GENETIC AND PROTEOMIC ANALYSIS OF THE P33ING1B TUMOUR SUPPRESSOR IN MELANOMA

by

ERIC I. CAMPOS

B.Sc. with Honours, University of Ottawa, 2000

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Experimental Medicine Program

THE UNIVERSITY OF BRITISH COLUMBIA

April 2006

ABSTRACT

The *ING1* (Inhibitor of Growth) gene is the founding member of at least five related human genes associated with tumour suppressive properties. *ING* genes are evolutionarily conserved and express cofactors of histone acetyltransferases (HAT) and histone deacetylases (HDAC). The *ING1* locus encodes at least three detectable protein isoforms, including the well-studied $p33^{ING1b}$ protein. Through associated HAT and HDAC activity, $p33^{ING1b}$ is capable of regulating the transcription of various genes, including the $p21^{waf1}$ and cyclin B1 cell cycle regulators and the pro-apoptotic Bcl-2 family member Bax, leading to inhibition of cell cycle progression and sensitization of cells to apoptosis. $P33^{ING1b}$ also enhances the nucleotide excision repair of ultraviolet-damaged DNA. This work describes 1) the generation and use of rabbit polyclonal antiserum that can specifically recognize the $p33^{ING1b}$ isoform; 2) the status of the *ING1* gene in malignant human melanoma; and 3) the regulation of the $p33^{ING1b}$ protein through protein phosphorylation and degradation.

Mutational alterations were found within the *ING1* gene of nearly a fifth of the melanoma biopsies examined. Two common alterations within the *ING1* gene at codons 102 and 260 were found to be detrimental to $p33^{ING1b}$-mediated enhancement of nucleotide excision repair. Mutations within the *ING1* gene may also be indicative of a poorer 5-year patient survival. The *ING1* gene was also found to be over-expressed in melanoma cells and biopsies compared to normal melanocytes. There was, however, widespread loss of nuclear expression of $p33^{ING1b}$ in the melanoma tumours.

This thesis further describes the regulation of the $p33^{ING1b}$ protein through phosphorylation of serine 126 by the cyclin-dependent kinase 1 in the absence of DNA damage and by the checkpoint kinase 1 upon genotoxic stress. Although serine 126 is near the nuclear-localization sequence, it was not found to affect the sub-cellular localization of the $p33^{ING1b}$ protein. Phosphorylation did however alter the half-life of the protein. Serine to alanine site-directed mutagenesis of codon 126 or the inhibition of the Cdk1 kinase both resulted in a higher turnover rate of the $p33^{ING1b}$ protein. Although $p33^{ING1b}$ did incorporate ubiquitin moieties, it was found not to undergo proteolysis through the classical ubiquitin-proteasome pathway.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	Full Name
6-4PP	pyrimidine (6-4) pyrimidone photoproducts
AFP	alpha fetoprotein
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
ATR	ATM-related protein
Bax	Bcl2-associated X protein
CAT	chloramphenicol acetyltransferase
Cdk	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
Chk1/2	checkpoint kinase 1/2
CIP	calf intestinal alkaline phosphatase
CKII	casein kinase II
CPD	cyclobutane pyrimidine dimers
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
GSK3α	glycogen synthase kinase 3α
GST	glutathione S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase
HRP	horseradish peroxidase
h-SMG-1	human homologue of the *C. elegans* protein CeSMG-1
ING	inhibitor of growth
KLH	keyhole limpet hemocyanin
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
MAPK	mitogen-activated protein kinase
MDM2	mouse double minute
NER	nucleotide excision repair
NLS	nuclear localization sequence
NTS	nucleolus-targeting sequence
NuA3	nucleosome acetylating H3
NuA4	nucleosome acetylating H4
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline tween-20
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PCR domain	potential chromatin regulatory domain
PCR-SOEing	polymerase chain reaction splicing overlap extension
PDIM	phosphorylation-dependent interacting motif

PHD	plant homeodomain
PI3K	phosphatidylinositol-3 kinase
PIKK	phosphatidylinositol-3 kinase-related kinase
PIM	peptide-interacting motif
PIP	PCNA-interacting protein domain
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homolog
NiNTA	nickel-nitrilotriacetic acid
Rb	retinoblastoma
RIA	radioimmunoassay
RNA	ribonucleic acid
RNAi	RNA Interference
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SCC	squamous cell carcinoma
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
SSCP	single strand conformational polymorphism
Ub	ubiquitin
UV	ultraviolet
Waf1	wild-type p53-activated factor
XP	xeroderma pigmentosum

PREFACE

Melanoma results from the malignant transformation and uncontrolled proliferation of melanocytes, the melanin-producing cells normally found in the basal layer of the skin epidermis. Melanoma is one of the most deadly cutaneous neoplasms. With an incidence increase of 4.1 percent per year in the US in the past few years (according to the American Cancer Society, 2005), there is great interest in finding the underlying genetic causes leading to its formation. There are numerous risk factors for melanoma, including sun sensitivity, light skin tone (little pigmentation), family predisposition, immunosuppression and the number of typical and large congenital nevi (MacKie *et al.*, 1989). However there is a great deal to be learned on the genetic factors that contribute to the cellular transformation of melanocytes and on the regulation of tumour suppressors that may be involved.

Although the exact mechanism of melanoma development remains to be elucidated, ultraviolet (UV) radiation is thought to be an important environmental factor linked to the transformation of cutaneous melanocytes (reviewed by Jhappan *et al.*, 2003). There are two mutually inclusive cellular pathways thought to contribute to melanomagenesis: 1) oncogenic activation of the Ras - mitogen-activated protein kinase (MAPK) cascade; and 2) inactivation of pathways that promote hypo-phosphorylation of the retinoblastoma (Rb) tumour suppressor. For the first, various studies have observed *RAS* (notably *NRAS*) gene mutations in approximately 5-25% of melanomas, or in some cases *RAS* gene amplification (Gorden *et al.*, 2003; Demunter *et al.*, 2001). Most

mutations were found to constitutively activate Ras which in turn activates the MAPK (RAF - MEK - ERK), or the PI3K - AKT - NF-κB pathway known to cross-talk with the former (Ackermann *et al.*, 2005; Chudnovsky *et al.*, 2005; Wu *et al.*, 2003), both of which promote cellular growth. Alternatively melanoma cells may also activate the MAPK or PI3K pathways without constitutive Ras activation. Loss of heterozygosity within the *PTEN* locus was observed in 30-50% of melanomas (Wu *et al.*, 2003); however, patients with Cowden disease (inherited *PTEN* mutation) are not reported to be susceptible to melanoma. Although it has also been found that up to 70% of melanomas may express mutant B-Raf (Davies *et al.*, 2002, Uribe *et al.*, 2003), a similar proportion of normal melanocytic nevi were found to carry the same mutation (Uribe *et al.*, 2003) suggesting that other genetic factors may be necessary for melanocytic transformation.

There are several pathways leading to the regulation of the Rb protein. Once thought to be the 'melanoma gene' the *CDKN2A* locus encodes two tumour suppressors within different reading frames (Quelle *et al.*, 1995) that can influence Rb activity. $P16^{INK4a}$ sequesters CDKs to prevent Rb phosphorylation (Rb inhibits cell cycle progression when hypo-phosphorylated) and $p14^{ARF}$ prevents mdm2-mediated p53 degradation, thus also Rb phosphorylation (see introductory chapter). Germline mutations of the *CDKN2A* gene are observed in a high proportion of familial melanomas (Hussussian *et al.*, 1994), however somatic mutations of this gene in sporadic primary melanoma are rare (Chachia *et al.*, 2000). Unlike other skin cancers there is a low (less than 15%) incidence of *TP53* mutations in melanoma (Gwosdz *et al.*, 2006). Both Rb inactivation and the activation of the Ras-MAPK/PI3K pathway are increasingly thought

to contribute to melanomagenesis (Ackermann *et al.*, 2005; Chudnovsky *et al.*, 2005). Since p53 activation does lead to the hypo-phosphorylation and activation of Rb; and unlike other cancers p53, $p16^{INK4a}$, $p14^{ARF}$ and Rb are infrequent targets in sporadic primary melanoma, there is a great interest to find other melanoma susceptibility genes that may lead to the inactivation of the retinoblastoma protein. A novel family of tumour suppressors, the Inhibitor of Growth (ING) family, has recently been identified and found to directly cooperate with the p53 protein. The $p33^{ING1b}$ protein is also capable of up-regulating the expression of the $p21^{Waf1}$ CDK inhibitor thereby contributing to Rb activation. Much of the present thesis concentrates on the $p33^{ING1b}$ protein. In order to determine whether $p33^{ING1b}$ inactivation may contribute to the susceptibility of sporadic melanoma, we sought to examine the status the *ING1* gene in malignant melanoma. Since $p33^{ING1b}$ impedes cellular growth and enhances the repair of damaged DNA, the regulation of the $p33^{ING1b}$ protein is also explored in the later part of the thesis. The observations made throughout this work were at times surprising but nonetheless interesting and will further contribute to the understanding of cutaneous melanoma development and of the $p33^{ING1b}$ tumour suppressor protein.

ACKNOWLEDGMENTS

I would like to acknowledge the Michael Smith Foundation for Health Research, the UBC & Vancouver Hospital Foundation, the Natural Science and Engineering Research Council, the National Cancer Institute of Canada (NCIC), the UBC Faculty of Graduate Studies and the UBC Department of Medicine, Division of Dermatology for their support.

I also sincerely thank my family and friends, as well as past and present members of Dr. Li's laboratory (particularly Yvonne, Jason, Mei, Marco and William for all their help). I also thank my supervisor Dr. Gang Li as well as my supervisory committee: Drs Vince Duronio, Bill Salh, Chris Ong and Vincent Ho as well as Dr. Hao Xiao (ImmuneChem Pharmaceuticals Inc.) and Dr. Magda Martinka (Vancouver Hospital) for guidance and help.

CO-AUTHORSHIP STATEMENT

Certain experiments and reagents were performed or obtained in collaboration with others, or under industrial contract, as listed below:

Synthetic peptides were generated by the Nucleic Acid and Protein Service Unit (NAPS) unit at the University of British Columbia. The radioimmunoassay of DNA samples was performed by Dr. David L. Mitchell (University of Texas M.D. Anderson Cancer Center). The KinaseProfiler^{TM} kinase selectivity screening is a service offered by Upstate Inc. Mass spectrometry analysis of p33^{ING1b} was done in the laboratory of Dr. Jeffrey Smith (The Burnham Institute). Certain kinase assays were performed with the help of Dr. Marco Garate (University of British Columbia) as well as the analysis for half-life estimations; triplicates for the half-life time point experiments were also obtained with the help of Wei-Hung Kuo (University of British Columbia).

Permission to reproduce copyright material was granted for the following published articles in written or as part of the publishing agreement:

1- Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

2- Campos E.I., Martinka M., Mitchell D.L., Dai D.L. and Li G. Mutations of the ING1 tumour suppressor gene detected in human melanoma abrogate nucleotide excision repair. 2004. Int J Oncol 25:73-80.

3- Campos E.I., Xiao H. and Li G. Generation of a polyclonal antibody specifically against the p33(ING1b) tumour suppressor. 2004. J Immunoassay Immunochem 25:71-80.

4- Campos E.I., Cheung K.J. Jr., Murray A., Li S. and Li G. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. 2002. Br J Dermatol 146:574-80.

1. INTRODUCTORY CHAPTER

1.1 The $p33^{ING1b}$ protein: A Literature Review

The *ING1* gene was originally identified using a strategy developed by Gudkov *et al.* (1994), for the recovery genetic elements that can promote neoplastic growth deregulation when lost or inactivated. Specifically, through subtractive hybridization between normal human mammary epithelial cells and seven breast cancer cell lines, genetic suppressor elements that were lost in the cancer cell lines were isolated. These genetic fragments were then cloned in an inverse orientation and the resulting antisense constructs retained if found to possess oncogenic characteristics *in vivo*. The sequence of these oncogenic fragments were subsequently used to retrieve the full gene from a normal human fibroblast cDNA library; hence the cloning of the *ING1* gene (Garkatsev *et al.*, 1996). *ING1* has been cytogenetically mapped to 13q34 at 5Mb of the telomeric region of the chromosome (Zeremski *et al.*, 1997). The *ING1* gene is also the founding member of at least five additional related human genes, namely *ING2-5* and *INGX* (*INGX* encodes a truncated ING-like PHD domain and expression has yet to be detected) (He *et al.*, 2005; Shimada *et al.*, 1998; Nagashima *et al.*, 2003; Shiseki *et al.*, 2003). It is interesting to note that *ING* genes have been shown to flank important functional genes. For example, *ING1* is flanked by the *RAB20* gene which encodes a Ras family member and the Rho guanidine nucleotide exchange factor gene *ARHGEF7* (He *et al.*, 2005). However, a

A version of this chapter has been published in: Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

coordinated regulation of proximal genes within *ING*-containing clusters has yet to be explored. Most importantly, ING proteins are found to be highly conserved through evolution and have so far been observed among members of all four eukaryotic kingdoms (animal, plant, fungi and protista) (He *et al.*, 2005; Mayanagi *et al.*, 2005).

The *ING1* gene comprises three exons that encode three detectable alternatively spliced protein isoforms of 47 ($p47^{ING1a}$), 33 ($p33^{ING1b}$) and 24 ($p24^{ING1c}$) kDa (Gunduz *et al.*, 2000; Saito *et al.*, 2000), of which $p33^{ING1b}$ is the most abundant and ubiquitously expressed in human tissues (Saito *et al.*, 2000; Shimada *et al.*, 1998). The following sections review studies on the structure of the $p33^{ING1b}$ protein, its implication in the cell cycle, in apoptosis and in DNA repair, as well as the status of the *ING1* gene in human cancers.

1.1.1 Structure of the $p33^{ING1b}$ Protein

ING family proteins are defined by the presence of a carboxyl C4-H-C3 zinc finger known as the Plant Homeodomain (PHD), as well as by the presence of an uncharacterized KIQI/KVQL motif named the 'Potential Chromatin Regulatory (PCR) domain' (initially termed the MDS00105 motif) (Kawaji *et al.*, 2002; He *et al.*, 2005). Most ING proteins also comprise an amino terminal leucine zipper. The $p33^{ING1b}$ protein is one of the few family members that lack the leucine zipper. Conversely, the $p33^{ING1b}$ protein has some unique structural features. This ING protein is the only human isoform to possess an amino terminal proliferating-cell nuclear antigen (PCNA)-interacting

protein (PIP) domain. Although all human ING proteins have a Nuclear Localization Sequence (NLS), the $p33^{ING1b}$ isoform is one of the few to possess two functional nucleolus-targeting motifs within its NLS (Scott *et al.*, 2001a). Based on sequence alignment analysis, $p33^{ING1b}$ is also predicted to contain at least two additional domains: a 'N-REASP-C' N-terminal sequence termed the phosphorylation-dependent interacting motif (PDIM) due to its resemblance to the RSXpSXP binding motif found on 14-3-3 proteins (He *et al.*, 2005; Yaffe *et al.*, 1997); and a C-terminal peptide-interacting motif (PIM) (He *et al.*, 2005) thought to mediate protein-protein interactions due to the presence of various basic residues (He *et al.*, 2005) (Figure 1-1).

Unfortunately little effort has so far been done to characterize most of the $p33^{ING1b}$ domains. However, some data has been obtained on the role of the PHD domain. The PHD finger belongs to the Treble class of zinc-binding motifs and is most often found in chromatin-interacting proteins. Like the closely related FYVE zinc finger, the $p33^{ING1b}$ and $p33^{ING2}$ PHD domains can serve as nuclear phosphoinositide (PtdIns)-binding modules with a strong preference for PtdIns(5)P (Gozani *et al.*, 2003). Phosphoinositide binding may help regulate ING protein functions in at least two distinct manners based on observations made on the $p33^{ING2}$ PHD domain. First, overexpression of the lipid PIKIIβ kinase (which converts PtdIns(5)P into PtdIns(4,5)P_2) caused a sharp reduction of chromatin bound $p33^{ING2}$ and increase of free $p33^{ING2}$, while overexpression of the IpgD phosphatase had the opposite effect (Gozani *et al.*, 2003). Secondly, PIKIIβ overexpression inhibited $p33^{ING2}$-dependent acetylation of the p53 protein and enhancement of apoptosis without affecting overall $p33^{ING2}$ protein levels (Gozani *et al.*,

2003). These observations imply a regulatory role for the ING PHD zinc fingers as PtdIns receptors and modulators of ING protein localization and activity.

1.1.2 Biological Relevance of the $p33^{ING1b}$ Protein

The $p33^{ING1b}$ protein is a type II tumour suppressor. It is involved in a wide range of vital biological functions including cell cycle regulation, gene regulation, DNA repair and apoptosis. ING proteins lack enzymatic activity but serve as co-factors of histone acetyltransferases (HATs) and histone deacetylases (HDAC). HAT enzymes catalyze the transfer of an acetyl group from a given substrate onto the ε-$NH3^+$ groups of histone amino terminal lysine residues. This transfer alters local charges and increases histone hydrophobicity and is thought to affect chromatin dynamics. The $p33^{ING1b}$ protein has been co-purified with the p300 HAT and the Sin3 complex which includes HDAC1 and HDAC2 (Vieyra *et al.*, 2002; Kuzmichev *et al.*, 2002; Skowyra *et al.*, 2001). The role of ING proteins in histone acetylation was first clarified in yeast. The *Saccharomyces cerevisae* ING-family member Yng1 has been described as a core component of the NuA3 HAT complex (Howe *et al.*, 2002). Yng1 was found non-essential for NuA3 HAT activity or for the structural integrity of the complex, but did however regulate the substrate specificity of the complex. In the absence of Yng1, NuA3 was found capable of acetylating free histones but lacked enzymatic activity on nucleosomes; whereas Yng1-proficient cells exhibited specific NuA3 activity on nucleosomal histones 3 and 4 but not on free histones (Howe *et al.*, 2002). The same observation has now been extended to other ING family members (Campos *et al.*, 2004; Doyon *et al.*, 2006) as it is believed that

they may help recruit HAT and HDAC complexes onto chromatin; therefore underlying ING protein implication in transcriptional regulation, cell cycle checkpoints, apoptosis and DNA repair.

1.1.2.1 Transcriptional Regulation

Much of the anti-proliferative effect of the p53 tumour suppressor is attributed to the up-regulation of the cyclin-dependent kinase (CDK) inhibitor $p21^{Waf1}$ (el-Deiry *et al.*, 1993). $P33^{ING1b}$-dependent suppression of growth is only evident in the presence of wild-type p53 (Garkatsev *et al.*, 1998). In fact, expression of anti-sense $p33^{ING1b}$ has been shown to alleviate p53-dependent inhibition of growth (Garkatsev *et al.*, 1998). The activation of various p53-inducible promoters, including Waf1, MDM2 and Bax and the repression of AFP is now known to require both p53 and $p33^{ING1b}$ (Takahashi *et al.*, 2002; Kataoka *et al.*, 2003). Both proteins have been reported to physically associate (Garkatsev *et al.*, 1998; Leung *et al.*, 2002), and the expression of either antisense *ING1* or antisense *p53* has been shown to result in a three to five-fold repression of the Waf1 promoter (Garkatsev *et al.*, 1998). Transcriptional expression of $p21^{Waf1}$ is also known to correlate with the amount of $p33^{ING1b}$ in cells over-expressing p53 (Shimada *et al.*, 2002). While the exact mechanism of $p33^{ING1b}$-mediated transcriptional regulation remains to be elucidated, it is evident that associated HAT and HDAC activities are likely involved.

Like p53, $p33^{ING1b}$ is believed to mediate contacts with chromatin. Electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP)

experiments have shown that $p33^{ING1b}$ is capable of binding DNA either directly or indirectly (Kataoka *et al.*, 2003; Vieyra *et al.*, 2002). The PCR domain of $p33^{ING1b}$ is hypothesized to help associate this protein to various HAT and HDAC complexes, and is thought necessary for interactions with Sap30 (He *et al.*, 2005; Skowyra *et al.*, 2001), a component of the Sin3/HDAC complex involved in transcriptional repression. $P33^{ING1b}$ is also known to associate with the p300 transcriptional up-regulator HAT (Vieyra *et al.*, 2002) and the Sir2 transcriptional repressor HDAC (Kuzmichev *et al.*, 2002). There are additional clues suggesting that $p33^{ING1b}$ is a transcriptional regulator. First, $p33^{ING1b}$ accumulates within the nucleolus where it may up-regulate various ribosomal components (Takahashi *et al.*, 2002; Scott *et al.*, 2001a). Most importantly, microarray analysis of cells expressing antisense *ING1b* significantly altered the expression of various genes in murine epithelial cells (Takahashi *et al.*, 2002) (known $p33^{ING1b}$ transcriptional targets are listed in Table 1-1). In a study aimed to investigate $p33^{ING1b}$ repression of *AFP*, it was found that $p33^{ING1b}$ can directly act through specific promoter domains such as the *AFP* AT motif (Kataoka *et al.*, 2003). Although $p33^{ING1b}$ repression of *AFP* was stronger in p53-proficient HepG2 cells, it could also be observed in a p53-null isogenic cell line invoking the possibility of p53-dependent and p53-independent regulatory mechanisms. Although it remains to be tested, the authors of this study proposed that $p33^{ING1b}$ may repress *AFP* transcription by binding to the AT motif, thus excluding HNF1 (the primary *AFP* positive transcriptional regulatory factor) binding, and by increasing p53 acetylation through binding and inhibition of Sir2.

1.1.2.2 Cell Cycle Checkpoints

$P33^{ING1b}$ is reported to enhance both G_1 and G_2 cell cycle checkpoints (Campos *et al.*, 2004). $P21^{Waf1}$ (also know as Cip1) is a potent mediator of the G_1 cellular checkpoint (Harper *et al.*, 1993). The amino-terminus of $p21^{Waf1}$ avidly binds and disables cyclin dependent kinases (Cdks) by forming ternary complexes with the Cdks and associated cyclins (Chen *et al.*, 1995). This inhibition results in the hypo-phosphorylation of the retinoblastoma (Rb) tumour suppressor protein, which in turn leads to Rb binding and sequestration of E2F, a critical transcription factor for the G_1/S transition (reviewed by Harbour and Dean 2000). $P33^{ING1b}$ has been shown to enhance p53-dependent up-regulation of $p21^{Waf1}$ (Garkatsev *et al.*, 1998).

