000). More recently two new C-terminal p63 variants named p63 δ and ε have been identified bringing the total number of p63 isoforms to ten [Figure 1.14] (Mangiulli et al. 2009).

1.3.2.1 Transactivation domains

Biochemical studies of p63 show that, like p53, p63 proteins act as sequence-specific DNA-binding transcription factors. TAp63 isoforms activate the transcription of reporter genes from heterologous promoters through p53 DNA binding sites, as well as from endogenous promoters of p53 target genes such as p21 (Ghioni et al. 2002; Westfall et al. 2003; Yang et al. 1998). Structurally the γ forms of p73 and p63 most closely resemble p53 itself. However, whilst TAp63γ is as powerful as p53 in transactivation and apoptosis assays (Westfall et al. 2005), TAp63α is relatively weak

(Dohn et al. 2001; Ghioni et al. 2002; Yang et al. 1998). This is due to the presence of an additional domain in the C-terminus of α isoforms of p63 known as the transactivation inhibitory domain (TID) (Serber et al. 2002). The TID domain interacts with the TA domain of p63, limiting the transactivation capability of TAp63α (Serber et al. 2002).

1.3.2.2 Sterile alpha motif

In contrast to p53, the p63α proteins contain an additional region known as the sterile alpha motif (SAM) domain. This is a protein-protein interaction domain implicated in developmental processes (Schultz et al. 1997; Thanos and Bowie 1999). The TID confers the transcriptional repressor activity of ΔNp63α contained within the SAM domain. SAM containing proteins are more stable as the TID domain, bound to the TA domain, hides the sites used by the degradation pathway. The SAM domain plays a role in activation of p63, binding to other activating partners, such as kinases which phosphorylate the TA or TID domain and thus open the protein into an active form.

1.3.2.3 Proline rich domain

The N-terminal of the p63 protein contains a proline rich region which can bind to a number of cellular proteins. TAp63 contains two repeats of PXXP motif (P – proline and X – any amino acid) between amino acids 60 and 130. The transcriptional activity of TAp63 requires the presence of both transactivation domain and the proline-rich domain. Deletion of the proline-rich domain attenuates the ability of TAp63β to induce endogenous gene expression, confirming that the proline-rich domain is essential for the transactivation and apoptotic potential of TAp63 without affecting its ability to bind to DNA or inhibit cell proliferation (Helton et al. 2008).

1.3.3 p63 function

1.3.3.1 Epithelial tissues

p63 is essential for normal embryonic development. Major evidence linking p63 to development of the epidermis comes from analysis of the p63 knockout mouse phenotype. p63-/- mice die at birth with severe developmental abnormalities, including truncation of limbs, craniofacial malformations and defects in the epidermis (Laurikkala et al. 2006; Mills et al. 1999; Yang et al. 1999a). The mice die within hours of birth, presumably because of dehydration. These mice are characterised by severe defects in epidermis, teeth, breast and uroepithelial tissues, all structures dependent on epithelial-mesenchymal interactions. The phenotypes observed in two independent lines of p63-/- mice share remarkable similarities, however distinctions

between them reflect a role for p63 in two processes critical to normal epidermal morphogenesis: stem cell maintenance (Yang et al. 1999a), and commitment to differentiation and a stratified epithelium (Mills et al. 1999).

1.3.3.1.1 *Embryonic tissues*

TA and ΔN p63 isoforms are expressed during distinct stages of embryonic epidermal development. TAp63 isoforms are the first p63 isoforms expressed during embryogenesis and are required for commitment to epithelial stratification while simultaneously blocking a differentiation programme. A shift towards ΔNp63 isoforms during later stages is required to counterbalance the activity of TAp63 thereby allowing cells to respond to signals required for maturation of embryonic epidermis. Ectopic expression of a TAp63 isoform in a simple epithelium *in vivo* results in the induction of squamous metaplasia, confirming the role of p63 as a master molecular switch. p63 therefore plays a dual role: it initiates epithelial stratification during development and maintains the proliferative potential of basal keratinocytes in mature epidermis (Candi et al. 2006; Koster et al. 2004; Truong et al. 2006).

1.3.3.1.2 *Adult tissues*

Studies of p63+/- mice, in conjunction with conditional p63 deletion in stratified epithelia, uncovered a function for p63 in adult tissues, as these mice manifested decreased longevity associated with characteristics of accelerated ageing, including skin lesions and alopecia (Flores et al. 2005; Keyes et al. 2005). Evidence suggests that both isoforms of p63 are implicated in the ageing process; overexpression of ΔNp63α results in a premature ageing phenotype in mice through decreased expression of the deacetylase, Sirt1 (Sommer et al. 2006) and knockout mice selectively lacking TAp63 display a reduced lifespan accompanied by features of premature ageing including severe ulcerations, impaired wound healing, kyphosis, disrupted integrity of skin and hair follicles leading to hair loss (Su et al. 2009a; Su et al. 2009b). Further analysis found TAp63 expression in skin to include epidermal cells but also the dermal sheath and dermal papilla, niches for dermal precursor cells known as skin derived precursors (SKPs) (Fernandes et al. 2004; Su et al. 2009b). Lack of TAp63 in stem cells of these compartments results in cellular senescence and a similar response associated with hair loss is observed in ageing wild-type mice, suggesting that TAp63 is critical for maintaining both epidermal and dermal precursor cells in a healthy, functional state with intact genomes.

1.3.3.2 Germline

Primordial follicles are highly sensitive to radiotherapy and anti-cancer therapy, thus depletion of the follicular reserve and premature ageing occur during these treatments. A new function for p63 underlying the mechanism responsible for this loss has recently been proposed. TAp63α is constitutively expressed in mouse oocytes perinatally and protects the female germline by inducing oocyte death in response to DNA damage (Livera et al. 2008; Suh et al. 2006). TAp63 expression correlates with oocyte radiosensitivity and is essential for DNA-damage induced death within the primordial follicle, arguing a role for p63 as the 'guardian of the germline'. The mechanism underlying the DNA damage response involves c-abl kinase sensing the DNA insult and changing the cellular programme by activating TAp63 transcriptional activity towards apoptotic genes (Gonfloni et al. 2009).

1.3.4 Regulation of p63

1.3.4.1 Post-translational modifications

Post-translational modifications play an important role in regulating protein stability and function of p53 (Bode and Dong 2004) [section 1.2.3.1]. Regulation of p63 through post-translational modifications is less well defined but appears to result in rapidly changing p63 protein levels [Figure 1.18] (Ghioni et al. 2005; Huang et al. 2004).

1.3.4.1.1 *Phosphorylation*

Phosphorylation on serine / threonine residues has been reported to result in stabilisation of exogenous TAp63α and γ isoforms upon genotoxic treatment (Katoh et al. 2000; Okada et al. 2002) [Figure 1.18]. Like p53, ΔNp63α also exists as a phosphoprotein and upon UVR and chemotherapy ΔNp63α is phosphorylated at serine 66/68 (in TAp63α, ser 160/162), subsequently leading to ubiquitination and proteosomal degradation (Fomenkov et al. 2004; Westfall et al. 2005).

In female perinatal oocytes, TAp63 is phosphorylated upon DNA damage increasing its binding to p53 cognate DNA sites (Gonfloni et al. 2009; Suh et al. 2006). The cellular response to genotoxic stress requires phosphorylation of the tyrosine residues in the region of p63 flanked by the TA and the DNA-binding domains by the tyrosine kinase c-abl, resulting in accumulation of TAp63 and activation of p63-mediated transcription of pro-apoptotic genes (Gonfloni et al. 2009).

Phosphorylation of TAp63γ (but not ΔNp63γ) by IκB kinase β (IKKβ) is reported although the exact amino acid residue for phosphorylation was not determined (MacPartlin et al. 2005). Another kinase, Polo like kinase-1 (Plk-1), phosphorylates TAp63α at Serine-52 (in the transcriptional domain) in liver cells, leading to suppression of its transcriptional activities as a result of accelerated protein degradation (Komatsu et al. 2009) which is similar to decreased transcriptional activities reported for p53 (Ando et al. 2004) and p73α (Koida et al. 2008).

1.3.4.1.2 *Acetylation*

The transcriptional activator p300, is a well established acetylator of p53 family members and acts as a co-activator of both p53 and p73 (Irwin and Kaelin 2001a; Levrero et al. 2000; Yang and McKeon 2000). Similarly, p300 acetylates TAp63γ but not ΔNp63γ, and this acetylation is induced by DNA damage demonstrating a role for p300 to act as a co-activator of p63γ to mediate induction of p21 and subsequently p63-dependent cell cycle arrest (MacPartlin et al. 2005).

1.3.4.1.3 *Sumoylation*

Sumoylation of p63 regulates protein/protein interactions, intracellular localisation as well as protecting modified targets from ubiquitin-dependent degradation (Huang et al. 2004). ΔNp63α (but not β or γ) is conjugated both *in vitro* and *in vivo* by small ubiquitin modifier-1 (SUMO-1) and SUMO-2 (Ghioni et al. 2002; Huang et al. 2004). SUMO-1 modification is demonstrated on the only sumoylated lysine 637 in exon 14, in the TID domain within the p63 sequence (in p63α isoforms only) (Ghioni et al. 2002). This modification is crucial for regulating p63 protein stability. Expression of SUMO-1 destabilises wild-type p63 but not a sumoylation-deficient p63 variant ($p63^{K637R}$) in a proteosome-dependent process. Accordingly, the variant is less efficiently ubiquitinated than the wild-type p63 (Bakkers et al. 2005; Ghioni et al. 2005). More recently, ΔNp63α was preferentially sumoylated by SUMO-2 and p14(ARF) promoted this process, leading to proteosomal degradation (Vivo et al. 2009).

Sumoylation plays a critical role in controlling p63 activity during developmental processes since p63α mutations which abolish the sumoylation of p63α protein are associated with isolated split hand/foot malformation (Huang et al. 2004). The absence of sumoylation enhances transcriptional regulation of gene targets involved in bone and tooth development which contributes to the underlying molecular

mechanisms predisposing to the split hand/foot malformation phenotype (Huang et al. 2004; van Bokhoven and McKeon 2002).

1.3.4.1.4 *Ubiquitylation*

Protein degradation occurs through lysosomal and proteosomal pathways. Proteosomal degradation requires prior ubiquitylation of the protein which is a highly ordered multistep enzymatic process. The sequential enzymatic cascade involves an E1 activating enzyme, an E2 conjugating enzyme and an E3 ubiquitin ligase before transfer to the 26S proteosome and degradation into individual amino acids. Increasing evidence suggests that protein ubiquitination and degradation are crucial for p63 activity (Bakkers et al. 2005; Fomenkov et al. 2004; Rossi et al. 2006a; Rossi et al. 2006b). p63 isoforms appear to demonstrate a distinctly differing degree of ubiquitylation, suggesting that it might be a mechanism used to distinguish the specific activity of individual isoforms (Gallegos et al. 2008).

1.3.4.2 Itch

Itch is a Hect (homologous to the E6-associated protein C terminus)-containing Nedd4-like ubiquitin protein ligase shown to functionally associate with p63 by binding, ubiquitylating and degrading p63 (Melino et al. 2006; Rossi et al. 2006a). Itch interacts with both TAp63α and ΔNp63α but preferentially for the latter. Furthermore in Itch-/- primary keratinocytes, ΔNp63 levels are increased, suggesting a relevant role for Itch in regulating p63 *in vivo*.

Itch activity is also regulated at the post-translational level (Chang et al. 2006; Gallagher et al. 2006; Yang et al. 2006) adding additional levels of complexity to the function of this enzyme. In addition, Nedd-4 binding partner-1 (N4BP1) associates with itch, interfering with E3 binding to its substrates. By acting as a negative regulator of itch, N4BP1 causes inhibition of the polyubiquitylation of p63 by itch increasing steady state levels of p63 (Oberst et al. 2007).

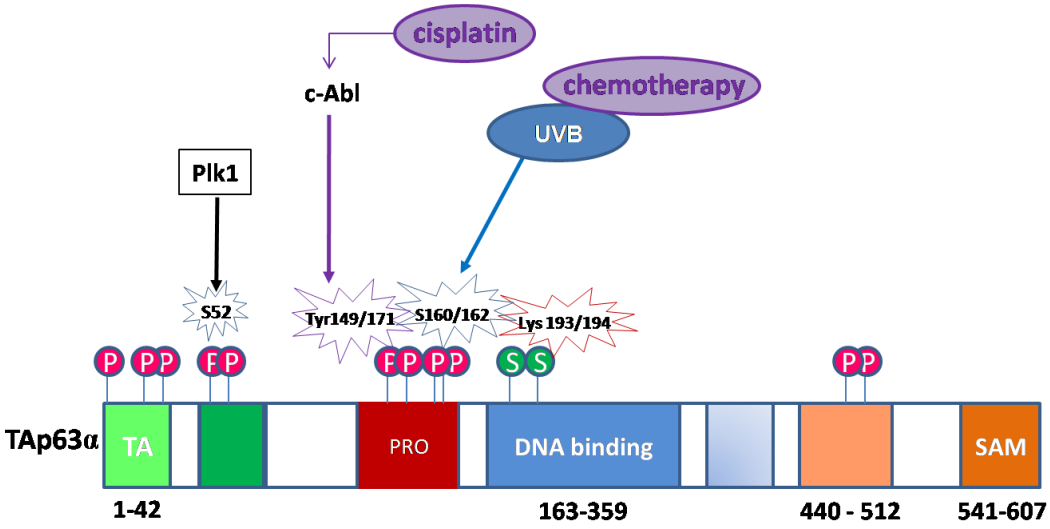


Figure 1.18: Post-translational modifications in p63. Regulation of p63 through post-translational modifications is not well established. A selection of phosphorylation sites (pink circles with P) and acetylation sites are shown (green circles of S) for TAp63α. Phosphorylation is the most investigated modification; upon UVR and treatment with various chemotherapeutic agents including cisplatin and paclitaxel, phosphorylation of serine 160/162 occurs in TAp63α subsequently leading to ubiquitination and proteosomal degradation. TA – transactivation domain, PRO – proline rich domain, SAM – sterile alpha motif.

1.3.4.3 WW domain containing E3 ubiquitin protein ligase 1 (WWP1)

WWP1 is an intrinsic E3 ubiquitin ligase targeting both TAp63α and ΔNp63α for ubiquitin-mediated proteosomal degradation, by binding p63α proteins through the WW/PY motif interaction, ubiquitinating and promoting p63α for proteosomal degradation (Li et al. 2008). In addition, WWP1 sensitised immortalised breast epithelial cells to chemotherapeutic drug-induced apoptosis through destabilisation of ΔNp63α and targeted endogenous TAp63α protein (in colon cancer cell lines) for degradation conferring cell survival in a p53-independent manner. To support these data, a negative correlation between WWP1 and p63 (mostly ΔNp63) was also reported in a panel of prostate and breast cancer cell lines (Li et al. 2008).

1.3.4.4 $SCF^{\beta TrCP1}$

SCF is an E3 ubiquitin ligase complex which works in conjunction with IKK kinase to degrade IκB and subsequently upregulate NFκB (Fuchs et al. 2004). βTrCP1 (β-Transducin Repeats Containing Protein-1) is one of the many substrate recognition components of the SCF complex (Fuchs et al. 2004). $SCF^{betaTrCP1}$ binds to both the N' and C' termini of TAp63γ (and to a lesser extent ΔNp63γ) which through ubiquitylation stabilises and activates TAp63γ to induce p21, which subsequently leads to cell cycle arrest (Gallegos et al. 2008).

1.3.4.5 Receptor for activated protein kinase C – 1 (RACK1)

RACK1 functions as one of the E3 ubiquitin ligases to promote ubiquitination and proteosomal degradation of ΔNp63α, regulating its levels in HNSCC (Fomenkov et al. 2004). One mechanism for the preferential degradation of ΔNp63 isoforms in response to genotoxic stress has been proposed to involve stratifin-mediated nuclear export of ΔNp63α followed by RACK1-mediated proteosomal degradation (Fomenkov et al. 2004).

1.3.4.6 Upstream signalling pathways

1.3.4.6.1 *Notch pathway*

Reciprocal negative regulation between Notch1 and p63 expression and activity has consequences for the balance between self-renewing and committed keratinocyte cell populations as well as carcinogenesis (Nguyen et al. 2006). Active Notch signalling suppresses p63 expression in keratinocytes *in vitro* and *in vivo*, while sustained p63 function inhibits the ability of Notch to promote cell cycle arrest and epidermal differentiation (Nguyen et al. 2006; Okuyama et al. 2008). TP63 is

regulated by Notch but it also controls the Notch expression and downstream notch signalling (Dotto 2009; Laurikkala et al. 2006; Nguyen et al. 2006). TP63 is also able to induce activation of this pathway in neighbouring cells by positive transcriptional regulation of the Notch ligands, Jagged 1 and Jagged 2 (Sasaki et al. 2002; Wu et al. 2003a). Induction of these genes by p63 is important for normal skin homeostasis. Moreover, Notch signalling has recently been shown to play a vital role in maintaining melanoblasts and melanocyte stem cells in skin (Moriyama et al. 2006) while Notch-1, -2 and Notch ligands are upregulated in melanoma tissue samples compared with benign melanocytic naevi (Massi et al. 2006).

1.3.4.7 microRNA regulation of TP63

Evidence is accumulating that the p53 family and microRNAs (miRNA) form a dense functional network: p53 family proteins are extensively regulated by miRNAs (Bailey et al. 2010) and act as regulators of miRNAs or downstream effectors which modulate anti-apoptotic and pro-apoptotic responses (Antonini et al. ; Georges et al. 2008).

p63 is expressed in a tissue-specific manner and a picture has recently emerged for regulation by specific miRNAs in this tissue context [Table 4.1]. Although a number of miRNAs have been reported, the two most widely investigated are miR-203 and miR-302. miR-203 was identified as a repressor of ΔNp63 in the skin (Lena et al. 2008; Yi et al. 2008) and miRNAs of the 302 cluster were identified using an immunofluorescence-based screen of germline cells, as novel antagonists of TAp63 (Scheel et al. 2009) [Figure 1.19]. These data gave rise to two classes of miRNAs which regulate the two major isoforms of p63; ΔNp63 isoforms which maintain the adult stem cell population in the skin are repressed by miR-203 during differentiation and TAp63-isoforms which mediate apoptosis in damaged oocytes prior to maturation, are kept in check by miR-302 [Figure 1.19]. Besides their physiological function, both miRNAs are also involved in disease; miR-203 is induced upon DNA damage in HNSCC and miR-302 is upregulated in germ cell tumours (Lena et al. 2008).

Increasing evidence from cancer biology continues to identify new miRNA regulators. In chronic lymphocytic leukaemia cells induction of miR-106b has been reported to be associated with downregulation of itch associated with a reciprocal accumulation of its pro-apoptotic substrate, p73, resulting in apoptosis (Sampath et al. 2009). It is

therefore possible in tumours where reciprocal regulation of itch and p63 are demonstrated, miR-106b may display a similar function.

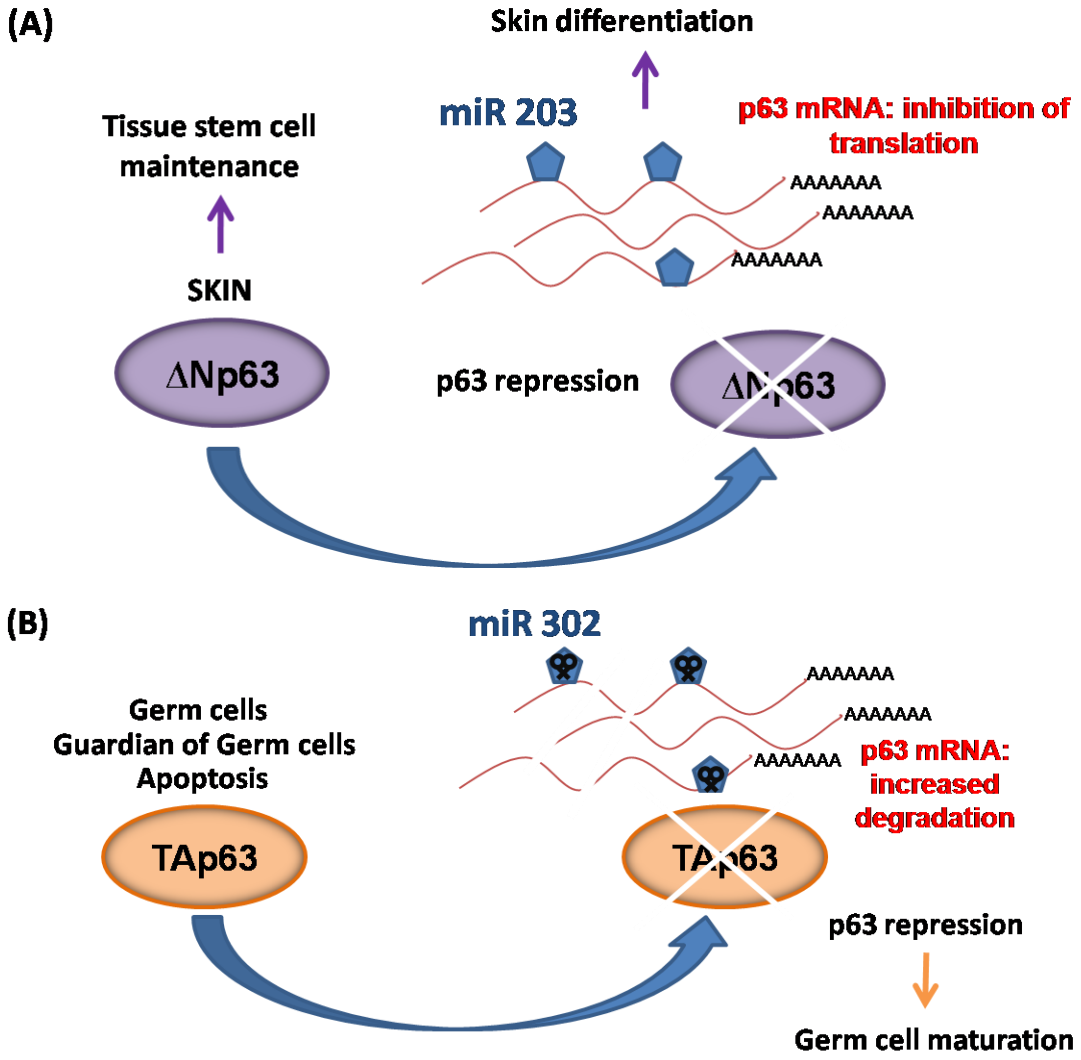


Figure 1.19: MicroRNA regulation of p63 isoforms. The most well established miRNAs regulating p63 are shown. (A) miR-203 was identified as a repressor of ΔNp63 in the skin (Lena et al. 2008; Yi et al. 2008) and (B) miR-302 was a novel antagonist of TAp63 (Scheel et al. 2009). These two classes of miRNAs regulate the two major isoforms of p63; miR-203 inhibits translation of p63 mRNA and miR-302 promotes degradation of p63 mRNA. ΔNp63 isoforms which maintain the adult stem cell population in the skin are repressed by miR-203 during differentiation and TAp63 isoforms which mediate apoptosis in damaged oocytes prior to maturation, are kept in check by miR-302. In addition, miRNAs are demonstrated in disease; miR-203 is induced upon DNA damage in HNSCC and miR-302 is upregulated in germ cell tumours (Lena et al. 2008). In addition, it is likely that there are as yet, unidentified miRNAs which regulate the expression of p63 isoforms in these tissue contexts. Figure adapted from Blandino and Moll (2009).

1.3.5 Role of p63 in response to DNA damage

A consistent picture emerges whereby certain forms of DNA damage induce an apoptotic response mediated, at least in part, through degradation of anti-apoptotic ΔN isoforms and stabilisation of pro-apoptotic TA isoforms. ΔNp63α transcript levels decline in epidermal tissue and mammary cell lines following treatment with UVB, cisplatin and doxorubicin (Harmes et al. 2003; Liefer et al. 2000). Following DNA damage, this may be mediated by the recruitment of ΔNp63α protein to a binding site in its own promoter, therefore repressing its own transcription (Harmes et al. 2003) or the downregulation of p63 allows p53 to protect the cell from perpetuating damaged DNA (Liefer et al. 2000). Dysregulated p63 expression would interfere with the protective role of p53, possibly by competing with p53 for binding to DNA targets and/or exclusion from the nucleus.

A recent study provided insight into the DNA-damage induced post-translational regulation of ΔNp63α protein stability by the ATM pathway suggesting that ATM has evolved to act as a master regulator in the skin stem cell. ATM acts to co-ordinately switch off ΔNp63α and switch on p53 after irradiation which is needed to attenuate the proliferative capacity of the stem cell in order for DNA damage to be repaired by the p53 response (Huang et al. 2008b). The expression of both p53 and ΔNp63α in basal cell populations raises the question of whether regulation of the pro-proliferative ΔNp63α and anti-proliferative p53 pathways are co-ordinated after DNA damage i.e. ΔNp63α protein is de-stabilised after DNA damage *in vitro* and *in vivo* systems whilst upregulation of p53 phosphorylation occurs at the CDK2 and ATM sites in basal skin cells *in vivo* (Finlan et al. 2006). Huang *et al.* (2008b) showed that ΔNp63α is sequentially phosphorylated after DNA damage by ATM, CDK2 and p70s6K leading to a dramatic decrease in ΔNp63α protein levels in HNSCC cells, an effect which is attenuated in the absence of these kinases. The data provided a co-ordinated mechanism for switching ΔNp63α off and p53 on involving the p53 activator ATM as the master switch in this axis to maintain genomic stability after DNA damage (Shiloh 2003). Similarly, as CDK2 has been implicated in stimulating p53 function after irradiation (Blaydes et al. 2001) this kinase pathway might also co-ordinate the activation of p53 and ΔNp63α protein degradation. These data suggest that at least two protein kinase pathways play dual roles in controlling p53 family function after DNA damage.

The tissue-specific response of p63 to DNA damage is variable. The only study investigating p63 response in melanocytes, reported expression of two isoforms:

TAp63β and either TAp63γ or ΔNp63β (undetermined) in mouse melanocytes and no endogenous upregulation in mouse melanoma cells. In this mouse model, p63 isoforms were not induced upon DNA damage (either IR or UVB) (Johnson et al. 2005; Kulesz-Martin et al. 2005). In contrast, ectopically expressed TAp63α and γ isoforms accumulate in leukaemic cells in response to UVB, UVC, doxorubicin and etoposide (Katoh et al. 2000; Okada et al. 2002). To support this, topoisomerase II inhibitors (doxorubicin and etoposide), but not UVB, induced endogenous expression of TAp63α (and p53-target genes p21, 14-3-3-σ, GADD45 and PIG3) but not ΔNp63 in mouse hepatocytes and human hepatocellular carcinoma cells (Petitjean et al. 2005).

1.3.6 p63 and apoptosis

TAp63 induces apoptosis in a similar way to p53, promoting both death receptor and mitochondrial apoptotic pathways (Gressner et al. 2005). In an inducible stable-cell system, TAp63β possessed the greatest ability to induce apoptosis, followed by TAp63γ and then by TAp63α, consistent with their transcriptional activity (Helton et al. 2008). In neuronal cells, TAp63γ alone is sufficient to promote neuronal apoptosis and essential for p53-induced apoptotic cell death (Jacobs et al. 2005).

In contrast, overexpression of ΔNp63α in mouse epidermis results in a reduction in UVB-induced apoptosis (Liefer et al. 2000); disruption of ΔNp63α in squamous carcinoma cells increases sensitivity to apoptosis-inducing agents (Westfall et al. 2005); and p63 does not appear to contribute to radiation-induced apoptosis in thymocytes (Senoo et al. 2004). Although ΔNp63α opposes p53-mediated apoptosis in baby hamster kidney cells (Yang et al. 1998), in H1299 cell lines and keratinocytes, both inducible TAp63α and ΔNp63α causes cell death associated with increased expression of p53 target genes (Dohn et al. 2001; Zhu et al. 2007).

The literature to date proposes that the p63 family of proteins display a diverse range of biological activities and impact cells in an isoform-dependent but also cell type- and stimulus-specific manner.

1.3.7 p63 and cancer

1.3.7.1 p63 mutations

The p63 gene maps to chromosome 3q27-28, a region that is frequently amplified in a variety of squamous cell cancers including those of the lung, cervix, ovary and skin (Casciano et al. 2002; Flores et al. 2002; Melino et al. 2003; Moll and Slade 2004;

Purdie et al. 2009; Westfall et al. 2003; Westfall and Pietenpol 2004; Wu et al. 2005; Yang et al. 1998). Germline mutations in TP63 (in the DNA binding domain) occur in human developmental syndromes and are associated with skin, hair, tooth and skeletal patterning defects (Brunner et al. 2002; Celli et al. 1999). These mutants vary in their ability to transactivate p53 and p63 target genes and it is likely that the phenotypic variations observed within p63-related syndromes are due to the differential effects of the mutants on canonical and non-canonical downstream signalling pathways of p63 (Khokhar et al. 2008). Somatic missense mutations of TP63 are rarely detected in human cancers supporting the notion that p63 is not a canonical tumour suppressor (Hagiwara et al. 1999; Ikawa et al. 1999; Irwin and Kaelin 2001b; Kato et al. 1999; Osada et al. 1998; Park et al. 2000; Sunahara et al. 1999; Tani et al. 1999; Trink et al. 1998).

1.3.7.2 p63 and carcinogenesis

p63 does however, play a complex role in tumourigenesis that is likely to be context specific. Two p63+/- mice models have led to conflicting hypotheses for the role of p63 in carcinogenesis [Table 1.7]. Both models were used to determine if mice with reduced p63 levels were predisposed to develop spontaneous tumours. The results were quite different with only heterozygote p63+/- mice developed by Yang and colleagues predisposed to develop spontaneous tumours (Flores et al. 2005). The alternative model by Mills and colleagues, generated p63+/- mice which were not predisposed to develop spontaneous tumours (Keyes et al. 2005), γ-irradiation induced lymphomas (Perez-Losada et al. 2005) or chemically-induced skin tumours (Keyes et al. 2005). In support of these findings, over 80% of HNSCC demonstrate p63 genomic locus amplification and/or overexpression of ΔNp63, signifying its role as a putative oncogene (Choi et al. 2002; Hibi et al. 2000; Hu et al. 2002; Massion et al. 2003; Mills 2006; Yang and McKeon 2000). Furthermore, overexpression of ΔNp63α preferentially represses induction of apoptosis by p73 mediated via transactivation of NOXA and PUMA (Rocco et al. 2006). In addition, ΔNp63 can inhibit p53, TAp63 and TAp73 transactivation and apoptosis (Liefer et al. 2000; Ratovitski et al. 2001; Yang et al. 1998), and overexpression is required to maintain a stem cell-like state, allowing continuous proliferation and promoting tumour growth (Deyoung and Ellisen 2007). These findings explain a selective advantage of overexpression of ΔN p63 isoforms in tumours indicating how they could act as oncogenes.

Table 1.7: Summary of phenotypic differences between two lines of independently generated p63 knockout mice. Adapted from Koster et al (2007)

Mouse model	Phenotype of p63+/- mice	Interpretation	Supporting evidence
Yang *et al* (2005)	Develop spontaneous tumours	p63 is a tumour suppressor	Loss of p63 expression occurs in a subset of advanced tumours and may lead to increased metastatic potential (Park, Lee et al. 2000; Urist, Di Como et al. 2002; Koga, Kawakami et al. 2003). TAp63 induces senescence and suppresses tumourigenesis *in vivo* (Guo et al. 2009).
Mills *et al* (2006)	Do not develop spontaneous tumours and are not predisposed to develop skin tumours	p63 is an oncogene	Upregulated expression of ΔNp63 expression is common in epithelial tumours (Mills 2006). p63 mutations are rare in human cancer (Mills 2006). Reactivated TAp63α expression accelerates skin tumour development and progression (Koster, Lu et al. 2006).

1.3.7.3 p63 expression and prognosis

Several studies have implicated p63 expression as a positive prognostic factor in cancer progression and outcome; p63 expression is associated with favourable prognosis in lung cancer and diffuse large B-cell lymphoma patients (Hallack Neto et al. 2009; Massion et al. 2003), overexpression of ΔNp63α in squamous cell carcinomas (lung and head/neck) is an indicator of favourable response to therapy and overall clinical outcome (Massion et al. 2003; Zangen et al. 2005) and loss of p63 expression in urothelial cancers is associated with progression to more invasive and metastatic tumours (Koga et al. 2003; Tuna et al. 2009; Urist et al. 2002).

In contrast, p63 expression is correlated with poor prognostic factors and outcome in breast cancer and follicular cell lymphoma (Fukushima et al. 2006; Ribeiro-Silva et al. 2003). Aberrant expression of p63 in the cytoplasm was demonstrated to be associated with increased prostate cancer-specific mortality up to 20 years after

diagnosis. Cytoplasmic expression was associated with reduced apoptosis and higher proliferative activity suggesting an oncogenic role in prostate cancer progression and survival (Dhillon et al. 2009).

One possible model to reconcile these findings would be that ΔNp63α contributes to the early stages of tumourigenesis while maintaining epithelial cell fate (Green et al. 2003; Truong et al. 2006). In contrast loss of p63 may mark tumours that have accumulated additional genetic events and have acquired mesenchymal properties, both of which are correlated with refractory clinical behaviour (Barbieri et al. 2006).

1.3.7.4 p63 and melanoma

The expression pattern of p63 isoforms has not been widely investigated in melanoma. Most studies have used immunohistochemistry techniques to investigate expression of p63 protein in melanoma using it as an example of negative reactivity [Table 3.11]. In two tissue microarrays, ΔNp63 was expressed in a small fraction; 2/59 and 2/25 human melanomas (Brinck et al. 2002; Reis-Filho et al. 2003a). In one study, 2/3 spindle cell melanomas expressed p63 (Morgan et al. 2008). A more recent study investigating the expression of p73 in primary and metastatic uveal melanomas also reported expression of TAp63 and ΔNp63 (using RT-PCR) in 12/18 and 1/18 uveal melanoma cell lines respectively (Kilic et al. 2008). Other reports have suggested that p63 is not expressed in melanoma *in situ* or invasive melanoma (Bourne et al. 2008; Sakiz et al. 2009).

1.4 HYPOTHESIS

Apoptotic dysregulation is a hallmark of melanoma pathogenesis and chemoresistance. Mutations in TP53 occur infrequently in melanoma and are not critical for tumour development. Nevertheless the TP53 apoptotic pathway is abrogated; this may alternatively result from either upstream or downstream TP53 pathway defects or from alterations in other members of the TP53 family, including the TP53 homologue, TP63. To date, there is no robust evidence explaining the mechanism(s) of inactivation or attenuation of p53 tumour suppression in melanomagenesis. A better understanding of dysregulation of the p53 pathway is necessary to delineate the molecular pathogenesis of melanoma and to develop more targeted chemotherapeutic strategies.

There is significant evidence to support a role for TP63 in carcinogenesis and the existence of multiple isoforms expressed in a tissue-specific manner warrants exploration of this p53 family member in melanoma. Literature to date has not thoroughly investigated expression or function of TP63 in melanoma.

Against this background, the hypothesis of this thesis is that TP63 has a biological role in melanoma, which negatively regulates apoptosis and thus ultimately contributes to the chemoresistance demonstrated in melanoma.

1.5 AIMS OF THIS THESIS

This thesis therefore aims to systematically explore:

(1) The biological role of p63 in melanoma
(2) The regulation of expression of p63 in melanoma
(3) The role of p63 in apoptosis and determine its function in relation to chemosensitivity of melanoma.

CHAPTER 2: MATERIALS AND METHODS

2.1 CELL AND TISSUE CULTURE

2.1.1 Primary melanocyte cultures

Neonatal human embryo melanocytes (NHEM 1 and 2; Cascade biologics) and human epithelial melanocytes from adults (HEMa 3; Cascade biologics) were maintained in 254 medium (Invitrogen™) supplemented with Human Melanocyte Growth Supplement-2 (Invitrogen™) (containing bovine pituitary extract, foetal calf serum (FCS), bovine insulin, bovine transferrin, basic fibroblast growth factor, hydrocortisone, heparin, and endothelin-1). In addition, HEMa lines (HEMa V3 and HEMa V4) developed from freshly isolated patient tissue samples (courtesy of V. Senatore) were maintained in DMEM:F12 media detailed below.

DMEM: F12 media (Ratio 3:1)

Supplemented with	FCS	20%
	Glutamine	1%
	Gentamicin	50 µg/ml
	TPA	100 µM
	hSCF	2 µg/ml
	Cholera Toxin	0.1 µM
	Endothelin	10 µM

TPA – tissue plasminogen activator, hSCF – human stem cell factor

2.1.2 Melanoma cell cultures

Established melanoma cell lines from the Centre for Cutaneous Research Cell bank [Table 2.1] and non-melanoma cell lines were used for experiments in this thesis [Table 2.3].

2.1.3 Cell culture conditions

Cells were grown at 37°C in 10% CO_2 / 90% air using the appropriate media. All melanoma cell lines used in experiments detailed in this thesis, with the exception of those detailed in table 2.2, were cultured in RPMI 1640 media supplemented with 10% FCS and 1% glutamine. Cell culture conditions for non-melanoma cell lines are detailed in Table 2.3.

2.1.4 Freezing and recovery of cells

Cells were washed in phosphate buffered saline (PBS) (PAA) and detached from flasks with trypsin:EDTA 1:1 (PAA), pelleted by centrifugation and resuspended in a solution of 60% FCS, 30% culture media and 10% dimethyl sulphoxide (DMSO). Aliquoted cells in cryotubes were stored in liquid nitrogen. To recover cells from frozen, vials were quickly thawed at 37°C and resuspended in the relevant media. Media was replaced 24 hours later to remove any traces of DMSO.

Table 2.1: Melanoma cell lines

Cell line		Provided by:	TP53 status	BRAF status
Radial growth phase cells (RGP)	WM35	D.C. Bennett (London)	wt	V600E
	WM1575	L. Lanfrancone (Milan)	wt	N/A
	WM1552C	L. Lanfrancone (Milan)	wt	V600E
	SBCl2	B.C. Giovanella (Texas)	wt	wt
Vertical growth phase cells (VGP)	WM793	Wistar Institute	wt	V600E
	WM115	Wistar Institute	wt	V600E
	WM278	Wistar Institute	wt	V600E
	VMM39	Kam. C Yeung	wt	wt
	1402P	M. Rodolfo (Milan)	mutant	V600E
	ME10538	M. Rodolfo (Milan)	wt	V600E
	5810P	M. Rodolfo (Milan)	wt	wt
	Mel 224	M. Rodolfo (Milan)	wt	wt
	Mel 505	Kam. C Yeung	wt	wt
Metastatic melanoma cells	26258M	M. Rodolfo (Milan)	wt	wt
	3988M	M. Rodolfo (Milan)	wt	V600E
	21768M	M. Rodolfo (Milan)	wt	wt
	Mel 501	Kam. C Yeung	wt	V600E
	Sk mel 5	Kam. C Yeung	wt	V600E
	DX3	J. Marshall (BLT)	wt	wt
	LT51	J. Marshall (BLT)	wt	wt
	WM1158	Wistar Institute	wt	V600E
	WM9	Wistar Institute	wt	V600E
	WM852	Wistar Institute	mutant	wt
	A375M	J. Marshall (BLT)	wt	V600E
	C8161	M. Hendrix	wt	wt
	Sk mel 31	Jiri Vachtenheim (Prague)	wt	wt
	MALME-3	Jiri Vachtenheim (Prague)	wt	V600E
	SK mel 24	Kam. C Yeung	wt	V600E
	HBL	G.E. Ghanem (Belgium)	wt	wt
	WM239A	Wistar Institute	wt	wt
	MM-AN	B. Gilchrest (Boston)	wt	N/A
	U1SO	T.K. DasGupta (Chicago)	Null	N/A

wt – wild-type, V600E mutation in BRAF [section 1.1.4.2.1], N/A – information not available.

Table 2.2: Culture media for melanoma cell lines.

Cell line	Culture media	Supplements*
WM1575	TU 2% #^	-
WM1552C	TU 2% #^	-
VMM39	DMEM	-
Mel 224	RPMI 1640	0.5% non-essential amino acids
Mel 505	RPMI 1640	0.5% non-essential amino acids
Mel 501	HAMS-F10	-
Sk mel 5	MEM	-
C8161	RPMI 1640	20mM Hepes+0.1% gentamicin sulphate
Sk mel 31	MEM	-
MALME-3	DMEM	-
SK mel 24	MEM	-
HBL	DMEM	-
MM-AN	DMEM	-
U1SO	MEM#	15% FCS, 1% glutamine, 1% non-essential amino acids, 0.1% gentamicin sulphate

* all media except cell lines marked with # are supplemented with 10% FCS & 1% glutamine

^TU 2% comprises 80% MCDB153 (Sigma), 20% Leibovitz's L-15 (Sigma), 2% FCS, 5 µg/ml insulin (Sigma), 1.68 mM calcium chloride (Sigma).

Table 2.3: Non-melanoma cell lines.

Name	Cell type	Source	Media used*
HaCaT	Immortalised keratinocytes	Karin Purdie (ICMS)	DMEM + 1% Penicillin/Streptomycin
HEK 293T	Immortalised embryonic kidney	Karin Purdie (ICMS)	DMEM + 1% Penicillin/Streptomycin
Phoenix™ retrovirus producer cells	Transformed HEK 293T cells (Packaging cell line)	Monika Cichon (ICMS)	DMEM
CaCo2	Human colonic adenocarcinoma cells	Cheen Khoo (Diabetes, ICMS)	MEM with Earle's salts

*All media was supplemented with 10% FBS and 1% L-glutamine (Invitrogen)

2.1.5 Transfection of mammalian cells

2.1.5.1 Liposomal mediated transfection

Human embryo kidney (HEK 293T) cells were seeded at 70-80% confluency, 24 hrs prior to use in 100 mm dishes. DNA was mixed at a ratio of 1 μg DNA : 2 μl Lipofectamine 2000 (Invitrogen™). 10 μg DNA and 250 μl DMEM media were incubated for 5 mins at room temperature. 20 μl of Lipofectamine 2000 and 250 μl DMEM media were incubated in another eppendorf for 5 mins at room temperature. Finally, DNA and Lipofectamine were mixed and incubated for a further 20 mins at room temperature. 5 mls of DMEM were added to each 100 mm plate. The DNA-Lipofectamine mixture was added to the cells. After 6 hrs, media was replaced with fresh DMEM. Cells were harvested 24 hrs after the transfection.

2.1.5.1.1 Melanoma cell line

Mel 505 cells were seeded at 50% confluency 16 hrs prior to use in 60 mm dishes. DNA was mixed at a ratio of 1 μg DNA: 3 μl FuGENE® 6 Transfection Reagent (Roche). FuGENE® 6 was added to serum-free media and vortexed. The mixture was left to incubate at room temperature for 5 mins. 5 μg DNA (TAp63α, TAp63β, TAp63γ) or 10 μg DNA (ΔNp63α, ΔNp63β, ΔNp63γ) was added to the FuGENE® 6-media mixture, briefly mixed and incubated at room temperature for 20 mins prior to addition to cells in drops. After 6 hrs, fresh serum-containing media was added to the cells.

2.1.5.1.2 Phoenix cells

Cells were seeded in two 60 mm dishes for each construct [Table 2.9] in addition to two control dishes. 1.5 million cells were seeded with a confluency of around 50%. The following day 1 ml of serum-free DMEM was mixed with 25 μl FuGENE® 6 in a bijou tube and left for 5 mins at room temperature. 10 μg of DNA was added and left to incubate at room temperature for a further 20 mins. Media was aspirated from the cells and the DNA/FuGENE® 6 mix was added to the plate and left to incubate at room temperature for 10 mins. 1 ml of serum-free DMEM was added and the plate was left in the incubator at growth conditions for 5 hrs after which a further 2 mls of complete DMEM was added to the plates.

2.1.5.2 Retroviral infection

Melanoma cells were seeded at 40-50% confluency in 6-well plates. After 16 hrs, media was aspirated and replaced with 1 ml / well of media supplemented with 5 μg polybrene (Millipore) for 10 mins at room temperature. Supernatant containing the

virus was defrosted and transferred to a bijoux tube where polybrene (5 µg/ml) was added. Polybrene-media was aspirated from the cells and replaced with 2 mls polybrene-supernatant mixture / well. The plate was centrifuged for 1 hour at 350 *g* at 32ºC and polybrene-supernatant mixture was aspirated. Cells were washed once in PBS. 2 mls of fresh media was added to each well and cells were transferred to 37ºC incubator and left for 48 hrs. After this time, selection of infected cells was undertaken using puromycin (0.9 – 1.25 µg/ml).

2.2 NUCLEIC ACID PROCEDURES

2.2.1 Plasmid DNA preparation

p63 plasmids were kindly donated by G. Melino (MRC, Leicester). The pcDNA3.1 vector with an HA epitope at the N-terminus of the protein was used. The cDNAs of the six isoforms were inserted using the NheI-NotI restriction sites [Figure 2.1].

2.2.1.1 Luria Bertani (LB) growth medium

To support bacterial growth, LB medium was used (containing 10 g/L Bacto-tryptone, 5 g/L yeast extract and 5 g/L NaCl). To ensure selectivity of bacterial growth 150 µg/ml of ampicillin was added to the media. LB agar was melted by heating in a microwave and cooling to 50ºC before pouring into 10 cm petri dishes. For selection of antibiotic resistant bacteria, ampicillin (150 µg/ml) was added and plates were stored at 4ºC. Cells were spread onto an agar plate in LB broth with ampicillin (50 µg/ml) and left overnight to incubate at 37°C.

2.2.1.2 Transformation of competent bacteria

Chemically competent *Escherichia coli (E. coli)* cells (One shot® TOP10; Invitrogen) were thawed on ice for 20 mins. For the transformation, 50 µl of competent *E.coli* cells were mixed with 20 ng DNA and held on ice for a further 20 mins. The mixture was subjected to heat shock for 30 s at 42°C followed by 1 min chill on ice. 500 µl of room temperature media was added and incubated at 37°C for 1 hr to allow expression of the antibiotic resistance marker. A 200 µl aliquot of transformed cell suspension was plated onto agar plates containing ampicillin and incubated overnight at 37ºC to allow colonies to form.

2.2.1.3 Storage of transformed bacteria

Transformed bacterial colonies were maintained for up to 2 weeks on agar plates, tightly sealed and stored at 4ºC. For long term storage, 850 µl of overnight cultures were added to 150 µl of glycerol and vortexed before storing at -20ºC.

2.2.1.4 Small scale plasmid DNA purification

Plasmid DNA was prepared from single colonies inoculated into 5 ml LB (containing 150 µg/ml ampicillin) overnight at 37ºC on a shaker. Cultures were pelleted at 8000 rpm for 3 mins and the pellet was either used immediately or frozen at -80ºC. The QIAprep® Spin MiniPrep Kit (Qiagen®) was used to isolate plasmid bacterial DNA from small scale culture according to the manufacturer's protocol. DNA was eluted in sterile water and stored at -20ºC.

2.2.1.5 Large scale plasmid DNA preparations

Bacterial colonies *(E.coli)* were grown overnight in 500 ml of LB broth (containing 100 µg/ml ampicillin) at 37°C on a shaker. Bacteria were pelleted by centrifugation at 4000 rpm for 15 mins using the Beckman JA-10 rotor. The Qiagen® Plasmid Maxi Kit (High Speed) protocol was used to isolate DNA from the large scale bacterial cultures according to the manufacturer's protocol. DNA was re-dissolved in 250 µl of sterile water and stored at -20ºC.

2.2.2 DNA quantification

The concentration of DNA in solution was determined spectrophotometrically (Nanodrop® ND-1000 Spectrophotometer) using water as a blank. The quality of RNA was assured by an $OD_{260/280}$ greater than 1.9.

2.2.3 RNA extraction and first strand cDNA synthesis

Total RNA was extracted from cells using RNeasy Mini Kit (Qiagen®) following the manufacturer's recommended protocol. From total RNA (500 ng – 1 µg) first strand cDNA was synthesised using Superscript™ III Reverse Transcriptase (Invitrogen™). For all reactions except for detection of ΔNp63 cDNA, oligo$(dT)_{12\text{-}18}$ primer (Invitrogen™) was used for cDNA synthesis. For generation of ΔNp63 cDNA, gene-specific primers were used to maximise detection of the gene.

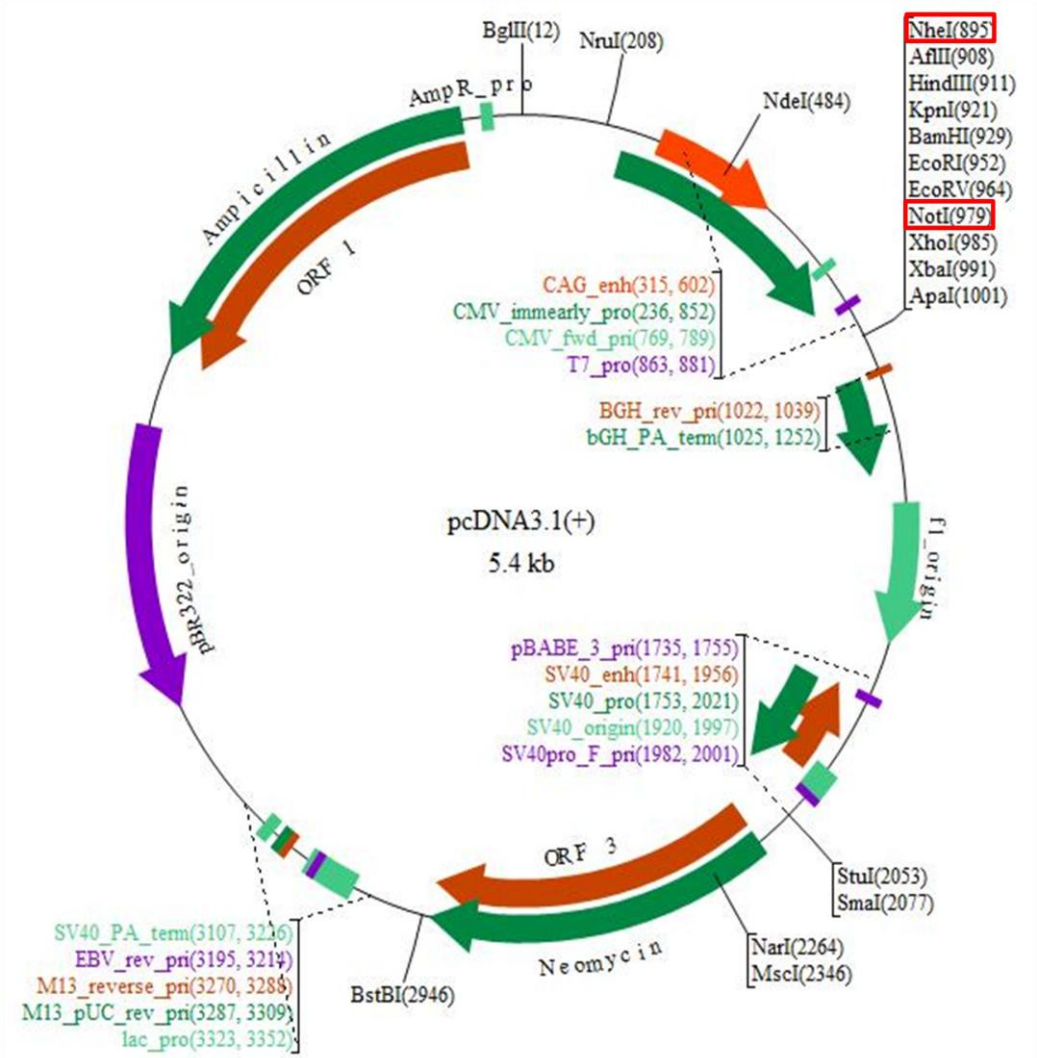


Figure 2.1: pcDNA3.1 vector. cDNAs of six isoforms of p63 (TAα, β, γ and ΔN α, β and γ) were inserted using the Nhel-NotI restriction sites (red boxes).

2.2.4 Reverse transcriptase - Polymerase chain reaction (RT-PCR)

2.2.4.1 Preparation of oligonucleotides

Oligonucleotides were supplied as dried pellet from Sigma-Aldrich® oligonucleotide synthesis service. Oligonucleotides were resuspended in DNase- and RNAse-free distilled water (Qiagen®) to make a 100 mM stock. These were stored at -20ºC.

2.2.4.2 PCR reaction

PCR was carried out using 1.1x ThermoStart™ Reddymix™ (Thermo Scientific) for TA and ΔN (all isoforms) and GAPDH reactions [Table 2.6]. Primer sequences (Borrelli et al. 2007; Koga et al. 2003; Yang et al. 1998) are shown in figures 2.2 and 2.3 and are detailed in table 2.4. The PCR reaction was consistently performed using the same thermocycler (DNA Engine Tetrad 2) [cycling protocol – Table 2.7].

2.2.4.3 Agarose gel electrophoresis

Agarose gels of 1-2% (wt/vol) were prepared in 100 ml of 1 x Tris-Borate-EDTA (TBE) (Sigma-Aldrich®) buffer in a microwave and allowed to cool before addition of ethidium bromide (final concentration 0.5 µg/ml). Reddymix™ PCR buffer contains an inert red tracker dye and DNA samples were loaded directly onto the agarose gel. TBE buffer was used as a running buffer for electrophoresis at 120 V. DNA fragments were visualised by UV light.

2.2.4.4 Optimisation

Hek293T and Mel 505 cells transfected with each of the p63 isoforms were used to assess the efficiency of the PCR reaction for each primer set. GAPDH was used as the housekeeping gene. RT negative samples and PCR negative (water only) samples were run with each PCR reaction as a control.

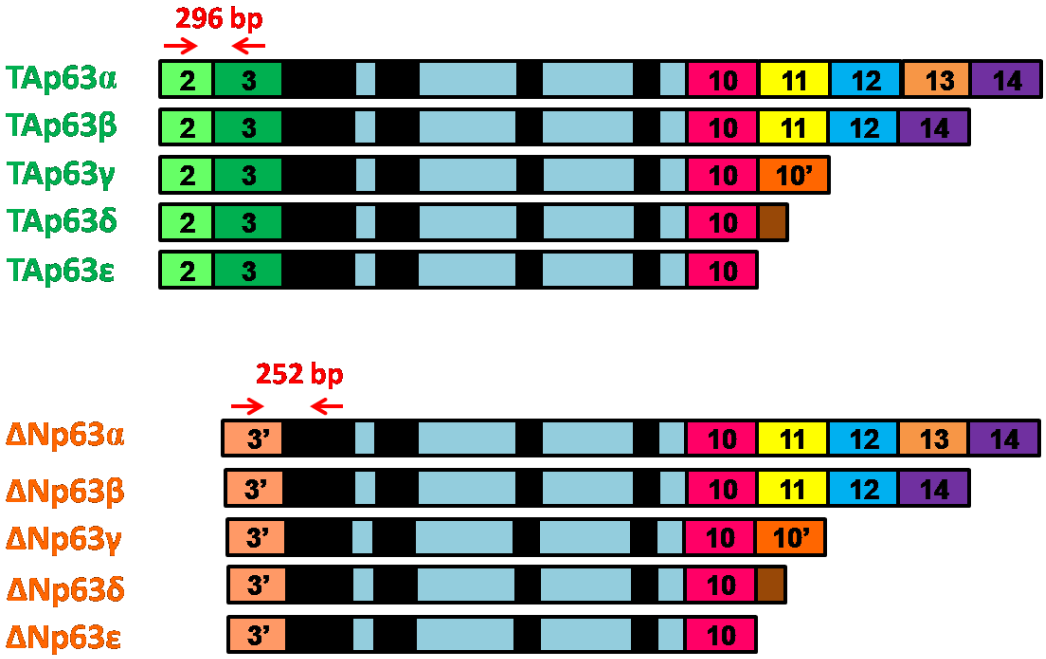


Figure 2.2: Specific annealing region of primers used to detect TP63 isoforms. Specific region of primer sets to anneal to p63 isoforms [Table 2.4]; top panel shows region used to detect all TA isoforms of p63 (296 bp product) (Koga et al. 2003) and lower panel shows region used to detect all ΔN isoforms of p63 (252 bp product) (Yang et al. 1998). Primer sets used for RT-PCR and Q-PCR.

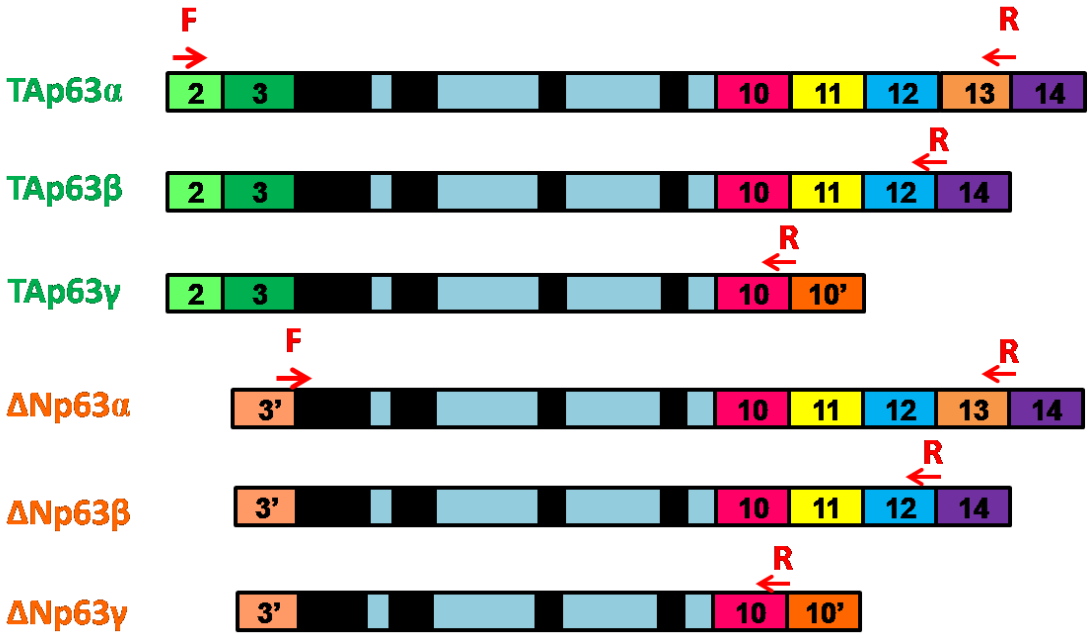


Figure 2.3: Specific annealing region of primers used to detect p63 splice variants. Primer set [Table 2.4] positions for annealing to TP63 are shown, to detect individual splice variants of p63 by RT-PCR. Primer sequences from Borrelli et al. (2007).

Table 2.4: Oligonucleotide primer sequences used for RT-PCR and Q-PCR#

Primer	Sequence (5' to 3')		Annealing temperature (ºC)
	Forward	Reverse	
TAp63 #	**GGTGCGACAAACAAGATTGAG**	**GAAGGACACGTCGAAACTGTG**	62.4
TAp63α^#	TTAGCATGGACTGTATCCGC	ACTTGCCAGATCATCCATGG	58.3
TAp63β^#	TTAGCATGGACTGTATCCGC	TCAGACTTGCCAGATCCTG	59
TAp63γ^#	TTAGCATGGACTGTATCCGC	AAGCTCATTCCTGAAGCAGG	54
ΔNp63 ~#	**GGAAAACAATGCCCAGACTC**	**GAAGGACACGTCGAAACTGTG**	60
ΔNp63α^#	CCAGACTCAATTTAGTGAGC	ACTTGCCAGATCATCCATGG	54
ΔNp63β^#	CCAGACTCAATTTAGTGAGC	TCAGACTTGCCAGATCCTG	56
ΔNp63γ^#	CCAGACTCAATTTAGTGAGC	AAGCTCATTCCTGAAGCAGG	54
p53	**GTCACTGCCATGGAGGAGCCGCA**	**GACGCACACCTATTGCAAGCAAGGGTTC**	67
∆Np73**	**CAACAAACGGCCCGCATGTTCCCC**	**GCGACATGGTGTCGAAGGTGGAGC**	60
GAPDH	CTCCTCCACCTTTGACGCTG	CCACCCTGTTGCTGTAGCCA	55
GUS#	AAACGATTGCAGGGTTTCAC	CTCTCGTCGGTGACTGTTCA	60

* (Koga et al. 2003); ^ (Borrelli et al. 2007); ~ (Yang et al. 1998); ** (Petitjean et al. 2008). **Primers in bold detect all isoforms of a gene**

Table 2.5 Oligonucleotide primer sequences used to detect C-terminal isoforms by Q-PCR (Mangiulli et al. 2009)

Primer	Sequence (5' to 3')		Annealing temperature (ºC)
	Forward	Reverse	
p63α	GATGGGCACCCACATGCCAAT	CAGCCCAACCTCGCTAAGAAAC	60
p63β	GATGGGCACCCACATGCCAAT	TTTCAGACTTGCCAGATCCTGAC	60
p63γ	CAGCAGCACCAGCACTTACTTC	CTATGGGTACACTGATCGGTTTG	60
p63δ	CAGCAGCACCAGCACTTACTTC	ATTTTCAGACTTGCCAGATCTGTTG	60
p63ε	CAGCAGCACCAGCACTTACTTC	AAGGTTGCAACTGAAAGAGGG	60

Table 2.6: Mastermix components for RT-PCR reaction.

Thermostart™ Reddymix™ Master Mix (x1)		Volume for 25 µl reaction (µl)
Thermostart™ Reddymix™	0.625 units ThermoStart™ Taq DNA polymerase	21
	1x ThermoStart™ reaction buffer	
	3 mM $MgCl_2$	
	0.2 mM each of dATP, dCTP, dGTP, dTTP	
Forward primer (10 µM)		1
Reverse primer (10 µM)		1
DNA template (100 ng)		2

Table 2.7: RT-PCR amplification cycling protocol

Cycle step	Duration of cycle	Temperature (ºC)	Cycles
Initial denaturation	15 mins	95	1
Denaturation	25 s	95	40
Annealing	35 s	50-60*	
Extension**	1 – 2 mins	72	
Final extension	5 mins	72	1

*annealing temperature variable [Table 2.4/2.5]

** extension time varied according to size of amplicon – Taq polymerase extended up to 1000 bp/min

2.2.5 Quantitative RT-PCR (Q-PCR)

2.2.5.1 Generation of standards

Primers were tested by RT-PCR as described [section 2.2.4]. The StrataPrep DNA gel extraction kit (Stratagene) was used to extract gel fractionated DNA from an agarose gel according to the manufacturers' instructions. The desired fragment was cut from the gel, placed in a microcentrifuge tube, combined with the DNA extraction buffer, incubated at 50°C, and transferred to a microspin cup containing a silica-based fibre matrix. The DNA bound to the fibre matrix in the microspin cup and contaminants were washed from the microspin cup with a wash buffer. The purified DNA was eluted from the fibre matrix with a low-ionic strength buffer and captured in a microcentrifuge tube. Double-stranded DNA ≥100 bp was retained. The concentration of purified products was quantified using a Nanodrop® Spectrophotometer and samples were diluted 1:2. Prior to each Q-PCR experiment original standards were serially diluted 1:5 to obtain 6 standards of decreasing concentration to create a standard curve.

2.2.5.2 Q-PCR reaction

Q-PCR reactions were set up in 96-well plates using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies). This Master Mix includes a SureStart Taq DNA polymerase with hot start capability and contains $MgCl_2$ at a concentration of 2.5 mM in the 1x solution. A passive reference dye (ROX) was added to the mix to compensate for non-PCR related variations in fluorescence. Reactions were set up by combining components in the order shown in Table 2.8 for 25 µl reactions. Experimental reactions were performed in triplicate and duplicate no-template controls were also run.

Once sample cDNA was added, the 96-well plate was briefly centrifuged to remove bubbles and ensure mixing. Data were collected by running a three-step cycling protocol using the AB7500 Fast Realtime PCR System (Applied Biosystems) [Table 2.9]. The temperature cycler was set to detect and report fluorescence during the annealing and extension step of each cycle. Formation of non-specific products was checked using gel analysis.

Table 2.8: Mastermix components for Q-PCR reaction

Master Mix (x1)	Volume (µl)
H_20	8.125
SYBR® green mix	12.5
Forward primer (5 µM)	1
Reverse primer (5 µM)	1
Rox reference dye (1:500)	0.375
Sample DNA	2

Table 2.9: Three-step cycling protocol for Q-PCR reaction

Cycles	Duration of cycle	Temperature (ºC)
1	10 mins	95
40	30 s	95
	1 min	50-60*
	30 s	72

*variable annealing temp [Table 2.4]

2.2.5.3 Analysis of Results and Normalisation

The comparative threshold (CT) method was used to determine the relative ratio of transcripts. Fold changes of expression were calculated using the formula $2^{-\Delta\Delta CT}$ where the CT represented the threshold cycles of the specific gene and the endogenous control, GUS. ΔCT was calculated as the average CT for target variant minus the average CT of endogenous GUS or GAPDH in each sample, and the ΔΔCT was calculated as the ΔCT of the sample minus the ΔCT of the calibrator. For expression of p63, the mean expression in five primary melanocyte cultures was used as the comparator. For shRNA and cell toxicity experiments, sh-scramble and untreated cells, were used as comparators, respectively.

2.2.6 Cloning

Cloning was undertaken to construct short hairpin RNA expression vectors for transfection into melanoma cell lines [section 2.1.5.2]. The vector used for cloning these sequences was the pSUPERIOR.retro.puro vector [Oligoengine; Figure 2.4]. Forward and reverse strands of the oligonucleotide sequences containing the siRNA-expressing sequence targeting p63 [Table 2.10] were annealed according to the

design strategy shown in Figure 2.5. 10 µl of the forward and reverse strand of the oligonucleotide sequences were blocked at 95ºC for 10 mins and then cooled down to room temperature. Phosphorylation was carried out using E kinase (New England Biolabs) using the following components:

Primer mix	2 µl
E kinase	1 µl
ATP	1 µl
Buffer	1 µl
H_2O	5 µl

Samples were incubated at 37ºC for 30 mins then 65ºC for 20 mins and stored at –20ºC.

2.2.6.1 Restriction enzyme digestion

10 µg of the pSUPERIOR.retro.puro vector was linearised by digestion with BglII and HindIII overnight at 37ºC in a water bath. To check that the digestion had worked, 2 µl of the products were run on a 1% agarose gel. Following digestion of the vector, 5' phosphate groups were dephosphorylated from the linearised plasmid by treatment with calf intestinal alkaline phosphatase 0.1 units (CIP, Promega) for 1 hr at 37ºC, to prevent re-circularisation of the plasmid. The digested vector was run out on a 1% agarose gel and the band visualised under a UV lamp was isolated and cut out using a scalpel. The resulting vector DNA was extracted by gel purification using StrataPrep® DNA gel extraction kit (Stratagene) according to the manufacturers protocol [section 2.2.5.1].

2.2.6.2 Ligation of DNA fragments

Ligation of the dephosphorylated vector and purified insert DNA was performed at a ratio of insert:vector DNA of 3:1. Insert and vector DNA were combined in the presence of T4 DNA ligase buffer and enzyme (Promega) to a final volume of 10 µl and incubated at 16ºC overnight. Half of the ligation mixture was used to transform competent bacterial cells as previously described [section 2.2.1.3].

<u>T4 DNA ligase reaction buffer (10X)</u>

Tris-HCl (pH 7.8 at 25°C)	300 mM
$MgCl_2$	100 mM
DL-Dithiothreitol (DTT)	100 mM
ATP	10 mM

2.2.6.3 Verification of plasmid constructs

To ensure that cloning was successful, constructs were directly sequenced using sequencing primers specific to sequences cloned into vectors used [Table 2.10]. The vector was transformed in bacteria.

2.2.7 RNA interference procedures

2.2.7.1 Transient transfection with short interfering RNA (siRNA)

Silencing of genes was achieved by transfection of DNA sequences [Table 2.10] into melanoma cells using HiPerFect transfection reagent (Qiagen®). A stable, fluorescent form of Cyclophilin B, siGLO Cyclophilin B Control siRNA (Dharmacon), was used to assess transfection efficiency. A pool of three validated Silencer® siRNA sequences (Applied Biosystems™) – siRNA-A; siRNA-B and siRNA-C, was used for silencing all p63 isoforms.

2.2.7.2 Stable infection with short hairpin RNA (shRNA)

The pSUPERIOR.retro.puro vector constructs containing annealed oligonucleotide sequences targeting all p63 isoforms and TAp63 and ΔNp63 individually [Table 2.10] was transfected into the packaging cell line Phoenix cells, before introducing the retrovirus into the melanoma cell lines. Infected cells were selected using puromycin (0.9 - 1.25 µg/ml) to establish a stable cell line for shRNA expression which is transcribed in cells from a DNA template as a single-stranded RNA molecule [Figure 2.6]. Effects on mRNA levels of p63 were subsequently assayed [section 2.2.5].

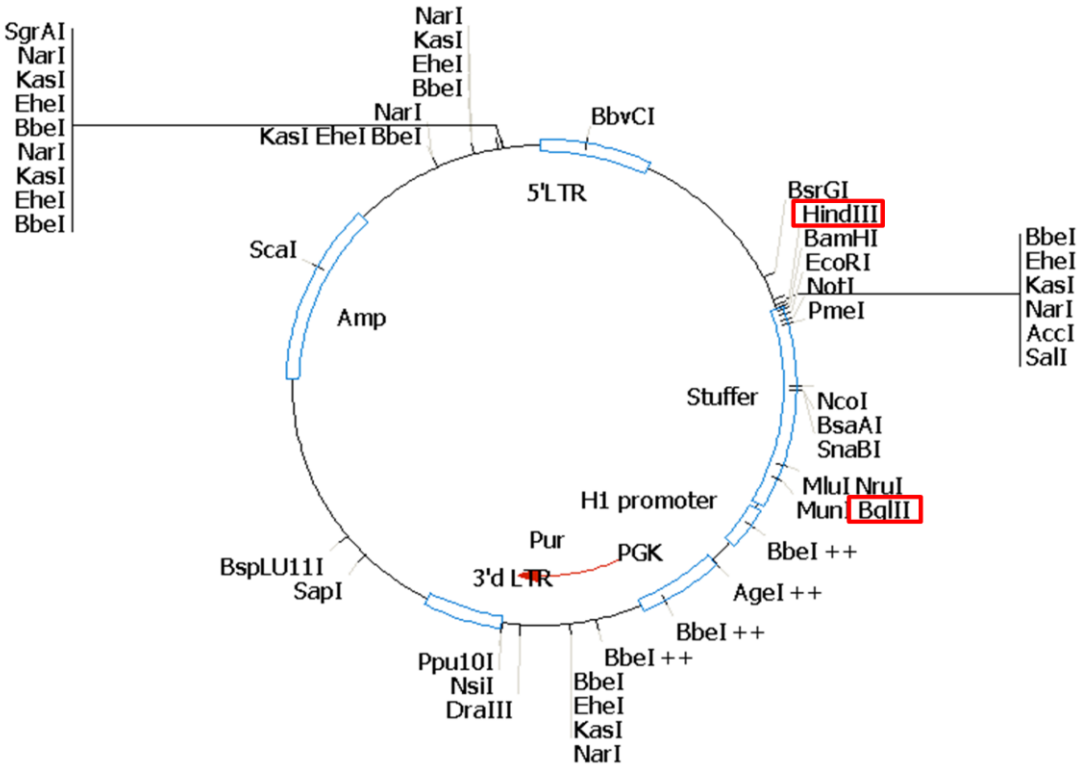


Figure 2.4: pcDNA for shRNA construct. The pSuperior.retro.puro vector was used for shRNA cloning of oligonucleotide sequences targeting p63 [Table 2.10]. Length of plasmid 7296 bp. Vector was linearised using digestion sites BGlII 2424 and HindIII 1441 (shown by red boxes).

Figure 2.5: Design strategy for creating short hairpin RNA template insert. Annealed complementary oligonucleotides are used to create a synthetic DNA duplex for cloning. This is the most commonly reported method for making shRNA constructs (74% of surveyed studies) which requires the synthesis, annealing and ligation of two complementary oligonucleotides into an expression vector. Image reproduced from McIntyre and Fanning (2006).

Table 2.10: RNAi targeting sequences

Gene target	Source	Target sequence
Itch (pool)	Dharmacon	GUUGGGAACUGCUGCAUUA
		CAACAUGGGACGUAUUUAU
		GAAAUUAAGAGUCAUGAUC
		CGAAGACGUUUGUGGGUGA
p63 siRNA-A*	Applied Biosystems™ (ID 217143)	CAUGCAAAGUUAACACACGAC
p63 siRNA-B*	Applied Biosystems™ (ID 4893)	GCACUUAAGUCACGGUUGGAC
p63 siRNA-C*	Applied Biosystems™ (ID 217144)	AAGUCAAGUCACCUUAUGCAG
p63 siRNA-D~	Mario Rossi, MRC Leicester	AACUUUGUGGAUGAACCAUCA
p63 siRNA-E#	Mario Rossi, MRC Leicester	AAGCCCAGACUCAAUUUAGUG

*siRNA sequences used in combination for siRNA-p63 pool

~ siRNA sequence designed to target TAp63 only

siRNA sequence designed to target ΔNp63 only

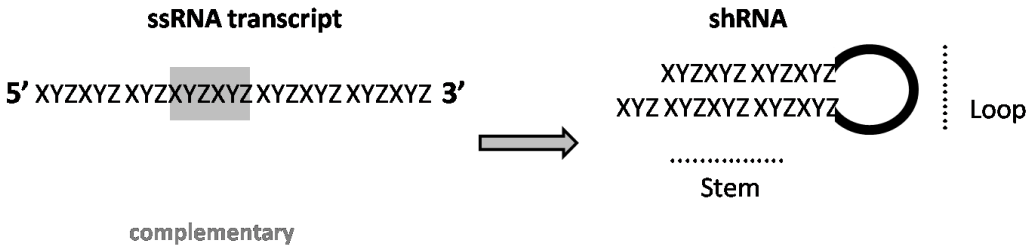


Figure 2.6: shRNA expression in cells. shRNA has the advantage of long-term and stable silencing with improved delivery of silencing to all cells. Expressed shRNA is transcribed in cells from a DNA template as a single-stranded RNA molecule (~50 – 100 bases). Complementary regions spaced by a small 'loop' cause the transcript to fold back on itself forming a 'short hairpin' - a stem-loop structure, in a manner analogous to natural microRNA. Recognition and processing by the RNAi machinery converts the shRNA into the corresponding siRNA. ssRNA – single stranded RNA. Figure adapted from McIntyre and Fanning (2006).

2.3 PROTEIN MANIPULATION

2.3.1 Preparation of whole cell lysates

Cells were trypsinized and washed with PBS followed by lysis in TGN buffer (150 µl/100 mm dish) in an Eppendorf. They were subjected to three rapid freeze/thaw cycles between dry ice and a 37°C water-bath. After a minimum of 30 mins on ice, lysates were clarified by centrifugation at 13 000 rpm for 15 mins at 4°C and then stored at -20ºC.

TGN Buffer (Total 50 ml)

Tris 1 M	2.5 ml
NaCl 2.5 M	3 ml
Glycerol	5 ml
Glycerophosphate 0.5 M	5 ml
Tween® 20	0.5 ml
Nonidet® P40	100 µl
H_20	33.9 ml

Complete mini protease inhibitor cocktail (PIC; Roche) 1 tab in 5 ml TGN buffer prepared freshly.

2.3.2 Preparation of mitochondrial extracts

This method was adapted from Mihara and Moll (2003) and Arnoult (2008). Three x 10 cm dishes of cells at a confluency of 70-80% were required to obtain sufficient levels of mitochondrial protein. All steps were performed on ice or at 4°C. Each plate was washed once with 1 ml ice cold PBS/1 mM EDTA before scraping the cells into a 15 ml falcon tube. Cells were pelleted at 750 *g* for 5 mins and then washed in ice cold PBS (3 mls). The cells were pelleted again for 5 mins at 750 *g*. The pellet was then resuspended in 400 µl of cold mitochondrial isolation buffer (MIB) buffer with protease inhibitor cocktail (PIC) (1:20). Cells were transferred to an ice-cold Dounce homogeniser (Wheaton) and homogenised with a minimum of 150 strokes, whilst monitoring under the microscope. Trypan blue staining was used to confirm cell membrane disruption. The solution was transferred to an Eppendorf and centrifuged for 5 mins at 800 *g* to isolate the nuclear fraction. The pellet was used in the nuclear isolation protocol [Section 2.3.3]. The supernatant was centrifuged for 30 mins at 10 000 *g* where the resulting pellet contained the mitochondrial fraction. The supernatant containing cytoplasmic proteins was added to the Eppendorf containing the nuclear

fraction which was to be used in the nuclear isolation protocol. The mitochondrial pellet was washed in 500 µl of 1 x MS buffer (+ PIC) and centrifuged again at 10 000 *g* for 15 mins. The mitochondrial fraction was resuspended in 50 µl of MIB buffer with 1% Triton-X 100. Protein concentration was determined [Section 2.3.4] and assayed using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

MIB buffer (stored at 4ºC)	
Mannitol	210 mM
Sucrose	70 mM
EDTA	1 mM
HEPES	10 mM

MS buffer (stored at 4°C)	
Mannitol	210 mM
Sucrose	70 mM
EDTA	5 mM
Tris-Cl, pH 7.6	5 mM

Hepes buffer (stored at 4ºC for <1 mth)	
Hepes-KOH, pH 7.4	20 mM
KCl	10 mM
$MgCl_2$	1.5 mM
EGTA pH7.4	1 mM
EDTA pH7.4	1 mM

RIPA buffer	
NaCl	150 mM
Tris pH 7.5	50 mM
Nonidet®-P40	1%
Sodium deoxycholate	0.05%
SDS	1%

PIC solution was freshly prepared (1 tab in 2 ml H_2O) and added to each solution (1:20).

2.3.3 Preparation of nuclear and cytoplasmic extracts

The nuclear pellet and cytoplasmic supernatant from the mitochondrial extract method was resuspended and centrifuged at 900 *g* for 5 mins at 4ºC. The supernatant was discarded. The pellet was resuspended in 300 µl nuclear isolation lysis buffer (NI) supplemented with PIC and incubated on ice for 15 mins. This was centrifuged at 3500 rpm for 10 mins at 4ºC. The supernatant was transferred to a new Eppendorf labelled cytoplasmic fraction. The pellet was resuspended again in 300 µl ice cold NI lysis buffer (+ PIC), centrifuged at 3500 rpm for 10 mins at 4ºC and supernatant was transferred to the cytoplasmic fraction Eppendorf. The nuclear pellet was washed in 1 ml ice-cold Tris-EDTA twice and centrifuged at 3500 rpm for 10 mins at 4ºC between each wash. The supernatant was discarded each time. The pellet was resuspended in 300 µl ice cold TGN buffer (+PIC) and incubated on ice for a total of 30 mins; vortexing every 10 minutes. The sample was centrifuged again at 15 000 rpm for 30 mins at 4ºC. The protein concentration of the supernatant

containing nuclear proteins was determined [section 2.3.4] and assayed using SDS-PAGE.

NI lysis buffer

Tris pH 6.5	10 mM
Sodium bisulphate	50 mM
$MgCl_2$	10 mM
Sucrose	8.6%
Triton X-100	1%
Made up with H_2O	

PIC solution was freshly prepared (1 tab in 2 ml H_2O) and added to each solution (1:20).

Tris-EDTA

Tris pH 7.4	10 mM
EDTA	13 mM

2.3.4 Protein concentration

Protein concentration of extracts was estimated using the Bio-Rad protein assay reagent, based on the Bradford dye-binding procedure (Bradford 1976). This colorimetric assay allows determination of protein concentration by reading spectrophotometric differences at a wavelength of 595 nm. Protein extracts were thawed on ice and 1 µl added to 200 µl of Bio-Rad reagent (1:5 dilution) in a 96-well plate. Solutions were left to incubate for 10 mins at room temperature. The Synergy HT Multi-Mode Microplate Reader (Bio-Tek) was used at 595 nm to measure the optical density of the samples. Protein concentration was calculated using the constant obtained from a bovine serum albumin (BSA) (1 mg/ml) standard curve, by plotting optical density generated by increasing BSA concentrations on the y-axis against quantity of protein in the BSA sample (0-15 µg) on the x-axis. Protein lysates at concentrations between 50 and 80 µg were loaded for separation using SDS-PAGE.

2.3.5 Sodium Dodecyl Sulphate (SDS) polyacrylamide gel electrophoresis

Gel mixture was prepared [Table 2.11] and poured between a sandwich of two clean glass plates separated by spacers, then overlaid with isopropanol (200 µl) and left to polymerise at room temperature. Once set, isopropanol was removed with residual

traces absorbed by blotting paper. An SDS stacking gel was prepared and added to the polymerised resolving gel, and a 10- or 15-lane well-forming comb was inserted, enabling loading of the protein sample. Stacking gels (3 ml) were prepared as described in Table 2.12. Once polymerised the gel was transferred to an electrophoresis running tank filled with 1 x SDS-PAGE running buffer, and the comb removed.

10x SDS-PAGE Running Buffer

Trizma Base 30 g
Glycine 144 g
SDS 10 g
Distilled water to 1 L

Prior to loading, the protein samples were denatured by boiling at 95ºC for 5 mins in 1 x NuPage® LDS Loading Buffer (Invitrogen™). Electrophoresis was performed at 100 V until separation of the Benchmark Protein marker (Invitrogen™) was visualised and then continued at 150 V. The gel was transferred to a solid support for immunodetection.

2.3.6 Transfer of proteins to solid support

Nitrocellulose transfer membrane (Protran®, Whatman®) was cut to size (9 x 7 cm) and directly wetted in 1 x Transfer Buffer prior to being placed on the SDS gel. Two sheets of 3 mm Whatman paper were used to cover the membrane and gel on either side, ensuring no air bubbles remained trapped between the layers. The apparatus was transferred in the transfer electrophoresis chamber (Hoefer) filled with 1 x Transfer buffer (with 20% methanol) for 3 hrs at a constant voltage of 55 V or for 18 hrs at 25 V.

10X TRANSFER BUFFER

Trizma Base 30 g
Glycine 144 g
Distilled water up to 1 L

Table 2.11: Volume of components for resolving gel

Resolving gel (10 ml total)	8% gel (ml)	10% gel (ml)	12% gel (ml)
30% acrylamide solution (National Diagnostics)	1.3	1.7	2
1M Tris pH 8.8	1.3	1.3	1.3
10% SDS	0.05	0.05	0.05
10% ammonium persulphate	0.05	0.05	0.05
TEMED (Sigma)	0.003	0.002	0.002
Distilled water	2.3	1.9	1.6

TEMED – Tetramethylethylenediamine

SDS – sodium dodecyl sulphate

Table 2.12: Volume of components for stacking gel

Stacking gel (3 ml total)	Volume (ml)
30% acrylamide solution (National Diagnostics)	0.5
1M Tris pH 6.8	0.38
10% SDS	0.03
10% ammonium persulphate	0.03
TEMED (Sigma)	0.003
Distilled water	2.1

TEMED - Tetramethylethylenediamine

SDS – sodium dodecyl sulphate

2.3.7 Immunodetection

Following protein transfer, membranes were blocked in either PBS-Tween 0.1% or TBS-Tween 0.1% supplemented with 5% (wt/vol) powdered milk (BioRad) for 1 hr at room temperature with gentle agitation. The membrane was incubated with the primary antibody [Table 2.13] diluted at the appropriate concentration in the same solutions, for 2 hrs at room temperature or overnight at 4ºC. Membranes were washed three times for 5 mins in PBS-Tween 0.1% at room temperature and incubated for 1 hr at room temperature with species specific horseradish peroxidase-conjugated secondary antibody (DAKO), at a dilution of 1:10,000 (vol/vol) in PBS-Tween 0.1% supplemented with 5% (wt/vol) powdered milk (BioRad) [Table 2.14]. Membranes were washed a further three times for 5 mins with PBS-Tween 0.1%, and bound secondary antibody was detected using an enhanced chemiluminescence method with ECL Plus Western blotting detection method (GE Healthcare) according to the manufacturer's protocol. Briefly the two components of the kit, solutions A and B, were mixed just prior to covering the air-dried membranes in a 40:1 proportion and incubated for 5 mins exposure. The reagent was then removed, membranes were briefly air dried and placed securely within a transparency in a film cassette and exposed for autoradiography.

TBS (10X)

Trizma Base	24.2 g
NaCl	80 g

Distilled water up to 1 L

Adjust pH to 7.5 with HCl

Where possible, a single membrane was probed for multiple proteins of varying molecular weight, in particular p63 and loading controls were always probed using the same membrane. A number of p63 antibodies detecting different amino acid regions of the p63 protein were used [Figure 2.7].