Initiation of the G_2 arrest requires phosphorylation of Cdk1 and is p53 and ING-independent. However like p53, $p33^{ING1b}$ is believed to help prolong the G_2 cell cycle checkpoint upon genotoxic stress. Several studies demonstrate that p53 can promote the maintenance of a G_2 cell cycle arrest by suppressing both Cdk1 and cyclin B expression (Imbriano *et al.*, 2005; Clifford *et al.*, 2003; Lakin and Jackson, 1999; Manni *et al.*, 2001; Innocente *et al.*, 1999). Cyclin B is the regulatory subunit of Cdk1 and is required for mitotic onset (reviewed by Porter and Donoghue, 2003). Microarray analysis of $p33^{ING1b}$-regulated genes identified *CCNB1* (cyclin B1 gene) as a repressed target of $p33^{ING1b}$ (Takahashi *et al.*, 2002), suggesting a similar involvement of $p33^{ING1b}$ in the cellular G_2/M checkpoint. This is substantiated by a few observations: First, overexpression of $p33^{ING1b}$ was shown to prolong adriamycin-induced G_2 arrest in the

p53-null H1299 non-small cell lung carcinoma cell line (Tsang *et al.*, 2003). Most importantly, reverse transcriptase polymerase chain reaction (RT-PCR) analysis of p53-deficient SAOS2 cells infected with *ING1b* also resulted in a decrease in cyclin B1 mRNA levels after 72 hours (Takahashi *et al.*, 2002). However, infection of both *p53* and *ING1b* constructs caused a marked reduction of cyclin B1 mRNA 24 h post-infection, suggesting that $p33^{ING1b}$ repression of cyclin B1 is enhanced in the presence of p53. Although $p33^{ING2}$ but not $p33^{ING1b}$ has been found to promote p53 K382 acetylation (Nagashima *et al.*, 2001) it remains to be determined whether $p33^{ING1b}$ may still promote acetylation of other p53 lysine residues to enhance p53 transcriptional regulation. This may be important since p53 protein lacking C-terminal lysine residues has minimal effects on cyclin B1 regulation and can promote G_1 but not G_2 cell cycle arrest (Nakamura *et al.*, 2002).

1.1.2.3 Apoptosis

Observations regarding developmental remodeling of regressing tails of *Xenopus laevis* tadpoles initially linked $p33^{ING1b}$ activity to programmed cellular death, or apoptosis. It was noted that the Xenopus homolog of $p33^{ING1b}$ was widely present in the receding tail of the tadpoles but was absent in the growing hind limbs (Wagner *et al.*, 2001). Antisense expression of *ING1b*, was subsequently shown to promote anchorage-independent growth and neoplastic transformation of mammalian cells as well as tumour formation in nude mice (Garkatsev *et al.*, 1996). Like in transcriptional regulation, $p33^{ING1b}$-dependent sensitization to apoptosis is largely influenced by p53.

Overexpression of $p33^{ING1b}$ was shown to have little effect on the survival of human and murine fibroblasts in the absence of p53; while expression of both $p33^{ING1b}$ and p53 were required for efficient suppression of colony formation (Garkatsev *et al.*, 1998). In agreement, ectopic expression of both $p33^{ING1b}$ and p53 was shown to synergistically induce apoptosis in various cultured cell lines, inhibit anchorage-dependent cell growth and reduce cell viability more efficiently than with p53 alone (Shinoura *et al.*, 1997; Shimada *et al.*, 2002). It is believed that $p33^{ING1b}$ is involved in the p53-mediated intrinsic apoptotic pathway since it can enhance the transcriptional up-regulation of the pro-apoptotic Bcl-2 family member Bax at the promoter level and consequently promotes changes in mitochondrial membrane potential favorable to cytochrome C release (Cheung and Li, 2002; Nagashima *et al.*, 2001).

1.1.2.4 DNA Repair

Various observations imply a role of the $p33^{ING1b}$ protein in nucleotide excision repair (NER). NER is one of the five major mammalian DNA repair pathways (the other four being 1- base excision repair for removal of deaminated bases; 2- mismatch repair in DNA replication; 3- non-homologous end-joining and 4- homologous recombination for the repair of double strand breaks). NER is the pathway responsible for the removal of bulky DNA lesions caused by intra-strand cross-linking such as those caused by UV irradiation (Eveno 1995). The most prominent UV-induced DNA lesions are cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP) (reviewed by Cadet *et al.*, 2005). $P33^{ING1b}$ was observed to be transcriptionally up-

regulated upon UV exposure in the MMRU melanoma cell line in a time and dose dependent manner (Cheung *et al.*, 2001). Most importantly, overexpression of $p33^{ING1b}$ was shown to enhance the repair of a reporter plasmid irradiated with ultraviolet light *ex vivo* and help reduce the amount of UV-induced genomic DNA lesions *in vivo* while antisense expression of *ING1b* had the opposite effect (Cheung *et al.*, 2001).

$P33^{ING1b}$-mediated enhancement of DNA repair is again dependent on wt p53. However unlike p53, $p33^{ING1b}$ is incapable of interacting with the core NER factors xeroderma pigmentosum complementation group A and B (XPA and XPB) (Cheung *et al.*, 2001). The exact mechanism of $p33^{ING1b}$-mediated enhancement of NER is currently under investigation and likely involves chromatin remodeling capabilities coupled to $p33^{ING1b}$-associated HATs (unpublished data). $P33^{ING1b}$ is also believed to enhance the repair of damaged DNA through interactions with PCNA. PCNA is a highly conserved protein which forms a homo-trimeric complex that can encircle DNA as part of larger multi-protein complexes involved in DNA replication and DNA repair (Maga and Hubscher, 2003). In NER, PCNA promotes the re-synthesis of damaged DNA strands by promoting DNA polymerase activity (Maga and Hubscher, 2003). The $p21^{Waf1}$ protein is known to bind and regulate PCNA activity thereby inhibiting DNA replication (Maga and Hubscher, 2003; Waga *et al.*, 1994). Through its PIP domain, $p33^{ING1b}$ is believed to competitively bind PCNA and displace $p21^{Waf1}$ to favor DNA repair over DNA replication (Scott *et al.*, 2001b).

1.1.3 Status of *ING1* in Human Malignancies

The status of the *ING1* gene is reported to vary in different human neoplasms (see Table 1-2). In most cancers observed to date, there are infrequent gene alterations but rather persistent loss of *ING1* gene expression (reviewed by Campos *et al.*, 2004). In a recent study, *ING1* expression was found to inversely correlate with the progression of astrocytomas (Vieyra *et al.*, 2003). *ING1* mRNA expression levels in pilocytic astrocytomas (WHO grade I) and in diffuse astrocytomas (WHO grade II) were significantly higher than in anaplastic astrocytomas and glioblastomas (WHO grades III and IV). Although expression of *ING1* was found to be independent of neuroblastoma progression, low *ING1* mRNA levels were associated with poor patient prognosis (Takahashi *et al.*, 2004). It is therefore not surprising that *ING1*-null mice are hypersensitive to DNA damaging events and are prone to lymphoma development (Kichina *et al.*, 2005).

Loss of *ING1* expression was also found to correlate with the invasiveness of bladder tumours as reduced levels of $p33^{ING1b}$ are found in most advanced cancers relative to early-stage transitional cell carcinoma of the bladder (Sanchez-Carbayo *et al.*, 2003). Intriguingly, $p33^{ING1b}$ expression was reported in this latter study to correlate with the survival of bladder cancer patients. Although the role of $p33^{ING1b}$ in bladder cancer needs further elucidation, this observation may be at least partly explained by the fact that much of this correlation is attributed to invasive cancers and also by the use of platinum-based chemotherapy in the treatment of this type of cancer (a combination of

methotrexate, vinblastine, doxorubicin, and cisplatin (MVAC) or of cisplatin, methotrexate, and vinblastine (CMV) (reviewed by Hussain and James, 2003). Platinum-based compounds act by creating intra-strand DNA cross-links (Bernges and Holler, 1991), which like UV-induced DNA photoproducts are also repaired through NER (Reardon *et al.*, 1999). Since NER can be enhanced by $p33^{ING1b}$ overexpression (Cheung *et al.*, 2001), the observed high levels of $p33^{ING1b}$ in this type of cancer may contribute to the repair of platinum-induced damage.

1.2 Protein Phosphorylation

Posttranslational modifications or the proteolytic cleavage or covalent addition of a modifying group to one or more amino acids, is a versatile mechanism to regulate protein function in space and time. There are over two hundred forms of posttranslational modifications known to exist (Banks *et al.*, 2000). Protein phosphorylation, once a mere phenomenon observed in glycogen metabolism (Rall and Sutherland, 1958), is now recognized as a ubiquitous and reversible transient mechanism capable of regulating protein activity. The added phosphoryl group is dianionic under most physiological conditions. The negative charges and capability to form extensive hydrogen bonds with the four phosphoryl oxygen atoms can have significant effects on protein conformation, interactions and functions. Up to two percent of the human genome is though to encode various protein kinases (Lander *et al.*, 2001). It is not surprising that protein phosphorylation is implicated in nearly all aspects of cellular function.

Various phosphorylation events are known to regulate the cellular response to DNA damage. For example, a number of protein kinases, notably the phosphatidylinositol-3 kinase-related kinase (PIKK) family members including ataxia-telangiectasia mutated (ATM), ATM-related protein (ATR), DNA-dependent protein kinase (DNA-PK), and the human homologue of the C. elegans protein CeSMG-1 (hSMG-1) are mobilized upon genomic injury and play critical roles initiating the G_2 cell cycle arrest (Bakkenist *et al.*, 2004; Brumbaugh *et al.*, 2004). ATM mainly responds to DNA strand breaks through altered chromatin topology which leads to auto-activation and subsequent phosphorylation of downstream modulators (Bakkenist *et al.*, 2004). Chemical agents such as the type II topoisomerase inhibitor doxorubicin are also reported to cause ATM auto-phosphorylation and activation through the generation of reactive oxygen species (ROS) (Kurz *et al.*, 2004). ATR is mainly activated through stalled replication forks, such as those resulting from ultraviolet-induced bulky DNA photo-lesions (Ward *et al.*, 2004; Unsal-Kacmaz *et al.*, 2002; Guo *et al.*, 2000). Upon activation ATM phosphorylates p53 and the damage checkpoint effector kinases Chk1 and Chk2, while ATR selectively phosphorylates p53 and Chk1 but not Chk2 (Helt *et al.*, 2005). Chk1 plays a critical role in inducing a G_2 cell cycle arrest, which allows genetic repair prior to mitotic onset. Chk1 phosphorylates and inhibits the protein phosphatase Cdc25 (Sanchez *et al.*, 1997), thus inhibiting the cyclin dependent kinase 1 (Cdk1) by preventing de-phosphorylation (Furnari *et al.*, 1997). Both Chk1 and Chk2 can also phosphorylate p53 on serine 20 (Shieh *et al.*, 2000; Hirao *et al.*, 2000). Mdm2 is an E3 recognin enzyme that can recognize the N-terminus of the p53 tumour suppressor and promote p53 degradation (Honda *et al.*, 1997; Kubbutat *et al.*, 1997). Phosphorylation of

p53 on serine 20 can prevent mdm2 binding and therefore p53 degradation (Unger *et al.*, 1999). There are no reports on phosphorylated $p33^{ING1b}$ residues prior to this thesis.

1.3 Protein Degradation

Protein degradation composes an important regulatory system necessary for proper cellular function. There are various pathways that regulate protein turnover. The most extensively studied proteolytic path involves ubiquitination of the target protein and subsequent degradation within a proteic complex known as the proteasome. In this model, ubiquitin (Ub) is transferred unto proteins through a series of well-orchestrated events to mark them for destruction. Typically, Ub is activated by proteins termed E1 enzymes, which hydrolyze ATP to form a complex with adenylated Ub. E1 proteins then transfer activated Ub unto E2 or Ub ligases and then onto the target protein through an E3 recognin enzyme which can recognize specific proteic targets (reviewed by Welchman *et al.*, 2005). Successive ligation of Ub onto an ubiquitinated residue (polyubiquitination) then marks proteins for degradation, unlike monoubiquitination which serves other regulatory functions (Welchman *et al.*, 2005). The proteasome is composed of a multicatalytic cylinder-like protease termed the 20S core particle which is flanked on both ends by a 19S regulatory particle that serve as 'lids' regulating the entry into the proteasome (Groll *et al.*, 2005). Several Ub-binding components then help 'thread' proteins into the core particle for proteolysis (Groll *et al.*, 2005).

The lysosome is another important proteolytic cellular component responsible for protein degradation. This organelle contains the highest concentration of proteases in the cell (de Duve, 1983) and is involved in both targeted and non-specific degradation of proteins. There are various digestive processes mediated by the lysosome such as receptor-mediated endocytosis, pinocytosis and phagocytosis of extracellular material, but also the micro- and macro-autophagy and chaperone-assisted transport and degradation of intracellular material. Micro- and macro-autophagy results from lysosomal internalization of the cytosol and can be selective or non-selective of its targets (Regiorri *et al.*, 2005). While selective-targeting of proteins through autophagy is poorly understood, non-selective autophagy is influenced by extracellular factors, such as nutrients and cytokines, including IFN-γ and the IL-3 growth factor (Gutierrez *et al.*, 2004; Lum *et al.*, 2005). Chaperone-mediated lysosomal degradation differs from autophagy since it does not require vesicular trafficking of the target proteins. Instead proteins are targeted to a receptor within the lysosomal membrane, the lysosome-associated membrane protein type 2a (lamp2a) receptor (Majeski and Dice, 2004). Proteins containing a "KFERQ" or related recognition sequences are bound by the Hsc70 chaperone and an associated protein complex, which then target the substrate to the lamp2a receptor where it is denatured and imported into the lysosome (reviewed by Majeski and Dice, 2004).

1.4 Thesis theme

The main objectives of this thesis are to: 1) better understand the status of the *ING1* gene in human melanoma; and 2) to determine if the $p33^{ING1b}$ tumour suppressor protein is subject to posttranslational regulation through protein phosphorylation and if so, when and how. The first objective was met by raising polyclonal antisera to specifically study the $p33^{ING1b}$ protein and by observing the expression pattern of this protein in human melanoma. Since $p33^{ING1b}$ closely associates with the p53 tumour suppressor and the *TP53* gene is rarely mutated in human melanoma, the *ING1* gene was also scanned for mutations in primary melanoma tumours and the *ING1* mutations studied for loss of function. The latter objective was met by demonstrating that the $p33^{ING1b}$ is a phospho-protein and identifying serine 126 as a phospho-residue. This work also identifies the kinases that phosphorylate serine 126 and addresses the effect of phosphorylation on the turnover rate of the $p33^{ING1b}$ through lysosomal degradation.

1.5 Hypotheses

The hypothesis for chapter 4 is:

1. The *ING1* gene is silenced or mutated in human melanoma.

The hypotheses for chapter 5 are:

2. The $p33^{ING1b}$ protein is phosphorylated upon genotoxic stress.
3. The DNA damage-responsive PIKK-related kinases ATM, ATR, DNA-PK or hSMG-1 phosphorylate $p33^{ING1b}$.
4. Protein phosphorylation regulates the $p33^{ING1b}$ protein.

1.6 Figures & Tables

Figure 1-1 Structural features of the $p33^{ING1b}$ protein. All ING proteins are defined by the presence of a C-terminal plant homeodomain (PHD) zinc finger motif which interacts with rare nuclear phosphoinositides populations and enhances binding to chromatin (Gozani 2003) and by a yet to be defined but highly conserved potential chromatin regulatory (PCR) domain (He 2005). The $p33^{ING1b}$ protein is also the only human ING protein to contain a functional PCNA-interacting (PIP) motif through which $p33^{ING1b}$ may help mediate DNA repair (Scott 2001b) and a truncated bromodomain still not known to mediate binding to acetylated residues. Like all mammalian ING proteins (except INGx), $p33^{ING1b}$ also contains a nuclear localization sequence (NLS); however $p33^{ING1b}$ and $p33^{ING2}$ are the only ING proteins to further contain two functional nucleolar translocation sequences within the NLS sequence (Scott 2001a). The $p33^{ING1b}$ protein is also predicted to contain a N-terminal sequence that resembles the RSXpSXP binding motif found on 14-3-3 proteins and a C-terminal lysine-rich sequence hypothesized to mediate protein-protein interactions (He 2005) (yet to be tested and therefore not shown). Numbers denote amino acid position.

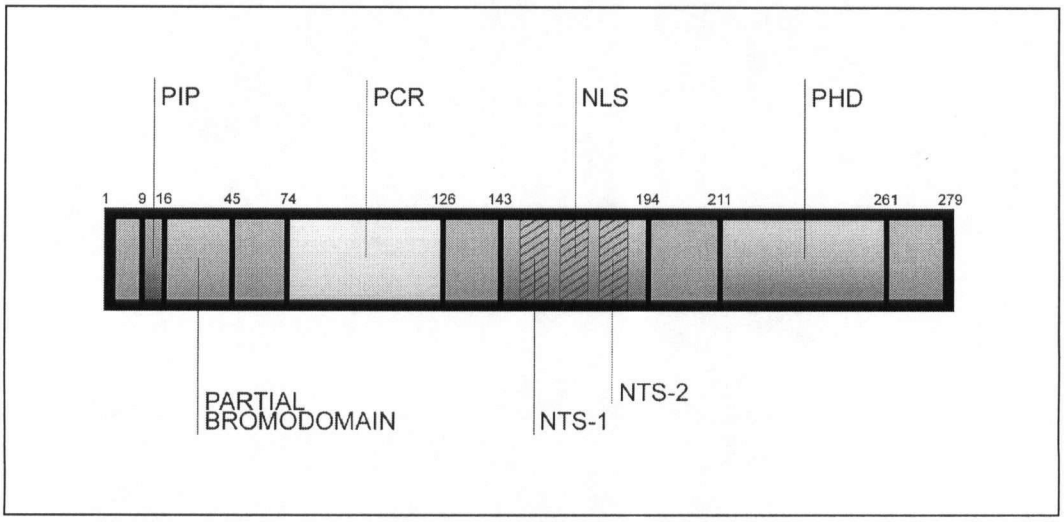


Table 1-1 List of $p33^{ING1b}$-regulated genes.

Target	Regulation	Reference
γ-actin	↓	Takahashi 2002
ζ-proteasome	↓	Takahashi 2002
AFP	↓	Takahashi 2002
Aldehyde dehydrogenase II	↓	Takahashi 2002
Cyclin B1	↓	Takahashi 2002
DEK	↓	Takahashi 2002
IGF-II receptor	↓	Takahashi 2002
Myosin light chain	↓	Takahashi 2002
Osomotic stress protein	↓	Takahashi 2002
Osteopontin	↓	Takahashi 2002
SDR	↓	Takahashi 2002
Serum albumin	↓	Takahashi 2002
TDE 1	↓	Takahashi 2002
TIS11	↓	Takahashi 2002
Bax	↑	Cheung 2002
Ef-2 (elongation factor-2)	↑	Takahashi 2002
Int-6	↑	Takahashi 2002
Waf1	↑	Garkavtsev 1998
RP L12	↑	Takahashi 2002
RP S7	↑	Takahashi 2002
RP S11	↑	Takahashi 2002
RP S29	↑	Takahashi 2002
TPT1	↑	Takahashi 2002

Table 1-2 Summary of reported *ING1* expression levels and gene alterations in human malignancies.

Tumour type	Expression ↓ or ↑	Rate (%)	Mutations (%)	References
Bladder cancer	↓	ND	ND	Sanchez-Carbayo *et al.* 2003
Brain tumour	↓	100	3.4	Vieyra *et al.* 2003
Brain tumour	↓	ND	0.0	Tallen *et al.* 2004
Breast cancer	↓	43.8	0.3	Toyama *et al.* 1999
Breast cancer	↓	70.8	ND	Tokunaga *et al.* 2000
Breast cancer	↓	80.2	ND	Nouman *et al.* 2003
Breast cancer cell lines	↓	100	0.0	Toyama *et al.* 1999
Esophageal SCC	↓	54.8	12.9	Chen *et al.* 2001
Esophagogastric junction adenocarcinoma	↓	63.2	5.3	Hara *et al.* 2003
Gastrointestinal cancers	↓	75.0	ND	Oki *et al.* 1999
Hepatocarcinoma	↓	54.7	ND	Ohgi *et al.* 2002
Lymphoblastic leukaemia	↓	76.5	ND	Nouman *et al.* 2002b
Lymphoid cancer cell lines	↓	56.3	0.0	Ohmori *et al.* 1999
lung cancer	↓	42.0	0.0	Kameyama *et al.* 2003
Basal cell carcinoma	↑	25.0	1.9	Chen *et al.* 2003
Brain tumour cell lines	↑	80.0	ND	Tallen *et al.* 2003
Malignant melanoma	↑	80.0	ND	Nouman *et al.* 2002a
Malignant melanoma	↑	96.3	19.6	Campos *et al.* 2004
Melanoma cell lines	↑	100	7.1	Campos *et al.* 2002
Oral SCC	↑	93.0	ND	Hoque *et al.* 2002
Colorectal carcinoma	ND	ND	0.0	Sarela *et al.* 1999
Gastrointestinal carcinoma cell lines	ND	ND	8.3	Oki *et al.* 1999
Head & neck SCC	ND	ND	13.0	Gunduz *et al.* 2000
Head & neck SCC	ND	ND	0.0	Sanchez-Cespedes *et al.* 2000
Various haematological malignancies	ND	ND	0.0	Bromidge and Lynas 2002
Myeloid leukemia	ND	ND	0.0	Ito *et al.* 2002
Non-small cell Oral SSC	ND	ND	0.0	Krishnamurthy *et al.* 2001
Ovarian cancer	ND	ND	0.0	Toyama *et al.* 1999

ND: Not Determined

1.7 References

Ackermann J., Frutschi M., Kaloulis K., McKee T., Trumpp A. and Beermann F. Metastasizing melanoma formation caused by expression of activated N-RasQ61K on an INK4a-deficient background. 2005. Cancer Res 65:4005-11.

Bakkenist C.J. and Kastan M.B. Initiating cellular stress responses. 2004. Cell 118:9-17.

Banks R.E., Dunn M.J., Hochstrasser D.F., Sanchez J.C., Blackstock W., Pappin D.J. and Selby P.J. Proteomics: new perspectives, new biomedical opportunities. 2000 Lancet 356:1749-56.

Bernges F. and Holler E. The reaction of platinum(II) complexes with DNA. Kinetics of intrastrand crosslink formation in vitro. 1991. Nucleic Acids Res 19:1483-9.

Bromidge T. and Lynas C. Relative levels of alternative transcripts of the ING1 gene and lack of mutations of p33ING1 in haematological malignancies. 2002. Leuk Res 26:631-5.