Table 2.13: Primary Antibodies used for protein detection

Target (name)	Primary antibody	Supplier	Dilution for WB	Conditions
Cox IV (12C4)	Mouse monoclonal	Molecular Probes	1:500	Room temp, 2 hrs
GAPDH (G9545)	Rabbit polyclonal	Sigma-Aldrich®	1:2500	Room temp, 2 hrs
Itch	Mouse monoclonal	BD Biosciences	1:500	4ºC O/N
Lamin A/C	Rabbit polyclonal	Cell signalling	1:500	4ºC O/N
Mt-Hsp70 (Grp75; JG1)	Mouse monoclonal	Abcam	1:500	4ºC O/N
p53 (DO-1)	Mouse monoclonal	Cancer Research UK	1:300	Room temp, 2 hrs
p63* (Ab-1 or Ab-4)	Mouse monoclonal	Neomarkers, CA	1:200 – 1:500	4ºC O/N
p63 * (4A4)	Mouse monoclonal	Santa Cruz	1:200	4ºC O/N
p63 * (H129)	Rabbit monoclonal	Santa Cruz	1:300	4ºC O/N
p63α* (H137)	Rabbit monoclonal	Santa Cruz	1:300	4ºC O/N
PARP	Rabbit polyclonal	Cell signalling	1:1000	4ºC O/N
β-Actin (A5441)	Ascites fluid	Sigma-Aldrich®	1:1500	Room temp, 2 hrs

WB – western blotting, PARP – poly-(ADP ribose) polymerase, GAPDH – glyceraldehyde 3-phosphate dehydrogenase

*Position of anti-p63 antibodies targeting p63 protein shown in figure 2.7

Table 2.14: Secondary Antibodies used for protein detection

Conjugated	Target	Secondary antibody	Source	Dilution
HRP	Mouse IgG	Goat polyclonal	Dako	1:10 0000
HRP	Rabbit IgG	Goat polyclonal	Dako	1:10 0000
HRP	Goat IgG	Rabbit polyclonal	Dako	1:10 0000

HRP - Horseradish peroxidase

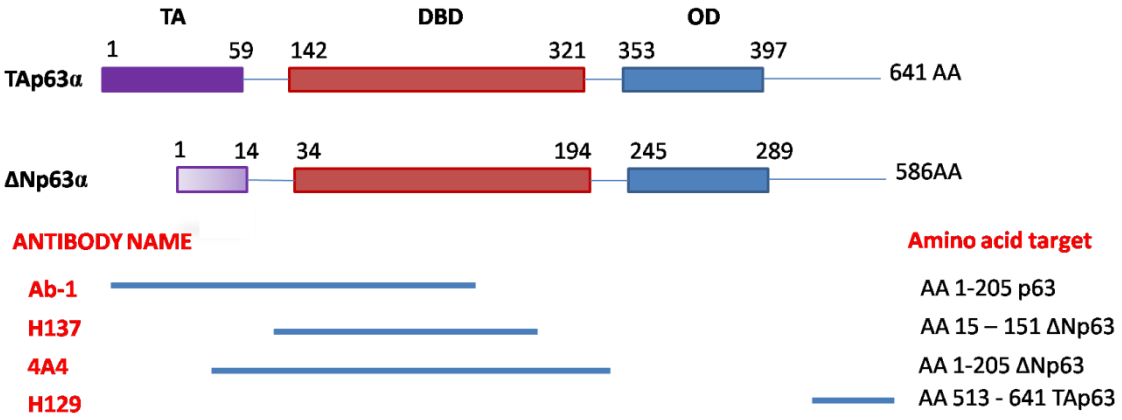


Figure 2.7: Specificity of different anti-p63 antibodies. Protein structure of the full length TA and ΔN isoforms of p63 shown in the top panel. Lower panel showing blue line which marks region of protein that the anti-p63 antibody (labelled in red on left) detects. The amino acid target of each antibody is shown on the right side of the corresponding blue line. AA – amino acid.

2.4 DNA DAMAGE ANALYSIS TECHNIQUES

2.4.1 DNA damaging agents

2.4.1.1 UVB irradiation

Cells were seeded at a density of $1x10^6$ cells / cm^2 in 100 mm culture dishes. Prior to irradiation, cells were washed with PBS, which was then completely aspirated. A CL-1000 ultraviolet cross linker (UVP) fitted with F8-T5 UVB lamps with peak output at 312 nm, was used to irradiate cells with a single dose ranging from 5 mJ/cm^2 to 50 mJ/cm^2 (depending on the experiment). Following irradiation, fresh media was replaced and cells were cultured as described until harvested.

2.4.1.2 Chemotherapeutic agents

Cells were seeded at a density of $1x10^6$ cells / cm^2 in 100 mm culture dishes. Prior to treatment with DNA damaging agents, cells were washed with PBS, which was then completely aspirated. Cells were treated with chemotherapeutic agents at various doses [Table 2.15]. All chemotherapeutic agents were diluted from a stock solution in distilled sterile water and then diluted to the final concentration in fresh media. Treated cells were harvested at different time points from 90 mins to 72 hrs.

2.4.2 Thiazolyl Blue Tetrazolium Bromide (MTT) assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used as a measurement of cell proliferation and thus indirect measure of cell death. MTT (Sigma-Aldrich®) is a yellowish solution which is converted to water-insoluble MTT-formazan of dark blue colour by mitochondrial dehydrogenases of living cells. These blue crystals are solubilised with DMSO and the intensity is measured colourimetrically.

Cells were seeded at a density of 7 500 cells / well in 96-well plates and treated with chemotherapeutic drugs at various doses. Media was removed from the cells and replaced with 200 µl MTT solution diluted in growth media (final concentration 0.6 mg/ml), and cells were incubated for 3 hrs. The solution was removed and 25 µl of Sorensen's glycine buffer was added to each well and left at room temperature for 10 mins. 200 µl of DMSO was added to each well and plates were gently agitated for a further 10 mins. Readings were taken using a spectrophotometer (Synergy HT Multi-Mode Microplate Reader; Bio-Tek) reading at wavelength of 690 nm and then at a wavelength of 570 nm. The former reading was deducted from the latter and normalised to the untreated cells. Experimental readings were performed in triplicate

and expressed as mean readings ± SEM for a minimum of two independent experiments.

Sorensen's Glycine Buffer (pH 10.5)

Glycine 0.1 mM

NaCl 0.1 M

Adjust pH using 0.1 M NaOH

Table 2.15: Chemotherapeutic agents

Drug	Source	Dose (µM)
Cisplatin	Oncon-Tain®, Mayne Pharma PLC	1 – 20
Etoposide	Medac UK, Pharmachemie BV	1 – 20
Doxorubicin	Teva UK, Pharmachemie BV	0.5 – 2
Paclitaxel	Peter Szlosarek, Institute of Cancer	0.5 – 2
Dacarbazine	Medac, Germany	100 – 1000 µg/ml
Tenovin-6	Sonia Lain, Karolinska Institute	5 – 15
Trichostatin A (TSA)	Sigma	500 – 1000
5-Azacytidine (5-Aza)	Sigma	5 – 10

2.5 FLOW CYTOMETRY METHODS

Flow cytometry methods are used to measure a variety of physical and chemical characteristics, such as cell size and shape, of whole cells or organelles as they travel in suspension past a source of light. Flow cytometry was used in a number of assays:

1) To analyse apoptotic cell death in response to DNA damage (Annexin V)
2) To detect the presence and translocation of intracellular proteins
3) To detect the presence of extracellular proteins (CD133)
4) To use fluorescence activated cell sorting (FACS) to separate cells

2.5.1 Flow cytometric analysis of apoptosis

In normal cells, phosphatidylserine (PS) residues are found in the inner membrane of the cytoplasmic membrane. During apoptosis, the PS residues are translocated in the membrane and are externalised. In general, this is an early event in apoptosis and considered a signal to neighbouring cells that a cell is ready to be phagocytosed. Annexin-V, a specific PS-binding protein was used to detect apoptotic cells.

Cells were treated with chemotherapeutic agents for 16-24 hrs, with or without prior DNA transfection or shRNA infection. Cells were trypsinized and all cells (living and dead) were pelleted. Cells were resuspended in 400 µl of 1 X Binding Buffer (BD Pharmingen™). 5 µl of Annexin V-FITC (BD Pharmingen™) was added and incubated in the dark, at room temperature for 15 mins. Samples were analysed on the flow cytometer (LSR II, Beckton Dickinson) within an hour. Viability dye was added just prior to reading; 4'-6-Diamidino-2-phenylindole (DAPI; 200 ng/ml) was used for experiments co-staining with anti-CD133 antibody; propidium iodide (PI, 5 µg/ml) was used for all other experiments.

2.5.1.1 Data analysis

The following controls were used to set up compensation and quadrants: unstained cells, cells stained with Annexin V-FITC only (no PI or DAPI), cells stained with PI or DAPI only (no Annexin V-FITC). Apoptotic cell population included Annexin V-FITC positive, PI/DAPI negative and Annexin V-FITC positive, PI/DAPI positive (right upper and lower quadrants of scatter plot). Results were analysed using the FACSDiva™ software (BD Biosciences) and expressed as mean ± SEM values of three independent experiments performed in triplicate.

10X Binding Buffer (stored at 4ºC)

Hepes (pH 7.4)	0.1 M
NaCl	1.4 M
$CaCl_2$	25 mM

2.5.2 Flow cytometric analysis of translocation of intracellular proteins

(Leverrier et al. 2007).

Upon induction of apoptosis, p53 translocates to mitochondria and nucleus from cytoplasm (Marchenko et al. 2000; Mihara and Moll 2003; Sansome et al. 2001). Translocation of p63 and p53 between subcellular compartments can be monitored by quantifying p63 or p53 fluorescence intensity by flow cytometry. Three different fluorescent stains were used:

1) MitoTracker Orange (CMTMRos, Molecular Probes) for the mitochondria
2) Hoechst (Bisbenzimide Hoechst 33342; Sigma-Aldrich®) for the nucleus
3) Secondary antibodies conjugated to Cy5 (Cy5-conjugated AffiniPure F(Ab)2 fragment goat anti-mouse IgG; Jackson ImmunoResearch Laboratories) for p63 or p53 labelling.

2.5.2.1 Analysis of whole cell antibody labelling

Cells were seeded in 100 mm plates at a confluency of 70-80% >16 hrs prior to treatment. Live cells were fluorescently stained with MitoTracker Orange (to label mitochondria) prior to treatment with chemotherapeutic agents. MitoTracker Orange stock solution (1 mM) was diluted (100 nM) in pre-warmed growth medium (1/10 000) and cells were incubated with 5 mls / 100 mm plate, for 30-40 mins under growth conditions. Media was aspirated and cells were washed twice in 1 X PBS to remove remaining MitoTracker Orange and replaced with pre-warmed growth medium. Cells were either untreated or treated with genotoxic agents and at specific time-points (3-16 hrs), media was discarded and cells washed twice in cold PBS. Cells were fixed with 500 µl PBS/0.25% paraformaldehyde (PFA – Sigma-Aldrich®) for 10 mins and harvested by scraping and transferring to an Eppendorf tube. Cells were pelleted at 9000 rpm for 1 min and resuspended in 1 ml PBS. At this point cells could be kept at 4°C for up to 24 hrs until the next stage of labelling. All the following steps were separated by a 1 min spin at 9000 rpm: cells were permeabilised using 1 ml PBS/0.01% Saponin (Sigma-Aldrich®) for 5 mins, then incubated on a wheel at 4°C for 30 mins with the primary antibody [Table 2.16] at a dilution of 1/50 in 200 µl PBS/0.01% Saponin. This was followed by incubation in 1 ml PBS/0.01% Saponin for 3 mins. Cells were incubated for 30 mins on a wheel at 4^{o}C with the secondary

Cy5 conjugated goat anti-mouse or anti-rabbit antibody at a dilution of 1/60 in 200 µl PBS/0.01% Saponin. Cells were washed in 1 ml PBS/0.01% Saponin containing Hoechst (20 µg/ml) diluted 1/2000 for 5 mins at room temperature and resuspended in 1 ml PBS before reading on a FACS flow cytometer (LSRII, Beckton Dickinson) [Section 2.5.2.3]. Data subsequently were analysed using FlowJo® V7.5 software (Tree Star, Inc).

2.5.2.2 Analysis of labelled cellular organelles

Cells were centrifuged at 9 000 rpm for 5 mins, the supernatant discarded and cells resuspended in Hepes buffer to determine the degree of translocation of p63 or p53 between organelles. This was achieved by homogenisation in Hepes buffer (750 µl) for 100 strokes using an ice-cold Dounce homogeniser (Wheaton). Homogenised cells were then re-analysed (1 x 10^6 events collected) using the same instrument settings to detect presence of p53-Cy5 or p63-Cy5 (530/30nm filter), MitoTracker Orange (575/26nm filter), Hoechst 33342 (440/40nm filter). Isotope controls were analysed for each experiment using primary mouse IgG2a antibodies (Santa Cruz).

Hepes Buffer

Hepes-KOH pH7.4	20 mM
KCl	10 mM
$MgCl_2$	1.5 mM
EGTA	1 mM
EDTA	1 mM

2.5.2.3 Flow cytometer

A Becton Dickinson LSRII fitted with 488, 405, 350-360 and 633nm lasers with FACS Diva software version 4.1.2 was used to acquire 30,000 whole cells using the Argon 488 nm laser line and a 575/26nm band pass filter to detect MitoTracker Orange; the UV 350-360 laser and a 440/40nm band pass filter was used to detect Hoechst 33342; the Red HeNe 633nm line and 660/20nm band pass filter was used to detect Cy5. No compensation was required because of the use of separate laser lines to detect fluorophores that did not display any spectral cross-talk.

Table 2.16: Flow cytometry primary antibodies

Target (name)	Primary antibody	Source
p53 (DO-1)	Mouse monoclonal	Cancer Research UK Laboratories
p63 (4A4)	Mouse monoclonal	Santa Cruz
IgG2a	Mouse monoclonal (isotype)	Santa Cruz
Phospho-p63 (Ser160/162)	Rabbit polyclonal	Cell signalling
IgG XP™	Rabbit monoclonal (isotype)	Cell signalling

2.5.3 Flow cytometric detection of extracellular proteins

Up to 1 x 10^7 cells (untreated, treated or infected with shRNA) were trypsinized and washed in PBS. Cells were centrifuged at 300 *g* for 10 mins and then resuspended in a 1:11 dilution of CD133/1 (AC133)-phycoerythrin [PE] conjugated antibody (Miltenyi Biotec) in 100 µl of PBS. Cells were refrigerated for 15 mins in the dark. Cells were washed again in 1 ml PBS and centrifuged for a further 10 mins at 300 *g*. The supernatant was completely aspirated and cells were resuspended in PBS before analysing samples on the flow cytometer (LSR II Becton Dickinson). For each cell line, mouse IgG isotype PE-conjugated was used for the baseline. Results were analysed using the FACSDiva™ software (BD Biosciences) and expressed as mean ± SEM values of three independent experiments performed in triplicate.

2.5.4 Fluorescence Activated Cell Sorting (FACS)

2.5.4.1 CD133 labelled cell populations [section 2.5.3]

Cells (either treated or untreated) were labelled with anti-CD133/1 (AC133)-PE antibody (Miltenyi Biotec) and passed through a cell strainer cap before reading on the FACS Aria™ Flow Cytometer (BD Biosciences) fitted with an Argon 488 nm laser. Sort gates were placed on CD133-positive and CD133-negative cells once compared to the isotype controls to isolate the two cell populations.

2.5.4.2 Subcellular fractions of homogenised cells labelled with fluorophores

Subcellular fractions identified from section 2.5.2.3 were sorted according to fluorophores using a FACS Aria™ Flow Cytometer (Becton Dickinson) fitted with an Argon 488 nm laser, violet diode laser 405nm and a red HeNe 633nm laser. A

homogenised mixture of intact whole A375M melanoma cells, naked nuclei and mitochondria labelled with Hoechst 33342, MitoTracker Orange and p63-Cy5 were sorted. Sort gates were placed on whole cells (double positive for Hoechst 33342^{+ve}/MitoTracker $Orange^{+ve}$), nuclei (Hoechst 33342^{+ve}/MitoTracker $Orange^{-ve}$) and mitochondria (Hoechst $33342^{weak+\ ve}$/MitoTracker $Orange^{+ve}$). Three-way sorting isolated whole cells, nuclei and mitochondria which were subsequently pelleted by centrifugation at 10 000 g for 30 mins. The supernatant was removed and the pellet was air dried onto glass slides. Cells were mounted in Vectashield (Vector Labs, CA) and overlaid with a cover slip sealed with clear colourless nail varnish. Images were acquired using an LSM 510 inverted confocal microscope (Carl Zeiss MicroImaging, Inc.)

2.6 CLINICAL RESOURCE

All cases of melanoma with a Breslow thickness greater than 1 mm, presenting within the North East London Cancer Network are referred to the Multidisciplinary Skin Cancer Clinic at Bart's and The London NHS Trust (BLT) in conjunction with all melanomas presenting in patients directly under the care of BLT. The supervising dermatologists are Professor Rino Cerio (Professor of Dermatopathology) and Dr Catherine Harwood (Senior Lecturer within the CR-UK Skin Tumour Laboratory). The team also comprises surgical, medical and clinical oncologists, a MacMillan melanoma nurse specialist and an oncology research nurse specialist. In 2009, over 120 new cases of melanoma were seen in this clinic; of these, 15 patients had stage III or IV disease (locoregional or distant metastases respectively), of whom 10 died from metastatic disease. Clinicopathological information, staging and outcome data are available for all patients. In addition, a large archive of clinicopathologically characterised, paraffin-embedded material representing all stages of melanoma development (benign melanocytic naevi, dysplastic naevi, melanoma *in-situ*, primary melanoma and melanoma metastases) was accessed.

2.6.1 Ethical approval

For analysis of archival melanoma tissue samples and to prospectively collect melanocytic lesions, a COREC application (REC approval number 07/QO604/23) was submitted on 12th March 2007 and ethical approval was granted on 9th May 2007 [Appendix 1]. Patients with melanocytic skin lesions (benign and malignant) were identified in the Skin Cancer Multidisciplinary clinic, and in general dermatology clinics when attending for their standard clinical care. Patients were requested to complete a clinician−delivered questionnaire, a clinical examination (part of routine

clinical care) and to provide a venous blood sample (20mls). All patients with known or suspected melanocytic lesions (malignant or benign) removed for clinical reasons, were approached to obtain fresh tissue samples from melanocytic lesions and peri-lesional tissue and blood samples for DNA extraction. Electronic medical records for patients who had a history of melanoma were also accessed to correlated expression of p63 with clinicopathological factors and patient outcome data.

2.7 IMMUNOCYTOCHEMISTRY METHODS

2.7.1 Fluorescent immunocytochemistry: cultured cells

Cultured cells were plated onto glass coverslips at a confluency of 150 000 cells/cm^2 in 12-well culture plates and allowed to attach overnight at 37°C. Media was discarded and replaced with media containing MitoTracker Orange (CMTMRos, Molecular Probes, dilution 1/10 000). Cells were incubated with MitoTracker Orange for 30-40 mins prior to washing twice in PBS followed by treatment with either UVB or pharmacological drug. Cells were washed in PBS for 5 mins at 5-24 hrs after treatment, fixed in 1 ml of 4% formaldehyde/PBS for 10 mins at room temperature, washed twice in fresh PBS for 5 mins and stored at 4°C in PBS.

2.7.1.1 Cell permeabilisation

PBS was removed from the cells and 1 ml of 0.1% Triton X-100 in PBS was added for 3 mins at room temperature to allow permeabilisation of the cell membranes. Cells were washed twice with PBS for 10 mins to remove any residual detergent.

2.7.1.2 Blocking

To avoid non-specific reaction with the secondary antibody, cells were incubated with 500 µl of 5% goat serum / PBS for 30 mins.

2.7.1.3 Detection

After removal of the serum, cells were incubated with primary antibody diluted in 5% goat serum / PBS overnight at 4°C [Table 2.17]. Cells were washed three times for 10 mins in PBS and incubated with secondary antibody for 1 hr at room temperature in the dark [Table 2.18]. Cells were washed again three times for 10 mins in PBS and incubated with 500 µg/ml DAPI (Invitrogen™) for 10 mins followed by two further washes in PBS for 10 mins at room temperature. Coverslips were mounted onto a glass slide using Vectashield Mounting Medium (Vector Laboratories, CA) to prevent photobleaching over time. Mounted slides were sealed with clear colourless nail varnish and stored at 4ºC protected from light.

2.7.2 Fluorescent immunocytochemistry: Tissue Microarray

A melanoma paraffin-embedded tissue array (Biomax US ME481) comprising a panel of 8 normal skin cores and 40 melanoma cores each of 1.5 mm diameter and 5 µM thickness were used for p63 immunohistochemistry [Figure 2.8; Appendix 2].

2.7.2.1 Preparation of tissue sections

The slide was initially baked at 60°C for 2 hrs. Tissue sections were subjected to deparaffinisation and rehydration using a xylene and ethanol (EtOH) series:

1. Xylene: x 2 at 5 mins each
2. 100% EtOH: x 2 at 5 mins each
3. 90% EtOH: x 1 at 3 mins
4. 70% EtOH: x1 at 3 mins
5. 50% EtOH: x1 at 3 mins
6. Distilled water: x1 at 5 mins
7. PBS: x1 at 5 mins

2.7.2.2 Antigen retrieval

The slide was incubated in citrate buffer, pH 6, placed in a microwave at 300 W for 5 mins for 3 cycles and subsequently cooled for 15 mins. It was returned to the microwave for 5 mins at 300 W, and again cooled to room temperature.

2.7.2.3 Blocking of section

The slide was incubated in 5 % goat serum / PBS for 2 hrs at room temperature.

2.7.2.4 Antibody incubation

The slide was placed in a humid chamber and incubated with the primary antibody (1:50 H137 and H129 anti-p63 antibodies) overnight at 4°C. It was washed in PBS buffer x 3 for 10 mins and incubated with the secondary antibodies [Table 2.18] for 1 hr at room temperature in 5% goat serum / PBS. It was washed twice for 10 mins in PBS followed by one wash for 10 mins in DAPI in PBS (500 µg/ml) and washed twice more for 10 mins in PBS to remove excess DAPI. The slide was then mounted using Vectashield (Vector Labs, CA) to prevent bleaching of the fluorophores.

2.7.3 Immunohistochemistry: Paraffin embedded tissue

Automated immunohistochemistry was performed on a selection of melanoma tissue samples collected from the pathology archive at Bart's and the London NHS Trust.

Cases were identified from the Skin Cancer Database held by the Skin Cancer Multidisciplinary Team. Clinical and demographic details for the naevi tissue samples and melanoma tissue samples are detailed in table 3.3 and Appendix 3, respectively. The RedMap™ kit was used with the Ventana Discovery® instrument to detect p63 reactivity (using anti-p63 antibodies – H129 or 4A4) in formalin-fixed paraffin-embedded (FFPE) tissue sections.

2.7.3.1 Preparation of FFPE sections

FFPE sections of melanocytic lesions were cut into 4 µM sections and placed on APES (3-Aminopropyltriethyoxysilane/acetone)-coated slides. This was carried out by the Royal London Hospital Experimental Pathology Service, Lincoln's Inn Fields Pathology Service and Bart's Pathology Service.

2.7.3.2 Immunohistochemical staining

Detection of proteins by immunohistochemistry was performed using paraffin-embedded tissue sections fixed with paraformaldehyde. Slides were subjected to the following steps:

1. Deparaffinisation — 75 ºC for 8 mins; 37 ºC for 2 mins
2. Long cell conditioner — 95 ºC for 8 mins
3. Medium cell conditioner #1 — 100 ºC for 4 mins
4. Mild CC1 (cell conditioner 1) buffer wash — 10 x 4 mins
5. Standard CC1 buffer — 37 ºC for 2 mins
6. Primary antibody 1:50 dilution – incubate for 60 mins
7. Universal secondary antibody – (1 drop) incubate for 30 mins
8. Blocker R1 – (1 drop) incubate for 2 mins
9. Counterstain with 1 drop SA-Alk Phos R (red chromogen) – incubate for 30 mins
10. Rinse slide x 4
11. Haematoxylin – (1 drop) incubate for 2 mins
12. Bluing reagent – (1 drop) incubate for 2 mins
13. Rinse x 3

2.7.3.3 Statistical analysis

Statistical analysis was undertaken in collaboration with Dr D. Mesher (Centre for Epidemiology, Mathematics and Statistics, QMUL). Clinicopathological variables were tabulated and compared with p63 status. For survival (overall and melanoma-specific

analysis), patients were censored at date of death or date of last follow-up. For recurrence and metastases, patients were censored at date of recurrence/metastases, date of death or date of last follow-up. Analysis of end points was performed by Cox proportional hazards model. Variables were examined univariately and subsequently a multivariate model was constructed using backwards stepwise selection method. A p-value was then presented comparing the multivariate model with and without p63 status included. All p-values were two-sided and all statistical analyses were carried out using Stata 10.0.

(http://www.biomax.us/tissue-arrays/Melanoma/ME481)

Figure 2.8: Tissue microarray slide. Haematoxylin and Eosin slide image of tissue microarray of melanoma samples and normal skin (x2). Magnified images supplied from website in Appendix 2.

Table 2.17: Summary of primary antibodies used for IHC/IF/immunogold EM

Antibody specificity	Supplier (antibody name)	Dilution for immunofluorescence
α-tubulin	Abcam	1:500
Calnexin	BD Biosciences	1:100
Cleaved caspase-3	Cell signalling (Asp175)	1:300
GM130	BD Biosciences	1: 100
HMB-45*	DakoCytomation	1:50
LAMP1	BD Biosciences	1:100
mtHsp70	**Abcam (Grp75)**	**1:50**
p53	Cancer Research UK (DO1)	1:50-100
p63*#	**Santa Cruz (H129)**	**1:50**
p63*	**Santa Cruz (4A4)**	**1:50**
p63α#	**Santa Cruz (H137)**	**1:50**
γ-H2AX	Cell signalling	1:500

*Antibodies used for immunohistochemistry, #used in combination for IF

Antibodies in bold are used for EM

IHC – immunohistochemistry, IF – immunofluorescence, EM – electron microscopy

Table 2.18: Antibodies used for fluorescent immunocytochemistry

Secondary antibody	Dilution
Alexa fluor ® 594 goat anti-rabbit SFX kit (Invitrogen™)	1:400
Alexa fluor ® 594 goat anti-mouse SFX kit (Invitrogen™)	1:400
Alexa fluor ® 488 goat anti-rabbit SFX kit (Invitrogen™)	1:400
Alexa fluor ® 488 goat anti-mouse SFX kit (Invitrogen™)	1:400

2.8 MICROSCOPY METHODS

2.8.1 Light microscopy

Images were acquired using the LSM 510 inverted confocal microscope (Carl Zeiss MicroImaging, Inc.). A Plan-Neofluor 10 x lens and a Plan-Apochromat 60 x lens were used to acquire images. Excitation was kept to a minimum to avoid bleaching of samples. Lasers used for confocal microscopy are detailed in Table 2.19.

Table 2.19: Lasers used in confocal microscopy

Dye	Wavelength (nm)	Laser unit
DAPI	405	Laser diode 405
FITC	488	Argon 2
Cy3/MitoTracker Orange	543	HeNe 1

DAPI - 4'-6-Diamidino-2-phenylindole

FITC – fluorescein isothiocyanate

2.8.2 Transmission electron microscopy

Electron microscopy experiments were performed at the Centre for Ultrastructural Imaging (CUI) at Kings College London. The experiment was supervised by the director of the CUI, Dr Alice Warley.

2.8.2.1 Immunogold localisation of p63

The Tokuyasu method for immunogold labelling (1980) was employed.

2.8.2.2 Preparation of cell extracts

Cells were seeded at 70% confluency in two 100 mm plates and treated with paclitaxel (2 µM) for 6 hrs. 8% formaldehyde + 0.2 % glutaraldehyde in 100 mM PIPES buffer (pH 7.2) was added to the media (1:1) and plates were refrigerated for 1 hr. Cells were scraped into an Eppendorf tube and centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and cells were resuspended in PIPES buffer for transport. The pellet was washed twice in PIPES buffer and the supernatant was discarded to ensured removal of all fixative. The pellet was resuspended in warm gelatin (10%) and centrifuged at 6000 rpm for 3 mins. The pellet of cells in gelatin was incubated at 4ºC for 30 mins to solidify the gelatin. Gelatin containing the cells was cut into small cubes approx 1 mm^3 which were incubated in 2.3 M sucrose at 4°C

overnight for cryoprotection. The cubes were mounted on specimen pins, excess sucrose was blotted away and the cubes were cryofixed by plunging into liquid nitrogen.

2.8.2.3 Cryosectioning

Cryosections, 90 nm thick, were cut from these blocks with glass knives at -100°C using a RMC MTXL ultramicrotome with a RMC CRX cryo-adaptation. Groups of sections were picked up on a droplet of 2.3 M sucrose and transferred onto Pioloform-coated nickel grids. The grids were floated onto standard buffer consisting of PBS + 1% BSA 0.1% sodium azide (pH 7.4).

2.8.2.4 Immunogold labelling.

Grids were incubated for 3 x 5 min on PBS-0.05 M glycine to remove any remaining aldehyde groups leftover from the fixation process before being washed in incubation buffer (PBS + 0.1% BSA-c (Aurion) + 0.1% azide, pH 7.4) and transferred onto droplets of primary antibody [Table 2.17] diluted in incubation buffer (1:50) for overnight incubation at 4ºC. The primary antibody was removed by passing over six droplets, of incubation buffer solution, 5 mins per droplet, before incubation for 1 hour at room temperature with the secondary antibody, diluted in incubation buffer (1:100). Secondary antibody was removed by passing grids over three droplets of PBS for 5 mins each and the reaction was fixed by exposing the grids to PBS-2% glutaraldehyde. The grids were then washed over distilled water 3 x 5 mins each. Sections were finally embedded in a solution of 2% methyl cellulose and 3% uranyl acetate in a 9:1 ratio.

2.8.2.5 Transmission electron microscopy of cryosections

Sections were examined and micrographs were obtained using a FEI T12 transmission electron microscope at an accelerating voltage of 120 kV.

2.9 GENE MICROARRAY ANALYSIS

2.9.1 Agilent array

2.9.1.1 Clinical samples

All samples and clinical data were collected with the Institute for Cancer Research and Treatment (IRCC) Ethics Committee's approval and patients' informed consent. This work was performed in collaboration with Giovanna Chiorino, (Fondo Edo Tempia, Biella, Italy). A total of 57 excisional biopsies were analysed [Table 2.20] which were obtained from an Italian patient population detailed in Scatolini et al. (2009).

2.9.1.2 Gene array technology

RNA extraction, microarray probe preparation, hybridisation and scanning are detailed in Scatolini M (2009). This was performed in Fondo Edo Tempia, Biella, Italy.

2.9.1.3 Microarray data analysis

Images were analysed in Fondo Edo Tempia, Biella, Italy using Feature Extraction software (Agilent Technologies) version 7.6. Output files containing feature and background intensities and the related statistical parameters for red and green signals were then loaded into the Resolver SE System (Rosetta Biosoftware, Seattle, WA) together with the scan images and the Agilent Human Whole Genome pattern file. Data processing and normalisation were performed using the Agilent Human Whole Genome platform specific error model. Replicated expression profiles were combined to form *ratio* experiments where each gene is associated with an expression fold-change and a p-value to assess the statistical significance of its modulation in the sample compared to the reference.

Analysis of intensities of p53 family members in these melanoma tissue samples compared with the Universal control was undertaken by the author of this thesis by extracting data from the complete microarray data analysis (Scatolini et al. 2009).

Table 2.20: Excisional biopsies used for Agilent gene array (Scatolini et al. 2009)

Tissue sample	Number of samples
Benign melanocytic naevi	18
Dysplastic naevi	11
RGP melanoma	8
VGP melanoma	15
Metastatic melanoma	5

RGP – radial growth phase

VGP – vertical growth phase

2.9.2 microRNA Microarray

2.9.2.1 miRNA extraction

Using the miRNeasy Mini Kit (Qiagen®), total RNA was extracted from cell pellets. Briefly this combined phenol/guanidine-based lysis of samples and a silica membrane–based purification of total RNA. Cells were homogenised in QIAzol Lysis Reagent which facilitated lysis of tissues, and inhibited RNases, removing most cellular DNA and proteins from the lysate by organic extraction. After addition of chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. RNA partitioned to the upper, aqueous phase, while DNA partitioned to the interphase and proteins to the lower, organic phase or the interphase. The upper, aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions for all RNA molecules ≥18 nucleotides. The sample was then applied to the RNeasy Mini spin column, where total RNA bound to the membrane, phenol and other contaminants were washed away. Total RNA was then eluted in RNase-free water.

2.9.2.2 Array technology

Total RNA quality and quantity was verified on the Nanodrop Spectrophotometer (Celbio) and on a Bioanalyzer (Agilent Technologies). Samples were labelled and hybridised according to Agilent Technologies miRNA Microarray System protocol. The following work was undertaken by Giovanna Chiorino, (Fondo Edo Tempia, Biella, Italy).

2.9.2.2.1 *Dephosphorylation*

Briefly, on the basis of spectrophotometer evaluation, an aliquot of each total RNA sample was diluted in nuclease-free water (final concentration 65 ng/µl) and read

again using the Nanodrop: for labelling, the reaction started from 100 ng diluted in a maximum of 2 µl. Samples were dephosphorylated with CIP at 30°C for 30 mins.

2.9.2.2.2 *Denaturation*

The reaction was stopped by adding 5 µl DMSO to each sample, incubating at 100°C for 10 mins and then immediately transferring to an ice-water bath to ensure the samples remain properly denatured and prevent the RNA from re-annealing.

2.9.2.2.3 *Ligation*

Labelling of samples was achieved by ligation. Samples were gently mixed by pipetting with Ligation Master Mix, briefly centrifuged and incubated at 16°C for 2 hrs. After the reaction, samples were completely dried in a vacuum concentrator at 50ºC for up to 1 hr.

Ligation Master Mix (Total volume 8 µl per reaction)

10x T4 RNAse ligation buffer	2 µl
pCp-Cyanine3	3 µl
T4 RNA ligase	1 µl
Nuclease free water	2 µl

2.9.2.2.4 *Hybridisation*

For hybridisation, samples were resuspended in nuclease-free water. 10 x GE Blocking Agent followed by Hybridisation buffer were added to each sample and mixed well. Reactions were incubated at 100°C for 5 mins and immediately transferred to an ice-water bath for 5 mins incubation.

Samples were loaded onto gasket slides (up to 8 samples each) and the miRNA glass arrays (Human miRNA Microarray Release 12.0, Agilent Technologies) were placed with the active surface facing the wells. The hybridisation chambers were assembled and tightly closed. Hybridisation was continued for 20 hrs in a hybridisation oven set to 55°C, with a rotation speed of 20 rpm. After incubation, hybridisation chambers were disassembled and arrays were washed in Gene Expression Washing buffer 1 (containing Triton X-10) for 5 mins, and pre-warmed Gene Expression Washing buffer 2 (containing Triton X-10) for 5 mins. These were air-dried and immediately scanned using the Dual-Laser Microarray Scanner B (Agilent Technologies).

2.9.2.3 Target prediction and network analysis

TIFF images were loaded into the Feature Extraction 9.5 (Agilent technologies) software and analysed using the microRNA grid template and protocol. Raw data were analysed with R, using the “invariant” procedure (Pradervand et al. 2009). Replicate probes were combined and then t-tests and Wilcoxon tests were applied to normalised log intensities, in order to detect differentially expressed miRs in each cell class. Analysis of raw data was undertaken as a collaboration between the author of this thesis and Giovanna Chiorino, (Fondo Edo Tempia, Biella, Italy).

CHAPTER 3: BIOLOGICAL ROLE OF TP63

3.1 BACKGROUND

3.1.1 p63 expression in cell systems

In the skin there is extensive evidence for the role of p63 in keratinocytes and its upregulation in the keratinocyte cancer squamous cell carcinoma (Choi et al. 2002; Hu et al. 2002; Park et al. 2000; Yang and McKeon 2000). However, there has been little research investigating its role in the melanocyte cell lineage (Brinck et al. 2002; Johnson et al. 2005; Kilic et al. 2008; Kulesz-Martin et al. 2005; Sakiz et al. 2009), and no previous study has rigorously examined the expression of this gene and its splice variants in melanoma.

3.1.2 Melanoma stem cell characterisation

Significantly increased expression of stem-cell markers have been demonstrated in primary and metastatic melanomas compared with benign naevi (Klein et al. 2007). During melanoma progression, stem-cell markers become more evident due to the increased dysregulation of stem-/progenitor-cell function and proliferation. The more extensively studied stem cell markers demonstrated in melanoma are detailed below:

3.1.2.1 CD166

Activated leucocyte adhesion molecule (CD166) is a member of the immunoglobulin super family and is a type 1 transmembrane protein involved in cell adhesion and cytoskeletal anchoring. CD166 is expressed on the surface of mesenchymal stem cells and in human melanoma cell lines (Swart et al. 2005). There is significantly greater expression of CD166 demonstrated in melanoma tissue samples when compared with benign naevi (Klein et al. 2007) which correlates with thickness of primary tumours (van Kempen et al. 2000) suggesting that CD166 might play an important role in melanoma cell invasion and disease progression.

3.1.2.2 Nestin

Nestin is an intermediate filament expressed in the cytoplasm of neuroepithelial stem cells (Dahlstrand et al. 1992; Lendahl et al. 1990). It is also expressed in migrating and proliferating cells during embryogenesis and various adult tissues undergoing regeneration, such as the central nervous system, liver, pancreas and gastrointestinal tract (Wiese et al. 2004). Upregulated expression of nestin is detected in metastatic melanoma when compared with primary tumours suggesting a correlation with high proliferative and migrational activity in this tumour (Klein et al. 2007; Rodolfo et al. 2004). More recently, a study using metastatic melanoma cell lines identified cells with heterogeneous morphology and antigenic characteristics which included a smaller cell population demonstrating nestin reactivity (Grichnik et al. 2006). This subset of cells showed a slower proliferative rate but was able to drive cellular expansion in culture, leading the authors to speculate that melanoma may be a tumour based on a mutant stem cell which expressed nestin, attempting to undergo normal developmental processes (Grichnik et al. 2006).

3.1.2.3 CD20

A population of melanoma cells that had the ability to grow in spheres (a growth pattern characteristic of stem cells), self-renew and differentiate into melanogenic, adipogenic, chrondrogenic, and osteogenic lineages were identified from metastatic melanoma cells (Fang et al. 2005). A subpopulation of these cells, expressing the haematopoietic marker CD20, was identified in melanoma tissue samples and in culture, formed larger spheres showing greater potential for mesenchymal differentiation. In addition, CD20 was identified by gene expression profiling as one of the top 22 genes associated with increased aggressiveness of tumours (Bittner et al. 2000), suggesting it is a marker of a potentially important tumourigenic subpopulation of melanoma cells with stem cell properties.

3.1.2.4 CD133

CD133, a transmembrane glycoprotein, also known as prominin-1, labels both normal and cancer stem cells. It has no known function but is expressed by developing epithelial cells and is rapidly downregulated upon differentiation (Corbeil et al. 2000). Expression of CD133 is demonstrated in primary human melanocytes and melanoma (Frank et al. 2005; Klein et al. 2007; Monzani et al. 2007). Monzani *et al*. (2007) determined that less than 1% of cells within metastatic melanoma tissue expressed CD133. Only CD133-positive cells collected from these biopsies induced tumours following xenografts in severe combined immunodeficiency mice, whereas the CD133-negative fraction failed to regenerate tumours, indicating that CD133-labelled cells are capable of recapitulating tumour growth. The expression of CD133 in melanomas supports the notion of a stem-cell component and CD133 has been used as a potential stem cell marker to isolate melanoma stem cells from cell lines, which are demonstrated to have self-renewal capacity, differentiation potential, and high tumorigenicity (Monzani et al. 2007).

Against this background suggesting that CD133 is a marker of a putative stem cell population, this thesis has focused on using CD133 to enrich for a melanoma stem cell fraction which could be analysed in relation to p63.

3.2 AIMS

The basis of the research undertaken in this chapter was to understand the biological role of p63 in melanoma. The specific aims were:

1. To establish the pattern of p63 isoform expression at mRNA and protein level, in primary melanocyte cultures and melanoma cell lines
2. To examine the expression of p63 protein in melanoma tissue samples
3. To analyse the association of p63 expression of melanoma with clinicopathological features of tumours and outcome in patients
4. To characterise melanoma stem cells using CD133 as a marker with respect to expression of p63

3.3 RESULTS

3.3.1 TP63 expression in melanoma cell lines

3.3.1.1 TP63 gene is expressed in melanoma cell lines: RT-PCR

Initial experiments determined expression of p63 in different cells in the skin. Primers designed to detect all splice variants of TAp63 (Koga et al. 2003) and ΔNp63 (Yang et al. 1998) were used for RT-PCR analysis [Figure 2.2]. Both TA and ΔN p63 isoforms were expressed in various skin phototypes in samples obtained from different body sites [Figure 3.1]. TAp63 and ΔNp63 were expressed in keratinocytes [Figure 3.1] and the relative expression of these isoforms was in keeping with reports that ΔNp63 is the predominant isoform expressed in keratinocytes forming the basal stem cell compartment of skin (Mills et al. 1999; Pellegrini et al. 2001; Senoo et al. 2007; Yang et al. 1999a). TAp63 was expressed at low levels in fibroblasts [Figure 3.1], also in keeping with previous literature reporting either low level or no expression of p63 in fibroblasts (Pignolo et al. 1998; Wenig 2003).

RT-PCR analysis failed to detect expression of either isoform in neonatal melanocytes (NHEM) or human adult epidermal melanocytes (HEMA) [Figures 3.1, 3.2] although it is possible that tissue culture conditions could affect gene expression. However both isoforms were expressed in a metastatic melanoma cell line (WM1158) prompting further analysis of TP63 in melanoma. Despite the apparent lack of p63 expression in primary melanocytes, TAp63 was detected by RT-PCR in both primary and metastatic melanoma cell lines [Figure 3.2A]. ΔNp63 was also detected in melanoma cell lines, particularly in metastatic cells, but overall expression levels were lower [Figure 3.2B].

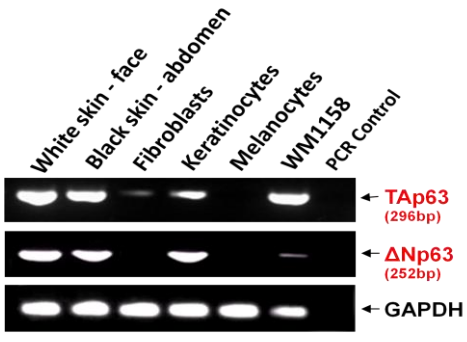


Figure 3.1: TP63 expression in human normal skin cellular components (RT-PCR). Both isoforms are expressed in skin from different body sites, primary keratinocyte cultures and WM1158 - a metastatic melanoma cell line. TP63 was not expressed in primary melanocyte cultures (NHEM) and barely detectable in fibroblasts. Controls included omission of cDNA. Size of amplified product shown in brackets. GAPDH was used as a loading control.

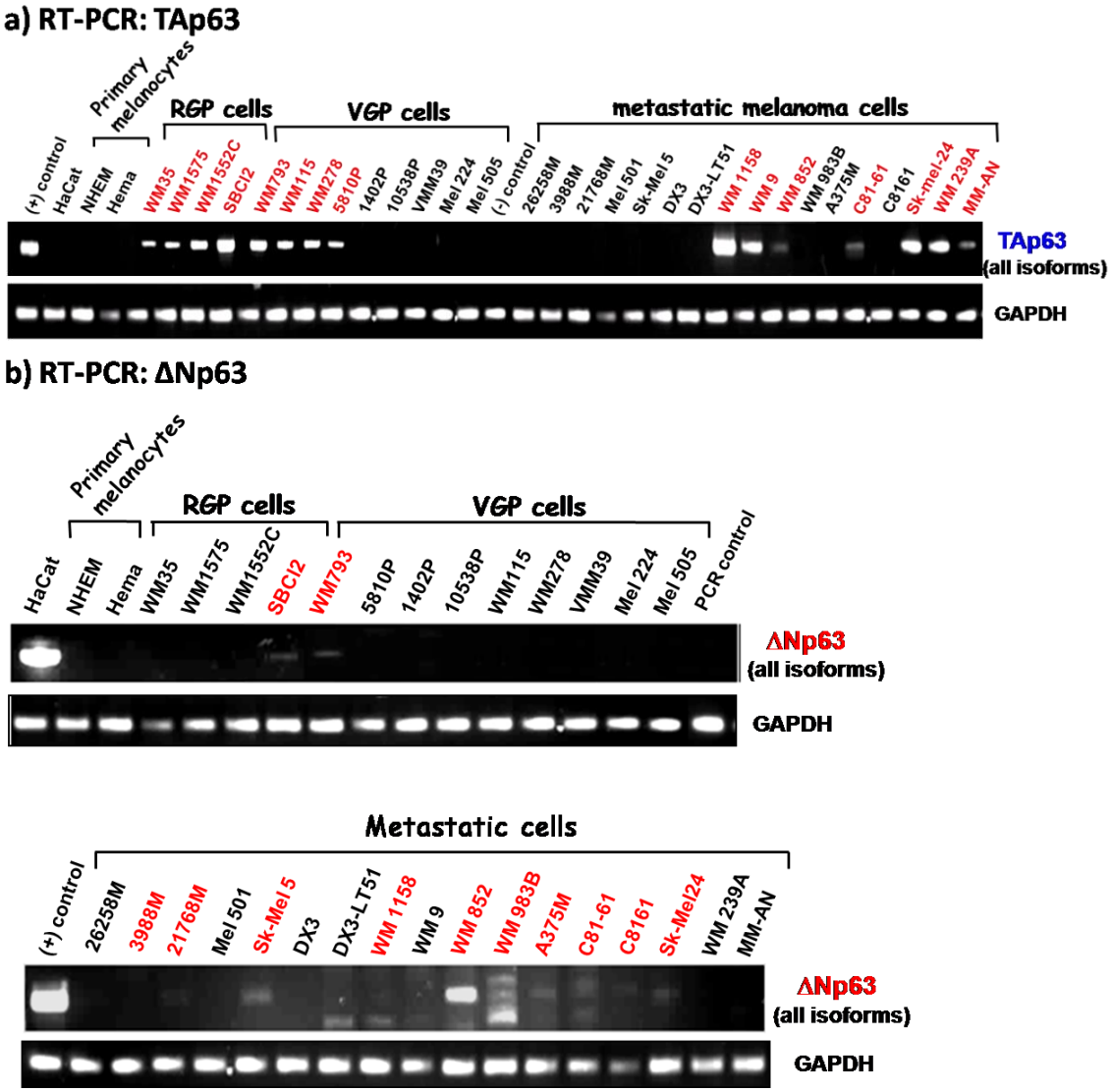


Figure 3.2: TP63 expression in primary melanocyte cultures and melanoma cell lines (RT-PCR). a) TAp63 expression was demonstrated predominantly in primary melanoma cell lines (RGP and VGP cells) but also in metastatic melanoma cell lines. b) HaCaT cells were used as a positive control for p63 mRNA. ΔNp63 was expressed predominantly in metastatic melanoma cell lines but at lower levels. TP63-positive cell lines are highlighted in red. Neither isoform was expressed in primary melanocyte cultures (shown are NHEM – NHEM1 and Hema – HEMa 3). PCR control included omission of cDNA. Positive control (+) of exogenous transfection of p63 plasmid in HEK 293T cells [Section 2.1.5.1] confirmed size of amplified product. Variation in p63 mRNA expression was observed in melanoma cells plated at varying confluencies and this was therefore explored further [section 4.3.3.1]. Data shown here are for cells plated at the same density and harvested consistently at 60-80% confluency. GAPDH was used for mRNA standardisation. RGP – radial growth phase, VGP – vertical growth phase.

3.3.1.2 Significant upregulation of TP63 mRNA is observed in melanoma cell lines: Quantitative RT-PCR

Although upregulation of TAp63 was readily detected using RT-PCR, the ΔN isoform was not consistently detected because of the low levels of mRNA. Quantitative RT-PCR (Q-PCR) was therefore used as a more specific and sensitive method for analysing expression of isoforms and for quantifying upregulation of TP63 compared with a physiological control - primary melanocyte cultures. Five primary melanocyte cultures (NHEM1, NHEM2, HEMa 3, HEMa V3 and HEMa V4) and 34 melanoma cell lines, representing various stages of disease progression, were screened for expression of TA and ΔNp63 mRNA [Figure 3.3]. Negative controls included samples without reverse transcriptase enzyme and without cDNA. HEK 293T cDNA was also used as a negative control as this cell line does not express TP63. HaCaT cells were used as a recognised positive control for expression of TP63.

TP63 gene expression levels in samples were standardised to the housekeeping gene, beta-glucuronidase (GUS). GUS was chosen because of its stable expression in melanoma cell lines and because it was a medium-expressed gene which was appropriate because of the medium-low expression levels detected for ΔNp63 in RT-PCR experiments. TP63/GUS expression levels in samples were compared to the mean of gene expression levels in the five primary melanocyte cultures to determine the extent of upregulation of p63 in melanoma cell lines [Figure 3.3]. Considering a threefold increase in gene expression as a stringent cut-off for significant upregulation, the proportion of melanoma cell lines demonstrating significant upregulation of the TP63 gene were tabulated [Table 3.1].

Overall 25/34 (74%) melanoma cell lines showed upregulation of TP63 compared with primary melanocyte cultures. Data from the Q-PCR analysis [Figure 3.3] supported findings from RT-PCR analysis of the same cell lines [Figure 3.2]. In general, upregulation of the two isoforms of TP63 appeared to be mutually exclusive. Simultaneous upregulation of both isoforms occurred infrequently (only 18%). These data raise the possibility that each isoform is redundant i.e. their function is overlapping or that the isoforms may interact with each other in a reciprocal manner in melanoma cell lines expressing TP63.

3.3.1.3 Expression of TP63 in melanoma cell lines is comparable to expression in HaCaT cells

The predominant isoform expressed in HaCaT cells is ΔNp63 and Q-PCR confirmed the huge expression of this isoform using primary melanocytes as a comparator [Figure 3.3B(ii) solid bar], and significantly greater expression of this isoform when TAp63 expression in HaCaT was used as the comparator [Figure 3.4A]. HaCaT cells are reported to express low levels of TAp63 (Laurikkala et al. 2006; Yang et al. 1998), but this is considerably greater when compared with melanocyte TAp63 expression levels [Figure 3.3A (ii) solid bar]. Although TP63 mRNA levels in primary melanocyte cultures were significantly lower than for HaCaT cells, detection of p63 was within 35 cycles of the Q-PCR reaction in 3/5 melanocyte cultures. When TAp63 expression was used as the comparator, there was greater expression of ΔNp63 in all three melanocyte cultures tested which was similar to the observation demonstrated in HaCaT cells [Figure 3.4A]. Although the level of TP63 mRNA in our primary melanocyte cultures is significantly less than the expression in keratinocytes, these data raise the possibility that ΔNp63 may have a role in the melanocyte lineage. Primary melanocytes may exhibit similarities to the role of p63 in the keratinocyte lineage in skin warranting further investigation of TP63 in melanoma.

To explore the relevance of upregulation of p63 in melanoma cell lines when compared with primary melanocytes, the expression data was re-analysed using HaCaT cells as a comparator for each of TA [Figure 3.4B] and ΔNp63 [Figure 3.4C]. Using HaCaT cells as a comparator, the majority of melanoma cell lines expressed TAp63 at higher levels, which is in keeping with reports that TAp63 is expressed at low levels in keratinocytes (Laurikkala et al. 2006; Yang et al. 1998). ΔNp63, normally expressed at very high levels in HaCaT cells was expressed to a greater extent in one melanoma cell line (Sk mel 31). This was supported by the semi-quantitative assessment of ΔNp63 expression using RT-PCR, which demonstrated overall lower expression levels of this isoform, when compared with TAp63 [Figure 3.2B].

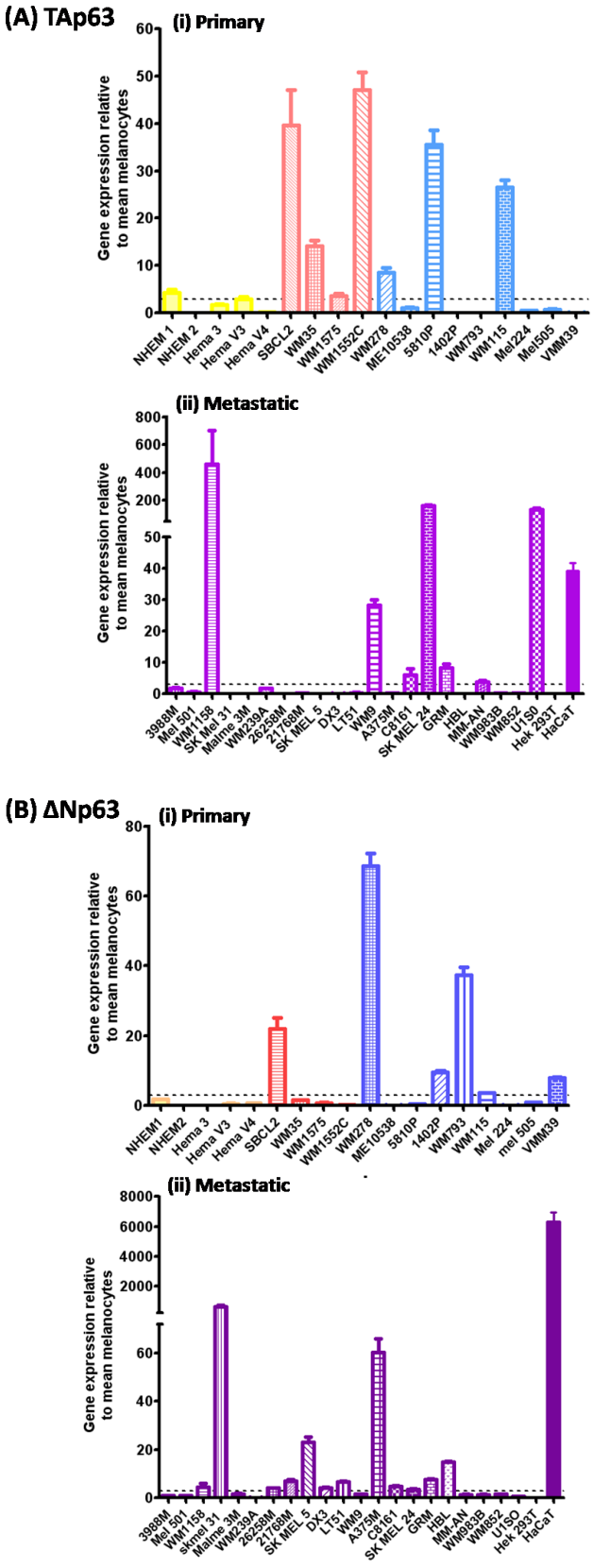


Figure 3.3: TP63 is upregulated in melanoma cell lines (Q-PCR). (A) TAp63 is upregulated in (i) primary melanoma cell lines and (ii) metastatic melanoma cell lines compared with the mean expression of TAp63/GUS in five primary melanocyte cultures (NHEM1, NHEM2, HEMa 3, HEMa V3 and HEMa V4). (B) ΔNp63 is upregulated in (i) primary melanoma cell lines and

(ii) metastatic melanoma cell lines compared with mean expression of ΔNp63 in five primary melanocyte cultures. HEK 293T cells did not express TP63 and HaCaT cells demonstrated upregulation of TAp63 and to a much greater extent, ΔNp63 (last two lanes in A and B (ii)). Dotted line marks threefold increase in gene expression compared with expression in melanocyte cultures. GUS was used as an endogenous control.

Table 3.1: Proportion of melanoma cell lines expressing TP63.

Stage of disease		**Number of cell lines demonstrating upregulation of gene (%)**			
		TAp63 only	**ΔNp63 only**	**Both**	**Total**
Primary	RGP (n=4)	3 (75)	0 (0)	1 (25)	4/4 (100%)
	VGP (n=9)	1 (11)	3 (33)	2 (22)	6/9 (67)
Metastatic (n=21)		4 (20)	8 (38)	3 (14)	15/21 (71)
Total (n=34)		**8 (24%)**	**11 (32%)**	**6 (18%)**	**25/34 (74)**

RGP: radial growth phase, VGP: vertical growth phase

Values are number of melanoma cell lines at each stage of disease demonstrating significant upregulation (in excess of threefold increase) of TP63 compared with primary melanocyte cultures; (percentage of cell lines within the category shown in brackets)

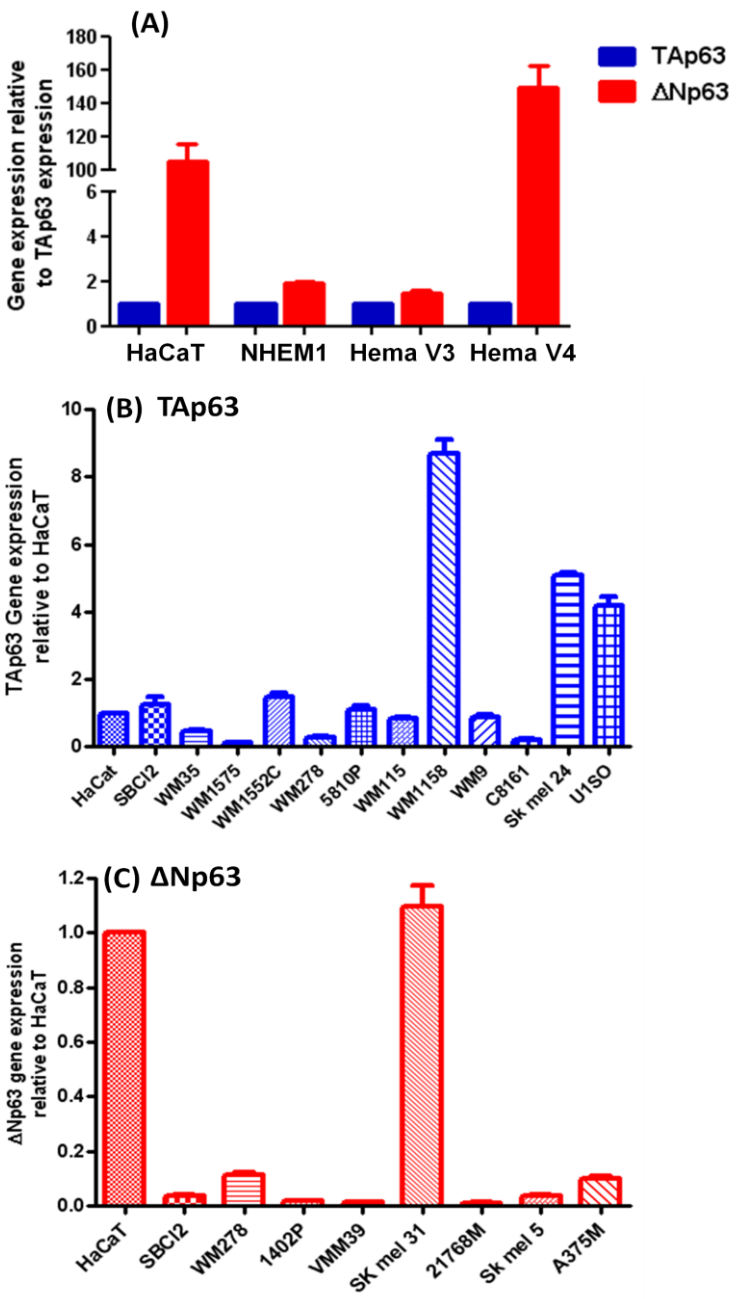


Figure 3.4: Comparative expression of TP63 mRNA to HaCaT cells (Q-PCR). (A) Expression of ΔNp63 in HaCaT cells using expression of TAp63 in same cell line as a comparator. Higher levels of ΔNp63 mRNA are expressed compared with TAp63 in HaCaT cells & three melanocyte cultures. Expression of (B) TAp63 and (C) ΔNp63 expression in a panel of melanoma cell lines (showing >3 fold upregulation of TP63 compared with primary melanocytes) to HaCaT cells. TAp63 expression is equal / higher in melanoma cell lines when compared with HaCaT cells. One melanoma cell line (Sk-mel 31) expressed greater ΔNp63 mRNA compared with HaCaT cells. mRNA levels were standardised against the housekeeping gene GUS. Values shown are mean +/- SEM of two independent experiments performed in triplicate.

3.3.1.4 TP63 splice variants are expressed in melanoma cell lines: RT-PCR

Tissue/cell type-specific expression has been reported for TP63 mRNA transcripts, suggesting a putative correlation with their differing cellular functions (Nylander et al. 2002). Despite only one or two isoforms being expressed in primary cell cultures e.g. keratinocytes, a more complex pattern of isoform expression is recognised in cancer cells. Having determined that melanoma cell lines express both TA and ΔNp63, it was necessary to establish expression of the splice variants. HEK 293T cells (normally do not express p63) were transfected with each of the six isoforms of p63 (TA and ΔN α, β and γ), to use as positive controls for optimisation of isoform-specific primers [section 2.1.5.1]. Isoform-specific primers (Borrelli et al. 2007) [Figure 2.3] were optimised, confirming successful transfection and specificity of the primers to detect only one individual isoform [Figure 3.5A]. A panel of melanoma cell lines expressing TAp63 &/or ΔNp63 were screened for expression of the splice variants. Although all six splice variants were differentially expressed in melanoma cell lines, TAp63β was the predominant variant detected in 6/7 cell lines tested [Figure 3.5B].

In addition to p63, the expression of p53 family members was also investigated in the same cell lines. All melanoma cell lines used in this screen were reported to harbour wild-type p53 [Table 2.1] and the RT-PCR data supported the expression of TP53 in all cell lines [Figure 3.5B]. ΔNp73 has been implicated in mediating chemoresistance in several cancers including melanoma (Muller et al. 2005; Tuve et al. 2006) and was therefore analysed in the same panel of cell lines. ΔNp73 was expressed in the three metastatic cell lines [Figure 3.5B] in keeping with reports that this gene is upregulated in metastatic melanoma and may contribute to melanoma progression (Tuve et al. 2006; Tuve et al. 2004).

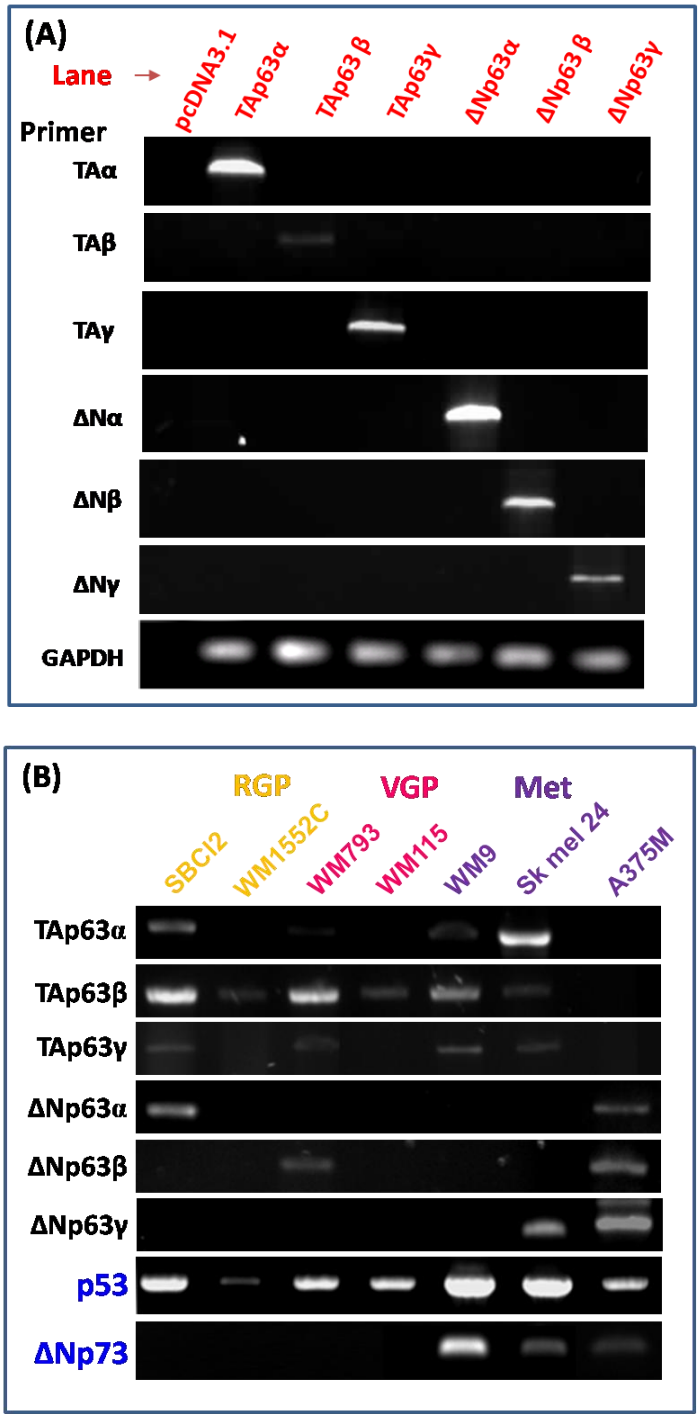


Figure 3.5: TP63 splice variant expression (RT-PCR). (A) Transfected isoforms of p63 in HEK 293T cells used to confirm specificity of isoform-specific primers. GAPDH used as a housekeeping gene for mRNA standardisation. (B) p63 splice variant expression in melanoma cell lines representing various stages of disease progression. TP53 is differentially expressed in all melanoma cell lines. ΔNp73 was expressed in metastatic melanoma cell lines only. RGP – radial growth phase, VGP – vertical growth phase, Met – metastatic. Results shown are representative of three independent experiments.

3.3.1.5 Differential expression of all TP63 splice variants in melanoma: Q-PCR

The recent discovery of two new p63 transcripts has added further complexity to the detection of splice variants of p63 (Mangiulli et al. 2009). As the size of the amplified products using primers to detect one of TA or ΔN at the N' terminus of TP63 and one of α, β, γ, δ or ε at the C' terminus exceeds 1kb, these primers could not be used for Q-PCR [Figure 2.4]. To detect p63 splice variants using Q-PCR, primers were used which were designed to only detect C-terminal variants (Mangiulli et al. 2009) [Table 2.3]. A panel comprising of one primary melanocyte culture (HEMa V4) and 6 melanoma cell lines (2 RGP, 1 VGP and 3 metastatic cell lines) were screened for the different C-terminal splice variants using HaCaT cells (reported to express all variants) as a comparator (Mangiulli et al. 2009). The δ and ε isoforms of p63 were detected within 35 cycles of amplification, in 3/6 and 4/6 melanoma cell lines respectively [Figure 3.6]. Furthermore, the expression levels of these two new splice variants appeared comparable to that of α, β and γ expression levels in two cell lines - SBCl2 and WM1158, both of which expressed both TA and ΔNp63. Although the splice variants were expressed at much lower levels compared to expression in HaCaT cells, this is the first evidence that both p63δ and p63ε are expressed in melanoma cell lines [Figure 3.6].

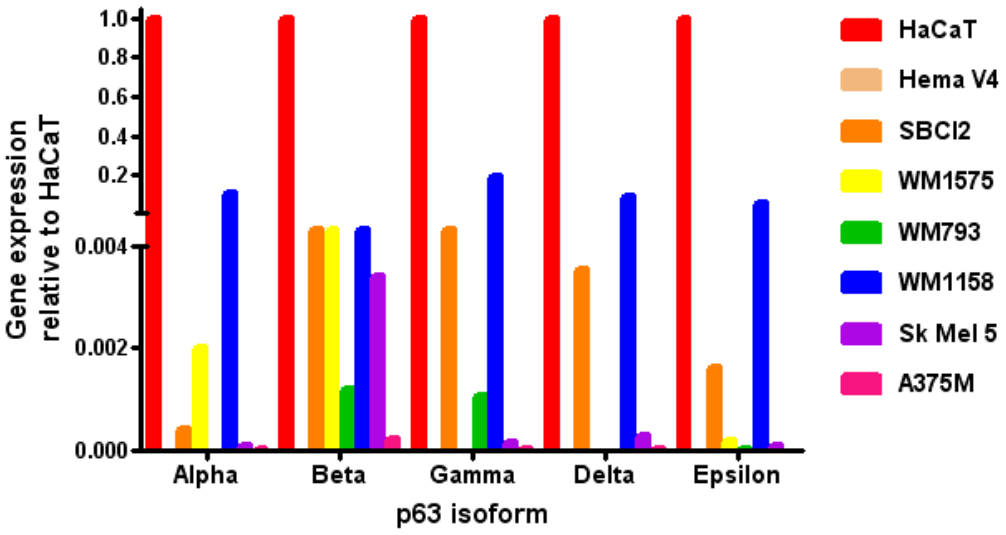


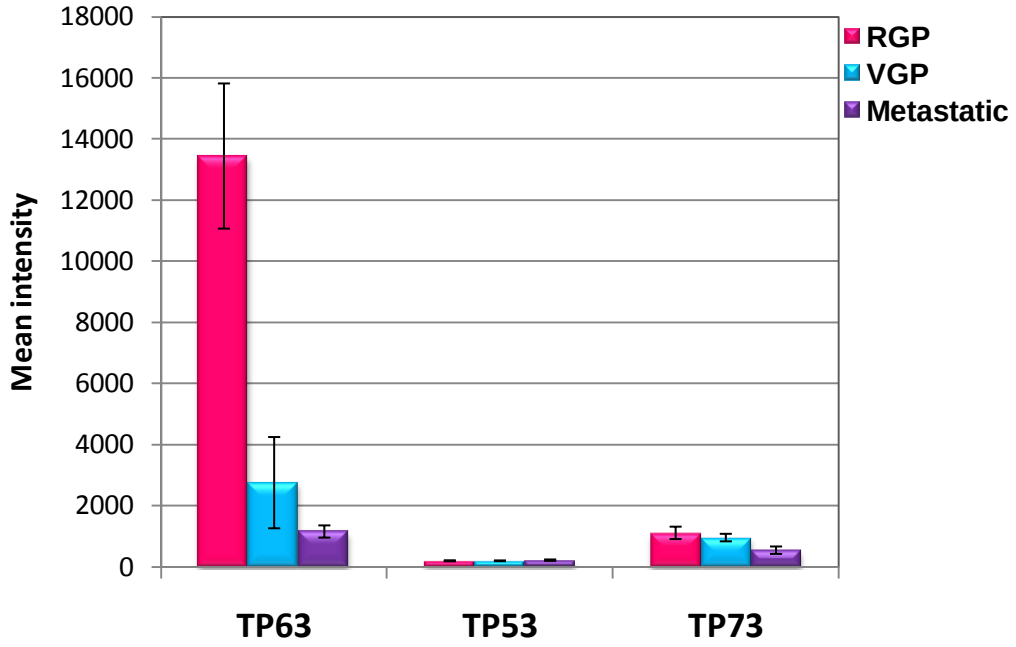
Figure 3.6: TP63 C-terminal splice variant mRNA expression in melanoma cell lines (Q-PCR). No expression of TP63 splice variants was detected in the primary melanocyte culture (HEMa V4). Differential expression of all five C-terminal splice variants is demonstrated in melanoma cell lines using HaCaT cells as a positive control and the comparator. Gene expression was standardised using the housekeeping gene, GUS.

3.3.2 TP63 gene is expressed during melanomagenesis

Having confirmed the expression of both TA and ΔNp63 isoforms and a differential pattern of expression of splice variants in melanoma cell lines, the TP63 gene profile was examined in melanoma tissue samples from a published cohort (Scatolini et al. 2009). This experiment was carried out in collaboration with Dr Giovanna Chiorino (Cancer Genomics Lab, Fondo Edo Tempia, Italy) and involved extracting data from analysis of a whole genome microarray [Section 2.9.1] (Scatolini et al. 2009).

Analysis of expression of p53 family members in the melanoma tissue samples were compared to a Universal Reference (BD™ Human Universal Reference Total RNA, CA). Upregulation of TP63 was detected at all stages of melanoma progression with marked upregulation in radial growth phase melanomas when compared with the Universal Reference [Figure 3.7]. TP53 and TP73 were not significantly upregulated during melanoma progression. A log10 ratio of the mean intensity of the gene in melanoma tissue samples compared with the mean intensity for a group of 29 naevi analysed, revealed significant upregulation of TP63 in primary melanomas (both RGP and VGP stages) suggesting a possible role in early melanomagenesis [Figure 3.7].

(A)



(B)

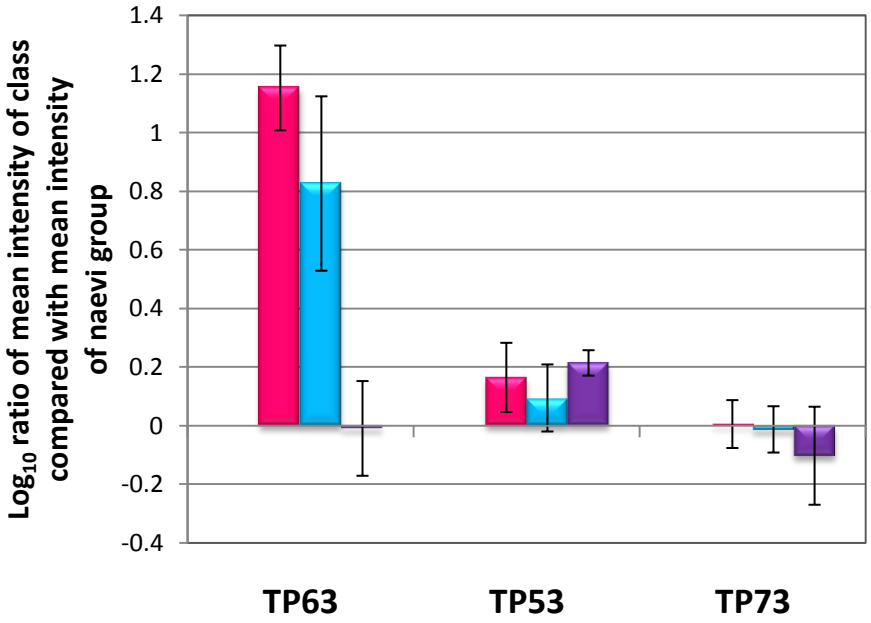


Figure 3.7: Gene expression of TP53 family members in melanoma tissue samples. (A) Analysis of TP53 family genes in melanoma tissue samples using gene microarray (Agilent) (Scatolini et al. 2009). Significant upregulation of TP63 demonstrated in primary melanoma (in particular RGP tumours) and to a lesser extent in metastatic melanomas, when compared with a Universal Reference. (B) Log_{10} ratio of mean intensity of TP53 family members compared with naevi confirms significant upregulation of TP63 in early stages of melanoma progression. RGP – radial growth phase, VGP – vertical growth phase.

3.3.3 p63 protein expression is demonstrated in melanoma

Having determined that TP63 was upregulated in both melanoma cell lines and melanoma tissue samples, the expression of p63 protein was investigated, using Western blotting for analysing melanoma cell lines, and immunohistochemistry for melanoma tissue samples.

3.3.3.1 p63 protein is expressed in melanoma cell lines

Protein was extracted from HEK 293T cells transfected with each of the six p63 isoforms [section 2.1.5.1]. Plasmids for the TA/ΔN δ or ε isoforms were not available. Figure 3.8A shows the expected molecular weights (kD) for each of the isoforms on a Western blot. Exogenously expressed plasmids were used as a reference for subsequent Western blots when detecting expression of p63. Actin was the initial housekeeping gene used for protein standardisation, but it demonstrated considerable variation between melanoma cell lines and was therefore replaced by glyceraldehyde 3-phosphate dehydrogenase (GAPDH). This exhibited stability amongst melanoma cell lines both for RNA and protein analysis. Moreover comparison with another housekeeping gene, α-tubulin, confirmed stability of GAPDH expression and validated its use as a loading control.

Antibody specificity for p63 was confirmed by using different antibodies to detect the six transfected isoforms; AB-1, AB-4 and 4A4 detected all p63 isoforms, H129 detected α isoforms only and H137 detected mainly ΔN isoforms of p63 [Figure 3.8B]. For most Western blotting experiments, AB-1 and AB-4 antibodies were used.

Five primary human melanocyte cultures tested did not express p63 protein [Figure 3.8C]. Melanoma cells, however, expressed variable combinations of TA and ΔN isoforms of p63 (detected using the anti-p63 antibody AB-4) [Figure 3.8C]. Although the ΔNp63δ plasmid was not available, it is possible, based on predicted molecular weight, that this isoform was also expressed in melanoma cell lines (Mangiulli et al. 2009). In keeping with findings of TP53 expression [section 3.3.1.4] these melanoma cell lines expressed p53 protein which is reported to be wild-type [Figure 3.8C].

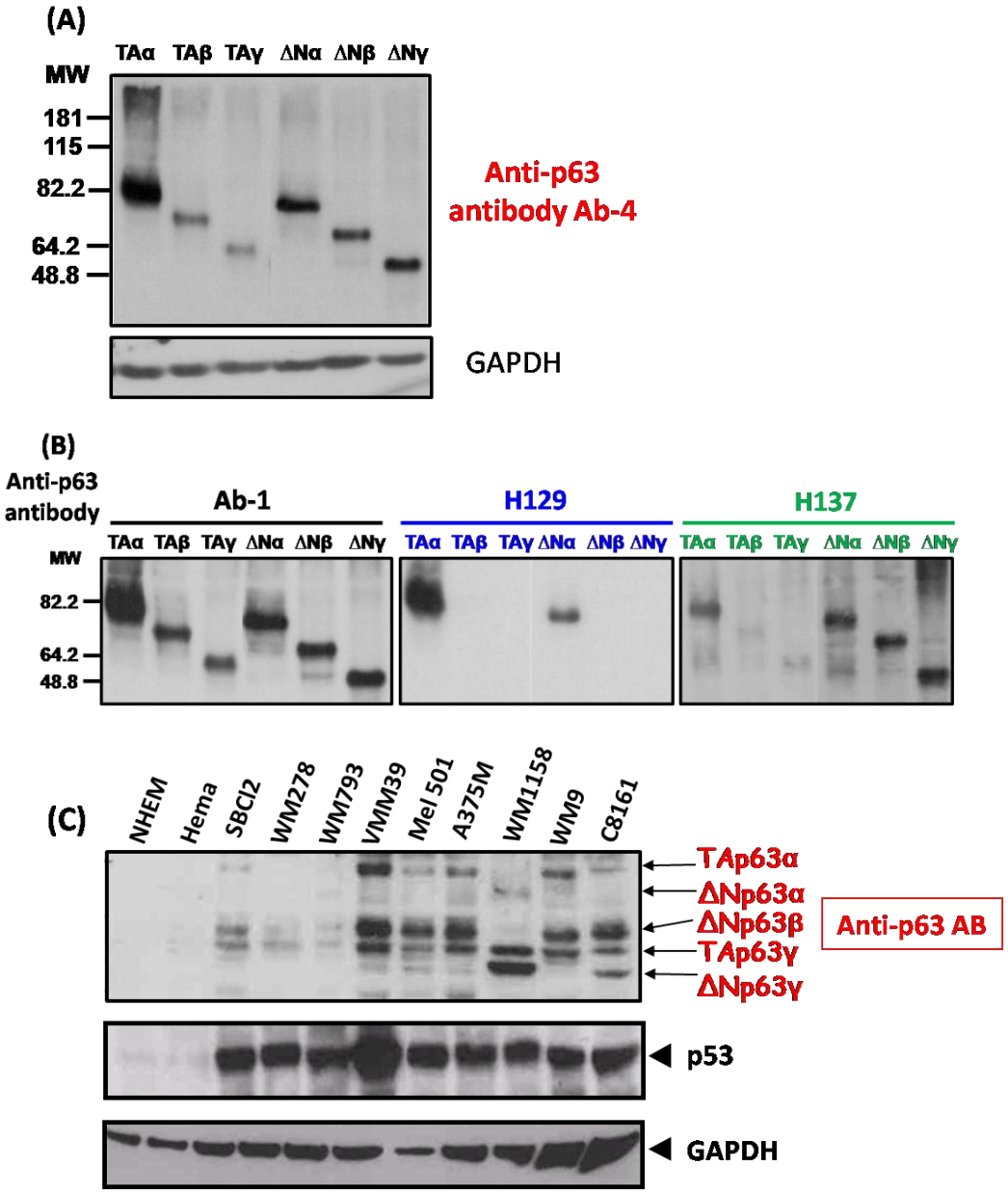


Figure 3.8: p63 protein expression (Western blotting). (A) Protein lysates of HEK 293T cells transfected with plasmids for six p63 isoforms were run on an SDS-PAGE gel and probed with anti-p63 antibody to determine molecular weights (MW) of each isoform. (B) Antibody specificity for p63 demonstrated using anti-p63 antibodies Ab-1, H129 (detects α isoforms) and H137 (detects ΔN isoforms predominantly although also detects TAα isoform). (C) Protein lysates (80 µg) from primary melanocyte cultures (NHEM – NHEM 2 and Hema – HEMa 3) and melanoma cell lines probed for p63 (using anti-p63 antibody AB-4 which detects all isoforms of p63) demonstrating upregulation and differential expression of p63 isoforms in melanoma but no expression in primary melanocyte cultures. Additional bands not corresponding to exogenously expressed isoforms in HEK 293T are observed in melanoma cell lines analysed. GAPDH was used as loading control.

3.3.3.2 p63 protein is localised to both nuclei and cytoplasm in melanoma cell lines

Having established the expression of p63 protein in melanoma cell lines, fluorescent immunohistochemistry was used to detect the cellular localisation of p63 protein in these cells. In basal cells of stratified epithelia such as skin, oral mucosa, cervix, vaginal epithelium, urothelium, prostate and breast, p63 is localised to the nucleus (Dellavalle et al. 2001; Di Como et al. 2002; el-Deiry et al. 1995). However cytoplasmic p63 has been reported in prostate cancer (Dhillon et al. 2009) and in lung adenocarcinoma (Narahashi et al. 2006).

Cells were fluorescently stained as outlined, using anti-p63 antibodies (H127 and H139) [section 2.7.1]. Slides were subsequently visualised using confocal microscopy. Exclusion of the primary antibodies confirmed specificity of p63 detection and HEK 293T cells which do not express p63 were used as a negative control [Figure 3.9]. To optimise the experimental conditions for fluorescent immunocytochemistry, HaCaT cells were used which demonstrated nuclear, non-nucleolar localisation of p63 [Figure 3.10]. Melanoma cell lines expressed p63 in both nuclei and cytoplasm and this finding was reproduced when using different anti-p63 antibodies.

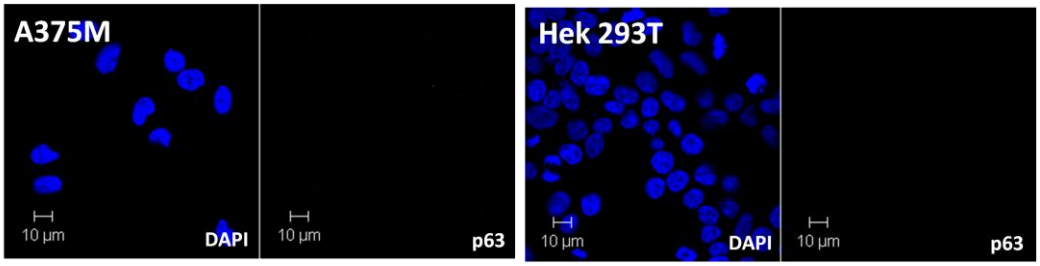


Figure 3.9: Antibody specificity used for immunofluorescence techniques. Left panel demonstrates A375M melanoma cells fluorescently stained as outlined [section 2.7.1] omitting the primary anti-p63 antibody. Right panel demonstrates no staining in HEK 293T cells (which do not express p63) when fluorescently stained using anti-p63 antibodies (H129/H137) confirming specificity of antibodies to detect p63 protein. DAPI (blue) was used to stain nuclei.

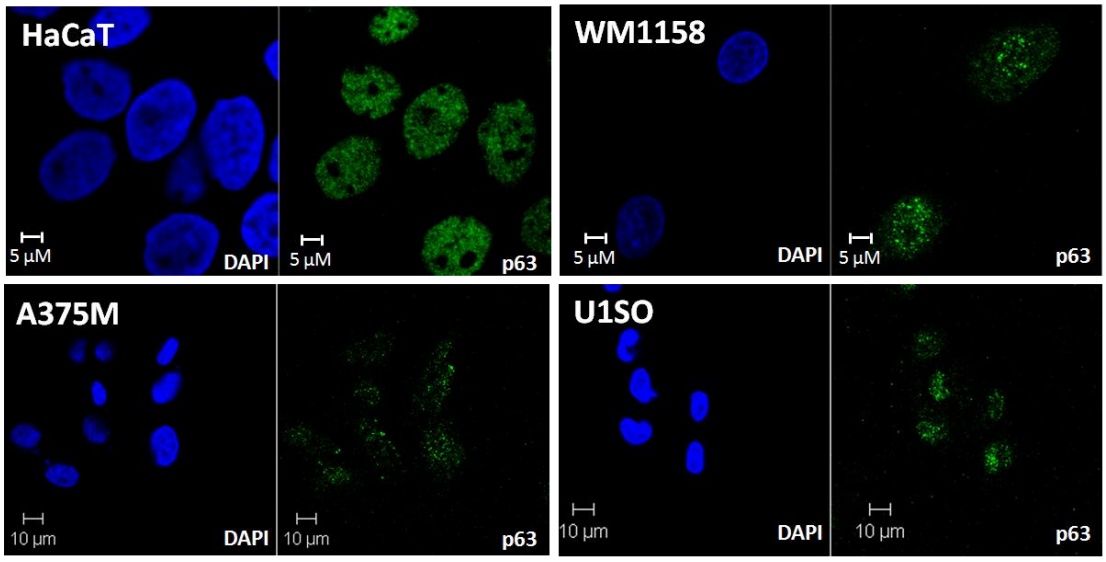


Figure 3.10: p63 protein expression in melanoma cell lines (IMF). HaCaT cells show nuclear, non-nucleolar staining of p63 (Alexa-488, green) using anti-p63 antibodies (H129/H137). p63 protein expression confirmed in nuclear and cytoplasmic compartments in three melanoma cell lines – two harbouring wild-type p53 (WM1158 and A375M) and one p53-null cell line (U1SO). DAPI used to stain nuclei blue.

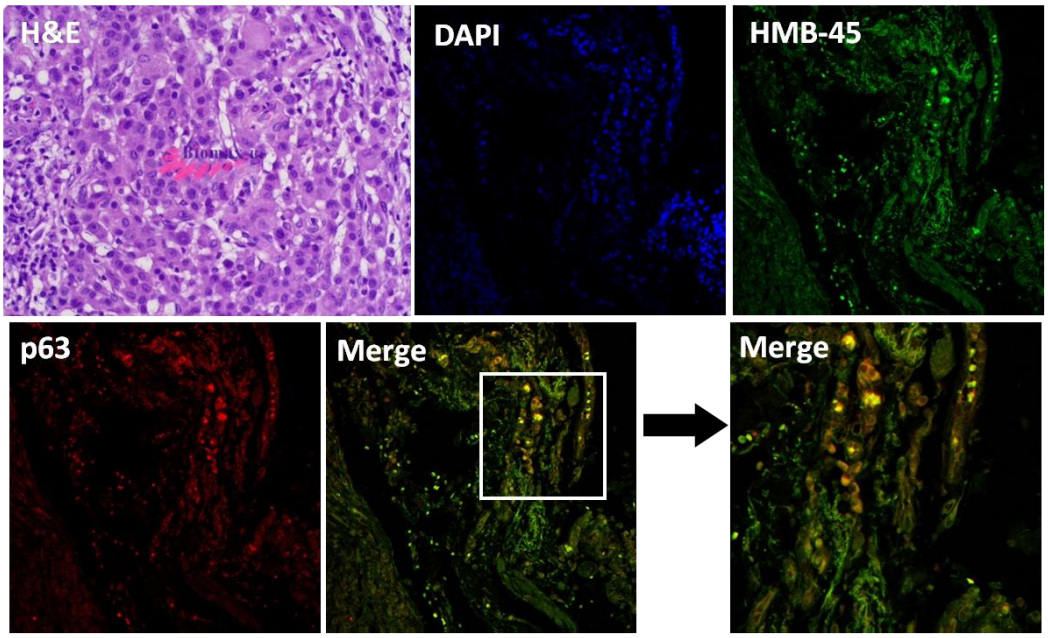
3.3.3.3 p63 is expressed in a melanoma tissue microarray

To extend findings which confirmed expression of p63 in melanoma cell lines, immunohistochemical expression of p63 was investigated in 40 primary melanoma tissue samples in a tissue microarray (TMA; Biomax) [section 2.7.2]. Two anti-p63 antibodies (H129 / H137) were used in combination to detect p63. HMB-45 is a highly specific marker routinely used in the diagnosis of primary and metastatic melanoma cells. This method uses monoclonal antibodies to a glycoprotein (gp100) present in cytoplasmic pre-melanosomes and is a highly specific immunohistochemical stain for primary and metastatic melanomas (Baisden et al. 2000; Colombari et al. 1988; Yaziji and Gown 2003). 16/40 (40%) samples in the TMA, strongly labelled p63 while co-staining with HMB-45, confirming expression of p63 in melanoma cells; two examples of positive expression of p63 protein in melanoma cell lines are shown [Figure 3.11].

Statistical analysis of clinical data available for the melanoma tissue array samples was undertaken [Table 3.2]. No significant differences between p63-positive and p63-negative melanomas were detected for age at diagnosis or gender of patients. A significant difference in body site between p63-positive and -negative melanomas was observed (p-value 0.044, chi-squared test), but a site predilection for p63-positive melanomas could not be determined due to the small sample size.

Although the majority of melanomas are HMB-45 positive, negative reactivity occurs in up to 20% cases (Al-Batran et al. 2005), mostly corresponding to rarer melanoma subtypes e.g. desmoplastic or spindle cell (Busam et al. 1998; Xu et al. 2002). In the TMA, 3/40 (7.5%) melanomas were HMB-45 negative but did demonstrate p63 reactivity. This could underestimate the number of p63-positive melanomas, particularly in light of data reporting 2/3 spindle cell melanomas express p63 (Morgan et al. 2008). Due to the nature of the TMA, co-expression studies simultaneously analysing other melanoma markers e.g. S100 and melan-A, are not possible, making it difficult to confirm the localisation of p63 to melanoma cells (and not keratinocytes or non-specific stromal tissue) in these three cases. Moreover, the poor histological detail of TMA images limits correlation of histopathological features of melanoma with p63-reactivity. To address this, the Bart's and the London NHS Trust archive was accessed, to obtain clinicopathologically characterised, formalin-fixed paraffin-embedded (FFPE) material representing all stages of melanoma development; benign melanocytic naevi, primary melanoma, recurrent melanoma and metastases, for analysis of p63 expression [Ethical approval number 07/QO604/23, section 2.6].

(A)



(B)

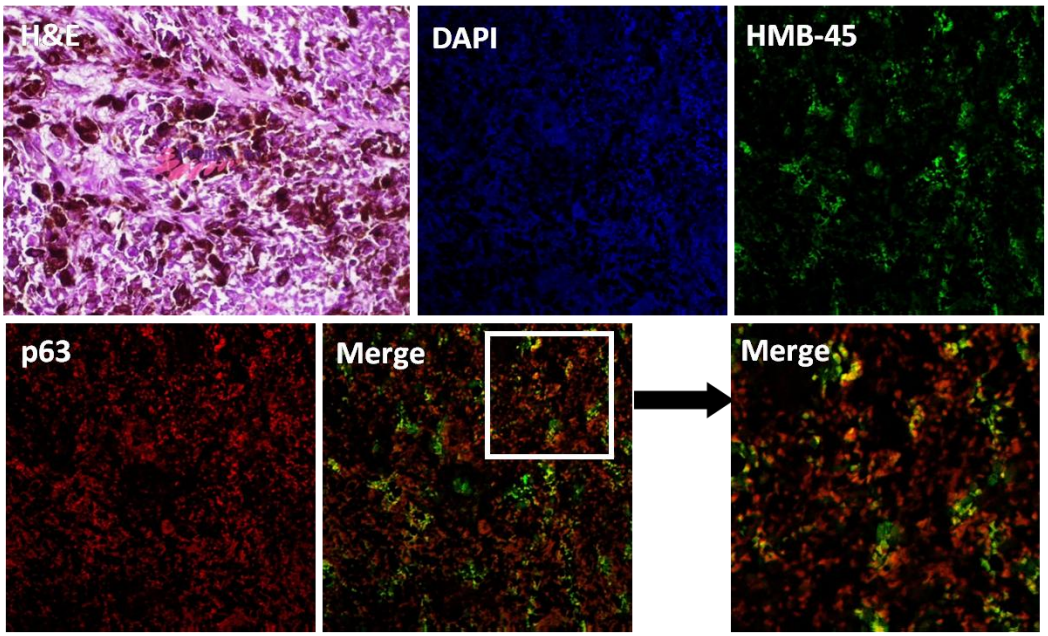


Figure 3.11: Expression of p63 in primary cutaneous melanomas (TMA). (A) Primary melanoma from left arm (81 y/o ♀). (B) Primary melanoma with heavy melanin deposition from right thumb (62 y/o ♀). Panels described left to right, top to bottom: H&E of melanoma, DAPI stains nuclei of melanoma cells (blue) (x20), HMB-45 labels melanoma cells (green), anti-p63 antibodies (H129/H137) labels cells within tumour for p63 (red), merged image demonstrates co-expression of HMB-45 and p63 (yellow) (x20), higher magnification confirms melanoma cells express p63 (yellow) (x40). H&E – haematoxylin and eosin.

Table 3.2: Statistical analysis of demographic data for p63-expressing primary melanoma tissue samples (TMA).

Demographic data	p63–ve tumours (n=24)	p63+ve tumours (n=16)	p-value
Mean age – yrs (SD)	54 (16.5)	56.3 (14.4)	0.64 (t-test)
Gender (M:F)	12 : 12	10 : 6	0.33 (χ-squared)
Site of melanoma			0.044* (χ - squared)
Trunk	6	9	
Limbs	4	3	
Acral	11	2	
Mucosal	3	2	

*statistically significant – chi squared test, SD – standard deviation

3.3.4 p63 is infrequently expressed in benign naevi

Paraffin-embedded samples of benign intradermal naevi (BIDN) were analysed using immunohistochemistry [section 2.7.3]. To confirm the specificity of staining, only sections with an internal control of epidermal keratinocytes positively labelled with p63 were included in the analysis. This comprised 12 BIDN samples excised from 9 individuals [Table 3.3]. Only 2/12 BIDN samples demonstrated positive labelling of p63 using the H129 antibody [Figure 3.12]. These two cases are highlighted in Table 3.3. Both the naevi were located on the back. For both individuals with p63-positively labelled BIDN, another naevus excised at the same time from a different body site demonstrated no p63 reactivity. These data implicate pathways within specific naevi, which might constitute early dysplasia involved in switching on this gene. No statistical analysis was undertaken in view of the small number of p63-positive BIDN samples.

Table 3.3: Demographic details of BIDN cases.

Sample ID	Patient ID	Gender	Age at diagnosis	Site	Staining intensity*
N5	2	F	33.89	Mid back	++
N6	2	F	33.89	L neck	
N7	3	F	61.28	L cheek	
N8	4	F	43.41	L neck	
N9	5	M	51.56	L lower back	
N10	6	M	40.21	L ear	
N11	7	F	45.10	L scalp	
N14	7	F	45.10	Back	+
N19	12	M	38.28	Lower back	
N21	12	M	38.28	R chin	
N27	14	F	61.46	R upper lip	
N32	18	M	70.18	R scapula	

*All samples demonstrated positive labelling of keratinocytes with p63 as internal control

Positive p63 reactivity demonstrated in naevi highlighted in pink. M – male, F – female, L – left, R – right, + weak, ++ moderate, +++ strong intensity staining.

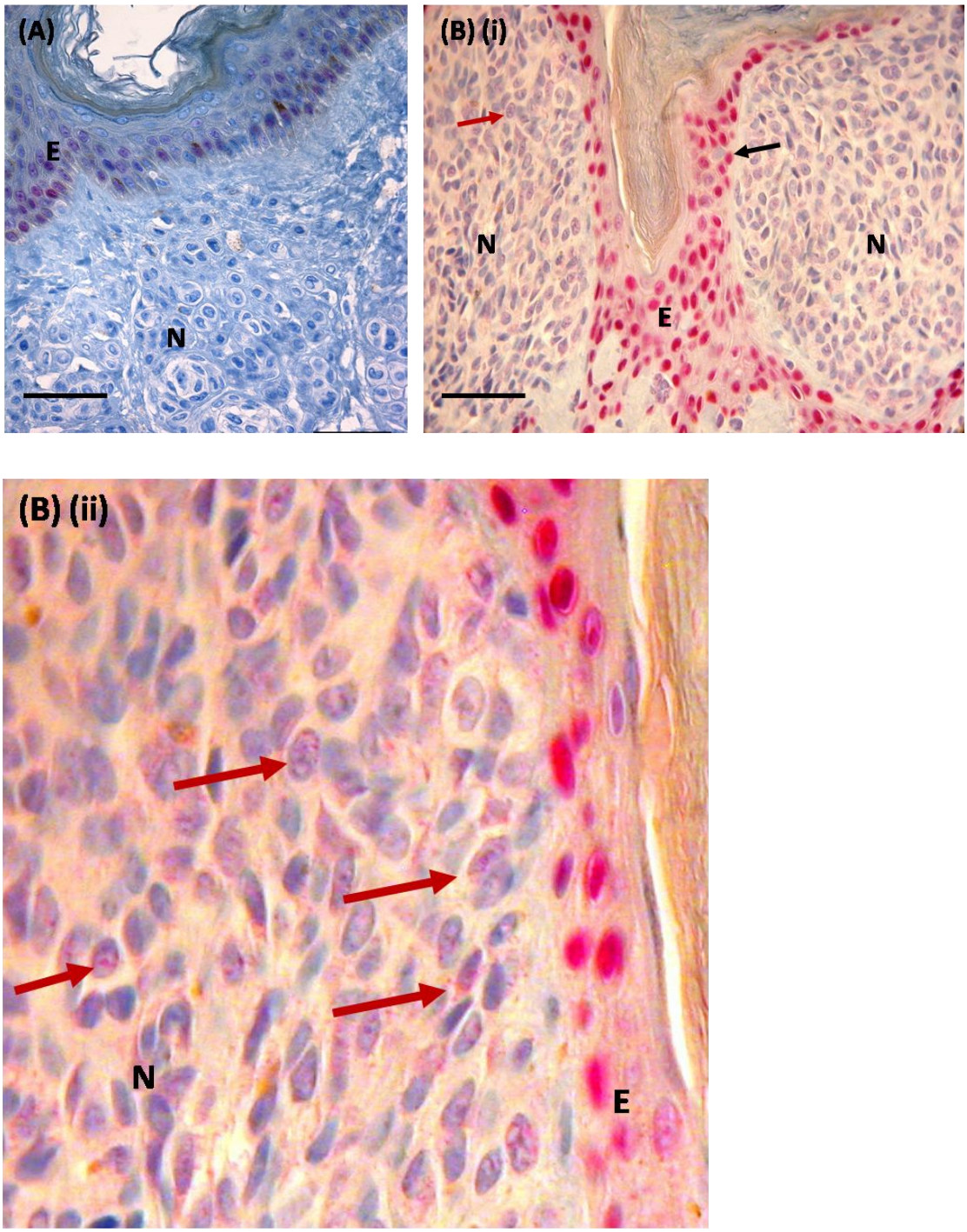


Figure 3.12: Immunostaining of p63 in BIDN. (A) Sample ID N11 [Table 3.3]. Well-circumscribed melanocytic naevus cells arranged in nests within the dermis denoted N. Strong nuclear p63 labelling of epidermal keratinocytes (denoted E) but negative p63 reactivity of naevus (N). (B) Sample ID N5 [Table 3.3], (i) Rounded, polygonal melanocytes grouped in nests within the dermis denoted N, demonstrating moderate intensity nuclear and cytoplasmic p63 staining of naevi cells shown by red arrow. Positive internal control for p63 staining of keratinocytes (black arrow) within epidermis denoted E. (ii) Higher magnification of naevi cells showing nuclear and cytoplasmic punctate p63 reactivity. Bar line 50 µm.

3.3.5 p63 is expressed in primary and metastatic melanoma tissue samples

The literature to date suggests that melanoma tissue samples, for the most part, do not express p63 [section 1.3.7.4]. However, fluorescent immunocytochemistry of melanoma tissue samples from a TMA have demonstrated at least 40% of primary melanomas from various body sites express p63. Lack of available outcome data for these melanomas prevented a prognostic correlation with p63-reactivity, to be undertaken. Immunohistochemistry of archival FFPE tissue overcomes this problem. In addition, histopathological analysis of tumours provides a more precise analysis of cellular localisation of p63 reactivity in melanoma cells.

3.3.5.1 Analysis of immunohistochemistry labelling of FFPE tissue samples

Melanoma tissue samples representing different stages of disease progression were selected from the archive of FFPE tissue samples at Bart's and the London NHS Trust. Clinical data were collected from patients attending the skin cancer multidisciplinary clinic and through accessing electronic medical records [Appendix 3]. Pathology reports confirmed the diagnosis of melanoma in all cases and slides were subsequently reviewed by a dermatopathologist (R.Cerio). Immunostaining was undertaken using the automated Ventana system at the Bart's Pathology Service (M.Ikram). Alkaline phosphatase (red) was used as the chromogen to avoid misinterpretation of a brown chromogen e.g. DAB with melanin pigmentation in melanoma. Initial optimisation was carried out using the anti-p63 antibodies, H129 and 4A4. As both antibodies demonstrated similar p63 reactivity, the H129 antibody (routinely used for the analysis of clinical samples and for clinical trials by the Bart's Pathology Service) was used for all tumour samples.

Positive reactivity to p63 in keratinocytes in the epidermis or ductal/glandular epithelia in the dermis, of skin samples were used as positive internal controls. Twenty-five sections cut more than one month prior to immunostaining demonstrated reduced or no reactivity but when sections were freshly cut and stained within two weeks, positive p63 reactivity of internal controls was observed [Figures 3.13A & B]. These findings were in keeping with reports in the literature of the negative effect of storage of sections on p63 immunoreactivity (Burford et al. 2009; Hameed and Humphrey 2005). Negative controls comprised omission of the primary antibody. Positive reactivity of p63 in melanoma tumour samples was confirmed when melanoma cells showed pink staining of nucleus and/or cytoplasm [Figures 3.13C & D]. Melanoma sections positively-labelled for p63 were reviewed by two dermatologists (RM and CAH) and one dermatopathologist (RC) blinded to clinical outcomes. Intensity of

melanoma cell staining was designated to a category - no staining, weak, moderate and strong [Figure 3.14]. Staining of melanoma cells was analysed by comparison with strong nuclear labelling of p63 in keratinocytes or ductal epithelia within the same skin tumour sample. Given the heterogeneity displayed by melanoma cells, there were cases where the proportion of cells demonstrating p63 reactivity was < 50% or cases where only focal staining was observed within a tumour (constituting <10% of melanoma cells). These cases were excluded from statistical analyses so as not to skew the data. Only tumours showing weak, moderate or strong intensity cytoplasmic reactivity in >50% of the melanoma were included for statistical analysis.

3.3.5.2 Cohort of melanoma tissue samples analysed

Overall, 44 primary melanomas, 11 recurrences and 24 metastatic tumours from 58 patients were suitable for analysis. Mean age at diagnosis for all melanomas examined was 58.9 yrs (range 21.9 – 86.6 yrs), M:F ratio 1:1.5 with a mean follow-up of 4.3 yrs (range 0.18 – 13.1 yrs). There were a total of 34 deaths (25 attributable to melanoma). For eight cases, matched primary and recurrent and/or metastatic tumours were available for analysis [section 3.3.5.5]. In addition one ocular melanoma and three metastatic tissue samples from the same individual were also tested. Ocular melanomas arise against a considerably different molecular and genetic background to cutaneous melanoma, and were therefore excluded from the analysis.

3.3.5.3 Primary melanoma tissue samples predominantly express cytoplasmic p63

Overall, 39/79 (49%) melanoma tissue samples showed positive reactivity for p63 at any intensity (using H129 anti-p63 antibody) [Table 3.4]. The proportion of primary tumours staining positive for p63 was greater than the proportion of metastatic tumours staining positive for p63 although no significant difference was detected in p63 expression at different stages of disease progression (p-value 0.31, global chi-squared). The majority of tumours showed weak cytoplasmic reactivity of p63 (n=19, 51%) with 41% (n=16) tumours showing moderate reactivity, and only three cases (8%) showing strong p63 reactivity. In cases where nuclear reactivity of p63 was observed, this constituted less than 5% of total melanoma cells within the tumour sample. Nuclear reactivity was observed in 23% (n=10) and 18% (n=6) of primary and recurrent/metastatic melanoma tissue samples, respectively [Figures 3.13C and 3.15(h)].

3.3.5.3.1 *p63 reactivity in primary melanomas is not significantly associated with clinicopathological factors*

24 out of 44 (55%) primary melanoma tissue samples showed p63 reactivity in the cytoplasm for >50% of tumour cells [Figure 3.15]. The mean age of diagnosis in patients harbouring p63-positive melanomas (61 yrs) was higher than the p63-negative tumours (56.3 yrs) with a M:F ratio of 1:1.4. There was an even distribution of p63-positive melanomas according to body site. Statistical analysis revealed no significant association between p63 expression of primary tumour with age at diagnosis, gender or site of melanoma [Table 3.5].

Analyses of histopathological determinants of prognosis for primary melanoma (including Breslow thickness, histological classification, growth phase, ulceration status and mitotic rate) did not demonstrate any significant association between these factors and expression of p63 [Table 3.6]. However, more than 50% of thicker tumours (Breslow thickness category T4 i.e. > 4 mm) were p63-positive compared with only 25% of p63-negative tumours. Nodular melanomas were over-represented in p63-positive tumours (almost 70%) compared with p63-negative tumours (45%), although this difference did not achieve statistical significance [Table 3.6].

Table 3.4: Expression of p63 in melanoma tissue samples

Tumour stage	p63-negative tumours (n=40)	p63-positive tumours (n=39)	P-value*
Primary (n=44)	20 (45)	24 (55)	0.31
Recurrence (n=11)	6 (55)	5 (45)	
Metastatic (n=24)	14 (58)	10 (42)	

Values shown are numbers of tumours (percentage of total in each category)

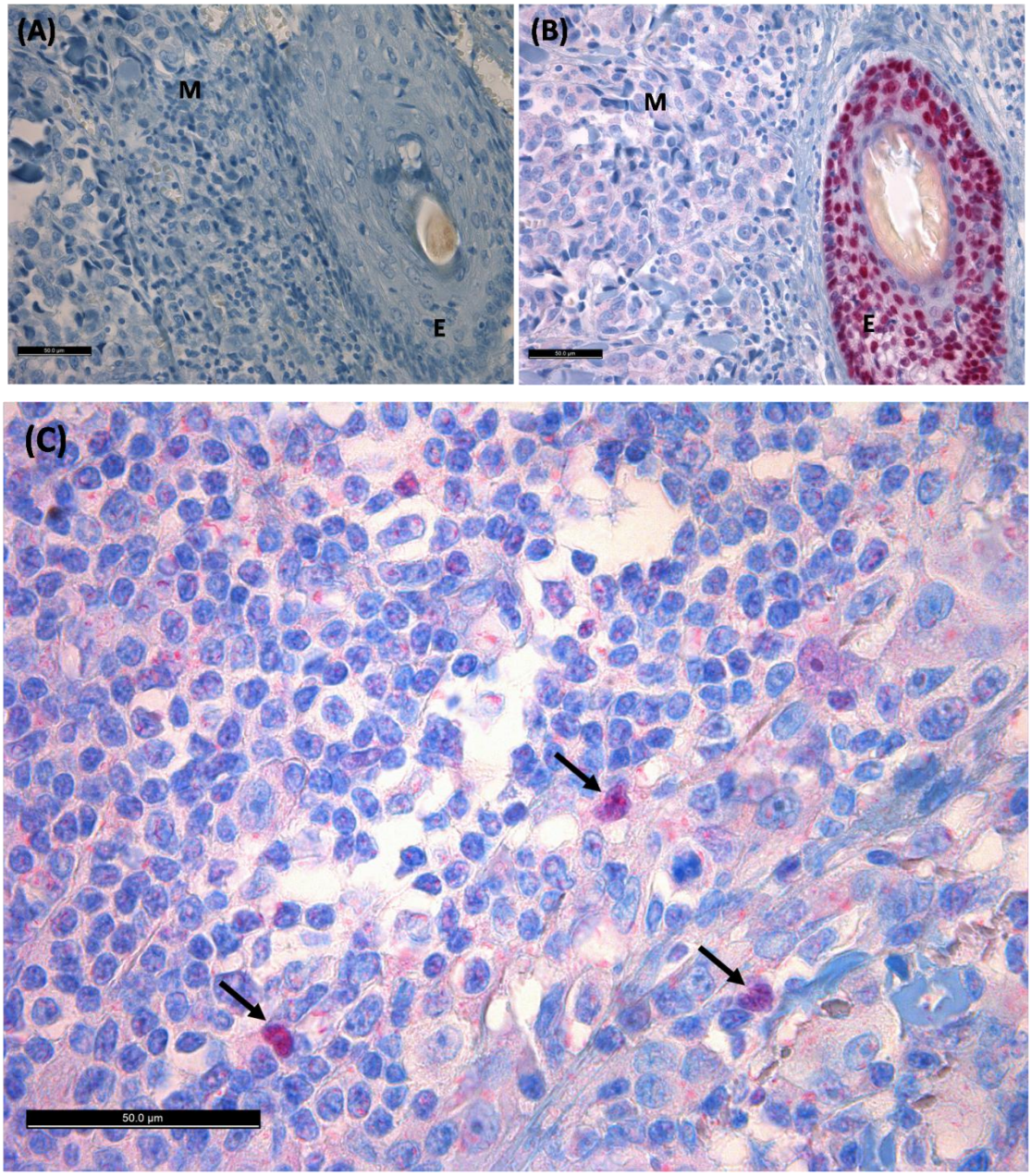
*p-value for odds comparing proportion of p63-positive tumours between primary and metastatic tumours, global chi-squared test

Table 3.5: Clinicopathological details of primary melanomas according to p63 status

	p63-negative tumours (n=20)	p63-positive tumours (n=24)	Total (n=44)	p-value*
Age at diagnosis (yrs)	56.3 (40.9, 74.6)	61.0 (46.9, 74.2)	59.4 (46.0, 74.2)	0.71
Gender				
Male	7 (35.0)	10 (41.7)	17 (38.6)	0.65
Female	13 (65.0)	14 (58.3)	27 (61.4)	
Site of Melanoma (n=44)				
Acral	4 (20.0)	4 (16.7)	8 (18.2)	-
Extremities	7 (35.0)	7 (29.2)	14 (31.8)	1.00
Head/Neck	2 (10.0)	5 (20.8)	7 (15.9)	0.40
Trunk	7 (35.0)	8 (33.3)	15 (34.1)	0.88

Values shown are number (percentage) or median (IQR) as appropriate

* p-values are odds comparing p63-negative with p63-positive tumours (chi-squared test)


(A)
M
E
(B)
M
E
(C)
50.0 µm

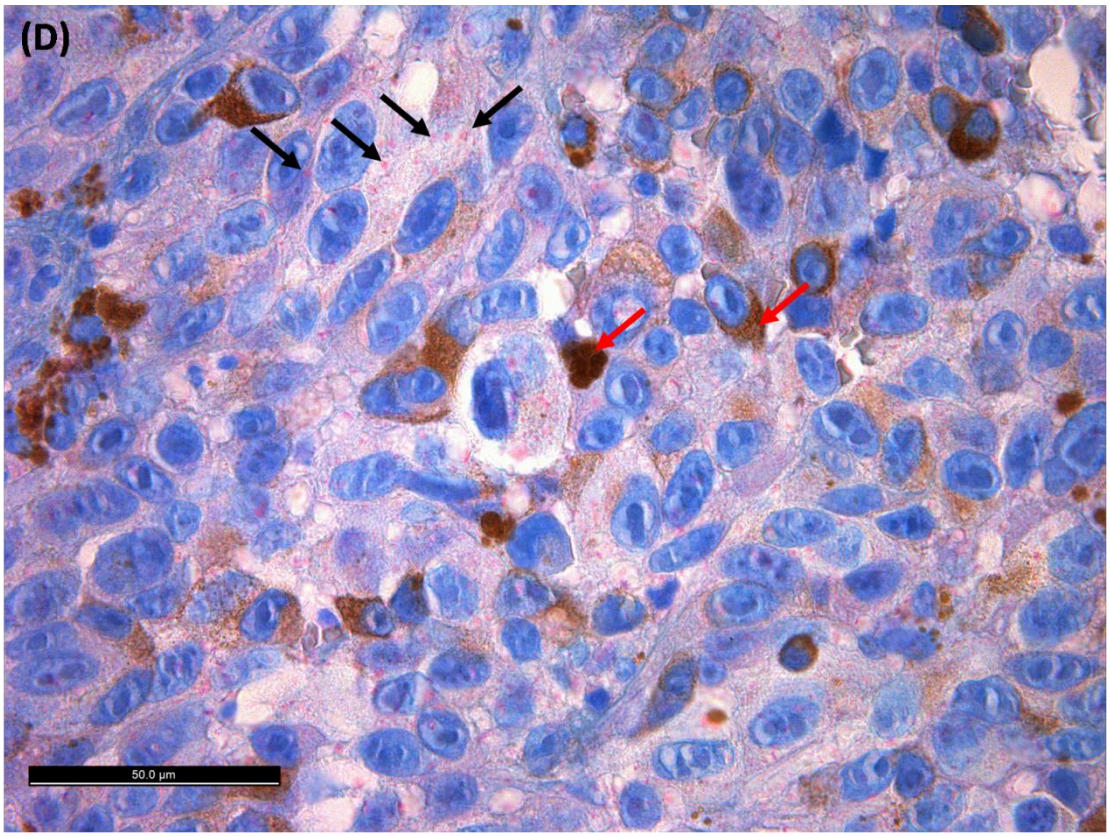


Figure 3.13: Punctate p63 reactivity in cytoplasm of melanoma cells. Example of primary nodular melanoma (22 y/o woman) on neck, Breslow thickness 3.5 mm, (A) sections cut three months prior to immunostaining show no p63 reactivity of keratinocytes in epidermis (denoted E) cut in cross-section and (B) section cut from same tumour block, one week prior to immunostaining showing strong nuclear p63 reactivity of keratinocytes in epidermis (denoted E) cut in cross-section and cytoplasmic stippling of p63 staining in melanoma cells (denoted M). These data confirmed the negative effect of storage on immunostaining (Burford et al. 2009) highlighting the potential for false-negative results. (C) Primary nodular melanoma (77 y/o woman) from left temple, Breslow thickness 4.5 mm showing cytoplasmic punctuate staining of p63 in melanoma cells in addition to nuclear staining in <5% of cells (black arrows). Co-localisation studies using macrophage markers could help to exclude the possibility that these cells are macrophages and not melanoma cells. (D) Metastatic acral lentiginous melanoma (84 y/o woman), Breslow thickness 10 mm showing punctate cytoplasmic p63 reactivity of melanoma cells shown by black arrows. Red chromogen of alkaline phosphatase is easily differentiated from brown pigment of melanin deposition (red arrows). Bar – 50 µm.

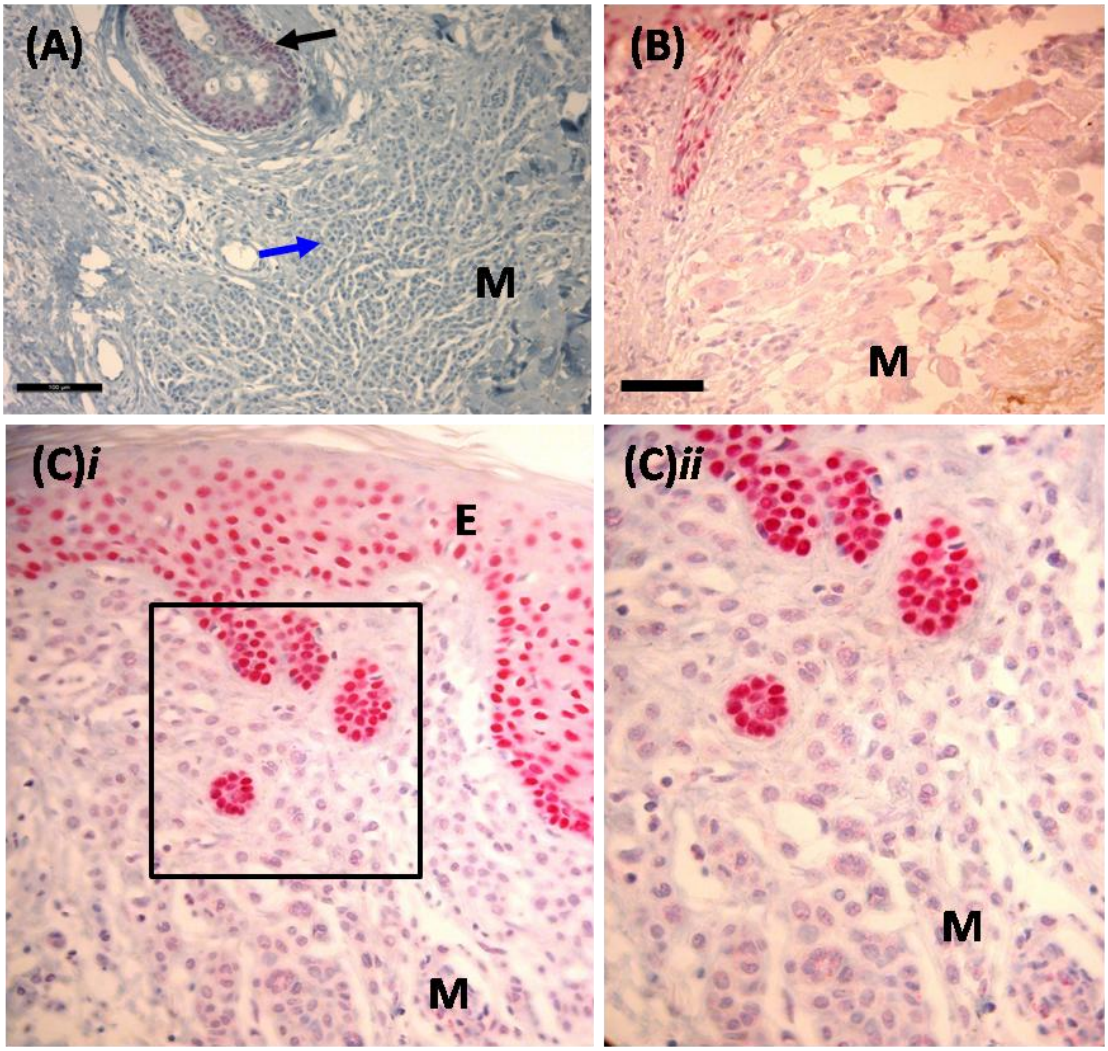


Figure 3.14: Intensity of p63 staining in melanoma tissue samples. (A) Negative p63 staining of primary superficial spreading melanoma on back (blue arrow), arising from intradermal naevus. Strong nuclear p63 staining of glandular epithelium in dermis (black arrow). (B) Moderate intensity cytoplasmic p63 reactivity of primary nodular melanoma (M), left foot, Breslow thickness 1 mm, high mitotic activity and strong nuclear p63 staining of epidermis (top left). (C) Cutaneous metastatic melanoma shown at (i) low power (x20) and (ii) higher magnification (x40) composed of round pleiomorphic melanoma cells demonstrating weak intensity cytoplasmic p63 reactivity. Comparison is made with strong nuclear reactivity of epidermal keratinocytes. M – melanoma cells, E – epidermal keratinocytes. Bar – 100 µm.

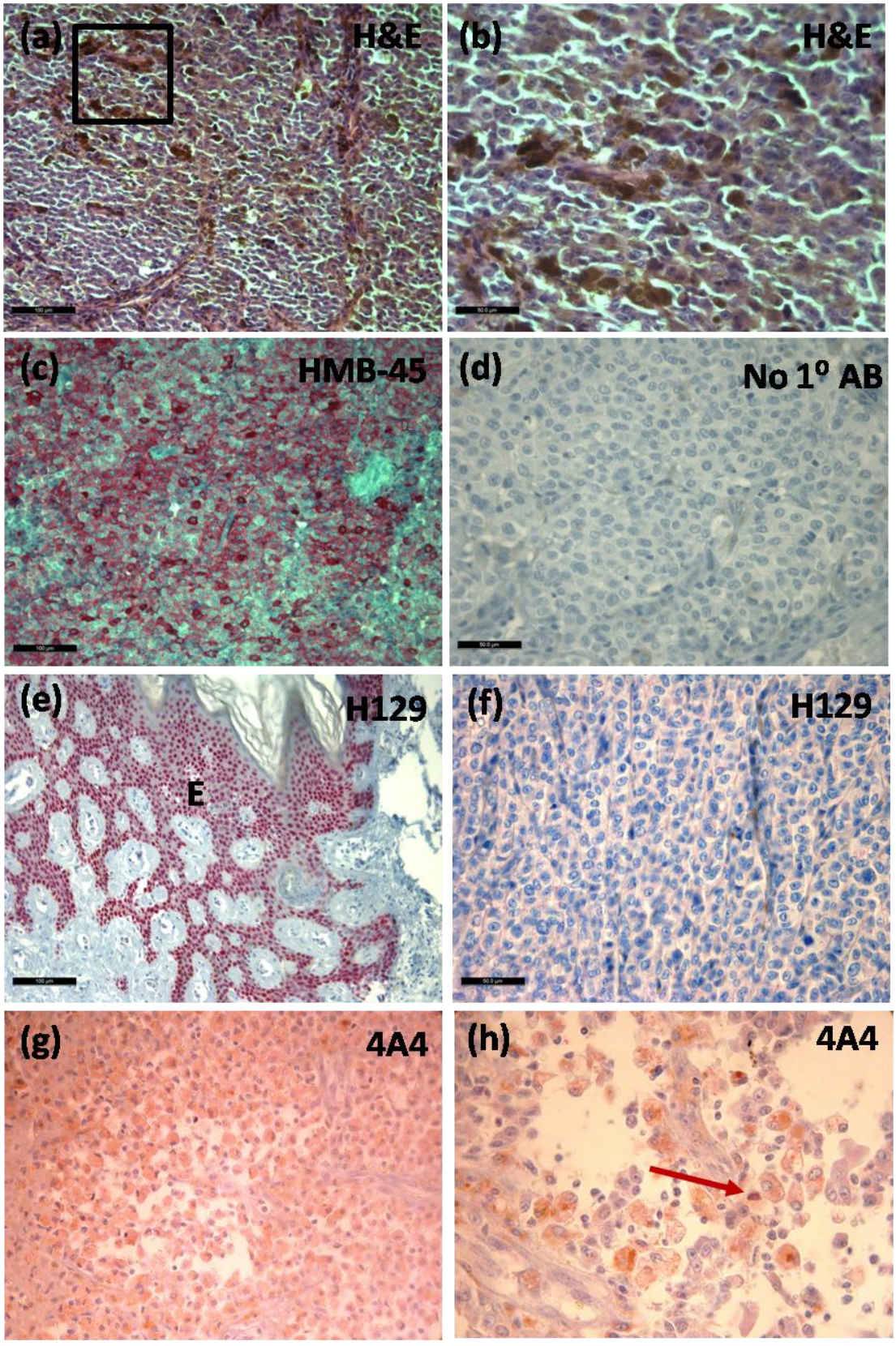


Figure 3.15: Immunohistochemistry analysis of FFPE primary melanoma tissue sample. (a) H & E of recurrent nodular melanoma on back (46.2 y/o male) demonstrating prominent melanocytic atypia and high mitotic activity, with minimal radial growth phase. (b) Magnification of black box from (a) demonstrating dermal melanoma cells with heavy melanin

deposition. (c) HMB-45 using alkaline phosphatase (red) chromogen demonstrates nuclear and cytoplasmic reactivity of melanoma cells. (d) Omission of primary antibody excludes non-specific staining by chromogen. (e) Anti-p63 antibody (H129) demonstrates strong nuclear expression of p63 in epidermal keratinocytes (E) in excised lesion containing melanoma. (f) Cytoplasmic p63 expression demonstrated in melanoma cells using H129 antibody. (g) Anti-p63 antibody 4A4 confirms similar positive cytoplasmic staining of p63 in melanoma cells of the same tumour (x20). (h) Higher magnification of dyscohesive melanoma cells (x40) stained using 4A4 antibody, demonstrates both cytoplasmic p63 and nuclear reactivity (red arrow), in <5% of melanoma cells within the tumour sample. Bar – 100 µm. H & E – haematoxylin and eosin.

Table 3.6: Histopathological details of primary melanomas according to p63 status

	p63-negative tumours (n=20)	p63-positive tumours (n=24)	Total (n=44)	p-value*
Breslow thickness, mm (n=43)				
0 – 1	5 (25.0)	4 (17.4)	9 (20.9)	0.24
1.01 – 2	3 (15.0)	1 (4.3)	4 (9.3)	
2.01 – 4	7 (35.0)	6 (26.1)	13 (30.2)	
>4	5 (25.0)	12 (52.2)	17 (39.5)	
Clarks Level (n=42)				
I	3 (15.8)	3 (13.0)	6 (14.3)	0.42
II	5 (26.3)	5 (21.7)	10 (23.8)	
III	10 (52.6)	11 (47.8)	21 (50.0)	
IV	1 (5.3)	4 (17.4)	5 (11.9)	
Histological classification (n=43)				
ALM	4 (20.0)	4 (17.4)	8 (18.6)	-
NMM	8 (40.0)	15 (65.2)	23 (53.5)	0.45
SSM	8 (40.0)	4 (17.4)	12 (27.9)	0.46
Growth phase (n=39)				
RGP	3 (16.7)	4 (19.0)	7 (17.9)	0.85
VGP	15 (83.3)	17 (81.0)	32 (82.1)	
Ulceration status (n=43)				
No	10 (50.0)	12 (52.2)	22 (51.2)	0.89
Yes	10 (50.0)	11 (47.8)	21 (48.8)	
Mitotic rate# (n=33)				
0	1 (6.7)	4 (22.2)	5 (15.2)	0.21
1 – 4	5 (33.3)	5 (27.8)	10 (30.3)	
5 – 10	6 (40.0)	8 (44.4)	14 (42.4)	
≥ 11	3 (20.0)	1 (5.6)	4 (12.1)	
Regression (n=39)				
No	19 (95.0)	19 (86.4)	38 (90.5)	0.34
Yes	1 (5.0)	3 (13.6)	4 (9.5)	
Microsatellites (n=43)				
No	15 (75.0)	20 (87.0)	35 (81.4)	0.31
Yes	5 (25.0)	3 (13.0)	8 (18.6)	

Clark level – defines depth related to skin structures: Level I: Melanomas confined to the epidermis, Level II: Penetration into the dermis, Level III: Melanoma invades deeper through dermis but still contained within skin, Level IV: Penetration of melanoma into fat, Level V: Penetration into the subcutis.

*t-test (paired); # number/mm^2 – categories chosen based on significant survival differences demonstrated between these groupings (Azzola et al. 2003).

ALM – acral lentiginous melanoma, NMM – nodular melanoma, SSM – superficial spreading melanoma, RGP – radial growth phase, VGP – vertical growth phase.

3.3.5.3.2 *Co-existing tumours in melanoma cases*

In three cases within the primary melanoma cohort, a second melanoma was reported – in two cases this occurred within four months of presentation of the melanoma examined, and in one case this preceded the primary melanoma examined, by 11 yrs. In all three cases, the second melanoma was of Breslow thickness < 1mm and all three tumours analysed demonstrated p63-reactivity. Additional co-existing tumours in this cohort of primary melanomas included 2 patients with co-existing breast carcinoma, 1 patient with non-Hodgkin's lymphoma, 1 patient with marginal zone lymphoma, 1 patient with renal cell carcinoma and 1 patient reporting co-existing lung adenocarcinoma and bladder carcinoma. It is well-reported that the risk of secondary cancers is elevated in patients with melanoma and these data from a small cohort, support this finding (Bellet et al. 1977; Bhatia et al. 1999; Goggins et al. 2001; Levi et al. 1997; Schmid-Wendtner et al. 2001; Swerdlow et al. 1995). Only melanomas from patients with co-existing breast carcinoma or lung adenocarcinoma demonstrated p63 reactivity.

3.3.5.3.3 *p63-reactivity affects recurrence rate of primary melanomas*

In the primary melanoma cohort, overall there were 19 deaths with 14 deaths attributable to melanoma. Mean follow up was 4.96 yrs (range 0.18 – 13.06 yrs) and mean time to recurrence (n=7) was 1.88 yrs (range 0.04 – 6.02 yrs). Univariate and multivariate analysis of prognostic factors, including p63 status, for time to recurrence was undertaken [Appendix 4]. Univariate analysis of p63 status for time to recurrence was not significant [Figure 3.16]. Multivariate analysis was then undertaken including only those factors that had a significant effect on outcome. This also failed to demonstrate p63-status to be a significant predictor of disease recurrence, although an upward trend was observed towards increased recurrence rates in the p63-positive cohort (HR 2.89; p-value 0.24, Cox proportional hazard ratio). The limitation of this analysis is the small sample size – only 7/44 cases in the cohort developed recurrent disease.

3.3.5.3.4 *p63-reactivity affects rate of metastases of primary melanomas*

Mean time to first metastasis in this cohort (n=20) was 2.07 yrs (range 0.1 – 4.69 yrs). Six of the twenty metastatic cases involved lymphatic spread only and 14 cases comprised metastatic disease to multiple organs. Univariate and multivariate analysis of predictors (including p63 status) of time to metastasis was undertaken [Appendix 5]. Univariate analysis of p63 status as a predictor of time to metastasis was not significant (HR 1.66, p-value 0.28, Cox proportional hazard ratio) [Figure 3.17].

Multivariate analysis demonstrated significantly shortened time to metastases associated with increasing Breslow thickness and ulcerated tumours in keeping with reported studies (Balch 2001). When these factors were adjusted for, p63-status was not significantly associated with shorter times to metastases, although again an upward trend for predicting increased metastatic rates was observed in the p63-positive cohort (HR 1.83; p-value 0.21, Cox proportional hazard ratio).

3.3.5.3.5 *p63-reactivity significantly predicts overall worse outcome in primary melanomas but not melanoma-specific mortality*

Univariate and multivariate analysis of prognostic factors including p63 status was undertaken for predicting time to overall death [Appendix 6]. Univariate analysis confirmed a significant predictive effect on outcome for increasing Breslow thickness, Clark level and site of melanoma in keeping with reported studies (Balch 2001). In addition, p63 status was also demonstrated to be a significant predictor of worse outcome (HR 2.79, p-value 0.05, Cox proportional hazard ratio) [Figure 3.18]. However, when these other factors were adjusted for, multivariate analysis of p63-status revealed p63 to be a non-significant predictor of time to overall death (HR 3.07; p-value 0.12, Cox proportional hazard ratio).

The mean time to melanoma-related death in this cohort was 3.53 yrs (range 0.51 – 6.55 yrs). Univariate and multivariate analysis of predictors (including p63 status), of death attributable to melanoma is shown in Table 3.7. Univariate analysis demonstrated increasing Breslow thickness, ulceration status and microsatellitosis as significant predictors of melanoma-death, in keeping with reported studies (Balch 2001). In addition, p63 status demonstrated positive (albeit non-significant) association with worse outcome (HR 2.43; p-value 0.13, Cox proportional hazard ratio) [Figure 3.19]. When these prognostic factors were adjusted for, multivariate analysis of p63-status showed p63 to be a non-significant predictor of death attributable to melanoma, although individuals with p63-positive tumours were more likely to die from melanoma than those with p63-negative tumours (HR 1.53; p-value 0.51, Cox proportional hazard ratio) [Table 3.17].

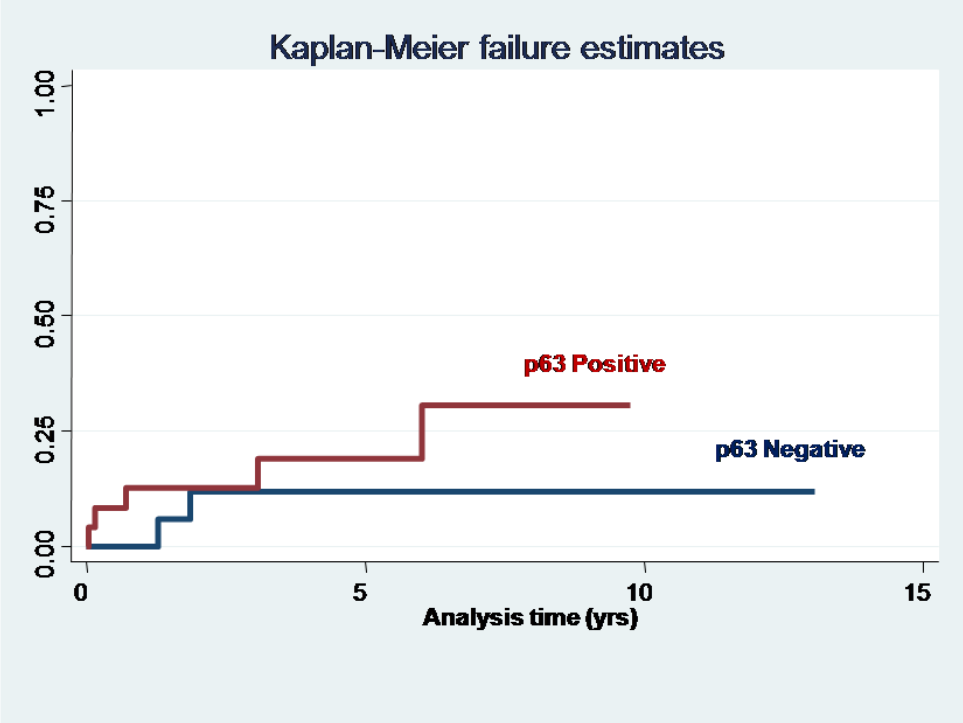


Figure 3.16: Kaplan-Meier failure estimates plot showing time to recurrence for primary tumours. Univariate analysis HR comparing p63-positive and p63-negative tumours was 2.28 (p-value 0.32, Cox proportional hazard ratio).

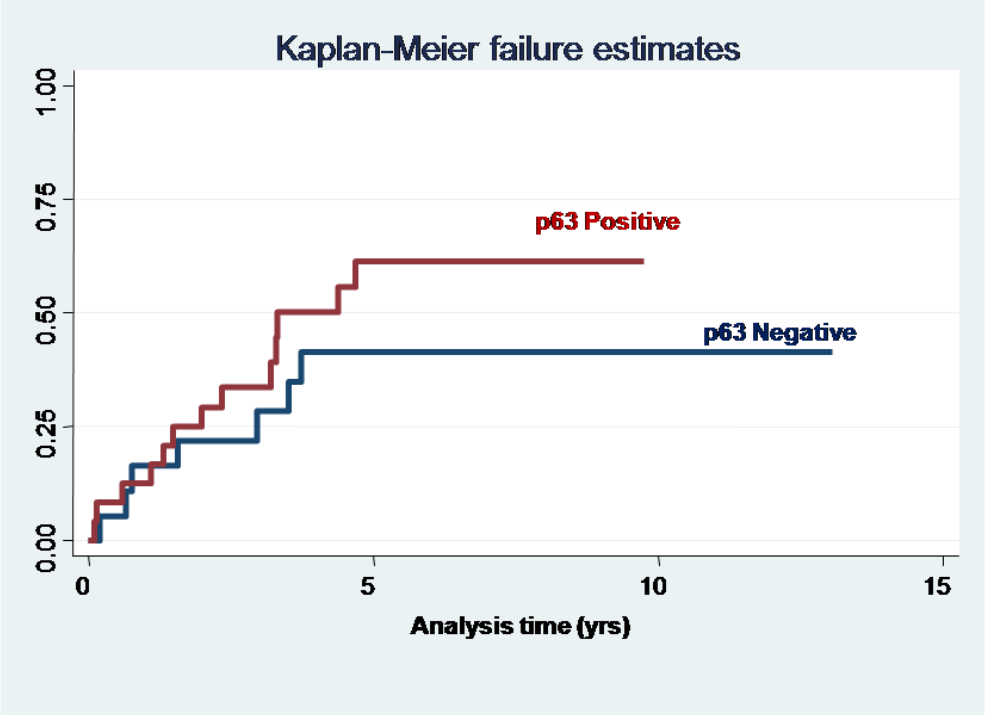


Figure 3.17: Kaplan-Meier failure estimates plot showing time to metastases for primary tumours examined. Univariate analysis HR comparing p63-positive and p63-negative tumours was 1.66 (p-value 0.28, Cox proportional hazard ratio).

Table 3.7: Kaplan Meier and Cox proportional hazard ratios for deaths from melanoma in patients with primary melanomas

	Univariate analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	**p-value***	**Hazard Ratio (95% CI)**	**p-value***
Age at diagnosis (yrs)	1.03 (1.00, 1.06)	0.06		
Gender				
Male	1			
Female	0.46 (0.19, 1.14)	0.09		
Site of Melanoma				
Acral	1		1	
Extremities	0.30 (0.06, 1.52)	0.15	0.20 (0.03, 1.47)	0.11
Head/Neck	1.87 (0.44, 7.91)	0.4	10.27 (1.31, 80.42)	0.03
Trunk	0.91 (0.24, 3.48)	0.89	4.42 (0.75, 26.14)	0.10
Breslow thickness (mm)				
0 – 1	1			
1.01 – 2	0.00 (0.00, .)	1		
2.01 – 4	5.46 (0.64, 46.78)	0.12		
> 4	9.91 (1.28, 76.59)	0.03		
Clarks Level				
I	1		1	
II	2.34 (0.24, 22.59)	0.46	2.27 (0.15, 33.91)	0.55
III	3.57 (0.45, 28.24)	0.23	5.39 (0.45, 65.21)	0.19
IV	9.41 (1.09, 80.94)	0.04	34.27(2.06,568.78)	0.01
Histological classification				
ALM	1			
NMM	2.77 (0.35, 21.70)	0.33		
SSM	0.60 (0.05, 6.73)	0.68		
Growth phase				
RGP	1			
VGP	4.60 (0.61, 34.94)	0.14		
Ulceration status				
No	1			
Yes	2.61 (0.96, 7.10)	0.06		

Mitotic rate (no/mm^2)*				
0	1			
1 – 4	1.23 (0.22, 6.75)	0.81		
5 – 10	2.26 (0.46, 11.04)	0.31		
≥ 11	3.07 (0.43, 21.96)	0.26		
Regression				
No	1			
Yes	0.00 (0.00, .)	1		
Microsatellites				
No	1		1	0.03
Yes	2.51 (0.88, 7.13)	0.08	2.94 (1.14, 7.52)	
p63 Status				
Negative	1		1	0.12
Positive	2.79 (0.99, 7.81)	0.05	3.07 (0.74, 12.83)	

*****number/mm^2 – categories chosen based on significant survival differences demonstrated between these groupings (Azzola et al. 2003).

ALM – acral lentiginous melanoma, NMM – nodular melanoma, SSM – superficial spreading melanoma, RGP – radial growth phase, VGP – vertical growth phase.

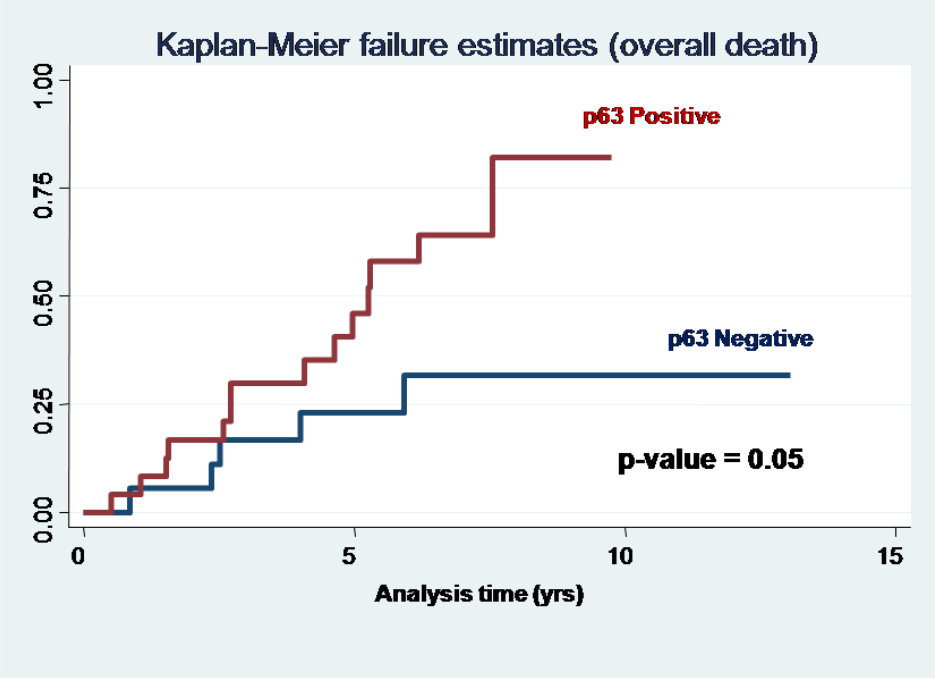


Figure 3.18: Kaplan-Meier plot for overall deaths in the primary melanoma cohort. p63-status was a significant predictor of worse overall outcome (univariate analysis HR 2.79, p-value 0.05, Cox proportional hazard ratio).

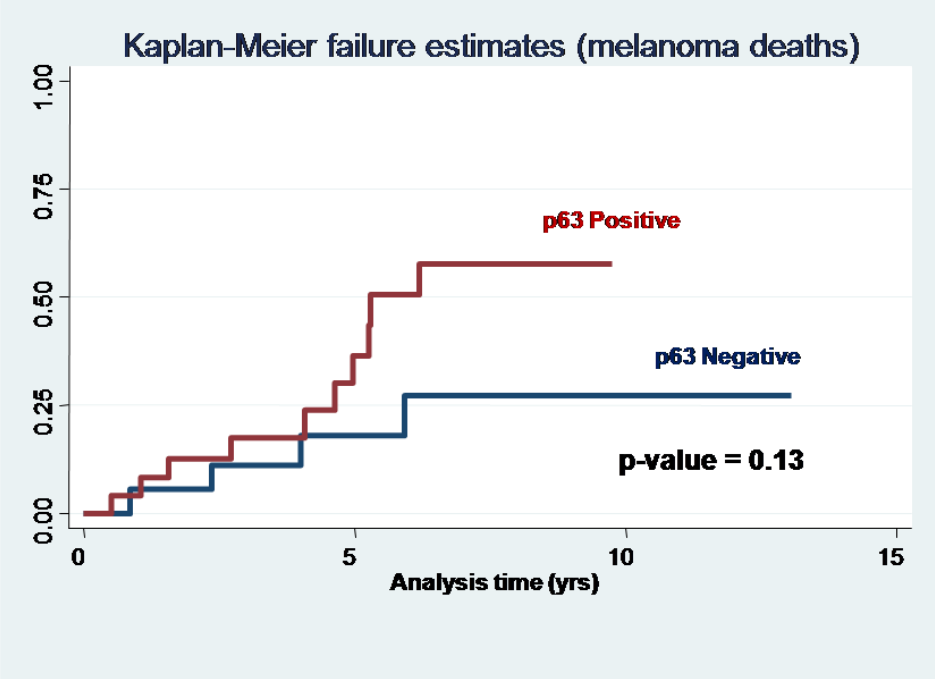


Figure 3.19: Kaplan-Meier plot for deaths attributable to melanoma in the primary melanoma cohort. p63-positive tumours show a markedly positive (albeit non-significant) association with worse melanoma-specific mortality (univariate analysis HR 2.43; p-value 0.13, Cox proportional hazard ratio).

3.3.5.4 Recurrent/metastatic melanoma tissue samples demonstrate positive reactivity to p63

3.3.5.4.1 *p63-reactivity in recurrent/metastatic melanomas do not show significant association with demographic features of tumours*

Thirty-five recurrent/metastatic melanoma tumour samples from 21 individuals were analysed. Overall 5/11 (45%) recurrent melanomas and 10/24 (42%) metastatic melanoma tissue samples showed weak/moderate positive staining for p63. p63-positive recurrent/metastatic melanoma samples were a younger cohort with a mean age at diagnosis of 60 yrs, compared with the p63-negative group which had a mean age at diagnosis of 65 yrs [Table 3.8]. There was no significant difference observed in the gender distribution between the two groups. Neither of the tissue samples from brain metastases, demonstrated p63 reactivity, and no significant difference for site distribution of melanoma was observed between the two groups (p-value 0.42, chi-squared) [Table 3.8].

3.3.5.4.2 *p63-reactivity in metastatic tumours is not a significant predictor of survival*

Mean follow up for this cohort was 1.87 yrs (range 0.19 – 6.43 yrs). Overall, there were 15 deaths, 13 were attributable to melanoma. Mean time to death from first recurrence/metastases in these 13 patients was 1.78 yrs (range 0.19 – 6.43 yrs). Univariate analysis of prognostic factors in both recurrent and metastatic tumours is shown in Table 3.9. A Kaplan-Meier plot showing failure estimates for individuals with metastatic disease was calculated using date of first recurrence/metastases to date of death. This showed a trend towards increased mortality in p63-positive metastatic melanoma samples (red line) compared with the p63-negative tumours (blue line) (univariate analysis HR 1.70, p-value 0.36, Cox proportional hazard ratio) [Figure 3.20]. Multivariate analysis to include significant predictors of outcome (including p63 status) is shown in the last two columns of Table 3.9. p63 status in recurrent/ metastatic tumours was a positive (albeit non-significant) predictor of worse outcome in the multivariate analysis (HR 1.25; p-value 0.72, Cox proportional hazard ratio) [Table 3.9].

Taken together, findings from immunohistochemistry analysis of melanoma tissue samples demonstrates p63 to be a positive predictor of shorter time to recurrence, shorter time to metastases, significantly worse overall outcome and worse outcome specifically due to melanoma. Moreover, p63-positive tumours correlate with an older

cohort of individuals with primary tumours and a younger cohort of individuals with metastatic tumours. The limitation of these data is the small number of tumours analysed which resulted in insufficient power to demonstrate statistically significant associations.

Table 3.8 Clinicopathological details for metastatic and recurrent tumours

	p63-Negative tumours (n=20)	**p63-Positive tumours (n=15)**	**Total (n=35)**	**p-value***
Age at diagnosis (yrs)	65.0 (57.5, 73.7)	60.2 (46.2, 62.4)	60.8 (49.7, 68.7)	0.07
Gender				
Male	8 (40.0)	8 (53.3)	16 (45.7)	0.43
Female	12 (60.0)	7 (46.7)	19 (54.3)	
Site of Melanoma				
Lymph	3 (15.0)	4 (26.7)	7 (20.0)	-
Brain	2 (10.0)	0 (0.0)	2 (5.7)	-
Lung	0 (0.0)	1 (6.7)	1 (2.9)	-
Skin#	15 (75.0)	10 (66.7)	25 (71.4)	0.42

Values shown are number (percentage) or median (IQR) as appropriate

* p-values are odds comparing p63 negative with p63 positive

#skin samples include 11 recurrent melanoma samples–p63-negative n=6, p63-positive n=5

Table 3.9: Death from metastases in patients with metastatic tumours

	Univariate analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	**p-value***	**Hazard Ratio (95% CI)**	**p-value***
Age at diagnosis (yrs)	1.01 (0.97, 1.05)	0.54	-	-
Gender				
Male	1	0.78	-	-
Female	0.85 (0.27, 2.65)		-	
Site of Melanoma				
Lymph	1		1	-
Brain	-	-	-	-
Lung	4.83 (0.43, 54.15)	0.2	4.13 (0.33, 52.67)	0.27
Skin	1.20 (0.31, 4.72)	0.79	1.16 (0.29, 4.62)	0.83
p63 staining				
Negative	1	0.36	1	0.72
Positive	1.70 (0.55, 5.30)		1.25 (0.38, 4.15)	

* p-values are odds comparing p63-negative with p63-positive, Cox proportional hazard ratio

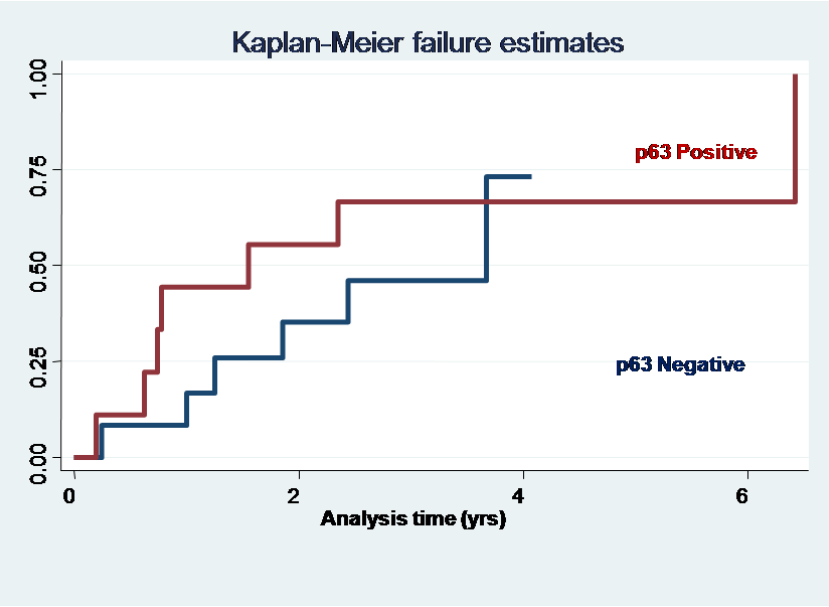


Figure 3.20: Kaplan-Meier plot showing failure estimates for recurrent/metastatic melanoma tissue samples. Failure estimates were calculated from date of first recurrence / metastasis to date of death. This shows a trend towards increased mortality in p63-positive metastatic melanoma samples (red line) compared with the p63-negative tumours (blue line) (univariate analysis HR 1.70, p-value 0.36, Cox proportional hazard ratio).

3.3.5.5 Matched primary-recurrent-metastatic melanoma tissue samples

For eight cases, matched primary and recurrent and/or metastatic tumours were analysed. In addition, one ocular melanoma and three metastatic tissue samples from the same individual were also examined [Table 3.10].

In 5/8 (62.5%) cases where additional tumours from the same individual were available, labelling of p63 was uniform between disease stages [highlighted in Table 3.10]. This included the case in which tumours representing all stages of disease were available, which demonstrated p63 reactivity in all three samples [Figure 3.21]. Breslow thickness of the primary tumour for this patient was not available as the original diagnosis was incorrect and only confirmed to be melanoma following review of the case when the patient developed a recurrence 3 years later.

Table 3.10: p63 reactivity in multiple tumours from single individuals.

Patient ID	Gender	Age at diagnosis (yrs)	Breslow thickness of 1°	p63 reactivity*		
				Primary	Recurrence	Metastasis (site)
1	M	46.9	7	-	-	N/A
2	F	76.7	4.5	+	-	N/A
3	M	46.7	N/A	+	+	+ (skin)
4	M	68.6	2.7	-	N/A	+ (lymph)
8	M	47.1	5.1	+	N/A	+ (lung)
25	F	84.0	10	+	-	N/A
26	M	63.7	6	-	N/A	- (lymph)
33	F	33.1	2.9	-	N/A	- (lymph)
59 (Ocular)	F	36.1	N/A	-	N/A	- (skin)
						+ (lung)
						+ (skin)

M – male, F – female

* (-) negative reactivity, (+) positive reactivity-any grade of intensity, N/A – not available. Tumours showing uniform labelling of p63 across stages of disease highlighted in pink

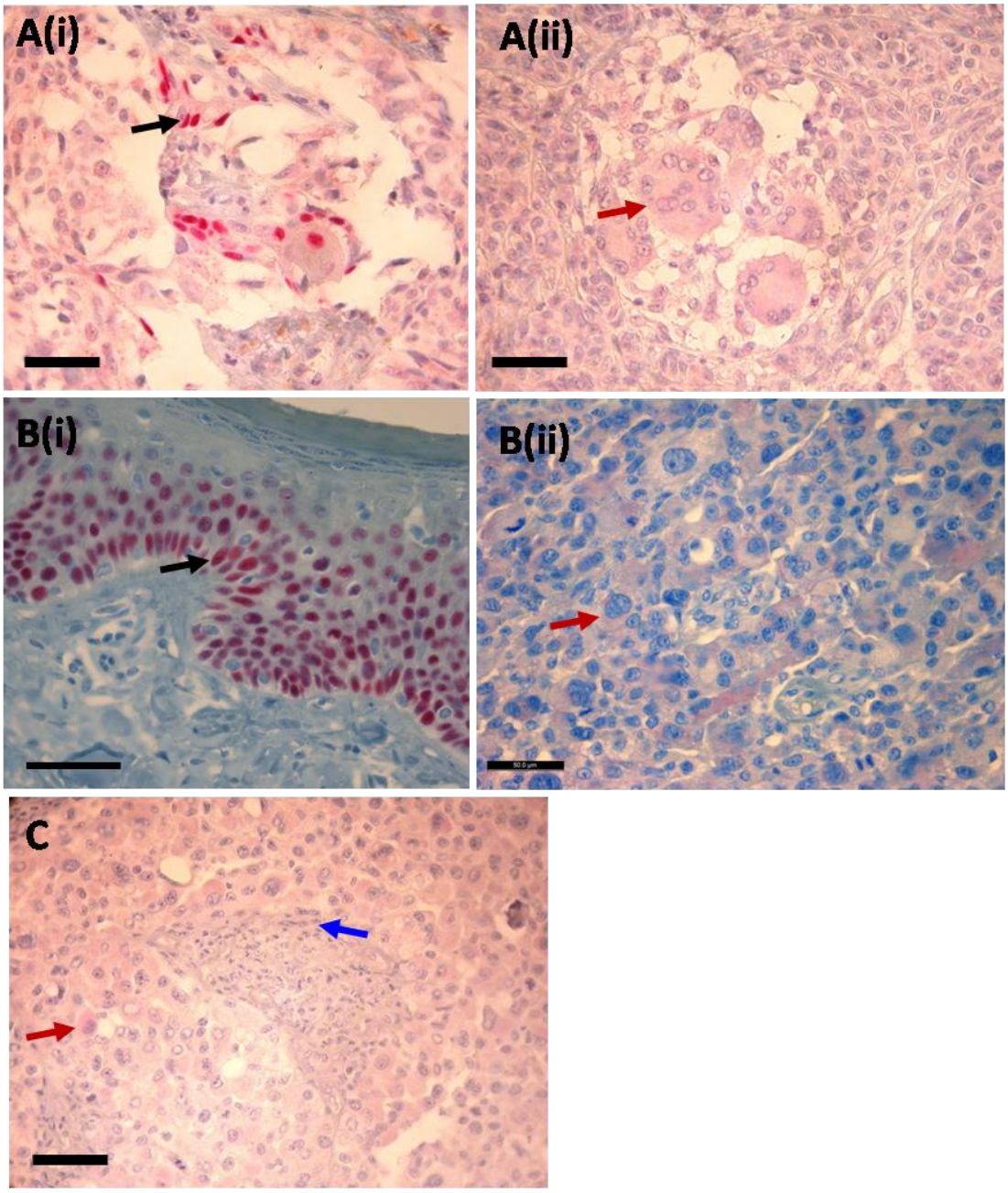


Figure 3.21: Series of p63-positive melanoma tissue samples from individual (male aged 46.7 yrs at diagnosis). (A) Primary naevoid melanoma (abdomen) showing (i) strong nuclear p63 staining of stromal epithelia (black arrow), (ii) moderate cytoplasmic p63 reactivity of giant cell melanocytes (red arrow). (B) Recurrence of amelanotic melanoma in abdomen showing (i) strong nuclear p63 labelling of epidermal keratinocytes and (ii) moderate cytoplasmic p63 reactivity of melanoma cells. (C) Cutaneous melanoma metastases demonstrating moderate intensity cytoplasmic p63 reactivity (red arrow) with sparing of necrotic tissue within tumour (blue arrow). Bar represents 50 µm.

3.3.6 Melanoma stem cell characterisation

There is growing evidence for tumour initiation from normal stem cells or their immediate descendents and perpetuation by cancer stem cells or tumour initiating cells (Burkert et al. 2006). Melanoma is now considered by many, to originate in the same way (Fang et al. 2005; Grichnik et al. 2006; Refaeli et al. 2009). Markers that allow enrichment for cancer stem cells from a heterogeneous tumour such as melanoma, are essential for purification, characterisation and eventual targeting of cancer stem cells. A specific antibody designated AC133, which recognises an epitope of the glycosylated form of the cell surface antigen CD133, has been widely used to detect cancer stem cells (Fang et al. 2005; Mizrak et al. 2008). This includes the identification of melanoma stem cells (Frank et al. 2005; Klein et al. 2007; Monzani et al. 2007), putative cancer stem cell populations from malignant tumours of brain (Singh et al. 2004), prostate (Collins et al. 2005), liver (Suetsugu et al. 2006), pancreas (Hermann et al. 2007), lung (Eramo et al. 2008) and colon (Shmelkov et al. 2008).

3.3.6.1 CD133 population is identified in melanoma cell lines

Initial screening of a selection of melanoma cell lines was undertaken to identify a putative stem cell fraction, by analysing expression of CD133 using the monoclonal antibody AC133 clone conjugated to phycoerythrin (PE), by flow cytometry detection of -PE [section 2.5.3]. Colon-derived epithelial cells (CaCo-2) expressing endogenous CD133 (Corbeil et al. 2000) were used as a positive control to confirm sensitivity and specificity of the antibody [Figure 3.22]. Mouse IgG1 PE–conjugated antibody was used as the isotype control. In 12/17 melanoma cell lines tested, the CD133-positive population was greater than 1% (indicated by the red line) [Figure 3.23]. No significant differences were detected between cell lines tested at different passages.

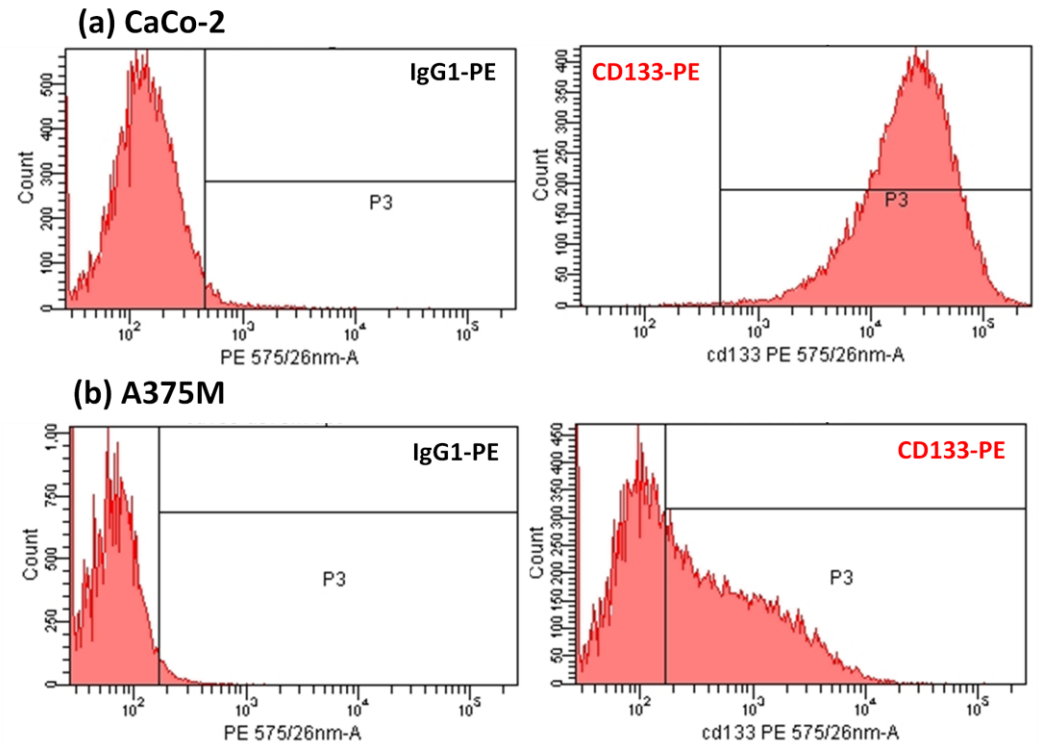


Figure 3.22: Flow cytometry analysis of CD133. Left panels show histogram (from flow cytometry analysis) of cells labelled with isotope-PE (IgG1-PE) and right panels show histograms for CD133-PE labelled cells. (a) Expression of CD133 detected in CaCo-2 cells demonstrates 100% positivity (right panel). (b) Significant shift of histogram comparing isotype and CD133 labelling in A375M cells demonstrates expression of CD133 in this melanoma cell line.

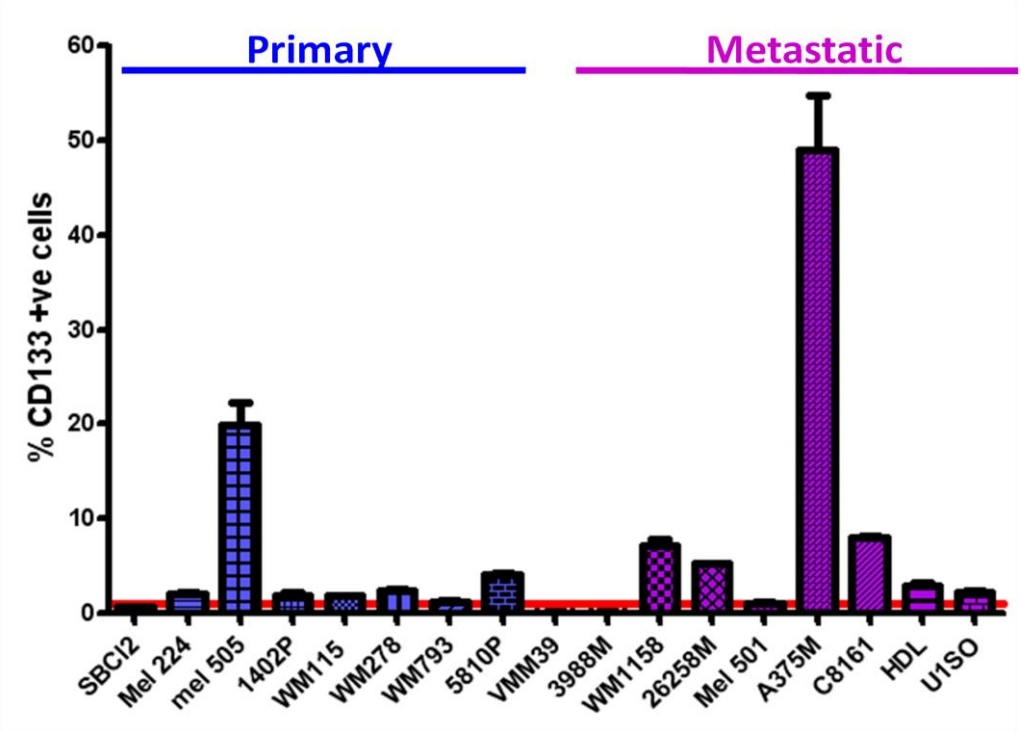


Figure 3.23: CD133 expression in melanoma cell lines. Panel of melanoma cell lines screened for expression of CD133 using flow cytometry. Baseline for expression using IgG1-PE isotope control was determined for each cell line. CD133 expression was detected using AC133-PE conjugated antibody. Red line denotes CD133-positive population greater than 1%. Marked expression of CD133 is detected in a proportion of both primary and metastatic melanoma cell lines. Values shown are mean percentage of CD133-positive cells +/- SEM of three independent experiments performed in triplicate.

3.3.6.2 TP63 is expressed in the melanoma stem cell fraction

p63 is an essential regulator of stem-cell maintenance in stratified epithelial tissues (Lee and Kimelman 2002; Mills et al. 1999; Parsa et al. 1999; Senoo et al. 2007; Truong et al. 2006; Yang et al. 1999a). It maintains stem-cell populations in epithelial tissues by directly promoting the proliferation of progenitor cells and their self-renewal in the skin epidermis and by stimulating cell survival in the thymus (Blanpain and Fuchs 2007). Studies in mice revealed that clonally derived prostate stem cells express p63 (Barclay et al. 2008). Moreover, in the human prostate, cells expressing CD133 possess stem cell-like features (Litvinov et al. 2006; Richardson et al. 2004), some of which are p63-enriched (Richardson et al. 2004) and rapidly give rise to further p63-expressing cells (Litvinov et al. 2006). Based on these data and the discovery of p63 expression in melanoma, a putative role for p63 in melanoma stem cells was proposed.

3.3.6.2.1 *Expression of p63 is significantly enriched in the CD133-positive population*

To address this issue and determine if p63 was expressed in the CD133-labelled stem cell fraction, the melanoma cell line with the highest level of CD133 expression, A375M, was analysed. Live A375M cells were labelled using CD133/1–PE conjugated antibody to detect the extracellular protein CD133 [section 2.5.3]. Labelled A375M cells were fixed, permeabilised and indirectly labelled using anti-p63 antibody (4A4) and Cy5-conjugated secondary mouse antibody to determine co-expression of CD133 with the intracellular protein p63. Isotype controls (Cy5-conjugated and PE-conjugated IgG mouse antibodies) were used as baseline comparators. Expression of p63 detected using flow cytometry was enriched in the CD133-positive population of A375M cells [Figure 3.24]. A histogram comparing endogenous levels of CD133, p63 and both proteins, showed the proportion of cells labelled with both CD133 and p63 was significantly increased when compared to individual populations [Figure 3.24(e)].

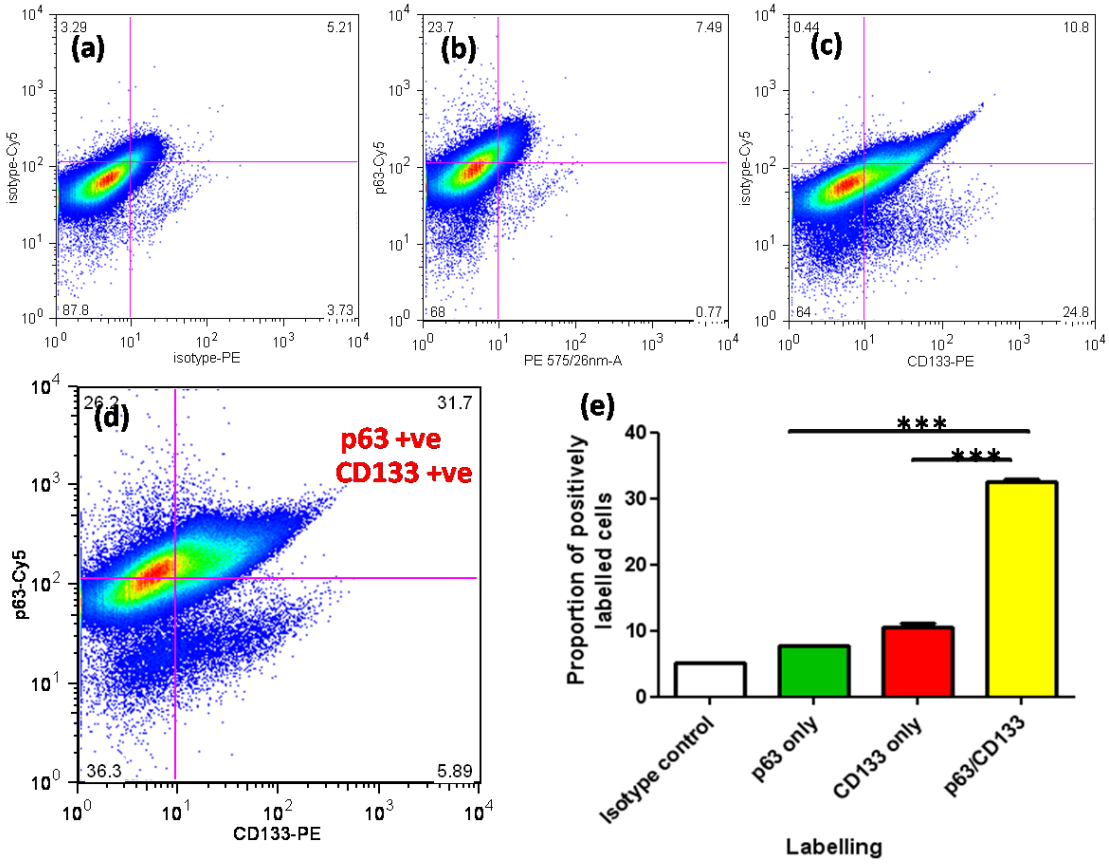


Figure 3.24: Expression of p63 is enriched in CD133-positive population in A375M melanoma cells. (a) Flow cytometry scatter plot of A375M cells labelled with isotope-PE and istotope-Cy5. (b) Flow cytometry scatter plot showing 7.5% expression of intracellular p63 in A375M cells (p63-positive cells lie in top right quadrant). (c) Expression of extracellular CD133 in A375M cells (10.8%) using CD133-PE conjugated antibody (CD133-positive population in the top right quadrant). (d) Marked increase in population of cells (31.7%) in upper right quadrant comprising A375M cells demonstrating double positivity to p63 and CD133. (e) Histogram showing data represented by the four flow cytometry scatter plot examples (a)-(d). Mean percentage of positively labelled cells +/- SEM of triplicates from three independent experiments is shown. Statistically significant differences were observed between co-expression of CD133 and p63 when compared with p63 alone (p-value 0.0003, paired t-test) and CD133 alone (p-value 0.0007, paired t-test).