Brumbaugh K.M., Otterness D.M., Geisen C., Oliveira V., Brognard J., Li X., Lejeune F., Tibbetts R.S., Maquat L.E. and Abraham R.T. The mRNA surveillance protein hSMG-1 functions in genotoxic stress response pathways in mammalian cells. 2004. Mol Cell 14:585-98.

Cadet J., Sage E. and Douki T. Ultraviolet radiation-mediated damage to cellular DNA. 2005. Mut Res 571:3-17.

Cachia A.R., Indsto J.O., McLaren K.M., Mann G.J., Arends M.J. CDKN2A mutation and deletion status in thin and thick primary melanoma. 2000. Clin Cancer Res 6:3511-5.

Campos E.I., Cheung K.J. Jr., Murray A., Li S. and Li G. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. 2002. Br J Dermatol 146:574-80.

Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

Campos E.I., Martinka M., Mitchell D.L., Dai D.L. and Li G. Mutations of the ING1 tumour suppressor gene detected in human melanoma abrogate nucleotide excision repair.
2004. Int J Oncol 25:73-80.

Chen B., Campos E. I., Crawford R., Martinka M. and Li G. Analyses of the tumour suppressor ING1 expression and gene mutation in human basal cell carcinoma. 2003. Int J Oncol 22:927-31.

Chen J., Jackson P.K., Kirschner M.W. and Dutta A. Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. 1995. Nature 374:386-8.

Chen L., Matsubara N., Yoshino T., Nagasaka T., Hoshizima N., Shirakawa Y., Naomoto Y., Isozaki H., Riabowol K. and Tanaka N. Genetic alterations of candidate tumour suppressor ING1 in human esophageal squamous cell cancer. 2001. Cancer Res 61:4345-9.

Cheung K.J. Jr and Li G. p33(ING1) enhances UVB-induced apoptosis in melanoma cells. 2002. Exp Cell Res 279:291-8.

Cheung K.J. Jr, Mitchell D., Lin P. and Li G. The tumour suppressor candidate p33(ING1) mediates repair of UV-damaged DNA. 2001. Cancer Res 61:4974-7.

Chudnovsky Y., Adams A.E., Robbins P.B., Lin Q., Khavari P.A. Use of human tissue to assess the oncogenic activity of melanoma-associated mutations. 2005. Nat Genet 37:745-9.

Clifford B., Beljin M., Stark G.R. and Taylor W.R. G2 arrest in response to topoisomerase II inhibitors: the role of p53. 2003. Cancer Res 63:4074-81.

Davies H., *et al.* Mutations of the BRAF gene in human cancer. 2002. Nature 417:949-54.

de Duve C. Lysosomes revisited. 1983. Eur J Biochem 137:391-7.

Demunter A., Stas M., Degreef H., De Wolf-Peeters C. and van den Oord J. Analysis of N- and K-ras mutations in the distinctive tumour progression phases of melanoma. 2001. J Invest Dermatol 117:1483-9.

el-Deiry W.S., Tokino T., Velculescu V.E., Levy D.B., Parsons R., Trent J.M., Lin D., Mercer W.E., Kinzler K.W. and Vogelstein B. WAF1, a potential mediator of p53 tumour suppression. 1993. Cell 75:817-25.

Eveno E., Bourre F., Quilliet X., Chevallier-Lagente O., Roza L., Eker A.P., Kleijer W.J., Nikaido O., Stefanini M. and Hoeijmakers J.H. Different removal of ultraviolet photoproducts in genetically related xeroderma pigmentosum and trichothiodystrophy diseases. 1995. Cancer Res 55:4325-32.

Furnari B., Rhind N. and Russell P. Cdc25 mitotic inducer targeted by chk1 DNA damage checkpoint kinase. 1997. Science 277:1495-7.

Garkatsev I., Kazarov A., Gudkov A. and Riabowol K. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. 1996. Nat Genet 14:415-20.

Garkavtsev I., Grigorian I.A., Ossovskaya V.S., Chernov M.V., Chumakov P.M. and Gudkov A.V. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. 1998. Nature 391:295-8.

Gorden A., Osman I., Gai W., He D., Huang W., Davidson A., Houghton A.N., Busam K. and Polsky D. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. 2003. Cancer Res 63:3955-7.

Gozani O., Karuman P., Jones D.R., Ivanov D., Cha J., Lugovskoy A.A., Baird C.L., Zhu H., Field S.J., Lessnick S.L., Villasenor J., Mehrotra B., Chen J., Rao V.R., Brugge J.S., Ferguson C.G., Payrastre B., Myszka D.G., Cantley L.C., Wagner G., Divecha N., Prestwich G.D. and Yuan J. The PHD Finger of the Chromatin-Associated Protein ING2 Functions as a Nuclear Phosphoinositide Receptor. 2003. Cell 114:99–111.

Groll M., Bochtler M., Brandstetter H., Clausen T. and Huber R. Molecular machines for protein degradation. 2005. Chembiochem 6:222-56.

Gudkov A.V., Kazarov A.R., Thimmapaya R., Axenovich S.A., Mazo I.A. and Roninson I.B. Cloning mammalian genes by expression selection of genetic suppressor elements: Association of kinesin with drug resistance and cell immortalization. 1994. Proc Nat Acad Sci USA 91:3744-8.

Gunduz M., Ouchida M., Fukushima K., Hanafusa H., Etani T., Nishioka S., Nishizaki K. and Shimizu K. Genomic Structure of the Human *ING1* Gene and Tumour-specific Mutations Detected in Head and Neck Squamous Cell Carcinomas. 2000. Cancer Res 60:3143-6.

Guo Z., Kumagai A., Wang S.X. and Dunphy W.G. Requirement for Atr in phosphorylation of Chk1 and cell cycle regulation in response to DNA replication blocks and UV-damaged DNA in Xenopus egg extracts. 2000. Genes Dev 14:2745-56.

Gutierrez M.G., Master S.S., Singh S.B., Taylor G.A., Colombo M.I. and Deretic V. Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages. 2004. Cell 119:753-66.

Gwosdz C., Scheckenbach K., Lieven O., Reifenberger J., Knopf A., Bier H. and Balz V. Comprehensive analysis of the p53 status in mucosal and cutaneous melanomas. 2006. Int J Cancer 118:577-82.

Hara Y., Zheng Z., Evans S.C., Malatjalian D., Riddell D.C., Guernsey D.L., Wang L.D., Riabowol K. and Casson A.G. ING1 and p53 tumour suppressor gene alterations in adenocarcinomas of the esophagogastric junction. 2003. Cancer Lett 192:109-16.

Harbour J.W. and Dean D.C. The Rb/E2F pathway: expanding roles and emerging paradigms. 2000 Genes Dev 14:2393-409.

Harper J.W., Adami G.R., Wei N., Keyomarsi K. and Elledge S.J. The p21 Cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. 1993. Cell 75: 805-16.

He G.H.Y., Helbing C.C, Wagner M.J., Sensen C.W. and Riabowol K. Phylogenetic Analysis of the ING Family of PHD Finger Proteins. 2005. Mol Biol Evol 22:104-16.

Helt C.E., Cliby W.A., Keng P.C., Bambara R.A. and O'Reilly M.A. Ataxia telangiectasia mutated (ATM) and ATM and Rad3-related protein exhibit selective target specificities in response to different forms of DNA damage. 2005. J Biol Chem 280:1186-92.

Hirao A., Kong Y.Y., Matsuoka S., Wakeham A., Ruland J., Yoshida H., Liu D., Elledge S.J. and Mak T.W. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. 2000. Science 287:1824-7.

Hoque M.O., Kawamata H., Nakashiro K., Omotehara F., Hino S., Uchida D., Harada K., Begum N.M., Yoshida H., Sato M. and Fujimori T. Dysfunction of the p53 tumour suppressor pathway in head and neck cancer. 2002. Int J Oncol 21:119-26.

Honda R., Tanaka H. and Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumour suppressor p53. 1997. FEBS Lett 420:25-7.

Howe L., Kusch T., Muster N., Chaterji R., Yates J.R. 3rd and Workman J.L. Yng1p modulates the activity of Sas3p as a component of the yeast NuA3 histone acetyltransferase complex. 2002. Mol Cell Biol 22:5047-53.

Hussain S.A. and James N.D. The systemic treatment of advanced and metastatic bladder cancer. 2003. Lancet Oncol 4:489-97.

Hussussian C.J., Struewing J.P., Goldstein A.M., Higgins P.A., Ally D.S., Sheahan M.D., Clark W.H. Jr., Tucker M.A. and Dracopoli N.C. Germline p16 mutations in familial melanoma. 1994. Nat Genet 8:15-21.

Imbriano C., Gurtner A., Cocchiarella F., Di Agostino S., Basile V., Gostissa M., Dobbelstein M., Del Sal G., Piaggio G. and Mantovani R. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. 2005. Mol Cell Biol 25:3737-51.

Innocente S.A., Abrahamson J.L., Cogswell J.P. and Lee J.M. P53 regulates a G2 checkpoint through cyclin B1. 1999. Proc Natl Acad Sci USA 96:2147-52.

Ito K., Kinjo K., Nakazato T., Ikeda Y. and Kizaki M. Expression and sequence analyses of p33(ING1) gene in myeloid leukemia. 2002. Am J Hematol 69:141-3.

Jhappan C., Noonan F.P. and Merlino G. Ultraviolet radiation and cutaneous malignant melanoma. 2003. Oncogene 22:3099-112.

Kameyama K., Huang C.L., Liu D., Masuya D., Nakashima T., Sumitomo S., Takami Y., Kinoshita M. and Yokomise H. Reduced ING1b gene expression plays an important role in carcinogenesis of non-small cell lung cancer patients. 2003. Clin Cancer Res 9:4926-34.

Kataoka H., Bonnefin P., Vieyra D., Feng X., Hara Y., Miura Y., Joh T., Nakabayashi H., Vaziri H., Harris C.C. and Riabowol K. ING1 represses transcription by direct DNA biding and through effects on p53. 2003. Cancer Res. 63:5787-92.

Kawaji H., Schonbach C., Matsuo Y., Kawai J., Okazaki Y., Hayashizaki Y. and Matsuda H. Exploration of novel motifs derived from mouse cDNA sequences. 2002. Genome Res 12:367-78.

Kichina J.V., Zeremski M., Aris L., Gurova K.V., Walker E., Franks R., Nikitin A.Y., Kiyokawa H., Gudkov A.V. Targeted disruption of the mouse ing1 locus results in reduced body size, hypersensitivity to radiation and elevated incidence of lymphomas. Oncogene In press.

Krishnamurthy J., Kannan K., Feng J., Mohanprasad B.K., Tsuchida N. and Shanmugam G. Mutational analysis of the candidate tumour suppressor gene ING1 in Indian oral squamous cell carcinoma. 2001. Oral Oncol 37:222-4.

Kubbutat M.H.G., Jones S.N. and Vousden K.H. Regulation of p53 stability by Mdm2. 1997. Nature 387:299-303.

Kurz E.U., Douglas P. and Lees-Miller S.P. Doxorubicin activates ATM- dependent phosphorylation of multiple downstream targets in part through the generation of reactive oxygen species. 2004. J Biol Chem 279:53272-81.

Kuzmichev A., Zhang Y., Erdjument-Bromage H., Tempst P. and Reinberg D. Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumour suppressor p33(ING1). 2002. Mol Cell Biol 22:835–48.

Lakin N.D. and Jackson S.P. Regulation of p53 in response to DNA damage. 1999. Oncogene 18:7644-55.

Lander E.S. *et al.* Initial sequencing and analysis of the human genome. 2001. Nature 409:860-921.

Leung K.M., Po L.S., Tsang F.C., Siu W.Y., Lau A., Ho H.T. and Poon R.Y. The candidate tumour suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. 2002. Cancer Res 62:4890-3.

Lum J.J., Bauer D.E., Kong M., Harris M.H., Li C., Lindsten T. and Thompson C.B. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. 2005. Cell 120:237-48.

MacKie R.M., Freudenberger T. and Aitchison T.C. Personal risk-factor chart for cutaneous melanoma. 1989. Lancet 2:487-90.

Maga G. and Hubscher U. Proliferating cell nuclear antigen (PCNA): a dancer with many partners. 2003. J Cell Sci 116:3051-60.

Majeski A.E. and Dice J.F. Mechanisms of chaperone-mediated autophagy. 2004. Int J Biochem Cell Biol 36:2435-44.

Manni I., Mazzaro G., Gurtner A., Mantovani R., Haugwitz U., Krause K., Engeland K., Sacchi A., Soddu S. and Piaggio G. NF-Y mediates the 23 transcriptional inhibition of the cyclin B1, cyclin B2, and cdc25C promoters upon induced G2 arrest. 2001. J Biol Chem 276:5570-6.

Mayanagi T., Amagai A. and Maeda Y. DNG1, a Dictyostelium homologue of tumour suppressor ING1 regulates differentiation of Dictyostelium cells. 2005. Cell Mol Life Sci 62:1734-43.

Nagashima M., Shiseki M., Miura K., Hagiwara K., Linke S.P., Pedeux R., Wang X.W., Yokota J., Riabowol K. and Harris C.C. DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. 2001. Proc Natl Acad Sci USA 98:9671-6.

Nagashima M., Shiseki M., Pedeux R.M., Okamura S., Kitahama-Shiseki M., Miura K., Yokota J. and Harris C.C. A novel PHD-finger motif protein, p47ING3, modulates p53-mediated transcription, cell cycle control, and apoptosis. 2003. Oncogene 22:343-50.

Nakamura S., Gomyo Y., Roth J.A. and Mukhopadhyay T. C-terminus of p53 is required for G(2) arrest. 2002. Oncogene 21:2102-7.

Nouman G.S., Anderson J.J., Lunec J. and Angus B. The role of the tumour suppressor p33ING1b in human neoplasia. 2003. J Clin Pathol 56:491-6.

Nouman G.S., Anderson J.J., Mathers M.E., Leonard N., Crosier S., Lunec J. and Angus B. Nuclear to cytoplasmic compartment shift of the p33ING1b tumour suppressor protein is associated with malignancy in melanocytic lesions. 2002(a). Histopathology 40:360-6.

Nouman G.S., Anderson J.J., Wood K.M., Lunec J., Hall A.G., Reid M.M. and Angus B. Loss of nuclear expression of the p33(ING1b) inhibitor of growth protein in childhood acute lymphoblastic leukaemia. 2002(b). J Clin Pathol 55:596-601.

Ohgi T., Masaki T., Nakai S., Morishita A., Yukimasa S., Nagai M., Miyauchi Y., Funaki T., Kurokohchi K., Watanabe S. and Kuriyama S. Expression of p33(ING1) in hepatocellular carcinoma: relationships to tumour differentiation and cyclin E kinase activity. 2002. Scand J Gastroenterol 37:1440-8.

Oki E., Maehara Y., Tokunaga E., Kakeji Y. and Sugimachi K. Reduced expression of p33(ING1) and the relationship with p53 expression in human gastric cancer. 1999. Cancer Lett 147:157-62.

Ohmori M., Nagai M., Tasaka T., Koeffler H.P., Toyama T., Riabowol K. and Takahara J. Decreased expression of p33ING1 mRNA in lymphoid malignancies. 1999. Am J Hematol 62:118-9.

Porter L.A. and Donoghue D.J. Cyclin B1 and CDK1: nuclear localization and upstream regulators. 2003. Prog Cell Cycle Res 5:335-47.

Quelle D.E., Zindy F., Ashmun R.A. and Sherr C.J. Alternative reading frames of the INK4a tumour suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. 1995. Cell 83:993-1000.

Rall T.W. and Sutherland E.W. Formation of a cyclic adenine ribonucleotide by tissue particles. 1958. J Biol Chem 232:1065-76.

Reardon J.T., Vaisman A., Chaney S.G. and Sancar A. Efficient nucleotide excision repair of cisplatin, oxaliplatin, and Bis-aceto-ammine-dichloro-cyclohexylamine-platinum(IV) (JM216) platinum intrastrand DNA diadducts. 1999. Cancer Res 59:3968-71.

Saito A., Furukawa T., Fukushige S., Koyama S., Hoshi M., Hayashi Y. and Horii A. p24/ING1-ALT1 and p47/ING1-ALT2, distinct alternative transcripts of p33/ING1. 2000. J Hum Genet 45:177-81.

Sanchez Y., Wong C., Thoma R.S., Richman R., Wu Z., Piwnica-Worms H. and Elledge S.J. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. 1997. Science 277:1497-501.

Sanchez-Carbayo M., Socci N.D., Lozano J.J., Li W., Charytonowicz E., Belbin T.J., Prystowsky M.B., Ortiz A.R., Childs G. and Cordon-Cardo C. Gene discovery in bladder cancer progression using cDNA microarrays. 2003. Am J Pathol 163:505-16.

Sanchez-Cespedes M., Okami K., Cairns P. and Sidransky D. Molecular analysis of the candidate tumour suppressor gene ING1 in human head and neck tumours with 13q deletions. 2000. Genes Chromosomes Cancer 27:319-22.

Sarela A.I., Farmery S.M., Markham A.F. and Guillou P.J. The candidate tumour suppressor gene, ING1, is retained in colorectal carcinomas. 1999. Eur J Cancer 35:1264-7.

Scott M., Boisvert F.M., Vieyra D., Johnston R.N., Bazett-Jones D.P. and Riabowol K. UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. 2001(a). Nucleic Acids Res 29:2052-8.

Scott M., Bonnefin P., Vieyra D., Boisvert F.M., Young D., Bazett-Jones D.P. and Riabowol K. UV-induced binding of ING1 to PCNA regulates the induction of apoptosis. 2001(b). J Cell Sci 114:3455-62.

Shieh S.Y., Ahn J., Tamai K., Taya Y. and Prives C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. 2000. Genes Dev 14:289-300.

Shimada Y., Saito A., Suzuki M., Takahashi E. and Horie M. Cloning of a novel gene (ING1L) homologous to ING1, a candidate tumour suppressor. 1998. Cytogenet Cell Genet 83:232-5.

Skowyra D., Zeremski M., Neznanov N., Li M., Choi Y., Uesugi M., Hauser C.A., Gu W., Gudkov A.V. and Qin J. Differential association of products of alternative transcripts of the candidate tumour suppressor ING1 with the mSin3/HDAC1 transcriptional corepressor complex. 2001 J Biol Chem 276:8734-9.

Shimada Y., Saito A., Suzuki M., Takahashi E. and Horie M. Cloning of a novel gene (ING1L) homologous to ING1, a candidate tumour suppressor. 1998. Cytogenet Cell Genet 83:232-5.

Shimada H., Liu T.L., Ochiai T., Shimizu T., Haupt Y., Hamada H. Abe T., Oka M., Takiguchi M. and Hiwasa T. Facilitation of adenoviral wild-type p53-induced apoptotic cell death by overexpression of p33(ING1) in T.Tn human esophageal carcinoma cells. 2002. Oncogene 21:1208-16.

Shinoura N., Muramatsu Y., Nishimura M., Yoshida Y., Saito A., Yokoyama T., Furukawa T., Horii A., Hashimoto M., Asai A., Kirino T. and Hamada H. Adenovirus-mediated transfer of p33ING1 with p53 drastically augments apoptosis in gliomas. 1999. Cancer Res 59:5521-8.

Shiseki M., Nagashima M., Pedeux R.M., Kitahama-Shiseki M., Miura K., Okamura S., Onogi H., Higashimoto Y., Appella E., Yokota J. and Harris C.C. p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. 2003. Cancer Res 63:2373-8.

Skowyra D., Zeremski M., Neznanov N., Li M., Choi Y., Uesugi M., Hauser C.A., Gu W., Gudkov A.V. and Qin J. Differential association of products of alternative transcripts

of the candidate tumour suppressor ING1 with the mSin3/HDAC1 transcriptional corepressor complex. 2001. J Biol Chem 276:8734-9.

Tallen G., Kaiser I., Krabbe S., Lass U., Hartmann C., Henze G. Riabowol K. and von Deimling A. No ING1 mutations in human brain tumours but reduced expression in high malignancy grades of astrocytoma. 2004. Int J Cancer 109:476-9.

Tallen G., Riabowol K. and Wolff J. E. Expression of p33ING1 mRNA and chemosensitivity in brain tumour cells. 2003. Anticancer Res 23:1631-5.

Takahashi M., Ozaki T., Todo S. and Nakagawara A. Decreased expression of the candidate tumour suppressor gene ING1 is associated with poor prognosis in advanced neuroblastomas. 2004. Oncol Rep 12(4):811-6.

Takahashi M., Seki N., Ozaki T., Kato M., Kuno T., Nakagawa T., Watanabe K., Miyazaki K., Ohira M., Hayashi S., Hosoda M., Tokita H., Mizuguchi H., Hayakawa T., Todo S. and Nakagawara A. Identification of the p33(ING1)-regulated genes that include cyclin B1 and proto-oncogene DEK by using cDNA microarray in a mouse mammary epithelial cell line NMuMG. 2002. Cancer Res 62:2203-9.

Tokunaga E., Maehara Y., Oki E., Kitamura K., Kakeji Y., Ohno S. and Sugimachi K. Diminished expression of ING1 mRNA and the correlation with p53 expression in breast cancers. 2000. Cancer Lett 152:15-22.

Toyama T., Iwase H., Watson P., Muzik H., Saettler E., Magliocco A., DiFrancesco L., Forsyth P., Garkavtsev I., Kobayashi S. and Riabowol K. Suppression of ING1 expression in sporadic breast cancer. 1999. Oncogene 18:5187-93.

Tsang F.C., Po L.S., Leung K.M., Lau A., Siu W.Y. and Poon R.Y. ING1b decreases cell proliferation through p53-dependent and -independent mechanisms. 2003. FEBS Lett 553:277-85.

Unger T., Juven-Gershon T., Moallem E., Berger M., Vogt Sionov R., Lozano G., Oren M. and Haupt Y. Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2. 1999. EMBO J 18:1805-14.

Unsal-Kacmaz K., Makhov A.M., Griffith J.D. and Sancar A. Preferential binding of ATR protein to UV-damaged DNA. 2002. Proc Natl Acad Sci USA 99:6673-8.

Uribe P., Wistuba I.I. and Gonzalez S. BRAF mutation: a frequent event in benign, atypical, and malignant melanocytic lesions of the skin. 2003. Am J Dermatopathol 25:365-70.

Vieyra D., Loewith R., Scott M., Bonnefin P., Boisvert F.M., Cheema P., Pastyryeva S., Meijer M., Johnston R.N., Bazett-Jones D.P., McMahon S., Cole M.D., Young D. and

Riabowol K. Human ING1 proteins differentially regulate histone acetylation. 2002. J Biol Chem 277:29832-9.