3.3.6.2.2 ∆*Np63 is enriched in CD133-positive populations*

To quantify p63 mRNA abundance in the CD133-positive population and to determine which isoform(s) of p63 were expressed in this fraction, mRNA expression of TA and ∆N p63 was analysed in both CD133-positive and -negative populations, using Q-PCR. Cells were labelled using anti-CD133/1–PE antibody (Miltenyi Biotec) and passed through a cell strainer cap before reading on the FACS Aria™ Flow Cytometer (BD Biosciences) fitted with an Argon 488 nm laser. Sort gates were placed on CD133-positive and CD133-negative cells once compared to the isotype controls to identify the two populations [Figure 3.25].

Three cell lines expressing high levels of extracellular CD133 were chosen; A375M, Mel 505 and WM1158. RNA was extracted from each cell line after fluorescence activated cell sorting (FACS) for the CD133-positive and negative populations [section 2.5.4.1]. Q-PCR analysis was undertaken for TAp63 and ∆Np63 mRNA, using the expression of these isoforms in the whole population (i.e. unsorted cells) as a comparator. In A375M cells there was no significant difference in expression of TA between unsorted cells and the CD133-negative population. Although the endogenous level of TAp63 in this cell line is low, no TAp63 was detected in the CD133-positive fraction.

In WM1158 cells, where the endogenous level of TAp63 was high, this was enriched in the CD133-negative population. In keeping with the data obtained using A375M cells, no TAp63 was detected in the CD133-positive fraction in WM1158 cells. In the third cell line tested, Mel 505, TAp63 was not detected in either unsorted or sorted cells [Figure 3.26]. In contrast, ∆Np63 expression was significantly increased in CD133-positive populations compared with CD133-negative populations for all three cell lines [Figure 3.26]. Taken together, these data demonstrate that ∆Np63 (and not TAp63) is the isoform enriched by the CD133-labelled stem cell fraction in melanoma cell lines and could therefore be considered a putative melanoma stem cell marker.

3.3.6.2.3 *Silencing of ∆Np63 results in reduction of CD133-positive population*

To further confirm that ∆Np63 was expressed by the CD133-positively labelled cell population (considered the melanoma stem cell fraction), CD133 expression levels were examined in A375M cells in which p63 was silenced by short-hairpin RNA to p63 (shRNA-p63) [section 2.2.7.2]. p63 siRNA target sequences were cloned into a pSUPERIOR.retro.puro vector (Oligoengine) [Figure 2.4]. As the method used to clone the shRNA constructs requires annealing of two long oligonucleotides the

chance of mutation due to synthesis error is high (Miyagishi and Taira 2004) and as a result sequencing confirmed successful cloning of target sequences A, C, D and E [Table 2.10]. Cloned vectors were transfected into the packaging cell line, Phoenix cells, before introducing the retrovirus into the melanoma cell line – A375M. Cells were selected using puromycin to establish a stable cell line for shRNA expression of shRNA-p63 or shRNA-scramble. Silencing of p63 by four different clones achieved >70% stable knockdown of ΔNp63, assessed by Q-PCR [Figure 3.27A]. Each of the four clones of A375M cells expressing shRNA-p63 were labelled using anti-CD133/1-PE antibody and analysed using the LSR II flow cytometer [section 2.5.3]. A significant decrease (p-value < 0.001, *Anova*) in the proportion of CD133-positive cells was demonstrated for each clone [Figure 3.27B]. Taken together, these data confirm that a putative melanoma stem cell population positively labelled with CD133 is detected in melanoma cell lines which enriches for ΔNp63 only, suggesting that it too could be a stem cell marker in melanoma.

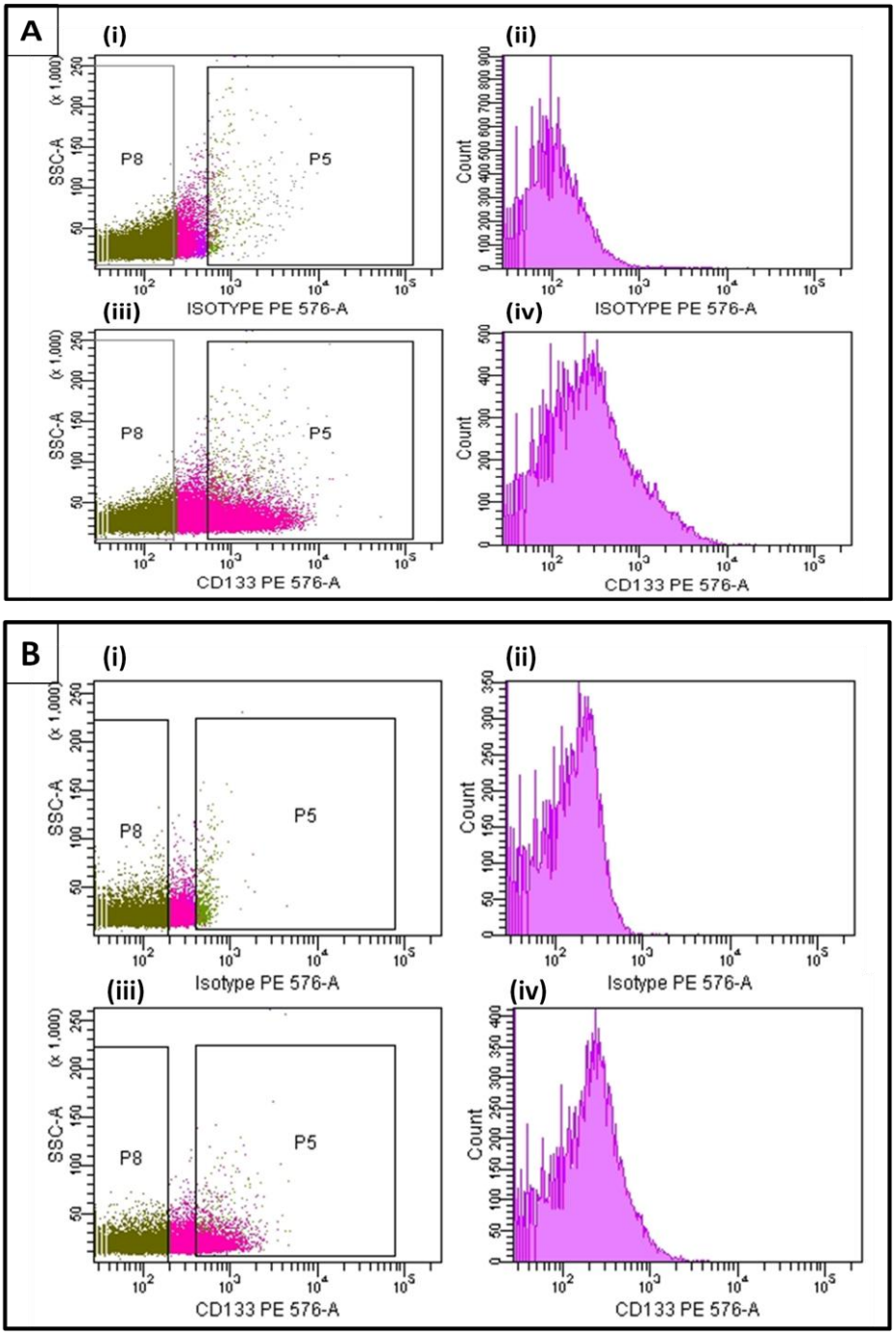


Figure 3.25: Fluorescence-activated cell sorting of (A) A375M cells and (B) Mel 505 cells. (i) Gate setting for isotype-PE control (set at 5% positive). (ii) Histogram plot for isotype control. (iii) Gate setting for CD133-PE. P5 is CD133-positive population and P8 is CD133-negative population with significant separation to ensure purity of fractions. (iv) Histogram plot showing CD133 positive population collected for mRNA analysis. Flow cytometry scatter plots are representative of four independent experiments performed for each cell line.

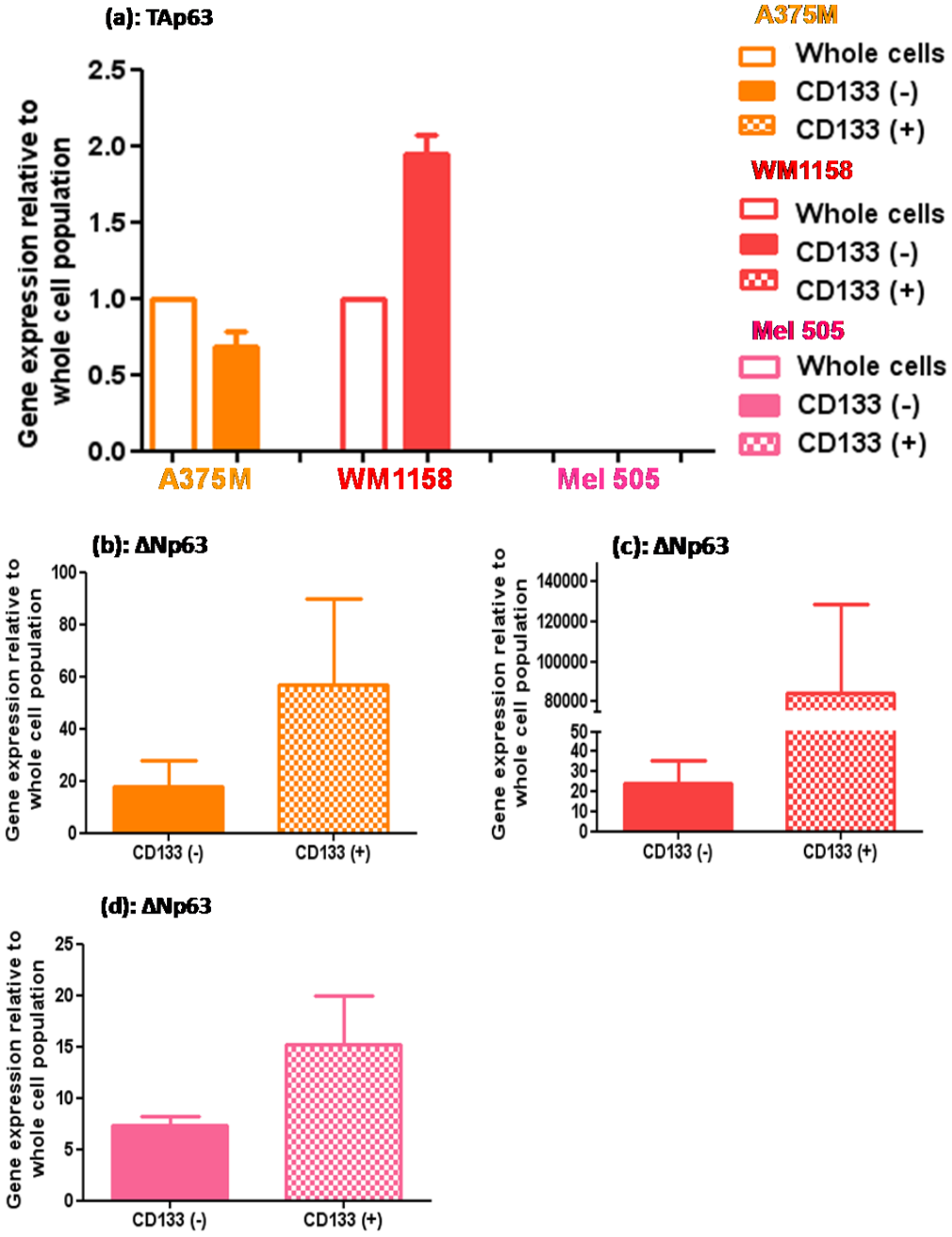


Figure 3.26: TP63 mRNA expression in CD133-populations following FACS (Q-PCR). (a) TAp63 expression in cell lines relative to expression in whole cell population i.e. unsorted cells. No TAp63 was detected in CD133-positive cells. Mel 505 cells do not express endogenous TAp63. ΔNp63 expression is significantly increased in CD133-positive populations when compared with expression in CD133-negative populations in (b) A375M cells (orange bars), (c) WM1158 cells (red bars) and (d) Mel 505 cells (pink bars). GUS was used as the housekeeping gene for mRNA standardisation. Values shown are mean expression +/- SEM of three independent experiments performed in triplicate.

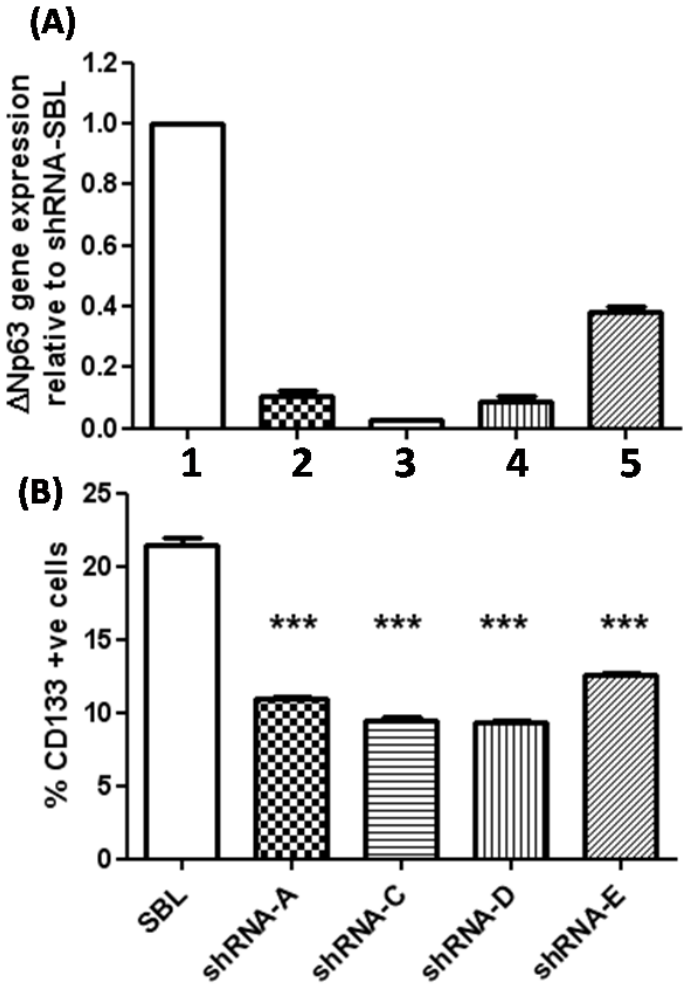


Figure 3.27: shRNA mediated silencing of p63 significantly depletes CD133-positive population. Histogram bars in (A) correspond to those in (B) directly below: lane 1 – shRNA-sbl, lane 2 – shRNA-A, lane 3 – shRNA-C, lane 4 – shRNA-D and lane 5 – shRNA-E. (A) ΔNp63 gene expression in A375M cells transfected with four different shRNA-p63 clones demonstrating significant depletion of the gene (Q-PCR). Expression of ΔNp63 in shRNA-sbl was used as the comparator. Values shown are mean expression +/- SEM of three independent experiments performed in triplicate. (B) Depletion of ΔNp63 by shRNA-p63 results in corresponding reduction of CD133-positive population (assessed using flow cytometry), which is significant for all four shRNA-p63 clones (p-values < 0.001, *one-way Anova*). SBL – scramble. Values shown are mean percentage of CD133-positive cells +/- SEM of three independent experiments performed in triplicate.

3.4 DISCUSSION

3.4.1 Biological role of p63 in the melanocyte lineage

The expression pattern of p63 has not been robustly investigated in the melanocyte lineage. Previous studies of expression of p63 in melanocytes are conflicting; mouse melanocytes express two isoforms of p63 – TAp63β and either TAp63γ or ΔNp63β (Kulesz-Martin et al. 2005) and cultured human eye melanocytes do not express TP63 (Kilic et al. 2008). Neither of these studies were adequate biological correlates for human cutaneous melanocytes; mouse melanocytes predominantly reside in the hair follicle within the dermis, and mice do not spontaneously develop melanoma (Bardeesy et al. 2000; Merlino and Noonan 2003); and the molecular biology of ocular melanoma is distinct to cutaneous melanoma (Belmar-Lopez et al. 2008; Sato et al. 2008; Shields et al. 2008). Expression of TP63 reported in these studies was analysed using RT-PCR. Data presented in this thesis used Q-PCR analysis, which displays increased sensitivity for detecting low expression levels of isoforms, has demonstrated that TP63 isoforms are infrequently expressed in five primary human melanocytes cultures tested, and when compared with melanoma cell lines and primary keratinocyte cultures, expression levels were significantly lower. In 2/5 primary melanocyte cultures where p63 mRNA was detected, the ΔNp63 isoform was elevated when compared with TAp63, in a similar manner to that observed for HaCaT cells. In normal skin, p63 has a role in keratinocytes to protect against UVB (Liefer et al. 2000) and melanocytes demonstrate resistance to common apoptotic stimuli through expression of apoptotic inhibitors (Bowen et al. 2003; Vancoillie et al. 1999). Data presented in this thesis raise the possibility that in melanocytes, the ratio of p63 isoform expression could contribute to protection against UVB damage in skin and warrants further investigation.

3.4.1.1 p63 is significantly expressed in melanoma

This is the first evidence demonstrating marked upregulation of both p63 mRNA and protein in melanoma cell lines. A differential expression of splice variants was demonstrated in melanoma cell lines suggesting that splicing mechanisms and/or transcription of these splice variants are regulated in melanoma. Without the availability of more potent isoform-specific and splice-variant specific antibodies one can only surmise from the observed molecular weight of exogenous isoforms on a Western blot analysis that a similar spectrum of p63 isoform expression might be found in melanoma tissue samples. Although these data are not able to define the roles of each individual isoform, it is likely that they have specific biological / biochemical activities. These activities may be overlapping and in cell lines where

both isoforms are expressed, (which constitutes a small proportion of the total number of cell lines expressing p63) there may be some redundancy of function, which requires further investigation.

Upregulated expression of p63 protein using immunohistochemistry in melanoma tissue samples compared with primary melanocytes and benign naevi samples, is in contrast to reported literature [Table 3.11]. There are a number of possible reasons for these conflicting data. The main discrepancy between immunohistochemistry results demonstrated in this thesis and that obtained by others could be explained by a difference in reagents and technique. There are multiple variables affecting immunostaining including length and type of fixation (Dapson 1993; Leong and Gilham 1989), sensitivity and specificity of antibody, chromogen and antigen retrieval systems (Bussolati and Leonardo 2008; Grabau 1998). A number of studies investigating p63 expression in melanomas used antibody dilutions in the range of 1:200 (Brinck et al. 2002; Di Como et al. 2002; Reis-Filho et al. 2003b; Sakiz et al. 2009) compared with the optimised dilution determined to be 1:50 which was used in this study. The most plausible explanation however, is the effect of storage of tissue samples on p63 reactivity; with increased storage times, a progressive and significant decrease in intensity of p63 staining was observed, with evidence of significant decline occurring as early as two weeks of storage (Burford et al. 2009; Hameed and Humphrey 2005). This effect was observed in our study [Figure 3.13] with 25 slides, initially failing to demonstrate p63 reactivity in an internal control (epidermal keratinocytes), but upon re-sectioning and staining within two weeks demonstrated nuclear p63 reactivity of keratinocytes. Therefore, for optimal detection of p63 in tissue samples, slides should be stained within two weeks of sectioning of tumour samples. In larger scale studies comprising multiple tumour types where array technology was not used, this is likely to have been a problem and could explain the higher proportion of p63-negative melanomas reported.

Most studies use the 4A4 murine monoclonal antibody, which is raised against a region in the core domain of p63 and recognises all isoforms of p63 by western blotting (Yang et al. 1998) but for immunohistochemistry has been cited to be most effective in detection of the ΔN isoform only (Nylander et al. 2002). However, mRNA analysis data presented here, suggest that a significant proportion of melanoma cell lines (in particular primary melanoma cells) express TAp63 [Table 3.1] and the expression levels are higher when compared to ΔNp63 expression which would otherwise be missed by the 4A4 antibody. Moreover, the use of the H129 antibody

(+/- H137) routinely used in diagnostic evaluation and clinical trials at Bart's and the London NHS Trust is likely to demonstrate increased sensitivity of detection of p63 protein. Finally, aberrant subcellular distribution of the p63 antigen inside melanoma cells could have been overlooked; some studies report using nuclear p63 reactivity only, as a determinant for positive staining (Brinck et al. 2002; Di Como et al. 2002; Dotto and Glusac 2006). Using these criteria, the majority of melanoma samples from the cohort studied in this thesis would have been considered p63-negative. Review of cases in the literature could thus, identify p63-reactive melanomas and to support this, one study shows a photomicrograph of a p63-positive melanoma, which although in monochrome, appears to show both nuclear and cytoplasmic reactivity but reported as nuclear only (Brinck et al. 2002).

3.4.1.2 Cytoplasmic p63 expression is associated with worse outcome in individuals with melanoma

These data provide the first evidence for significant expression of p63 in melanoma demonstrating predominant cytoplasmic localisation. Although p63 is reported to primarily be a nuclear protein (Dellavalle et al. 2001; Di Como et al. 2002; el-Deiry et al. 1995), aberrant cytoplasmic expression is reported in lung and prostate cancers (Dhillon et al. 2009; Narahashi et al. 2006). Data presented in this thesis support the latter, demonstrating cytoplasmic p63-reactivity in 42 – 55% of melanoma tissue samples depending on stage of disease progression. Analysis of the p63-positive population demonstrated an increased frequency of thicker primary tumours, occurring more frequently on the head/neck in an older cohort of individuals in the p63-positive primary tumours but without reaching statistical significance. Moreover, p63-positive primary tumours demonstrated a positive trend towards shorter time to recurrence (Univariate Cox proportional HR 2.89) and shorter time to metastases (Univariate Cox proportional HR 1.83) and this trend persisted when adjusting for other significant predictors of survival in the same cohort. In addition, individuals with p63-postive tumours had significantly worse overall outcome (p-value 0.05, Cox proportional hazard ratio) and demonstrated trend towards worse outcome specifically due to melanoma (Univariate Cox proportional HR 2.43).

Analysis of recurrent/metastatic tumours showed p63-positive tumours arising in a younger cohort of patients were also associated with poorer outcomes when compared with the p63-negative tumours (Univariate Cox proportional HR 1.7). The consistent positive association between p63-positivity and poor predictors and measures of outcome without achieving statistical significance might be addressed by

evaluating a larger cohort of melanoma cases. A power calculation using death from melanoma as the primary end-point was performed. For 80% power, 200 melanomas from individuals with follow-up over 5 yrs need to be analysed, to detect a 5% significant difference in outcomes between p63-positive and p63-negative tumours.

The relocalisation of p63 to the cytoplasm demonstrated in at least 50% of melanoma tumour samples, suggests an alteration of stability and/or function of the protein which could impact apoptotic pathways. The inverse correlation between cytoplasmic p63 reactivity and outcomes in melanoma suggests a possible oncogenic effect of p63 mediated through interaction with other molecules in the cytoplasm. These data warrant further investigation of subcellular localisation of p63 in the cytoplasm. This could be achieved by co-localisation studies with subcellular markers. Sequestration of p63 to the cytoplasm links the protein to apoptosis and investigation of other apoptotic markers in these tumours could be assessed for correlation. A possible role for p63 in apoptosis will be investigated in the following chapters of this thesis.

Table 3.11: Summary of expression of p63 in melanoma tissue samples

Reference	Archival tissue / Array	p63 Antibody	p63 reactivity (%)
Di Como et al. (2002)	TMA	4A4^ (Santa Cruz)	0/15 (0)*
Brinck et al. (2002)	TMA	4A4^ (Santa Cruz)	2/59 (3)*
Reis-Filho et al. (2003a)	TMA	4A4^ (Neomarkers)	2/25 (8)
Bourne et al. (2008)	Mucosal melanomas	4A4 (DAKO)	0/7 (0)
Dotto and Glusac (2006)	Desmoplastic melanomas	4A4 (DAKO)	0/8 (0)*
Morgan et al. (2008)	Spindle cell melanoma	4A4 (Dako Corps)	2/3 (66.7)[#]
Sakiz et al. (2009)	Melanoma *in situ*	4A4 + Y4A3 ^ (Neomarkers)	0/7 (0)*
Kanner et al. (2010)	Melanoma	4A4 (DAKO)	1/20 (5)*

TMA – tissue microarray, ^ p63 antibody used in 1:200 dilution, *Only nuclear expression of p63 was accepted as positive, # no comment made by authors regarding subcellular localisation of p63-reactivity nor any photomicrographs shown.

3.4.2 CD133 is used to identify a putative stem cell population

Analysis of a panel of melanoma cell lines demonstrated a putative stem cell fraction (12/17 cell lines) in varying proportions (range 1% - 40%), identified by CD133 labelling using flow cytometry. However, the role of CD133 as a putative stem cell marker in melanoma requires further investigation. Monzani et al. (2007) compared abilities of CD133-positive versus CD133-negative melanoma cells to initiate tumour formation *in vivo*. Primary tumour-initiating properties were exclusively contained within melanoma cell subsets characterised by expression of CD133, whereas CD133-negative melanoma cells were found to lack tumourigenicity (Monzani et al. 2007). In contrast to findings by Fang et al (2005), more tumourigenic CD133-positive melanoma subsets were confined to adherent cell populations, whereas melanoma sphere associated cells were devoid of CD133 (Monzani et al. 2007). The role of CD133 as a genuine stem cell marker is thus, debated (Fang et al. 2005; Jaksch et al. 2008) and more extensive studies including secondary tumour formation and stringent self-renewal and differentiation experiments are needed to draw definitive conclusions about CD133 as a candidate melanoma stem cell marker.

Future work following on from that presented here, is required to confirm the cancer stem cell characteristics of CD133-positive cells in the melanoma cell lines analysed in this chapter; namely sphere formation, expression of cancer stem cell markers, self renewal and differentiation potential, and tumourigenicity *in vivo* (Frank et al. 2005; Grichnik et al. 2006; Klein et al. 2007; Quintana et al. 2008; Refaeli et al. 2009; Schatton and Frank 2008; Schatton et al. 2008; Zabierowski and Herlyn 2008). In addition, characterisation of these stem cells for other putative melanoma stem cell markers would add support to the validity of CD133 as a melanoma stem cell marker.

3.4.2.1 ΔNp63 is a putative melanoma stem cell marker

p63 identifies epidermal stem cells in the skin (Pellegrini et al. 2001) and more recently, ΔNp63 specifically is reported to confer stemness in skin (Yi et al. 2008). Moreover, reports that a subset of CD133-positive prostate stem cells expresses TP63 (Litvinov et al. 2006; Parsons et al. 2009; Richardson et al. 2004) led to our proposal that p63 may also be a putative marker of melanoma stem cells. Flow cytometry analysis confirmed co-expression of extracellular CD133 and intracellular p63. FACS sorting of CD133-positive cells confirmed the putative melanoma stem cell population labelled with CD133 enriched only for ΔNp63, with no expression of TAp63. These data were confirmed in three cell lines providing the first evidence for

ΔNp63 as a putative stem cell marker in melanoma or at least plays a possible role in regulating the CD133-positive cells.

The limitations of these data are the controversy regarding the role of CD133 in melanoma cells discussed earlier [section 3.4.2]. To further address this issue, other stem cell markers e.g. CD20 and nestin, could be examined to verify enriching of ΔNp63 expression in these stem cell populations, thus confirming a role for p63 in identifying stem cells in melanoma. In addition, expression of cancer stem cell markers can be modulated by different experimental and environmental conditions which induce expression of stem-cell like surface markers and interfere with gene expression (Greijer et al. 2005). Therefore, to provide *in vivo* proof of concept, CD133-positive cells should be isolated from tumour samples and analysed for expression of ΔNp63. These experiments would strengthen the proposal that ΔNp63 is indeed, a putative stem cell marker which could be utilised for novel chemotherapeutic strategies.

CHAPTER 4: REGULATION OF EXPRESSION OF TP63

4.1 BACKGROUND

4.1.1 Epigenetic events in melanoma

Epigenetic modulation refers to changes in gene expression without alterations of DNA sequence, comprising chromatin organisation and modification (Egger et al. 2004). Epigenetic events can be divided into two major classes: DNA methylation and histone modifications. DNA is packaged into nucleosomes comprising a histone octamer (H2A, H2B, H3 and H4) around which 146 bp of DNA is wrapped (LaVoie 2005). The flexible histone N-terminal tails protrude from the nucleosome and are subject to covalent modifications including acetylation, arginine and lysine methylation, sumoylation, neddylation, ubiquitination and phosphorylation. These modifications dictate the histone code read by the gene regulatory machinery, allowing the cell to either facilitate or inhibit transcription of target genes (Berger 2002; Fischle et al. 2003; Rountree et al. 2001). The interplay between DNA methylation and histone modifications has a profound effect on epigenetic regulation of gene expression patterns and is thus an important factor in tumourigenesis (Li 2002). The precise positioning of nucleosomes and pattern of associated histone modifications play a major role in determining access to DNA, thus chromatin

modification and remodelling are key determinants of gene expression and DNA replication and repair [Figure 4.1].

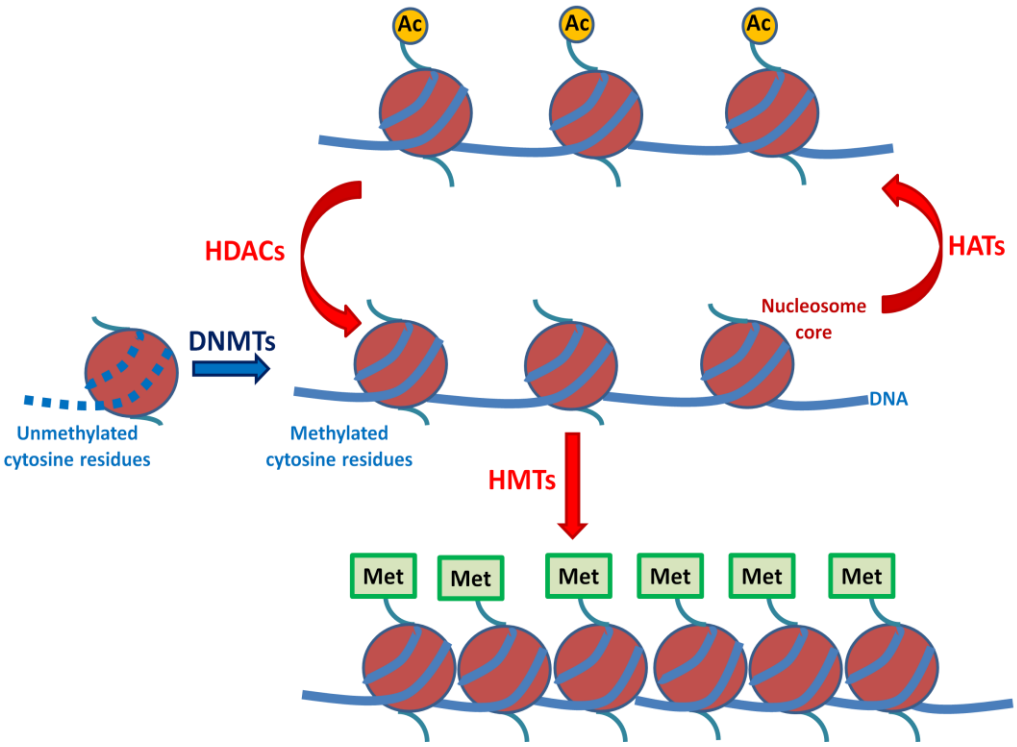


Figure 4.1: Chromatin organisation and modification. DNA is methylated by DNMTs. Methylated cytosine residues in DNA (solid blue line) subsequently allows recruitment of histone enzymes e.g. HDACs, HATs and HMTs. Targeted HAT and HDAC activities determine acetylation status of chromatin. Acetylation establishes a structure that permits ATP-dependent chromatin remodelling factors to open promoters. Deacetylation, frequently followed by histone methylation, may form a solid base for highly repressive structures, such as heterochromatin. Acetylated histone tails are shown as yellow circles. Methylations are indicated as green rectangles. HAT - histone acetyltransferase; HDAC - histone deacetylase; HMT - histone methyltransferase, DNMT- DNA methyltransferase

4.1.1.1 DNA methylation

The transfer of a methyl group to the carbon 5 position of cytosines, almost always in the context of CpG dinucleotides, is the only known epigenetic modification of DNA itself. Increased methylation of CpG islands (CpG rich regions of DNA usually, although not exclusively, associated with gene promoters), is a common mechanism of epigenetic gene silencing. Both hypermethylation of CpG islands located in the promoters of tumour suppressor genes and global hypomethylation seem to play a role during cancer development. DNA methylation occurs via covalent modification of cytosine and is catalysed by DNA methyltransferases (DNMT) which add a methyl group to the 5' carbon of the cytosine residues (Goll and Bestor 2005). Methylated DNA can then be bound by methyl-binding proteins which function as adaptors between methylated DNA and histone modifying enzymes [Figure 4.1]. Histone modifying enzymes then covalently modify the amino-terminal residues of histones to induce the formation of chromatin structures that repress gene transcription (Jenuwein and Allis 2001).

DNMTs also add methyl groups to cytosines within a CpG island (Baylin 2005). CpG islands located in the promoter regions of tumour suppressor genes are mainly unmethylated in normal somatic cells, whereas during cancer development aberrant hypermethylation of the CpG islands can occur, effectively silencing transcription of selected tumour suppressor genes (Robertson 2005b). However, in contrast to inherited inactivating gene mutations, epigenetic gene silencing through promoter methylation must be actively maintained by continuous re-methylation during each cell division and DNA replication cycle. This offers the opportunity to use DNA methyltransferase inhibitors as tools for cancer treatment with the aim of reversing aberrant hypermethylation of tumour suppressor genes.

The first DNA methyltransferase inhibitor synthesised was 5-azacytidine (5-Aza), a derivative of the nucleoside cytidine. It is incorporated into DNA and methylated by DNMT together with the regular cytosines. The 5-azacytosine residues however, form a covalent DNA-protein complex which leads to inactivation of the DNMT and consequently demethylation of DNA (Goffin and Eisenhauer 2002; Lyko and Brown 2005). Treatment with DNA methyltransferase inhibitors has demonstrated limited success in melanoma (Abele et al. 1987; Goffin and Eisenhauer 2002) but is likely to represent a powerful tool in combination with other therapeutic approaches. To date, in melanoma approximately 50 genes have been identified which are regulated, at

least in part, by promoter methylation and have been shown to be silenced during disease development and progression (Rothhammer and Bosserhoff 2007).

4.1.1.2 Chromatin remodelling

Chromatin remodelling mediates the dynamic structural changes in chromatin organisation that occurs through the cell cycle. It can be achieved by the concerted action of two different types of enzymes: ATP-hydrolysing enzymes and enzymes that covalently modify histones (van Grunsven et al. 2005).

4.1.1.2.1 *Histone modifications: Acetylation*

Histones are nuclear proteins which are necessary for the organisation of DNA into chromatin. The acetylation of lysine residues in the histone tails is one of the best characterised chromatin modifications and generally, acetylated histones are associated with transcriptionally active chromatin. Acetylation relieves the positive charges of histones leading to relaxation of the chromatin that enables interaction of transcription regulatory proteins with chromatin. Moreover, histone tails acetylated at specific residues provide recognition sites for transcriptional co-factors (Dhalluin et al. 1999; Zeng and Zhou 2002). Similarly chromatin deacetylation is linked to transcriptional repression. The acetylation status of histones is not a permanent event and is regulated by (i) histone acetyltrasferases (HATs) which transfer an acetyl group to the lysine residue, and (ii) histone deacetylases (HDACs) which remove acetyl groups. Consequently there is a dynamic and complex interplay occurring between acetylated and deacetylated regions of histones (Carrozza et al. 2003; Grant 2001; Huang et al. 2003; Santos-Rosa and Caldas 2005).

HDACs reverse the effects of HATs resulting in deacetylation of specific lysine residues which is a necessary pre-requisite for modification by methylation and ubiquitination. Deacetylation of histones promotes condensation of chromatin which reduces accessibility and consequently leads to repression of gene transcription (Huang et al. 2003). HDACs are subdivided into three different classes: class I which contains human HDACs 1-3 and 8, class II which contains human HDACs 4-7, 9 and 10 and class III, the Sirtuin family of NAD+ dependent HDACs (Boyle et al. 2005; de Ruijter et al. 2003; Holbert and Marmorstein 2005).

The function of HATs and HDACs is often deregulated in tumour cells leading to altered gene transcription (Minucci et al. 2001). HDAC inhibitors induce accumulation of hyperacetylated nucleosome core histones resulting in transcriptional activation of

genes. In addition, they induce acetylation of non-histone proteins providing an alternative mechanism for their chemotherapeutic activity in cancers (Gu and Roeder 1997). HDAC inhibitors (e.g. trichostatin A, depsipeptide FR901228 and SAHA) have shown potential as therapeutic agents *in vivo* and in clinical trials in conjunction with current therapeutic approaches (Kelly et al. 2003; Qiu et al. 1999; Sandor et al. 2002) but have not been extensively used because of their global effect on multiple signalling pathways.

Upregulation of TAp63α and adenoviral-mediated p63γ expression upon treatment with HDAC inhibitors is reported in different cell lines including testicular cancers (Sasaki et al. 2008; Sayan et al. 2009; Scheel et al. 2009). Furthermore, induction of TAp63α coincides with its cleavage, sensitising colon cancer cells to apoptosis (Sasaki et al. 2008; Sayan et al. 2007). Based on these reports it seemed reasonable to investigate the effects of acetylation on p63 expression in melanocytes and melanoma by treatment of cells with HDAC inhibitors.

4.1.1.2.2 *Histone modifications: Methylation*

Methylation of histones is generally associated with transcriptional repression with methylation of lysine-9 on histone-H3 required to maintain heterochromatin (Noma et al. 2001; Peters et al. 2002). In contrast, methylation of lysine-4 on histone-H3 can be stimulatory for transcription. The different combinations of histone modifications at different residues may act synergistically or antagonistically to influence gene expression (Jenuwein and Allis 2001). Histone methyltransferases (HMT) are the enzymes responsible for catalysing transfer of one to three methyl groups to lysine and arginine residues of histone proteins. Histone methylation has a role in epigenetic regulation since DNA is bound more tightly to methylated histones and consequently gene transcription is repressed [Figure 4.1]. The development of HMT inhibitors is at an early pre-clinical stage. However there is evidence to suggest that DNA methyltransferase inhibitors have significant influence on the methylation status of histones (Kondo et al. 2003).

Data from this thesis have demonstrated significant upregulation of p63 in up to 50% of melanoma cell lines and melanoma tissue samples. However, individual expression of TA and ΔN isoforms varied, and only 18% of melanoma cell lines tested expressed both isoforms. A possible explanation for the differential pattern of isoform expression could be epigenetic modulation. The purpose of the following experiments was to explore this by using agents which modulate the acetylation and

methylation of TP63. Under certain circumstances, p63 expression has been demonstrated to be modulated by methylation although this has not been rigorously examined (Park et al. 2000). To explore this in melanoma, cells which do not express p63 were treated with the DNA methyltransferase inhibitor 5-Azacytidine (5-Aza) in a dose-dependent manner, to examine the possibility of restoration of p63 expression.

4.1.2 MicroRNA regulation of TP63 gene expression

MicroRNAs (miRNA) are ~22 nucleotide-long small RNA molecules encoded in the genome that have a profound effect in controlling gene expression. MicroRNAs bind to their target mRNAs and downregulate their stability and/or translation. By binding to its target mRNA with complete complementarity, the miRNA can lead to degradation of the target, and by binding to targets with incomplete complementarity, in the 3' UTR regions, this leads to translational suppression of target genes (Esquela-Kerscher and Slack 2006; Kim and Nam 2006; Meltzer 2005). It is currently estimated that 30% of all human genes may be regulated by microRNAs (Lewis et al. 2005). Each miRNA is predicted to have many targets, and each mRNA may be regulated by more than one miRNA (Lewis et al. 2003; Lim et al. 2005; Rajewsky 2006). Specific over- or under-expression has been shown to correlate with particular tumour types (Lu et al. 2005; Volinia et al. 2006). miRNA over-expression results in downregulation of tumour suppressor genes whereas their under-expression leads to oncogene upregulation (Calin et al. 2004; Croce and Calin 2005; Gregory and Shiekhattar 2005; McManus 2003).

A complex interplay between miRNAs and epigenetics has recently been established; miRNAs target key enzymes involved in establishing epigenetic memory e.g. HDACs (Tuddenham et al. 2006) and epigenetic mechanisms affect expression of miRNAs (Saito et al. 2006). The interaction of epigenetics and miRNAs implies that epigenetic modulators may exert anti-tumour effects on two levels: by switching on tumour-suppressor genes that are silenced epigenetically, but also switch on tumour-suppressor miRNAs which downregulate target oncogenic mRNAs.

The regulation of p63 in different tissues is substantiated by reports of tissue-specific miRNA regulation of p63 expression [section 1.3.4.7, Table 4.1]. As this is the first demonstration of p63 in melanoma, it is pertinent to explore the regulation of this gene by miRNAs.

Table 4.1: Summary of MicroRNA regulation of p63

microRNA	Isoform targeted	Cell type	Reference
miR-203	ΔNp63	Epithelia	(Lena et al. 2008; Yi et al. 2008)
miR-302	TAp63	Germline	(Scheel et al. 2009)
miR-21	TAp63	Glioblastoma Cervical cancer	(Papagiannakopoulos et al. 2008)
miR-92	ΔNp63β	Myeloid cells	(Manni et al. 2009)
miR-34a	ΔNp63	SCC cell lines	(Chiorino, personal communication)

SCC squamous cell carcinoma

4.1.3 Regulation of p63 protein expression

Expression and regulation of p63 in human cancers is still a relatively unexplored field. In contrast to frequent p53 mutations, p63 mutations in human cancer are rare (Hagiwara et al. 1999; Ikawa et al. 1999; Irwin and Kaelin 2001b; Kato et al. 1999; Osada et al. 1998; Sunahara et al. 1999; Tani et al. 1999). However, overexpression of wild-type p63 isoforms is detected in epithelial cancers (Choi et al. 2002; Hibi et al. 2000; Hu et al. 2002; Massion et al. 2003; Mills 2006; Yang and McKeon 2000) and this thesis has provided the first evidence of expression of p63 in a significant proportion of melanoma cell lines and tumour samples [sections 3.3.3 and 3.3.5].

Studies have demonstrated that p63 mRNA and protein can be regulated by a number of factors including DNA damage, interaction with oncoproteins and complex signalling pathways (Huang et al. 2008b; Liefer et al. 2000; Nguyen et al. 2006; Petitjean et al. 2008). Post-translational modifications are important in regulating the stability of p63 [section 1.3.4.1]. p63 activity is also regulated through its physical interaction with a number of binding partners including Itch, WWP1 and RACK1 (Fomenkov et al. 2004; Li et al. 2008; Rossi et al. 2006b). Moreover, there is evidence that the p53 family members interact in a manner that is likely to be tissue-specific (Flores et al. 2002; Jacobs et al. 2005; Senoo et al. 2004) and truncated variants of p63 and p73 are able to negatively regulate full-length isoforms of all members (Melino et al. 2002; Melino et al. 2003; Moll and Slade 2004; Yang et al. 1999a; Zaika et al. 2002).

4.1.3.1 Stability of p63 protein

Despite the potent transactivation capabilities of p63, analysis of its expression both in melanoma cells and other cell systems, is limited by the availability of sensitive antibodies and transcripts of very low abundance. Stability of p63 proteins appears to be inversely correlated with their transactivation ability. TAp63γ, the most potent transactivator of the p63 isoforms, is often undetectable by Western blot, even in amounts that have robust transcriptional activity (Koster et al. 2004; Serber et al. 2002). TAp63α has a lower transactivation potential, and is more easily detected, but deletion of the C-terminal TID from TAp63α increases its transcriptional activity and reduces protein expression levels (Serber, Lai et al. 2002).

Following transient transfection in hepatocellular carcinoma cell lines, TAp63 transcripts were rapidly degraded, with a half life of ~1 hr, the most rapidly degraded form being TAp63α (Petitjean et al. 2008). Among the ΔN isoforms, ΔNp63α also showed a rapid turnover, whereas ΔNp63γ had a half life of at least 8hrs. ΔNp63β (the predominant isoform expressed in melanoma cell lines) was reported to show inconsistent degradation patterns in liver cells (Petitjean et al. 2008).

There is evidence that stability of p63 isoforms in different tissue-types is proteosome-dependent (Okada et al. 2002; Osada et al. 2001; Petitjean et al. 2008; Rossi et al. 2006a; Yin et al. 2002). The peptide aldehyde inhibitor MG-132 inhibits proteosomes, calpains and certain lysosomal cysteine proteases (Lee and Goldberg 1998; Tawa et al. 1997) and has been widely used to demonstrate the dependence of protein stability on the proteosome and will be used in experiments presented in this chapter.

4.2 AIMS

The aim of this chapter was to investigate the regulatory mechanisms governing the expression of p63 in melanoma. The objectives were:

a) To explore the epigenetic regulation of gene expression by examining the response of TP63 to histone deacetylase inhibitors and a DNA methyltransferase inhibitor
b) To investigate the regulation of TP63 by microRNAs using array technology
c) To study mechanisms which regulate p63 protein stability

4.3 RESULTS

4.3.1 Epigenetic regulation of TP63

4.3.1.1 Epigenetic modulation is infrequent in primary melanocyte cultures

Initial experiments were carried out in primary melanocyte cultures to determine the effect of HDAC inhibitors on expression of p63 isoforms. Initially the pan-HDAC inhibitor, trichostatin A (TSA) was used (Finnin et al. 1999). A differential effect was observed in primary melanocyte cultures upon treatment with low (500 µM) and high (1 mM) doses of TSA. In two out of four primary melanocyte cultures tested (NHEM 1 and NHEM 2), no upregulation of either TA or ΔN p63 was observed upon treatment with TSA. In the other two cell lines tested (HEMa V3 and V4), TAp63 was not detected in either untreated or treated HEMa cells using Q-PCR. In contrast, expression of ΔNp63 was upregulated upon treatment with TSA in HEMa V3 cells, although the same effect was not observed in HEMa V4 cells [Figure 4.2]. To investigate the possible role of methylation, melanocyte cultures were treated with 5-Aza in a dose and time-dependent manner. Neither restoration of TAp63 nor upregulation of ΔNp63 expression was detected in the four melanocyte cultures tested. Taken together, these data suggest that in primary melanocyte cultures, epigenetic modulation of p63 is an infrequent event.

4.3.1.2 Epigenetic modulation of TP63 occurs in melanoma cell lines

To investigate hypermethylation as a possible epigenetic mechanism for p63 silencing in melanoma cell lines, a search was performed using *Ensembl (www.ensembl.org)* and *USC Genome (genome.ucsc.edu)* to identify CpG islands in the promoter region. This revealed no large CpG islands in the promoter of p63 suggesting that it was unlikely to be regulated by methylation of its own promoter. It is possible, however, that p63 could be subject to modulation of the chromatin (or the histone code) (Brown and Strathdee 2002) or by promoter methylation of upstream targets (Raman et al. 2000).

Epigenetic mechanisms were explored in five melanoma cell lines which variably expressed TA and/or ΔN p63 [Figure 4.3]. Initially sub-lethal doses of TSA (500 µM – 1 mM) or 5-Aza (5-10 µM) were determined from optimisation experiments using various melanoma cell lines. Because DNA methylation and histone acetylation can act synergistically to silence tumour suppressor genes in cancer cell lines, the DNA methyltransferase inhibitor was used in combination with the HDAC inhibitor to enhance reversal of epigenetic silencing (Cameron et al. 1999). TSA and 5-Aza were

administered to cells individually for 24 – 48 hrs or in combination at low doses. In the latter case each treatment was either given at the same time or in succession, with or without a media change between treatments.

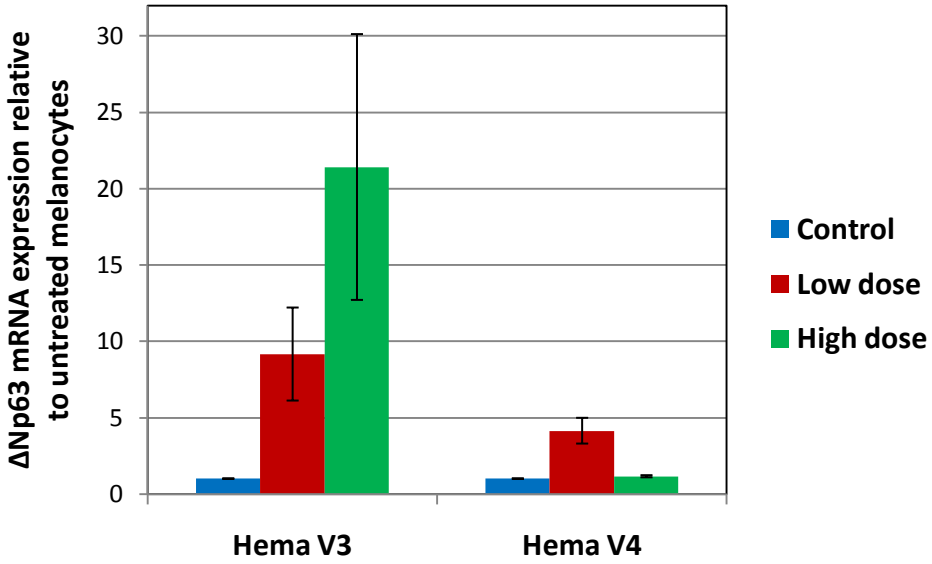


Figure 4.2: Epigenetic modulation of p63 in primary melanocyte cultures. Treatment of primary melanocyte cultures with high dose (1 mM) and low dose (500 µM) of TSA. ΔNp63 mRNA was analysed using Q-PCR demonstrating variable upregulation as a result of acetylation in HEMa V3 and V4 cells. Values shown are mean expression +/- SEM of three independent experiments performed in triplicate.

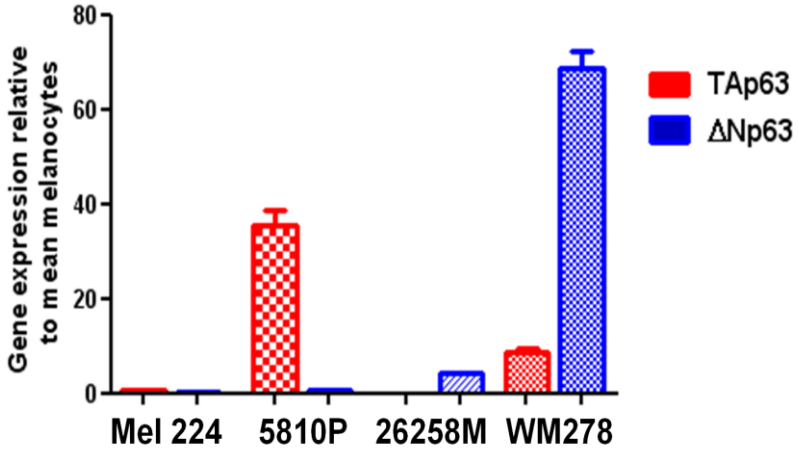


Figure 4.3: mRNA analysis of p63 isoform expression in melanoma cell lines used for subsequent epigenetic modulation experiments (Q-PCR). mRNA expression (+/- SEM) of p63 isoforms was determined relative to the mean expression of isoforms in five primary melanocyte cultures. Four cell lines were analysed, expressing no p63 (Mel 224), TAp63 only (5810P), ΔNp63 only (26258M) and both isoforms of p63 (WM278). GUS was the housekeeping gene used for mRNA standardisation. TAp63 – red bars, ΔNp63 – blue bars.

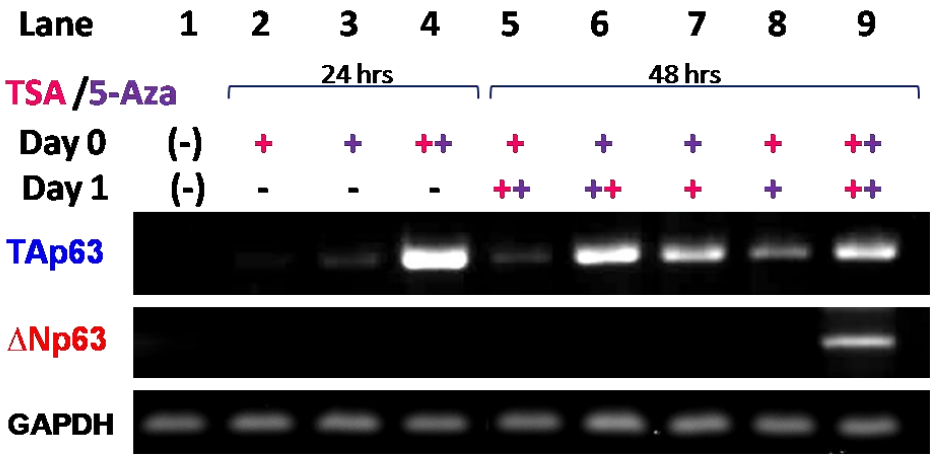
4.3.1.3 Epigenetic modulation induces *de novo* synthesis of p63

To explore the modulation of p63 in melanoma cell lines, initial experiments were performed in cells which did not express p63. To determine if restoration of p63 protein resulted from acetylation or demethylation, expression of TA and ΔNp63 in Mel 224 cells was analysed in the first instance using RT-PCR [Figure 4.4A]. Using the expression of the gene in untreated cells as the comparator, Q-PCR confirmed these results; TAp63 was induced by high dose TSA in the presence or absence of 5-Aza [Figure 4.4B]; restoration of ΔNp63 expression was much less pronounced and only significantly detected upon treatment with both TSA and 5-Aza for 48 hrs [Figure 4.4C]. In contrast, maximum restoration of TAp63 occurred within 24 hrs suggesting that the kinetics of modulation of the two isoforms differs in melanoma cells [Figure 4.4B]. A similar phenomenon was observed in Mel 505 cells with marked restoration of TAp63 expression induced by TSA, and ΔNp63 induced by the combination of TSA and 5-Aza at 48 hrs (data not shown).

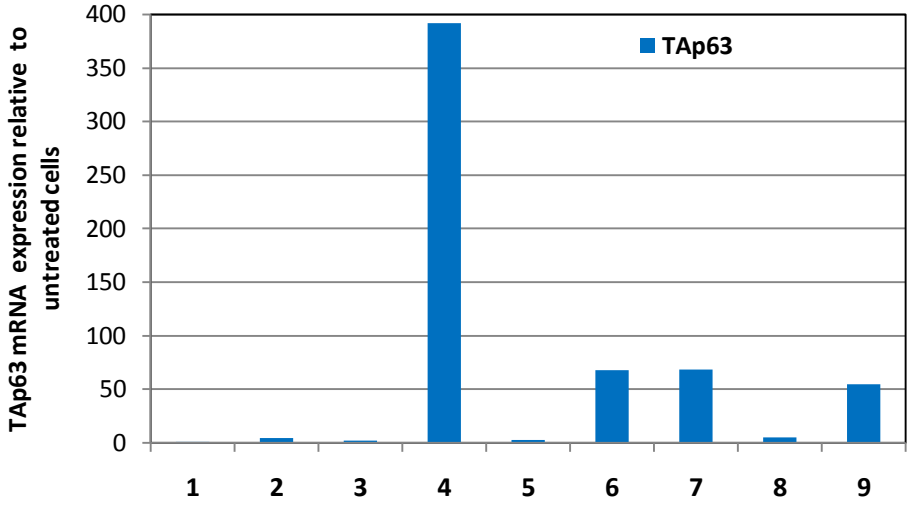
To localise the expression of p63 protein after treatment with high dose 5-Aza and TSA (24 – 48 hrs), immunohistochemistry and confocal microscopy analysis were performed [section 2.7.1, Figure 4.5A]. p63 protein was detected in Mel 224 and Mel 505 cells treated with both 5-Aza and TSA [Figure 4.5A]. In addition, a class III specific HDAC inhibitor tenovin-6 (Dr S Lain, Karolinska Institute, Sweden), was also able to induce expression of p63 protein but to a lesser extent (data not shown). This finding is in keeping with that of a recent report showing greater efficiency of pan-HDAC inhibitors in upregulating TAp63 compared with class specific inhibitors (Sayan et al. 2009).

To confirm restoration of p63 protein, Western blotting of the same cells treated with TSA and/or 5-Aza was undertaken. Protein lysates were run on a gel and probed with anti-p63 antibody [Figure 4.5B]. This demonstrated efficient restoration of p63 expression with upregulation of mainly TA isoforms, supporting data from RT-PCR experiments.

(A)



(B)



(C)

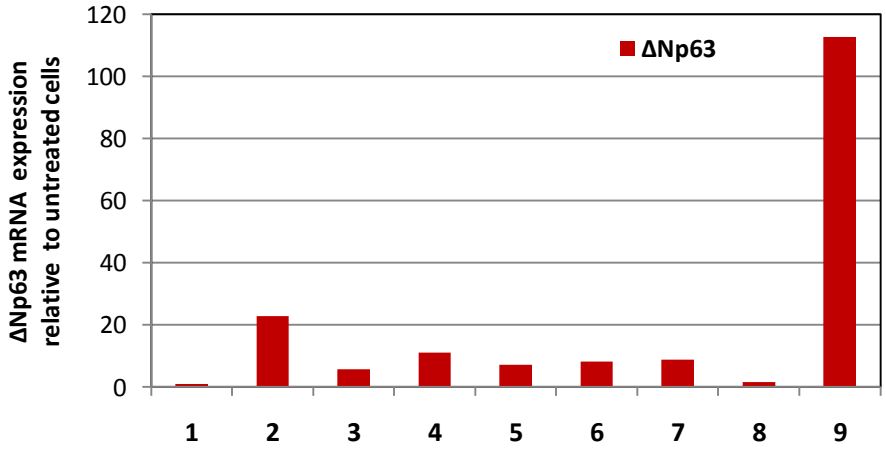


Figure 4.4: Epigenetic modulation of p63 mRNA (Mel 224 cells). (A) RT-PCR shows no endogenous expression of TA or ΔNp63 (lane 1). Upregulation of TA and ΔN p63 isoforms is observed upon treatment with TSA (pink) and 5-Aza (purple). (B) Q-PCR showing restoration

of expression of TAp63 in response to TSA (lane 4) and in conjunction with 5-Aza (lanes 6-9). Bars correspond to lane numbers shown in (A). Expression of TAp63 in treated cells is relative to expression in untreated cells using GUS as an endogenous comparator for all samples. (C) Q-PCR showing marked restoration of expression of ΔNp63 only upon prolonged treatment with TSA and 5-Aza in combination (lane 9). Bars correspond to lane numbers shown in (A). Expression of ΔNp63 in treated cells is relative to expression in untreated cells using GUS as an endogenous comparator for all samples. TSA – trichostatin A, 5-Aza – 5-Azacytidine.

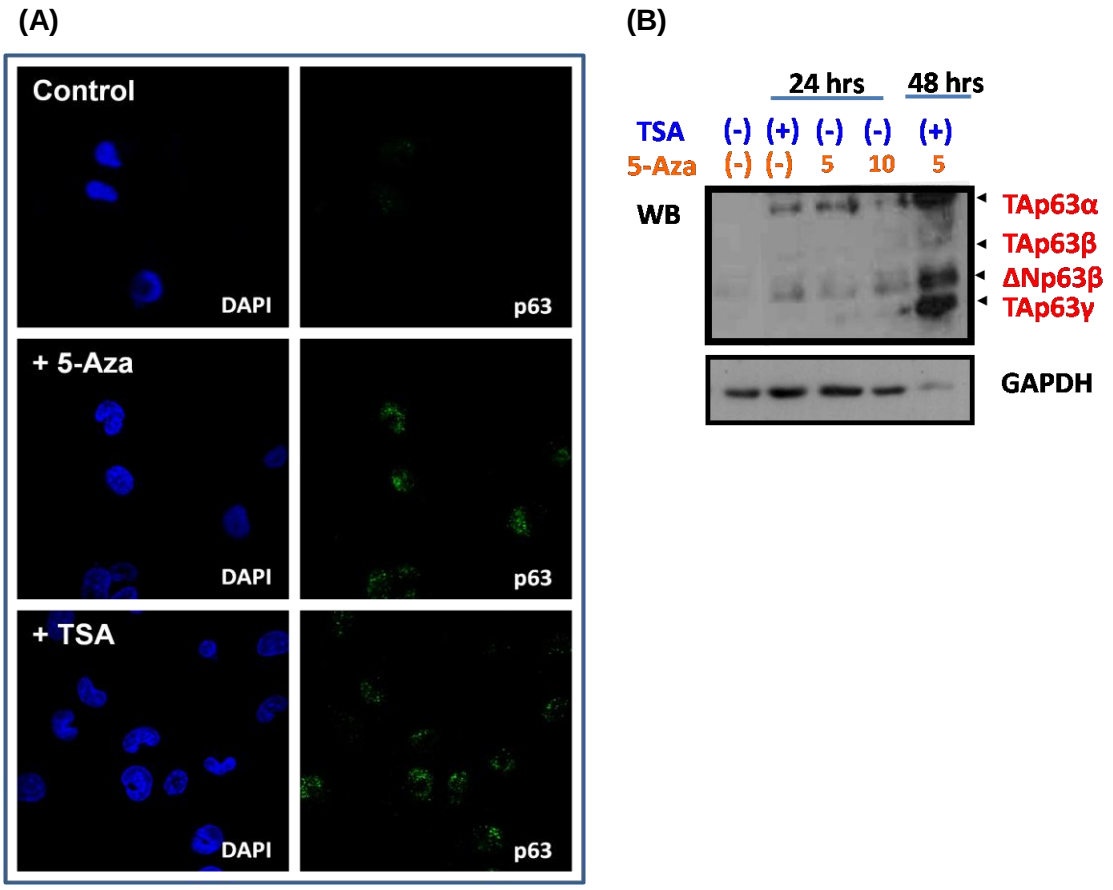


Figure 4.5: Epigenetic modulation of p63 protein expression (Mel 224 cells). (A) Confocal microscopy images showing undetectable levels of p63 (green) by immunofluorescence in untreated cells (top panels), and upregulation of p63 protein (all isoforms detected using H129 & H137 anti-p63 antibodies) demonstrated in cells treated with 5-Aza (10 µM – middle panels), and upon treatment with TSA (500 µM – bottom panels). DAPI (blue) was used to stain nuclei. (B) Western blot showing no p63 expression in untreated cells [lane 1 (-)] but upregulation of different isoforms of p63 (detected using anti-p63 antibody Ab-4 which detects all isoforms of p63) in response to treatment with TSA (500 µM – lane 2) or 5-Aza (5 – 10 µM lanes 3 and 4) for 24 hrs and in combination for 48 hrs (lane 5). P63 isoforms determined by comparing molecular weight of exogenously expressed isoforms in HEK 293T cells [section 2.1.5.1] (data not shown here). TSA – trichostatin A, 5-Aza – 5-Azacytidine.

4.3.1.4 Epigenetic mechanisms do not induce *de novo* synthesis of ΔNp63

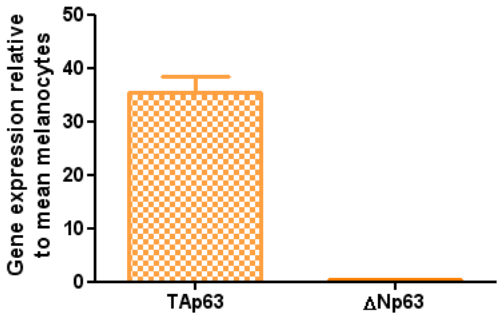
To explore the role of epigenetic mechanisms in modulating specific isoforms of p63, a melanoma cell line expressing only TAp63 (5810P) was investigated [Figure 4.6A]. This was undertaken primarily to determine if restoration of the unexpressed isoform (ΔNp63) occurred in response to HDAC inhibition and/or demethylating agents. Moderate upregulation of TAp63 was observed at 24 hrs following treatment with TSA and 5-Aza individually but to a much lesser degree when the two agents were given in combination [Figure 4.6B]. No re-expression of ΔNp63 was observed in this cell line upon treatment with either agent individually or in combination up to 72 hrs. These data suggest that silencing of ΔNp63 in a cell line already expressing TAp63 is not a result of methylation or deacetylation.

4.3.1.5 Epigenetic mechanisms increase endogenous expression of ΔNp63

To determine if the silencing of TAp63, when it is the unexpressed isoform occurs as a result of epigenetic mechanisms, the melanoma cell line 26258M (which expressed no TAp63) was subjected to HDAC inhibition and/or demethylation and analysed using Q-PCR [Figure 4.7A]. Whilst significant restoration of TAp63 expression was observed upon treatment for 24 hrs with TSA alone and in combination with 5-Aza, a modest upregulation of the ΔN isoform was only observed when cells were treated with the combination for 48 hrs [Figure 4.7B]. Upon treatment with low dose TSA and 5-Aza in succession for 72 hrs, both TAp63 and ΔNp63 were efficiently upregulated in 26258M cells [Figure 4.7C].

These data confirm earlier results that TAp63 expression can be epigenetically modulated in a cell line with no detectable endogenous TAp63. These findings are in keeping with those reported for this isoform of p63 (Sayan et al. 2009). However this is the first evidence that epigenetic modulation of ΔNp63 also occurs. Compared with TAp63, upregulation of ΔNp63 was less pronounced and required up to 48 hrs to observe an effect, suggesting that the mechanism regulating this isoform is a slower process.

(A)



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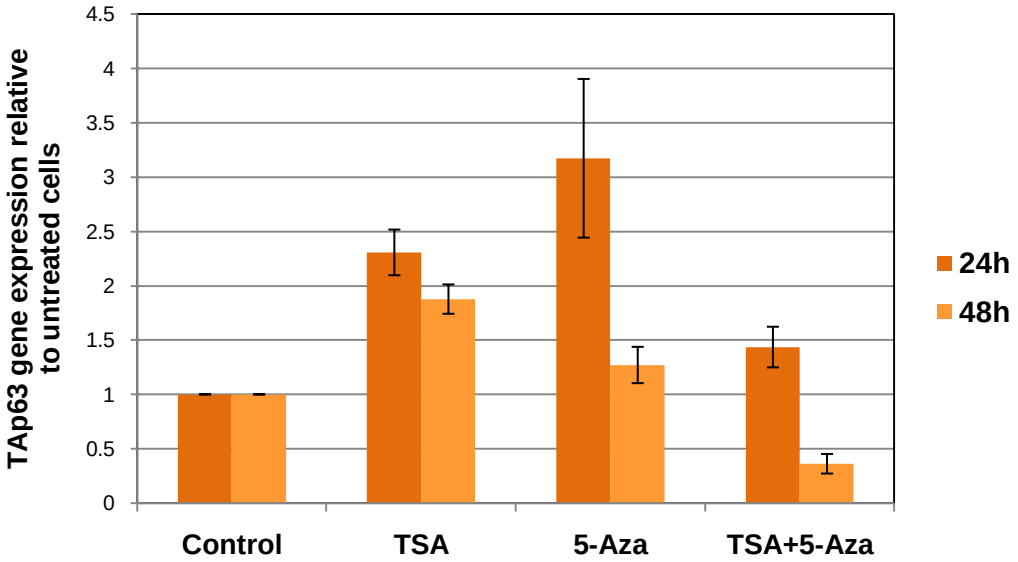


Figure 4.6: Modulation of TAp63 in a melanoma cell line expressing no endogenous ΔNp63 (5810P). (A) Gene expression of TA and ΔNp63 relative to the mean expression of the genes in five primary melanocyte cultures demonstrating upregulated TAp63 but no endogenous ΔNp63. (B) Histogram showing significantly greater upregulation of TAp63 expression upon treatment with TSA and 5-Aza at 24 hrs treatment in 5810P cells. The combination of TSA and 5-Aza treatment has little effect on the expression of TAp63 at any time-point. Values shown are mean expression +/- SEM of two independent experiments performed in triplicate. TSA – trichostatin A, 5-Aza – 5-Azacytidine.

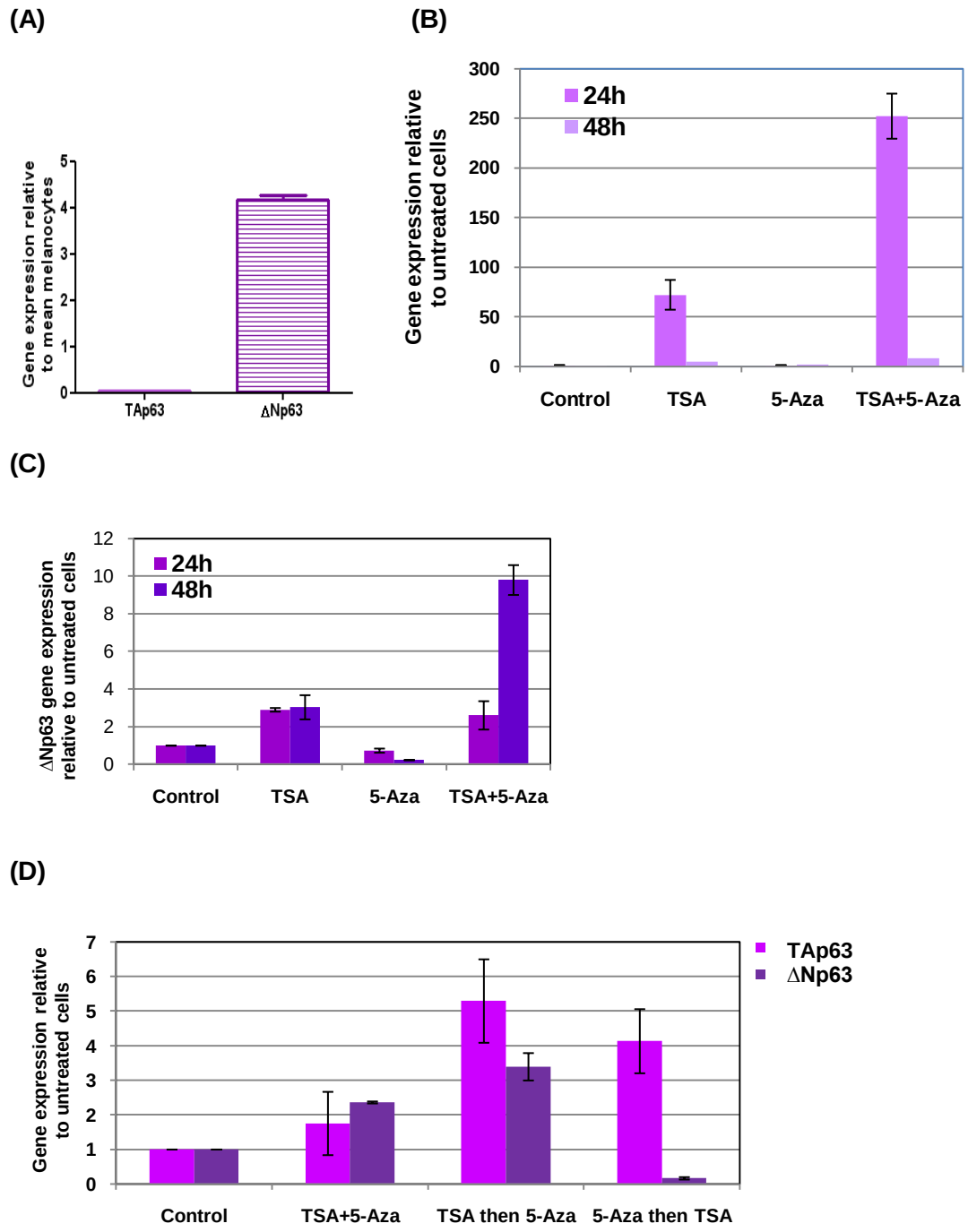


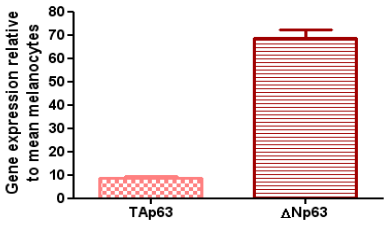
Figure 4.7: Epigenetic modulation of TP63 (26258M). (A) p63 mRNA expression relative to mean expression of p63 in five melanocyte cultures, showing upregulation of ΔNp63 but not TAp63 in this cell line. (B) TAp63 mRNA expression restored in response to TSA with similar effect upon combination TSA and 5-Aza treatment. 5-Aza does not restore expression of TAp63. (C) ΔNp63 mRNA is upregulated in response to TSA alone and in combination with 5-Aza, with marked increases observed at 48 hrs. (D) TA (light purple bars) and ΔNp63 (dark purple bars) mRNA response to combination treatment of TSA and 5-Aza, given concurrently or in 24 hrs succession. Maximal modulation occurs for TAp63. TSA treatment, 24 hrs prior to addition of 5-Aza, had the greatest effect on mRNA upregulation of both isoforms. Values shown are mean expression +/- SEM of two independent experiments performed in triplicate.

4.3.1.6 Epigenetic modulation of p63 is predominantly acetylation

To complete the analysis of epigenetic modulation of p63, the final group of cell lines tested were those expressing both isoforms of p63. TSA treatment of the melanoma cell line WM278, resulted in significant upregulation of TA and ΔNp63 at 24 and 48 hrs respectively [Figure 4.8]. 5-Aza treatment had little effect on the expression of either isoform. A modest upregulation of TA and ΔN p63 was observed upon treatment with the combination of treatments at 24 and 48 hrs respectively [Figure 4.8].

These data confirm the previous results that modulation of both TA and ΔN p63 occurs predominantly as a result of acetylation. The most readily induced isoform of p63 by HDAC inhibitors was TAp63. Epigenetic modulation of ΔNp63 was a more gradual process which did not occur consistently. Modulation of p63 gene expression did not appear to be due to the direct effects of gene methylation since demethylating agents alone had little effect on the expression of either isoform of p63. The combination of HDAC inhibition and demethylation of melanoma cells under certain circumstances had an additive effect on gene re-expression. This can be explained by the HDAC inhibitor allowing increased access of the transcription factors to the demethylated gene as a result of increased levels of histone acetylation and the consequent chromatin re-modelling (Egger et al. 2007).

(A)



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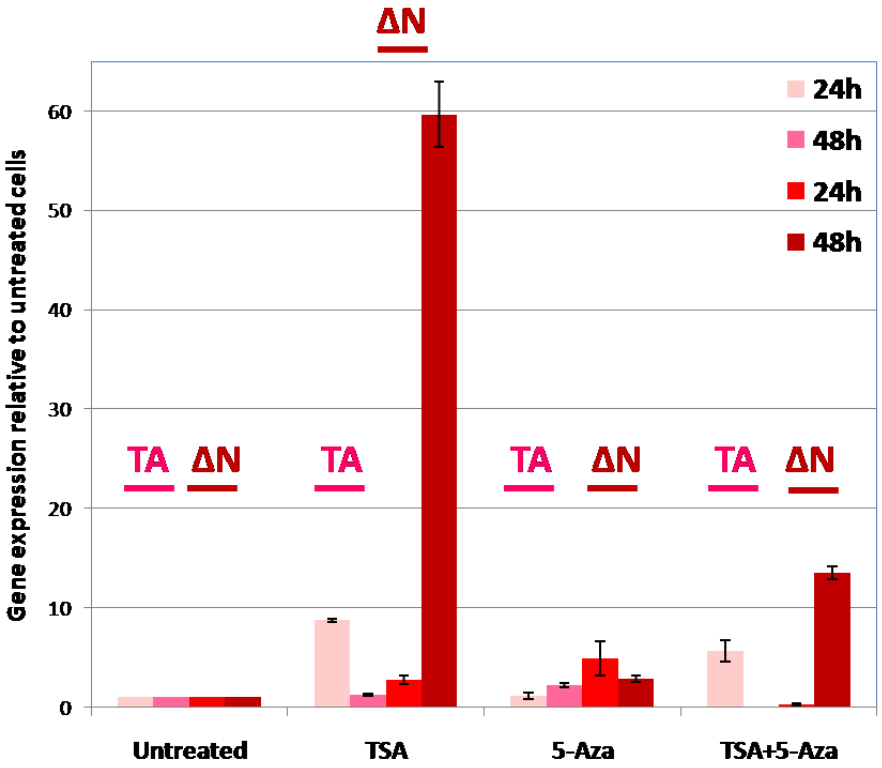


Figure 4.8: Epigenetic modulation of TP63 mRNA (WM278). (A) TP63 mRNA expression relative to mean expression of TP63 in five melanocyte cultures showing upregulation of both TAp63 and ΔNp63 in this cell line. (B) TAp63 mRNA expression is significantly upregulated in response to TSA at 24 hrs but little effect was observed upon treatment with 5-Aza. ΔNp63 mRNA expression is markedly upregulated in response to TSA alone, and to a lesser extent when treated in combination with 5-Aza at 48 hrs. 5-Aza has little effect in modulating ΔNp63 mRNA. Values shown are mean expression +/- SEM of two independent experiments performed in triplicate. TSA – trichostatin A, 5-Aza – 5-Azacytidine.

4.3.2 microRNA regulation of TP63

To evaluate the dysregulation of microRNAs which could account for variable expression of p63 in melanoma cell lines screened [section 3.3.1.2], a panel of cell lines consisting of four categories were chosen for analysis: p63-null cell lines and three categories comprising of melanomas expressing p63: p63 (both TA and ΔN isoforms), TAp63 only and ΔNp63 only [Table 4.2]. Samples were labelled and hybridised according to Agilent Technologies miRNA Microarray System protocol [section 2.9.2]. TIFF images were loaded into the Feature Extraction 9.5 (Agilent technologies) software and analysed using the microRNA grid template and protocol. Replicate probes were combined and then t-tests and Wilcoxon tests were applied to normalised log intensities, in order to detect differentially expressed miRNAs in each cell class. The array itself was undertaken by Dr G Chiorino and Dr P Ostana (Cancer Genomics Lab, Italy) but the raw data were analysed in collaboration with Dr P Ostana.

Comparisons were performed to explore the regulation of expression of p63 by miRNA. Melanoma cells which did not express p63 were used as the reference for all comparisons. An absolute log(fold change) > 0.58 was chosen as it corresponds to an absolute fold change >~ 1.5. This cut-off point was chosen since small changes in miRNA expression can be relevant for their function in the cell. Dysregulation of miRNAs with a statistically significant p-value but a small log(FC) were excluded as the change in expression was too small to be considered relevant. miRs with a marked change in the absolute log(fold change) but which did not reach statistical significance were deemed false positives and also excluded.

Table 4.2: Expression of p63 isoforms in a panel of melanoma cell lines analysed using microRNA array (Agilent Technologies). Cell lines were grouped according to expression of TA and/or ΔN isoform of p63 and highlighted with colour; lighter shading within a group represents primary melanoma cell lines and darker shading within a group represents metastatic cell lines.

Cell line		Stage of disease	TAp63 expression	ΔNp63 expression
p63-null	Mel224	Primary	-	-
	Mel505		-	-
	Mel501	Metastatic	-	-
p63 – both isoforms	SBCl2	Primary	+	+
	WM278		+	+
	WM1158	Metastatic	+	+
TAp63 only	WM115	Primary	+	-
	WM239A	Metastatic	+	-
	Sk mel 24		+	-
	C8161		+	-
ΔNp63 only	WM793	Primary	-	+
	1402P		-	+
	A375M	Metastatic	-	+
	HBL		-	+

4.3.2.1 Novel miRNAs identified to regulate p63 expression

Initially the absolute log(fold change) for miRNAs was analysed by comparing p63 null melanoma cell lines (Mel 224, Mel 505, Mel 501) with all three groups of p63-expressing melanoma cell lines together (TAp63 only, ΔNp63 only and TA and ΔNp63 only). The only miRNAs significantly upregulated in p63-null cell lines were miR18a and miR18b suggesting they were putative negative regulators of p63 expression. miR-18a demonstrated significant upregulation when comparisons were made with TAp63-expressing cell lines and both isoform-expressing cell lines [Table 4.3].

To investigate if p63 mRNA is a target of miR-18a, the 3' UTR mRNA sequence of p63 was analysed by computational algorithms utilising distinct parameters to predict the probability of a functional miRNA binding site within the mRNA target. Kuhn et al (2008) recommend at least two algorithms to predict a miRNA binding site before additional validation experiments should be pursued. Bioinformatic algorithms were therefore utilised to predict miRNA binding sites within the 3' UTR of p63 mRNA and a putative binding site was found (http://www.microrna.org and http://mirdb.org/miRDB/download.html) [Figure 4.9].

4.3.2.2 Tissue-specific miRNAs reported to regulate p63 could act in melanoma

Analysis of the reported miRNAs deemed to regulate p63 [Table 4.1] revealed two candidate miRNAs (miR-21, miR-34a) which were dysregulated between melanoma cell lines lacking p63 and those expressing p63 [Table 4.4, Appendix 7]. Unexpectedly, these two reported microRNAs targeting p63 were significantly upregulated in melanoma cell lines variably expressing isoforms of p63 [Table 4.4]. Although the more commonly studied mechanism of regulation by microRNAs is a direct interaction, a less well understood, indirect effect to activate gene expression has also been documented (Krutzfeldt et al. 2006) [Figure 4.10]. A possible explanation for the unexpected upregulation of miR-34a and miR-21 in p63-expressing cell lines could be an effect mediated through inhibition of a transcriptional repressor of p63 [Figure 4.10].

Analysis of the 3'UTR of TP63 using the same algorithms failed to demonstrate alignment for miR-34a (http://www.microrna.org). Putative intermediate targets of miR-34a which are known to transcriptionally repress p63 highlighted Notch 1, Notch 2 and JAG1 as possible candidates (PicTar; http://pictar.bio.nyu.edu and TargetScan Human 5.1; http://www.targetscan.org) which are implicated in regulating ΔNp63

levels in keratinocytes (Nguyen et al. 2006). In support of this hypothesis, a recent report has confirmed targeting of notch 1 / notch 2 in glioblastoma by miR-34a (Li et al. 2009). To add complexity to this hypothesis, miR-21 maintains cell cycle progression in glioblastoma cells through regulation of TAp63 (Papagiannakopoulos et al. 2008) and a very recent report demonstrated ΔNp63 transcriptionally represses miR-34 in murine keratinocytes contributing to cell cycle progression (Antonini et al.). These data clearly show that microRNA regulation of the different isoforms of TP63 mRNA targets is complex and likely to be significantly different between primary cells and cancer cells. Growing evidence supports the co-ordinate regulation of single genes by multiple miRNAs (Krek et al. 2005) implying that significant work is still required to delineate the regulation of p63 in melanoma cells.

Table 4.3: MicroRNAs upregulated in p63-null melanoma cell lines compared with three groups of p63-isoform expressing melanoma cell lines.

	p63 (-) vs. all p63 (+) groups		p63 (-) vs. both TA and ΔNp63		p63 (-) vs. TAp63		p63 (-) vs. ΔNp63	
	P-value	Log(FC)	P-value	Log(FC)	P-value	Log(FC)	P-value	Log(FC)
miR-18a	0.012	0.77	0.037	0.89	0.036	0.91	0.062	0.54
miR-18b	0.047	0.74	0.205	0.86	0.039	0.83	0.112	0.55

Numbers in red show miRNAs significantly upregulated in p63-null melanoma cell lines (p63(-))

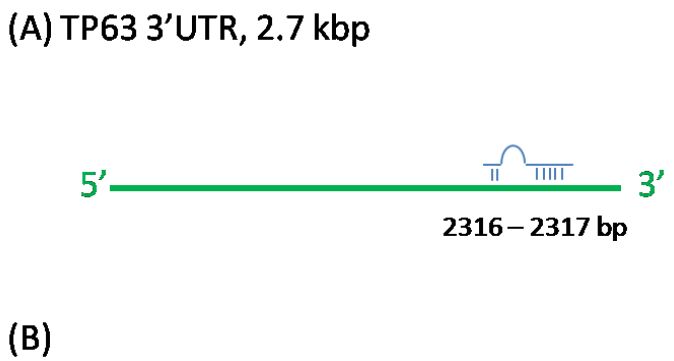


Figure 4.9: Putative binding of miR-18a to TP63 (http://www.microrna.org). (A) miR-18a target site within the 3'UTR of p63 mRNA. (B) Alignment of hsa-miR-18a and TP63 3' UTR demonstrating putative binding.