Vieyra D., Senger D.L., Toyam T., Muzik H., Brasher P.M., Johnston R.N., Riabowol K. and Forsyth P.A. Altered subcellular localization and low frequency of mutations of ING1 in human brain tumours. 2003. Clin Cancer Res 9:5952-61.

Waga S., Hannon G.J., Beach D. and Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. 1994. Nature 369:574-8.

Wagner M.J., Gogela-Spehar M., Skirrow R.C., Johnston R.N., Riabowol K. and Helhbing C.C. Expression of novel ING variants is regulated by thyroid hormone in the Xenopus laevis tadpole. 2001. J Biol Chem 276: 47013-20.

Ward I.M., Minn K. and Chen J. UV-induced Ataxia-telangiectasia-mutated and Rad3-related (ATR) Activation Requires Replication Stress. 2004. J Biol Chem 279:9677-80.

Welchman R.L., Gordon C. and Mayer R.J. Ubiquitin and ubiquitin-like proteins as multifunctional signals. 2005. Nat Rev Mol Cell Biol 6:599-609.

Wu H., Goel V. and Haluska F.G. PTEN signaling pathways in melanoma. 2003. Oncogene 22:3113-22.

Yaffe M.B., Rittinger K., Volinia S., Caron P.R., Aitken A., Leffers H., Gamblin S.J., Smerdon S.J. and Cantley L.C. The structural basis for 14-3-3:phosphopeptide binding specificity. 1997. Cell 91:961-71.

Zeremski M., Horrigan S.K., Grigorian I.A., Westbrook C.A. and Gudkov A.V. Localization of the candidate tumour suppressor gene ING1 to human chromosome 13q34. 1997 Somat Cell Mol Genet 23:233-6.

2 MATERIALS AND METHODS

2.1 Cell Culture and Transfection

Fourteen melanoma cell lines were used for this study. The MMAN, MMRU, RPEP and PMWK cell lines were kind gifts from Dr R. Byers, Boston University School. The Sk-mel-2 and Sk-mel-5 cell lines were obtained from the Tissue Bank at the National Institutes of Health, U.S.A. The MEWO, Sk-mel-3, Sk-mel-24, Sk-mel-93, Sk-mel-110, KZ-2, KZ-13 and KZ-28 cell lines were kind gifts from Dr A.P. Albino (Memorial Sloan-Kettering Cancer Center, New York, U.S.A.). Normal human epithelial melanocytes and fibroblasts were purchased from Clonetics. All melanoma cell lines and human fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Canadian Life Technologies), 100 units/ml penicillin and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37°C. Normal melanocytes were maintained in melanocyte growth medium (Clonetics) at 37°C in a 5% CO_2 atmosphere.

Cells were grown to 50–60% confluency prior to transfection. Transient transfections were done using Effectene reagent (Qiagen) as directed by the manufacturer at a ratio of 1 mg DNA to 25 ml Effectene. ATM siRNA (Ambion) was introduced into cells using Lipofectine (Invitrogen) reagent according to the manufacturer's protocol. The pCI-*ING1b* plasmid was a kind gift from Dr. K. Riabowol (University of Calgary). The p*ING1b*-FLAG plasmid was constructed by inserting BglII and KpnI restriction sites up- and down-stream the *ING1b* cDNA by PCR (forward primer: 5'-

GAAGATCTACCATGCTGAGTCCTGCCAAC-3'; reverse primer: 5'-CGGGGTACCCCTGTTGTAAGCCCTCTC-3') and re-ligating in the pCMV-FLAG vector. BamHI and EcoRI restriction sites were also introduced upstream and downstream of pCI-*ING1b* by PCR (forward primer: 5'-CTCGAGGATCCCTGCAGC-3'; reverse primer: 5'-CGAATTCCTACCTGTTGTAAGCCC-3') to sub-clone into the pGEX-2T glutathione-S-transferase (GST) vector (Amersham Bioscience).

2.2 Generation of Polyclonal Antibodies

Antibodies were generated as previously described (Campos *et al.*, 2004). Briefly, the synthetic peptides N--LSPANEQLHLVNC--C and N--ELGDTAGNpSGKAGADRP--C (where p denotes phosphorylation) corresponding to the N-terminus of the $p33^{ING1b}$ protein and a $p33^{ING1b}$ peptide containing phosphorylated serine 126 were conjugated to keyhole limpet hemocyanin (KLH). Twenty milligrams of KLH were initially denatured by boiling in the presence of 5% (v/v) sodium dodecyl sulfate (SDS) and 10 mM sodium carbonate. The solution was then activated with N-hydroxysuccinimide-iodoacetate ester (NHS-Iac) at room temperature for 10 min. Activated KLH was then purified by passing though a G15 sephadex bead column. Five milligram of the ING1b peptide were dissolved in 400 ml of 10 mM sodium phosphate [pH 8.5] and conjugated to the iodoacetate-activated KLH by incubating overnight at room temperature with gentle rotation. The concentration of the KLH conjugated ING1b protein solution was adjusted to 1 mg/ml in PBS. Two New Zealand rabbits were immunized with 1 mg of the

conjugated ING1b peptide in Freund's complete adjuvant, and every 2 weeks subsequently in Freund's incomplete adjuvant.

Ten milliliters of sepharose CL beads were washed with 100 ml of 10 mM sodium carbonate and then incubated for 20 min with 100 mg of sodium m-periodate at room temperature with gentle rotation. The beads were then washed with 0.1 M citrate buffer and conjugated with 10 mg of the synthetic $p33^{ING1b}$ peptides (N-terminal for anti-$p33^{ING1b}$ antisera and phosphorylated peptide for anti-*p*-S126 antisera) by rotating at room temperature for 30 min. Ten milligrams of sodium cyanoborohydride was then added to the beads left to rotate at room temperature overnight. The peptide-conjugated beads were extensively washed with PBS prior to use. Approximately 80 ml of the antiserum was passed through the affinity column. The column was then washed with 100 mL of PBS followed by 50 ml of 1 M NaCl. The specific anti-$p33^{ING1b}$ antibodies retained in the column were eluted with 3% acetic acid. Antibodies were then precipitated with 30% of ammonium sulphate and reconstituted with PBS. Anti-*p*-S126 serum was further affinity purified after incubating with glutathione agarose beads-bound recombinant GST-$p33^{ING1b}$ at 4°C overnight with gentle rocking and the non-bound fraction kept for use.

2.3 Drug Treatments and Ultraviolet Irradiation

The topoisomerase inhibitors doxorubicin and etoposide were dissolved directly into culture media at 0.1-1 μg/ml and 0.25 μg/ml respectively. The okadaic acid phosphatase

inhibitor was used at a working concentration of 100 μM in culture media. The kinase inhibitors wortmannin, staurosporine, roscovitine, kenpaullone and H-89 were used at 100 mM, 50 μM, 50 μM, 10 μM and 25 μM working concentrations respectively. The 80S cytosolic ribosome inhibitor cycloheximide was diluted to 20 μg/ml directly into the culture media. The proteasome inhibitor MG132 was also used at 50 μM in culture media. All drugs were obtained from Sigma. Ultraviolet irradiation was performed by removing media and exposing cells to 20 mJ/cm^2 UVB (290–320 nm) light using a bank of four unfiltered FS40 sunlamps (Westinghouse).

2.4 Single-strand Conformation Polymorphism

SSCP was initially developed to identify genetic polymorphisms (Orita *et al.*, 1989) and was adapted as previously described (Chen *et al.*, 2003). DNA was isolated from hematoxylin- and eosin-stained 12 μm sections of paraffin-embedded tumour blocks. Normal and tumour tissues were dissected under a microscope, and the paraffin removed through a series of xylene washes prior to DNA extraction. DNA from both fixed tissues and cultured cells was extracted using the DNeasy Tissue Kit (Qiagen) according to the manufacturer's protocol. All three exons of the *ING1* gene were then amplified by PCR using the following primers: forward 5'-AGCAGCTCCACCTGGTGAAC-3' and reverse 5'-ACTGAAGCGCTCGTAGCACT-3' (exon 1a); forward 5'-TGGCTGTGATGTCCTTCGTG-3' and reverse 5'-AGGCCAGGAGGAGAACCAAC-3' (exon 1b set 1); forward 5'-GTGTGGTTGGTTCTCCTC-3' and reverse 5'-GGATCACTGCTACTGCTA-3' (exon 1b set 2); forward 5'-

CGAGAAGATCCAGATCGTGA-3' and reverse 5'-GGCTTGTCAGACTGCGCTAC-3' (exon 2 set 1); and forward 5'-GACCTCCAAGAAGAAGAAGC-3' and reverse 5'-CCATGGTCTTCTCGTTCTCC-3' (exon 2 set 2). DNA samples were denatured at 94°C for 3 min and amplified by 40 cycles of 30 sec denaturing at 94°C, 1 min primer annealing at 60°C (55°C for set 2 of both exon 1b and exon 2) and 1 min extension at 72°C with a final 5 min extension following the last cycle. PCR samples were then diluted 1:4 in SSCP loading buffer (95% formamide, 0.05% bromo-phenol blue, 20 mM NaOH), denatured at 80°C for 20 min and quickly chilled on ice prior loading onto a 6% non-denaturing polyacrylamide gel. Electrophoresis was carried out at 80 V at 4°C in a water-cooled apparatus overnight and the DNA visualized by staining with ethidium bromide.

2.5 DNA Sequencing

Genomic DNA from samples exhibiting band-shifts in the SSCP analysis were re-amplified for direct sequencing. Products were sequenced using the Big Dye Terminator Kit on a ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequences were aligned and compared to the ING1 gene under the GeneBank accession nos. AB024401, AB024402 and AF078835 (differing by known SNPs).

2.6 Western Analysis

Whole cell extracts were obtained by scraping cells in cold PBS and centrifuging cells at 500 × *g* for 2 min at 4°C. Cell pellets were solubilized by adding 3 volumes of lysis buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 1% Nonidet P-40) in the presence of a premixed protease inhibitors (Roche) and a Ser/Thr phosphatase inhibitor cocktail mix (Sigma) where applicable. Samples were left on ice for 15 min and sonicated twice for 10 s using a Microson sonicator (Heat Systems - Ultrasonics) at setting 8. Lysates were then centrifuged at 12,000 × *g* for 30 min at 4°C and supernatants kept for analysis.

Nuclear extracts were obtained by washing cells with cold PBS and scraping cells off plates. Cells were then centrifuged at 500 × *g* at 4°C for 2 min. The volume of the packed pellet was estimated and four volumes of NP-40 lysis buffer (50 mM Tris-HCl, 10 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40) containing the abovementioned inhibitors. Samples were left on ice for 5 min, and centrifuged at 4°C, 500 × *g* for 2 min. The supernatant was discarded, and the pellet was washed once in four volumes of NP-40 lysis buffer. The supernatant was discarded, and the packed nuclei solubilized with approximately three volumes of nuclear extraction buffer (20 mM Hepes [pH 7.9], 0.5 M NaCl, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol) containing protease inhibitors, left on ice for 30 min and sonicated as previously described. Extract and then centrifuged at 12,000 × *g* for 30 min at 4°C and supernatant kept for analysis.

Concentration of proteins was determined using the DC Protein Assay (Bio-Rad) system. Fifty μg/lane of proteins were separated by 15% SDS-PAGE and electrotransferred onto polyvinylidene difluoride filters (Bio-Rad). Low-*bis* band shift assays were performed using gels containing a 118.5:1 acrylamide:*bis* crosslinker ratio instead of the usual 37.5:1. Filters were incubated with primary antiserum at room temperature for 1 h or at 4°C overnight followed by three washes in 0.04% Tween-20 PBS for 5 min each and subsequent incubation with horseradish peroxidase-conjugated secondary antiserum for 1 h at room temperature. Blots were washed and signals detected with SuperSignal enhanced chemiluminescence (Pierce).

The following antibodies were used in this study: anti-ATM, anti-Chk1, anti-Cdk1 antibodies (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-ING1 (PharMingen), anti-β-actin (Sigma). Densitometry analysis was carried using the Quantity One software (Bio-Rad). Intensity of the signal of interest was corrected for the different amounts of cellular protein loaded on the gel by using β-actin as the input control.

2.7 Semi-quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted with TriZol reagent (Canadian Life Technologies) and the concentrations were determined by UV spectrophotometry. Five micrograms of total RNA were reverse transcribed into cDNA using Superscript II RNase H reverse transcriptase (Canadian Life Technologies) in a 20 μl reaction using oligo d(T) primers as

prescribed by the manufacturer. *ING1* mRNA was amplified from 2 μl of the RT-PCR reaction using the following primers: forward 5'-GATCCTGAAGGAGCTAGACG-3', reverse 5'-AGAAGTGGAACCACTCGATG-3' using Taq DNA polymerase (Qiagen) as prescribed. Amplification was carried at an annealing temperature of 50°C as previously described.

2.8 Immunohistochemistry

Six-micron slides were cut from paraffin-embedded blocks of melanoma biopsies. Tissues were de-waxed by heating at 55°C for 30 min followed by three 5 min washes in xylene. Samples were re-hydrated by washing for 5 min each in 100%, 90% and 70% ethanol and 30 min in PBS. Antigen retrieval was performed by microwaving the samples for 5 min at full power in a 10 mM sodium citrate solution (pH 6.0). Endogenous peroxidase activity was quenched with a 0.3% H_2O_2 solution for 10 min and non-specific antibody binding blocked by incubating with 10% pre-immune goat serum in PBS for 20 min. Proteins were immunolabeled with primary antisera and subsequently HRP-conjugated secondary antisera diluted 1:500 in blocking serum. Labeling was finalized by developing with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories).

2.9 Immunofluorescence

MMRU cells were grown on coverslips at a density of 2×10^5 cells/well in a 6-well plate. Twenty four hours following transfection cells were simultaneously fixed and extracted in 2% paraformaldehyde, 0.5% Triton X-100 in PBS for 30 min at 4°C. Cells were incubated in 10% pre-immune goat serum diluted in PBS for 1 h at room temperature to minimize non-specific signal. Indirect immunolabeling was performed by incubating fixed cells on primary antibody diluted 1:50 in PBS at room temperature for 1 h, washing 3 times with PBS and incubating for 1 h at room temperature with Cy3 or Cy5-conjugated secondary antiserum (Jackson ImmunoResearch) diluted 1:500 in PBS. Cells were counterstained with 650 ng/ml Hoechst 33258 diluted in PBS to visualize DNA. Slides were visualized under a Zeiss Axioplan 2 microscope.

2.10 Site-directed mutagenesis

Site-directed mutagenesis was performed by splicing overlap extension (PCR-SOEing) (Ho *et al.*, 1989) using the high-fidelity Pfx DNA polymerase (Invitrogen) using 2× Pfx buffer in the final recommended reaction. Mutagenesis of the *ING1b* coding sequence was performed on the p*ING1b*-FLAG or the pCIneo-*ING1b*-FLAG vectors using the following primer pairs: 5'-GAGAACCTCACGCGGCA-3' (forward) and 5'-TGCCGCGTGAGGTTCTCC-3' (reverse) for R102L; 5'-AGAGCGAGAAGACCATGGA-3' (forward) and 3'-CCATGGTCTTCTCGCTCTC-3' (reverse) for N260S; and 5'-GGCAACGCAGGCAAGGC-3' (forward) and 5'-

CCTTGCCTGCGTTGCCC-3' (reverse) for S126A. All constructs were verified by DNA sequencing.

2.11 DNA Repair Assays

Use of the host-cell reactivation assay to assay for cloned DNA repair genes was first described by Henderson *et al.* (1989). We followed this protocol using the chloramphenicol acetyltransferase (*CAT*) gene as a reporter in the plasmid pCMVcat (kind gift from Dr. Lawrence Grossman, Johns Hopkins University). The reporter plasmid was irradiated *ex vivo* at 40 mJ/cm^2 using a ultraviolet crosslinker. The damaged reporter was then co-transfected (as abovementioned) with empty pCIneo or pCIneo plasmids expressing wild-type or mutant p33^{ING1b}. Cells were collected 40 h after transfection, resuspended in 30 µl of a 250 mM Tris-Cl [pH 7.8] and 5 mM EDTA solution and cell-free extracts obtained by repeatedly freezing the samples in liquid nitrogen and centrifuging the lysates at 12,000 × *g* for 15 min. Supernatants were then individually mixed with 7.5 µl of 5 mM chloramphenicol, 1 µl of 2.5 mM [^{3}H]-acetyl-CoA and 16.5 µl distilled water. The chloramphenicol acetyltransferase reaction was allowed to proceed for 90 min at 37°C. The organic phase was then isolated, dried and the amount of radioactivity that was transferred onto the chloramphenicol determined using a scintillation counter. All reactions were performed in triplicates.

Measurement of damaged DNA using the radioimmunoassay (RIA) technique was first described by Hsu *et al.* (1981). RIA was performed using rabbit antibodies

capable of recognizing UV-induced pyrimidine (6-4) pyrimidinone DNA photoproducts (6-4PPs). DNA used for RIA was obtained from MMRU cells expressing wild-type or mutant $p33^{ING1b}$ and irradiated with 20 mJ/cm^2 UVB or ambient light for an equivalent amount of time. DNA was isolated 4 h after UV irradiation by standard phenol:chloroform extractions. Two milligrams of heat-denatured DNA samples were then incubated with 5 pg of poly(deoxyadenylated TMP; labeled over 5×10^8 cpm/μg by nick translation with [^{32}P] dideoxythymidine 5'-triphosphate) in a total volume of 1 ml of 10 mM Tris [pH 7.8], 150 mM NaCl, 1 mM EDTA, and 0.15% gelatin. 6-4PP antibodies were then added at a dilution that yielded 30–60% binding to labeled ligand, and then immunoprecipitated using with anti-rabbit secondary antisera. After centrifugation the pellet was dissolved in tissue solubilizer (Amersham) and radioactivity quantified by scintillation. The experiment was then repeated in the presence of increasing amounts of an unlabeled competitor that inhibits antibody binding to the radiolabeled ligand. Sample inhibition was then extrapolated through a dose-response curve to determine the number of photoproducts in 10^6 DNA bases (6-4PPs/Mb DNA). Salmon sperm DNA (Sigma) irradiated with increasing doses of UV was used as an internal standard control.

2.12 Immunoprecipitation and Phosphatase Treatment

Immunoprecipitations were performed from nuclear extracts with 2 μg/ml of either anti-Cdk1 or anti-Chk1 antibodies (Santa-Cruz Biotech.) or anti-FLAG M2 antibodies (Sigma) with end-to-end rotation at 4°C overnight. Samples were further incubated with 20 μl (50% slurry) protein A or protein G-agarose beads (Pharmacia) with rotation for 1 h

at 4°C and beads washed 3 times with 500 µl PBS. Alkaline calf intestinal phosphatase (CIP) (New England Biolabs) treatment of immunoprecipitated $p33^{ING1b}$ was done on-beads in NEBuffer-3 and 10 U CIP per immunoprecipitation. Reaction was carried at 37°C for 2 h and stopped upon addition of sample buffer.

2.13 Enzyme-linked Immunosorbent Assay

A 96-well ELISA plate was incubated with 100 µl of phosphorylated or non-phosphorylated synthetic peptide corresponding to amino acids 118-134 of $p33^{ING1b}$ diluted at 50 µg/ml in 50 mM Na_2CO_3 at room temperature overnight, and then blocked with 5% milk PBS-T at room temperature for 20 min. Plates were then incubated with 100 µl of the purified anti-*p*-S126 antibody at different dilutions at room temperature for 60 min. The plates were then washed with PBS-T, and incubated with a HRP-conjugated goat anti-rabbit antibody (Santa-Cruz Biotech). 100 µl of the HRP substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Moss Inc.) was added to develop for 15 min and the reaction stopped with 50 µl of 0.1 N HCl. O.D. was measure at 450 nm on a Titertek plate reader.

2.14 Purification of Recombinant $p33^{ING1b}$

Recombinant $p33^{ING1b}$ was expressed from the pGEX-ING1b vector in *E. Coli* BL-21 DE3. Bacteria was grown to an optical density of 0.6 and induced with isopropyl-β-D-thiogalactopyranoside (Sigma) for 4 h at room temperature. Bacteria was then pelleted

by centrifugation and incubated in resuspension buffer (25 mM Tris [pH 7.5], 150 mM NaCl, 5 mM β-mercaptoethanol and protease inhibitors) and lysed for 30 min on ice in the presence of 50 ng/ml lysozyme prior to freeze-thaw cycles. Lysate was further incubated on ice for 30 min in the presence of 50 ng/ml DNase I (Sigma) and 5 mM $MgCl_2$ and for another 30 min with 1% Nonidet P-40 at 4°C with gentle rotation. Lysates were then centrifuged at 12,000 × *g* for 30 min at 4°C. Supernatant was recovered and GST-$p33^{ING1b}$ affinity purified using glutathione agarose beads (Sigma). Beads were extensively washed with PBS and GST cleaved from the fusion protein by incubating the ING1b-bound beads with 50 U thrombin (Sigma) at room temperature for 12 h.

2.15 Mass Spectrometry

$P33^{ING1b}$ protein samples were resolved by SDS-PAGE and excised from the gel. Proteins were then digested with AspN using an Abimed Digest Pro robot, and the resultant peptides dried. A newly described methodology (Xu *et al.*, 2005) was used to reduce the ionization of the positively charged ions, therefore enabling easier detection of negatively (i.e. phosphorylated) ions. After the digested protein was dried, it was resuspended in methanolic hydrochloric acid and incubated for 3 h at room temperature. The solution was dried and the peptides were next resuspended in 0.1% trifluoroacetic acid, 30% acetonitrile. The matrix used for this preparation was 50 mM 2,6-dihydroxyacetophenone in 90% methanol and 100 mM diammonium citrate in Milli-Q water. These two solutions were mixed in equal volumes and then this mixture was mixed at a 1:1 ratio with the sample. The mass spectra were acquired on an Applied

Biosystems Voyager DE-Pro in both positive ion, reflector detector mode and negative ion, reflector detector mode. The spectra were calibrated using the autolytic trypsin peaks at m/z 842.5099 and 2211.0968. The results of the mass spectroscopic analysis were compared to an in silico digest using the PAWS program. This method adds +14 to D, E and C resides and can add +15 to N and Q residues. The phosphorylated peptides were also subjected to on-target phosphatase treatment. Approximately 1 µl of 0.05 U/µl CIP was spotted onto each sample on the MALDI target. The target was placed in a humidified chamber at 37°C for 2 h. The CIP was blotted and allowed to dry. The samples were then run on the mass spectrometer and a loss of -80 (HPO_3) or -98 (H_3PO_4) was expected of the phosphorylated samples.