Table 4.4: Dysregulation of microRNAs previously reported to regulate p63 expression in other tissue contexts. Two microRNAs were downregulated in p63-null melanoma cell lines compared with three groups of p63-isoform expressing melanoma cell lines.

ID	p63 (-) vs. all p63 (+) groups		p63 (-) vs. both TA and ΔNp63		p63 (-) vs. TAp63		p63 (-) vs. ΔNp63	
	P-value	Log(FC)	P-value	Log(FC)	P-value	Log(FC)	P-value	Log(FC)
miR-21#	0.1276	-1.5098	0.0648	-2.0974	**0.0485**	**-2.2922**	0.7727	-0.2866
miR-34a*	**0.0003**	**-2.6902**	**0.0331**	**-2.6124**	**0.0174**	**-3.0419**	**0.0066**	**-2.3968**
	0.0089	**-3.9134**	**0.0177**	**-4.0316**	**0.0074**	**-4.3374**	**0.0142**	**-3.4006**

Negative values (in green) represent decreased log(FC) in p63-null melanoma cell lines. p-values in green demonstrate significant downregulation of miRNA in p63 null cell lines.

(Papagiannakopoulos et al. 2008)

*Dr G Chiorino, personal communication

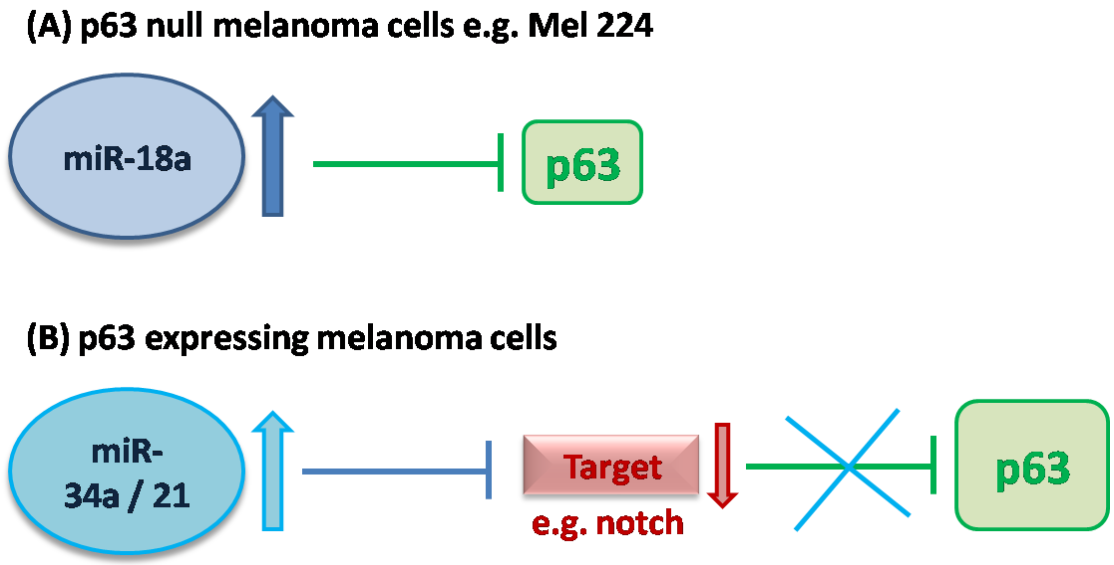


Figure 4.10: Two putative microRNA regulatory mechanisms to explain expression of p63 in melanoma cell lines. (A) Direct regulation of p63 mRNA by miR-18a mediated through mRNA degradation or inhibition of mRNA translation. (B) Indirect effect of microRNAs on p63. miR-34a and/or miR-21 could regulate p63 indirectly through inhibition of mRNA of a transcriptional repressor e.g. notch (Li et al. 2009). This results in derepression of p63 thus increased expression of p63.

4.3.3 Regulation of p63 stability

To investigate the stability of p63 mRNA and protein in melanoma, general factors applicable to cell culture conditions and specific factors previously reported to regulate p63 in other cell systems, were explored.

4.3.3.1 Cell culture conditions

Cell density is a well-recognised phenomenon influencing gene and/or protein expression. ΔNp63 mRNA and protein levels increase as a function of confluence in human corneal epithelial cells, peaking at 100% confluence (Robertson et al. 2008). In addition, a number of E3 ubiquitin ligases are demonstrated to interact with p63, targeting it for degradation [section 1.3.4.2 – 1.3.4.5] and polo-like kinase-1-related TAp63 protein degradation is negatively regulated by increased cell density of Hep3B liver cells (Komatsu et al. 2009). There appear to be no data examining the relationship between cell density and p63 in cancer cell lines. Analysis of p63 mRNA using RT-PCR in melanoma cell lines revealed low levels of p63 expressed in cells plated sparsely, suggesting that expression of p63 could be a function of confluency, and was therefore explored using RT-PCR and western blotting.

4.3.3.1.1 *Cell density does not induce synthesis of TP63 in TP63-null cell lines*

To investigate cell confluency as a determinant of TP63 gene expression, cells were seeded at increasing density varying from $1x10^4$ to $3x10^6$ cells/60 mm dish, and expression of TA and ΔN p63 was assessed using RT-PCR [section 2.2.4]. Initial experiments were performed in primary melanocyte cultures which did not express endogenous TP63. Increasing cell density from 40 to 100% had no effect on expression of TP63 in three primary melanocyte cultures tested (NHEM 2, HEMA V3 and V4), suggesting that cell density does not increase *de novo* synthesis of p63 [Figure 4.11]. In keeping with this finding, when a melanoma cell line which did not express endogenous p63 (Mel 224), was seeded at increasing cell density (including post-confluency), p63 mRNA levels did not increase [Figure 4.11].

4.3.3.1.2 *TP63 mRNA expression is a function of cell density*

Further experiments were performed in melanoma cell lines with endogenous expression of TAp63 only (WM239A) and ΔNp63 only (A375M), which demonstrated increasing cell density positively correlated with expression of the endogenous isoform [Figure 4.12]. To exclude the possibility that cell density regulation of p63 was cell-line specific, three other cell lines (Mel 501, ME10538 and 1402P) were tested and a melanoma cell line expressing both isoforms (WM1158) was also examined.

These results confirmed that mRNA expression increased as a function of cell density in melanoma cell lines for endogenously expressed p63 isoforms [Figure 4.12]. When cells were left in culture for 24 hrs post-confluence TP63 levels declined (data not shown). These findings were in keeping with that reported for ΔNp63 in human corneal epithelia (Robertson et al. 2008). p63 expression reached a maximal plateau in melanoma cells plated at 60% confluency which was then consistently maintained for all subsequent experiments.

4.3.3.1.3 *p63 protein expression is a function of cell density*

Protein expression of p63 was not consistently reproducible for melanoma cell lines. One explanation is the poor sensitivity and specificity of the anti-p63 antibodies used to detect endogenous p63, which recognised undefined bands on a Western blot which did not correspond to the molecular weights of exogenously transfected p63 isoforms [section 3.3.3]. This poses a problem when there are a number of isoforms of p63 expressed at low levels which are very close in molecular weight on a Western blot. In light of data which showed mRNA expression influenced by cell density, the same experiment was repeated to establish if confluency affected protein expression, with the aim of addressing the issue of poor antibody sensitivity. Protein lysates from cells seeded at increasing density were harvested for Western blotting analysis, and probed with the anti-p63 antibody Ab-1. Increasing stability of isoforms of p63 was demonstrated in response to increasing cell density with evidence of equal protein loading confirmed by GAPDH [Figure 4.12].

In summary, these data demonstrate that confluency does not affect *de novo* expression of p63 but levels of p63 gene and protein expression in melanoma cells increases throughout the log growth phase with the optimum confluency determined to exceed 60%.

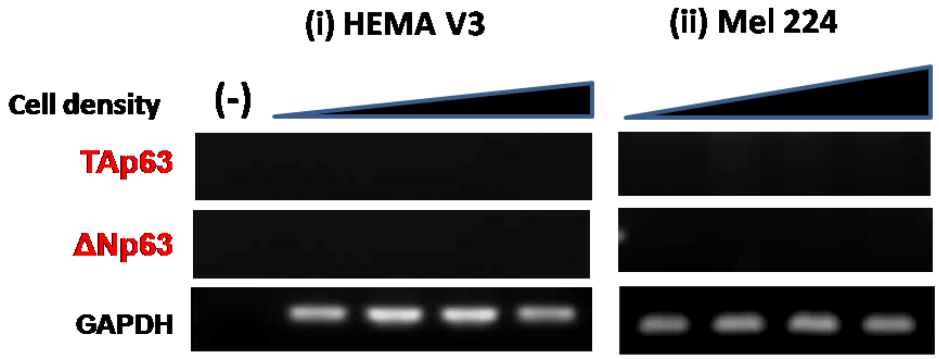
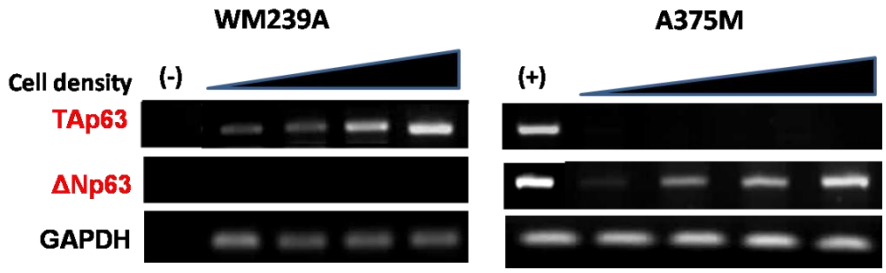


Figure 4.11: Cell density has no effect on *de novo* synthesis of p63. Increasing cell density of (i) the primary melanocyte culture - Hema V3 and (ii) Mel 224 cells (which did not express endogenous p63) had no effect on gene expression. (-) = PCR control with no cDNA. GAPDH was used as a loading control.

(A)



(B)

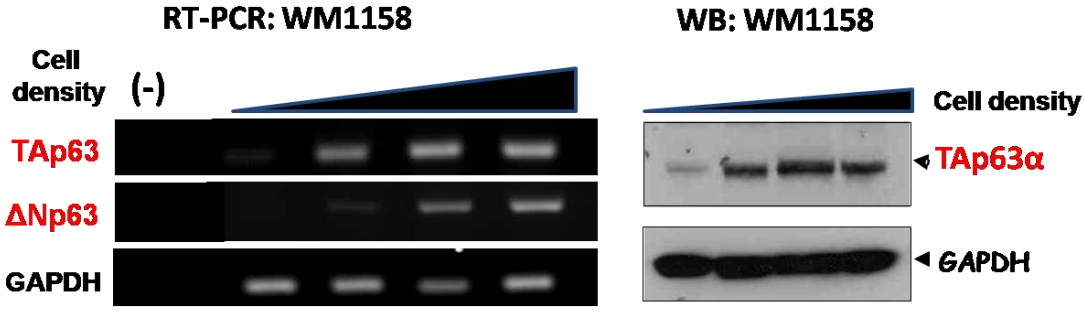


Figure 4.12: TP63 expression is a function of cell density. Cells were seeded at increasing densities and harvested 24 hrs later when cells were at a minimum confluency of 30% to a maximum confluency of 100%. (A) TAp63 mRNA levels increase as a function of confluency in WM239A melanoma cells and ΔNp63 mRNA levels demonstrate a similar phenomenon in A375M cells. (B) In WM1158 cells both isoforms of TP63 mRNA increase as a function of confluency, with optimal confluency determined to be 60%, and this is confirmed by increased p63 protein levels shown in right panel. GAPDH was used a loading control. (-) = RT-PCR control with no cDNA, (+) = TAp63 plasmid exogenously transfected in HEK 293T cells to confirm successful PCR reaction.

4.3.3.2 Stability of p63 protein is dependent on the proteosome

To explore stability of p63 isoforms in melanoma cell lines, metastatic melanoma cells (WM9, WM278, WM1158) were treated with peptide aldehyde inhibitor, MG-132 (1-30 μM). These experiments showed stabilisation of endogenous p63, although this was not consistently demonstrated for all melanoma cell lines [Figure 4.13]. The stability of transfected p63 isoforms was tested using MG-132. Mel 505 cells transiently transfected with all six isoforms of p63 failed to demonstrate consistent stabilisation of transfected isoforms upon treatment with MG-132 (data not shown). Taken together, these findings indicate that proteosomal degradation may be one of many possible mechanisms affecting the stability of p63 protein in melanoma cells.

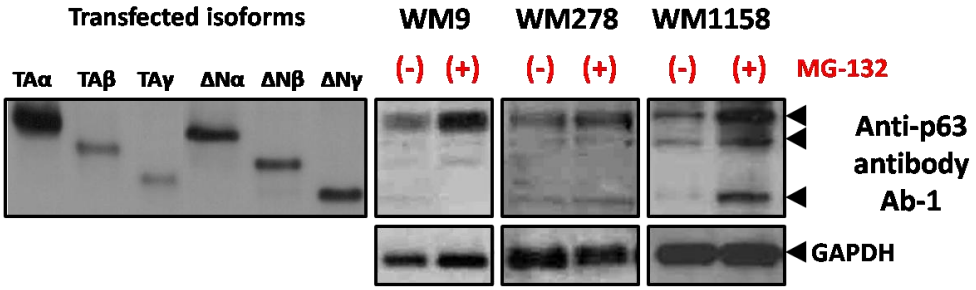


Figure 4.13: Stability of p63 is partially dependent on the proteosome in melanoma cell lines. Cells were plated at a confluency of 1 x 10^6 / 10 cm, treated 16 hrs later and harvested at 6 – 24 hrs. The identity of isoforms was established by their co-migration with exogenously expressed p63 isoforms in HEK 293T cells (left panel). Melanoma cell lines were treated (+) with MG-132 (20 μM) and protein lysates were run on a Western blot probed using anti-p63 antibody (Ab-1) demonstrating possible stabilisation of isoforms of p63 at 24 hrs. GAPDH was used as a loading control. Western blot representative of experiments performed in triplicate.

4.3.3.3 Stability of p63 protein is dependent on the E3-ubiquitin ligase itch

As a result of data supporting proteosome-mediated degradation of p63 in melanoma cell lines, a number of E3-ubiquitin ligases explored in relation to p63 degradation can be considered [sections 1.3.4.2 – 1.3.4.5]. However, the association of p63 with Itch is the most widely investigated of these proteins (Fomenkov et al. 2004; Gallegos et al. 2008; Li et al. 2008; Rossi et al. 2006b). Itch can bind, ubiquitylate and degrade p63, preferentially interacting with ΔN p63 (Melino et al. 2006; Rossi et al. 2006b). Itch is co-expressed with p63 in the epidermis and, by controlling its ubiquitin-dependent degradation, provides evidence for a functional association *in vivo*. The downregulation of p63 by itch protein plays an important role in epithelial homeostasis (Rossi et al. 2006a). There are no reports of itch protein expression in the melanocyte lineage or in melanoma.

Initial experiments were performed to determine the expression of itch in this lineage. Primary melanocyte cultures and melanoma cells were seeded at equal densities and lysates were harvested to investigate the endogenous expression of itch. Itch was not expressed at detectable levels in three primary melanocyte cultures tested (NHEM, HEMa 3 and HEMa V3). To investigate the relationship between itch and p63 levels, melanoma cell lines were analysed showing itch was expressed in melanoma irrespective of the stage of disease progression [Figure 4.14]. A reciprocal relationship between expression of p63 and itch was observed in 3/9 (33%) melanoma cell lines tested. These data suggest that regulation of p63 expression in a subset of melanoma cell lines could occur through the E3-ubiquitin ligase, itch.

To explore the relationship between itch and p63 further, melanoma cell lines with high endogenous expression of itch were selected for transient silencing of the gene using RNAi technology to determine the effect on p63 protein. A pool of four siRNA targets of itch (Dharmacon) was used to deplete the gene [section 2.2.7.1, Table 2.10]. Initial experiments were performed in Mel 505 cells to determine the minimum dose of siRNA required to significantly deplete itch which was 20 nM [Figure 4.15A]. A time-dependent experiment revealed that this silencing was maintained until 96 hrs [Figure 4.15B]. Western blot analysis using anti-p63 antibody showed upregulation of p63 protein in Mel 505 cells which were depleted of itch, suggesting a role for itch in regulating p63 protein [Figure 4.15B].

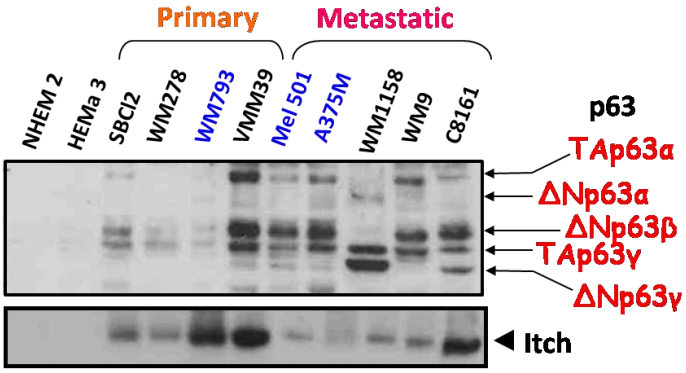
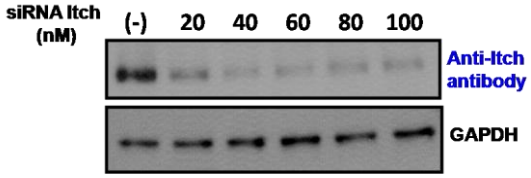


Figure 4.14: Expression pattern of itch and p63 proteins. A panel of primary melanocyte cultures and melanoma cell lines were analysed using Western blotting for itch and p63. Itch was not expressed in primary melanocyte cultures (NHEM 2, HEMa 3) but was differentially expressed in melanoma cell lines representing different stages of disease progression. Analysis of p63 expression (using anti-p63 antibody AB-4) in the same lysates demonstrates a reciprocal pattern of expression with itch (using anti-itch antibody) in 3/9 cell lines highlighted in blue. GAPDH confirmed equal loading of proteins (not shown).

(A)



(B)

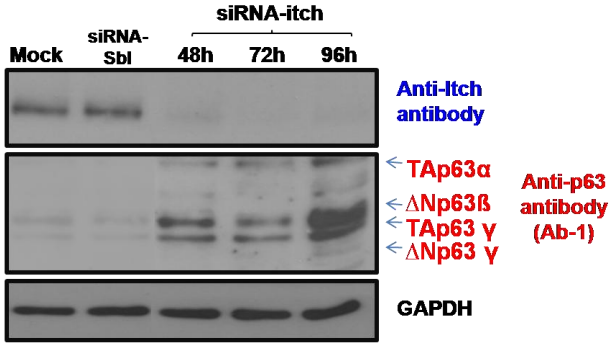


Figure 4.15: Itch depletion results in upregulation of p63 protein (Mel 505 cells). A pool of four siRNA-itch sequences was used to silence itch in melanoma cell lines (example shown is mel 505 cells). (A) Significant depletion of itch protein was achieved at a dose of 20 nM at 24 hrs. (B) Depletion of itch protein was maintained for 96 hrs following transfection with siRNA-itch when compared with siRNA-scramble and mock transfected cells. Expression of p63 in the same cell line showed significant upregulation of p63 isoforms in itch-depleted cells. GAPDH was used as a loading control for proteins. The identity of isoforms was established by their co-migration with exogenously expressed p63 isoforms in HEK 293T cells (data not shown). siRNA-Sbl = siRNA-scramble sequence.

4.4 DISCUSSION

4.4.1 TP63 is modulated by acetylation

To understand why 25/34 (74%) melanoma cell lines expressed p63 with a differential expression of isoforms and 26% of melanoma cell lines did not express endogenous p63, epigenetic silencing of p63 was investigated. No CpG islands were detected in the promoter of TP63. Melanoma cell lines treated with demethylating agents did not induce *de novo* expression of TA or ΔN p63 suggesting that methylation does not influence p63 expression. Moreover, demethylating agents had little effect, if any, on endogenous p63 mRNA and protein levels. Acetylation, however, was an important mode of modulation of p63. TSA was able to efficiently induce *de novo* expression of TAp63 and upregulate endogenous TAp63 levels in melanoma. Modulation of ΔNp63 by acetylation was a more gradual process which did not occur consistently. *De novo* synthesis of ΔNp63 was not shown upon treatment with any epigenetic modulator. Moreover, a recent study has suggested that the induction of p63 depends on p53 (Sayan et al. 2009) and although this was not directly tested, all cell lines tested harboured wt-p53, supporting this hypothesis.

The limitation of these data is that only two HDAC inhibitors were used to demonstrate upregulation of p63. Although the focus of these experiments was to investigate global epigenetic mechanisms regulating p63, evidence provided here that acetylation is important warrants further attention. Investigating the effect of different HDAC inhibitors on endogenous expression of p63 in melanoma would further delineate the mechanism of acetylation. To confirm that HDAC inhibitors also function by modifying protein stability, p63 levels should be assessed using Western blotting upon treatment with HDAC inhibitors in the presence and absence of cyclohexamide. This would confirm that HDAC inhibitors contribute to increased expression of TAp63 through both *de novo* synthesis of the gene and increased protein stability.

4.4.2 Novel miRNAs identified in melanoma which could regulate expression of p63

4.4.2.1 miRNA-18a

The only microRNA upregulated in p63-null cell lines when compared with those expressing p63 was miR-18a, identifying it as a potential novel negative regulator of p63. miR-18a is a member of the miR-17-92 cluster which is an example of a miRNA cluster with oncogenic properties (Mendell 2008). It comprises 7 mature miRNAs: miR-17-5p, -17-3p, -18a, -19a, -19b, -20a and -92a which are transcribed as one

common primary transcript. This cluster is located within ~1kb of an intron of the C13-25Orf locus on human chromosome 13q31, a region frequently amplified in solid cancers, lymphoma and uveal melanoma (Hayashita et al. 2005; Hughes et al. 2005; Ota et al. 2004; Volinia et al. 2006). Limited information is reported regarding the physiological and pathophysiological role of miR-18a; a pro-proliferative effect is reported in hepatoma cells but inhibition of proliferation was reported in breast cancer cells, providing evidence of a single miRNA exhibiting activities which depend on the cellular context (Dews et al. 2006; Hayashita et al. 2005; Liu et al. 2009b; Matsubara et al. 2007). Of relevance, miR-92 was demonstrated in murine myeloid cells to increase cellular proliferation by downregulating ΔNp63β, but in HaCaT cells was also able to repress ΔNp63α (Manni et al. 2009).

Preliminary findings from the miRNA array require further validation [Section 4.4.2.2]. A limitation of the methods employed to study the regulation of p63 by microRNAs, is that the cell lines analysed correspond to tumour samples from different individuals, which raises the possibility that other perturbations in the molecular pathway could account for the observed differences. Melanoma displays significant molecular heterogeneity indicating the need to approach results from this microRNA array with caution. One strategy that could be used to address this problem would be to analyse a cell line endogenously expressing TA and/or ΔN isoforms of p63 and compare this with the same cell line in which the isoform is silenced by RNAi technology, thus providing more specific and p63-centered results.

4.4.2.2 Future experimental validation of miR-18a

For miR-18a to repress the expression of its biological target, the co-expression of miR-18a and p63 would need to be established in melanoma cell lines using Q-PCR. Further validation of the role of miR-18a to suppress p63 would involve confirming increased expression of miR-18a in p63-null cells (Mel 224, Mel 505 and Mel 501) relative to p63-expressing cells using TaqMan Q-PCR assays. The experimental approach to confirm the interaction between miR-18a and p63 mRNA would involve cloning the 3' UTR of p63 downstream of a luciferase or green fluorescent protein open reading frame sequence containing the reporter plasmid. The recombinant plasmid and the microRNAs of interest (in this case miR-18a but also the reported microRNA regulators of p63 should also be considered) would be transiently transfected into a melanoma cell line and the luciferase or fluorescence is measured 24-48 hr after transfection.

Finally, to confirm the hypothesis that p63 is a true target of miR-18a, modulation of miRNA concentration should correspond to a predictable change in the amount of protein encoded by the target mRNA (p63). Transient overexpression of miR-18a in melanoma cell lines expressing high levels of p63 e.g. WM1158 should result in downregulation of p63 mRNA and protein. Conversely, transfection with antagomir-18a into p63-null cell lines e.g. Mel 224 should derepress the target gene p63 and increase its levels. In addition, experiments should be undertaken to determine the effect of miRNAs which were previously validated in other tissue-specific contexts [Table 4.1] on p63 protein to determine if these are relevant in a melanoma-specific context. This would involve analysing p63 mRNA and protein following transfection of these reported miRNAs e.g. miR-34a in melanoma cell lines already expressing p63 to demonstrate a downregulation. To explore the indirect effect of this miRNA [section 4.3.2.2] mediated through notch-1, downregulation of this protein would need to be established and demonstration of the reciprocal relationship with p63 expression confirmed. The converse experiments using antagomirs against miR-34a could be used to upregulate expression of p63 (via notch-1) in p63-null cell lines. These experiments would form the basis of investigations to prove that miRNA-mediated regulation of p63 expression in melanoma cells equates to altered biological function and disease progression.

4.4.3 Stability of p63 is dependent on the proteosome

Expression of p63 mRNA and protein was influenced by cell density with an optimal confluency determined to exceed 60%. This cell density was then used in all subsequent experiments described in this thesis. Since cell density had no effect on *de novo* synthesis of p63, one can speculate that the increased expression of p63 was mediated either through increased stability of the protein or inhibition of degradation. This was not addressed in this thesis and to establish this, the stability of isoforms could be determined using the cyclohexamide chase assay.

Preliminary data presented here suggest that the proteosome could partially contribute to stability of the p63 protein. This is in keeping with findings from the literature (Okada et al. 2002; Osada et al. 2001; Petitjean et al. 2008). Investigation of the most widely investigated E3-ubiquitin ligase itch, demonstrated a reciprocal pattern of expression with p63 in a small proportion of melanoma cell lines examined. To explore the relationship between itch and p63, itch was efficiently depleted using siRNA which resulted in upregulation of isoforms of p63. These data demonstrate that itch could be a negative regulator of p63 expression in a subset of melanoma cell

lines. This relationship needs to be explored in melanoma tissue samples to demonstrate *in vivo* proof. Future experiments should investigate the influence of reported E3 ubiquitin ligases e.g. WWP1, which could be involved in regulating expression of p63 in melanoma (Fomenkov et al. 2004; Fuchs et al. 2004; Li et al. 2008).

It was not possible to delineate which mechanism(s) contributed to the regulation of p63 as these were investigated in isolation. However, most significantly, the data presented thus far, suggest that regulation of expression of p63 in melanoma cell lines is a highly complex process, involving a variety of mechanisms. These would include epigenetic modulation (particularly acetylation), melanoma-specific miRNA regulation, protein stability and degradation by ubiquitin ligases. It is likely that multiple regulatory mechanisms are active in any one cell line. This reflects the heterogeneity of melanoma which is a cancer where tightly controlled regulatory mechanisms are likely to contribute to the aggressive and chemoresistant nature of the disease.

CHAPTER 5: ROLE OF TP63 IN CHEMORESISTANCE

5.1 BACKGROUND

5.1.1 Response of p63 to DNA damage

p63 exhibits several transcriptional and stress-response properties similar to those of p53 in a cell-specific manner (Petitjean et al. 2008). A differential response to genotoxic agents is exhibited by p63 isoforms, with literature to date suggesting that the apoptotic response is mediated at least in part by degradation of ΔN isoforms and stabilisation of TA isoforms. These data are predominantly based on findings reported in epidermal keratinocytes in the skin (Harmes et al. 2003; Huang et al. 2008b; Liefer et al. 2000). In cancer cells, accumulation of ectopically expressed and endogenous TA isoforms of p63 is demonstrated in leukaemic cells (Katoh et al. 2000; MacPartlin et al. 2008; Okada et al. 2002; Petitjean et al. 2005) and hepatocellular carcinoma (Petitjean et al. 2005), respectively. Moreover, TAp63 induces apoptosis in response to DNA damage (Gressner et al. 2005) and this effect

is more pronounced when there is accumulation of the isoform as a result of epigenetic modulation (Sayan et al. 2007; Sayan et al. 2009). In light of the demonstration that TAp63 accumulates in melanoma cell lines upon epigenetic modulation, **the aim of the initial section of this chapter was to determine the response of p63 isoforms to genotoxic stress, and to characterise its putative role in apoptosis.**

5.1.2 TP53 family and the mitochondrial apoptotic pathway

Although p53 and p73 have been closely linked to apoptosis, there are limited data physically connecting the other p53 family member, p63, to the mitochondrial apoptotic pathway. Exogenous transfection of TAp63α in hepatoma cells resulted in alteration of the mitochondrial membrane, induction of pro-apoptotic genes and activation of both extrinsic and intrinsic apoptotic pathways (Gressner et al. 2005), but there is no evidence thus far, demonstrating physical translocation of p63 to the mitochondria to achieve this effect.

In contrast, translocation of p53 to the mitochondria is well characterised. In response to genotoxic stress, mdm-2 mediated monoubiquitylation of p53 promotes its translocation to the mitochondria, directly inducing mitochondrial apoptosis (Marchenko et al. 2007). Upon arrival at the mitochondria, p53 undergoes de-ubiquitylation by mitochondrial HAUSP which generates the apoptotically active non-ubiquitylated p53 (Marchenko et al. 2007). Stress-induced mitochondrial p53 translocation is a rapid process, detectable within 30 mins and peaking at 2 hrs (Erster et al. 2004; Marchenko et al. 2000). In contrast, nuclear p53 export is a slow process, requiring 3 – 8 hrs (Stommel and Wahl 2004). Mitochondrially translocated p53 arises from a distinct cytoplasmic pool. Taken together, these reported findings suggest the presence of two largely independent pools of pre-existing p53 in unstressed cells, cytoplasmic and nuclear, which simultaneously respond to genotoxic stress.

5.1.2.1 Caspase cleavage of the TP53 family

Induction of apoptosis by the TP53 family members appears to depend on cleavage of proteins which alters their transcriptional activity (Sayan et al. 2008; Sayan et al. 2006; Sayan et al. 2007). p53 was the first member of the p53 family demonstrated to be a caspase target. Cleavage localised p53 fragments to the mitochondria resulting in apoptosis induction which was at least partly due to mitochondrial damage (Sayan et al. 2006). More recently, cleavage of the C-terminal TI domain of both TA and ΔN

p63α isoforms (at amino acid 458) by activated caspases directly or indirectly following treatment with UVB, staurosporine, cisplatin, etoposide and taxol was also demonstrated to induce apoptosis (Sayan et al. 2007). Cleavage of TAp63α resulted in marked increase in its transcriptional activity and increased sensitivity to apoptosis. Although the transcriptional activity of ΔNp63α was unaffected, its inhibitory effect on TAp63 isoforms was relieved. Moreover, p63 cleavage fragments demonstrated specific cellular localisation, namely the N-terminus fragments remained in the nucleus, the C-terminal fragment was largely cytoplasmic and mitochondrial localisation of either full length p63 or cleaved fragments was not demonstrated (Sayan et al. 2007). Taken together, the authors concluded that the effects of caspase cleavage of p53 are mitochondrial and non-transcriptional whereas the effects of caspase cleavage of p63 are nuclear and transcriptional. Full length p73 and caspase-cleaved p73 fragments are also able to translocate to the mitochondria (Sayan et al. 2008). Moreover, caspase-generated p53 fragments which localise to the mitochondria interact with full length p73/cleaved p73 in/on mitochondria providing a possible mechanism for p73 entry to the mitochondria (Sayan et al. 2006).

Demonstration of cytoplasmic p63 expression in melanoma tissue samples [section 3.3.5] suggests that p63 has an extranuclear role which could involve the mitochondrial apoptotic pathway. It seems plausible that if both p53 and p73 can translocate to the mitochondria then p63 should also be able to. It is possible that this is a tissue specific phenomenon and can only be demonstrated using endogenous p63 in a biological system such as melanoma. **This work therefore aims to link p63 to the mitochondrial apoptotic pathway by demonstrating its physical translocation to the mitochondria in response to genotoxic stress.**

5.1.3 TP53 family and chemosensitivity

5.1.3.1 p53 and chemosensitivity

Chemosensitivity in some cancers is dependent on the complex interactions between the three p53 family members. The role of wt-p53 as a determinant of chemosensitivity in melanoma is conflicting; in two studies melanoma cells typically expressing wt-p53 protein sensitised cells to DNA damaging agents when compared with those harbouring mutant p53 (Li et al. 2000; Li et al. 1998) whereas the p53 stabilising compound CP-31398 failed to chemosensitise either wt or mutant p53 melanoma cells suggesting that p53 is not a determinant of response to therapy.

Small molecular weight p53 isoforms (p53β, Δ40p53, Δ133p53β) have been identified and show differential subcellular localisation (nuclear and cytoplasmic) in melanoma cells (Avery-Kiejda et al. 2008). In response to cisplatin, these isoforms are upregulated and differentially alter transcription of pro-apoptotic p53 target genes providing an additional mechanism which regulates sensitivity to DNA damaging agents (Avery-Kiejda et al. 2008).

5.1.3.2 p63 and chemosensitivity in melanoma

p63 has been linked to chemosensitivity in a number of tumours; in head and neck squamous cell carcinoma, ΔNp63 was a key determinant of therapeutic response (Rocco et al. 2006) and expression correlated with clinical response to cisplatin (Zangen et al. 2005); in breast cancer, p63 expression positively correlated with chemosensitivity to cisplatin (Rocca et al. 2008); in hepatoma cells, transfection of TAp63α sensitised cell lines to chemotherapy and transient depletion led to chemoresistance (Gressner et al. 2005). Significant expression of p63 has not previously been demonstrated in melanoma and therefore correlation with chemosensitivity in this cancer has not been investigated.

5.1.3.3 p73 and chemosensitivity in melanoma

The third p53 family member, p73, is essential for apoptosis induced by various chemotherapeutic agents (summarised in Muller M 2007). ΔNp73 interacts with mutant p53 and/or TAp73 and confers chemoresistance in squamous cancers (Bergamaschi et al. 2003a; Gasco and Crook 2003; Irwin 2004; Meier et al. 2006; Muller et al. 2005; Strano and Blandino 2003). Upregulation of ΔNp73 has been demonstrated in metastatic melanoma. The balance between ΔNp73 and TAp73 is a factor determining chemosensitivity in melanoma and adenoviral expression of TAp73β sensitised cells to chemotherapies (Tuve et al. 2006), although there are no findings reporting a role for other p73 isoforms.

5.1.3.4 Interactions within the p53 family

In addition, there is intense debate on whether, and how, p63 (and p73) interact with p53 in apoptosis and tumour suppression (Benchimol 2004). Several complementary regulatory loops exist between p53 family members that provide tight control over p63 activities. Induction of ΔNp73 expression by TAp63 isoforms was demonstrated in hepatocellular carcinoma cells (Petitjean et al. 2008). ΔNp73 has been demonstrated to be a transcriptional target of TAp63α and γ, (and p53 and TAp73) contributing to the fine balance of transcriptional activities of p63 (Kartasheva et al.

2002; Lanza et al. 2006). ΔNp73 has also been demonstrated to negatively regulate TAp63γ transcriptional activity (Grob et al. 2001; Kartasheva et al. 2002). Co-operativity among the three family members has been reported in mouse embryo fibroblasts and in primary neuronal cultures (Flores et al. 2002) but in thymocytes and hepatocellular carcinoma, p53-dependent apoptosis occurs independently of p63 and p73 (Petitjean et al. 2005; Senoo et al. 2004). More recently, chemotherapy was shown to engage all three members of the p53 family and promote cell death in response to DNA damage via both intrinsic and extrinsic apoptotic signalling pathways (Seitz et al. 2009).

5.1.4 Chemosensitivity testing in melanoma

Dacarbazine is currently the standard treatment regimen in metastatic melanoma (Roberts et al. 2002), with reported response rates of up to 20% (Schadendorf et al. 2006; Therasse et al. 2000). Taxane-based regimens are considered for second-line chemotherapy in melanoma (Gogas et al. 2004b; Hodi et al. 2002; Legha et al. 1990; Rao et al. 2006; Ugurel et al. 2006). Higher response rates (12 – 41%) were observed when taxanes were given in association with other agents, such as dacarbazine, cisplatin or carboplatin (Bafaloukos et al. 2002; Hodi et al. 2002; Rao et al. 2006; Ugurel et al. 2006). Platinum compounds have shown modest activity as single agents in patients with metastatic melanoma (Atkins 1997; Glover et al. 2003; Mortimer et al. 1990).

Melanoma demonstrates notorious chemoresistance when compared with other solid cancers (Soengas and Lowe 2003) and an incomplete picture exists for the role of the p53 family as determinants of chemosensitivity. **This thesis has provided the first evidence for the expression of p63 isoforms in melanoma and proposes a role for p63 in determining the chemosensitivity of melanoma. This will be explored in this chapter through depletion of endogenous p63 and determining the effect on sensitivity to standard chemotherapeutic agents.**

5.1.5 A putative role for p63 in melanoma stem cells

The chemo- and radio-resistance of melanoma and its high recurrence rates are related to the genetic heterogeneity and genomic instability of the tumour. Over the last few years, a subpopulation of cells – cancer stem cells – have been demonstrated in melanoma and are considered to be the source of the primary tumour mass, of tumour recurrence and metastases and deemed responsible for drug resistance. Although mounting evidence supports the existence of cancer stem

cells, their characterisation is limited by difficulties related to identifying and separating these relatively rare cells. To date, markers used to distinguish cancer stem cells from other tumour cells are predominantly based on knowledge of embryonic stem cell surface markers or cancer drug resistance markers. Assuming that cancer stem cells have significantly different biological properties compared with the other cancer cells, the poor efficacy of current therapies can be explained because the majority were developed by testing activity against the bulk of cancer cells which might not be the cells that drive tumour growth.

In melanoma, CD133 is a putative marker of melanoma stem cells (Frank et al. 2005; Frank et al. 2003; Klein et al. 2007; Monzani et al. 2007) which is differentially expressed in a subset of melanoma cell lines tested in this thesis. The findings from the work presented in chapter 3, demonstrated enriched expression of ΔNp63 (deemed to confer stemness in keratinocytes) in the CD133-positive population of melanoma cells [section 3.3.6.2]. **The work undertaken in this chapter aims to explore a link between the co-expression of p63 and CD133 by investigating the function of this isoform of p63 within the CD133-positive population.**

5.2 AIMS

This part of the thesis hypothesises that p63 may at least in part, account for the dysregulation of the p53 apoptotic pathway and, as a consequence, contribute to the chemoresistance exhibited by melanoma. The aims were:

1) To explore the response of p63 to DNA damage in melanoma cell lines
2) To investigate the role of p63 in chemotherapeutic-induced apoptosis
3) To determine the relationship between p63 and p53 in response to DNA damage in melanoma cells
4) To determine the contribution of p63 to chemoresistance of melanoma cells
5) To explore the role of the ΔNp63 isoform in CD133-positive melanoma cells

5.3 RESULTS

5.3.1 TP63 is responsive to DNA damaging agents in melanoma

5.3.1.1 TP63 mRNA is upregulated upon DNA damage

Melanoma cell lines were treated with various chemotherapeutic agents and RNA was extracted at various time-points (range 1.5 hrs to 48 hrs) [section 2.2.3]. Upon treatment, TP63 mRNA was not upregulated in primary melanocyte cultures (NHEM, Hema 3, V3 or V4 – data not shown). Upregulation of both TA and to a lesser extent, ΔN isoforms of TP63 mRNA was demonstrated in melanoma cell lines expressing endogenous p63 and those that did not [Figure 5.1]. Maximal upregulation was observed upon treatment with doxorubicin and paclitaxel, although marked stabilisation of p63 isoforms was also demonstrated upon treatment with cisplatin and etoposide. No upregulation of TP63 was observed with dacarbazine in any of the melanoma cell lines tested.

In addition, stabilisation of other p53 family members, TP53 and ΔNp73 in response to genotoxic agents was investigated using RT-PCR. The response to different chemotherapeutic agents was assessed in melanoma cell lines expressing wt-p53 e.g. A375M, and the p53-null cell line U1SO [Figure 5.1C]. Both TA and ΔNp63 were induced by all drugs, despite endogenous expression of only one isoform. No TP53 was detected in the U1SO cells (confirming p53-null status), and marked stabilisation of TP53 was not demonstrated in A375M cells. ΔNp73, previously reported to contribute to chemoresistance in melanoma (Tuve et al. 2006) was assessed in the same melanoma cell lines prior to undertaking functional experiments. ΔNp73 was not observed in treated or untreated U1SO cells, but marked upregulation was observed in A375M cells upon treatment with cisplatin, etoposide and doxorubicin but not with paclitaxel [Figure 5.1C]. These data demonstrate the complex interplay between the p53 family members in response to different chemotherapeutic agents which could be cell line-dependent, but also provide a basis for further analysis of p63 protein induction in melanoma cell lines.

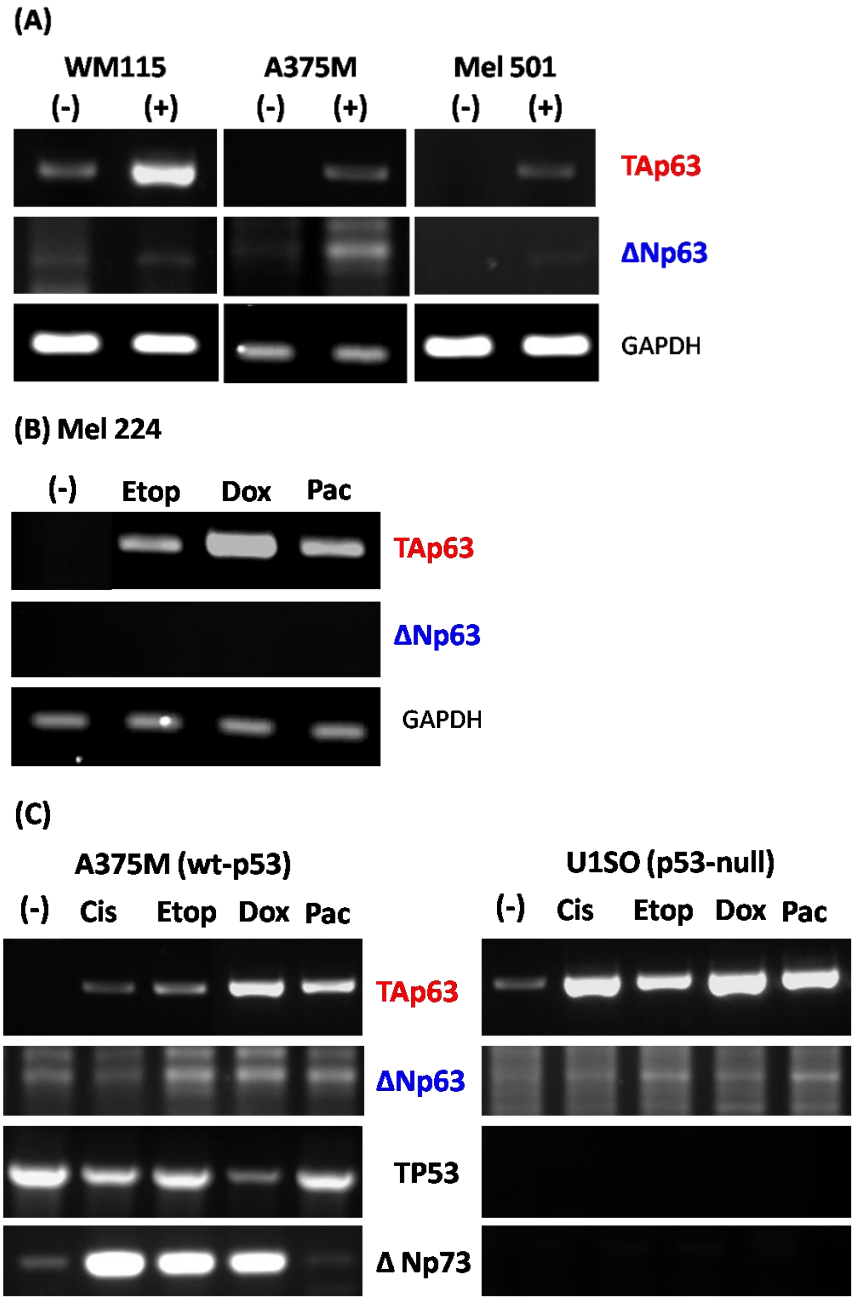


Figure 5.1: TP63 is stabilised in response to DNA damage. (A) TAp63, and to a lesser extent ΔNp63, was upregulated in primary (WM115) and metastatic (A375M and Mel 501) melanoma cell lines, upon treatment with cisplatin (10 μM) for 24 hrs. (B) In melanoma cell lines not expressing endogenous p63 (Mel 224 shown here), upregulation of TAp63 was consistently observed. GAPDH was the housekeeping gene used for mRNA standardisation. (C) Stabilisation of TAp63 (and to a lesser degree, ΔNp63) in response to various chemotherapeutic agents (cisplatin and etoposide 10 μM, doxorubicin and paclitaxel 1 μM) occurs independently of p53 status of melanoma cells. Stabilisation of wt-TP53 mRNA is not consistent although ΔNp73 upregulation was observed in A375M cells. No stabilisation of either protein was observed in U1SO cells. Data shown is representative of four independent experiments. Cis – cisplatin, Etop – etoposide, Dox – doxorubicin, Pac – paclitaxel.

5.3.1.2 p63 protein is stabilised upon DNA damage in melanoma

The response of p63 to DNA damage in keratinocytes is well established; the predominant isoform endogenously expressed in keratinocytes (and HaCaT cells) ΔNp63, a nuclear protein, upon UVB treatment is downregulated which is deemed necessary for epidermal apoptosis (Harmes et al. 2003; Huang et al. 2008b; Liefer et al. 2000). Overexpression of TAp63α in keratinocytes (under physiological conditions expressed at low levels) also significantly prevents apoptosis in UVB irradiated keratinocytes (Ogawa et al. 2008), despite the fact that it can induce apoptosis in other cell systems (Gressner et al. 2005; Yang et al. 1998). Taken together, the role of p63 in keratinocytes is to protect against UVB induced damage.

To confirm the specificity of p63 antibodies and experimental conditions for immunofluorescence microscopy, initial experiments were undertaken in HaCaT cells. In keeping with the literature (Liefer et al. 2000), characteristic downregulation of p63 was observed [Figure 5.2]. To evaluate the response of p63 to DNA damage in the melanocyte lineage, primary melanocyte cultures (Hema V3) were treated with UVB radiation and various chemotherapeutic agents (cisplatin, etoposide, doxorubicin, dacarbazine) from 6-48 hrs. No stabilisation or re-activation of p63 was observed in melanocyte cultures despite induction of DNA damage and induction of an apoptotic pathway, as demonstrated by positive γ-H2AX and cleaved caspase staining, respectively [Figure 5.3]. These data were confirmed in another primary melanocyte culture (Hema V4) upon treatment with the same chemotherapeutic agents (data not shown).

Experiments were extended to include melanoma cell lines which endogenously expressed TP63. Expression of p63 was predominantly nuclear but also cytoplasmic and upon DNA damage, upregulation of p63 was observed in both compartments, with stabilisation occurring as early as 2 hrs, and in some cell lines persisting for 48 hrs [Figure 5.4]. This effect was observed in more than one cell line suggesting that this was a general phenomenon in melanoma cells [Figure 5.5: example of WM1158 cells].

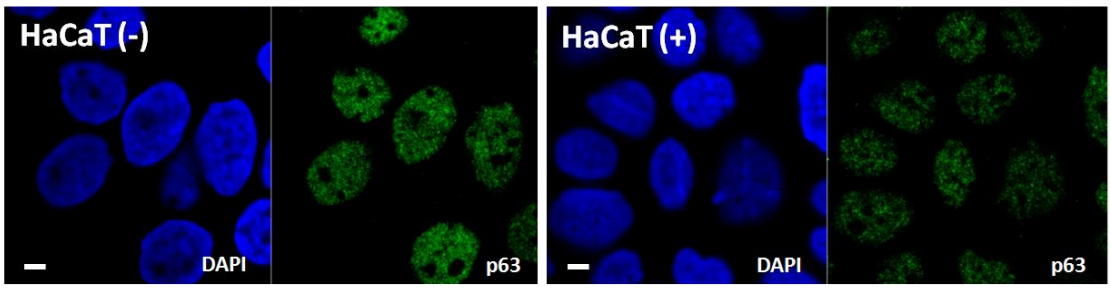


Figure 5.2: Downregulation of p63 in response to UVB in HaCaT cells (IMF). In untreated cultures (-), p63 is a nuclear, non-nucleolar protein. Upon treatment with UVB (50 mJ/cm^2 for 6 hrs) downregulation of p63 was observed, confirming specificity of the anti-p63 antibodies (H129 and H137) and optimisation of immunostaining technique. DAPI was used to label nuclei (blue). Images are representative of three independent experiments. Bar 20 µm.

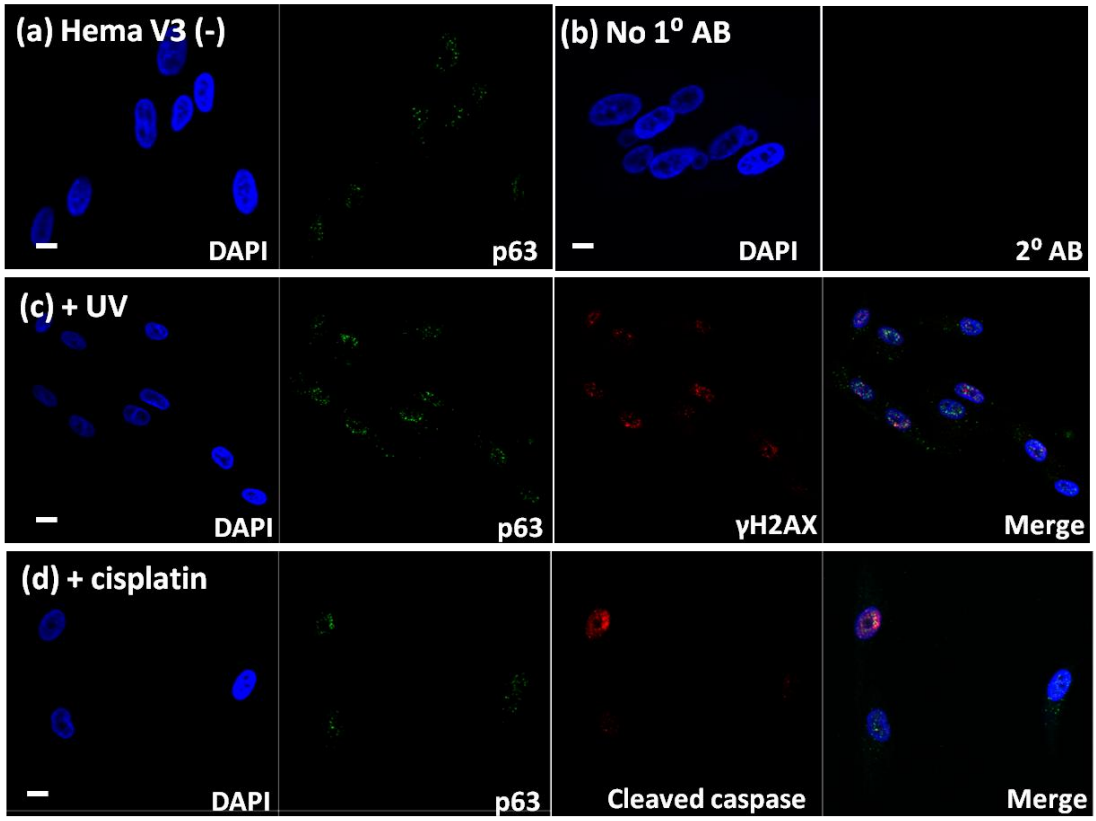


Figure 5.3: p63 is not induced by DNA damage in primary melanocyte cultures (IMF). (a) Untreated (-) Hema V3 melanocytes express almost no endogenous p63 protein. (b) Omission of primary antibody confirms specificity of anti-p63 antibodies (H129, H137 in combination). (c) Hema V3 cells treated with UVB (50 mJ/cm^2 for 24 hrs) showing evidence of induction of DNA damage confirmed by γH2AX staining (red) but no stabilisation of p63 (green). (d) Upon treatment with cisplatin (10 µM for 24 hrs), no upregulation of p63 was observed in Hema V3 cells despite evidence of apoptosis induction by positive cleaved caspase staining (red). DAPI was used to stain nuclei (blue). Images are representative of three independent experiments. Bar – 10 µm.

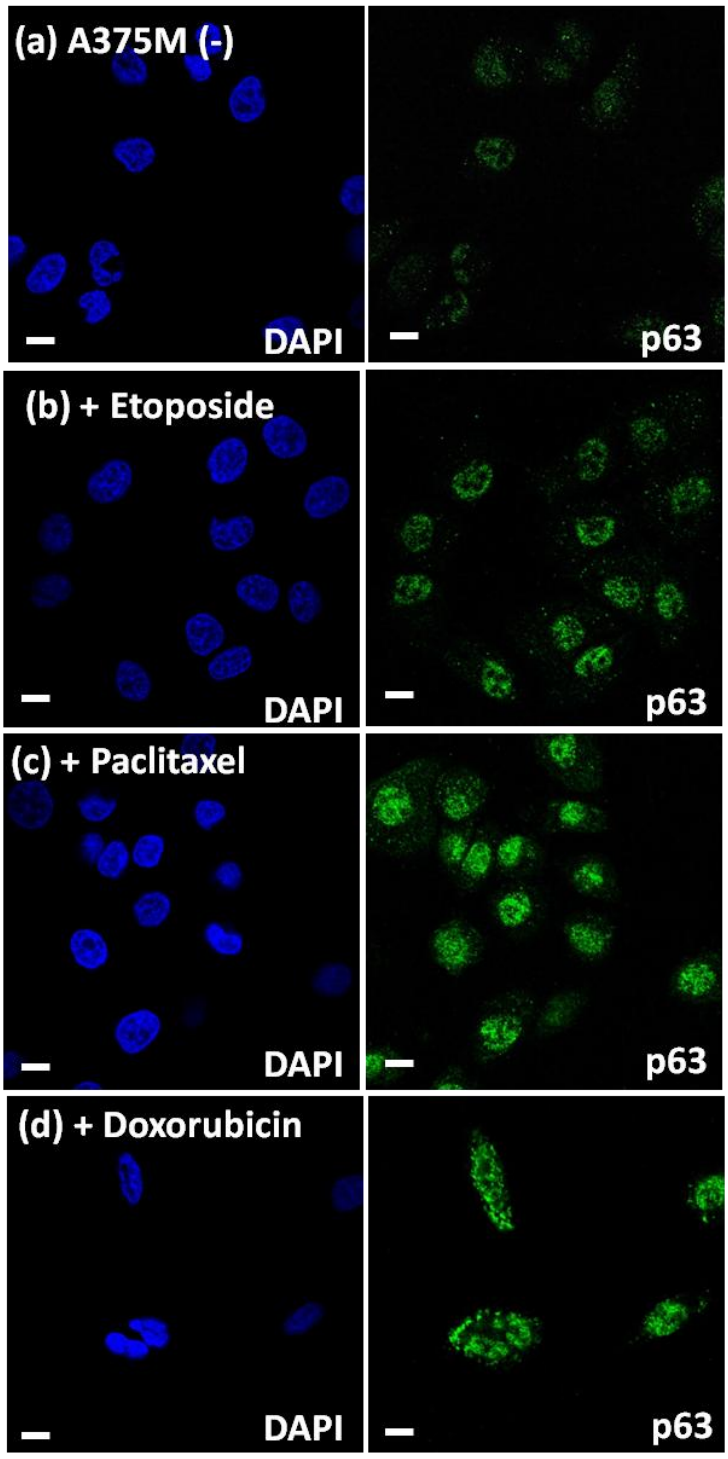


Figure 5.4: Stabilisation of p63 protein in A375M cells upon genotoxic stress (IMF). (a) Untreated A375M cells (-) have low levels of endogenous p63 (detected by anti-p63 antibodies H129 and H137) which is largely nuclear. Significant upregulation of p63 (green) in both nuclear and cytoplasmic compartments of A375M cells was observed upon treatment with (b) etoposide (20 µM) (c) paclitaxel (2 µM) and (d) doxorubicin (2 µM) for 6 hrs. DAPI was used to stain nuclei (blue). Images are representative of three independent experiments. Bar 10 µm.

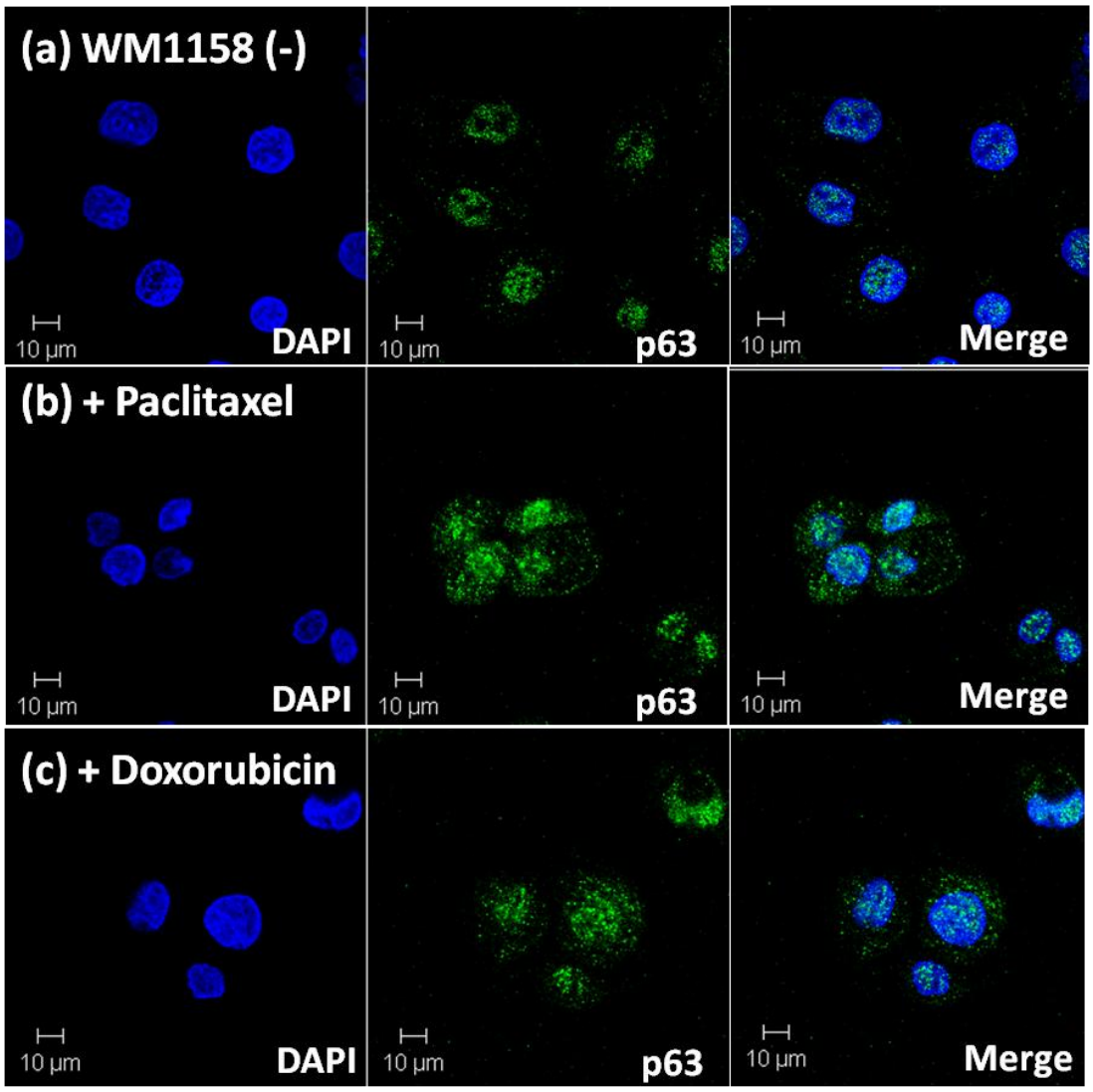


Figure 5.5: Stabilisation of p63 protein upon DNA damage (IMF – WM1158 cells). (a) Untreated WM1158 cells (-) express predominantly nuclear endogenous p63 (using anti-p63 antibodies H129, H137). Upon treatment with (b) paclitaxel (2 µM) and (c) doxorubicin (2 µM) for 6 hrs, p63 (green) is efficiently stabilised in both nuclear and cytoplasmic compartments. DAPI was used to stain nuclei (blue). Images are representative of three independent experiments.

5.3.1.3 DNA damage induces differential stabilisation of p63 protein isoforms

To further investigate the response of p63 protein to genotoxic stress, Western blotting analysis using the anti-p63 antibody AB-4 (which detects all isoforms of p63) was undertaken. GAPDH was used as a loading control. Despite recent reports that GAPDH participates in oxidative stress-induced cell death in neuroblastoma cells (Nakajima et al. 2009), different loading controls including α-tubulin were used as a comparison, consistently demonstrating stability of the GAPDH protein in melanoma cell lines treated with different chemotherapeutic agents. Treatment with UVB radiation and chemotherapeutic agents (cisplatin, etoposide, doxorubicin) resulted in a differential stabilisation of p63 splice variants in melanoma cell lines [Figure 5.6]. Upregulation corresponded at least in part, to induction of apoptosis, determined by cleavage of poly (ADP ribose) polymerase (PARP) [Figure 5.6]. The pattern of stabilisation of isoforms varied between cell lines. A number of slower migrating bands were consistently observed and could be explained by poor specificity of the antibody, post-translational modification of smaller isoforms of p63 or cleaved products of p63 (Sayan et al. 2007). The recent discovery of new isoforms of p63 (Mangiulli et al. 2009) also raises the possibility that these bands are p63δ or p63ε isoforms but one can only speculate this since exogenously expressed plasmids for these isoforms in a p63-null cell line were not available for confirmation.

5.3.2 p53 protein is stabilised in response to DNA damage

To determine the response of p53 protein in response to DNA damaging agents, Western blotting analysis was carried out using the anti-p53 antibody DO-1. p53 was upregulated in response to genotoxic stress in melanoma cell lines. A dose- and time- dependent upregulation was observed in 3/6 melanoma cell lines examined although this was not consistent (data not shown). These data confirm stabilisation of p53 protein occurs in response to chemotherapeutic agents, in keeping with previous reports (Satyamoorthy et al. 2000) but did not delineate a relationship between p63 and p53 or uncover their contribution to chemoresistance in melanoma cells.

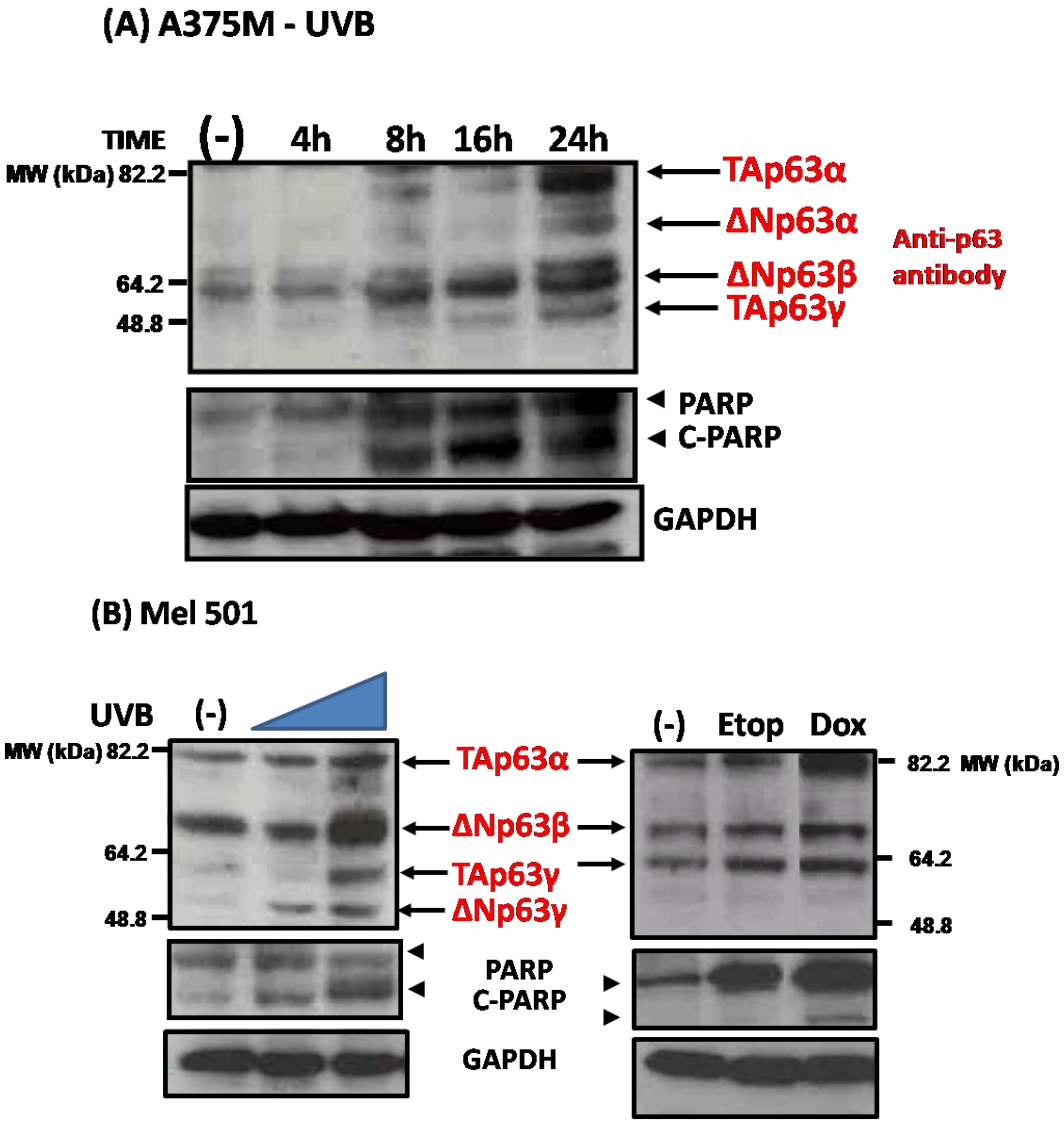


Figure 5.6: Upregulation of p63 isoforms in response to DNA damage (WB). (A) A375M cells were treated with UVB (50 mJ/cm^2) and collected for analysis at increasing time-points demonstrating upregulation of several isoforms of p63 (detected using anti-p63 antibody AB-1). (B) Stabilisation of p63 isoforms was demonstrated in Mel 501 cells upon treatment with various genotoxic agents (UVB 25 and 50 mJ/cm^2; etoposide 10 µM and doxorubicin 1 µM) for 24 hrs. For both cell lines, upregulation of p63 was partially associated with induction of apoptosis as demonstrated by cleavage of PARP (c-PARP). A number of slower migrating bands were observed and possible explanations are discussed in section 5.3.1.3 including poor antibody specificity, post-translational modification of small isoforms of p63, cleaved products of p63 (Sayan et al. 2007), or recently discovered new p63 delta or epsilon isoforms (Mangiulli et al. 2009). GAPDH was used as a loading control. Molecular weight of standardised protein ladder shown (kDa). Western blots shown are representative of three independent experiments performed in four different melanoma cell lines (A375M, WM793, Mel 501, WM1158). UVB – ultraviolet B radiation, Etop – etoposide, Dox – doxorubicin.

5.3.3 Translocation of p63 upon DNA damage

5.3.3.1 p63 demonstrates aberrant extranuclear expression

Stabilisation of p63 within the cytoplasmic compartment of melanoma cell lines raised the question of relocation of p63 to different subcellular compartments. To investigate this, a number of subcellular markers were used; α-tubulin (cytoskeleton), calnexin (endoplasmic reticulum), GM130 (Golgi body), Lamp1 (lysosomes), MitoTracker Orange and mtHsp70 (mitochondria). A375M cells were treated with chemotherapeutic agents for 24 hrs prior to fixing [section 2.7.1]. In untreated A375M cells, endogenous p63 protein showed no obvious co-localisation with any of the cellular markers. Upon treatment with paclitaxel or doxorubicin, no co-localisation with α-tubulin [Figure 5.7] or LAMP1 was observed (data not shown). In contrast, partial co-localisation of calnexin and p63 was observed in treated A375M cells [Figure 5.7]. However, organisation of the endoplasmic reticulum (ER) in treated cells appeared significantly different to untreated cells i.e. ER co-localisation with the nuclei was observed, in keeping with reports of re-organisation of the ER and Golgi body demonstrated to occur as part of the ER-stress response (Corazzari et al. 2007). Moreover, cleavage of calnexin is reported, which alters its structure and possibly localisation (Takizawa et al. 2004) suggesting that the co-localisation of p63 with calnexin is unlikely to be genuine.

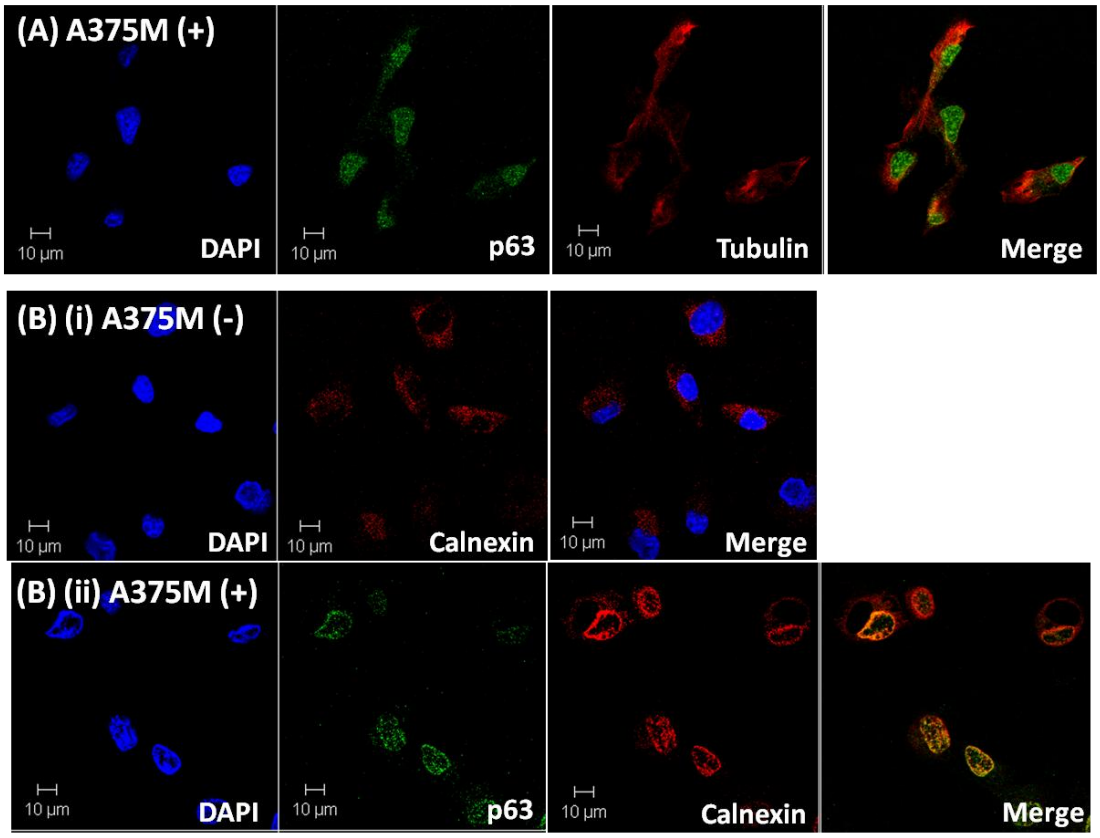


Figure 5.7: Subcellular compartment labelling in A375M cells. (A) Upon treatment with paclitaxel (2 µM), p63 protein (green) stabilises in the cytoplasm but does not co-localise with tubulin (red). (B) (i) In untreated A375M cells (-) calnexin (red), a marker of the endoplasmic reticulum (ER) demonstrates no co-localisation with the nuclei. (ii) A375M cells treated with paclitaxel (2 µM) demonstrates stabilisation of p63. Re-organisation of the ER as shown by calnexin (red), appears to co-localise with both p63 (green) and the nuclei (blue) demonstrating organisation of the ER in response to stress. Similar findings were observed when cells were treated with doxorubicin (2 µM) (data not shown). DAPI was used to label nuclei (blue). Images are representative of three independent experiments.

5.3.3.2 Upon DNA damage p63 relocates to the mitochondrial compartment

To investigate a connection between stabilisation of extranuclear p63 and induction of the intrinsic apoptotic pathway, relocation of p63 to the mitochondrial compartment was examined. This was considered in light of evidence that a fraction of wt-p53 translocates to the mitochondria in response to various apoptotic stimuli (Marchenko et al. 2000; Mihara and Moll 2003; Sansome et al. 2001), and more recent reports that p73 is also capable of translocation to the mitochondria (Sayan et al. 2008).

For direct visualisation of p63, cells were incubated with MitoTracker Orange (for mitochondrial labelling) prior to treatment with various DNA damaging agents. Cells were fixed at various time-points, fluorescently stained using anti-p63 antibodies (either H127 and H139 in combination or using 4A4) and subsequently slides were visualised using confocal microscopy [section 2.7.1]. Figure 5.8 shows subcellular localisation of p63 in untreated WM1158 cells and those treated with paclitaxel and doxorubicin at different time-points. The merged image in untreated cells demonstrates localisation of p63 largely confined to the nucleus [Figure 5.8] but following treatment with paclitaxel (6 hrs) there is stabilisation of p63 (shown by a greater intensity of green) in the cytoplasm and more specifically in the mitochondria [Figure 5.8]. A more pronounced effect was demonstrated upon treatment with doxorubicin (1 μM) persisting up to 24 hrs. Dose-dependent stabilisation of p63 was demonstrated upon treatment with cisplatin (10–30 μM) or etoposide (10–30 μM) (data not shown) resulting in greater translocation of p63 to the mitochondria, without induction of cell death.

5.3.3.3 Partial relocation of p63 to the mitochondria is a rapid and persistent process

Extending these observations to other cell lines, e.g. A375M cells, revealed a similar phenomenon showing translocation of p63 to the mitochondria occurring as early as 2 hrs following treatment with paclitaxel (2 μM) and demonstrated to persist up to 24 hrs [Figure 5.9]. Taken together, these data confirm that p63 is upregulated in response to genotoxic stress and is localised to both nuclei and mitochondria. The physical localisation of p63 to the mitochondria has previously never been reported but appears to be a general phenomenon occurring in melanoma cell lines.

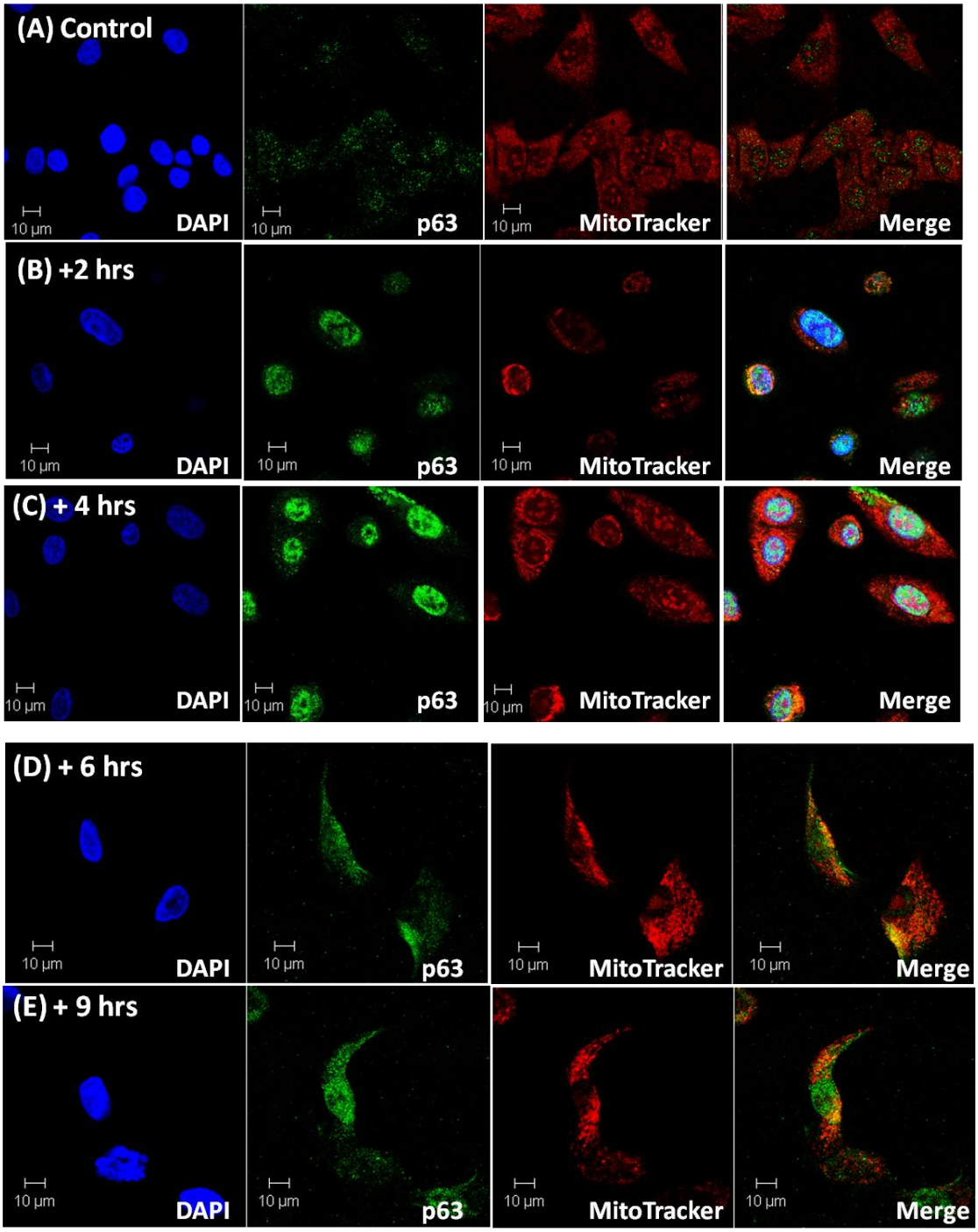


Figure 5.8: p63 relocates to the mitochondria upon genotoxic stress (IMF). A375M cells were labelled with MitoTracker Orange (red) prior to treatment with paclitaxel (2 µM) and fixed at various time-points. p63 is largely nuclear in (A) untreated A375M cells and there is stabilisation in both nuclear and cytoplasmic compartments at increasing time-points: (B) 2 hrs, (C) 4 hrs, (D) 6 hrs and (E) 9 hrs. Merged images (far right panels) demonstrate significant co-localisation (yellow) of p63 (green) and MitoTracker Orange (red) confirming p63 relocates to the mitochondria in response to genotoxic stress as early as 2 hrs. DAPI was used to label nuclei (blue). Images are representative of three independent experiments.

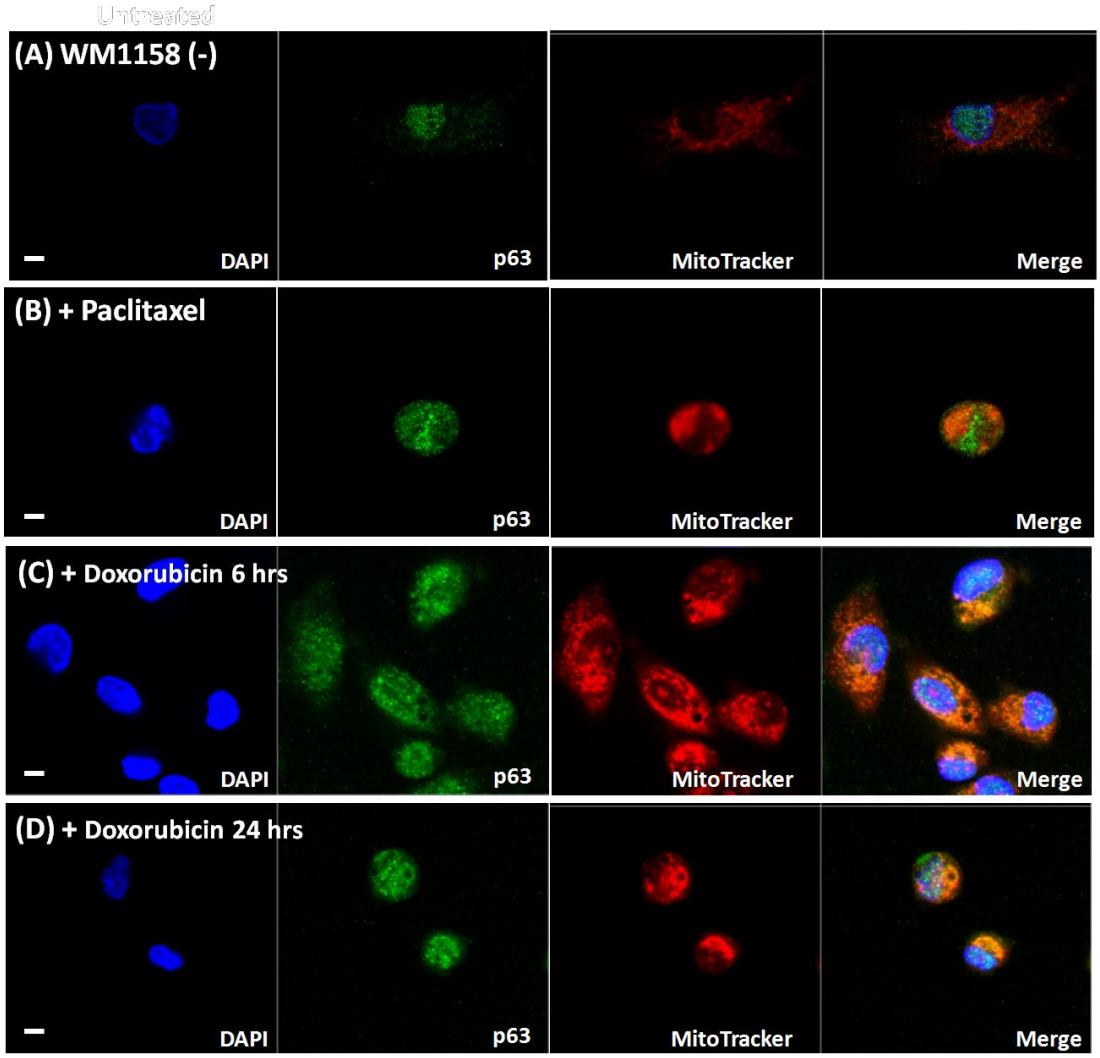


Figure 5.9: Relocation of p63 to the mitochondria is a general phenomenon (IMF). WM1158 cells were labelled with MitoTracker Orange (red) prior to treatment with (B) paclitaxel (2 µM) and doxorubicin (1 µM) and fixed for immunofluorescence staining at (C) 6 hrs and (D) 24 hrs. p63 is largely nuclear in (A) untreated WM1158 cells (-) and there is stabilisation in both nuclear and cytoplasmic compartments upon treatment with different chemotherapeutic agents at increasing time-points. Merged images (far right panels) demonstrate significant co-localisation (yellow) of p63 (green) and MitoTracker Orange (red), confirming p63 relocates to the mitochondria in response to genotoxic stress in WM1158 cells. DAPI was used to label nuclei (blue). Images are representative of three independent experiments. Bar 10 µm.

5.3.3.4 Upon genotoxic stress p63 translocates to the mitochondrial membrane and within the matrix

Having established that p63 was demonstrated to localise to the mitochondria, it was necessary to determine whether p63 was localised on/around the mitochondrial membranes or within the mitochondrial matrix. To achieve this, immunogold localisation of p63 was assessed using transmission electron microscopy [section 2.8.2]. Treatment with paclitaxel was chosen as this consistently resulted in upregulation of p63. A time-point of 6 hrs was chosen based on immunofluorescence microscopy experiments that demonstrated significant upregulation of p63 within this time-frame in the absence of cell death. The primary antibodies used to detect p63 were anti-p63; H129, H137, combination of H129 and H137 and 4A4 [Table 2.17]. Because MitoTracker Orange could not be used to label the mitochondria in transmission electron microscopy, an alternative marker for mitochondria – mitochondrial heat shock protein-70 (mtHsp70) was used. To confirm that the anti-mtHsp70 antibody was a specific mitochondrial marker, cells were fluorescently labelled with MitoTracker Orange prior to fixation. Cells were indirectly labelled with anti-mtHsp70 antibody and Alexa-488 conjugated secondary antibody [section 2.7.1]. Confocal microscopy demonstrated good co-localisation between MitoTracker Orange and mtHsp70, suggesting that mtHsp70 would be a valid marker for mitochondria for use in transmission electron microscopy experiments [Figure 5.10].

Treated A375M and WM1158 cells were pelleted, fixed and processed for transmission electron microscopy [section 2.8.2]. Ultrastructurally mitochondrion is an organelle formed by a peripheral and inner membrane. The peripheral membrane encloses the entire contents of the mitochondrion, and the internal membrane forms a series of cristae (folds) which project inwards towards the interior space of the organelle. The area enclosed by the inner membrane is the mitochondrial matrix. In the classic electron micrograph, mitochondria appear as solitary and individual organelles. However recent evidence supports a mitochondrial network whereby mitochondria display large, elongated and branched structures (Benard et al. 2007; Legros et al. 2004) extending throughout the cytosol (Amchenkova et al. 1988). The mitochondrial morphology is continuously modified by functional requirements to adapt to different cell demands. Mitochondria can exhibit continuous shape changes such as branching, bending and retractions, a change in shape or increase in number of cristae and may fuse or increase in size to form giant mitochondria. In cancers, mitochondria can display considerable heterogeneity within a single cell (Arismendi-Morillo 2009; Collins and Bootman 2003).

Mitochondria were identified by their characteristic ultrastructure and confirmed by the presence of immunogold particles secondary to anti-mtHsp70 antibody [Figures 5.11, 5.12]. Mitochondria demonstrated considerable heterogeneity within a single melanoma cell, in keeping with reported findings (Collins and Bootman 2003). Upon treatment with paclitaxel, immunogold particles of p63 were demonstrated in mitochondria of both cell lines using the various anti-p63 antibodies [Figures 5.11, 5.12]. Immunogold particles of p63 were also demonstrated in the nucleus supporting the immunofluorescence microscopy data confirming stabilisation of p63 in this compartment [section 5.3.3.2]. Furthermore there was no association of the immunogold particles with other subcellular structures identified by their characteristic ultrastructure e.g. the Golgi body [Figure 5.11]. Negative controls were visualised by excluding the primary antibody [Figures 5.11, 5.12].

Taken together, transmission electron microscopy experiments confirm localisation of p63 within the mitochondrial matrix, the cytoplasm and the nucleus of melanoma cell lines, upon treatment with paclitaxel. This suggests that upon genotoxic stress, p63 is able to translocate between the three compartments to effect its function.

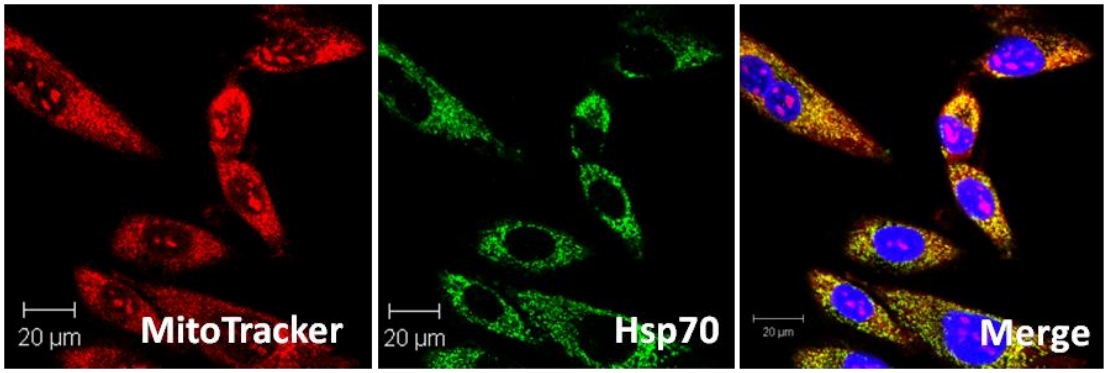
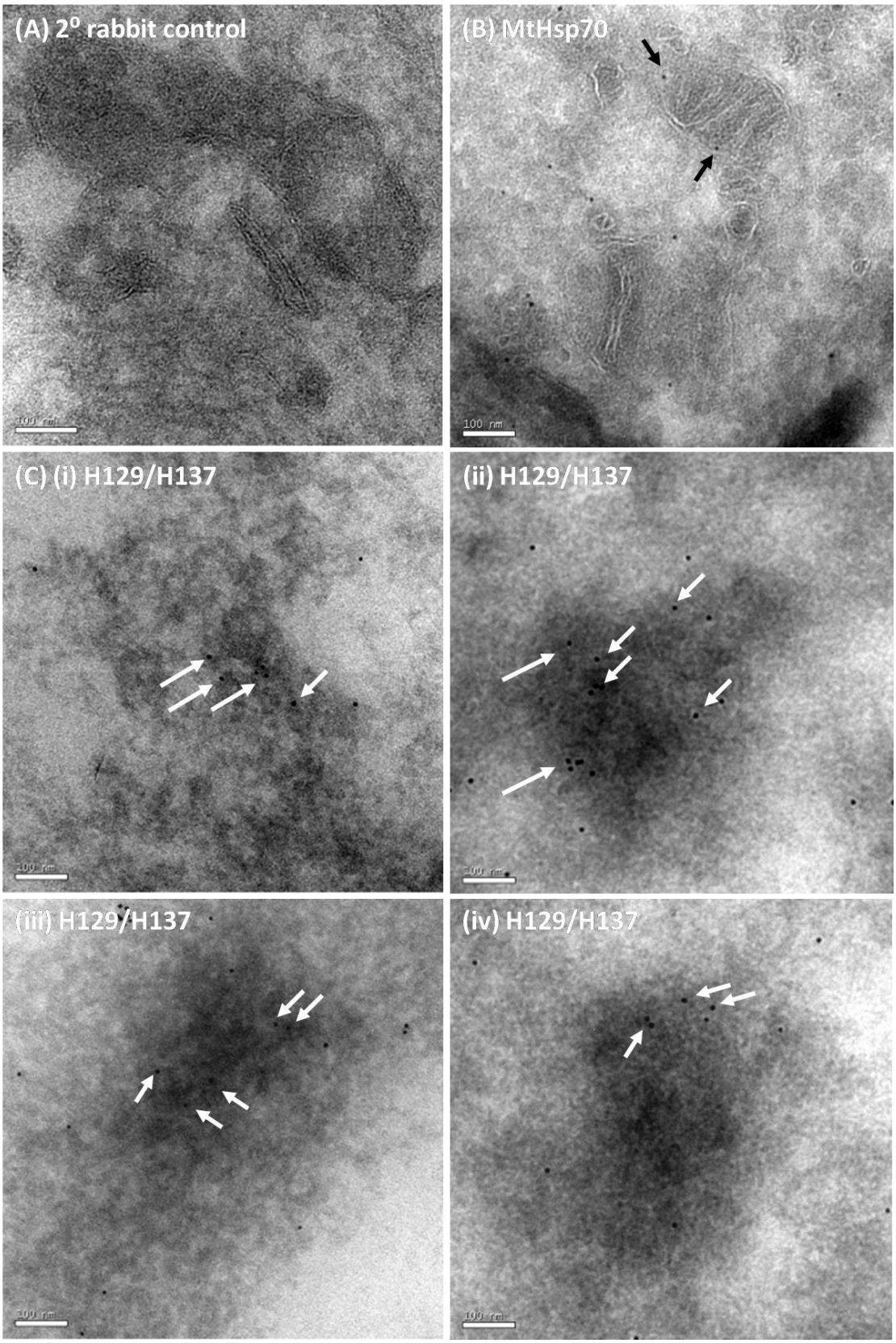


Figure 5.10: Hsp70 is a valid mitochondrial marker (IMF). A375M cells were labelled with MitoTracker Orange (red) to label mitochondria prior to fixation and immunostaining using anti-mtHsp70 antibody (green). Merged image (far right panel) demonstrates co-localisation (yellow) of Hsp70 with MitoTracker Orange, demonstrating the validity of using mtHsp70 as a marker for mitochondria in electron transmission microscopy experiments. Images are representative of three independent experiments.


(A) 2° rabbit control
(B) MtHsp70
(C) (i) H129/H137
(ii) H129/H137
(iii) H129/H137
(iv) H129/H137

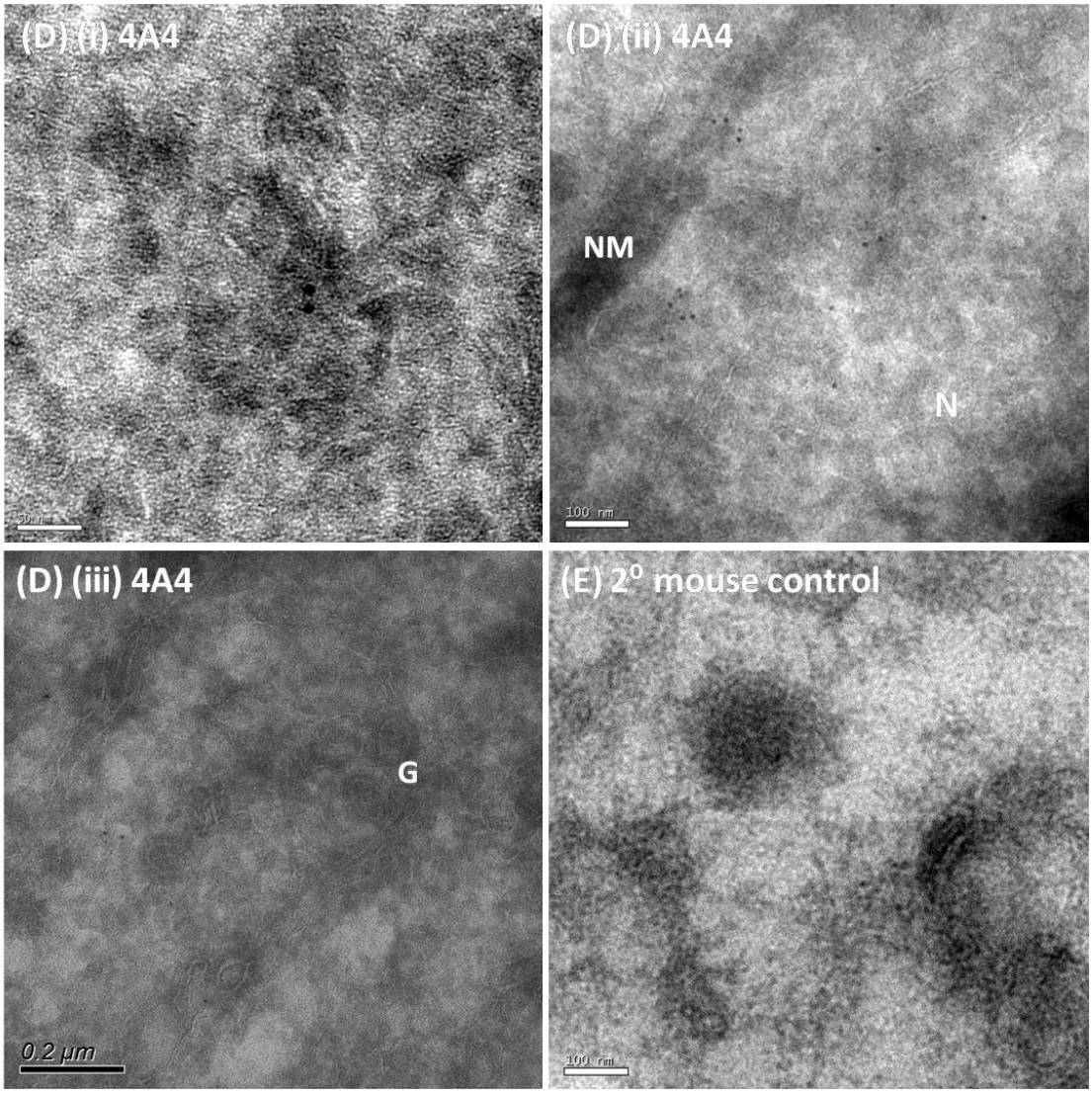


Figure 5.11: Transmission electron micrographs of immunogold labelling of p63 in mitochondria (A375M cells). (A) Mitochondria characterised by double membrane and cristae projections. Negative control comprises exclusion of primary antibody, to confirm specificity. Bar – 100 nm (B) Mitochondrion confirmed by electron dense matrix surrounded by double membrane with cristae projections showing immunogold labelled-mtHsp70 localisation within the mitochondrial matrix (black arrows), bar – 100 nm (C) (i-iv) Electron dense mitochondria characterised by remarkably electron dense mitochondrial matrix and cristae. Images demonstrate immunogold localisation of p63 (using combination of anti-p63 antibodies H129 and H137) in cytosol, on mitochondrial membranes and within mitochondrial matrix (white arrows), bar (i) and (iii) 100 nm, (ii) and (iv) – 200 nm (D) Electron micrographs showing immunogold labelling of p63 (using 4A4 anti-p63 antibody) in (i) electron dense mitochondrion matrix, bar – 50 nm (ii) nucleus labelled N, nuclear membrane denoted NM, bar – 100 nm and (iii) no p63 localised to the Golgi apparatus (labelled G), bar – 0.2 µm. (E) Negative control for mouse primary antibodies using only secondary anti-mouse antibody to confirm specificity, bar – 100 nm.

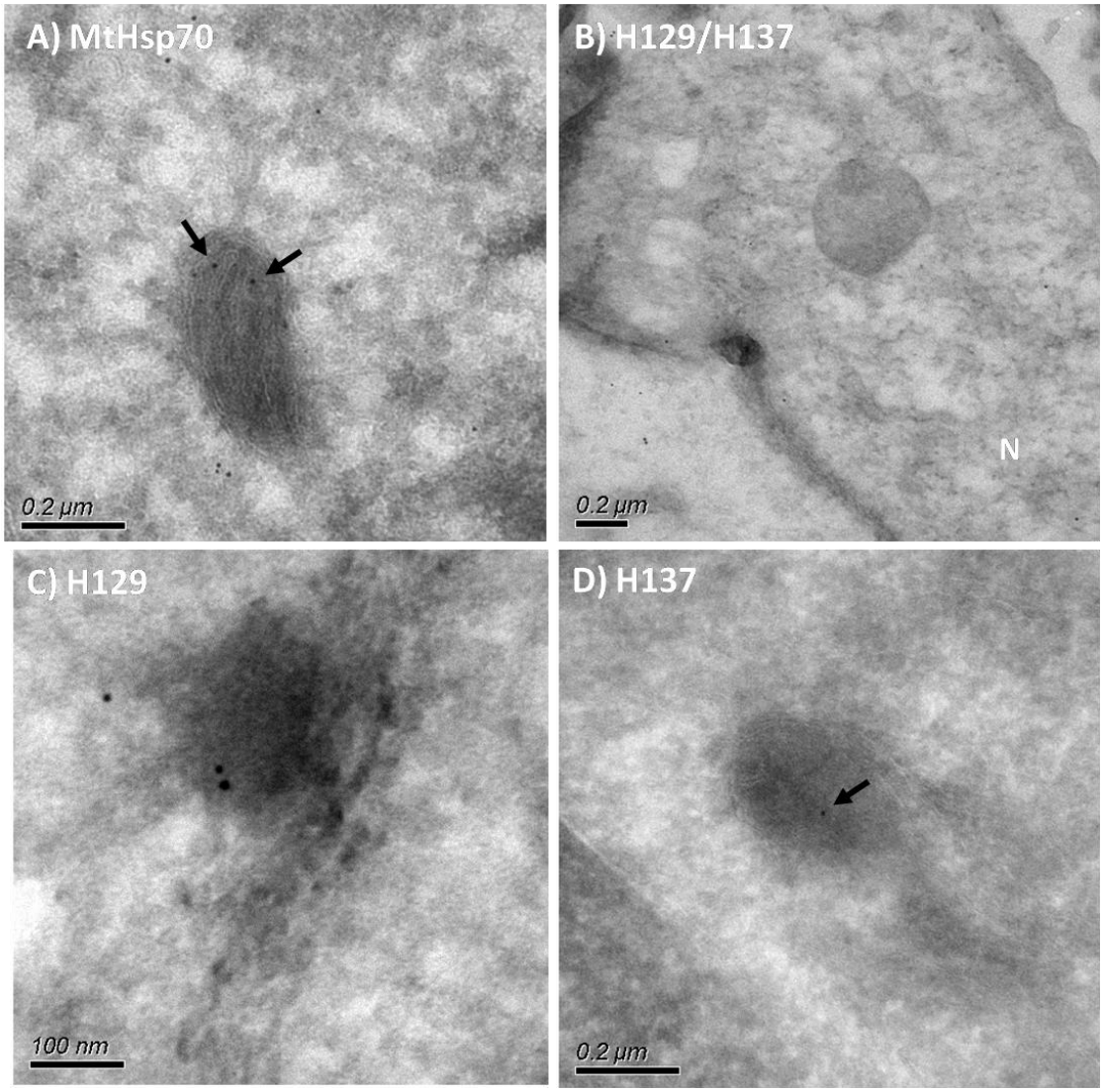


Figure 5.12: Transmision electron micrographs of immunogold labelling of p63 in mitochondria (WM1158 cells). (A) Mitochondrion visualised by electron dense matrix surrounded by double membrane with cristae projections showing immunogold labelled-mtHsp70 localisation within the mitochondrial matrix (black arrows). (B) Electron micrograph showing both nuclear (labelled N) and extranuclear localisation of p63. (C) Electron dense mitochondria characterised by remarkably electron dense mitochondrial matrix and cristae. Image demonstrates immunogold localisation of p63 (using combination of anti-p63 antibody H129) in transit to mitochondria and within mitochondrial matrix. (D) Electron micrograph showing immunogold labelling of p63 (using H137 anti-p63 antibody) within electron dense mitochondrion matrix (arrow), Cristae are visible.

5.3.3.5 p63 splice variants are differentially localised in melanoma cells

To further clarify the expression pattern of p63 isoforms in cell organelles and to explore translocation of these isoforms to the mitochondria, protein lysates were analysed following the subcellular fractionation technique [section 2.3.2]. Melanoma cells were treated with chemotherapeutic agents (etoposide 30 µM, doxorubicin 2 µM and paclitaxel 2 µM) and harvested at various time-points. Using Western blotting, subcellular fractionated protein lysates were probed with anti-p63 antibody (AB-4) which detected all isoforms of p63. Although the subcellular fractionation technique has a recognised disadvantage of contamination of fractions, the aim of this technique was to enrich for proteins of interest within mitochondrial and nuclear fractions. Purity of fractions was confirmed by incubation with anti-PCNA (nuclear), anti-COX-IV or anti-mtHsp70 (mitochondrial) and anti-GAPDH (cytosolic) antibodies.

Different splice variants of p63 were expressed in different cellular compartments. In untreated WM1158 cells, TAp63α was largely expressed in the nuclear fraction and TAp63β was largely expressed in the mitochondrial compartment, with upregulation of each isoform in their respective compartments in response to various chemotherapeutic agents [Figure 5.13].

5.3.3.6 p63 splice variants are differentially stabilised in subcellular compartments

In untreated A375M cells the expression pattern observed was different. Both ΔNp63 α and β were localised in the nucleus and mitochondria, with stabilisation of both isoforms of p63 in both compartments upon treatment with etoposide, paclitaxel and doxorubicin [Figure 5.14]. In this situation, p63 isoforms could be stabilised in their respective compartments or partially translocate from nucleus to mitochondria upon genotoxic stress, thus influencing apoptotic pathways in melanoma. In addition, treatment with chemotherapeutic agents revealed a slower migrating band in the mitochondrial fraction (arrow) which may be ΔNp63δ (Mangiulli et al. 2009) or a post-translational modification of ΔNp63γ. p63 has been demonstrated to be a phosphoprotein (Westfall et al. 2005) and UVB irradiation results in rapid induction of ΔNp63α followed by phosphorylation of ser-66/68 which leads to its degradation and inactivation by 6 hrs (Papoutsaki et al. 2005; Westfall et al. 2005). This prevents apoptosis through activation of the Akt survival pathway (Ogawa et al. 2008). Probing with anti-phospho-p63 antibody failed to confirm this on a Western blot. However the only commercially available anti-phospho-p63 antibody detects phosphorylation at ser 160/162 in TAp63α alone and in A375M cells the predominant isoform expressed

is ΔNp63, so an alternative phosphorylation site, e.g. ser-66/68 is a more likely explanation for the slow migrating band on the blot.

Very low expression levels of p63 protein were detected using Western blotting in the cytosol. This could be a result of instability of the p63 isoforms within this compartment and/or the transient nature of the isoforms within the cytosol while migrating between nucleus and/or mitochondria upon genotoxic stress.

5.3.3.7 Putative cleavage of p63 is demonstrated upon DNA damage

ΔNp63 isoforms are upregulated in A375M cells upon treatment with chemotherapeutic agents but additional bands, not corresponding to specific splice variants of p63, were also consistently identified [Figure 5.14]. To explore this further, AB-4 antibody (which detects all isoforms of p63) was used to probe for p63 in A375M cells treated with doxorubicin [Figure 5.14B]. Putative cleaved fragments of p63 were detected in nuclear, cytoplasmic and mitochondrial lysates. Nuclear and cytoplasmic localisation of an N-terminal cleaved fragment (molecular weight ~35kD) and nuclear and mitochondrial localisation of a C-terminal fragment (molecular weight ~20kD) was observed [Figure 5.14B]. A similar pattern of putative cleaved products of p63 was detected in melanoma cell lines upon treatment with etoposide and paclitaxel (data not shown).

5.3.3.8 Detection of p53 protein in subcellular compartments

Endogenous wt-p53 protein in melanoma accumulates after genotoxic stress and retains its transcriptional activity (Kichina et al. 2003; Satyamoorthy et al. 2000). Although it is well established that p53 relocates to the mitochondria inducing both the transcriptional-dependent and transcriptional-independent apoptotic pathways (Marchenko et al. 2000; Mihara and Moll 2003), there is little evidence of this in melanoma. A recent study of melanocytes demonstrated translocation of p53 to the mitochondria upon UVA irradiation, but not UVB (Waster and Ollinger 2009). Subcellular fractionated protein lysates of A375M cells and WM1158 cells treated with different chemotherapeutic agents were probed for p53 (using DO-1 antibody) revealing predominant stabilisation of p53 in the nucleus, with less pronounced stabilisation in mitochondria [Figures 5.13 and 5.15]. In support of this, immunofluorescence microscopy of the same cells demonstrated predominant nuclear stabilisation upon treatment with doxorubicin and paclitaxel [Figure 5.16]. These findings suggest that in melanoma, p53 displays nuclear stabilisation in response to genotoxic stress with possible translocation to the mitochondria.

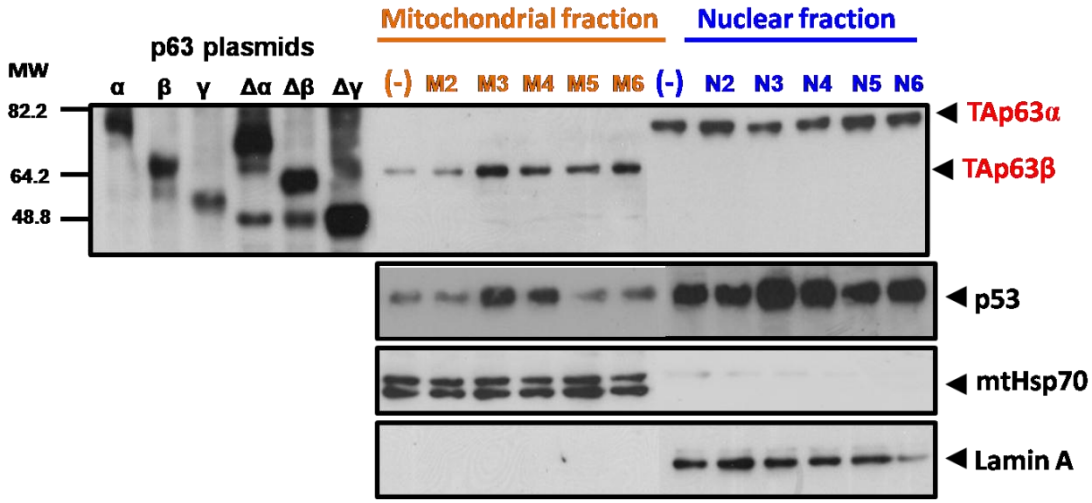
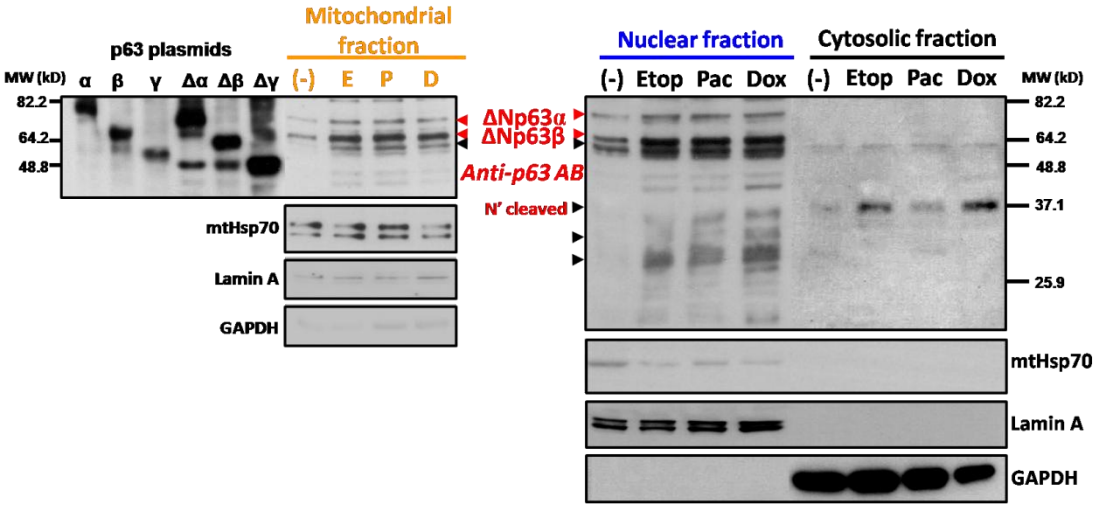


Figure 5.13: Differential subcellular expression of p63 splice variants (WB). Untreated WM1158 cells (-) shows localisation of specific isoforms in different subcellular compartments; TAp63α in nuclear fraction and TAp63β in mitochondrial fraction. This was confirmed by comparing molecular weights to exogenously transfected p63 plasmids in HEK 293T cells (far left lanes 1-6). Upon treatment with various chemotherapeutic agents (M2/N2 cisplatin, M3/N3 doxorubicin, M4/N4 paclitaxel, M5/N5 etoposide and M6/N6 UVB, corresponding to mitochondrial (M) and nuclear (N) fractions of the same cells), stabilisation of the endogenous isoform was observed. p53 expression analysed in the same lysates (using anti-p53 antibody DO-1) demonstrates marked stabilisation in the nuclear fractions and much less in the mitochondria. mtHsp70 was used as a marker for mitochondrial protein loading and lamin-A was used for loading of nuclear proteins. Western blot data is representative of cellular fractionation experiments performed in triplicate. MW – molecular weight in kDa.

(A)



(B)

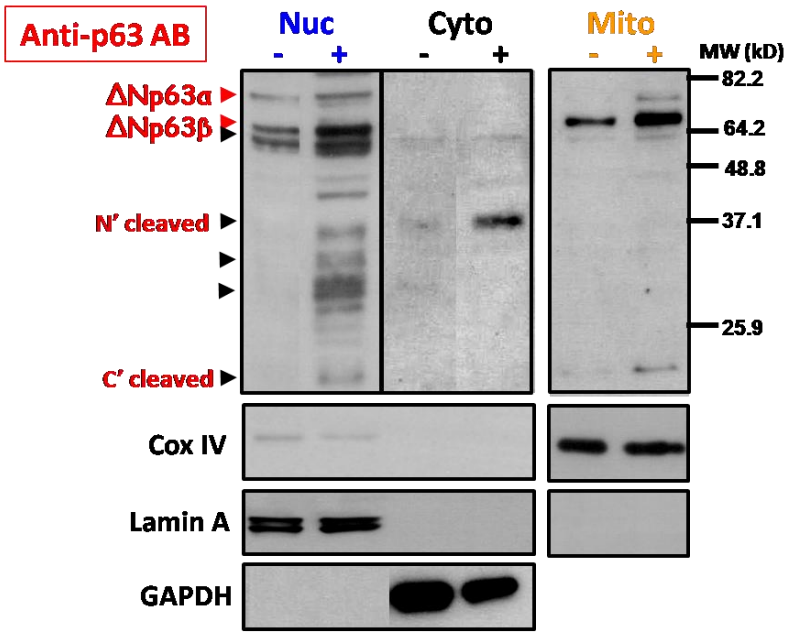


Figure 5.14: Subcellular fractionation of A375M cells. (A) Left panel demonstrates molecular weight markers of exogenously transfected p63 plasmids in HEK 293T cells (using anti-p63 antibody AB-4). This was used to confirm the expression of ΔNp63α and β isoforms in the mitochondrial fraction of untreated A375M cells (-) and upregulation upon treatment with etoposide (lane E), paclitaxel (lane P) and doxorubicin (lane D). In addition, bands not corresponding to any of the standard six plasmids were also observed (denoted by black arrows) including a putative N–terminal cleaved p63 product around 37 kD (labelled in red). The right panel demonstrates stabilisation of the same p63 isoforms in the corresponding nuclear fraction of A375M cells treated with the same chemotherapeutic agents. (B) Subcellular fractionation of A375M cells treated with (+) and without (-) doxorubicin demonstrating differential pattern of isoform expression in subcellular compartments. ΔNp63β

is significantly upregulated upon DNA damage in mitochondrial and to a lesser extent, in nuclear fractions. Additional protein bands are noted (black arrows) in the nuclear fraction. A slower migrating band in the cytoplasmic fraction (~37kD) could correspond to the N terminal cleaved product of p63 (Sayan et al. 2007), and another at ~20kD (labelled in red) in both mitochondrial and nuclear fractions (labelled in red), could correspond to the C-terminal cleaved p63 product deemed to be important for induction of apoptosis (Sayan et al. 2007). Other bands could correspond to other, as yet unidentified, cleaved products, could be smaller undiscovered molecular weight splice variants of p63 or could be non-specific. mtHsp70/COX IV, lamin A and GAPDH were used as loading controls for mitochondrial, nuclear and cytoplasmic fractions, respectively. Western blot data is representative of cellular fractionation experiments performed in triplicate. Nuc – nuclear fraction, Cyto – cytoplasmic fraction, Mito – mitochondrial fraction, MW – molecular weight, Etop – etoposide, pac – paclitaxel, dox – doxorubicin.

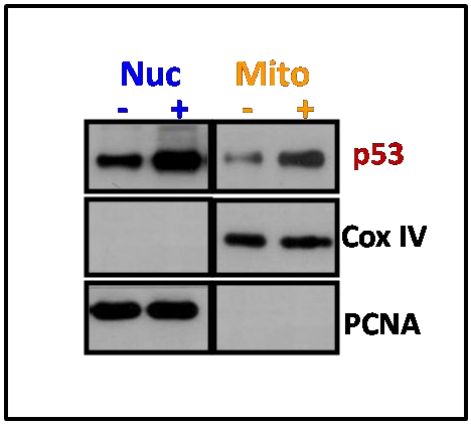


Figure 5.15: Stabilisation of p53 in subcellular fractions of A375M cells. Untreated (-) and treated (+) cells with doxorubicin (1 µM) for 6 hrs were fractionated prior to Western blot analysis of p53 (using anti-p53 antibody DO-1). Stabilisation of p53 protein was observed in both nuclear and mitochondrial fractions in response to DNA damage. COX IV and PCNA were used as loading controls for purity of mitochondrial and nuclear fractions, respectively. PCNA – proliferating cell nuclear antigen, Nuc – nuclear fraction, Mito – mitochondrial fraction.

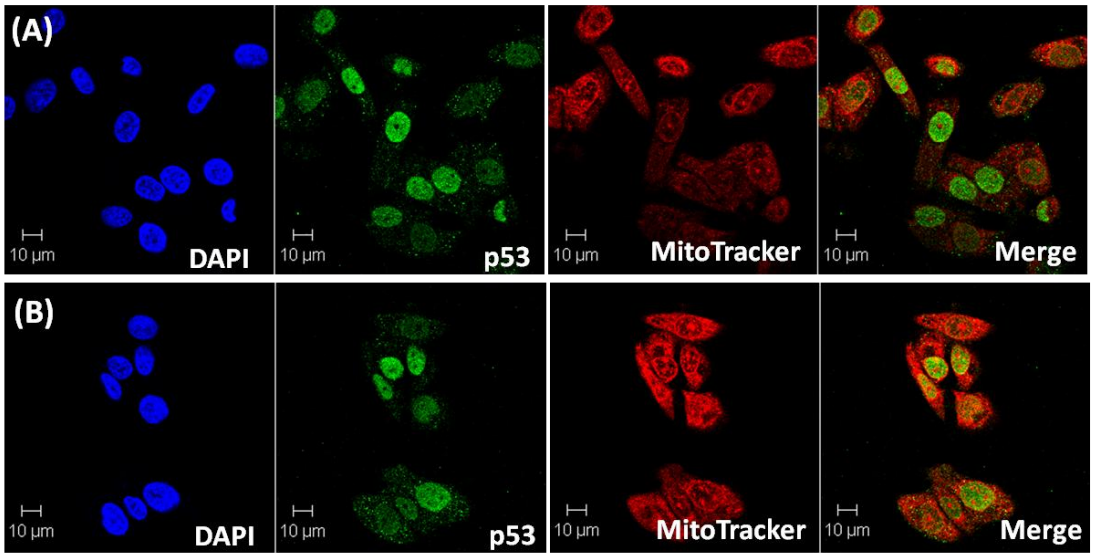


Figure 5.16: Stabilisation of nuclear wt-p53 in A375M cells (IMF). Endogenous expression of p53 (using anti-p53 antibody DO-1) demonstrates that it is a nuclear protein in A375M cells (not shown). Cells were labelled with MitoTracker Orange (red) to label mitochondria prior to treatment and immunostaining for p53 (green). Upon treatment with (A) doxorubicin (2 µM) and (B) paclitaxel (2 µM) for 6 hrs, p53 (green) is predominantly stabilised in the nuclei (labelled by DAPI - blue) and to a lesser extent in mitochondria. A similar pattern was observed upon treatment for 2 – 8 hrs (data not shown). Images are representative of three independent experiments.

5.3.4 Quantification of translocation of p63

Subcellular fractionation enriches for p63 protein within each fraction but does not allow for assessment of changes in concentration of p63 in response to genotoxic stress. To quantify the relative concentration of p63 in different cellular compartments, a new method was developed as an alternative to the fractionation technique (Leverrier et al. 2007). The advantages of this method include: increased sensitivity, requirement for fewer cells, marginal spill over between cellular compartments, and reproducible results which allow quantification of relative protein concentration within a cellular compartment. Moreover, data from quantification studies can be validated using confocal microscopy to confirm purity of the fractions [section 5.3.4.1].

Flow cytometry analysis for quantification of protein translocation was undertaken as outlined [section 2.5.2.1]. Live cells were incubated with MitoTracker Orange to fluorescently label mitochondria. Cells were labelled with anti-p63 or IgG mouse isotype primary antibody and Cy5-conjugated anti-mouse secondary antibody followed by Hoechst (to label the nuclei). The anti-p63 antibody used (4A4) detected all isoforms of p63 and is unable to distinguish between TA and ΔN isoforms. Intact A375M cells, characterised by their double positivity to Hoechst and MitoTracker Orange labelling, were analysed [Figure 5.17]. Cells were disrupted using a Dounce homogeniser and the resulting homogenates were re-analysed by flow cytometry. For each cell line, preliminary control experiments included MitoTracker Orange or Hoechst fluorescently labelled cells only, showing location of the fractions in the homogenised sample [Figure 5.18]. Hoechst-positive and MitoTracker Orange-negative population were defined as free nuclei; Hoechst-negative and MitoTracker-positive population were defined as free mitochondria and the final region gated comprised intact cells [Figure 5.18]. After gating for whole cell, mitochondrial and nuclear populations, fluorescence intensity of p63 shown by Cy5 histograms were analysed using the FloJo software [Figure 5.19].

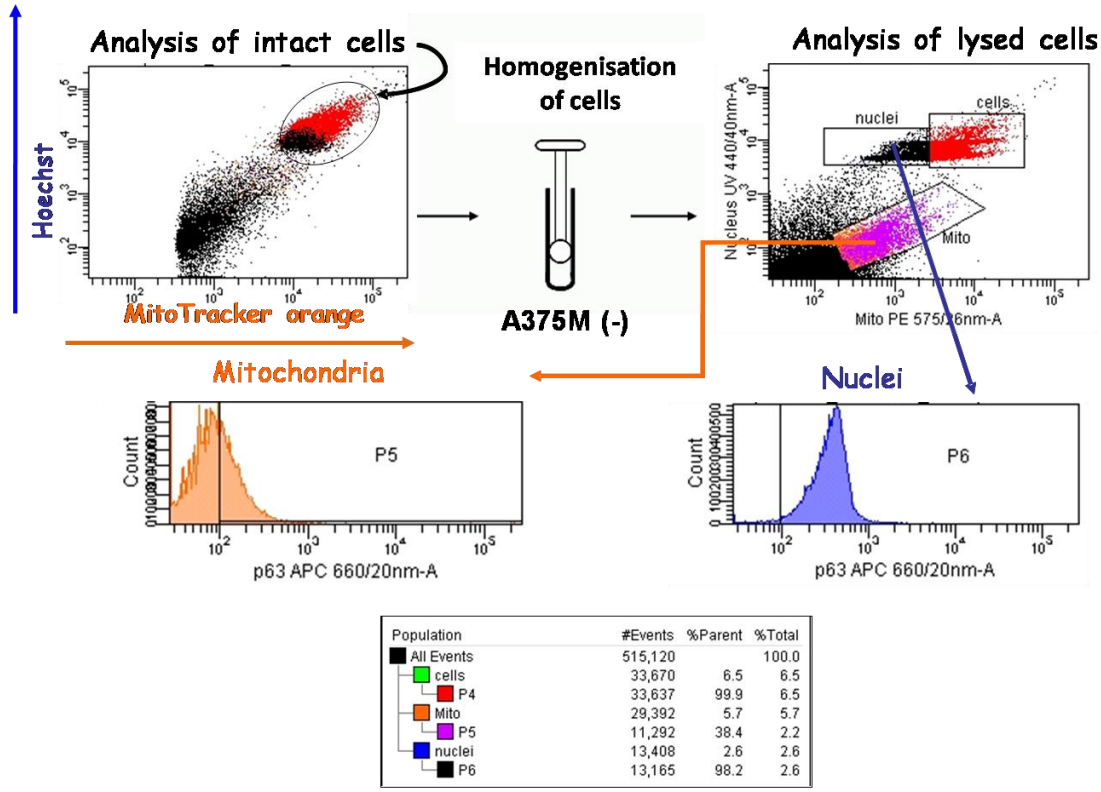


Figure 5.17: Quantitative measurement of p63 localisation in untreated A375M cells using flow cytometry. Intact A375M cells were analysed using the LSR II flow cytometer to confirm positivity to all dyes - Hoechst, MitoTracker and Cy5-conjugated antibody labelling (top left panel – position of intact cells on plot shown). Cells were disrupted using a Dounce homogeniser and resulting homogenates were re-analysed using the same gating and settings (top right panel – position of different cellular fractions shown). Hoechst-positive and MitoTracker-negative population are defined as nuclei (represented by blue histogram - middle right panel), and Hoechst-negative and MitoTracker-positive population are defined as mitochondria (represented by orange histogram - middle left panel). In each of these populations, p63-Cy5 signal is analysed (representative example shown in bottom panel) and relative change in Cy5 signal when comparing untreated (-) and treated cells is determined to quantify increase/decrease of protein of interest in the different subcellular compartments.

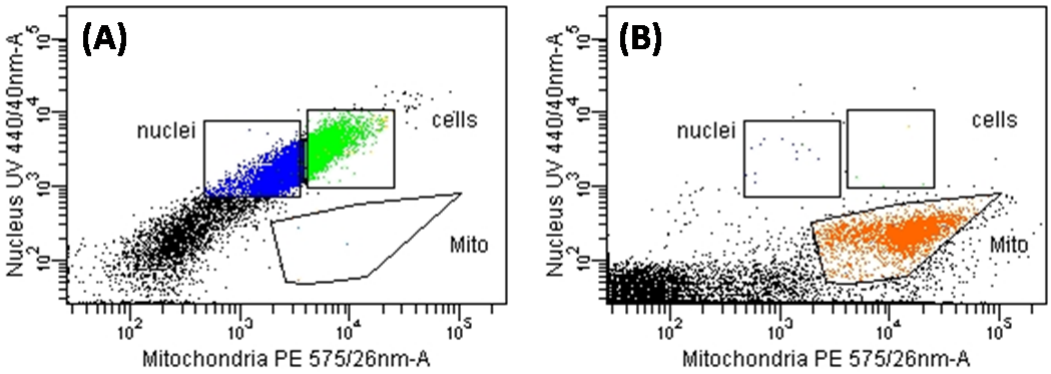


Figure 5.18: Example of single fluorescence labelling of fractionated cells confirming localisation on flow cytometry scatter plot. Untreated A375M cells were homogenised and analysed using flow cytometry according to labelling with (A) Hoechst to show position of gating of nuclei and cells and (B) MitoTracker Orange to show position of gating for free mitochondria.

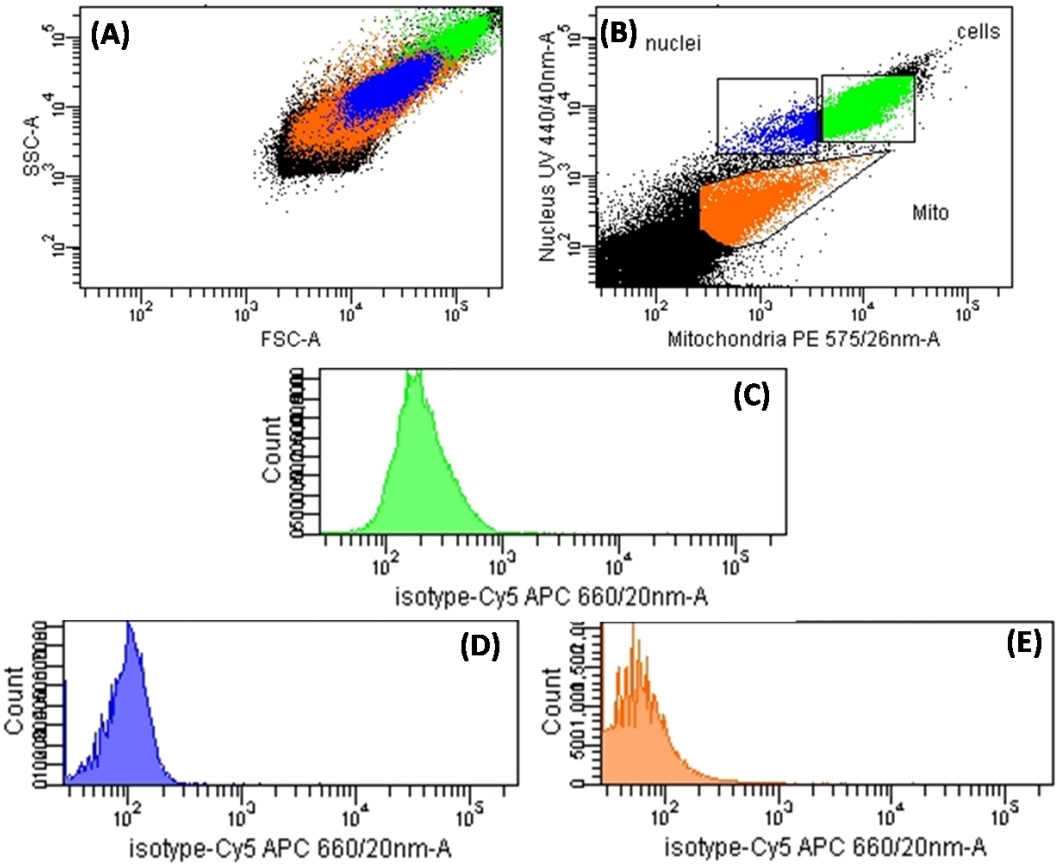


Figure 5.19: Optimisation of flow cytometry fractionation technique using isotope-Cy5 labelling (A375M cells). (A) Analysis of untreated lysed cells labelled with isotope-Cy5 showing position of cells in scatter plot, using LSR II flow cytometer. (B) Position of gating for subcellular fractions shows the isotope control levels in (C) whole cells, (D) nuclei and (E) mitochondria, depicted as histograms. Representative images shown from at least three independent experiments performed.

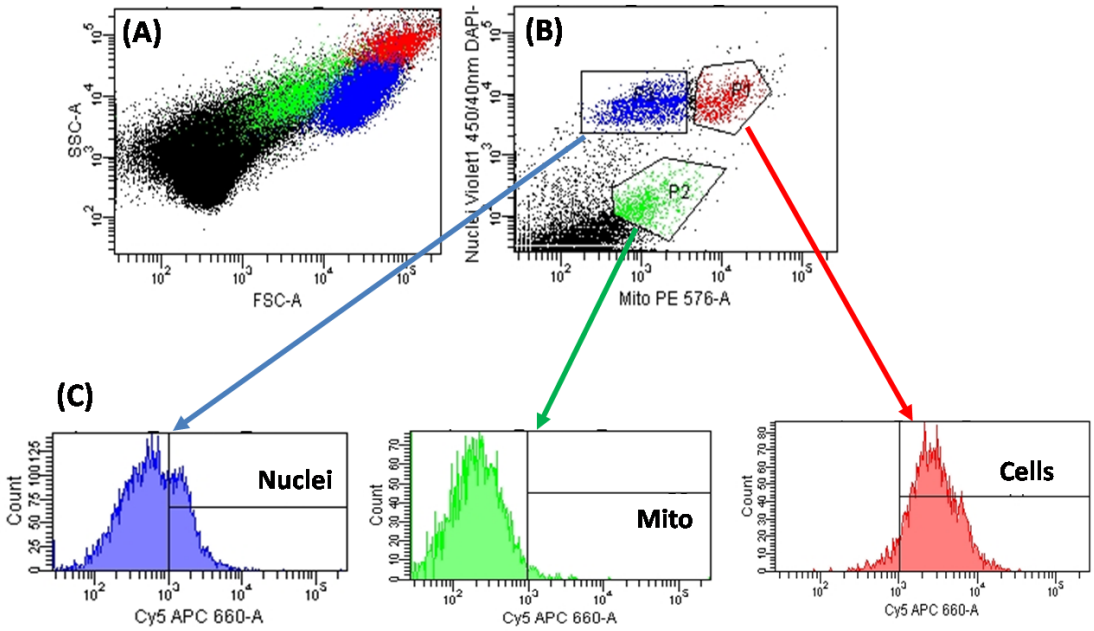


Figure 5.20: Flow cytometry analysis of p63-Cy5 in fractionated A375M cells. Untreated A375M cells were lysed and labelled as described [section 2.5.2.2]. (A) Scatter plot showing size distribution of A375M whole cells (red), nuclei (blue) and mitochondria (green). (B) Scatter plot of Hoechst vs. MitoTracker Orange demonstrating organelle distribution; blue gated region (Hoechst +ve) shows the nuclei; red marked area (Hoechst +ve / MitoTracker Orange +ve) shows whole cells; green marked area (Hoechst weak+ve / MitoTracker Orange +ve) shows mitochondria. (C) Histograms demonstrating level of p63-Cy5 expression corresponding to whole cells, nuclei and mitochondria gated in (B). Representative images shown from at least four independent experiments performed. Mito – mitochondria.

5.3.4.1 Validation of method

To establish purity of the nuclear and mitochondrial fractions, homogenised cells were sorted according to Hoechst and MitoTracker Orange labelling using the FACSAria™ cell sorter. The sorted fractions were then re-analysed to confirm purity of each fraction [Figure 5.21]. The sorted mitochondrial fraction was then centrifuged at 13 000 g for 30 mins at 4ºC and the supernatant discarded. The mitochondrial isolates fluorescently labelled with MitoTracker Orange were mounted on a glass slide in Vectashield to prevent bleaching of the fluorescent signal. Coverslips were secured using clear nail varnish and the isolates were visualised using confocal microscopy [Figure 5.21]. The nuclear fraction was prepared in the same way except centrifugation performed at 4 500 rpm for 15 mins at 4ºC before mounting the isolates onto a slide. Imaging of the intact cells, nuclei and mitochondria by confocal microscopy validated the analysis gates used to separate fractions comprising of pure nuclei and mitochondria and not fragments of cells [Figure 5.22].

Further verification of this technique included detection of p63 protein by visualising p63-Cy5 in mitochondrial and nuclear fractions using confocal microscopy, following fluorescence activated cell sorting (FACSAria™ flow cytometer). Cy5 was not routinely used for immunofluorescence microscopy because it is prone to photobleaching. Nevertheless it was possible to detect p63-Cy5 in both nuclear and mitochondrial sorted fractions which confirmed the purity of fraction and validated the method as a means of quantification of protein within each cellular fraction [Figure 5.22].

Cells treated with chemotherapeutic agents (etoposide, paclitaxel and cisplatin) were prepared using the same method. Doxorubicin is a red compound with an intrinsic fluorescence. Its excitation and emission wavelengths are 480 nm and 550 nm and therefore because of spectral cross-talk with Hoechst could not be used for this quantification experiment despite data from Western blotting and immunofluorescence microscopy suggesting that it may be the most potent inducer of p63 in melanoma. The dose of drug used for the flow cytometry fractionation assays was the optimal dose required to induce p63 without inducing cell death. For each cell line this was optimised by observing the morphology of cells following treatment [Figure 5.23] in conjunction with data from immunofluorescence microscopy and western blotting analyses of p63 protein expression.

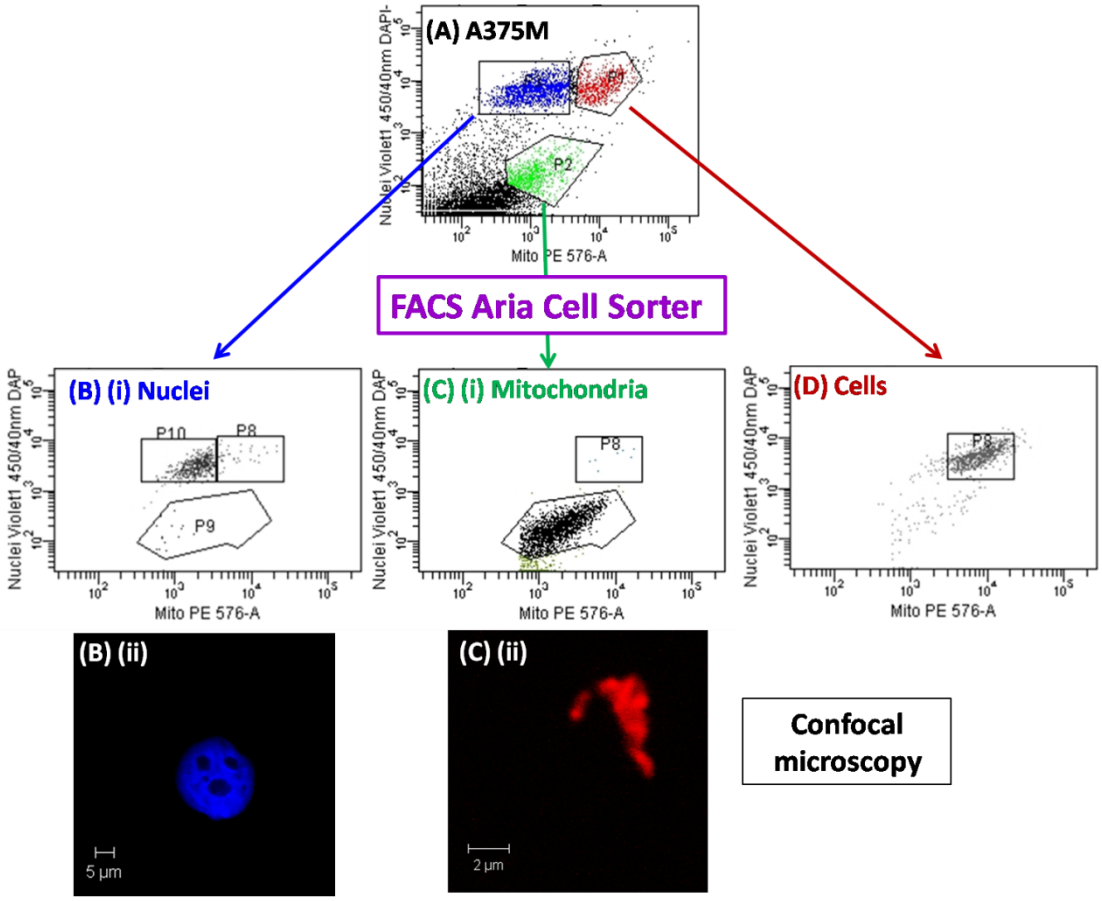


Figure 5.21: Validation of flow cytometry fractionation technique. A375M cells were labelled as outlined and homogenised prior to analysis using the FACS Aria Cell Sorter (A). Gated fractions - whole cells, nuclei and mitochondria were subjected to FACS and then reanalysed demonstrating purity of (B)(i) nuclear, (C)(i) mitochondrial and (D) whole cell fractions. Scatter plots demonstrating background signal noise from buffers (PBS and HEPES) are shown in Appendix 8. Sorted cell fractions were then prepared as outlined [section 5.3.4.1] and (B)(ii) nuclear and (C)(ii) mitochondrial isolates were visualised using confocal microscopy confirming purity of the sorted fraction. Image examples provided are from one of five independent experiments performed.

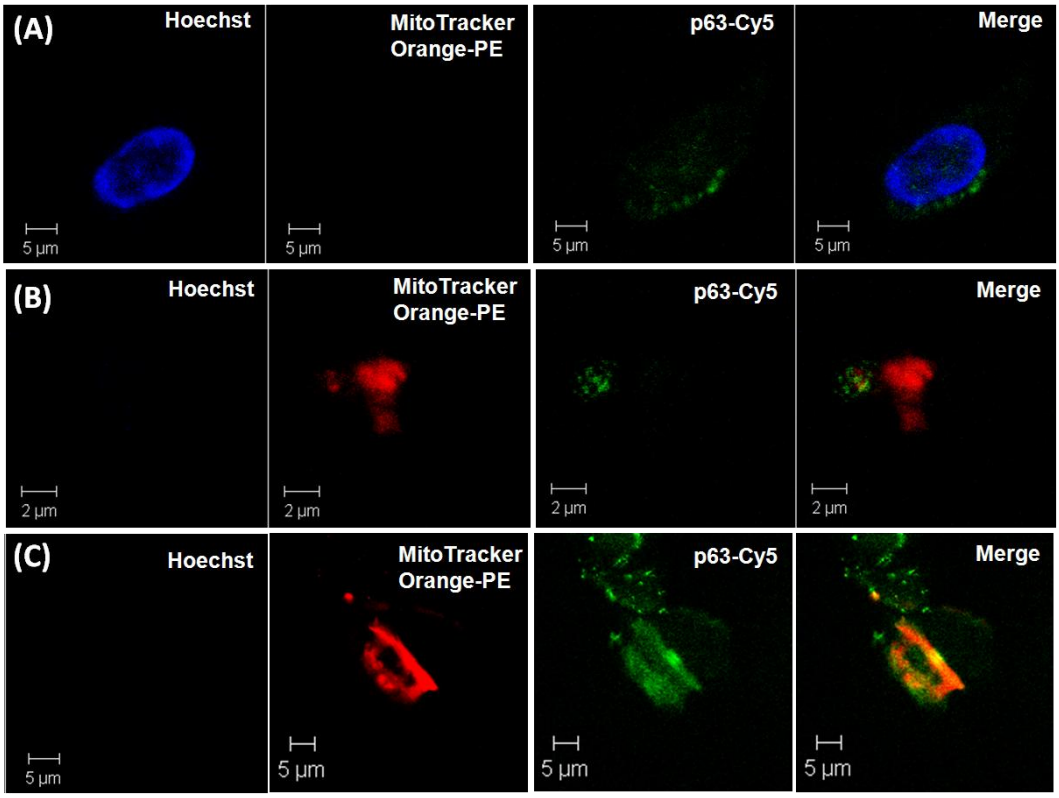


Figure 5.22: p63-Cy5 expression in cellular fractions obtained from FACS of labelled A375M cells. Cells were labelled with fluorescence markers for nuclei (Hoechst) and MitoTracker Orange (mitochondria) and p63-Cy5 (green) to label p63 in A375M cells. Following homogenisation, cells were subjected to FACS sorting and fractionated isolates were prepared as outlined [section 5.3.4.1]. Confocal microscopy analysis demonstrated p63-Cy5 expression in pure (A) nuclear and (B) mitochondrial fractions of A375M cells. (C) Giant mitochondria show co-localisation (yellow) of p63-Cy5 and MitoTracker Orange (red) confirming validity of the flow cytometry method of quantification of p63 translocation to the mitochondria. Images are representative of five independent experiments.

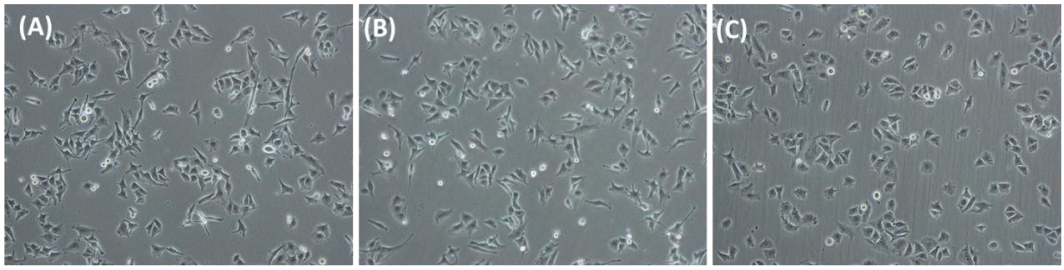


Figure 5.23: Effect of chemotherapy on A375M cells used in mitochondrial relocation experiments. Morphology of A375M cells (using phase contrast microscopy) treated with various chemotherapeutic agents (A) untreated, (B) etoposide 20 µM and (C) paclitaxel 2 µM for 6 hrs, demonstrating absence of apoptosis and minor changes in morphology of cells upon treatment with paclitaxel. Doxorubicin was not used for flow cytometry experiments because of cross-over of intrinsic fluorescence of the drug with Hoechst emission wavelength. Magnification x20.

5.3.4.2 Genotoxic agents induce a differential p63 expression profile

A375M cells were treated in a time-dependent manner to determine the effect of treatment with etoposide (30 µM) on expression of p63 using flow cytometry. To quantify the degree of p63 stabilisation upon treatment, the percentage positive events were combined with the relative fluorescent intensity of the Cy5 signal. Expression of p63 was determined by comparing the expression of p63 to the IgG mouse isotype control for each treatment, using the same Cy5-conjugated secondary antibody. The relative concentration of p63 in cells (considered total p63), nuclei and mitochondria in A375M cells upon treatment was calculated by comparing concentration of p63-Cy5 for each treatment to the untreated sample. p63 was stabilised to a maximum at 6 hrs with a reduction thereafter to 24 hrs [Figure 5.24]. When cells were lysed and re-analysed, expression of nuclear p63 increased linearly up to 24 hrs but the stabilisation profile of mitochondrial p63 was similar to the profile for total p63 [Figure 5.24].

The same cells were treated with paclitaxel to determine if a similar phenomenon occurred. When compared with etoposide, treatment with paclitaxel resulted in marked stabilisation of p63 in all cellular compartments of A375M cells [Appendix 9]. To investigate if the increased induction of p63 upon treatment with paclitaxel compared with etoposide, in the mitochondrial fraction was attributed to a differential splicing pattern for p63 proteins, Western blot analysis of the mitochondrial fraction of A375M cells was undertaken. This demonstrated stabilisation of ΔNp63 isoforms (detected using anti-p63 antibody AB-4) [Figure 5.25B]. When the same lysates were probed with anti-p63 antibody H129, reported to detect cleaved p63 (Sayan et al. 2007), significant stabilisation of a putative cleaved product (MW ~20 kD) was observed upon treatment with paclitaxel which could provide a possible explanation for the significantly greater upregulation of p63 detected by the flow cytometry quantification [Figure 5.25A].

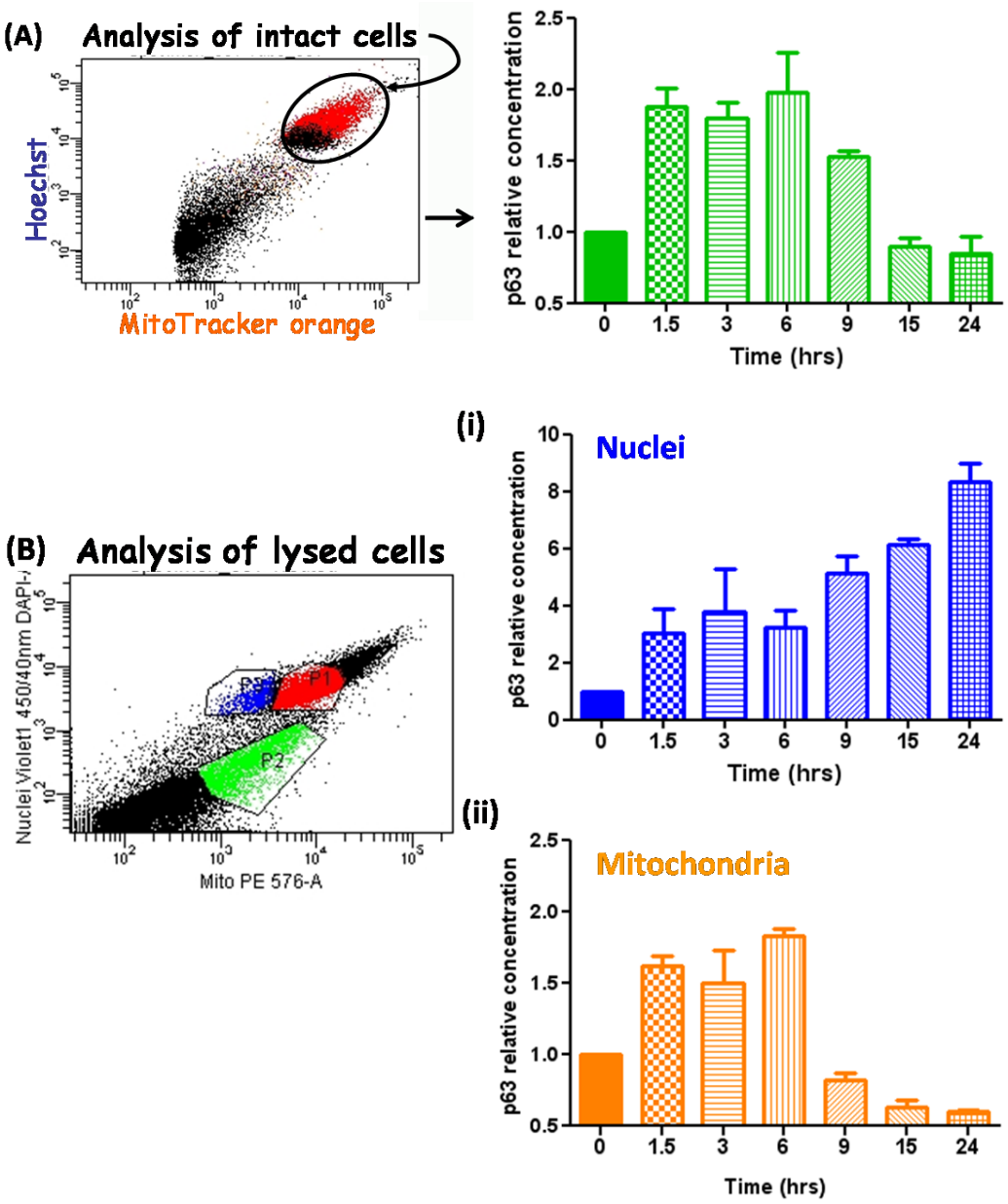


Figure 5.24: Profile of relative change in p63 concentration upon treatment with etoposide. (A) Scatter plot (left panel) demonstrating intact A375M cells analysed for p63-Cy5 expression in whole cells upon treatment with etoposide from 0-24 hrs. Histogram (right panel) demonstrates maximal upregulation of p63 in A375M cells occurs within 6 hrs of treatment. (B) Scatter plot demonstrates analysis of lysed cells and gating of subcellular fractions. (i) Upregulation of p63-Cy5 in the nuclei increases linearly up to 24 hrs of treatment with etoposide. (ii) Upregulation of p63-Cy5 in the mitochondrial fraction occurs rapidly within 6 hrs and then decreases considerably thereafter to 24 hrs. Data show mean expression of p63 +/- standard error of mean for three independent experiments performed in triplicate.

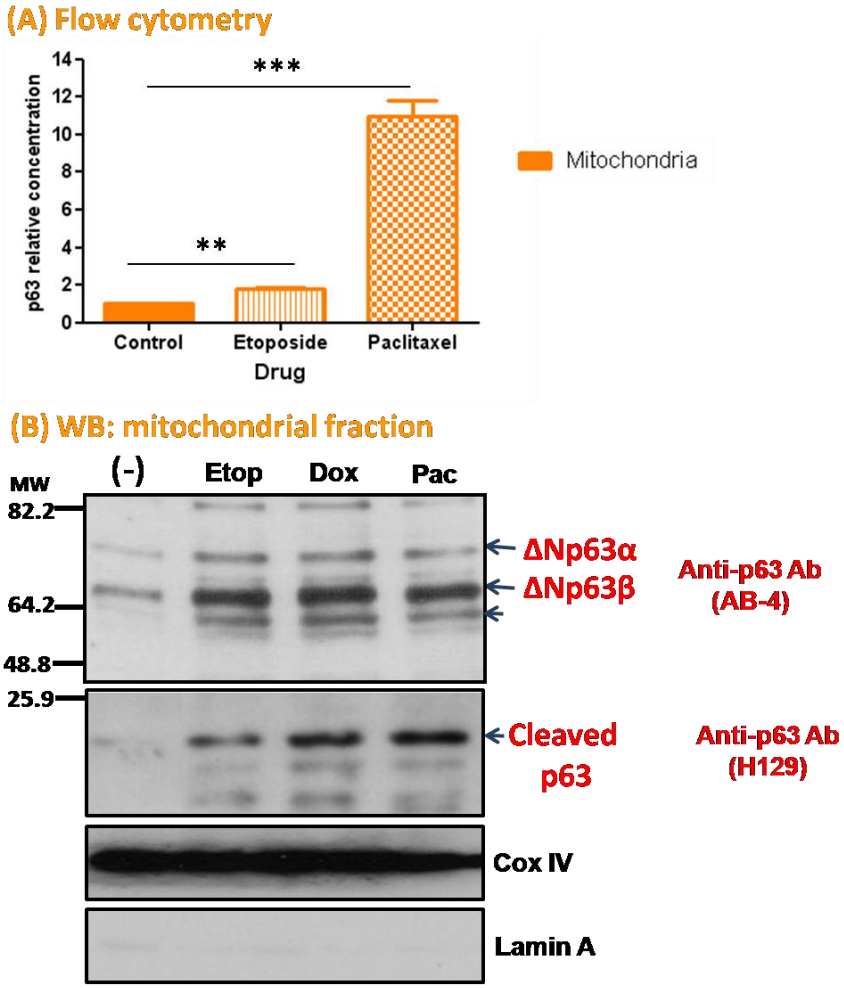


Figure 5.25: p63 isoforms are stabilised in the mitochondrial fraction. (A) Flow cytometry analysis showing relative concentration of p63-Cy5 in A375M cells upon treatment with etoposide and paclitaxel. Significant upregulation of p63-Cy5 was demonstrated for treatment with both etoposide (p-value 0.0075 *t-test*) and paclitaxel (p-value 0.0003 *t-test*). Data show mean expression of p63 +/- standard error of mean for three independent experiments performed in triplicate. (B) Western blot analysis of untreated cells (-) and those treated with etoposide (Etop), doxorubicin (Dox) and paclitaxel (Pac) demonstrates stabilisation of ΔNp63 isoforms (detected using anti-p63 antibody AB-4). When the same lysates were probed with anti-p63 antibody H129, reported to detect cleaved p63, significant stabilisation of this putative cleaved product was observed upon treatment with paclitaxel which could provide a possible explanation for the significantly greater upregulation of p63 detected by the flow cytometry quantification in (A). Cox IV and Lamin A confirmed purity and loading of mitochondrial and nuclear lysates respectively. MW – molecular weight.

5.3.4.3 Stabilisation of p63 occurs in the nucleus with partial translocation to the mitochondria upon genotoxic stress

Flow cytometry experiments were extended to determine if stabilisation of p63 was a general phenomenon occurring in melanoma cell lines. p63 expression was determined in WM1158 cells by comparing intensity of fluorescence for p63 to the IgG mouse isotype control for each treatment, using the same Cy5-conjugated secondary antibody [Appendix 10]. The relative concentration of p63 in cells (considered total p63), nuclei and mitochondria in fractionated WM1158 cells upon treatment were analysed as described earlier by calculating the change in concentration of p63-Cy5 for each treatment relative to the untreated sample [Figures 5.26, 5.27]. Upon treatment of WM1158 cells (metastatic melanoma cell line) with paclitaxel (2 µM), maximal stabilisation of total p63 expression was demonstrated at 6 hrs, in keeping with the results observed with A375M cells [Figure 5.28]. Overall paclitaxel induced greater translocation of p63 to the mitochondria in WM1158 cells too, when compared with etoposide [Figure 5.28].

To confirm that translocation of p63 to the mitochondria and nuclei was a general phenomenon not confined to metastatic melanoma cell lines, the experiments were extended to include primary melanoma cells in radial growth phase (SBCl2). Upregulation of p63 was demonstrated using flow cytometry analysis in fractionated SBCl2 cells upon treatment with cisplatin, etoposide and paclitaxel [Figure 5.29]. Significant stabilisation of mitochondrial p63 (and to a lesser extent, nuclear p63) was observed in SBCl2 cells upon treatment with different chemotherapeutic agents (cisplatin and paclitaxel) at 6 hrs [Figure 5.29].

Taken together, these data demonstrate stabilisation of p63 in nucleus and mitochondria occurs in response to genotoxic stress in melanoma cell lines representing different stages of disease progression. Translocation of p63 in the mitochondrial compartment resulted in a concomitant increase in nuclear p63 although in most cases the relative increase in mitochondrial p63 was significantly greater than the increase in nuclear p63. This suggests significant mobilisation of p63 to the mitochondria in response to genotoxic stress. These data support observations for p53 (Erster et al. 2004; Marchenko et al. 2007), showing recruitment of p63 to the mitochondria is a rapid process (occurring as early as 2 hrs), and stabilisation of p63 in the nuclei is a more gradual process, (continuing to increase up to 24 hrs) following exposure to genotoxic stress.

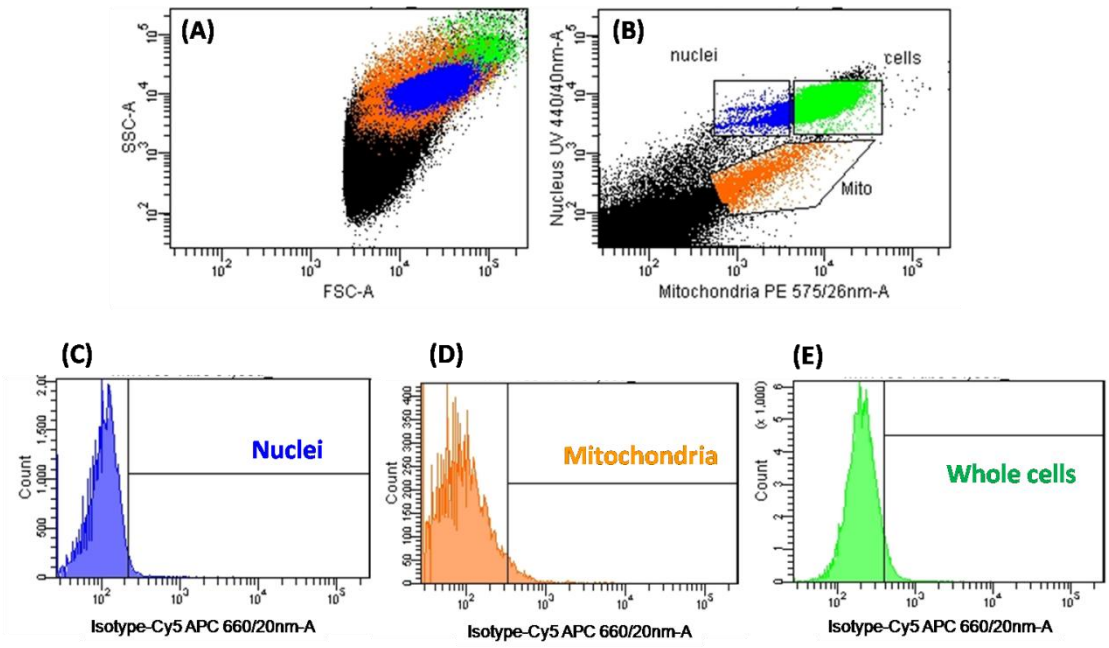


Figure 5.26: Isotope-Cy5 labelling of WM1158 cells (flow cytometry fractionation study). Untreated WM1158 cells labelled with isotope-Cy5 were lysed and analysed (A) using the LSR II flow cytometer. (B) Gating for subcellular fractions shows the isotope control levels in (C) nuclei, (D) mitochondria and (E) whole cells depicted as histograms. Representative images shown from at least three independent experiments performed.

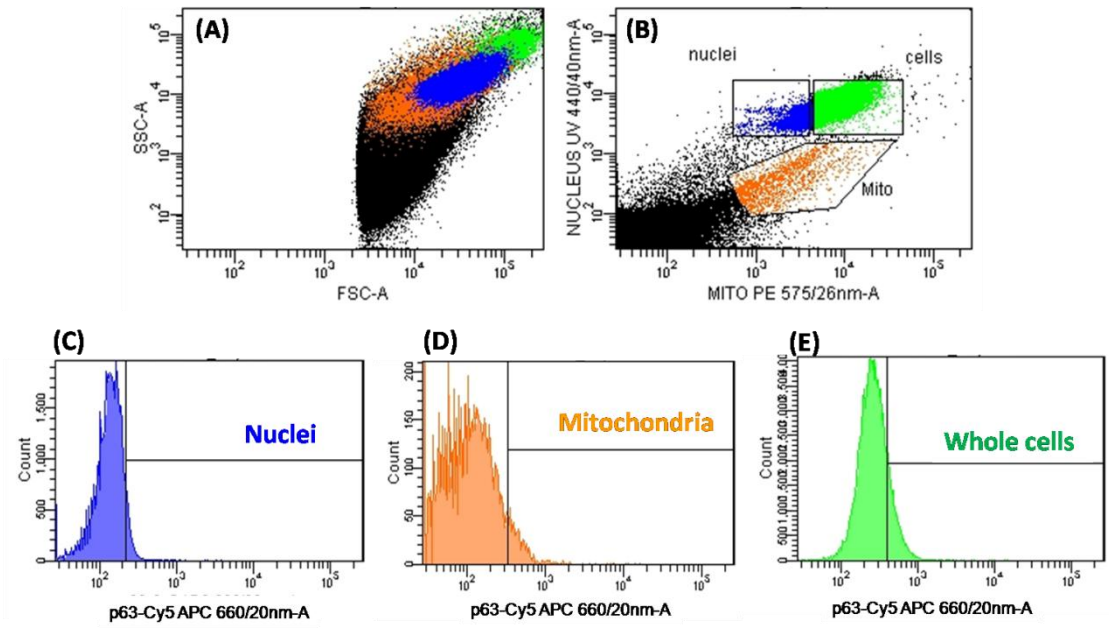


Figure 5.27: p63-Cy5 labelling of WM1158 cells (flow cytometry fractionation study). Untreated WM1158 cells labelled with p63-Cy5 were lysed using the Dounce Homogeniser and analysed (A) using the LSR II flow cytometer. (B) Gating for subcellular fractions shows the p63-Cy5 levels in (C) nuclei, (D) mitochondria and (E) whole cells, depicted as histograms. Representative images shown from at least three independent experiments performed.

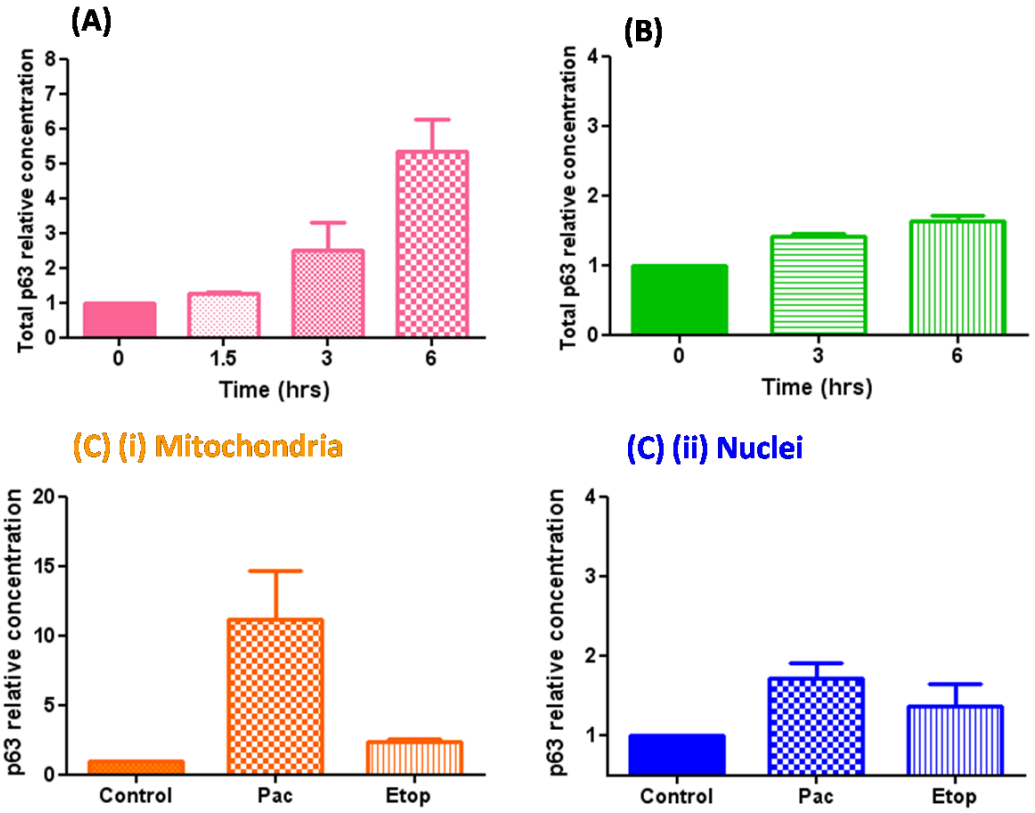


Figure 5.28: Stabilisation of p63 in mitochondrial and nuclear fractions in WM1158 cells (using flow cytometry fractionation technique). WM1158 cells were treated in a time-dependent manner with (A) paclitaxel and (B) etoposide showing upregulation of p63 in whole WM1158 cells occurs in a linear relation up to 6 hrs for both treatments. (C) Analysis of relative changes in subcellular fractions of treated cells demonstrates upregulation of p63 in (i) mitochondria and (ii) nuclei, upon treatment with paclitaxel and to a lesser extent, with etoposide. Data showing mean protein expression +/- SEM for at least three independent experiments performed in duplicate.

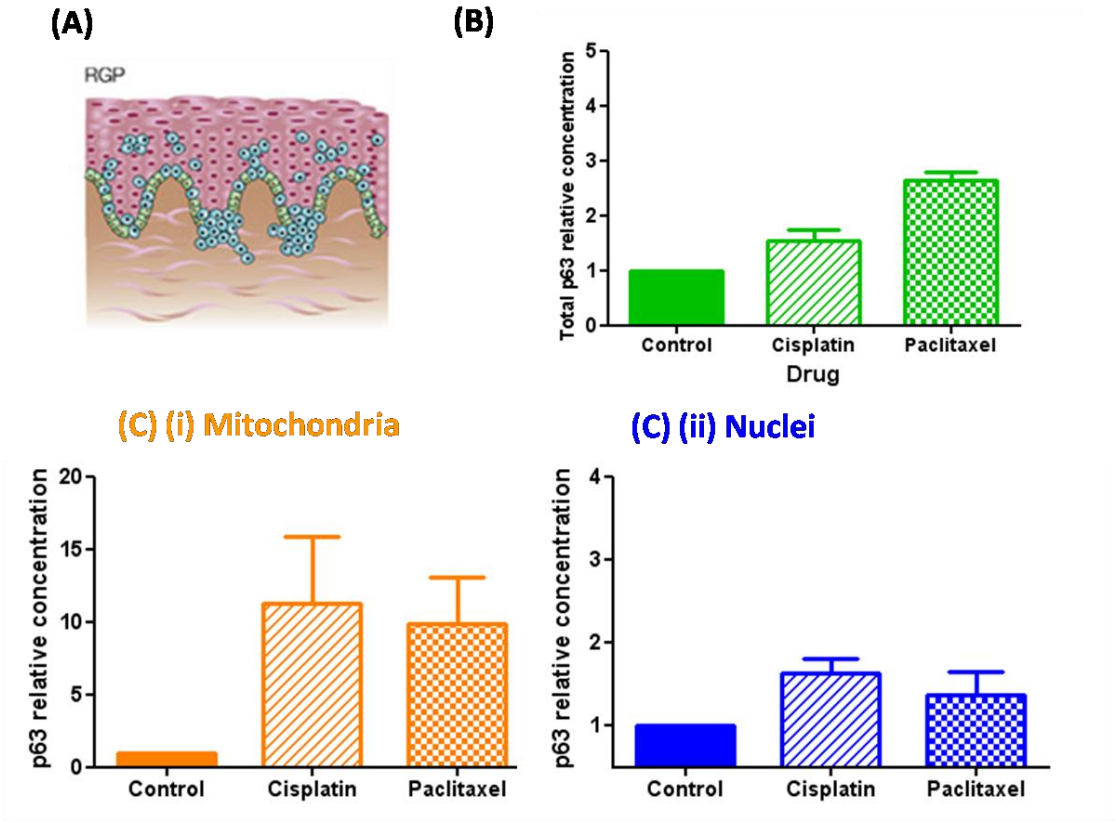


Figure 5.29: Stabilisation of p63 in subcellular fractions in SBCL2 cells (analysed using flow cytometry fractionation technique). (A) Pictorial representation of radial growth phase (RGP) of melanoma illustrating horizontal growth pattern of SBCl2 cells. (B) Upregulation of p63 was demonstrated in whole cells upon treatment (6 hrs) with cisplatin (30 µM), paclitaxel (2 µM) and etoposide (30 µM) (data not shown for latter). (C) Analysis of relative changes in subcellular fractions of treated cells demonstrates upregulation of p63 in (i) mitochondria and (ii) nuclei upon treatment with cisplatin and paclitaxel. Similar findings were observed upon treatment with etoposide (data not shown). Data shows mean protein expression +/- SEM for at least three independent experiments performed in duplicate.

5.3.4.4 Phosphorylation of p63 occurs in response to genotoxic stress

Post-translational modifications of p63 significantly alter p63 protein levels with a number of studies reporting phosphorylation of p63 to achieve this effect (MacPartlin et al. 2005; Osada et al. 1998; Suh et al. 2006; Westfall et al. 2005). TAp63α is phosphorylated in response to γ-irradiation (Suh et al. 2006) and TAp63γ (but not ΔNp63γ) is phosphorylated by IKKβ kinase in response to γ-irradiation or TNF-α (MacPartlin et al. 2008). Phosphorylation of TAp63γ increases its expression levels which is postulated to occur through loss of inhibition of ubiquitylation and thus degradation of the protein (MacPartlin et al. 2008).