2.16 *In vitro* Kinase Assay

Initial kinase predictions were performed with NetPhosK 1.0 (Blom *et al.*, 2004). The peptide ELGDTAGNSGKAGADRP corresponding to $p33^{ING1b}$ amino acids 118-134 was sent for KinaseProfiler™ (Upstate) kinase selectivity screening service and assayed as substrate for rabbit CaMKII, human Cdk1-Cyclin B, human Chk1, human CK2 and human GSK3α.

Chk1, Cdk1 and ATM were immunoprecipitated as aforementioned. After washing with PBS, beads were washed twice more with 50 µl kinase buffer (20 mM MOPS [pH 7.2], 25 mM ß-glycerol phosphate, 1 mM dithiothreitol, 1 mM $CaCl_2$) the beads were resuspended in 20 µl kinase buffer. Kinase assays were performed by mixing

0.5 mM of substrate peptide (full length $p33^{ING1b}$ or the LGDTAGNSGKAGADRPK peptide corresponding to amino acids 119-135 of $p33^{ING1b}$ diluted in kinase buffer to above mentioned immunoprecipitates and 5 µl of 1:10 diluted [gamma-^{32}P]ATP mixture (Perkin-Elmer) in the presence of phosphatase inhibitors. The peptide used in the kinase assays differs slightly from the one used in the KinaseProfiler™ screen because of a change in pI (4.56 to 8.59) and therefore better binding to P81 paper. Reactions were incubated at 30°C for 30, 90 or 120 min and stopped by transferring a 25 µl aliquot onto the center of phosphocellulose P81 paper. P81 squares were washed with 0.75% phosphoric acid three times and once with acetone prior to scintillation counting. Radioactive labeling of full-length $p33^{ING1b}$ was performed in the same manner but using 5 µg of MMRU nuclear cell extracts as the kinase source and incubating the reactions for 30 min. Reactions were stopped upon the addition of loading buffer and resolved by SDS-PAGE prior to autoradiography. PKC kinase assays were performed using a PKC assay kit (Upstate) comparing ING1b peptide to control peptide as indicated by the manufacturer.

2.17 References

Campos E.I., Xiao H. and Li G. Generation of a polyclonal antibody specifically against the p33(ING1b) tumour suppressor. 2004. J Immunoassay Immunochem 25:71-80.

Chen B., Campos E. I., Crawford R., Martinka M. and Li G. Analyses of the tumour suppressor ING1 expression and gene mutation in human basal cell carcinoma. 2003. Int J Oncol 22:927-31.

Blom N.T., Sicheritz-Ponten T., Gupta R., Gammeltoft S., and Brunak S. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. 2004 Proteomics 4:1633-49.

Henderson E.E., Valerie K., Green A.P., and de Riel J.K. Host cell reactivation of CAT-expression vectors as a method to assay for cloned DNA-repair genes. 1989 Mutat Res 220:151-60.

Ho S.N., Hunt H.D., Horton R.M., Pullen J.K., and Pease L.R. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. 1989 Gene 77:51-9.

Hsu I.C., Poirier M.C., Yuspa S.H., Grunberger D., Weinstein I.B., Yolken R.H., and Harris C.C. Measurement of benzo(a)pyrene-DNA adducts by enzyme immunoassays and radioimmunoassay. 1981 Cancer Res 41:1091-5.

Orita M., Iwahana H., Kanazawa H., Hayashi K., and Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. 1989 Proc Natl Acad Sci USA 86:2766-70.

Xu C.F., Lu Y., Ma J., Mohammadi M., and Neubert T.A. Identification of phosphopeptides by MALDI Q-TOF MS in positive and negative ion modes after methyl esterification. 2005 Mol Cell Proteomics 4:809-18.

3 GENERATION OF RABBIT POLYCLONAL ANTISERA DIRECTED AGAINST THE $P33^{ING1B}$ PROTEIN

3.1 Rationale

In order to fully study the cellular function of a protein, it is crucial to have the right tools that enable its detection and purification. At the onset of this thesis there were two commercially available antibodies capable of recognizing $p33^{ING1b}$. The first, a rabbit monoclonal was raised against full length recombinant $p33^{ING1b}$ and was not affinity purified (Garkatsev *et al.*, 1996). Since the *ING1* gene expresses at least three protein variants with identical carboxyl-termini and the carboxyl-terminus further shares a high degree of homology with other ING proteins, it is not surprising that this antiserum lacked specificity. This antibody has since been discontinued by the manufacturer. The second antibody is a mouse monoclonal (Garkatsev *et al.*, 1997), which is specific for $p33^{ING1b}$ but that lacks avidity in various basic molecular assays. For this reason we envisaged to raise a rabbit polyclonal antisera that could specifically recognize the $p33^{ING1b}$ protein.

A version of this Chapter has been published in: Campos E.I., Xiao H. and Li G. Generation of a polyclonal antibody specifically against the p33(ING1b) tumour suppressor. 2004. J Immunoassay Immunochem 25:71-80.

3.2 Results & Discussion

3.2.1 Immunization and Affinity Purification

The carboxyl-terminus of all three ING1 proteins is encoded by exon 2 of ING1. The peptides largely differ at their amino-terminus, encoded by exon 1b for $p47^{ING1a}$, exon 1a for $p33^{ING1b}$ and exon 2 for $p24^{ING1c}$ (Figure 3-1). A 12-amino acid peptide corresponding to the amino-terminus of $p33^{ING1b}$ was therefore used to raise rabbit antisera (Figure 3-1). A glycine residue found within this part of the protein was not included in the peptide to avoid the formation of a cyclic structure in the synthetic peptide upon synthesis. A cysteine residue was also added at the carboxyl terminus to allow conjugation to the highly immunogenic keyhole limpet hemocyanin (KLH) carrier protein to serve as an immune stimulant and carrier for the synthetic $p33^{ING1b}$ hapten. SELDI mass spectrometry confirmed the purity of the synthesized peptide. The observed molecular weight of the peptide corresponds to the predicted molecular weight of 1437.56 (Figure 3-2) and minimal impurity was detected. New Zealand rabbits were immunized with the conjugated N-terminal $p33^{ING1b}$ peptide and after 6 weeks of immunization the serum was tested for an immune response by ELISA. The serum was capable of detecting a bacterially produced recombinant GST-$p33^{ING1b}$, but not the GST tag alone (Figure 3-3). Since the serum demonstrated strong immunogenic properties against $p33^{ING1b}$ peptides in the ELISA, further serum was collected and affinity purified.

3.2.2 Specificity of the Affinity-purified anti-p33^{ING1b} Antiserum

The affinity-purified polyclonal antibody was found to be highly specific for the p33^{ING1b} protein in biochemical and immunocytochemical assays. The specificity of this antibody was first assessed by western analysis. Protein extracts were obtained from untransfected MMRU cells or MMRU cells transfected with vectors encoding a FLAG-tagged or an untagged p33^{ING1b} protein. The extracts were resolved by SDS-PAGE, transferred to PVDF membranes and probed with the affinity purified antibody (Figure 3-4). The antibody demonstrated high specificity and avidity. As expected, the FLAG fusion protein was detected at a slightly higher molecular weight compared to untagged p33^{ING1b}. Importantly, endogenous p33^{ING1b} was also detected with this antibody in untransfected cells. The FLAG epitope was only detected in extract from cells expressing FLAG-tagged p33^{ING1b} when the membrane was stripped and re-probed using an anti-FLAG antibody. Endogenous and exogenous p33^{ING1b} protein could also be detected through indirect immunofluorescence using this N-terminal specific polyclonal antibody (Figure 3-5). Consistant with previous reports, the p33^{ING1b} protein was largely nuclear and the FLAG-tagged exogenous protein co-localized with the FLAG epitope.

Antibodies are essential tools that help dissect the biochemical functions and properties of proteins. Designing an antibody that specifically recognized the p33^{ING1b} protein was especially important to this study, since all proteins encoded by the *ING1* gene share an identical carboxy-terminus that is also highly conserved in other ING family members. Furthermore, isoforms encoded by the *ING1* gene do have certain

antagonistic effects, notably in HAT/HDAC associations and in the transcriptional regulation of certain genes (Campos *et al.*, 2004). For these reasons the generation of this antibody has evident advantages over the use of an antibody that recognizes all ING1 protein variants; especially in immunocytochemical studies where multiple proteins that are recognized by the same antibody cannot be differentiated.

3.3 Figures

Figure 3-1 Peptide used for the generation of a $p33^{ING1b}$-specific polyclonal antibody. Sequences of the $p47^{ING1a}$, $p33^{ING1b}$, and $p24^{ING1c}$ proteins (GeneBank accession numbers BAA82887, BAA82886 and BAA83496, respectively) are compared. The shaded carboxyl-terminal protein sequence encoded by exon 2 of the *ING1* gene is common to all three protein isoforms. The boxed sequence in the amino-terminus of the $p33^{ING1b}$ protein highlights the sequence of the synthetic peptide that was used to immunize rabbits to raise a $p33^{ING1b}$-specific polyclonal antibody. The glycine residue was omitted to avoid bending of the peptide during synthesis. A cysteine residue was also added to the carboxyl-terminus of the synthetic peptide to facilitate its conjugation to keyhole limpet hemocyanin.

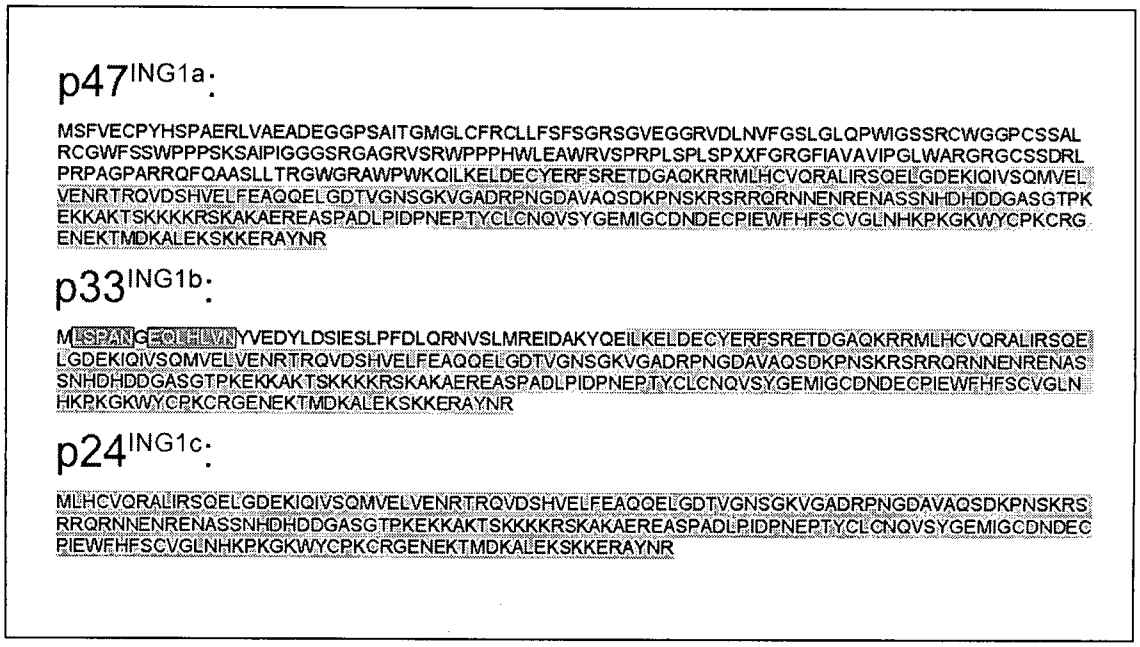


Figure 3-2 Purity of the peptide used to raise a $p33^{ING1b}$-specific polyclonal antiserum. Purity was assessed by SELDI mass-spectrometry. The predicted molecular weight of the synthetic 'LSPANEQLHLVNC' peptide is of 1437.56. The observed weight of the synthetic peptide is of 1437.3 and contains little impurities.

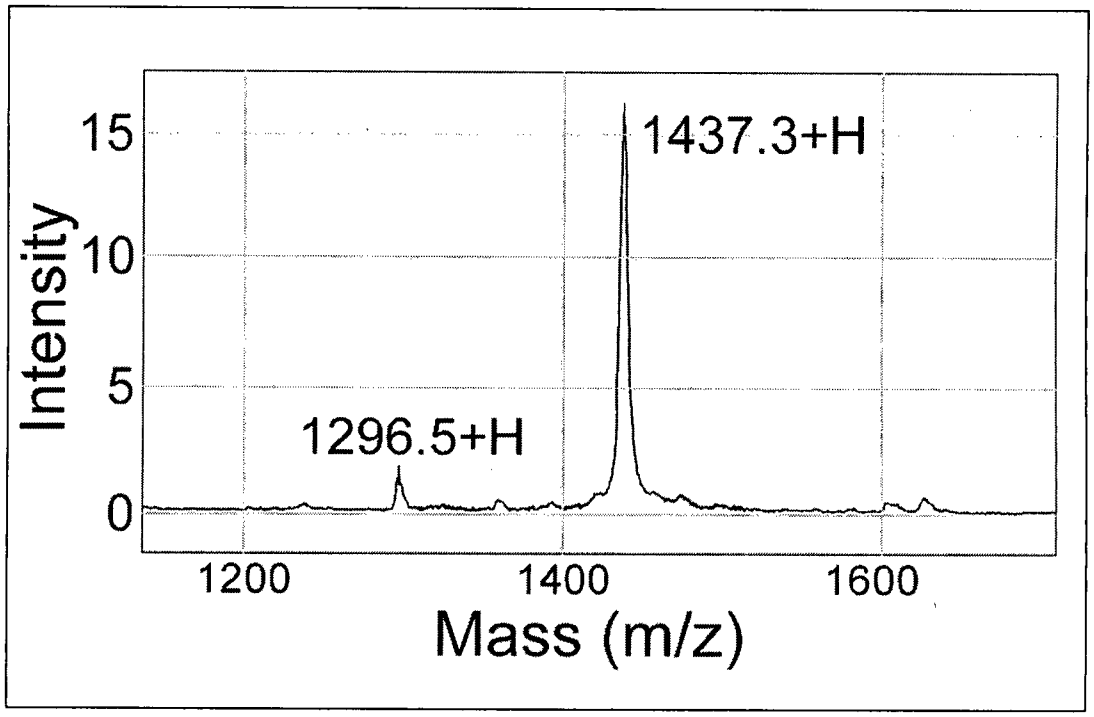


Figure 3-3 Enzyme-linked immunosorbent assay of serum from rabbits inoculated with a synthetic peptide corresponding to a sequence of the $p33^{ING1b}$ protein. The ELISA plate was pre-coated with serial dilutions of bacteria-expressed recombinant GST tag (left column) or GST-$p33^{ING1b}$ (right column), and incubated with 0.2 μg/ml of total serum and a HRP-tagged goat anti-rabbit antibody subsequently. The numbers on the right of the plate correspond to the dilution of the GST and GST-$p33^{ING1b}$ proteins on each row. The last row 'C' contained buffer only. Bound HRP was detected by adding a *o*-phenylenediamine dihydrochloride substrate. Immunized rabbits did develop $p33^{ING1b}$-specific antibodies and was therefore further purified by affinity chromatography.

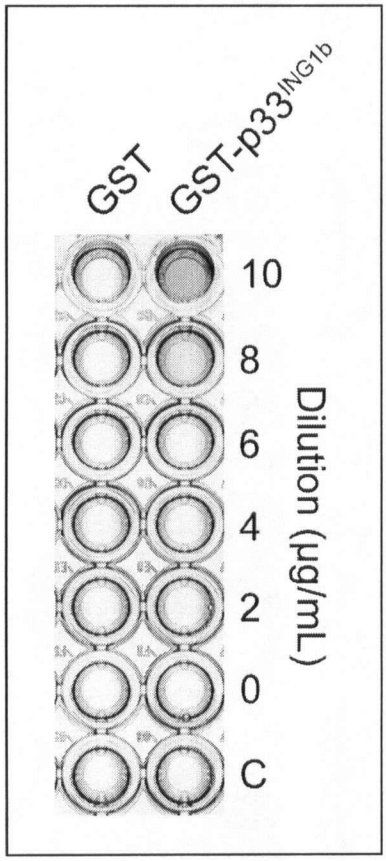


Figure 3-4 Western analysis of p33^{ING1b}-expressing MMRU cells using the rabbit polyclonal p33^{ING1b}-specific antibody. Lanes were loaded with 50 μg of protein extracts from untransfected cells (control) or cells transfected with p*ING1b*-FLAG or pCI-*ING1b* plasmids. Membranes were probed with the rabbit anti-p33^{ING1b} antibody diluted to 0.4 ng/ml and then striped and re-probed with an anti-FLAG antibody. FLAG-tagged p33^{ING1b} appears slightly higher that the untagged counterpart. The p33^{ING1b} polyclonal antibody can also detect endogenous protein.

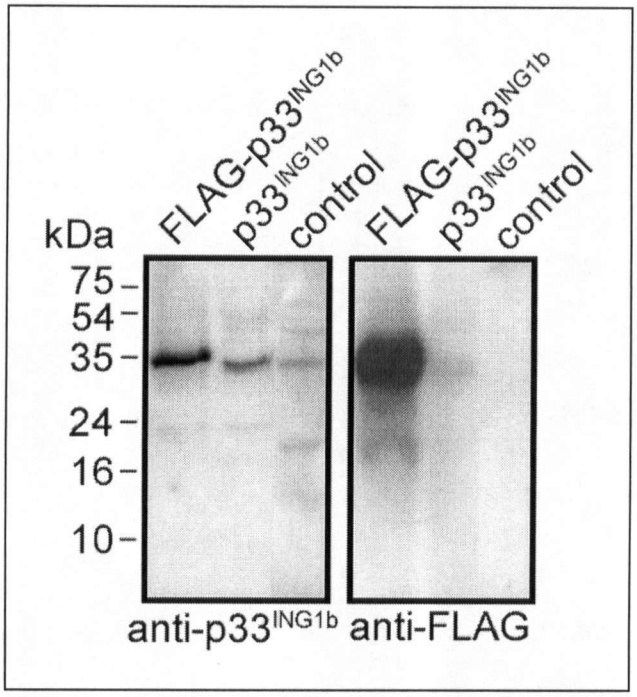
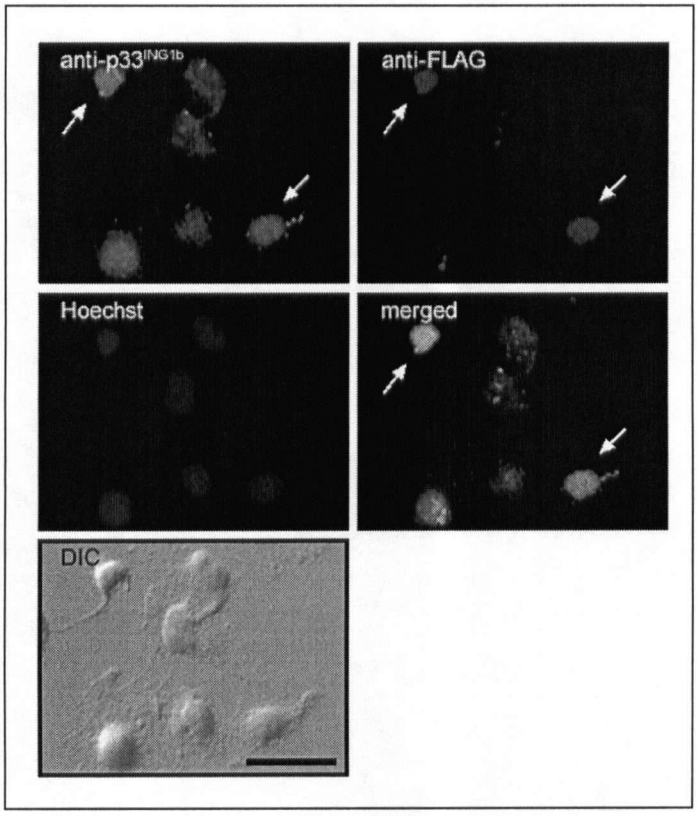


Figure 3-5 Epifluorescent images of MMRU cells transiently transfected with the pING1b-FLAG plasmid and immunolabeled using the anti-$p33^{ING1b}$ rabbit polyclonal and anti-FLAG antibodies. The $p33^{ING1b}$ protein was found to be mainly nuclear using both antibodies. Both endogenous and exogenous $p33^{ING1b}$ was detected with the $p33^{ING1b}$-specific rabbit polyclonal antibody while exogenous protein was detected using both $p33^{ING1b}$- and FLAG-specific antibodies. Cells were counterstained with Hoechst 33258 and further photographed under Nomarski's differential interference contrast (DIC). Scale bar = 50 μm.



3.4 References

Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

Campos E.I., Xiao H. and Li G. Generation of a polyclonal antibody specifically against the p33(ING1b) tumour suppressor. 2004. J Immunoassay Immunochem 25:71-80.

Garkavtsev I., Boland D., Mai J., Wilson H., Veillette C. and Riabowol K. Specific monoclonal antibody raised against the p33ING1 tumour suppressor. 1997. Hybridoma 16:537-40.

Garkatsev I., Kazarov A., Gudkov A. and Riabowol K. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. 1996. Nat Genet 14:415-20.

4 STATUS OF THE *ING1* GENE IN HUMAN MELANOMA

4.1 Rationale

Cutaneous malignant melanoma is a skin cancer that results from the transformation and uncontrolled proliferation of melanocytes. Of all types of cancer, melanoma has seen the highest increase in incidence in recent years. According to the American National Cancer Institute (NCI), the lifetime risk of developing melanoma jumped from 1 in 1,500 in 1935 to roughly 1 in 74 in 1999 within its Caucasian population (Brochez and Naeyaert, 2000; Ries *et al.*, 2002). Melanoma is also a highly heterogeneous disease (Freitas *et al.*, 2004) and displays a high degree of chemoresistance (Serrone and Hersey, 1999). Exposure to sunlight is widely thought to be an important risk factor in the genesis of cutaneous melanoma (Gilchrest *et al.*, 1999). This is further supported by a study that suggests a significantly higher melanoma development in sun-exposed body parts compared to non-exposed parts (Brochez and Naeyaert, 2000). Indeed biological evidence strongly suggests a role of UV light in DNA damage and pathogenesis of melanoma (Brochez and Naeyaert, 2000; Gilchrest *et al.*, 1999). Recent evidence suggests that $p33^{ING1b}$ plays an important role in the removal of UV-induced DNA damage and is transcriptionally up-regulated after UV irradiation (Cheung *et al.*, 2001).