Probing with anti-phospho-p63 antibody failed to identify phosphorylation of p63 upon DNA damage in A375M cells on a Western blot, probably because of involvement of an alternative phosphorylation site [section 5.3.3.6]. However, possible phosphorylation of TAp63 in WM1158 cells was not detected despite involvement of the correct phosphorylation site (ser 160/162). This could be attributed to poor stability of the protein in its phosphorylated form or the poor sensitivity of the antibody to detect the very small fraction of phosphorylated protein on a Western blot. Flow cytometry techniques were therefore used as a more sensitive method to detect phosphorylation of TAp63 (at ser 160/162) in WM1158 melanoma cells upon treatment with DNA damaging agents.

WM1158 cells treated with paclitaxel (2 μM) for 1.5-6 hrs were indirectly labelled with anti-phospho-p63 antibody (detecting phosphorylation of serine 160/162 in TAp63) conjugated to Cy5 before analysing on the flow cytometer (LSRII) [section 2.5.2.1]. IgG rabbit isotype control conjugated to Cy5 was used as a control for each treatment. Exposure to paclitaxel resulted in upregulation of total phosphorylated p63 [Figure 5.30]. To investigate the kinetics of p63 and phosphorylated-p63 in different cellular compartments, both forms of p63 were analysed in the same experiment. Protein expression in untreated cells was used as the comparator. The phosphorylated form of p63 detected in treated cells constituted a small fraction of total p63 stabilised [Figure 5.30]. This was in keeping with reports of phosphorylation of p63 in keratinocytes and human squamous cell carcinoma cells upon treatment with paclitaxel (Westfall et al. 2005). Stabilisation of phosphorylated p63 was maximal at 90 mins and 3 hrs in the nuclear and mitochondrial fractions, respectively. When compared with total p63, a marked upregulation of phosphorylated-p63 protein was detected in the nucleus suggesting that in this compartment the phosphorylated form may have an important functional role.

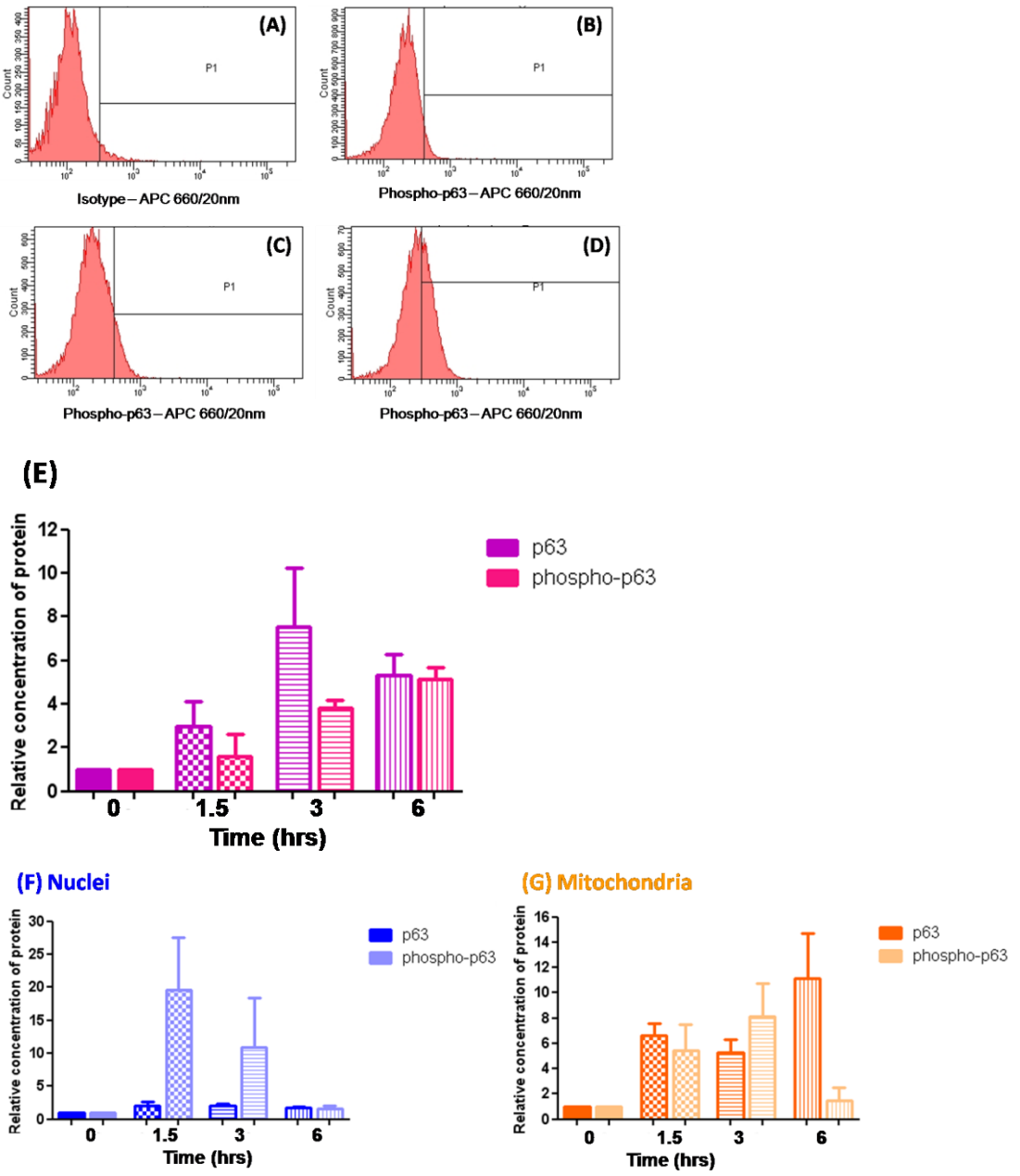


Figure 5.30: Subcellular relocation of total p63 and phosphorylated-p63 (WM1158 cells). Histograms showing flow cytometry analysis of untreated cells which are (A) labelled with rabbit isotope-APC and (B) labelled with phosphorylated-p63-APC. Shift of histogram to the right demonstrates upregulation of phosphorylated-p63 upon treatment with paclitaxel for (C) 3 hrs and (D) 6 hrs. (E) Comparison of p63-Cy5 and phosphorylated-p63 assessed in whole cells demonstrates upregulation of the phosphorylated form of p63 up to 6 hrs treatment with paclitaxel. Analysis of subcellular lysates following homogenisation demonstrates maximal phosphorylation of p63 occurring within 90 mins and 3 hrs in the (F) nuclei and (G) mitochondria, respectively. Data showing mean protein expression +/- SEM for at least three independent experiments performed in duplicate. Phospho-p63 – phosphorylated p63.

5.3.4.5 Translocation of p63 to the mitochondria is dependent on wt-p53

Stabilisation of p63 in melanoma in response to genotoxic stress, raises the possibility that p63 may substitute for, or affect the apoptotic function of, wild-type p53. To determine the possible contribution of p63 on p53 apoptotic activity in apoptosis in these cells, immunofluorescence microscopy of p53-null melanoma cells (U1SO) was undertaken. Q-PCR analysis of these cells showed expression of TAp63 [Figure 3.3]. Upon treatment with paclitaxel, stabilisation of TAp63 was predominantly observed in the nuclei of U1SO cells [Figure 5.31].

The flow cytometry fractionation experiment was initially used to analyse whole U1SO cells. This confirmed upregulation of total p63 protein levels upon treatment with paclitaxel (2 µM) and etoposide (30 µM) suggesting that stabilisation of p63 occurred independent of p53 [Figure 5.32A]. In contrast, FACS analysis of fractionated U1SO cells demonstrated limited upregulation of p63 in either nuclear or mitochondrial fractions [Figure 5.32]. Taken together, these data suggest that p63 requires the presence of wt-p53 for significant translocation to the mitochondria from the nucleus and/or cytosol.

To further understand the contribution of p53, to stabilisation of p63 in the mitochondria, a melanoma cell line expressing wt-p53 (A375M) was compared to the p53-null cell line (U1SO). Treatment with paclitaxel induced significantly greater total p63 and mitochondrial p63 in the wt-p53 melanoma cell line (A375M) compared with the p53-null cell line (U1SO) [Figure 5.33], suggesting that translocation of p63 to the mitochondria may depend on wt-p53.

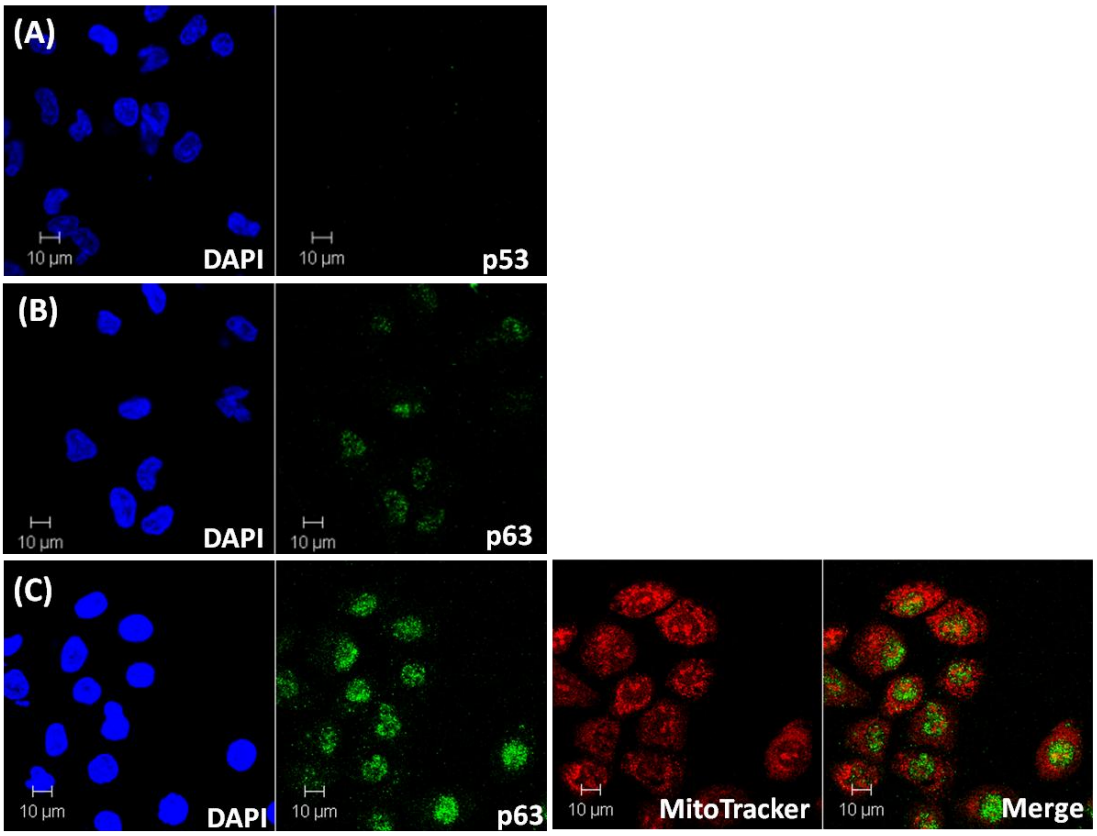


Figure 5.31: Expression of p53 family members in p53-null melanoma cells (U1SO). (A) p53-null cells were confirmed by negative staining for p53 (using anti-p53 antibody DO-1). (B) In keeping with Q-PCR results [Figure 3.3], U1SO cells express p63 (TA isoform), which was predominantly nuclear. (C) Upon treatment with paclitaxel (2 µM) for 6 hrs, marked upregulation of p63 (green) was observed in the nucleus and cytoplasm. Using MitoTracker Orange to fluorescently label mitochondria, no significant localisation of p63 to the mitochondria was observed (Merge panel). DAPI was used to label nuclei (blue). Images are representative of three independent experiments.

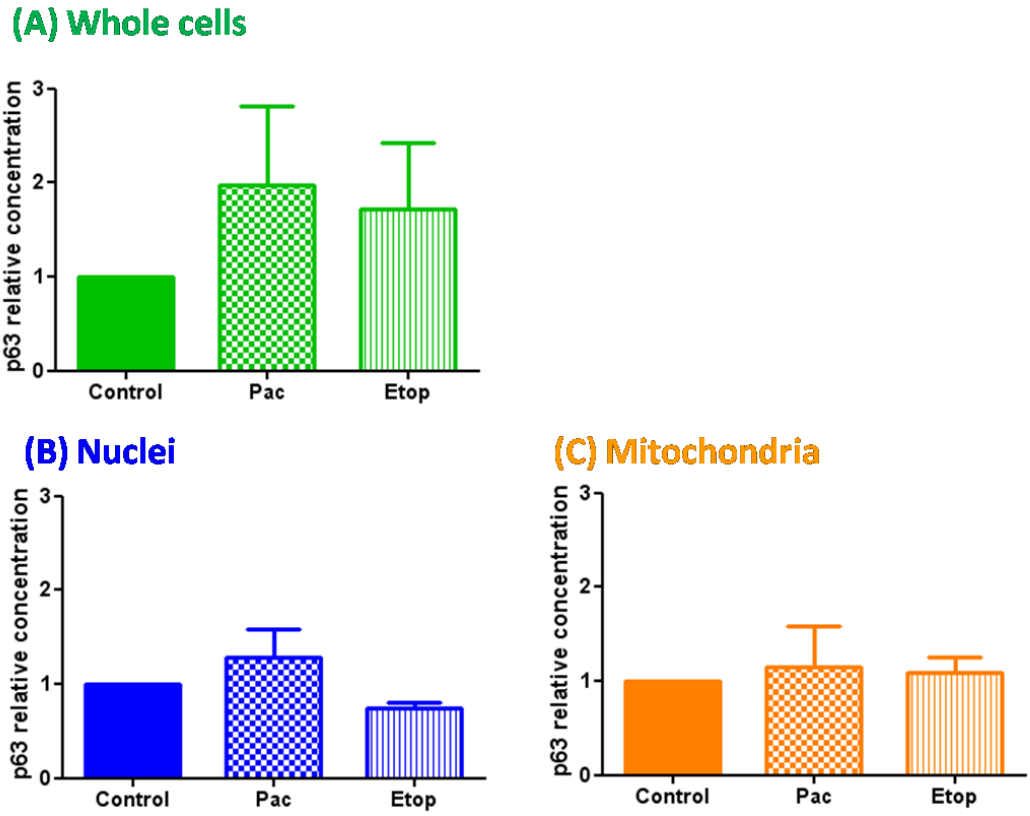


Figure 5.32: p63 requires wt-p53 to translocate to the mitochondria. TP53-null cells (U1SO) were analysed using the flow cytometry fractionation technique, to detect relative expression of p63 in (A) whole cells, demonstrating stabilisation in cells upon treatment with paclitaxel and etoposide. Lysed cells were analysed for expression of p63 in (B) nuclei and (C) mitochondria. Non-significant changes in p63 were detected in p53-null cells upon treatment with paclitaxel (nuclear and mitochondrial p-values 0.58 and 0.43, respectively, t-test) and etoposide (nuclear and mitochondrial p-values 0.67 and 0.06, respectively, t-test). Pac – paclitaxel, etop – etoposide. Data shows mean protein expression +/- SEM for at least three independent experiments performed in duplicate.

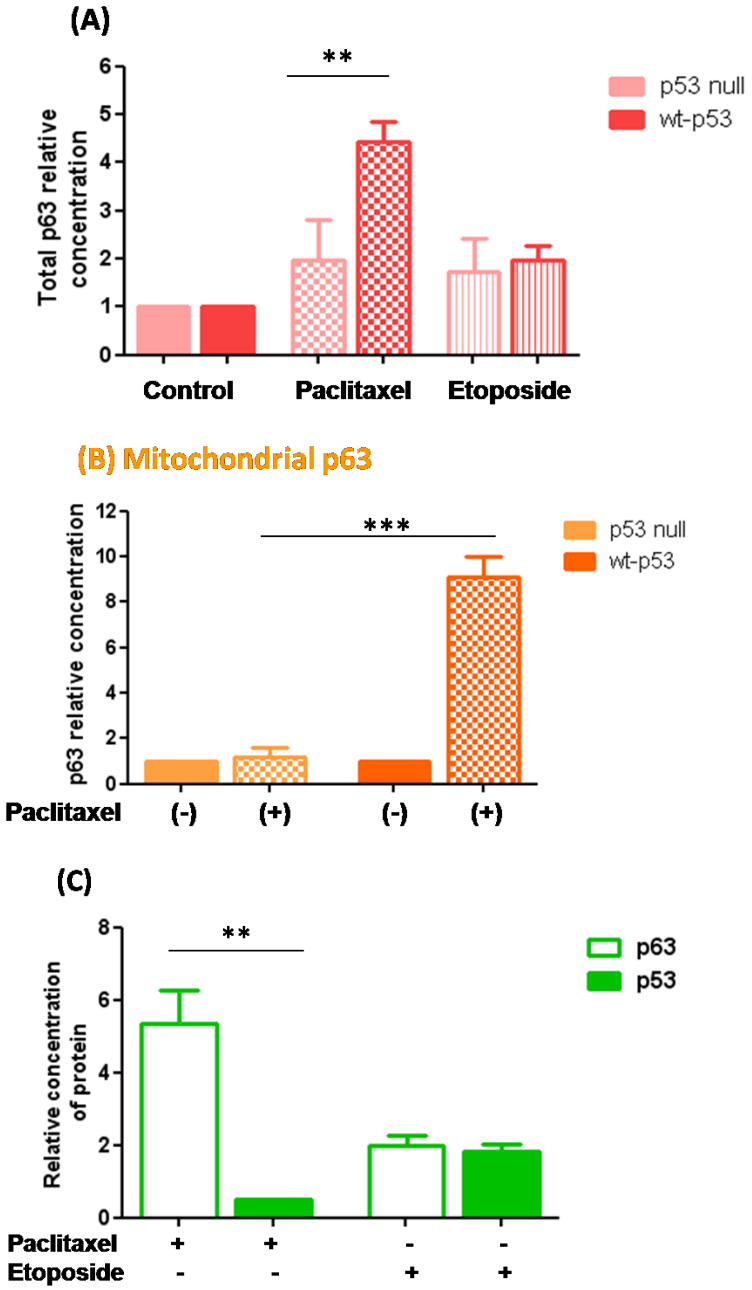


Figure 5.33: p63 translocation to the mitochondria requires wt-p53 (using flow cytometry fractionation technique). (A) Significantly greater p63 is upregulated in wt-p53 expressing melanoma cells, when compared with p53-null cells (p-value 0.0421, t-test) but no difference was observed for etoposide (p-value 0.4105, t-test), despite upregulation of p63 observed in both cell lines. (B) Analysis of p63-Cy5 expression in the mitochondrial fraction of wt-p53 expressing cells and p53-null cells upon treatment with paclitaxel, supported significant upregulation of p63 in the former (p-value 0.0001, t-test). (C) A significant difference between total p53 and p63 concentrations upregulated upon treatment with paclitaxel was observed (p-value 0.0345, t-test) but not for etoposide (p-value 0.6365, ns, t-test) suggesting that p63 was implicated in mediating toxicity of paclitaxel. Data shows mean protein expression +/- SEM for at least three independent experiments performed in duplicate.

5.3.4.6 Translocation of p53 to the mitochondria requires p63

It is well established that p53 translocates to the mitochondria inducing apoptosis (Marchenko et al. 2000; Mihara and Moll 2003; Sayan et al. 2006) and Western blotting data have demonstrated that this may also occur under certain conditions in melanoma cells [Figure 5.15]. Flow cytometry fractionation studies were therefore undertaken to quantify translocation of wt-p53 to the mitochondria in response to the same chemotherapeutic agents in melanoma cells and to further delineate the relationship between p53 and p63.

Upregulation of both p53 and p63 was demonstrated using flow cytometry in A375M cells upon treatment with chemotherapeutic agents [Figure 5.33C]. Significantly greater upregulation of total p63 compared with total p53, was observed in response to paclitaxel, a drug previously reported to utilise p63 to effect apoptosis (Westfall et al. 2005). To quantify translocation of p53 in the presence and absence of p63, A375M cells expressing shRNA-scramble were analysed using flow cytometry fractionation studies and compared to A375M cells expressing shRNA-p63 (clone D) [section 2.2.7.2]. A375M cells were incubated with MitoTracker Orange and treated with paclitaxel and etoposide at different time-points. Early time-points (treatment up to 6 hrs) were chosen because p53 is reported to translocate to the mitochondria rapidly (Erster et al. 2004; Marchenko et al. 2000).

Cells were fixed, permeabilised and labelled with anti-p53 or IgG mouse isotype antibody and secondary Cy5-conjugated anti-mouse antibody followed by incubation with Hoechst [Section 2.3.2]. No marked upregulation of total, nuclear or mitochondrial p53 was detected upon treatment with paclitaxel or etoposide at 1.5 hrs and 3 hrs (data not shown). Stabilisation of total p53 was observed in A375M cells upon treatment with etoposide but not paclitaxel [Figure 5.34B]. In A375M cells depleted of p63 (shRNA-p63), significantly greater accumulation of total p53 was observed, for all treatments [Figure 5.34B]. In conjunction with this, p53 stabilisation occurred in the nucleus upon treatment with etoposide but in the absence of p63, greater p53 stabilisation was observed upon treatment at 6 hrs [Figure 5.34C]. In contrast, depletion of p63 resulted in decreased translocation of p53 to the mitochondria [Figure 5.34D]. These data suggest that p53 requires p63 for its translocation to the mitochondria but its stabilisation in the nucleus is inhibited by p63.

Taken together with experiments using the p53-null cell line, these findings demonstrate stabilisation of both p63 and p53 in the nucleus and translocation of both to mitochondria in melanoma cell lines, upon genotoxic stress. The mechanism for the latter process appears to be one of co-dependence, whereby depletion of one protein limits translocation of the other. Furthermore, these data support an interaction between p63 and p53 suggesting a putative anti-apoptotic role for p63 which might explain the failure of wt-p53 in melanoma.

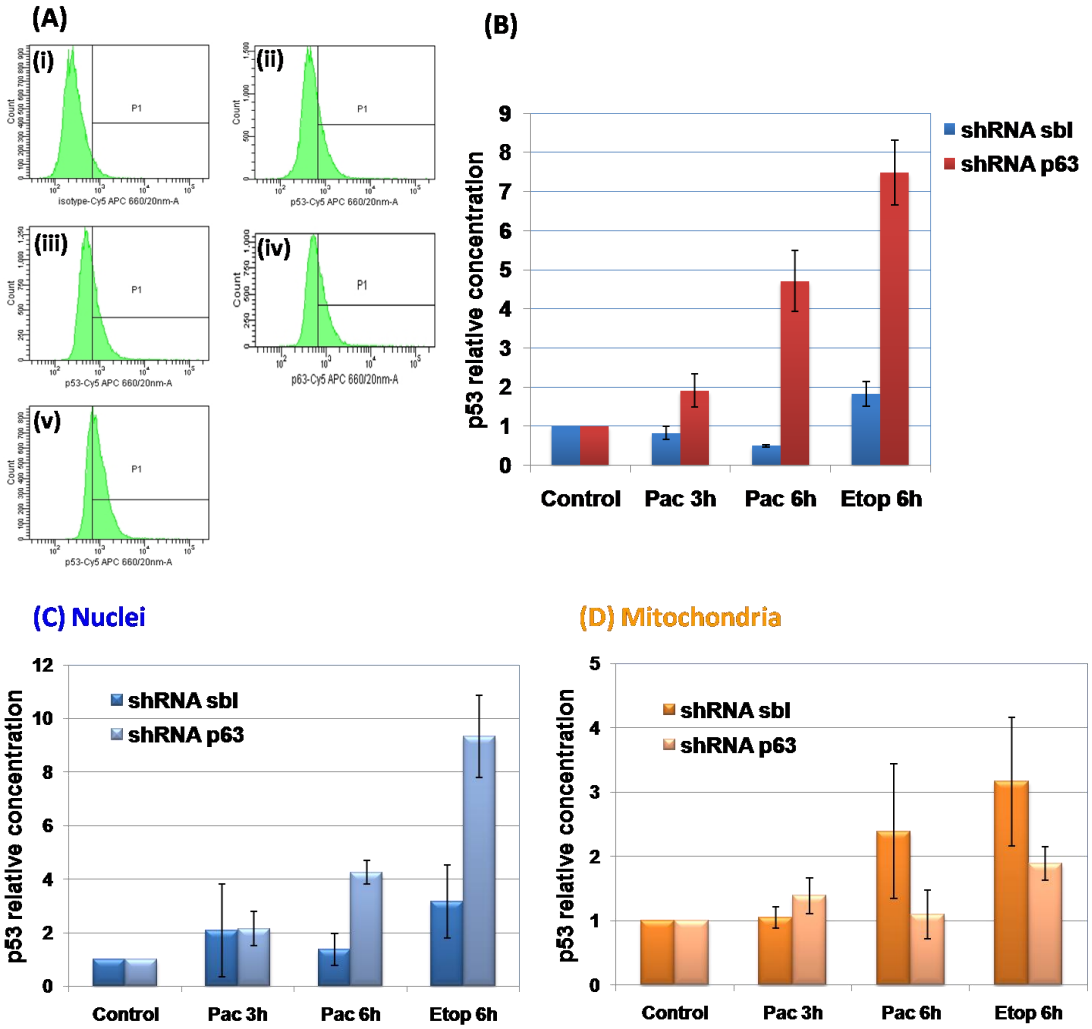


Figure 5.34: Silencing p63 affects wt-p53 response to DNA damage (using flow cytometry fractionation technique). (A) Representative flow cytometry histograms depicting (i) isotope-Cy5 expression in untreated A375M cells silenced for p63, and p53-Cy5 expression in (ii) untreated, and treated A375M cells silenced for p63 with (iii) paclitaxel for 3 hrs, (iv) paclitaxel for 6 hrs and (v) etoposide for 6 hrs. (B) Stabilisation of total p53 was observed in A375M cells upon treatment with etoposide but not paclitaxel. shRNA-p63 cells show significantly greater accumulation of total p53, for all treatments. (C) p53 stabilisation occurred in the nucleus upon treatment with etoposide but in the absence of p63, greater p53 stabilisation was observed upon treatment at 6 hrs. (D) Depletion of p63 led to reduced translocation of p53 to the mitochondria suggesting that p53 requires p63 for its translocation to the mitochondria but its stabilisation in the nucleus is inhibited by p63. Data showing mean protein expression +/- SEM for at least three independent experiments performed in duplicate. Pac – paclitaxel, Etop – etoposide.

5.3.5 p63 is a determinant of chemosensitivity in melanoma

5.3.5.1 Melanoma cell lines display differential sensitivity to chemotherapeutic agents

The translocation of p63 to the mitochondria has been demonstrated by various methods and links it to a putative role in apoptosis. To investigate this, initial experiments assessed the chemosensitivity of a panel of melanoma cell lines to determine if a differential sensitivity might be explained by p63 expression. Initial experiments to assess the effect of different chemotherapeutic agents were performed using the MTT assay [section 2.4.2]. Although this assay is a metabolic analysis of cells, it is an established method with high throughput and reproducibility, used to provide an indirect measure of cell death. Moreover, chemosensitivity testing using the MTT assay has proven to be clinically useful, with correlation of chemosensitivity of drugs and clinical response reported in tumours (Furukawa et al. 1991).

Various chemotherapeutic agents were chosen based on their varied mode of actions [Table 1.6]. Dacarbazine (DTIC) is licensed for first-line treatment of metastatic melanoma (Roberts et al. 2002). Both cisplatin and paclitaxel are used as second line treatments in metastatic melanoma. Recently there has been great interest in activators of p53, particularly in tumours which harbour wt-p53 where the protein appears inactive e.g. MDM2 inhibitors (Vassilev 2007). Tenovin-6 is a small-molecule p53 activator discovered in a cell based screen which demonstrated potent reduction of cell viability in cancer cell lines (Lain et al. 2008). This new drug (provided by Dr S Lain, Karolinska Institute) was tested in a panel of melanoma cell lines to determine its effect on cell viability and compare its efficacy to the more traditional chemotherapies. A dose-dependent reduction in cell viability was observed in cells treated with tenovin-6 [Figure 5.35]. A 30% decrease in cell viability in neonatal human epidermal melanocytes (NHEM) was observed upon treatment with tenovin-6 but increasing doses of the drug did not influence this effect [Figure 5.36A]. A significantly greater reduction in cell viability was observed in metastatic melanoma cell lines upon treatment with tenovin-6 when compared with primary melanoma cells [Figure 5.36].

Cell viability was assessed in a panel of primary melanoma cell lines (Mel 224, Mel 505, WM115, VMM39) and metastatic melanoma cell lines (A375M, WM9, C8161, Mel 501) with differential p63 expression, in response to various chemotherapeutic agents. Dose-dependent reductions in cell viability were observed upon treatment

with various chemotherapeutic agents at 48 hrs. Figure 5.36 is a representative example of dose-dependent toxicity demonstrated in WM1158 cells. Differential sensitivity to chemotherapeutic agents was observed in cell lines treated with the same chemotherapeutic agents [Figure 5.37]. Tenovin-6 demonstrated comparable effects on reduction of cell viability in metastatic melanoma cell lines to paclitaxel and doxorubicin [Figure 5.37B]. Although dacarbazine is licensed for use in the treatment of metastatic melanoma it did not appear to significantly affect apoptosis *in vitro,* confirming previous reports (Mhaidat et al. 2007) [Figure 5.37].

5.3.5.2 p63 mRNA expression correlates with sensitivity to cisplatin and tenovin-6 in melanoma cell lines

Sensitivity of a panel of melanoma cell lines to cisplatin and tenovin-6 determined using the MTT assay, was plotted against the relative mRNA expression of TAp63 and ΔNp63 (from Q-PCR data – section 3.3.1.2) [Figure 5.38]. Non-parametric Spearman correlation analysis was performed demonstrating a significant correlation between expression of TAp63 mRNA and ΔNp63 mRNA with sensitivity to cisplatin [Figures 5.38A, B]. In addition, a significant correlation between expression of TAp63 mRNA and sensitivity to tenovin-6 was also demonstrated [Figure 5.38C]. All cell lines included in the analysis harboured wild-type p53, excluding the possibility that p53 might account for differences.

5.3.5.3 p53 is unlikely to be a determinant of chemosensitivity in melanoma

Although the MTT assay is a useful screening tool to assess cell viability, it is not a direct measure of apoptosis. Therefore a more sensitive method – the Annexin V assay – was used to directly measure apoptosis. Apoptotic cells included Annexin V-FITC+ve/PI -ve and Annexin V-FITC+ve/PI+ve. Four melanoma cell lines (three wt-p53 and one p53-null) were examined following treatment with cisplatin and paclitaxel [Figure 5.39]. Cisplatin induced apoptosis more efficiently than paclitaxel and in metastatic cell lines compared with the primary cell line, Mel 505 [Figure 5.39E]. No significant differences in apoptosis were detected between p53-null (U1SO) and wt-p53 (WM1158) melanoma cells which both expressed TAp63 (cisplatin p-value 0.055, paclitaxel p-value 0.1, Mann Whitney test), suggesting that the presence of p53 was not a determinant of chemosensitivity in these cell lines [Figure 5.39E].

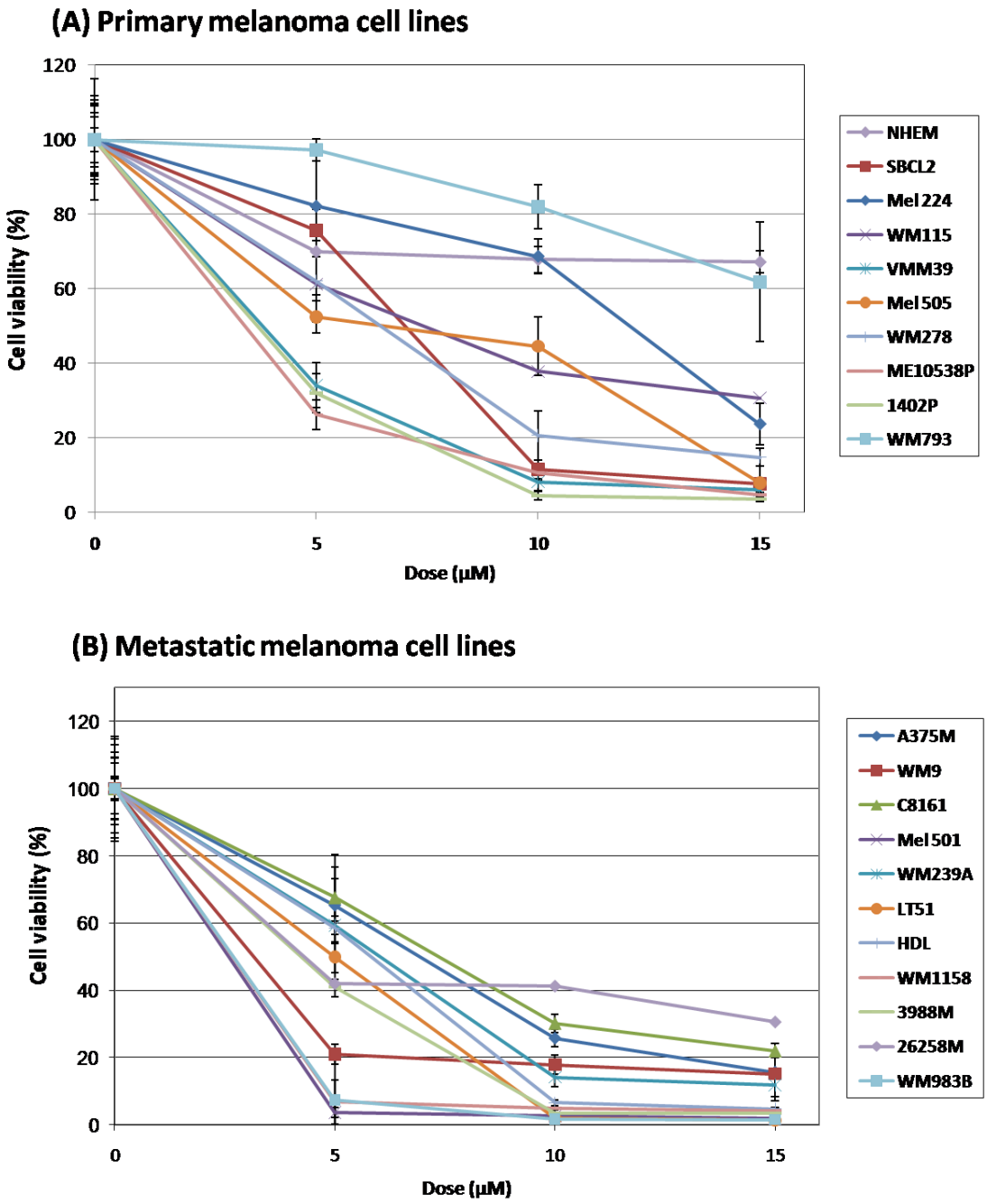


Figure 5.35: Dose-dependent toxicity of tenovin-6 in melanoma cell lines. Cell viability was determined using the MTT assay for (A) a primary melanocyte culture (NHEM) and primary melanoma cell lines, and (B) metastatic melanoma cell lines, treated with increasing doses of tenovin-6 for 48 hrs. Tenovin-6 demonstrated increased efficiency in reducing cell viability in metastatic melanoma cell lines compared with the primary cells providing *in vitro* evidence that this drug should be tested *in vivo.* Data show mean cell viability +/- SEM of three independent experiments performed in triplicate.

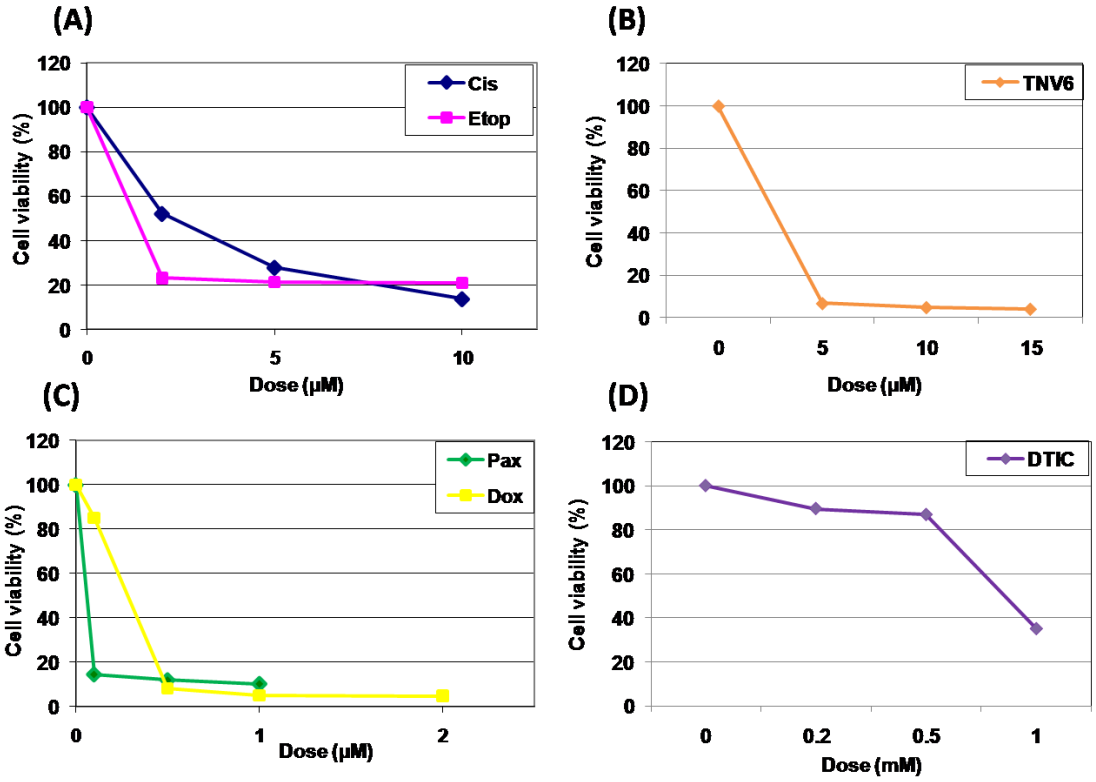


Figure 5.36: Dose-dependent reduction in cell viability observed upon exposure to chemotherapeutic agents (MTT assay). WM1158 cells were seeded at a uniform density of 7500 cells/well in a 96-well plate. Sixteen hours later, cells were treated with chemotherapeutic agents. Cells were treated for 48 hrs with increasing doses of (A) cisplatin and etoposide, (B) tenovin-6, (C) paclitaxel and doxorubicin and (D) dacarbazine. All treatments induced a dose-dependent reduction in cell viability for WM1158 with the exception of dacarbazine. Data shows mean of three independent experiments performed in triplicate. Cis – cisplatin, Etop – etoposide, TNV6 – tenovin-6, Pax – paclitaxel, Dox – doxorubicin, DTIC – dacarbazine.

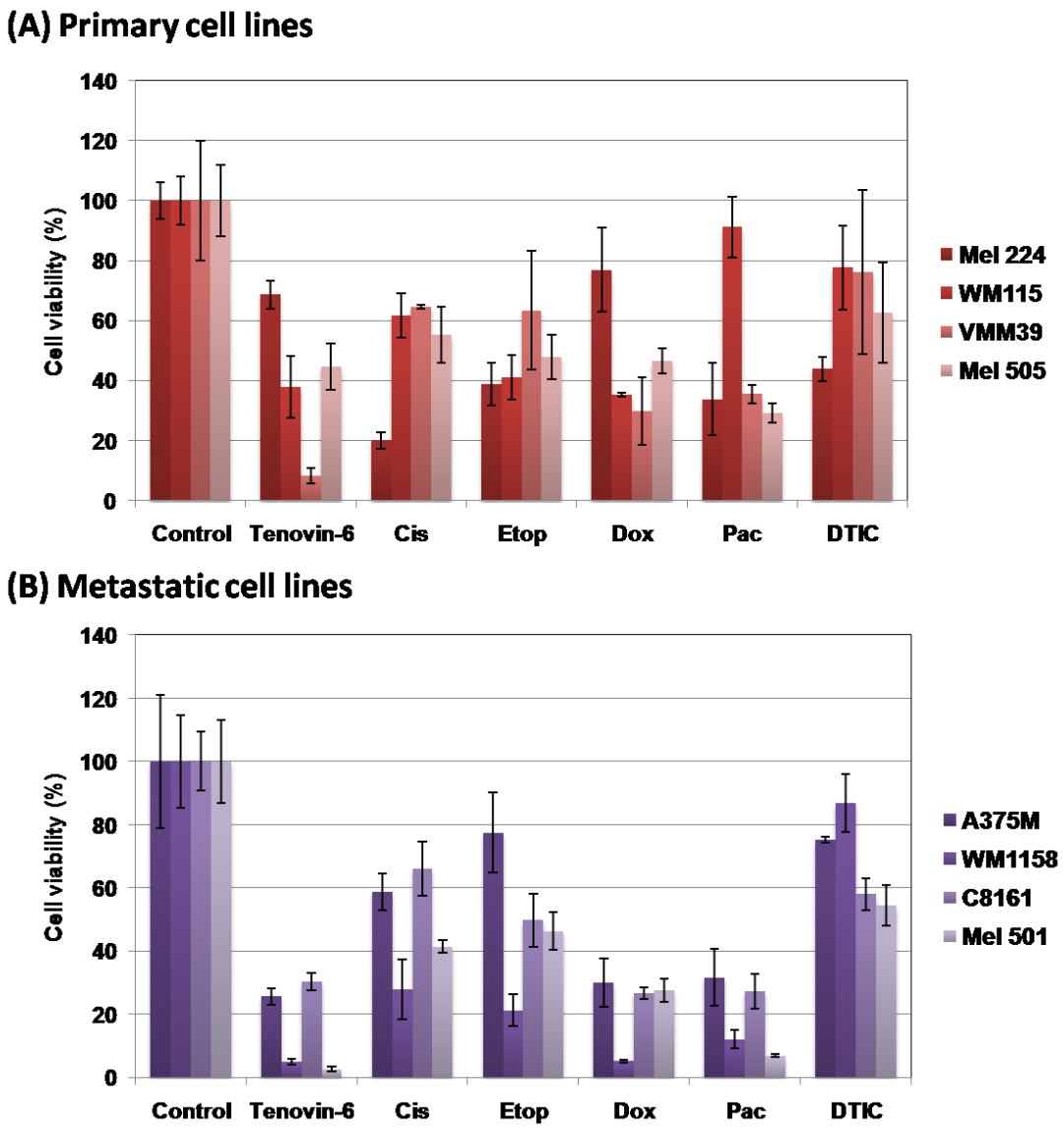


Figure 5.37: Differential sensitivity of melanoma cell lines to chemotherapeutic agents. A panel of (A) primary melanoma cell lines and (B) metastatic melanoma cell lines were treated with various chemotherapeutic agents (tenovin-6 10 µM, cisplatin 5 µM, etoposide 5 µM, doxorubicin 1 µM, paclitaxel 1 µM and dacarbazine 500 µM) and cell viability was measured using the MTT assay. In general, metastatic melanoma cell lines were more sensitive to the chemotherapeutic agents tested, compared with primary melanoma cell lines. A differential sensitivity to drugs was demonstrated. Tenovin-6 showed significant toxicity in metastatic cell lines but dacarbazine showed little reduction in cell viability when used to treat either primary or metastatic melanoma cell lines. Data shown are mean cell viability +/- SEM of three independent experiments performed in triplicate. Cis – cisplatin, Etop – etoposide, Dox – doxorubicin, Pac – paclitaxel, DTIC – dacarbazine.

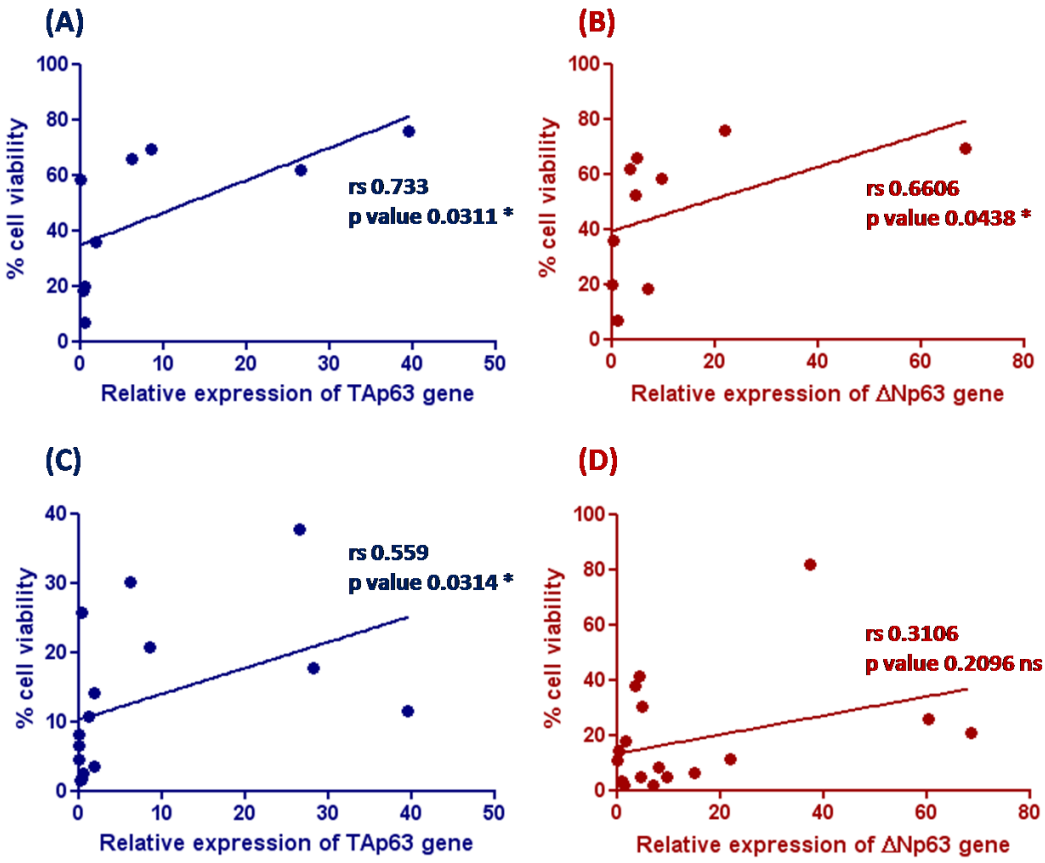


Figure 5.38: TP63 expression is a determinant of chemosensitivity. Sensitivity of melanoma cell lines (Mel 224, WM115, VMM39, A375M, C8161, Mel 501, 26258M, WM9) to (A), (B) cisplatin and (C), (D) tenovin-6 was plotted against the relative expression of (A), (C) TAp63 and (B), (D) ΔNp63. Significant correlation was demonstrated between expression of TAp63 and chemoresistance to (A) cisplatin and (B) tenovin-6. ΔNp63 also demonstrated significant correlation with chemoresistance to cisplatin (D). Gene expression was determined using Q-PCR relative to GUS (endogenous housekeeping gene used for standardisation) compared with mean expression of the gene in five primary melanocyte cultures [section 2.2.5].

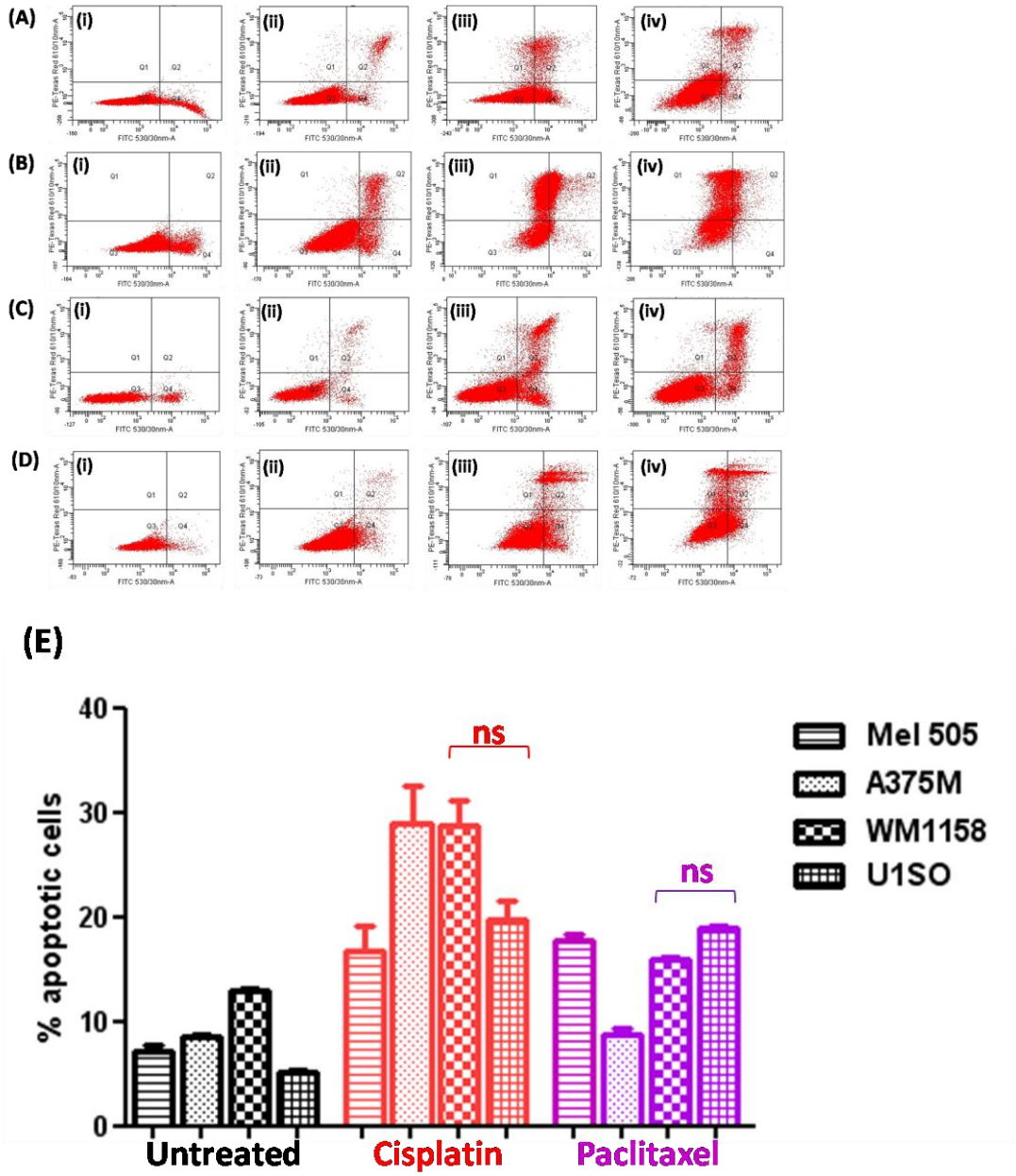


Figure 5.39: Chemosensitivity of melanoma cell lines is not dependent on wt-p53. Cells were treated with chemotherapeutic agents for 24 hrs, trypsinised and all cells (living and dead) were pelleted and analysed using the Annexin-V assay [section 2.5.1]. Flow cytometry profiles are representative of three independent experiments undertaken in (i)(ii) untreated cells, (iii) treatment with cisplatin (10 µM) and (iv) treatment with paclitaxel (2 µM) showing the percentage of apoptotic cells in (A) Mel 505 cells, (B) A375M cells, (C) WM1158 cells and (D) U1SO (p53-null) cells. Panel (i) demonstrates Annexin-V only flow cytometry profile and panel (ii) demonstrates Annexin-V/DAPI labelled cell profile. (E) Histogram showing mean % of apoptotic cells +/- SEM for three independent experiments performed in triplicate. No significant difference between % apoptotic cells was demonstrated between WM1158 cells and U1SO cells (cisplatin p-value 0.055, paclitaxel p-value 0.1, t-test).

5.3.5.4 Depletion of p63 increases chemosensitivity in melanoma

To definitively demonstrate the putative role of p63 in mediating chemosensitivity of melanoma cells, experiments to silence p63 were performed in melanoma cells using RNA interference technology. This involves small non-coding RNAs, which associate with nuclease-containing regulatory complexes and then pair with complementary messenger RNA targets, thereby preventing the expression of mRNAs. Five sequences were used to silence p63 [Figure 5.40]. Three sequences (A, B and C) were designed to knockout both TA and ΔN p63. Sequence D was designed to knockout TAp63 alone as the target sequence was in exon 3, not found in ΔNp63 which has a different promoter. Sequence E was designed to knockout ΔNp63 alone, by targeting exon 3' which is only found in ΔN isoforms as a result of promoter P2 [Figure 5.40].

5.3.5.4.1 *siRNA-p63 increases chemosensitivity in melanoma cell lines*

Silencing of TP63 in WM1158 cells was initially achieved by transfection of a pool of 3 siRNA sequences (siRNA-A, -B and -C) targeting p63 [section 2.2.7.1] and comparing response to cells transfected with siRNA-sbl. Depletion of both TA and ΔN isoforms of p63 by the siRNA-p63 pool [Figure 5.41A(i)] resulted in a significant reduction in cell viability upon treatment with chemotherapeutic agents, when compared to cells transfected with siRNA-scramble [Figure 5.41A(ii)]. These data provided preliminary evidence that depletion of p63 could sensitise melanoma cells to chemotherapy.

The predominant endogenous isoform expressed by WM1158 cells was TAp63 [section 3.3.1]. To determine if the sensitising effect of depletion of p63 was due to depletion of predominantly the TA isoform alone, the same experiment was undertaken in WM278 cells which expressed significant endogenous levels of both TA and ΔN p63. The same pool of three siRNA-p63 sequences was used to deplete both isoforms of p63 resulting in over 70% knockdown of the gene when assessed using Q-PCR [Figure 5.41B(i)]. Cells depleted of p63 were treated with different chemotherapeutic agents and using the MTT assay significant decreases in cell viability were demonstrated in p63 silenced cells [Figure 5.41B(ii)]. When p63 was silenced using a different siRNA-p63 clone (D), greater than 70% knockdown of the gene was achieved and this depletion resulted in significantly increased sensitivity (assessed using the MTT assay) upon treatment with different chemotherapeutic agents [Figure 5.41C]. Significant depletion of both TA and ΔN isoforms was achieved by clone shRNA-D despite its design to target TAp63. On further

examination parts of the target sequence D exhibited sequence similarity in exon 3 and 10 in ΔNp63, providing a possible explanation for the silencing effect demonstrated for this isoform [Figure 5.40(ii)]. An alternative explanation is a regulatory loop may exist between the TA and ΔN isoforms whereby silencing of the TA isoform has a knock-on effect on stability/expression of the ΔN isoform.

Taken together, these data suggest that resistance to chemotherapy observed in melanoma cells could be mediated by the presence of both TA and ΔNp63 isoforms.

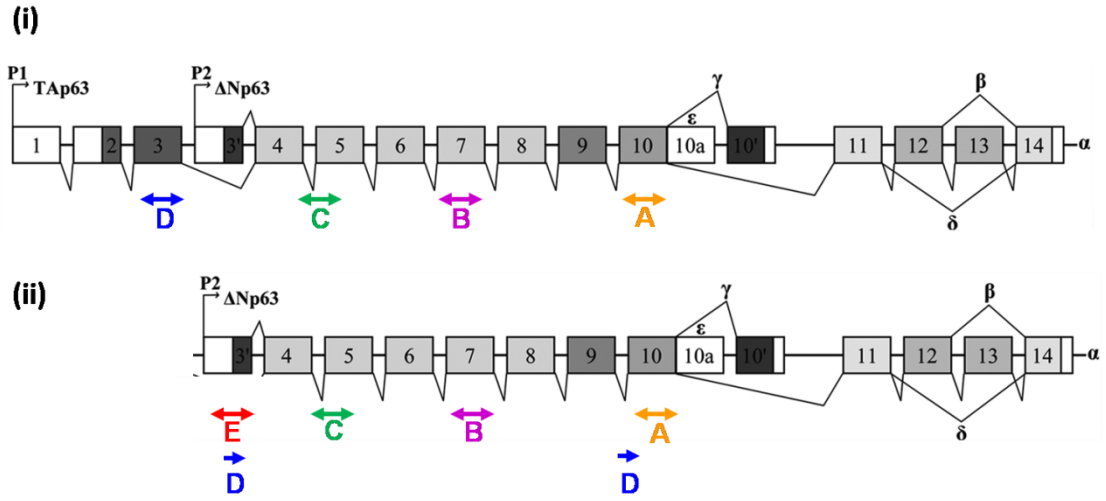


Figure 5.40: RNAi target sequences for silencing TP63. Pictorial representation of (i) TAp63 and (ii) ΔNp63 gene demonstrating targeted sequences employed by various RNAi oligonucleotides (A – E). Sequences A, B and C were designed to target regions in both TA and ΔN isoforms of p63. Sequence D designed to specifically target TAp63 demonstrates sequence similarity in two exons of (ii) ΔNp63. Sequence E was designed to specifically target ΔNp63.

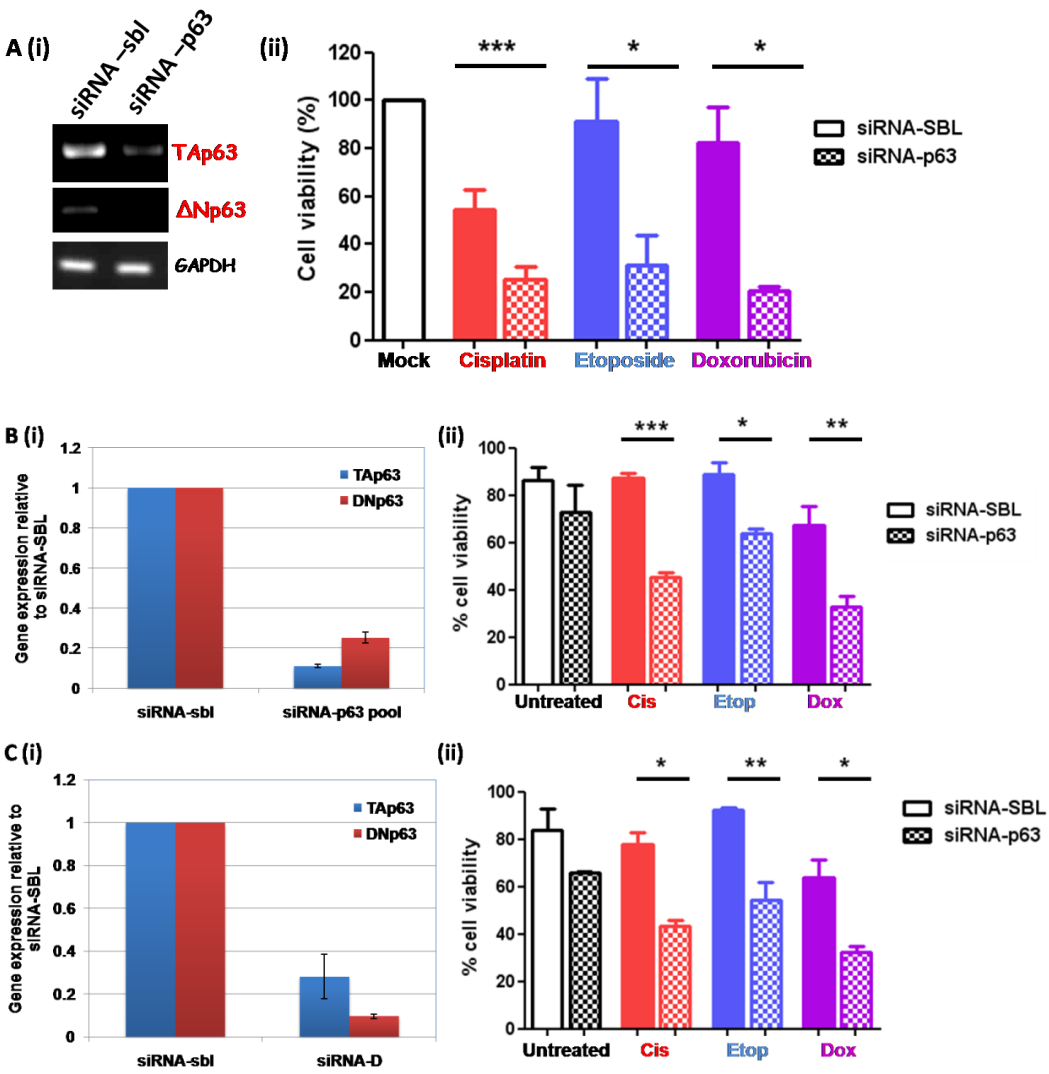


Figure 5.41: siRNA-p63 increases chemosensitivity in melanoma cells. (A) (i) Significant depletion of TAp63 and ΔNp63 was observed following transfection of WM1158 cells with siRNA-p63 pool of 3 sequences (A, B and C) using RT-PCR. (ii) WM1158 cells transfected with either siRNA-scramble sequence or siRNA-p63 pool were seeded in 96-well plates and assessed using the MTT assay [section 2.4.2] and treated for 48 hrs with different chemotherapeutic agents. Cells depleted of p63 demonstrated significant reduction in cell viability in response to treatment with cisplatin, etoposide (both 5 μM) and doxorubicin (1 μM) for 48 hrs when compared with cells transfected with siRNA-scramble (siRNA-SBL). (B) Significant depletion of TAp63 and ΔNp63 was observed following transfection of WM278 cells with siRNA-p63 pool using Q-PCR. (ii) WM278 cells depleted of both isoforms of p63 demonstrated significant reduction in cell viability in response to treatment with cisplatin, etoposide and doxorubicin for 48 hrs when compared with cells transfected with siRNA-SBL. (C) Significant depletion of TAp63 and ΔNp63 was observed following transfection of WM278 cells with siRNA-D sequence using Q-PCR. (ii) WM278 cells depleted of both isoforms of p63 by a different siRNA target sequence (D) confirmed significant reduction in cell viability in response to the same chemotherapeutic agents when compared with cells transfected with siRNA-SBL. Cis – cisplatin, Etop – etoposide, Dox - doxorubicin.

5.3.5.4.2 *shRNA-mediated depletion of p63 increases chemosensitivity of melanoma cell lines*

Although siRNA was effective in silencing p63 (transfection efficiency 60-70%) for up to 72 hrs, short hairpin RNA (shRNA) has the advantage of long-term and stable silencing with improved delivery of silencing to all cells. By using shRNA to silence p63, a more sensitive method to detect apoptosis (Annexin V assay) could be used to confirm that depletion of p63 genuinely affects chemosensitivity in melanoma. As the method used to clone the shRNA constructs requires annealing of two long oligonucleotides the chance of mutation due to synthesis error is high (Miyagishi M 2004) and as a result sequencing confirmed successful cloning of target sequences A, C, D and E [Table 2.10].

The morphology of A375M cells depleted of p63 using four different shRNA-p63 clones was not dissimilar to those stably expressing shRNA-scramble [Figure 5.42] and cell growth was not significantly different. The effects on mRNA expression of p63 in A375M cells with p63 silenced were assayed [Figure 5.43A]. All clones significantly silenced ΔNp63 which was the predominantly expressed isoform of p63 in A375M cells. No difference in TAp63 and ΔNp63 mRNA expression was observed between untransfected A375M cells and A375M cells stably expressing scramble sequences, confirming the shRNA-scramble had no effect on p63 gene expression. To confirm that the effects demonstrated in these experiments were mediated by p63 depletion and not attributed to depletion of other p53 family members, the expression levels of ΔNp73 and p53 were examined using RT-PCR. No marked reduction of p53 or ΔNp73 was observed in A375M cells expressing shRNA-p63 clones [Figure 5.43B].

Apoptosis was assessed at 24 hrs, using the Annexin-V assay, in A375M cells expressing each of the four clones of shRNA-p63 upon treatment with different chemotherapeutic agents. A significant increase in apoptosis was observed with all shRNA-p63 clones when compared with shRNA-sbl in cells treated with cisplatin [Figure 5.44]. To extend this experiment, another cell line (WM1158) in which the predominant isoform expressed was TAp63 was tested. No difference in morphology was observed between cell lines expressing the four shRNA-p63 clones A, C, D and E [Figure 5.45]. Significant depletion of both TA and ΔN isoforms was demonstrated by clone shRNA-D despite its design to target TAp63 only [Figure 5.46A]. mRNA analysis in untransfected cells confirmed no effect of shRNA-sbl on expression of

p63. Increased chemosensitivity to cisplatin and paclitaxel was similarly demonstrated by shRNA-p63 clone D in WM1158 cells [Figure 5.46C].

Although one could argue for possible depletion of ΔNp73 by the shRNA clones in A375M cells accounting for the increased sensitivity to cisplatin, ΔNp73 was not detected in WM1158 cells [Figure 5.46A(ii)]. These data therefore support the interpretation that the sensitising effect of p63 depletion demonstrated in A375M cells was attributed to silencing of p63 alone. To summarise these data, depletion of both TA and ΔN p63 results in increased chemosensitivity of melanoma cells, implicating p63 as an anti-apoptotic factor in melanoma.

5.3.5.4.3 *p63 does not mediate sensitivity to dacarbazine*

To determine if the sensitising effect of p63 depletion might have a clinically relevant therapeutic application, experiments were extended to include dacarbazine, a drug currently licensed for use in the treatment of metastatic melanoma. Dose dependent experiments in WM1158 cells, revealed no increased sensitivity to the drug at various doses [Figure 5.47]. These data support earlier experiments using the MTT assay demonstrating that dacarbazine does not utilise an apoptotic pathway mediated by p63 to achieve its therapeutic effect in melanoma [Figure 5.37].

5.3.5.4.4 *Both TA and ΔN isoforms contribute to chemoresistance in melanoma*

One clone (shRNA-E) designed to target ΔNp63 at exon 3' [Figure 5.40] had an unexpected effect on gene expression by significantly increasing expression of TAp63 but silencing ΔNp63 in both A375M and WM1158 cells [Figure 5.48]. In both these cell lines, this clone did not affect sensitivity to either cisplatin or paclitaxel [Figure 5.48]. Taken together, these chemosensitivity experiments demonstrate depletion of p63 is capable of sensitising melanoma cells to cisplatin and possibly paclitaxel. To achieve this effect depletion of both TA and ΔN p63 is required, implicating an oncogenic role for both isoforms in melanoma.

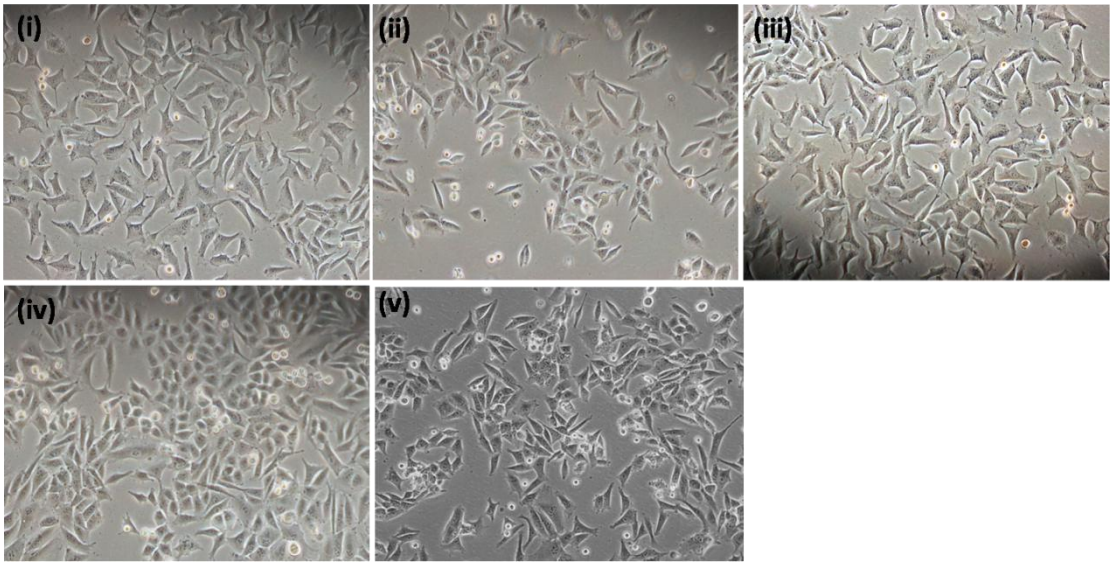


Figure 5.42: Morphology of A375M cells transfected with shRNA-p63 target sequences. Phase contrast microscopy images of A375M cells transfected with (i) shRNA-scramble, and shRNA-p63 sequences (ii) shRNA-A, (iii) shRNA-C, (iv) shRNA-D, (v) shRNA-E demonstrated no obvious change of morphology or proliferation rate following depletion of p63. Magnification x20.

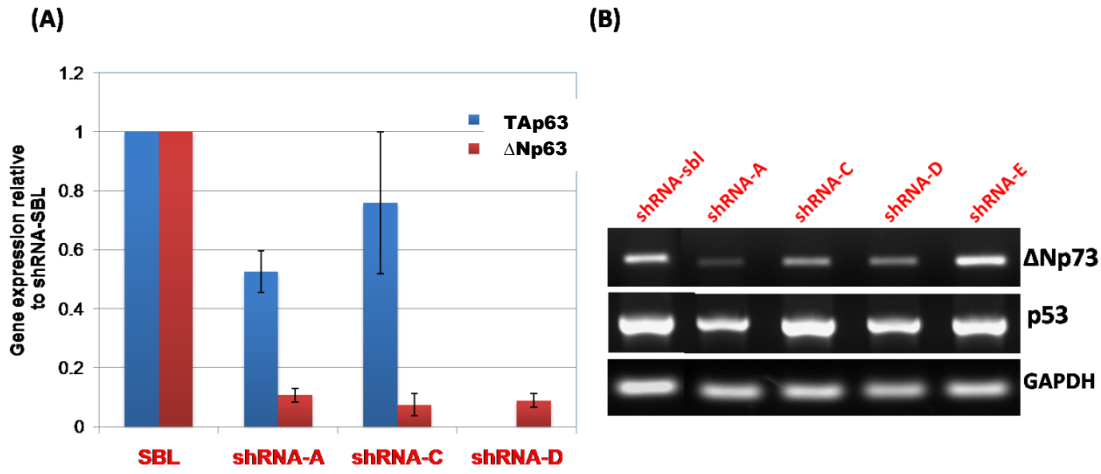


Figure 5.43: Expression of p53 family members following transfection with shRNA-p63 (A375M cells). (A) Q-PCR of cells transfected with three different shRNA-p63 sequences demonstrates significant reduction of ΔNp63 for all three when compared with shRNA-Scramble transfected cells. Significant TAp63 reduction was achieved using shRNA-D although endogenous expression of TAp63 in A375M cells was already very low. GUS was used as the housekeeping gene for standardisation. Data shown is mean +/- SD expression of gene from three independent experiments. (B) Using RT-PCR, depletion of ΔNp73 was noted in cells depleted of p63 but no marked depletion of p53 was demonstrated. GAPDH was used as the housekeeping gene for mRNA standardisation.

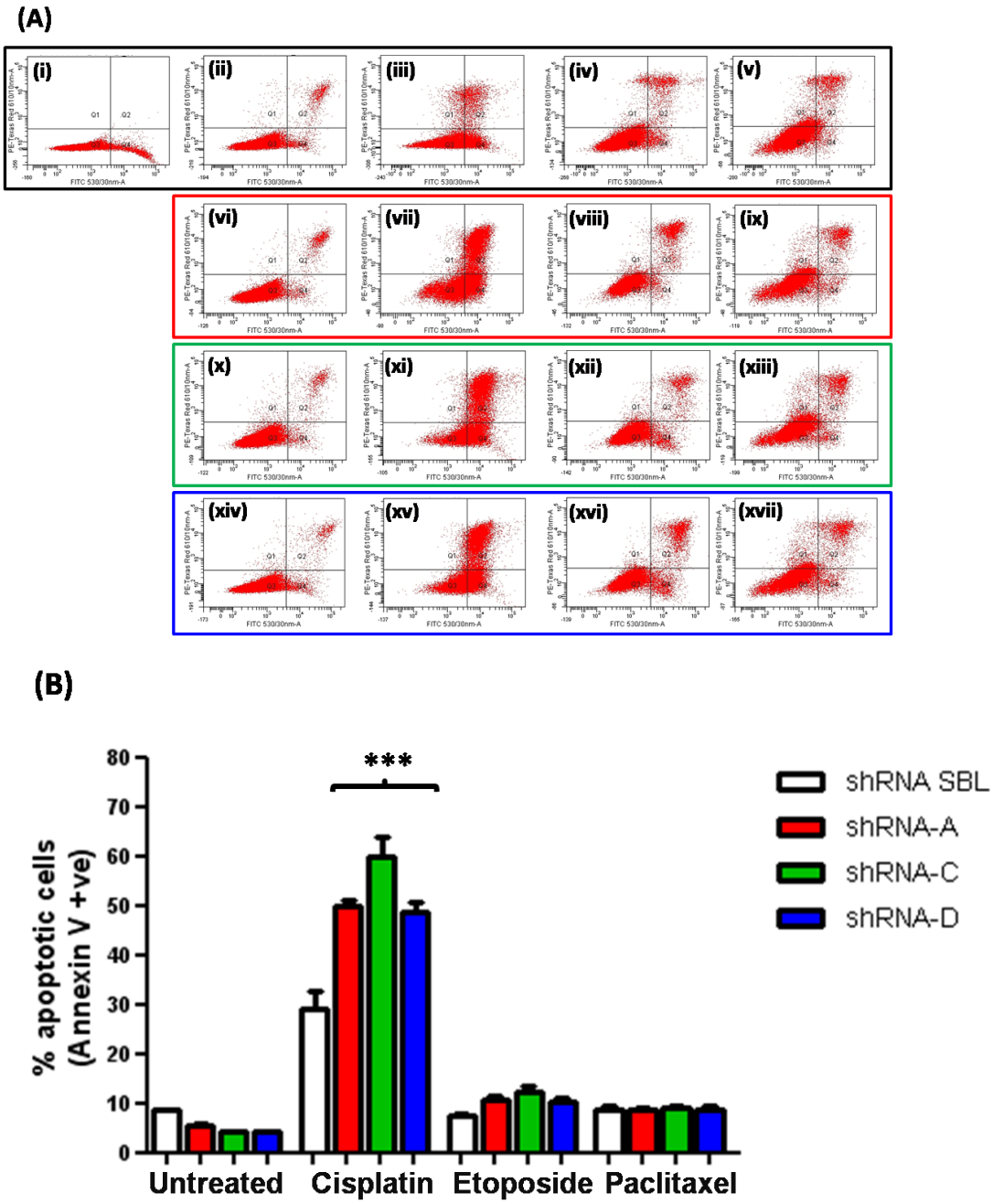


Figure 5.44: Depletion of p63 confers significant chemosensitivity to cisplatin. (A) A375M cells transfected with shRNA clones (i-v) shRNA-scramble, (vi – ix) shRNA-A, (x-xiii) shRNA-C and (xiv – xvii) shRNA-D were treated with cisplatin (10 µM), etoposide (10 µM) and paclitaxel (2 µM). Flow cytometry scatter plots demonstrate Annexin-V assay of apoptosis in A375M cells with p63 silenced by different shRNA clones. (i) Annexin-V only, scatter plot of untreated shRNA-scramble cells. (ii, vi, x, xiv) Annexin-V/DAPI plot for untreated cells with different shRNA clones. (B) The percentage of apoptotic cells (Annexin-V positive) are displayed in the graph as the mean ± SEM for three independent experiments performed in duplicate. Significantly increased apoptosis was detected between shRNA-SBL and shRNA-p63 clones was shown demonstrating that silencing p63 increases chemosensitivity to cisplatin (p-value <0.0001, *one way Anova*).

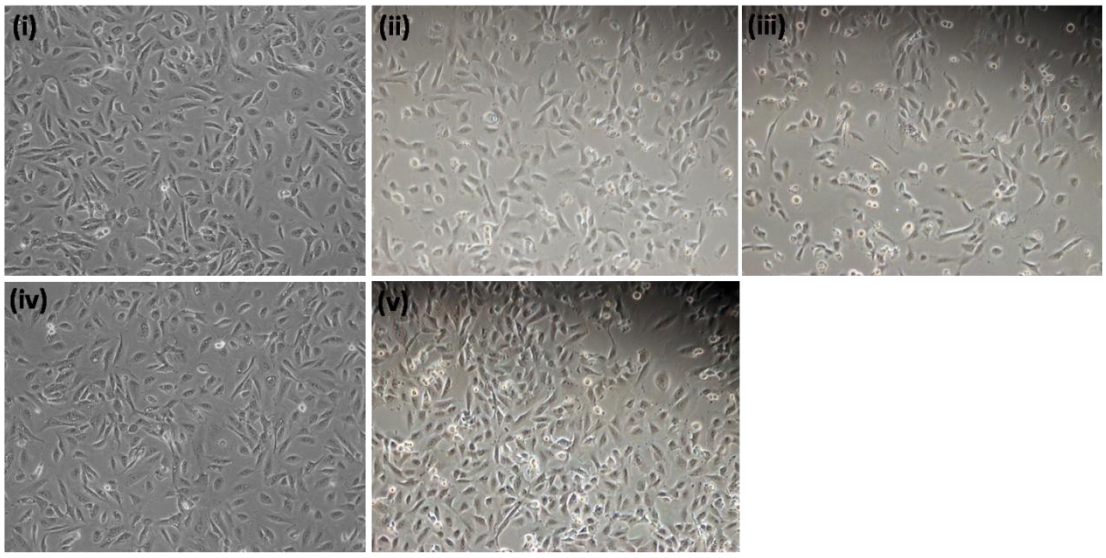


Figure 5.45: Morphology of WM1158 cells transfected with shRNA-p63 target sequences. Phase contrast microscopy images of WM1158 cells transfected with (i) shRNA-scramble, and shRNA-p63 sequences (ii) shRNA-A, (iii) shRNA-C, (iv) shRNA-D, (v) shRNA-E demonstrated no obvious change of morphology following depletion of p63. Magnification x20.

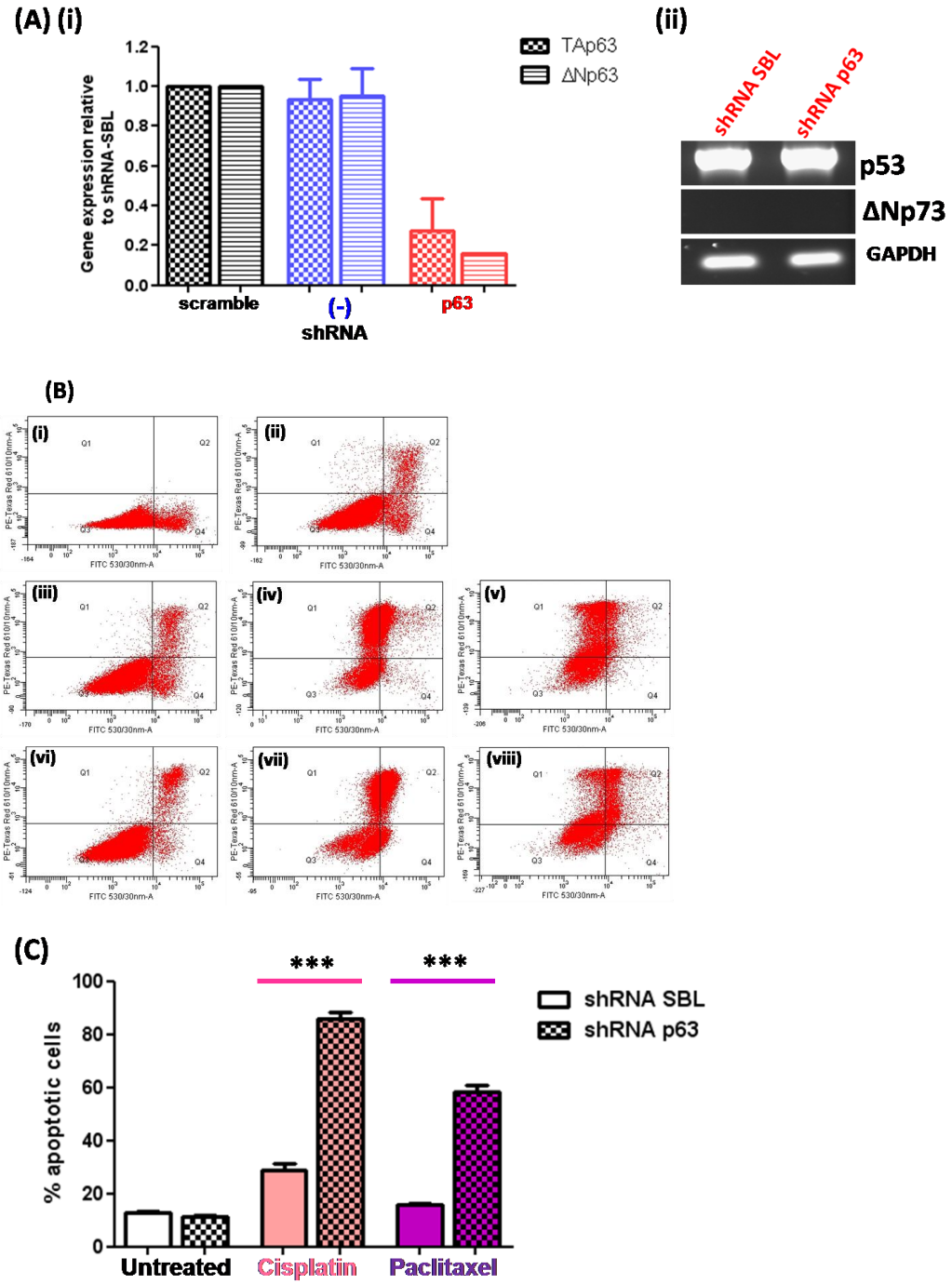


Figure 5.46: Depletion of p63 by shRNA-p63 clones increases chemosensitivity. (A) TP53 family members were examined in WM1158 cells. (i) No difference in p63 was observed between cells transfected with shRNA-scramble and mock transfection (-) but significant reduction of both TA and ΔNp63 was achieved by shRNA-p63 clone D assessed by Q-PCR. GUS was used as the endogenous comparator. Data shows mean +/- SD of three independent experiments. (ii) Using RT-PCR shRNA-p63 had no effect on TP53 mRNA. ΔNp73 was not expressed in WM1158 cells. GAPDH was used as the housekeeping gene for standardisation. (B) Flow cytometry scatter plots showing Annexin V apoptosis assay for

WM1158 transfected with (i – v) shRNA-SBL, (vi – viii) shRNA-p63, (i-iii and vi) untreated and treated with (iv and vii) cisplatin, or (v and viii) paclitaxel. (i) Annexin V only shRNA-SBL cells, (ii) Annexin V / DAPI scatter plot for shRNA-SBL cells. (C) The percentage of apoptotic cells (Annexin-V positive – Q2 + Q4) are displayed in the graph as the mean ± SEM for three independent experiments performed in duplicate. Significantly increased apoptosis was detected in shRNA-p63 WM1158 cells when compared with shRNA-scramble cells upon treatment with either cisplatin (p-value 0.0009, t-test) or paclitaxel (p-value 0.0037, t-test).

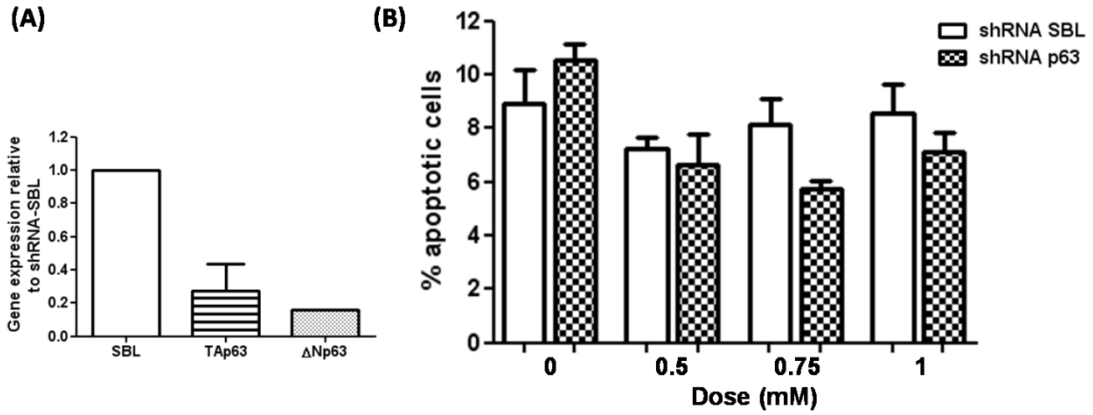


Figure 5.47: Depletion of p63 has no effect on chemosensitivity to dacarbazine. (A) Significant reduction of both TA and ΔNp63 was achieved by shRNA-p63 clone D in WM1158 cells assessed by Q-PCR when compared to shRNA-scramble (shRNA-SBL). GUS was used as the endogenous comparator. Data shows mean +/- SD of three independent experiments. (B) The percentage of apoptotic cells (Annexin-V positive) in transfected cell upon treatment with dacarbazine at increasing doses are displayed in the graph as the mean ± SEM for three independent experiments performed in duplicate. No significant difference in apoptosis was detected between WM1158 cells depleted of p63 when compared with shRNA-scramble cells upon treatment with any dose of dacarbazine (500 µM p-value 0.742; 750 µM p-value 0.2051 and 1 mM p-value 0.5073, t-test).