A version of this chapter has been published in: Campos E.I., Cheung K.J. Jr., Murray A., Li S. and Li G. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. 2002. Br J Dermatol 146:574-80. and in: Campos E.I., Martinka M., Mitchell D.L., Dai D.L. and Li G. Mutations of the ING1 tumour suppressor gene detected in human melanoma abrogate nucleotide excision repair. 2004. Int J Oncol 25:73-80.

Furthermore, $p33^{ING1b}$ closely cooperates with the p53 tumour suppressor (reviewed by Campos *et al.* 2004) also known to enhance the repair of UV-induced DNA lesions. Since p53 is rarely mutated in human melanoma (Gwosdz *et al.*, 2006) and *ING1*, a gene that cooperates with p53 in apoptosis and DNA repair is also reported to be lost in various cancers (see Table 1-2), we therefore hypothesized that $p33^{ING1b}$ might be down-regulated or mutated in human melanoma.

4.2 Results

4.2.1 *ING1* Gene Expression in Human Melanoma

To determine if *ING1* expression is altered in human melanoma the $p33^{ING1b}$ protein levels were first assessed in fourteen human melanoma cell lines. Results from western analysis demonstrated that $p33^{ING1b}$ was clearly overexpressed in all 14 melanoma cell lines compared to normal cultured human epithelial melanocytes (Figure 4-1). Densitometric readings corrected for total protein loading confirmed that the levels of $p33^{ING1b}$ protein in the melanoma cell lines ranged from a twofold to up to a 15-fold increase compared to normal human melanocytes (Figure 4-1). To investigate if the accumulation of $p33^{ING1b}$ results from aberrant *ING1* gene transcription mRNA levels were also assessed by semi-quantitative RT-PCR. All cell lines were found to overexpress the *ING1* gene at the transcriptional level, while *ING1* mRNA levels were nearly undetectable in normal melanocytes (Dr. K.J. Cheung, personal communication). In order to determine if $p33^{ING1b}$ also accumulates in cutaneous malignant melanoma,

twenty seven biopsies were collected from the 1995-2001 archives of the Pathology Department of the Vancouver General Hospital. Levels of $p33^{ING1b}$ were assessed in the 27 malignant melanoma biopsies by immunohistochemistry (Figure 4-2). The intensity of the staining was rated as negative (0), weak (1+), moderate (2+) or strong (3+). Negligible or no nuclear expression of $p33^{ING1b}$ protein was detected in normal melanocytes from normal skin biopsies. Among the 27 biopsies, 20 (74.1%) showed high levels of $p33^{ING1b}$, 6 (22.2%) had moderate expression and only one sample (3.7%) had undetectable $p33^{ING1b}$ protein. Normal melanocytic nevus, but not normal epidermal melanocytes where also found to contain high levels of the $p33^{ING1b}$ protein (Figure 4-2).

Although $p33^{ING1b}$ was highly expressed in the melanoma biopsies, all twenty seven biopsies also showed a curious phenomenon. Unlike normal melanocytes and normal melanocytic nevi, all melanoma biopsies contained melanoma cells in which $p33^{ING1b}$ was expressed in an alternate sub-cellular compartment. Unlike melanocytes, all melanoma cells expressed cytoplasmic $p33^{ING1b}$ and all biopsies contained large cancerous populations that no longer displayed nuclear $p33^{ING1b}$. Curiously, normal epidermal keratinocytes also expressed cytoplasmic $p33^{ING1b}$. Figure 4-2 compares normal nuclear $p33^{ING1b}$ in normal nevus melanocytes and cytoplasmic accumulation of $p33^{ING1b}$ in a malignant melanoma biopsy. Since all biopsies contained a given proportion of the cell population which there no longer was nuclear $p33^{ING1b}$, cells with cytoplasmic but no nuclear $p33^{ING1b}$ and cells with both nuclear and cytoplasmic $p33^{ING1b}$ were counted. In all of the melanoma biopsies only 10-20% of the counted melanoma

cells retained some nuclear staining, while all cells expressed cytoplasmic $p33^{ING1b}$ (Figure 4-6).

4.2.2 *ING1* Gene Mutation in Human Melanoma

There are reported correlations between p53 tumour suppressor overexpression and *TP53* gene mutations in certain types of cancers (Kandioler-Eckersberger *et al.*, 2000; Lukas *et al.*, 2000). For this reason the mutational status of the *ING1* gene was further assessed in the fourteen melanoma cell lines and in 46 malignant melanoma biopsies. Single strand conformational polymorphism (SSCP) analysis was used to screen for mutations within the *ING1* gene. SSCP requires PCR amplification of the entire *ING1* gene in fragments under 300 bp using a high-fidelity DNA polymerase and non-denaturing PAGE of single-stranded PCR products. Since the electrophoresis is performed under non-denaturing conditions, single-stranded DNA is free to fold into a given secondary structure and therefore migrates with a given velocity that differs of the same denatured or double-stranded product (Orita *et al.*, 1989). When there are single base alterations or insertions and deletions, the single-stranded products uptake a slightly different secondary structure detectable as mobility shifts in the PAGE (Orita *et al.*, 1989).

A band-shift indicative of DNA base alterations, deletion and /or addition was detected in the PCR product corresponding to exon 1a of the *ING1* gene in the Sk-mel-110 cell line. All other band-shifts occurred in PCR products corresponding to exon 2 of the *ING1* gene of the Sk-mel-24 cell line and of 9 of the 46 biopsies (Figure 4-3).

Samples demonstrating band-shifts in the SSCP assay were further sequenced to confirm and identify genomic alterations within the *ING1* gene (Figure 4-4). All samples, but the SK-mel-110 cell line contained at least one missense or nonsense mutation within exon 2 of the *ING1* gene. The SK-mel-110 cell line only contained silent mutations. Sequencing results were compared to published *ING1* sequence and SNPs (GeneBank accession numbers: AB024401; AB024402 and AF078835). Sequencing of melanoma sample DNA was further compared to DNA from normal human fibroblasts and from adjacent normal epidermal cells (for the melanoma biopsies). No mutations were found in the matched normal skin suggesting that these mutations were of somatic origin. No mutations were found in exon 1b of the *ING1* gene, which encodes the $p47^{ING1a}$ isoform. Table 4-1 recapitulates the sequencing results in all melanoma biopsies and cell lines with aberrant electromobility in the SSCP assay. Interestingly, the mutations were restricted to two areas encoded by exon 2 (within the PCR and the PHD domains of $p33^{ING1b}$). Of further interest, two melanoma patients harbored the same mutation at codon 102 (R102L) and two patients and one melanoma cell line harbored the same mutation within codon 260 (N260S) (Figure 4-4). Overlapping sequencing signals in certain melanoma biopsies (exemplified by an overlapping T and G in the bottom-left sequencing sample of the figure) may result from heterozygosity but most likely from the presence of normal tissue found within the tumour biopsy (i.e. blood vessels and cells, etc.) that could not be removed under microscopic dissection using a needle.

4.2.3 Correlations between *ING1* Mutation and Clinical Parameters

The biopsies collected from the 46 melanoma patients were from 24 males and 22 females which ranged from 26 to 91 years old, with an average of 55 years. The melanomas were classified into Clark's level of invasiveness (I - V) based on the following parameters: Clark's I, melanoma in situ; Clark's II, the tumour has spread to the upper dermis; Clark's level III, the tumour involves most of the upper dermis; Clark's level IV, the tumour has spread to the lower dermis; and Clark's level V, the tumour has spread beneath the dermis. Only 27 of the 46 biopsies were large enough for both immunohistochemistry and mutational analysis of the ING1 gene.

No correlations were found between *ING1* mutation and gender, tumour site or Clark's level of invasion (Table 4-2). Interestingly, the *ING1* mutation rate was significantly higher in patients aged of 40 years or younger (6/12, 50%) than those older than 40 years of age (3/34, 8.8%) ($P<0.01$, χ^2 test). Five-year survival data was also obtained for 34 of the 46 patients. Kaplan-Meier survival curves were plotted for tumour biopsies which contained *ING1* mutation and for those with wild-type *ING1* (Figure 4-7). Our data revealed a trend in which patients with *ING1* mutation have a poorer 5-year survival ($P=0.06$, log-rank test) in which half of patients harboring *ING1* mutations in the melanoma cells had died of the disease compared to only 18% of the patients with no *ING1* mutation in the melanoma cells. However, due to the relatively small number of cases with *ING1* mutations, this trend could not be considered statistically significant in

our analysis. Finally, there was no correlation between *ING1* mutation and levels of $p33^{ING1b}$ detected by immunohistochemistry (Figure 4-6).

4.2.4 Effect of *ING1* mutation on DNA repair

To investigate the effect of *ING1* mutation on the biological functions of the tumour suppressor, site-directed mutagenesis was performed to obtain constructs expressing FLAG-tagged $p33^{ING1b}$ mutants with point mutations at codon 102 (arginine to leucine) or codon 260 (asparagines to serine) since they occurred more than once and represent two areas prone to mutations (Figure 4-5). Figure 4-8 confirms successful mutagenesis of the two mutant plasmid constructs. All constructs further had comparable expression levels when transiently expressed in the MMRU melanoma cell line, as demonstrated by western blotting (Figure 4-8).

A host-cell-reactivation assay (Figure 4-9) was used to determine whether the R102L and N260S point mutations have any effect on $p33^{ING1b}$-mediated enhancement of UV-damaged DNA. The pCMV*cat* plasmid, which encodes the *CAT* reporter gene, was exposed to 40 mJ/cm^2 UV-C *ex vivo* and then co-transfected into MMRU cells with either empty vector or vectors expressing wt or mutant $p33^{ING1b}$. The CAT activity, which indirectly reflects the repair efficiency of UV-damaged reporter plasmid (Figure 4-9), was then measured after 40 hours. The CAT activity was 10-fold higher in cells overexpressing wild-type $p33^{ING1b}$ compared to cells transfected with empty vector (Figure 4-9), confirming previous reports on $p33^{ING1b}$-mediated enhancement of DNA

repair (Cheung *et al.*, 2001). The CAT activity of cells transfected with R102L or N260S mutant $p33^{ING1b}$ was however comparable to that of cells transfected with empty vector suggesting that these two mutations are detrimental to $p33^{ING1b}$-mediated enhancement of DNA repair (Figure 4-9). Co-transfection of wild-type $p33^{ING1b}$ with either one of the two mutants rescued the deficiency in DNA repair further suggesting that these are not dominant negative mutations (Figure 4-9).

To confirm that the R102L and N260S alterations of $p33^{ING1b}$ can abolish $p33^{ING1b}$ enhancement of UV-damaged DNA repair *in vivo*, a radioimmunoassay with antibodies that directly recognize UV-induced 6-4PP was performed (Figure 4-9). MMRU cells were transfected with empty vector, wild-type or mutant $p33^{ING1b}$ and exposed to ultraviolet radiation (20 mJ/cm^2 UVB) the next day. Genomic DNA was then collected from cells before, immediately after or four hours after exposure to UV light. The RIA assay helped determine the amount of 6-4PPs remaining in each sample. The results demonstrated that cells overexpressing wild-type $p33^{ING1b}$ had 47% less 6-4PPs 4 hours following exposure to UV irradiation compared to cells transfected with empty vector. However, the $p33^{ING1b}$-mediated enhancement of DNA repair was greatly reduced in cells transfected with the R102L or N260S mutant forms with only 18.6% and 18.5% less 6-4PPs compared to the empty vector control (Figure 4-9).

4.3 Discussion

The $p33^{ING1b}$ plays an important role in the cellular stress response to UV radiation. Expression of the *ING1* gene is responsive to cellular exposure to UV light in cultured melanoma cells (Cheung *et al.*, 2001), but most importantly it induces cell cycle arrest (Garkatsev *et al.*, 1998) an important step to allow cells to repair sub-cellular damage. The key observation that $p33^{ING1b}$ drastically enhances the removal of damaged DNA by nucleotide excision repair (Cheung *et al.*, 2001) confirmed that like p53, $p33^{ING1b}$ is a guardian of genomic integrity in the cells. Since $p33^{ING1b}$ closely cooperates with p53 to indirectly promote the hypo-phosphorylation of the Rb tumour suppressor (see introductory chapter) and p53 is rarely mutated in human melanoma (Gwosdz *et al.*, 2006), we therefore hypothesized that the *ING1* gene might be silenced or mutated in human cutaneous melanoma. Although nearly a fifth of the melanoma biopsies observed did contain a missense mutation there were a few surprising results, such as the high levels of $p33^{ING1b}$ in melanoma cells, which command a revision of the initial hypothesis.

The expression level of $p33^{ING1b}$ in human tumours appears to be dependent on the tissue type. Diminished expression was reported in lymphoid tumour cell lines, gastric carcinomas, breast cancer, esophageal squamous cell cancer, hepatocellular carcinoma and adenocarcinomas of the esophagogastric junction. However, higher $p33^{ING1b}$ expression was observed in neuroblastoma and brain cancer cell lines and in basal cell and oral squamous cell carcinomas on top of the here-reported melanoma cell lines and melanoma biopsies (Table 1-2). Although the results in this chapter argue that

the expression of the *ING1* gene is clearly not silenced in cutaneous human melanoma silenced, nearly 85% of all melanoma cells within the melanoma biopsies had little accumulation of $p33^{ING1b}$ within the nucleus. This observation was further confirmed by an independent report in which melanoma cells are found to relocate $p33^{ING1b}$ in an alternative sub-cellular compartment (Nouman *et al.*, 2002a). Furthermore, observation of the same phenomenon has now been reported in brain tumours (Vieyra *et al.*, 2003), childhood acute lymphoblastic leukemia (Nouman *et al.*, 2002b), seminoma (Nouman *et al.*, 2002c), papillary thyroid carcinoma (Nouman *et al.*, 2002c) and ductal breast carcinoma (Nouman *et al.*, 2002c). The nuclear to cytoplasmic compartment shift occurs independently of *ING1* gene alterations (Figure 4-6). It is interesting to note that there are three reported missense mutations within the NLS, some observed in cancers reported to have mislocalized $p33^{ING1b}$ protein (Hara *et al.*, 2003; Vieyra *et al.*, 2003). However, none of these mutations were observed in melanoma. The cytoplasmic translocation in melanoma may perhaps be due to posttranslational modifications and/or interactions with proteins that can alter $p33^{ING1b}$ sub-cellular localization, such as in the case of de-phosphorylation and subsequent mdm2-mediated p53 nuclear to cytoplasmic shuttling (Kubbutat *et al.*, 1997). Although the reason for the nuclear to cytoplasmic compartment shift in melanoma cells, and other types of cancer, has yet to be identified it can certainly contribute to a functional inactivation of $p33^{ING1b}$ since most of its functions are attributed to its role in histone acetylation and deacetylation. This is an important observation that certainly merits further investigation.

In this study 46 primary melanoma biopsies and 14 melanoma cell lines were further screened for *ING1* mutations. SSCP and sequencing analysis revealed that the *ING1* gene was mutated in 9 of 46 (19.6%) of melanoma primaries and 2 of 14 (14.3%) melanoma cell lines. This mutation rate is the highest among all human cancers examined so far (Table 1-2) after head and neck SCCs (3/23, 13%) (Gunduz 2000), and esophageal SCCs (4/31, 12.9%) (Chen *et al.*, 2001). In the majority of cancers studied to date *ING1* is however rarely mutated. The relatively high *ING1* mutation in human melanoma supports the notion that $p33^{ING1b}$ plays an important role in cellular stress response to UV radiation and melanomagenesis. There where six G to T transversions among the 10 missense mutations identified through sequencing (Table 4-1). G to T transversions frequently result from oxygen free radical damage in DNA (Reid *et al.*, 1991). The G to T transversions in the *ING1* gene of melanoma biopsies may therefore result from oxidative damage associated with solar UV, as G to T mutation has been found in N-Ras and p53 genes in UV-induced skin cancers (Pierceall *et al.*, 1992; Seidl *et al.*, 2001). However, the fact that no *ING1* mutations were found in sun-exposed sites (face, ear and neck) (Table 4-2) adds to the subtleness of UV role in melanoma gene mutation. Since the etiology of melanoma suggests that exposure to sunlight in early childhood may be an important factor in adult melanoma formation (Gilchrest *et al.*, 1999), the site of the tumour with *ING1* mutation may not correlate with adult exposure but rather with childhood recreational exposures (e.g., sunburn). One interesting finding from this study is that melanomas from younger individuals (40-years or less) had a significantly higher *ING1* mutation rate than those over 40-years of age (Table 4-2). Although the cause for this age-related mutation rate and its biological significance have

yet to be determined, our findings could indicate an involvement of *ING1* mutation in melanomagenesis since melanoma affects younger patients unlike non-melanoma skin cancers which are directly related to cumulative sun exposure and mainly diagnosed in older populations. However, the lack of correlation between *ING1* mutation and Clark's level of progression argues otherwise; or at least that *ING1* mutation is not a common event leading to melanomagenesis. Cytoplasmic shuttling of $p33^{ING1b}$ may therefore be a more critical event contributing to melanoma formation and/or progression since all melanocytic nevus (often considered precursors to melanoma formation) observed clearly contained predominantly nuclear $p33^{ING1b}$ (Figure 4-2).

However, cancer formation is a multi-step process and involves the activation of growth promoting genes and inactivation of cell cycle regulators and promoters of genomic stability. Since $p33^{ING1b}$ plays an important role in the cellular stress response to UV radiation, *ING1* mutations would certainly contribute to genomic instability and promote cellular transformation. All reported *ING1* mutations to date are located within exon 2 (Figure 4-5). *ING1* mutations in cutaneous melanoma were found to be clustered within the PHD domain, which can regulate the binding of ING proteins to chromatin (Gozani *et al.*, 2003) and a region reported to interact with components of the sin3 HDAC complex and is believed important in $p33^{ING1b}$-mediated acetylation (Kuzmichev *et al.*, 2002). The unique locations of *ING1* mutations therefore lead to the hypothesis that they may affect $p33^{ING1b}$-mediated enhancement of DNA repair. Using a host-cell-reactivation assay and a radioimmunoassay specific for UV-induced 6-4PP DNA lesions the two most common mutations, R102L and N260S, were indeed found to abrogate

$p33^{ING1b}$ enhancement of NER (Figures 4-9 and 4-10). Since cancer development is a multi-step process, the defects in NER caused by *ING1* mutation would have severe consequences on melanoma formation, progression and prognosis of melanoma patients. For instance, the reduced capacity of NER caused by *ING1* mutation will increase the genomic instability by failing to repair UV-induced DNA damage in crucial genes which are important for regulating cell cycles, leading to unnecessary cell divisions and propagation of UV-damaged DNA. Since $p33^{ING1b}$ also promotes UV-induced apoptosis (Cheung and Li, 2002), mutation of the *ING1* gene may reduce the removal of cells containing severely damaged DNA by apoptosis. Increased genomic instability could further lead to tumour progression. This may explain why higher percentage of patients with *ING1* mutations (50%) died within five years compared to 18% of the patients who retained wild-type *ING1*. *ING1* mutation occurs in only 20% of the melanoma biopsies, and therefore cannot be a sole critical contributing factor in melanoma progression. $P33^{ING1b}$ closely cooperates with the p53 protein and resides within in a pathway capable of regulating Rb phosphorylation. It is interesting to note that like $p33^{ING1b}$, p53 is also overexpressed but rarely mutated in human malignant melanoma (Gwosdz *et al.*, 2006; Sparrow *et al.*, 1995). Exploration of the functionality of this pathway and further experiments exploring the relation between *ING1/TP53* expression and mutations and $p21^{Waf1}$ regulation, Rb phosphorylation and cell cycle progression would certainly prove interesting.

4.4 Figures

Figure 4-1 Western analysis of $p33^{ING1b}$ protein levels in fourteen melanoma cell lines. (A) Fifty micrograms of total cell extracts were resolved by SDS-PAGE and blotted with anti-$p33^{ING1b}$ antibody. Normal human epithelial melanocytes (NHEM) were used as control and β-actin as the internal loading control. (B) Densitometric measurement of protein expression levels in (A) corrected for total loading. Compared to NHEM all fourteen melanoma cell lines accumulated high levels of $p33^{ING1b}$ protein.

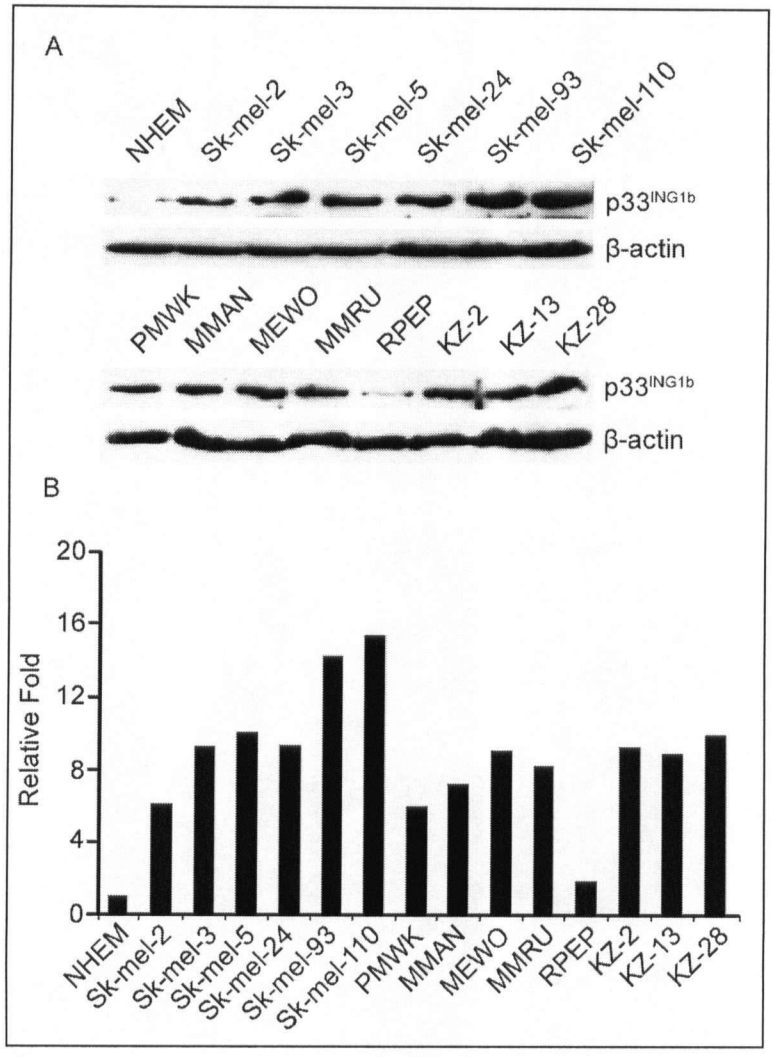


Figure 4-2 Immunohistochemical analysis of $p33^{ING1b}$ expression in normal epidermis (A), a normal melanocytic nevus (C), and in a typical cutaneous malignant melanoma tumour (E). Although some cytoplasmic $p33^{ING1b}$ was found in normal epithelial keratinocytes no or little cytoplasmic $p33^{ING1b}$ was detected in normal melanocytes (panel A indicated by arrows). There was evident nucleolar accumulation of $p33^{ING1b}$ in various keratinocytes and basal cells in the normal epidermis but low levels of nuclear $p33^{ING1b}$ in normal melanocytes. Benign melanocytic nevi were often found to stain strongly for $p33^{ING1b}$; however $p33^{ING1b}$ was largely confined to the nucleus in these cells (indicated by arrows in panel C). Virtually all melanoma tumours were found to accumulate high amounts of $p33^{ING1b}$, however most melanoma cells displayed a loss of nuclear $p33^{ING1b}$ staining. The black arrows in panel E point at melanoma cells expressing high $p33^{ING1b}$ levels in both nucleus and cytoplasm while blue arrows point to melanoma cells having lost nuclear expression of $p33^{ING1b}$; exemplary of typical heterogeneity in this type of cancer. Tissues were indirectly immunolabeled using anti-$p33^{ING1b}$ antibodies and subsequent HRP-DAB staining. Tissues were counterstained with hematoxylin to visualize DNA. Primary antibody was omitted in tissues in panels B, D and F (from the same biopsies as in panels A, B and C respectively). Magnification, 250×.

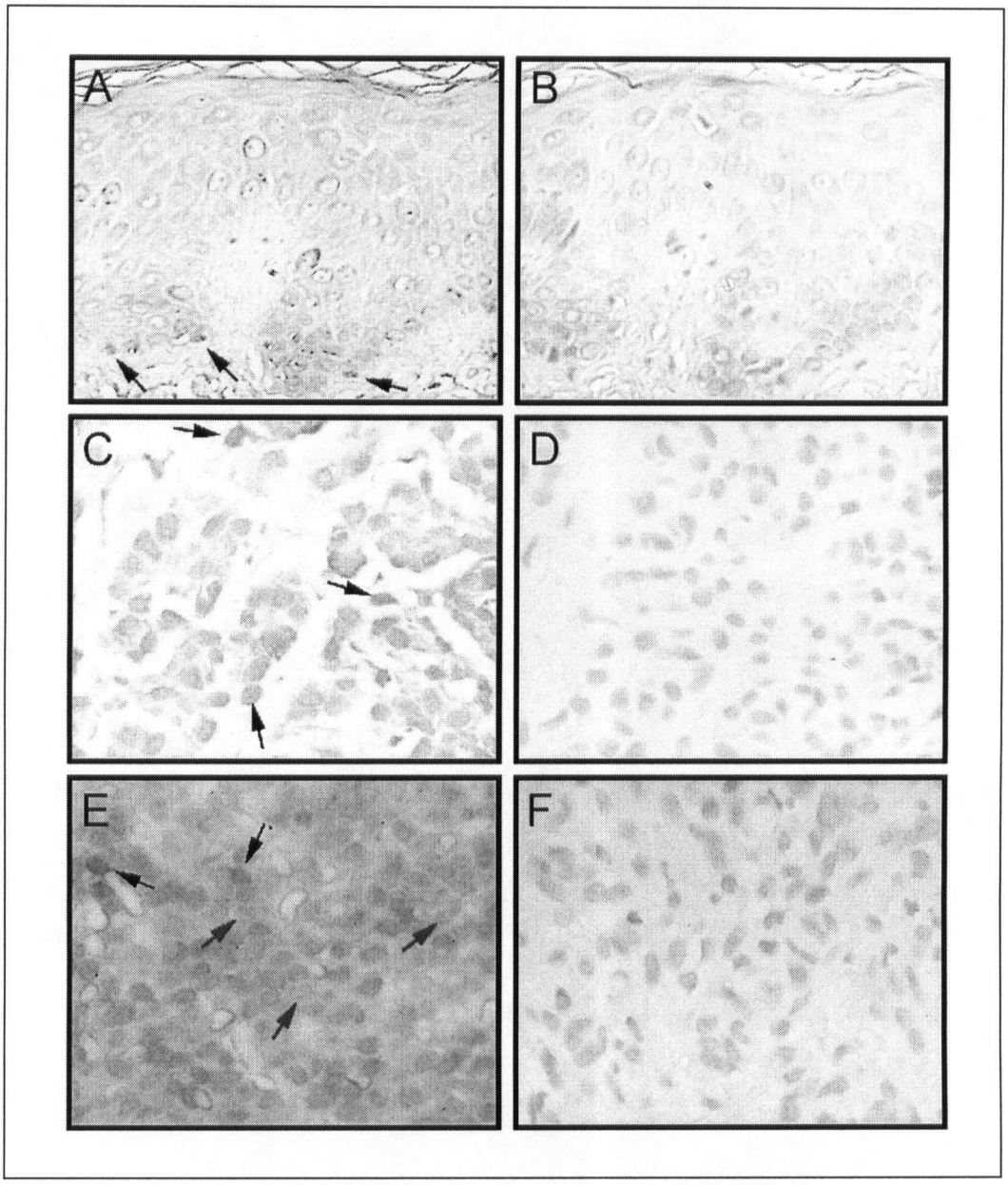

A
B
C
D
E
F

Figure 4-3 Single-strand conformational polymorphism (SSCP) analysis of the *ING1* gene in human melanoma cell lines (A) and cutaneous malignant melanoma biopsies (B). DNA from cultured normal human fibroblasts (NHFB) containing wild-type *ING1* was used as a reference to detect mutations in the melanoma cell lines. Sequences from the first and last halves of exon 2 were separately screened for mutations (Exon 2(a) and Exon 2(b) respectively). DNA extracted from normal (N) and tumour (T) tissues from the same patient (represented by a different number) were compared for *ING1* alterations. No mutations were detected in exons 1a and 1b of the *ING1* gene in genomic DNA from the melanoma cell lines. Arrowheads highlight altered mobility of single-stranded DNA in given samples, indicative of potential *ING1* gene alterations.

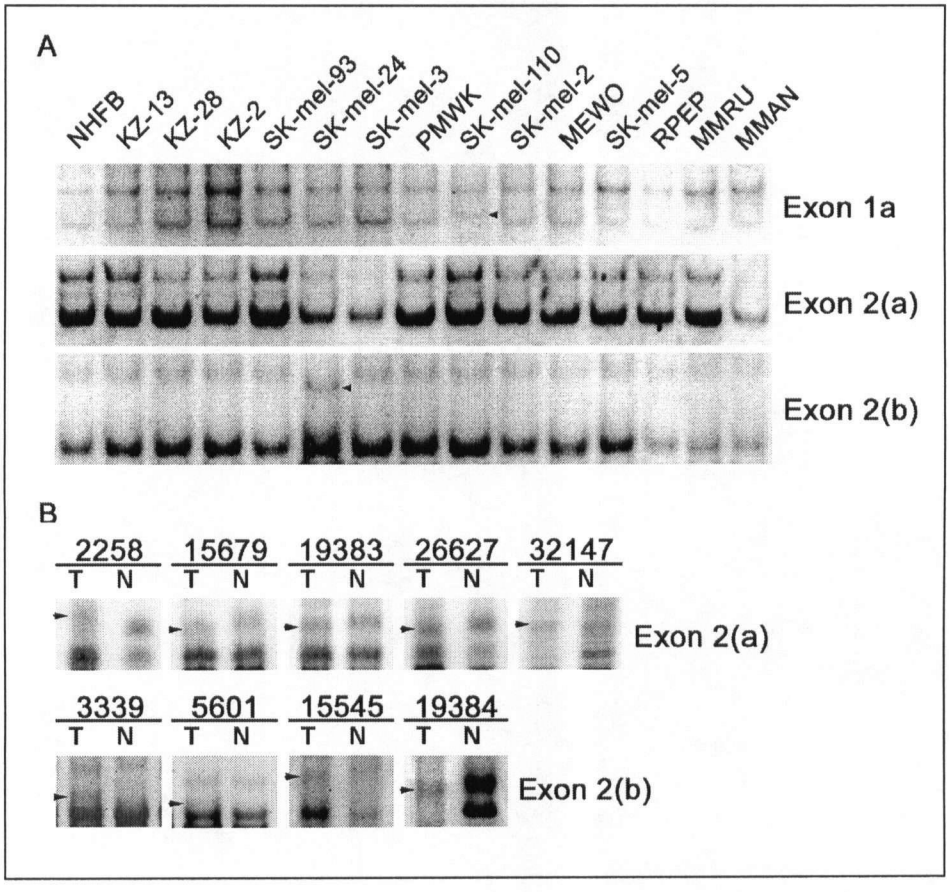


Figure 4-4 Representative sequencing analysis of samples with aberrant SSCP migration. All samples were found to contain at least one point mutation. (A) Sequencing of the *ING1* gene in two melanoma cell lines, Sk-mel-24 and Sk-mel-110 (left and right panels respectively). A missence mutation was found to change asparagine of codon 260 of the $p33^{ING1b}$ protein to a serine in the Sk-mel-24 cell line. The Sk-mel-110 cell line was the sole sample sequenced not to contain a missence or nonsense mutation. (B) Missence mutations of codons 102 and 260 of the $p33^{ING1b}$ protein in the melanoma biopsies number 32147 and 5601 respectively. The *ING1* gene of melanoma cell lines and of melanoma biopsies was compared to that of cultured normal human fibroblasts (NHFB) and normal tissue from the same patients, respectively.

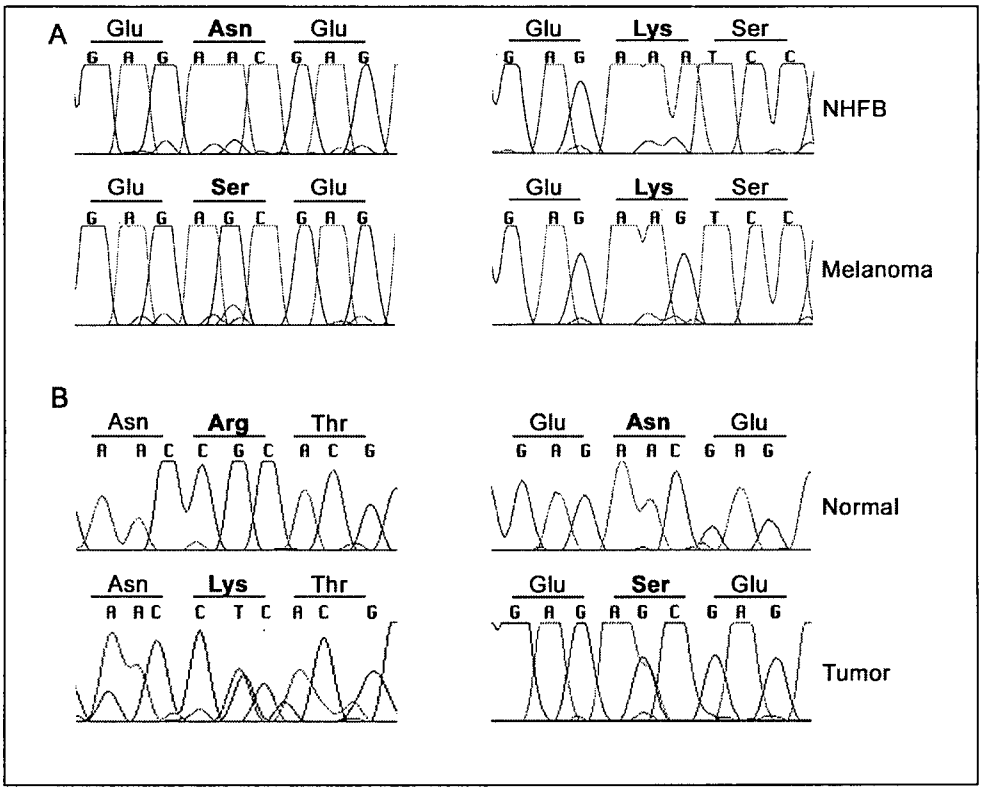


Figure 4-5 Location and relative frequency of mutations affecting the $p33^{ING1b}$ protein. Codons (represented by numbers) in black are indicative of mutations occurring in human cutaneous malignant melanoma and human melanoma cell lines. Codons in grey represent mutations found in breast cancer, gastrointestinal cancers, head and neck squamous cell carcinoma, esophageal squamous cell cancer, basal cell carcinomas, adenocarcinoma of the esophagogastric junction and exocrine pancreatic carcinoma. These alterations do not include reported polymorphisms, but a nonsense mutation (codon 253) is included. Codons 1-56 are encoded by exon 1a and codons 57-279 are encoded by exon 2 of the *ING1* gene.

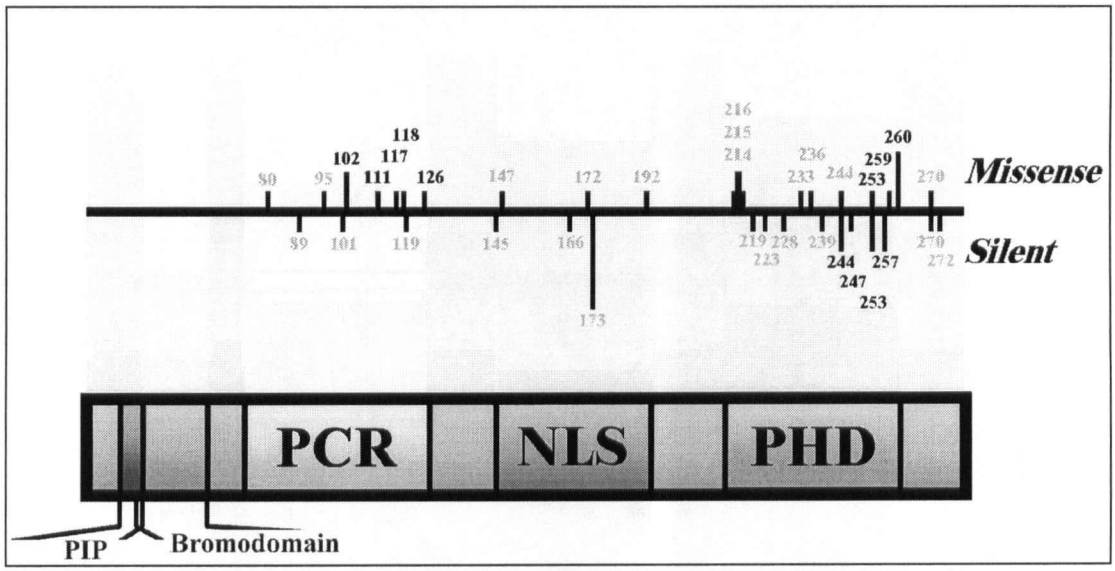


Figure 4-6 Correlative analysis of immunohistochemical parameters of $p33^{ING1b}$ and *ING1* gene mutations in human cutaneous malignant melanoma biopsies. There was little difference in $p33^{ING1b}$ intensity staining between samples with mutant and wild-type *ING1* (top panel). There also was little difference between the amount of cells expressing nuclear $p33^{ING1b}$ in melanoma biopsies with wild-type and mutant *ING1* (bottom panel) suggesting that $p33^{ING1b}$ expression and localization occurs independently of *ING1* gene alterations.

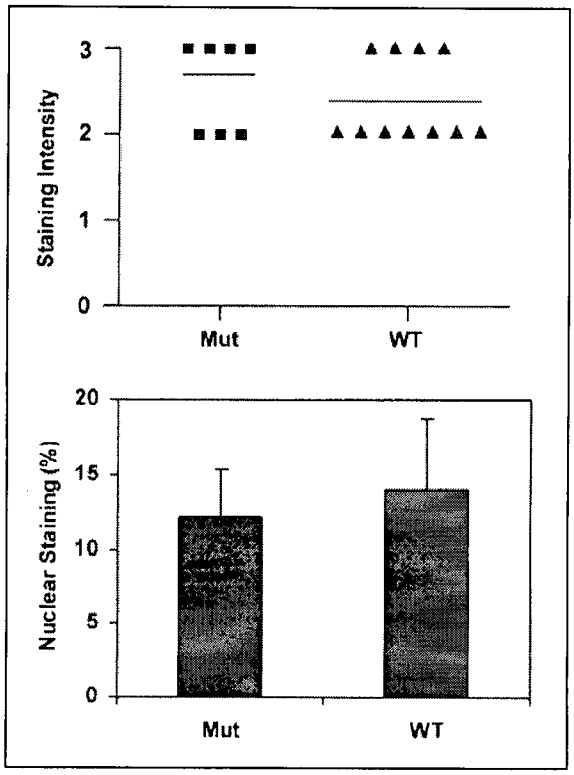


Figure 4-7 Kaplan-Meier curves illustrating a potential correlation between *ING1* mutation and 5-year patient survival. Patients with missense or nonsense *ING1* alterations had a poorer 5-year survival than patients with a wild-type *ING1* gene. Within five years 50% of patients with *ING1* gene mutations died of the disease compared to 18% of patients with no *ING1* gene alterations. Due to the relatively small sample number this result only borders statistical significance ($p = 0.06$, log-rank test).

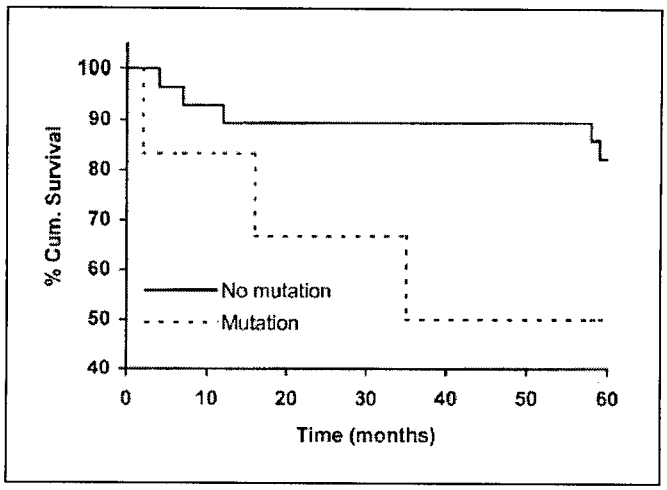


Figure 4-8 Site-directed mutagenesis of the pCIneo-*ING1b*-FLAG plasmid. Point mutations were introduced into two separate plasmids to mimic the R102L and N260S mutations (panels A and B, respectively). (C) Western analysis of total cell extracts from MMRU cells transiently transfected with an empty vector or the pCI-*ING1b*-FLAG, pCI-*ING1b*-FLAG-R102L or the pCI-*ING1b*-FLAG-N260S plasmids. The FLAG epitope could be detected from all but the vector-transfected cells. $P33^{ING1b}$ was expressed in all cases and was clearly overexpressed in cells transfected with either *ING1b*-expressing vector.

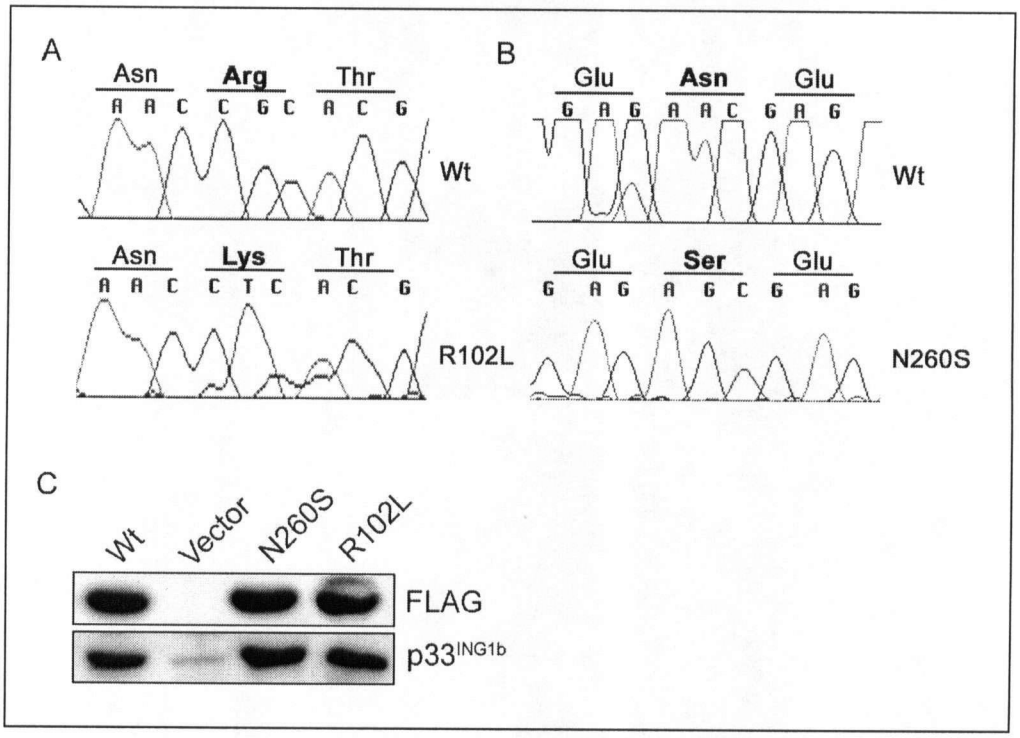


Figure 4-9 *ING1* mutations found in human cutaneous malignant melanoma can abrogate $p33^{ING1b}$-dependent repair of UV-damaged DNA. (A) Effect of *ING1* gene mutation on the repair of a UV-damaged reporter plasmid DNA by the host-cell-reactivation assay. Undamaged or UV-damaged pCMV-*CAT* reporter plasmids were co-transfected with undamaged empty vector or vectors expressing wild-type or mutant $p33^{ING1b}$ in MMRU cells. Cells were collected after a 40 h period, and the activity of the CAT enzyme assayed *in vitro* by measuring its capability to transfer a radiolabeled acetyl group into an appropriate substrate. The CAT activity was determined by scintillation counting and expressed as net dpm damage dose/net dpm zero dose. Experiments were performed in triplicates. (B) Effect of $p33^{ING1b}$ R102L and N260S mutations on the repair of UV-damaged genomic DNA by RIA. MMRU cells transfected with either empty vector or a vector expressing wild-type or mutant $p33^{ING1b}$. Twenty-four hours later cells were exposed to 20 mJ/cm^2 UVB light and genomic DNA collected harvested after 4 h. The percentage of remaining 6-4PPs was then measured using antisera specific for 6-4PPs. Data represents the average of two independent experiments.