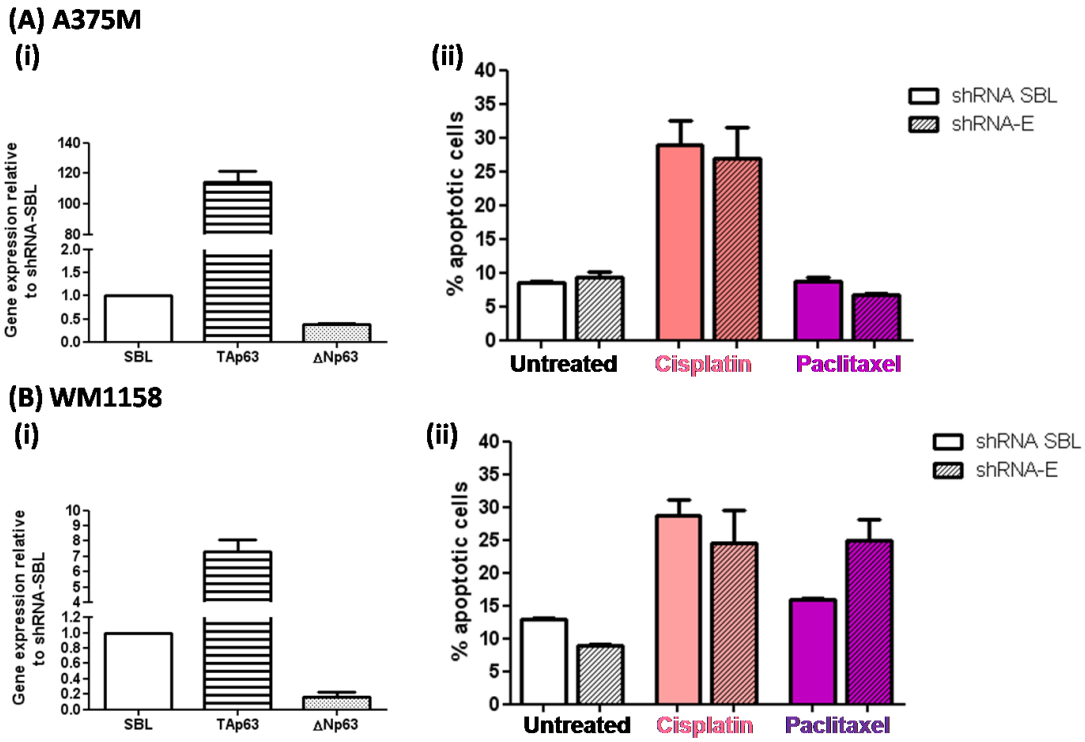


Figure 5.48: Depletion of both TA and ΔN isoforms of p63 are required to chemosensitise melanoma. Significant reduction of ΔNp63 but marked upregulation of TAp63 was achieved by shRNA-p63 clone E in (A) (i) A375M cells and (B) (i) WM1158 cells assessed by Q-PCR when compared to shRNA-scramble (shRNA-SBL). GUS was used as the endogenous comparator. Data shows mean +/- SD of three independent experiments. The percentage of apoptotic cells (Annexin-V positive) in (A) (ii) A375M cells and (B) (ii) WM1158 cells upon treatment with cisplatin (10 µM) and paclitaxel (2 µM) for 24 hrs are displayed in the graph as the mean percentage of apoptotic cells ± SEM for three independent experiments performed in duplicate. No significant difference in apoptosis was detected between either A375M or WM1158 cells with depletion of ΔNp63 only, when compared with shRNA-scramble cells upon treatment with either cisplatin or paclitaxel. (A375M treated with cisplatin p-value 0.6973, paclitaxel p-value 0.6531; WM1158 treated with cisplatin p-value 0.3187, paclitaxel p-value 0.1273; t-test).

5.3.5.5 Transfection of p63 isoforms confers chemoresistance in melanoma

As silencing of p63 conferred sensitivity to chemotherapeutic agents (in particular cisplatin), experiments were performed to determine if expression of p63 isoforms in a p63-null cell line conferred resistance to treatment. Expression vectors for each p63 isoform were transiently transfected into Mel 505 cells which express almost no endogenous p63 [Section 2.1.5.1.1]. Transfection efficiency was determined to be approximately 40-50% as confirmed using immunofluorescence microscopy (data not shown). Transfected cells were assayed for protein and mRNA expression of p63. All exogenous p63 isoforms were expressed and detected albeit at a variable level for each isoform [Figure 5.49]. To further investigate any relationship between p63 and ΔNp73, the expression of ΔNp73 was assessed in Mel 505 cells transfected with each isoform. Both TA and ΔN β and γ isoforms induced expression of ΔNp73. Although the TA isoforms have been reported to induce expression of ΔNp73 (Petitjean et al. 2008) this is the first report that the ΔN isoforms also possess this ability [Figure 5.49]. Apoptosis was assessed (using the Annexin-V assay) in Mel 505 cells transfected with each isoform [Figure 5.50] and upon treatment with cisplatin (30 μM), etoposide (30 μM) and paclitaxel (2 μM) [Figure 5.51]. All isoforms conferred resistance to at least one of the drugs.

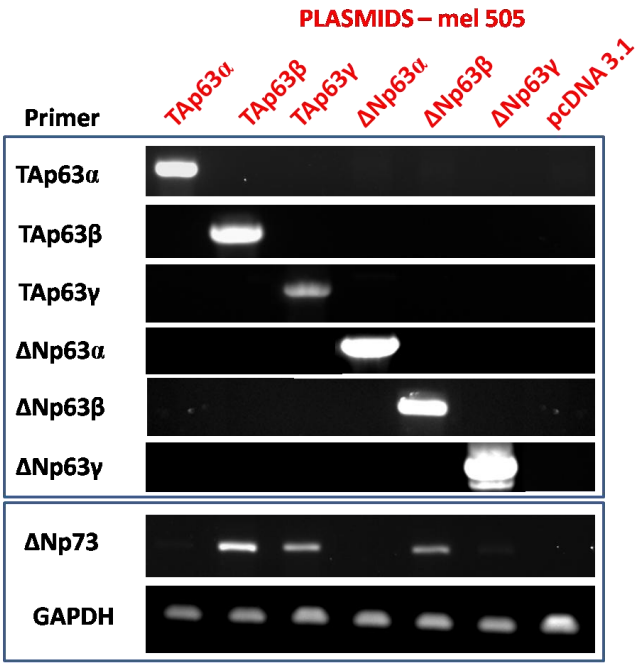


Figure 5.49: Successful transfection of Mel 505 cells with p63 plasmids. Plasmids for six isoforms of p63 were successfully transfected into Mel 505 melanoma cells and using isoform specific primers confirmed specificity using RT-PCR. ΔNp73 expression was also induced by transfection of all TA isoforms of p63 (TAα to a very low extent) but also ΔNp63 β and γ

isoforms. Gene expression standardisation was confirmed using GAPDH as the housekeeping gene. The plasmid pcDNA 3.1 was transfected for control.

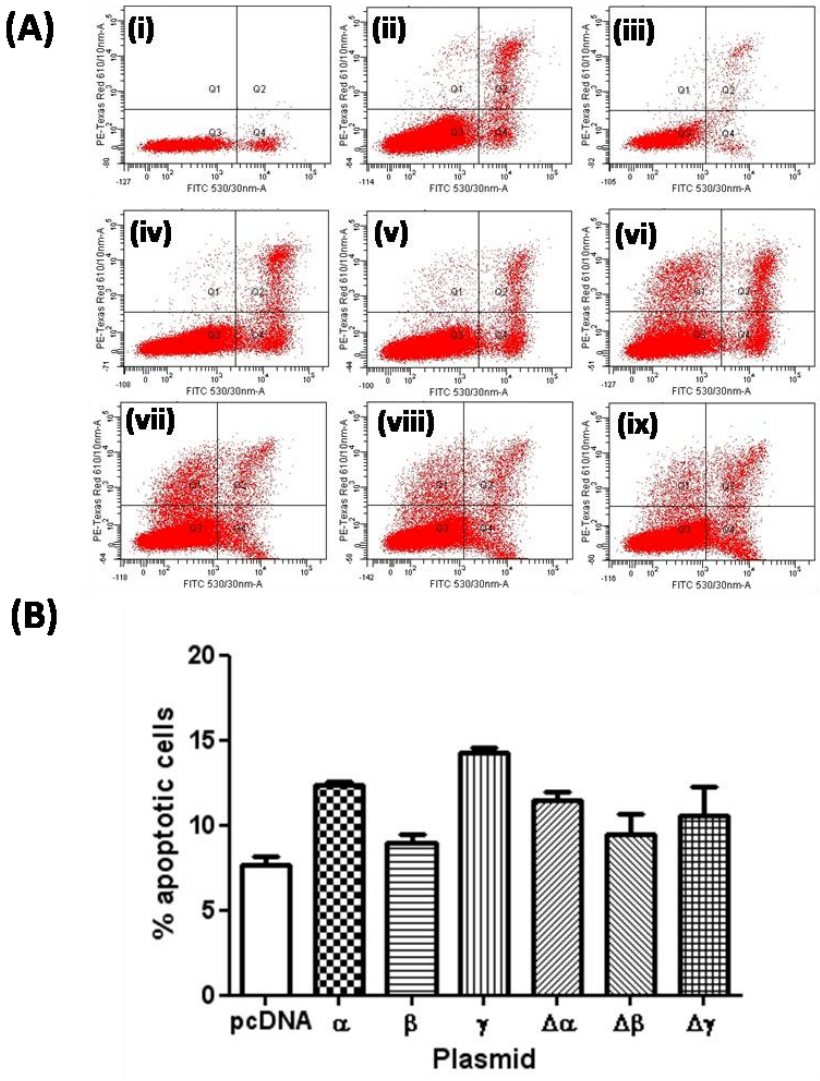


Figure 5.50: Apoptosis assay in Mel 505 cells transfected with p63 isoforms. (A) Representative flow cytometry scatter plots showing untreated Mel 505 cells (i) Annexin-V only plot for untransfected cells, Annexin-V/DAPI plots for (ii) untransfected cells, transfected cells with (iii) pcDNA3.1 cells (iv) TAα isoform, (v) TAβ isoform, (vi) TAγ isoform, (vii) ΔNα isoform, (viii) ΔNβ isoform, (ix) ΔNγ isoform. (B) The percentage of apoptotic cells (Annexin V positive – Q2 + Q4) for Mel 505 cells transfected with plasmids for each isoform is shown. Data shown are mean percentage of apoptotic cells +/- SEM of three independent experiments each performed in triplicate.

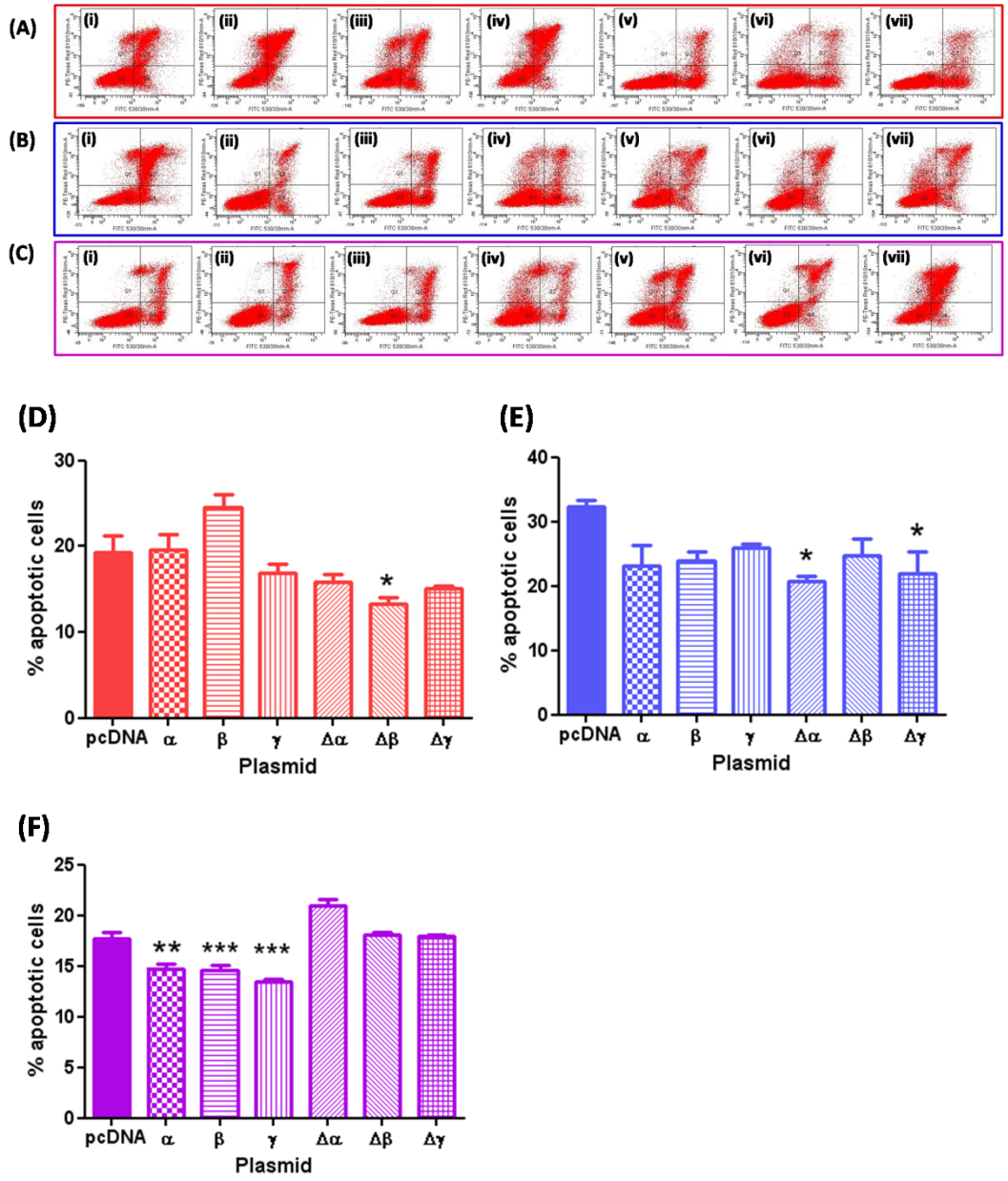


Figure 5.51: Chemoresistance in melanoma demonstrated by p63 isoforms. (A), (B), (C) Representative Annexin-V/DAPI flow cytometry scatter plots showing mel 505 cells transfected with (i) pcDNA3.1, (ii) TA α isoform, (iii) TAβ isoform, (iv) TAγ isoform, (v) ΔNα isoform, (vi) ΔNβ isoform, (vii) ΔNγ isoform and treated with (A) cisplatin (10 μM), (B) etoposide (10 μM) and (C) paclitaxel (1 μM) for 24 hrs. The percentage of apoptotic cells (Annexin V positive – Q2 + Q4) for Mel 505 cells transfected with plasmids for each isoform is shown upon treatment with (D) cisplatin, (E) etoposide and (F) paclitaxel. Significant reduction in apoptosis upon treatment with cisplatin was demonstrated by transfection with ΔNp63β (p-value < 0.05, ANOVA). Significant reduction in apoptosis upon treatment with etoposide was demonstrated by transfection with ΔNp63α and ΔNp63γ (p-values < 0.05, ANOVA). Significant reduction in apoptosis upon treatment with paclitaxel was demonstrated by transfection with all TAp63 isoforms (p-values < 0.05, ANOVA). Data shown are mean values +/- SEM of three independent experiments each performed in triplicate.

5.3.6 CD133 has a role in chemoresistance of melanoma

5.3.6.1 CD133 labels cells involved in apoptosis in melanoma

Evidence demonstrating ΔNp63 enrichment in the CD133-positive melanoma stem cell population and a role for p63 to confer chemoresistance in melanoma cells, led to a series of experiments to link the CD133 stem cell population (enriched with p63) to apoptosis. In light of data confirming upregulation of p63 in response to chemotherapeutic agents [section 5.3.2] and enrichment of the CD133 population with ΔNp63 [section 3.3.6.2], it was necessary to determine if upregulation of the CD133 population occurred. Expression of CD133-PE was measured using flow cytometry compared to IgG-PE mouse isotype controls. Using two melanoma cell lines with low endogenous levels of CD133, upregulation was detected upon DNA damage with paclitaxel and UVB [Figure 5.52]. These data provide a possible source of chemoresistance in melanoma through upregulation of ΔNp63 in the CD133-positive cell fraction. In head and neck cancer, ΔNp63 has been demonstrated to be an anti-apoptotic, anti-differentiation and pro-survival factor (Rocco et al. 2006; Sniezek et al. 2004) and these data suggest that the ΔN isoform is acting in this way in the CD133-enriched population.

5.3.6.2 CD133-population mediates chemoresistance in melanoma

To link the upregulation of CD133+ve expression levels (and ΔNp63) to apoptosis, the percentage of apoptotic cells was assayed using Annexin-V in CD133-positive and -negative populations. A375M cells (either treated or untreated) were labelled with anti-CD133/1 (AC133)-PE antibody (Miltenyi Biotec) and passed through a cell strainer cap before analysing on the FACS Aria™ Flow Cytometer (BD Biosciences). Sort gates were placed on CD133-positive and CD133-negative cells after comparing with the isotype control. Despite upregulation of the CD133-positive population in response to various genotoxic agents (cisplatin, paclitaxel and UVB), significantly more apoptotic cells were consistently detected in the CD133-negative populations [Figure 5.53] suggesting that resistance to apoptosis was mediated at least in part, by CD133-positive melanoma stem cells (which are enriched for ΔNp63).

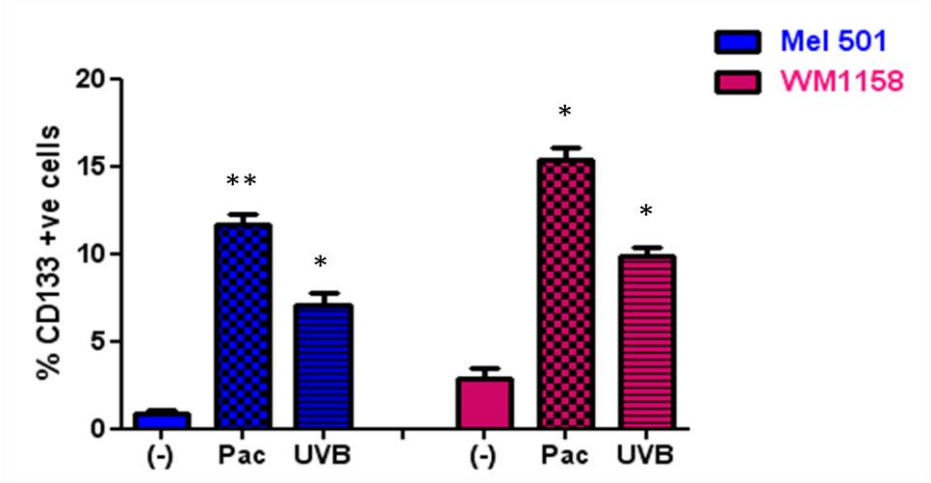


Figure 5.52: Upregulation of CD133-positive fraction upon DNA damage. Metastatic melanoma cell lines (Mel 501 and WM1158) were treated with paclitaxel (1 µM) and UVB (40 mJ/cm^2) for 16 hrs and then labelled with CD133-PE and analysed using flow cytometry. Significant upregulation of CD133-positive Mel 501 cells were detected upon treatment with paclitaxel (p-value 0.0018, t-test) and UVB (p-value 0.0193, t-test) and CD133-positive WM1158 cells upon treatment with paclitaxel (p-value 0.0109, t-test) and UVB (p-value 0.0215, t-test). Data shown are mean values +/- SEM from three independent experiments performed in duplicate.

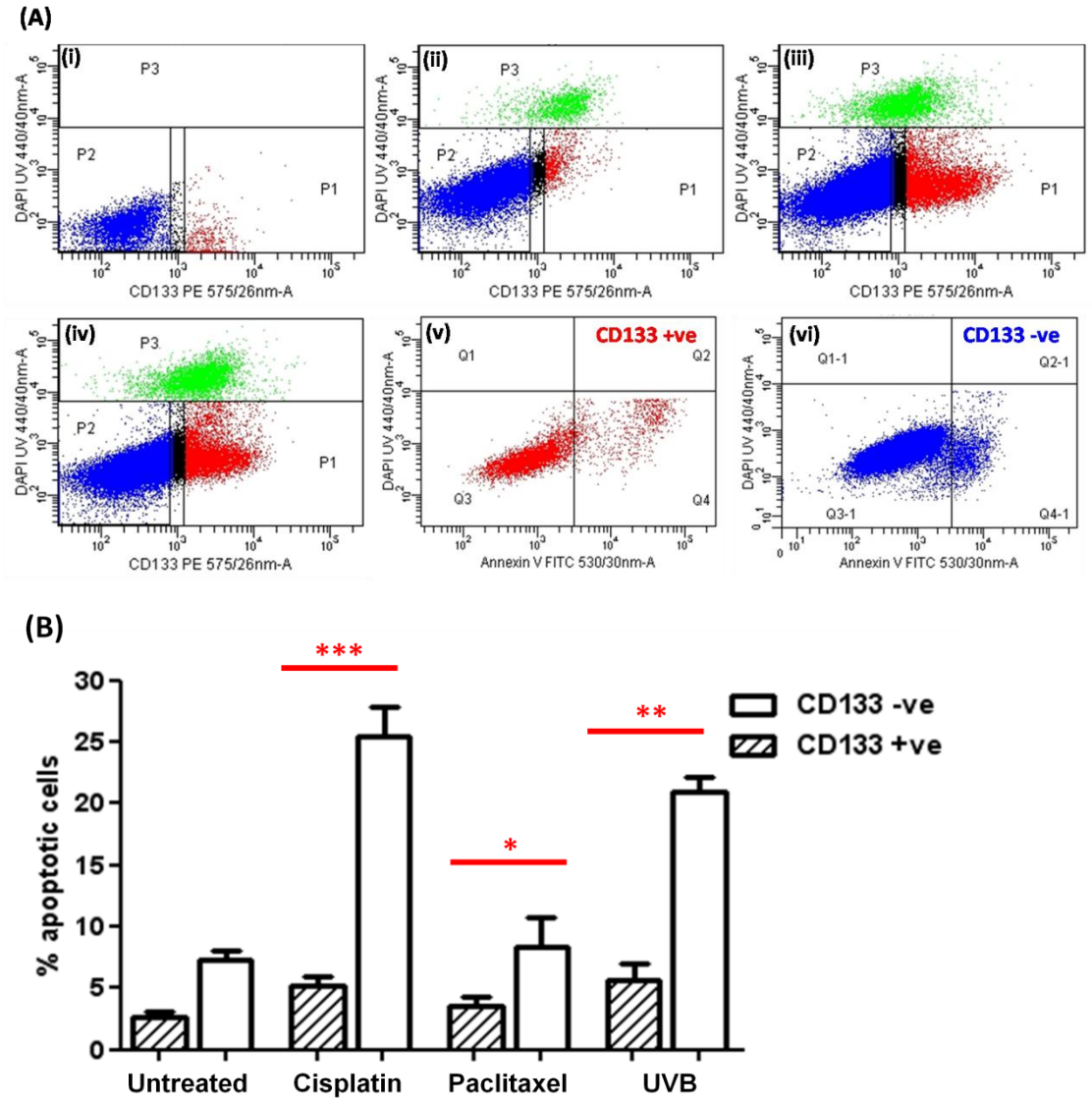


Figure 5.53: CD133-positive population of cells confer resistance to apoptosis. (A) Representative examples of flow cytometry scatter plots of A375M cells treated with cisplatin: (i) Annexin-V only, isotope-PE labelled untreated cells, (ii) Annexin-V/DAPI and isotope-PE labelled untreated cells, (iii) Annexin-V/DAPI and CD133-PE labelled untreated cells, (iv) Annexin-V/DAPI and CD133-PE labelled cells treated with cisplatin (Gating – P1=CD133-positive and P2=CD133-negative, P3=necrotic cells), (v) Annexin-V FITC plot for CD133-positive population, (vi) Annexin-V FITC plot for CD133-negative population. (B) The percentage of apoptotic cells (Annexin-V positive) for CD133-positive (hatched bars) and CD133-negative (unhatched bars) is shown. Significantly fewer apoptotic cells are detected in the CD133-positive population of cells upon treatment with cisplatin 10 µM (p-value <0.0001, t-test), paclitaxel 1 µM (p-value 0.0435, t-test) and UVB radiation 40 mJ/cm^2 (p-value 0.0039, t-test) for 16 hrs. Data shown are mean values +/- SEM of three independent experiments each performed in triplicate.

5.4 DISCUSSION

5.4.1 p63 is linked to the mitochondrial apoptotic pathway in melanoma

Upregulation of p63 (and p53) is demonstrated in melanoma cell lines upon exposure to genotoxic agents. Both TA and ΔN p63 isoforms were stabilised in response to DNA damage. The specific isoforms stabilised appear to be cell-line dependent but the main isoform consistently upregulated was TAp63, supporting evidence demonstrated in other cancer cell lines (Petitjean et al. 2005). Endogenous p63 was predominantly expressed in the nucleus but also in the cytoplasm. Stabilisation of p63 protein was observed in both compartments and, using mitochondrial markers, cytoplasmic p63 was observed to partially localise to the mitochondria. The stabilisation of p63 in both nuclear and mitochondrial compartments upon genotoxic stress in melanoma cells suggests transcriptional and non-transcriptional roles for the protein.

5.4.2 Mitochondrial translocation of p63 was demonstrated using various methods

Translocation of p63 to the mitochondria upon genotoxic stress was confirmed using a variety of techniques and provides evidence linking p63 to apoptosis in melanoma.

5.4.2.1 Microscopy-based experiments

Immunofluorescence microscopy utilising MitoTracker Orange, confirmed localisation of p63 to the mitochondria upon DNA damage. The limitation of this method is the inability to confirm whether p63 enters the mitochondrial matrix or attaches to its membrane. Although a z-stack analysis was performed in an attempt to visualise this, the resolution needed to detect this level of detail was not possible with confocal microscopy. Transmission electron microscopy (EM) was therefore used to confirm the presence of p63, at mitochondrial membranes and within the mitochondrial matrix, using the characteristic ultrastructure of mitochondria to confirm this, in conjunction with detection of a mitochondrial protein, mtHsp70. Stabilisation of p63 upon genotoxic stress was demonstrated in nuclear, cytoplasmic and mitochondrial compartments of the cell suggesting that the protein shuttles between the three depending on the stimulus and raises the possibility that distinct subcellular pools of p63 could exist. EM experiments were undertaken by analysing endogenous protein expression in intact melanoma cells and therefore have the advantage of demonstrating physiological translocation of p63 upon genotoxic stress, unlike the more artificial methods previously utilised to demonstrate translocation of other p53 family members to isolated mitochondria (Sayan et al. 2008).

5.4.2.2 Protein analysis of subcellular fractions

Western blotting has historically been used to detect proteins in subcellular fractions. In melanoma cell lines, Western blotting revealed two consistent findings; a) the presence of specific TA isoforms in the nuclear (TAα) and mitochondrial (TAβ) compartments with independent stabilisation of both, suggesting specific isoforms translocate to the mitochondria in response to DNA damage; b) the same ΔN isoforms (α and β) present in both compartments which are upregulated in response to DNA damage suggesting a functional role for these isoforms in both nucleus and mitochondria. It is unclear as to which splice variants are able to translocate to the mitochondria, although, these findings demonstrate that both TA and ΔN isoforms are stabilised at the mitochondria upon DNA damage. Moreover, it appears that both p63β isoforms are localised to the mitochondria and in some cell systems, p63β is the strongest transactivator of endogenous target genes e.g. p21, mdm2 and PIG3 (Ghioni et al. 2002; Helton et al. 2008). Further analysis by transfection of individual splice variants of p63 in melanoma cell lines could help to determine the downstream target gene effects using luciferase assays, and thus delineate more clearly, the specific roles of p63 splice variants at the mitochondria.

There are a number of well-recognised limitations of Western blotting cited; poor reproducibility, degradation of proteins and the lack of purity of fractions. However, cleavage of p63 could only be demonstrated using Western blotting, based on the molecular weights of the cleaved products (Sayan et al. 2007). These findings suggest that upon DNA damage, cleavage of p63 does occur in melanoma cell lines, and both N-terminal and C-terminal cleaved products are observed in the nuclear fraction. The cleaved fragments are then released and the N-terminal cleaved fragment was observed in the cytoplasm and the C-terminal fragment was observed in the mitochondria. This is the first report that both full-length isoforms of p63 and a putative cleaved form of p63 can translocate to the mitochondria. These data should, however, be approached with caution since interpretation of Western blotting was based on estimated molecular weights from a previous report (Sayan et al. 2007). To overcome this, each of the p63 cleaved fragments should be cloned into a vector and transiently expressed in melanoma cell lines to confirm their molecular weights, thus providing more definitive conclusions. Downstream target gene effects following observations of cleavage of p63 should be analysed to determine if cleaved products in melanoma also have a function in apoptotic signalling.

Further work is also required to clarify the localisation of cleavage products of p63 in melanoma cell lines. Detection of C-terminal cleaved fragments of p63 are reported using the H129 antibody (Sayan et al. 2007). It is therefore possible that significant upregulation of p63 detected by immunofluorescence microscopy, following treatment with chemotherapeutic agents, could also include upregulation of cleaved p63, detected by the use of a combination of the antibodies - H129 (detecting largely C-terminal cleaved p63) and H137 (detecting largely N-terminal cleaved p63). This could lend support to the evidence demonstrating that cleavage of p63 occurs in melanoma in response to genotoxic agents, but without antibodies to specifically detect cleaved fragments, immunofluorescence microscopy cannot be used to confirm the findings demonstrated by Western blotting. Although it is difficult to draw conclusions from these data, protein analysis is clearly a method needed to detect cleavage of proteins, post-translational modifications and isoform expression in different cellular compartments.

5.4.2.3 Flow cytometry analysis of fractionated cells

This was a new method (Leverrier et al. 2007) developed in this thesis to overcome the disadvantages of Western blotting and to determine the relative quantification of proteins in subcellular fractions. A multi-laser instrument (BD LSR II) allowed the use of fluorescent dyes and antibodies, which precluded the need for electronic compensation and thus removed any possibility of incorrect interpretation of the degree of translocation. This was achieved by choosing dyes only excited by specific laser lines; Hoechst 33342 was excited by a UV laser line, MitoTracker Orange excited by 488 nm laser and Cy5 excited at 633nm by the Red HeNe laser diode. Mitochondrial dye MitoTracker Orange was chosen as this dye appeared from confocal imaging to be specific for mitochondria, does not leach out from the organelle upon cell death and has no ‘cross-talk’ with other dyes used in the assay. The use of Cy5 to detect the presence of proteins in the cell organelles again precluded any compensation for ‘cross-talk’ between the UV and argon ion laser excited dyes used in this assay. Another question arising from this method is the specificity of the organelle probes. In this assay, Hoechst 33342 binds to A-T bases in DNA and thus not only labels the nucleus but also mitochondrial DNA (mitoDNA). Sorting of the fractions confirmed purity and use of confocal microscopy validated the technique as a method for separating the subcellular fractions and quantifying the presence of Cy5 in each fraction to determine relative concentrations of the protein of interest [section 5.3.4.1].

5.4.2.3.1 *p63 translocates to the mitochondria*

These data provided a quantitative analysis of translocation of p63 to the mitochondria which occurred fairly rapidly compared with a more gradual stabilisation of the protein in the nucleus, implicating p63 in the mitochondrial apoptotic pathway and a nuclear transcriptional pathway. This finding was demonstrated in three different melanoma cell lines suggesting that the translocation of p63 to the mitochondria in response to genotoxic stress is a general phenomenon. Although data suggest that p63 has an anti-apoptotic role in melanoma, further work is needed to clarify the downstream apoptotic effects of translocation of p63 to the mitochondria. This could be achieved by analysing the apoptotic pathway upon genotoxic stress in the presence and absence of p63.

5.4.2.3.2 *Phosphorylated p63 localises to both nuclei and mitochondria*

Analysis of phosphorylation of p63 revealed that phosphorylation occurs within three hours of exposure to genotoxic agents, predominantly in the nucleus but also in the mitochondria. This may affect stability of the protein and/or its transactivation abilities. Phosphorylation of p63 in keratinocytes has no effect on subcellular localisation (Westfall et al. 2005) and phosphorylation of p53 may have a role in targeting the protein to the mitochondria (Mancini et al. 2009; Nemajerova et al. 2005). Based on these data, one can speculate that, analogous to p53, phosphorylation of p63 may contribute to the stability of the protein in the nucleus and may assist in targeting the protein to the mitochondria.

The limitations of these data are that only one method was used to demonstrate this mechanism - flow cytometry. The lack of available phospho-antibodies specific for different phosphorylation sites on p63 prevents the confirmation by Western blotting. By exploring the specific splice variants in subcellular compartments and identifying the specific phosphorylation sites involved, the effect on protein stability of p63 could be delineated. The significant phosphorylation in the nucleus could regulate stability of the protein but also affect transcription of p63 (and/or p53) downstream target genes, which have not been investigated in this thesis. Moreover, a number of post-translational mechanisms are described for p63 [section 1.3.4.1] and these have not been considered in this thesis. It is possible that modifications including acetylation and sumoylation which regulate stability of p63, may also have specific roles in subcellular locations which require further consideration.

5.4.2.3.3 *Translocation of p63 and p53 to the mitochondria is a co-dependent process*

Flow cytometry fractionation experiments demonstrated that p63 prevented accumulation of total and nuclear p53 in response to DNA damaging agents, suggesting a possible role for p63 in melanoma to inhibit p53 transcriptional function. This could be explained in A375M cells where the predominant isoform is ΔNp63, which has been demonstrated to inhibit p53 (Crook et al. 2000). In contrast, translocation of p63 to the mitochondria was dependent on p53, and vice versa. Using a p53-null cell line, p63 was shown to depend on the presence of wt-p53, to translocate to the mitochondria upon genotoxic stress.

One limitation of the latter experiment is the use of different cell lines. Although chemosensitivity was not significantly different between the two cell lines when assessed using the Annexin V assay [Figure 5.39], a more convincing experiment would be to compare p63 responses against the same isogenic background. To achieve this it is necessary to stably silence TP53 in A375M cells and compare the p63 response in A375M cells (with wt-p53) against A375M cells depleted of p53. An alternative experiment would be to assess p63 translocation in A375M cells upon treatment with pifithrin α, a chemical inhibitor of p53 specifically interfering with the interaction between p53 and its mitochondrial binding partners (Strom et al. 2006).

Nevertheless, these data lead to speculation that p63 could interfere with both the transcription-dependent and possibly transcription-independent apoptotic pathway of wt-p53 in melanoma. To prove this, the negative effect of p63 on recruitment of p53 to the mitochondria would need to be established, by demonstrating reduced transcriptional activation of p53-induced pro-apoptotic genes and thus decreased mitochondrial outer membrane permeabilisation and release of cytochrome C (Chipuk and Green 2006).

5.4.3 p63, CD133 and chemosensitivity

5.4.3.1 Both isoforms of p63 (TA and ΔN) are determinants of chemosensitivity

Both siRNA and shRNA-mediated depletion of p63 revealed that the expression of both TA and/or ΔNp63 isoforms confer resistance to chemotherapy in melanoma cell lines and provide the evidence for an oncogenic role of p63 in this tumour. Confirmation of this comes from data using shRNA-clone E which depleted ΔNp63 but resulted in significant upregulation of TAp63 and consequentially had no effect on chemosensitivity [Figure 5.48]. Although both TA and ΔNp63 isoforms can act as

transcription factors when homodimerised, the ΔN isoforms can hetero-oligomerize with the TAp63 isoforms (as well as other p53 family members) and modify their activity *in vitro* (Chan et al. 2004; Choi et al. 2002; Hibi et al. 2000; Serber et al. 2002). ΔNp63α is reported to efficiently inhibit the function of TAp63 and p53 (Serber et al. 2002). It is therefore possible that silencing ΔNp63 in melanoma cells, by shRNA-clone E could result in loss of inhibition of TAp63, thus increasing its expression level. Overall, the increase in TAp63 levels negates any effect that depletion of ΔNp63 may have on chemosensitivity. One can further speculate that the effect of silencing ΔNp63 and subsequent upregulation of TAp63 may not be a direct interaction but instead, mediated through microRNA regulatory mechanisms yet to be elucidated.

The net anti-apoptotic effect of translocation of TA and/or ΔNp63 to the mitochondria is likely to be mediated through inhibition of pro-apoptotic effectors e.g. p53 in the mitochondria, although the two proteins appear to shuttle together. Moreover, the inhibitory effect of p63 (especially ΔNp63) on stabilisation of p53 in the nuclei, is likely to result in a reduction of transcription of p53-dependent pro-apoptotic genes. Analysis of the effect on p53-induced downstream target genes would confirm a mechanism by which p63 contributes to oncogenesis in melanoma.

5.4.3.2 All splice variants of p63 are potential determinants of chemoresistance

Transfection of p63 isoforms in a melanoma cell line demonstrated each isoform to confer resistance to chemotherapies. However, a specific pattern of isoform effect was not established. One possible reason is that transfection of plasmids was transient, and although Western blotting of cells 48 hrs after transfection demonstrated expression of the p63 isoforms, a significant percentage of untransfected cells would mask the chemoresistance mediated by p63-transfected cells. Moreover, the process of liposomal-mediated transfection is itself a stressor for cells, which could result in the alteration of signalling pathways, thus affecting the sensitivity to genotoxic stress. Cases where resistance was demonstrated but did not reach statistical significance could be explained by this, but also by the low transfection efficiency, thus underestimating the true resistance p63 confers. To overcome this problem, isoforms cloned with GFP-tagged plasmids recognised by flow cytometry, could be used to detect apoptosis in a transfected cell only. This would ensure that differences in apoptosis observed between cell lines were solely attributed to the effects mediated by p63.

5.4.3.3 ΔNp63 is a marker of chemoresistance in a putative stem cell population

Earlier data demonstrated enrichment of the ΔNp63 isoform in CD133-positive cells [Section 3.4.2]. The fraction of CD133-positive cells increases upon exposure to chemotherapeutic agents and preferentially survives genotoxic stress, thus delineating a specific role for ΔNp63 to achieve an anti-apoptotic function in a subset of melanoma stem cells. Evidence from this thesis suggests that ΔNp63 is an independent predictor of chemoresistance in melanoma cell lines. However, CD133-positive glioblastoma cell lines prepared from patients also demonstrated resistance to chemotherapeutic agents as a result of significantly higher levels of expression of anti-apoptotic genes Bcl-2, Bcl-xL, FLIP and XIAP compared with the corresponding CD133-negative cells (Liu et al. 2006). Future work to investigate upregulation of other anti-apoptotic factors should be undertaken in CD133-positive melanoma cells. It is possible that this may be a downstream effect of ΔNp63 (Dohn et al. 2001) and this could be explored further by analysing these factors in a FACS sorted CD133+ve/p63-ve population.

Furthermore, upregulation of the ATPase pump, adenosine triphosphate-binding cassette-5 (ABCB5) was demonstrated in CD133-positive cells, including progenitor cells of human epidermal melanocytes and a sub-population of melanoma cells (Frank et al. 2003) and these CD133+ve/ABCB5+ve melanoma cells demonstrated resistance to doxorubicin (Frank et al. 2005). In this thesis, the ABCB5 status of the melanoma cell lines screened was not determined. It is possible that p63 is another marker of chemoresistance in the CD133-positive population, but its relation to ABCB5 expression needs to be clarified.

The role of CD133 in melanoma cells is not fully characterised and correlation of differentiation ability and tumourigenicity by comparing CD133-positive and CD133-negative fractions is yet to be demonstrated. To define the biological significance of CD133 enrichment and to confirm its role in chemosensitivity, *in vivo* proof is also required to show increased resistance of CD133-positive cells to chemotherapeutic agents compared with most tumour cells (which are CD133-negative). This could be achieved by isolating melanoma tissue from patients, demonstrating high levels of both ΔNp63 and CD133 and then demonstrating a resistance to apoptosis in these isolated cells.

Despite the limitations discussed above, these data provide the preliminary evidence needed to embark upon translational experiments. If melanoma stem cell populations

are indeed associated with chemoresistance and melanoma progression in patients, specific targeting of melanoma stem cells via a molecular marker such as CD133 or ΔNp63 could provide a more potent and selective means for targeted melanoma therapy. Cancer stem cell surface molecules e.g. CD133 have been used in clinical applications including diagnostic identification in melanoma biopsies and as putative prognostic indicators (Gupta et al. 2009). Furthermore they provide potential targets for antibody-based therapies that selectively eliminate these cells. By specifically inhibiting CD133-positive cells, thus downregulating p63 (specifically ΔNp63), melanoma cells could be sensitised to chemotherapies improving overall survival in patients.

CHAPTER 6: CONCLUDING REMARKS

6.1 SUMMARY OF FINDINGS

6.1.1 TP63 has an oncogenic role in melanoma

This is the first study to systematically explore the expression, regulation and functional role of p63 isoforms in melanoma. Upregulation of p63 was demonstrated in primary and metastatic melanoma cell lines compared with primary melanocyte cultures. Differential expression of isoforms of p63 was observed in 74% of melanoma cell lines – 24% expressed TAp63 only, 32% ΔNp63 only and both isoforms were expressed in 18% of cell lines [section 3.3.1]. All splice variants of p63 were expressed in melanoma, including the more recently identified δ and ε variants. The predominant variant expressed was TAp63β, but all variants were observed in melanoma. Although data are not able to define the role of each individual isoform, it is likely that they have specific biological / biochemical activities. These activities may be overlapping and the small proportion of cell lines which express both TA and ΔN isoforms suggests that there may be redundancy of function. In keeping with this, functional data analysing the role of p63 in mediating genotoxic stress suggests that both isoforms contribute to the chemoresistance observed in melanoma [section 5.3.5].

Upregulation of p63 was demonstrated in melanoma tissue samples compared with benign intradermal naevi, in 40% of melanomas in a tissue microarray and 49% of FFPE archival tissue samples [sections 3.3.3 to 3.3.5]. p63 reactivity was not significantly associated with clinical or histological features of the primary tumours. Expression of cytoplasmic p63 reactivity demonstrated tendency towards increased recurrence rates and time to metastases. Moreover, p63 reactivity was a significant

predictor of overall death (HR 2.79, p-value 0.05, Cox proportional hazard) and although the trend persisted, it was not a significant predictor of melanoma-deaths (HR 2.43, p-value 0.13, Cox proportional hazard). This aberrant expression of p63 from nucleus to cytoplasm appears to be an important mechanism for the oncogenic effect of this gene in melanoma.

6.1.2 Diverse mechanisms regulate p63 expression in melanoma

Regulation of expression of p63 and its stability in melanoma is shown to be a multi-factorial process. Epigenetic modulation of p63 by HDAC inhibitors was demonstrated in melanoma cells, in keeping with recent reports (Sayan et al. 2009). A novel miRNA – miR-18a has been identified to regulate the tissue-specific expression of p63 in melanoma [section 4.3.2.2]. Numerous miRNAs are recognised to target single mRNAs and evidence suggests that miRNA regulation of p63 is no different (Blandino and Moll 2009; Lena et al. 2008; Manni et al. 2009; Papagiannakopoulos et al. 2008). Previously identified regulators of p63 were also dysregulated in melanoma cell lines expressing p63, thus increasing the complexity of p63 regulation. Stability of p63 protein appears to be dependent on the proteosome in melanoma, which implicates a number of E3 ubiquitin ligases that could be involved in degradation of p63. Data presented in this thesis show itch to be one modulator of p63 expression but it is likely that other reported ligases (Fomenkov et al. 2004; Gallegos et al. 2008; Li et al. 2008; Rossi et al. 2006a; Rossi et al. 2006b) are also involved and deserve investigation. The mechanisms identified in this study warrant a detailed analysis of their interplay and interaction with other factors determining the tissue-specific expression of this gene in melanoma.

6.1.3 p63 is an anti-apoptotic effector in melanoma

Upon genotoxic stress, both TA and ΔN isoforms of p63 are stabilised in the nucleus and partially translocate to the mitochondria [section 5.3.3]. In conjunction with data showing p63 confers chemoresistance [section 5.3.5], one can speculate that for p63 to achieve this effect in the mitochondria, it could perform one/more of the following functions:

a) Direct anti-apoptotic effect in the mitochondria
b) Inhibition of apoptosis by indirectly inhibiting pro-apoptotic effectors
c) Interference with translocation of pro-apoptotic effectors to the mitochondria
d) Direct or indirect induction of anti-apoptotic regulators in the mitochondria

Analysis of the effect of genotoxic stress on wt-p53 revealed that, in the presence of p63, translocation to the mitochondria occurs but accumulation in the nucleus is inhibited. The latter may be a result of either inhibition of entry of p53 into the nucleus or more likely, due to an inhibitory effect on stability of wt-p53 through induction of ubiquitin ligases e.g. MDM2. In the absence of p63, p53 does not readily translocate to the mitochondria, and vice versa. These data imply a co-dependent mechanism of translocation of p63 and p53 to the mitochondria. This could be a result of hetero-oligomer formation or co-translocation by molecular chaperones recognised to transport p53, e.g. hsp90 or hsp70 (Walerych et al. 2004; Walerych et al. 2009; Whitesell and Lindquist 2005) [Figure 6.1]. The failure of wt-p53 to activate apoptotic pathways in melanoma could therefore be explained by the inhibition of wt-p53 pro-apoptotic function by p63 in two ways: inhibition of the p53 mitochondrial (transcription-independent) apoptotic pathway and/or inhibition of accumulation of wt-p53 in the nucleus and thus, the transcriptional-dependent apoptotic pathway.

These data identified an unexpected result implicating both TA and ΔN isoforms in mediating chemoresistance in melanoma. The oncogenic effect of both TA and ΔN isoforms of p63 is reported in other tumour types (Koster et al. 2006; Marchini et al. 2008; Mills 2006). In addition, the majority of squamous cell cancers express high levels of ΔNp63 (Mills 2006) and ΔN isoforms are thought to regulate the transcriptional activity of TA isoforms either by the formation of inactive hetro-oligomers or by competition for specific response elements on DNA. This latter property also allows ΔNp63 to act antagonistically towards p53, thus providing a theoretical mechanism of p53 functional inactivation during tumourigenesis. In melanoma, this thesis provides evidence in favour of preferential upregulation of TP63 over TP53 mutation. In contrast, TAp63 has been reported to be pro-apoptotic in other cell types (Gressner et al. 2005). However, data from this thesis, have demonstrated that shRNA-p63 clone-E which depleted ΔNp63 resulted in derepression of TAp63 and had no effect on sensitivity to chemotherapeutic agents. The anti-apoptotic effect of TAp63 in melanoma could be an indirect effect although this is yet to be established.

Mechanisms controlling export of p63 between cellular compartments were not investigated. One can speculate that post-translational mechanisms may be involved. There is evidence that MDM4 mediates mitochondrial relocalisation and anchors phosphorylated p53 to BCl2 (Mancini et al. 2009). By analogy with p53, the data presented in this thesis also show phosphorylated p63 at the mitochondria, although

the function of this is, as yet, undetermined. There is robust evidence for ubiquitination of p53 enabling shuttling to the mitochondria (Marchenko et al. 2007) and a more recent model hypothesises that nuclear export of p53 could occur by co-operation of sumoylation with monoubiquitination (Stehmeier and Muller 2009b). Equally, it is likely that post-translational mechanisms of p63 are responsible for targeting it to the mitochondria and these could interact with modifications already reported for targeting p53 to the mitochondria.

6.1.4 Summary

Taken together, these data propose an oncogenic role for p63 in melanoma, whereby expression of p63 in melanoma imparts chemoresistance to tumours and is associated with worse outcome in patients with melanoma. The oncogenic role of both TA and ΔNp63 isoforms in melanoma could be explained in part, by dysregulation of the transcriptional and non-transcriptional function of wt-p53 [Figure 6.2]. Ultimately, these data could provide a novel therapeutic approach aimed at counteracting expression of p63, and sensitising melanoma to standard chemotherapeutic agents.

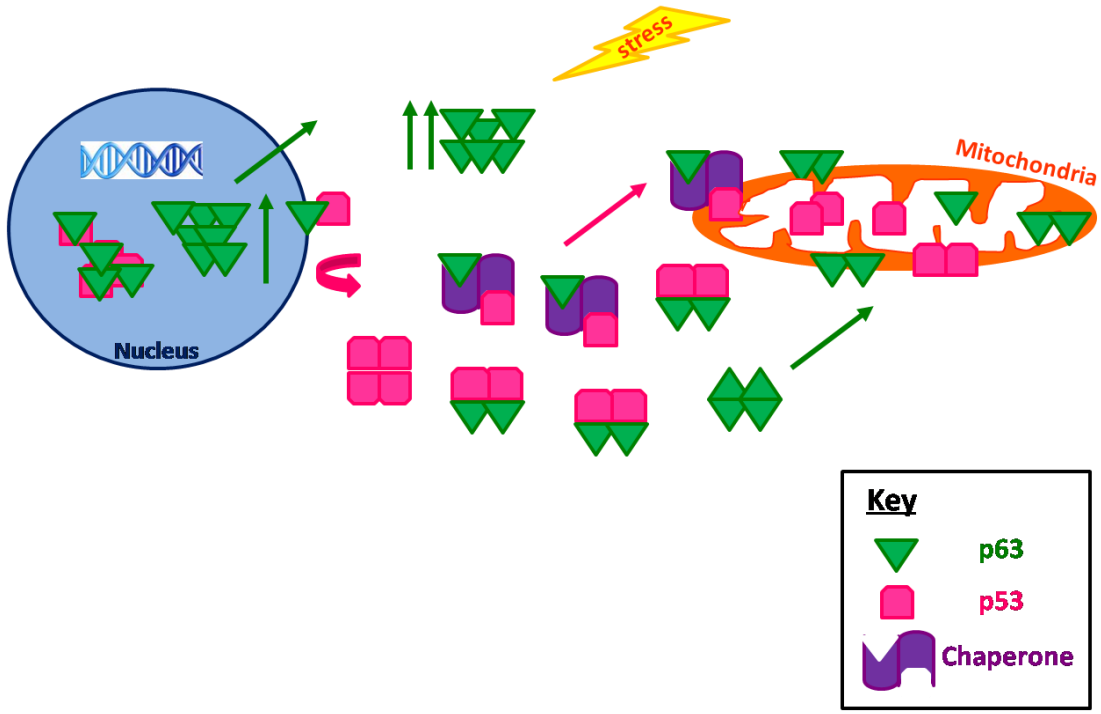


Figure 6.1: p63 and p53 response in melanoma to chemotherapeutic agents. Upon genotoxic stress, stabilisation of p63 occurs in the nucleus with translocation via the cytoplasm to the mitochondria. Data presented in this thesis support a co-dependent mechanism for p53 and p63 to shuttle to the mitochondria which could involve the formation of hetero-oligomers or involve a molecular chaperone e.g. hsp70 or hsp90 (Walerych et al. 2004; Walerych et al. 2009; Whitesell and Lindquist 2005). Stabilisation of p63 in the nucleus results in transcription of downstream target genes but additionally, through an unknown mechanism, prevents the accumulation of nuclear wt-p53. This could be a result of inhibition of entry into the nucleus or through increased protein degradation. This could provide an explanation for failure of wt-p53 activity commonly observed in melanoma.

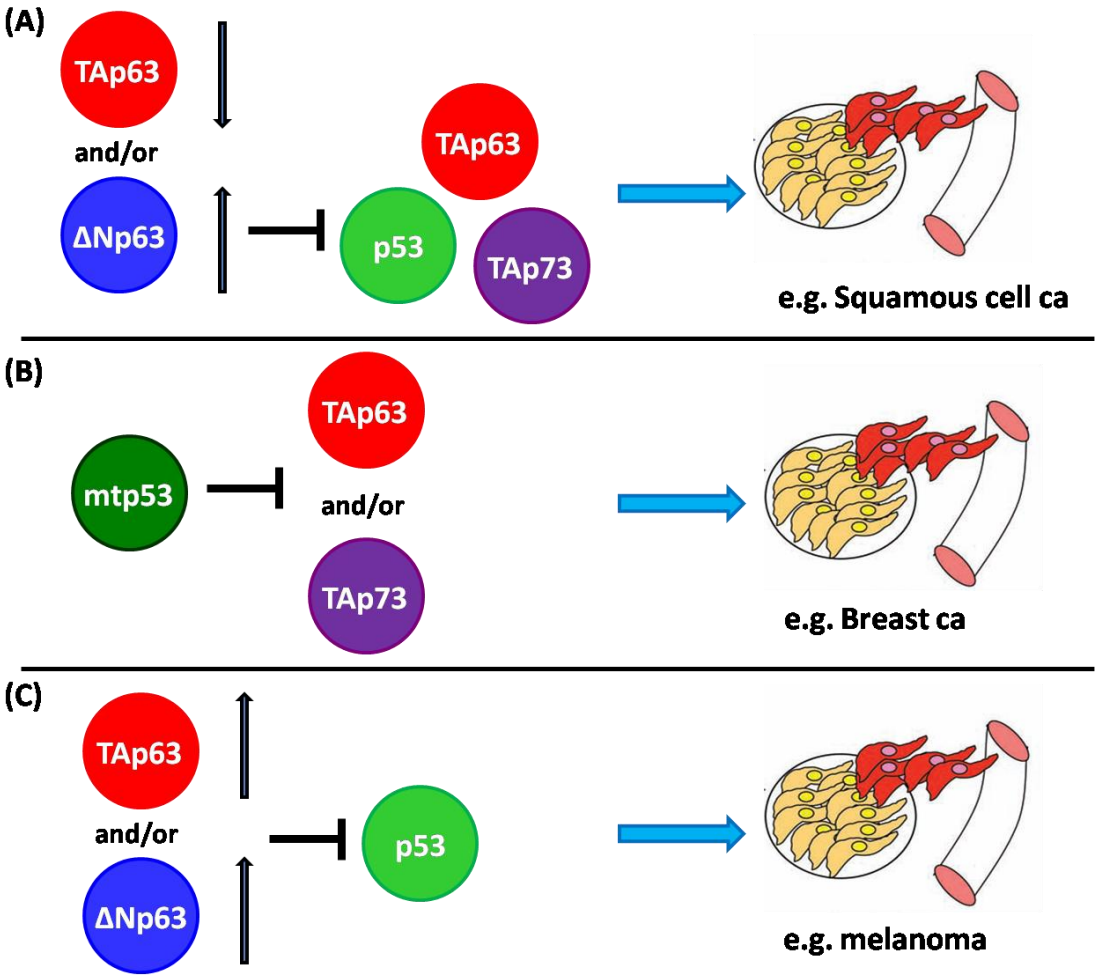


Figure 6.2: Models for p63 in carcinogenesis. Schemes (A) and (B) are models for p63 as a tumour suppressor in cancers but scheme (C) depicts the model demonstrated in this thesis where p63 is an oncogene. (A) Downregulation or loss of TAp63 and/or overexpression of ΔNp63 leads to inhibition of functions of TAp63, p53 and TAp73 and results in invasive and metastatic tumours such as squamous cell carcinoma of lungs, head and neck or bladder carcinoma. (B) Mutant p53 binds to TAp63 and TAp73 inhibiting their function leading to the development of an invasive and metastatic tumour such as breast carcinoma. (C) Overexpression of TA and/or ΔNp63 has a negative effect on wt-p53 function which in melanoma leads to a more aggressive and chemoresistant cancer. Ca – carcinoma, mtp53 – p53 harbouring mutation in DNA binding domain. Figure adapted from Flores (2007).

6.2 THERAPEUTIC APPLICATIONS

6.2.1 HDAC inhibitors

Melanoma is a notoriously chemoresistant cancer. By demonstrating a role for p63 in mediating chemosensitivity, these novel findings have provided one possible explanation for this. Exposure to a variety of chemotherapeutic agents, including HDAC inhibitors, resulted in stabilisation of p63 isoforms. Although the sensitivity to apoptosis mediated by HDAC inhibitors was not examined, one can speculate that the upregulation of p63 is likely to mediate resistance to apoptosis, similar to that demonstrated for other chemotherapeutic agents including cisplatin and paclitaxel. HDAC inhibitors have been used in clinical trials but with limited success (Drummond et al. 2005; Sandor et al. 2002), and this has been attributed to the widespread effect of epigenetic modulation of multiple genes in various molecular pathways. These data suggest that in melanoma, one example is the upregulation of oncogenic p63 by acetylation, suggesting a possible deleterious effect of this therapy in a subset of tumours.

6.2.2 Identification of novel inhibitors of p63

Evidence that upregulation of both TA and ΔN p63 contributes to the chemoresistance of melanoma, presents a novel therapeutic approach which would involve downregulation of p63 levels and activation of apoptosis. Depletion of p63 has only been demonstrated using one currently available pharmacological agent, imatinib (Gonfloni et al. 2009; Ongkeko et al. 2006). Imatinib is a tyrosine kinase inhibitor with a number of targets including platelet-derived growth factor receptor (PDGFR) (Buchdunger et al. 2002), c-abl (Buchdunger et al. 2000; Druker and Lydon 2000) and c-kit (Heinrich et al. 2000). Of relevance, this drug has shown considerable promise in clinical trials for managing metastatic mucosal and acral melanomas (Hodi et al. 2008; Jiang et al. 2008; Kim et al. 2008). In these subtypes of melanoma, imatinib is used to target c-kit mutations which occur with increased frequency (Beadling et al. 2008). The mode of action may be to deplete p63 is through inhibition of c-abl, which is reported to stabilise both TAp63 (Gonfloni et al. 2009) and ΔNp63 (Ongkeko et al. 2006). *In vitro* demonstration of depletion of p63 isoforms by imatinib is required, but if proven, this would identify a further subset of patients with cutaneous melanoma, who could benefit from this drug.

Sequence-specific gene silencing using RNA interference technology has revolutionised basic scientific research and has been used in this thesis to delineate a role for p63 in melanoma. Development of this technique for therapeutic purpose

has opened new horizons for targeted therapy in cancer and is currently being evaluated in pre-clinical and clinical trials. Approaches for systemic and localised delivery of siRNA are being investigated (Ozpolat et al. ; Sanguino et al. 2008) and this method could offer one possible route of targeted depletion of p63 in melanoma.

6.2.3 Cancer stem cell therapy

Data presented in this thesis demonstrate a role for a putative stem cell population labelled with CD133 and enriched for ΔNp63, mediating chemoresistance to cisplatin and paclitaxel in melanoma [section 5.3.6]. CD133-positive tumour initiating cells have demonstrated resistance to chemo- or radiotherapy in other cancer types (Bao et al. 2006; Frank et al. 2005; Hambardzumyan et al. 2006). There is theoretical evidence that a small proportion of cells with stem-like features escape the effect of established chemotherapeutic agents. These cells then give rise to secondary tumours that are highly resistant to further treatment, since they were positively selected by the original therapy. Since the function of CD133 is unknown, it is not clear if CD133 is just a marker of chemoresistant cells (which in melanoma co-express ΔNp63) or whether high expression of CD133 in tumour initiating cells could contribute to the resistance to therapy. Nevertheless, targeted monoclonal antibody therapy directed against the CD133-positive cell fraction in melanoma could render this cancer sensitive to standard chemotherapeutic agents, and if proven to be true tumour initiating cells, then also providing the potential for cure [Figure 6.3].

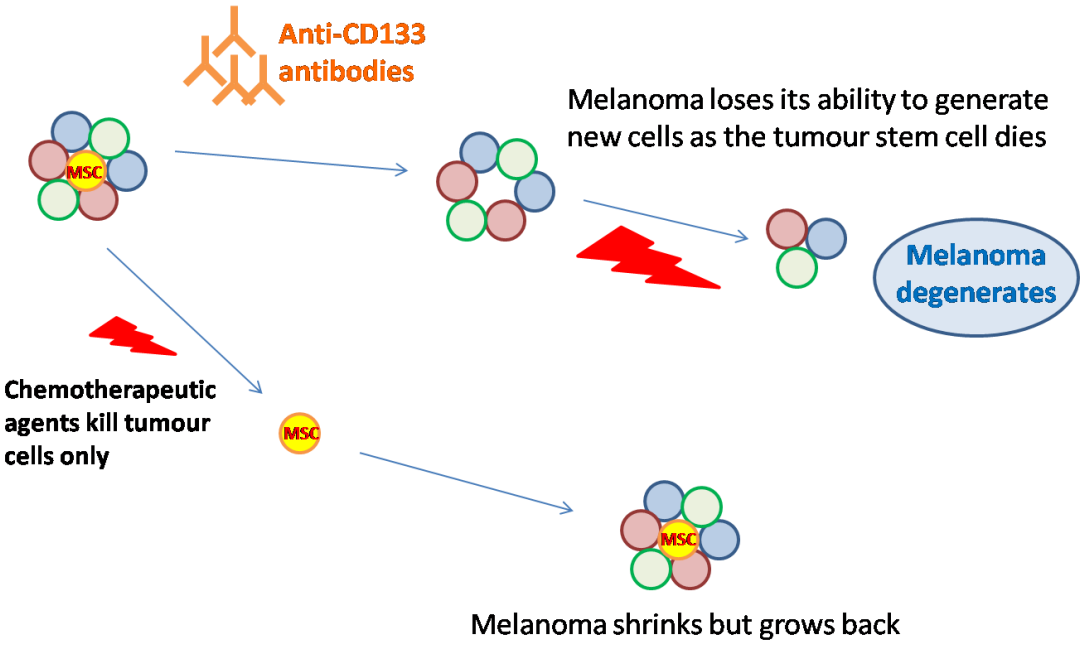


Figure 6.3: Therapeutic potential of anti-CD133 antibodies. Conventional therapies may shrink tumours by killing mainly cells with limited proliferative potential. If the putative melanoma stem cells are less sensitive to these therapies then they will remain viable after therapy and re-establish the tumour (lower path). In contrast, if therapies are targeted against melanoma stem cells, then effective killing of stem cells renders the melanoma unable to grow or metastasise. The example shown is anti-CD133 antibodies which could target ΔNp63-positive cells in melanoma, subsequently leading to (a) sensitisation of melanoma cells to conventional chemotherapies e.g. cisplatin/paclitaxel and/or (b) degeneration of the tumour by targeting a putative stem cell population. Evidence suggests that even if cancer stem cell-directed therapies do not shrink tumours initially, they are more likely to eventually lead to cures. MSC – melanoma stem cells.

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East London & The City HA Local Research Ethics Committee 2

LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION

For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.

REC reference number:	07/Q0604/23	**Issue number:**	0	**Date of issue:**	09 May 2007
Chief Investigator:	Dr Catherine A Harwood				
Full title of study:	Molecular mechanisms in the pathogenesis and treatment resistance of malignant melanoma				

This study was given a favourable ethical opinion by East London & The City HA Local Research Ethics Committee 2 on 09 May 2007. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.

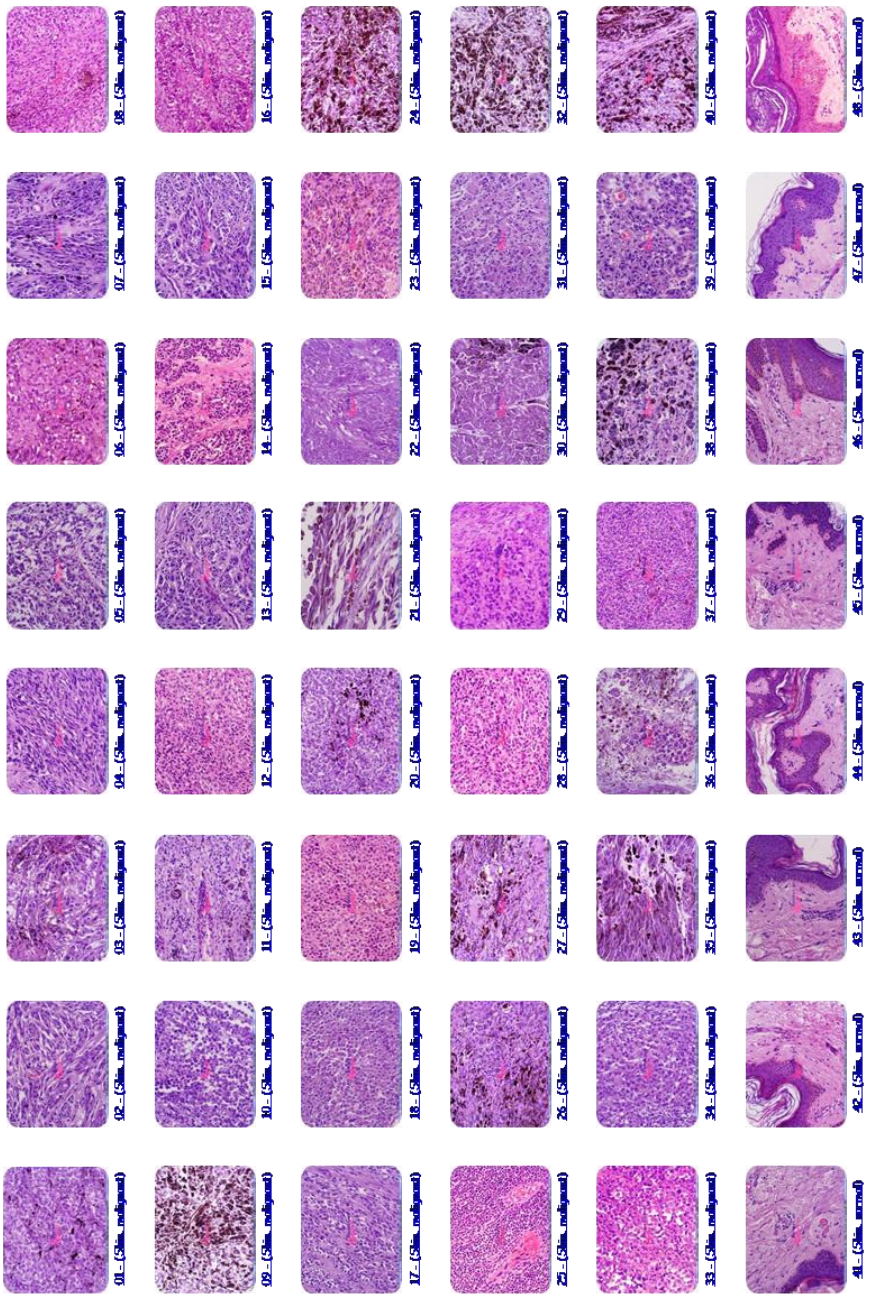
Principal Investigator	*Post*	*Research site*	*Site assessor*	*Date of favourable opinion for this site*	*Notes* [1]
Dr Rubeta N H Matin	Clinical Research Training Fellow	Barts and the London NHS Trust / Queen Mary University of London	East London & The City HA Local Research Ethics Committee 2	09/05/2007	

Approved by the Chair on behalf of the REC:

..... (Signature of Chair/Co-ordinator)
(delete as applicable)

..... (Name)

Appendix 2: Melanoma tissue array with normal skin tissue, 48 cores (ME481 US Biomax).



Patient ID	p63 status	Gender	Age at diagnosis	Site group	Breslow (mm)	Clarks level	Histo classification	Growth phase	Ulceration	Mitotic Rate category	Regress-ion	Micro-satellites
1	N	M	46.93	Acral	7	V	ALM	VGP	Yes	3	No	No
4	N	M	68.63	Head/Neck	2.7	IV	NMM		No	3	No	No
5	N	F	84.05	Trunk	6	IV	NMM	VGP	No	1	No	Yes
9	N	F	78.62	Acral	4	IV	ALM	VGP	Yes	3	No	Yes
13	N	F	36.73	Trunk	1.5		NMM		Yes		No	No
14	N	F	52.25	Trunk	0.5	III	SSM	RGP	No		No	No
16	N	F	65.03	Extremities	2.3	IV	SSM	VGP	No	3	No	No
20	N	M	56.69	Extremities	3.72	IV	NMM	VGP	No	2	No	Yes
21	N	M	77.23	Extremities	8	III	NMM	VGP	Yes	4	No	Yes
26	N	M	63.76	Extremities	6	IV	NMM	VGP	Yes	3	No	No
29	N	F	86.80	Acral	3.2	III	ALM	VGP	Yes	4	No	No
32	N	F	45.13	Extremities	1.7	IV	NMM	VGP	Yes	3	No	No
33	N	F	33.08	Acral	2.9	IV	ALM	VGP	Yes	2	No	No
34	N	M	35.88	Trunk	0.7	III	NMM	VGP	No	2	No	No
35	N	F	72.02	Extremities	1	II	SSM	VGP	No		Yes	No
36	N	F	33.55	Trunk	0.31	II	SSM	RGP	No		No	No
37	N	F	56.07	Trunk	0.35	II	SSM	RGP	No	2	No	No
39	N	M	34.47	Head/Neck	10	III	SSM	VGP	Yes	4	No	Yes
40	N	F	48.31	Trunk	3.5	IV	SSM	VGP	No		No	No
42	N	F	78.46	Extremities	2	IV	SSM	VGP	Yes	2	No	No

2	P	F	76.71	Head/Neck	4.5	IV	NMM	VGP	No	2	No	No
3	P	M	46.65	Trunk								
6	P	M	51.05	Trunk	6	III	NMM	VGP	Yes	4	No	Yes
7	P	F	69.12	Extremities	5.5	V	NMM	VGP	Yes	1	No	No
8	P	M	47.13	Trunk	5.1	IV	NMM	VGP	Yes		No	No
10	P	F	74.42	Extremities	5	IV	NMM	VGP	No	2	No	No
11	P	M	59.44	Trunk	2.6	IV	NMM		No		No	No
12	P	F	48.00	Extremities	2.3	IV	NMM		Yes	2	No	No
15	P	M	33.64	Acral	1	III	ALM	VGP	No	3	No	No
17	P	M	66.69	Trunk	5	III	NMM	VGP	No	2	No	No
18	P	M	76.51	Head/Neck	7.2	V	NMM	VGP	No		No	No
19	P	F	70.07	Trunk	4	IV	NMM	VGP	Yes	3	No	No
22	P	F	45.42	Acral	0.5	II	ALM	RGP	No	1	No	No
23	P	F	74.15	Acral	4	IV	ALM	VGP	Yes	3	No	No
24	P	F	59.37	Extremities	13.5	IV	NMM	VGP	Yes	3	No	No
25	P	F	84.02	Acral	10	IV	ALM	VGP	Yes	3	No	No
27	P	M	40.13	Extremities	4.1	III	SSM	VGP	No	3	Yes	No
28	P	F	71.59	Trunk	6.5	V	NMM	VGP	No	3	No	No
30	P	M	86.02	Head/Neck	6	V	NMM	VGP	Yes			Yes
31	P	M	76.71	Head/Neck	2.7	IV	NMM	VGP	Yes	3	No	No
38	P	F	33.40	Extremities	0.9	II	SSM	RGP	No	1	Yes	No
41	P	F	21.87	Head/Neck	3.5	IV	NMM	VGP	Yes		No	Yes

43	P	F	62.65	Extremities	1.5	III	SSM	RGP	No	1	Yes	No
44	P	F	56.06	Trunk	0.5	II	SSM	RGP	No	2	No	No

Appendix 3: Demographic details of patients with primary melanoma.

Appendix 4: Univariate and multivariate analysis of clinicopathological factors with time to recurrence in patients with primary tumours.

	Univariate analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	p-value*	Hazard Ratio (95% CI)	p-value*
Age at diagnosis (years)	1.03 (0.98, 1.08)	0.23		
Gender				
Male	1			
Female	0.73 (0.16, 3.28)	0.68		
Site of Melanoma				
Acral	1			
Extremities	0.07 (0.01, 0.68)	0.02		
Head/Neck	0.23 (0.03, 2.09)	0.19		
Trunk	0.08 (0.01, 0.74)	0.03		
Breslow thickness				
0-1 mm	1			
1.01- 2 mm	-	-		
2.01-4 mm	-	-		
>4 mm	-	-		
Clarks Level				
I	1			
II	-	-		
III	-	-		
IV	-	-		
Histological classification				
ALM	1		1	
NMM	0.06 (0.01, 0.58)	0.01	0.05 (0.00, 0.46)	0.01
SMM	0.09 (0.01, 0.82)	0.03	0.08 (0.01, 0.85)	0.04
Growth phase				
RGP	1			
VGP	7.8e+14 (0.00, .)	1		
Ulceration status				
No	1			
Yes	2.70 (0.48, 15.10)	0.26		
Mitotic rate				
0	1			
1-4	-	-		
5-10	-	-		
>11	-	-		
Regression				
No	1			

Yes	1.31 (0.15, 11.68)	0.81		
Microsatellites				
No	1			
Yes	1.05 (0.12, 9.00)	0.97		
P63 Status				
Negative	1		1	
Positive	2.28 (0.44, 11.79)	0.32	2.89 (0.49, 16.97)	0.24

Appendix 5: Univariate and multivariate analysis of clinicopathological factors with time to metastasis in patients with primary tumours.

	Univariate analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	p-value*	Hazard Ratio (95% CI)	p-value*
Age at diagnosis (years)	1.02 (0.99, 1.05)	0.14	-	-
Gender				
Male	1		-	-
Female	0.75 (0.31, 1.85)	0.54	-	
Site of Melanoma				
Acral	1		-	-
Extremities	0.38 (0.11, 1.36)	0.14	-	-
Head/Neck	0.68 (0.16, 2.87)	0.6	-	-
Trunk	0.52 (0.16, 1.69)	0.28	-	-
Breslow thickness				
0-1 mm	1		-	-
1.01- 2 mm	0.00 (0.00, 0.00)	.	-	
2.01- 4 mm	3.76 (0.78, 18.17)	0.1	-	
>4 mm	4.05 (0.88, 18.55)	0.07	-	
Clarks Level				
II	1		-	-
III	2.33 (0.24, 22.37)	0.47	-	
IV	4.80 (0.62, 37.02)	0.13	-	
V	7.28 (0.75, 70.68)	0.09	-	
Histological classification				
ALM	1		-	-
NMM	0.72 (0.25, 2.11)	0.55	-	-
SMM	0.24 (0.05, 1.01)	0.05	-	-
Growth phase				
RGP	1		-	-
VGP	5.27 (0.70, 39.90)	0.11	-	
Ulceration status				
No	1		1	
Yes	2.99 (1.13, 7.90)	0.03	3.19 (1.20, 8.47)	0.02
Mitotic rate				
0	1		-	-
1-4	0.63 (0.11, 3.79)	0.62	-	
5-10	2.31 (0.50, 10.64)	0.28	-	
>11	1.98 (0.28, 14.11)	0.49	-	
Regression				
No	1		-	-
Yes	0.00 (0.00, 0.00)	.	-	
Microsatellites				
No	1		-	-
Yes	2.29 (0.82, 6.37)	0.11	-	

P63 Status				
Negative	1		1	
Positive	1.66 (0.66, 4.18)	0.28	1.83 (0.71, 4.69)	0.21

Appendix 6: Univariate and multivariate analysis of clinicopathological features and overall outcome in primary tumours.

	Univariate analysis		Multivariate Analysis	
	Hazard Ratio (95% CI)	**p-value***	**Hazard Ratio (95% CI)**	**p-value***
Age at diagnosis (years)	1.03 (1.00, 1.06)	0.06		
Gender				
male	1			
female	0.46 (0.19, 1.14)	0.09		
Site of Melanoma				
Acral	1		1	
Extremities	0.30 (0.06, 1.52)	0.15	0.20 (0.03, 1.47)	0.11
Head/Neck	1.87 (0.44, 7.91)	0.4	10.27 (1.31, 80.42)	0.03
Trunk	0.91 (0.24, 3.48)	0.89	4.42 (0.75, 26.14)	0.10
Breslow thickness				
0-1 mm	1			
1.01 – 2mm	0.00 (0.00, .)	1		
2.01 – 4 mm	5.46 (0.64, 46.78)	0.12		
>4 mm	9.91 (1.28, 76.59)	0.03		
Clarks Level				
I	1		1	
II	2.34 (0.24, 22.59)	0.46	2.27 (0.15, 33.91)	0.55
III	3.57 (0.45, 28.24)	0.23	5.39 (0.45, 65.21)	0.19
IV	9.41 (1.09, 80.94)	0.04	34.27 (2.06, 568.78)	0.01
Histological classification				
ALM	1			
NMM	1.18 (0.33, 4.19)	0.8		
SMM	0.20 (0.03, 1.24)	0.08		
Growth phase				
RGP	1			
VGP	4.60 (0.61, 34.94)	0.14		
Ulceration status				
No	1			
Yes	2.61 (0.96, 7.10)	0.06		
Mitotic rate				
0	1			
1-4	1.23 (0.22, 6.75)	0.81		
5-10	2.26 (0.46, 11.04)	0.31		
>11	3.07 (0.43, 21.96)	0.26		
Regression				
No	1			
Yes	0.00 (0.00, .)	1		
Microsatellites				

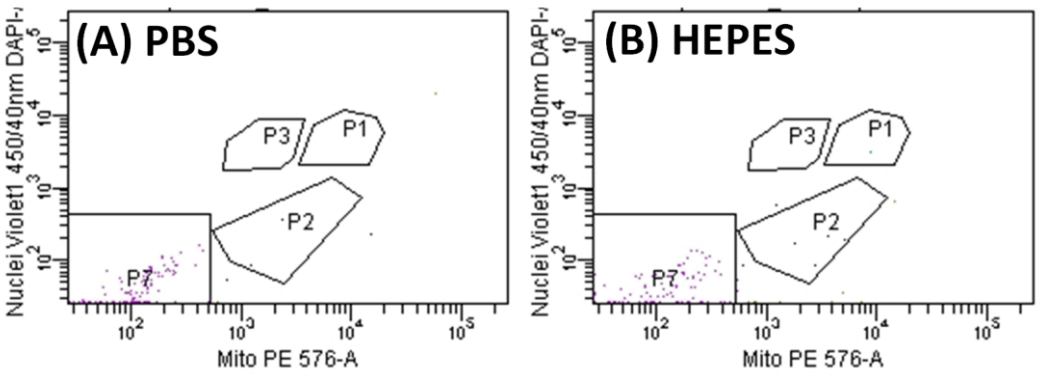
No	1		1	0.03
Yes	2.51 (0.88, 7.13)	0.08	2.94 (1.14, 7.52)	
P63 Status				
Negative	1		1	0.12
Positive	2.79 (0.99, 7.81)	0.05	3.07 (0.74, 12.83)	

Appendix 7: Analysis of reported tissue-specific miRNAs dysregulated in melanoma.

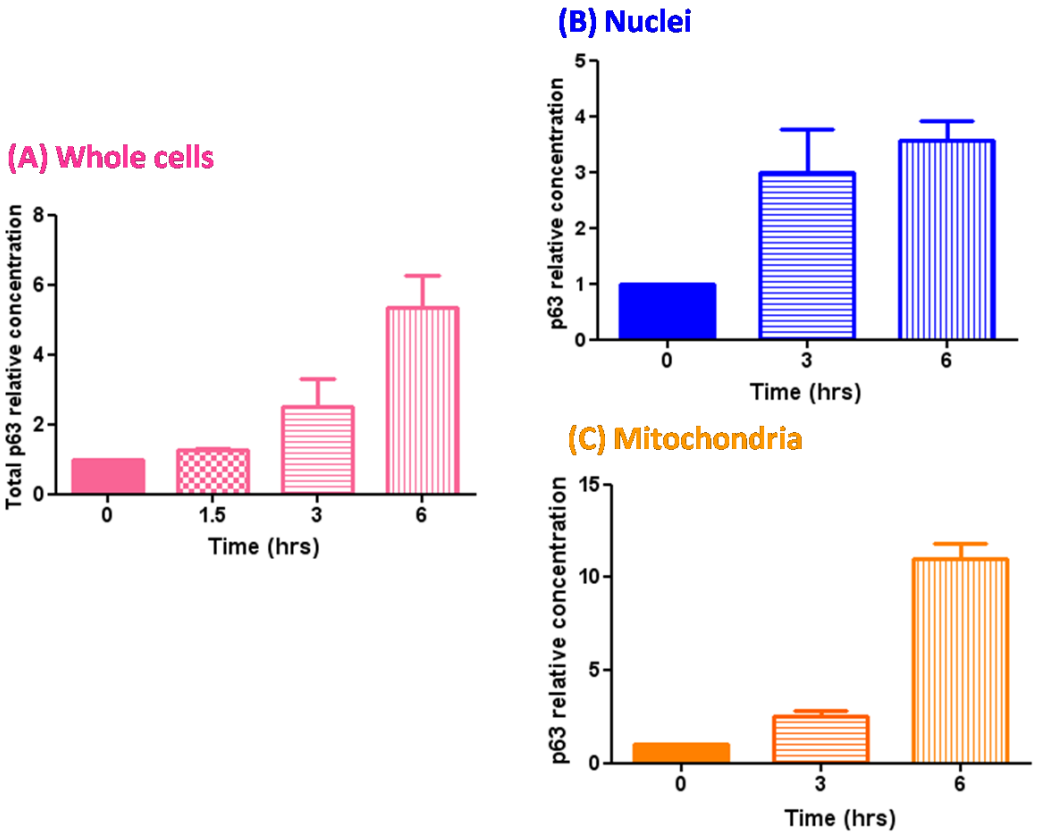
ID	miR	P value	logFC
A_25_P00010975	hsa-miR-21	0.1276	-1.5098
A_25_P00010976	hsa-miR-21	0.1984	-1.4753
A_25_P00012085	hsa-miR-34a	0.0003	**-2.6902**
A_25_P00012086	hsa-miR-34a	0.0089	**-3.9134**
A_25_P00012030	hsa-miR-92a	0.3212	0.5045
A_25_P00012031	hsa-miR-92a	0.3329	0.5007
A_25_P00012707	hsa-miR-92b	0.5518	-0.0407
A_25_P00012708	hsa-miR-92b	0.4672	0.0361
A_25_P00012709	hsa-miR-92b	0.9418	0.0044
A_25_P00012710	hsa-miR-92b	0.4685	0.0445
A_25_P00010163	hsa-miR-302d	0.0273	0.1097
A_25_P00010505	hsa-miR-302c*	0.0365	0.0848
A_25_P00010506	hsa-miR-302c*	0.0452	0.1076
A_25_P00010618	hsa-miR-302b	0.0016	0.1478
A_25_P00010982	hsa-miR-302a	0.0377	0.0775
A_25_P00013492	hsa-miR-302a*	0.0042	0.1035
A_25_P00013493	hsa-miR-302a*	0.0189	0.1112
A_25_P00013511	hsa-miR-302b*	0.0280	0.1077
A_25_P00013512	hsa-miR-302b*	0.0126	0.1107
A_25_P00013513	hsa-miR-302b*	0.0220	0.0765
A_25_P00013516	hsa-miR-302d*	0.0103	0.1061
A_25_P00010628	hsa-miR-203	0.0318	0.1431
A_25_P00010629	hsa-miR-203	0.3686	0.1698

Values highlighted in green are significantly downregulated in p63-expressing melanoma cell lines (both isoforms) compared with p63-null.

Appendix 8: Flow cytometry scatter plots demonstrating analysis of buffers. Flow cytometry scatter plots demonstrating background noise signals following fluorescence activated cell sorting of A375M cells. (A) PBS buffer analysis using FACS Aria Cell Sorter. (B) HEPES buffer analysis using FACS Aria Cell Sorter. Images are representative of three independent experiments carried out in triplicate.



Appendix 9: Relative change in p63 concentration in A375M cells treated with paclitaxel for 1.5 – 6 hrs. (A) Histogram showing relative increase in p63-Cy5 is maximal at 6 hrs treatment with paclitaxel in intact A375M cells. Upregulation of p63-Cy5 is maximal in both (B) the nuclei and (C) mitochondria at 6 hrs.



Appendix 10: Single fluorescence labelling of WM1158 cells fractionated. Untreated WM1158 cells were labelled with (A) MitoTracker Orange and (B) Hoechst only prior to homogenisation to confirm localisation on flow cytometry plot. Flow cytometry analysis demonstrates (i) size of cells and (ii) gating of subcellular fractions within scatter plots. (A) Hoechst labelled nuclei enabling gating for cells and nuclei and (B) MitoTracker Orange enabled gating for free mitochondria.