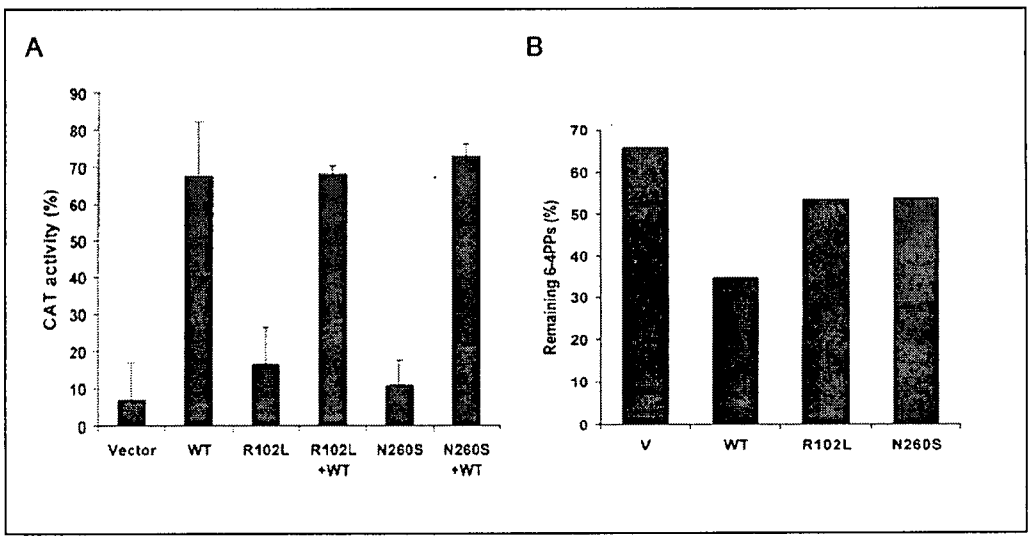


Table 4-1 Summary of *ING1* gene mutations found by SSCP-DNA sequencing in the human melanoma cell lines Sk-mel-24 and Sk-mel-110.

Cell line	Codon	Nucleotide	Amino acid	Type of alteration
Sk-mel-24	239	TCG→TCC	Ser→Ser	Silent
	244	AAT→AAC	Asn→Asn	Silent
	247	CCC→CCA	Pro→Pro	Silent
	253	TGT→TGC	Cys→Cys	Silent
	257	CGG→CGT	Arg→Arg	Silent
	260	AAC→AGC	Asn→Ser	Missense
	270	AAA→AAG	Lys→Lys	Silent
	272	AAA→AAG	Lys→Lys	Silent
Sk-mel-110	89	GTG→GTA	Val→Val	Silent
	101	GAC→GAT	Asp→Asp	Silent

Table 4-2 Summary of *ING1* gene mutations found by SSCP-DNA sequencing in the human cutaneous malignant melanoma biopsies. Codons represent those of the $p33^{ING1b}$ protein and not other ING1 isoforms.

Case no.	Sex	Age	Site	Clarks level	Codon	Nucleotide	Amino acid	Type of Alteration
32147	F	29	Abdomen	I	102	CGC→CTC	Arg→Leu	Missense
3339	M	33	Scalp	II	257	CGG→CGT	Arg→Arg	Silent
					260	AAC→AGC	Asn→Ser	Missense
26627	F	38	Shoulder	II	102	CGC→CTC	Arg→Lys	Missense
15545	M	75	Calf	III	257	CGG→CGT	Arg→Arg	Silent
					259	GAG→GAT	Glu→Asp	Missense
15679	F	26	Arm	III	126	AGC→AAC	Ser→Asn	Missense
19383	F	35	Calf	III	117	CAG→CAT	Gln→His	Missense
19384	F	40	Abdomen	III	244	AAT→AAC	Asn→Asn	Silent
					247	CCC→CCA	Pro→Pro	Silent
					253	TGT→TGA	Cys→*Stop*	Nonsense
5601	M	56	Shoulder	IV	253	TGT→TGC	Cys→Cys	Silent
					260	AAC→AGC	Asn→Ser	Missense
2258	M	64	Back	IV	111	GAG→GAT	Glu→Asp	Missense
					118	GAG→GAT	Glu→Asp	Missense

Table 4-3 Correlations between *ING1* gene mutation in human cutaneous malignant melanoma and clinical parameters. P-value is based on $\chi 2$ or Fisher's exact test. Sun-exposed sites include face, ear and neck while sun-protected sites indicate other body sites.

	ING1 mutation			
	+(%)	-(%)	Total	P-value
Age				
≤40	6 (50)	6 (50)	12	<0.05
>40	3 (8.8)	31 (91.2)	34	
Sex				
Male	4 (16.7)	20 (83.3)	24	
Female	5 (22.7)	17 (77.3)	22	>0.05
Clarks level				
I1	(14.3)	6 (85.7)	7	>0.05
II	2 (18.2)	9 (81.8)	11	
III	4 (33.3)	8 (66.7)	12	
IV	2 (14.3)	12 (85.7)	14	
Site				
Sun-exposed	0 (0)	10 (100)	10	
Sun-protected	9 (25)	27 (75)	36	>0.05

4.5 References

Brochez L. and Naeyaert J.M. Understanding the trends in melanoma incidence and mortality: where do we stand? 2000. Eur J Dermatol 10:71-6.

Campos E.I., Cheung K.J. Jr., Murray A., Li S. and Li G. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. 2002. Br J Dermatol 146:574-80.

Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

Campos E.I., Martinka M., Mitchell D.L., Dai D.L. and Li G. Mutations of the ING1 tumour suppressor gene detected in human melanoma abrogate nucleotide excision repair.
2004. Int J Oncol 25:73-80.

Chen L., Matsubara N., Yoshino T., Nagasaka T., Hoshizima N., Shirakawa Y., Naomoto Y., Isozaki H., Riabowol K. and Tanaka N. Genetic alterations of candidate tumour suppressor ING1 in human esophageal squamous cell cancer. 2001. Cancer Res 61:4345-9.

Cheung K.J. Jr, Mitchell D., Lin P. and Li G. The tumour suppressor candidate p33(ING1) mediates repair of UV-damaged DNA. 2001. Cancer Res 61:4974-7.

Cheung K.J. Jr and Li G. p33(ING1) enhances UVB-induced apoptosis in melanoma cells. 2002. Exp Cell Res 279:291-8.

Doyon Y., Cayrou C., Ullah M., Landry A.J., Cote V., Selleck W., Lane. W.S., Tan S., Yang X.J. and Cote J. ING tumour suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. 2006. Mol Cell 21:51-64.

Freitas Z.F., Rodrigues E.G., Oliveira V., Carmona A.K. and Travassos L.R. Melanoma heterogeneity: differential, invasive, metastatic properties and profiles of cathepsin B, D and L activities in subclones of the B16F10-NEX2 cell line. 2004. Melanoma Res 14:333-44.

Garkavtsev I., Grigorian I.A., Ossovskaya V.S., Chernov M.V., Chumakov P.M. and Gudkov A.V. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. 1998. Nature 391:295-8.

Gilchrest B.A., Eller M.S., Geller A.C. and Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. 1999. N Engl J Med 340:1341–8.

Gozani O., Karuman P., Jones D.R., Ivanov D., Cha J., Lugovskoy A.A., Baird C.L., Zhu H., Field S.J., Lessnick S.L., Villasenor J., Mehrotra B., Chen J., Rao V.R., Brugge J.S., Ferguson C.G., Payrastre B., Myszka D.G., Cantley L.C., Wagner G., Divecha N., Prestwich G.D. and Yuan J. The PHD Finger of the Chromatin-Associated Protein ING2 Functions as a Nuclear Phosphoinositide Receptor. 2003. Cell 114:99–111.

Gunduz M., Ouchida M., Fukushima K., Hanafusa H., Etani T., Nishioka S., Nishizaki K. and Shimizu K. Genomic Structure of the Human *ING1* Gene and Tumour-specific Mutations Detected in Head and Neck Squamous Cell Carcinomas. 2000. Cancer Res 60:3143-6.

Gwosdz C., Scheckenbach K., Lieven O., Reifenberger J., Knopf A., Bier H. and Balz V. Comprehensive analysis of the p53 status in mucosal and cutaneous melanomas. 2006. Int J Cancer 118:577-82.

Hara Y., Zheng Z., Evans S.C., Malatjalian D., Riddell D.C., Guernsey D.L., Wang L.D., Riabowol K. and Casson A.G. ING1 and p53 tumour suppressor gene alterations in adenocarcinomas of the esophagogastric junction. 2003. Cancer Lett 192:109-16.

Kandioler-Eckersberger D., Ludwig C., Rudas M., Kappel S., Janschek E., Wenzel C., Schlagbauer-Wadl H., Mittlbock M., Gnant M., Steger G. and Jakesz R. TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. 2000. Clin Cancer Res 6:50-6.

Kubbutat M.H.G., Jones S.N. and Vousden K.H. Regulation of p53 stability by Mdm2. 1997. Nature 387:299-303.

Kuzmichev A., Zhang Y., Erdjument-Bromage H., Tempst P. and Reinberg D. Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumour suppressor p33(ING1). 2002. Mol Cell Biol 22(3):835-48.

Lukas J., Niu N. and Press M.F. p53 mutations and expression in breast carcinoma in situ. 2000. Am J Pathol 156:183–91.

Nouman G.S., Anderson J.J., Mathers M.E., Leonard N., Crosier S., Lunec J. and Angus B. Nuclear to cytoplasmic compartment shift of the p33ING1b tumour suppressor protein is associated with malignancy in melanocytic lesions. 2002(a). Histopathology 40:360-6.

Nouman G.S., Anderson J.J., Wood K.M., Lunec J., Hall A.G., Reid M.M. and Angus B. Loss of nuclear expression of the p33(ING1b) inhibitor of growth protein in childhood acute lymphoblastic leukaemia. 2002(b). J Clin Pathol 55:596-601.

Nouman G.S., Angus B., Lunec J., Crosier S., Lodge A. and Anderson J.J. Comparative assessment expression of the inhibitor of growth 1 gene (ING1) in normal and neoplastic tissues. 2002(c). Hybrid Hybridomics 21:1-10.

Orita M., Iwahana H., Kanazawa H., Hayashi K. and Sekiya T. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. 1989. Proc Natl Acad Sci USA 86:2766-70.

Pierceall W.E., Kripke M.L. and Ananthaswamy H.N. N-ras mutation in ultraviolet radiation-induced murine skin cancers. 1992. Cancer Res 52:3946-51.

Reid T.M., Fry M. and Loeb L.A. Endogenous mutations and cancer. 1991. Princess Takamatsu Symp 22:221-9.

Ries L.A., *et al.* SEER cancer statistics review,1973-1999. Bethesda,Md.: National Cancer Institute, 2002.

Seidl H., Kreimer-Erlacher H., Back B., Soyer H.P., Hofler G., Kerl H. and Wolf P. Ultraviolet exposure as the main initiator of p53 mutations in basal cell carcinomas from psoralen and ultraviolet A-treated patients with psoriasis. 2001. J Invest Dermatol 117:365-70.

Serrone L. and Hersey P. The chemoresistance of human malignant melanoma: an update. 1999. Melanoma Res 9:51-8.

Sparrow L.E., Soong R., Dawkins H.J., Iacopetta B.J., Heenan P.J. p53 gene mutation and expression in naevi and melanomas. 1995. Melanoma Res 5:93-100.

Vieyra D., Senger D.L., Toyam T., Muzik H., Brasher P.M., Johnston R.N., Riabowol K. and Forsyth P.A. Altered subcellular localization and low frequency of mutations of ING1 in human brain tumours. 2003. Clin Cancer Res 9:5952-61.

5 PHOSPHORYLATION OF THE P33^{ING1B} PROTEIN

5.1 Rationale

Posttranslational modifications, such as protein phosphorylation and acetylation, are fundamental mechanisms through which intracellular events can be rapidly and effectively regulated. A number of protein kinases, notably various phosphatidylinositol 3 kinase (PI3K)-related kinase family members have been identified as transducers which initiate a series of phosphorylation events in response to DNA damage (Bakkenist and Kastan, 2004). For example, p53 activation and stabilization occurs upon activation of the PI3Ks ATM and ATR. Serine 15 of p53 is directly phosphorylated by both ATM and ATR, which in turn is thought to enhance its transactivating activity (Bakkenist and Kastan, 2004). Phosphorylation of p53 serine 20 is also important for p53 stabilization because the phosphate group sterically interferes with binding of the E3 Ub recognin enzyme mdm2 which contributes to p53 degradation (Shieh *et al.*, 2000; Hirao *et al.*, 2000). The Chk1/2 kinases are key regulators of the G_2/M checkpoint which are activated by ATM and ATR (Bakkenist and Kastan, 2004).

The p33^{ING1b} protein is transcriptionally up-regulated by UV-induced genotoxic stress in a time- and dose-dependent manner (Cheung *et al.*, 2001). Not only does p33^{ING1b} closely cooperate with the p53 protein but it also enhances the nucleotide

A version of this chapter will be submitted for publication.

excision repair of UV-damaged DNA (Campos *et al.*, 2004; Cheung *et al.*, 2001). However no regulatory mechanisms for the $p33^{ING1b}$ protein have yet been described. Since protein phosphorylation is a crucial mechanism for the activity of many tumour suppressors, and forms a major mechanism through which various cellular stress response events are orchestrated upon DNA damage; it is therefore credible to hypothesize that $p33^{ING1b}$ is subject to protein phosphorylation upon genotoxic stress and that phosphorylation of $p33^{ING1b}$ may represent a key mechanism for the regulation of its activity.

5.2 Results

5.2.1 $P33^{ING1b}$ is a Phospho-protein

To test the hypothesis that the $p33^{ING1b}$ protein is subject to posttranslational modifications upon genotoxic stress, we first performed a low-*bis* SDS-PAGE comparing protein extracts of untreated and topoisomerase II inhibitor-treated human MMRU melanoma cells. Since the transiently expressed FLAG-tagged $p33^{ING1b}$ appeared as a doublet in untreated cells, it is likely that some posttranslational modification event occured early on after translation (Figure 5-1). The upper FLAG signal would represent posttranslational modified (i.e. phosphorylated) $p33^{ING1b}$, due to altered charged interactions between phosphopeptides and SDS, while the lower signal would represent unmodified $p33^{ING1b}$. Increasing doses of the DNA-damaging agents etoposide and doxorubicin resulted in altered mobility of $p33^{ING1b}$. Both topoisomerase II inhibitors

lead to a net accumulation of a higher molecular weight protein variant 24 h after exposure, suggestive of a stress-induced posttranslational modification.

To further investigate if the upper FLAG signal was due to protein phosphorylation, we incubated the cells in the presence of the cell permeable phosphatase inhibitor okadaic acid or the kinase inhibitors wortmannin and staurosporine. Okadaic acid, a potent inhibitor of protein phosphatase 1A and 2A, caused the disappearance of the bottom (presumably native) proteins (Figure 5-1), indicating that the upper band was likely a result of protein phosphorylation. Furthermore, the addition of the serine-kinase inhibitor staurosporine dramatically reduced the appearance of the upper band (phosphorylated proteins) (Figure 5-1). Wortmannin an inhibitor of phosphoinositide kinase-3 (PI-3K) and PI-3K related kinases also reduced the upper band albeit to a lesser extent.

To further confirm that $p33^{ING1b}$ is a phospho-protein, we performed western blot analysis using antibodies directed against phosphorylated serine residues. Transiently expressed FLAG-tagged $p33^{ING1b}$ was immunoprecipitated from doxorubicin-treated MMRU cells with a mouse anti-FLAG antibody. The immunoprecipitate was divided and half incubated in an alkaline phosphatase reaction, while the other half was incubated in a mock reaction. Western analysis using antibodies directed against phosphorylated serine residues indicated that at least some serine residue(s) within the $p33^{ING1b}$ protein can be phosphorylated (Figure 5-2). This was further supported by the loss of phospho-serine signal, but not of the overall $p33^{ING1b}$ signal, that ensued following a phosphatase

treatment suggesting that $p33^{ING1b}$ is phosphorylated on at least one serine residue *in vivo*. Radiolabeling of GST-tagged $p33^{ING1b}$ further confirmed that $p33^{ING1b}$ can also incorporate phosphate groups *in vitro* using cell extracts as the kinase source (Figure 5-2).

5.2.2 Serine 126 is Phosphorylated upon Genotoxic Stress

Chemical manipulations that utilize differences in survey modes when doing MALDI-TOF mass spectrometry (Xu *et al.*, 2005) allowed the identification of serine 126 as a phosphorylatable residue. The modified sample preparation enhances the detection of negative ions, therefore eliminating the need to phospho-enrich samples. It has the advantage of direct mass measurement analysis of any modification that adds a negative charge to a peptide (e.g., phosphorylation) without the background signal of acidic residues. Since the negative charges associated with carboxylate groups of glutamic and aspartic acid residues can be removed through methyl esterification, it is used to suppress the ion intensity of non-phosphorylated acidic peptides in a negative ion mode, thereby enhancing phospho-peptide detection. The analysis was performed on recombinant GST-$p33^{ING1b}$ exposed to either untreated or doxorubicin-treated MMRU cell extracts. Minimal phosphorylation of serine 126 was observed when extracts from untreated cells were used to phosphorylate recombinant $p33^{ING1b}$ (Figure 5-3 top panels) compared to doxorubicin-treated MMRU cell extracts (Figure 5-3 bottom panels), indicating that this is a stress-responsive phospho-residue. To confirm that serine 126 is a *bona fide* phosphorylation site, the samples were subjected to an alkaline phosphatase treatment

prior to a second round at MALDI-TOF (Figure 5-3 right panels). Such treatment resulted in a reduction of the 1044.8 m/z peak corresponding to the putative phospho-peptide DTAGNpSGKAGA to 964 m/z due to a neutral loss of 80 phosphates (HPO_3) group. β-casein, a protein containing multiple phospho-residues, was used as CIP positive control (Dr. J. Bush, personal communication).

To further confirm that serine 126 is phosphorylated *in vivo*, rabbit polyclonal antibodies were raised against a phosphorylated peptide and used in western analysis of extracts from cells subjected to genotoxic events. The antibody was sequentially affinity purified using a synthetic phosphorylated serine 126 peptide corresponding to amino acids 118-134 and subsequently using non-phosphorylated recombinant full-length $p33^{ING1b}$. In order to test the specificity of the antibody, the sera was incubated with a phosphorylated or non-phosphorylated peptide corresponding to amino acids 118-134 of $p33^{ING1b}$ and the antibody-peptide binding analyzed by ELISA (Figure 5-4). The binding of the antibody to phosphorylated peptide was much stronger (> 8-fold at 1:2000 dilution) than to non-phosphorylated peptide. To further test the specificity of the antibody, a western analysis was done comparing bacterially-expressed recombinant $p33^{ING1b}$ left untreated or phosphorylated using MMRU cell extracts. In eukaryotes, protein phosphorylation occurs mainly as phosphoester in serine, threonine, and tyrosine residues. However, while prokaryotic cells are known to have serine/threonine protein kinases (Kenelly *et al.*, 1998), phosphorylation events largely result from a two-component system in which a phosphate group is transferred from a histidine residue of the kinase protein onto an aspartate residue of the substrate protein (Hoch, 2000). It is

therefore not surprising that the polyclonal anti-*p*-S126 antibody did not recognize bacterially-produced recombinant $p33^{ING1b}$ unless it was phosphorylated *in vitro* using eukaryotic cell extracts.

To determine the kinetics of serine 126 phosphorylation within endogenous $p33^{ING1b}$ protein upon genotoxic events, we exposed MMRU cells to 20 mJ/cm^2 UVB or 1 μg/ml doxorubicin and monitored phosphorylation levels at various time points. Since $p33^{ING1b}$ is up-regulated by DNA-damaging events (Cheung *et al.*, 2001), cycloheximide was added to the media to prevent *de novo* protein synthesis. Using the anti-*p*-S126 antibody, over two-fold increase in serine 126 phosphorylation in endogenous $p33^{ING1b}$ were observed as quickly 1 minute following UVR, and 60 minutes after exposure to doxorubicin (Figure 5-4). However, a basal level of serine 126 phosphorylation was detected in the untreated asynchronous cells, suggesting that phosphorylation of this residue also occurs under non-stress physiological conditions. Cycloheximide alone had little effect on the phosphorylation of serine 126.

5.2.3 Serine 126 Phosphorylation Does not Alter $p33^{ING1b}$ Sub-cellular Localization

Altered expression and sub-cellular localization as well as gene mutation of the $p33^{ING1b}$ tumour suppressor have been found in various cancer types (Campos *et al.*, 2004). Since serine 126 is 16 residues from the nuclear localization sequence we considered important to examine the localization of the protein in the context of protein phosphorylation. In order to prevent phosphorylation, serine 126 was mutated to alanine by site-directed

mutagenesis (Figure 5-5). Serine to alanine substitutions can be used to prevent phosphorylation since the alanine cannot be phosphorylated and its methyl group side chain is unlikely to participate in most hydrophobic or hydrophilic interactions. The S126A mutant was then transfected into MMRU melanoma cells and compared to wild-type protein by immunofluorescent staining to examine the protein sub-cellular localization. Both wild-type $p33^{ING1b}$ and the S126A mutant showed nuclear signals, suggesting that phosphorylation of serine 126 does not modulate the cellular localization of the protein (Figure 5-6).

5.2.4 Phosphorylation of Serine 126 Increases the Half-life of $p33^{ING1b}$

The use of the kinase inhibitors staurosporine and wortmannin reduced the level of posttranslationally-modified $p33^{ING1b}$ but also overall protein levels (Figure 5-1). These results implicate a role for serine 126 phosphorylation in protein stability. To further explore this possibility the turnover rate of $p33^{ING1b}$ was assessed in MMRU cells by overexpressing FLAG-tagged protein and harvesting cycloheximide-treated cells at fixed time intervals (Figure 5-7). Densitometry analysis of SDS-PAGE blots established a half-life at approximately 16.8 h for wild-type $p33^{ING1b}$ compared to 5.7 h for its S126A mutant counterpart in MMRU cells (Figure 5-7). The intensity of $p33^{ING1b}$ bands were corrected for total protein loading and ploted as a natural logarithm of remaining $p33^{ING1b}$ in function of time (based on three independent experiments). It therefore reasonable to deduce that phosphorylation at this site enhances protein stability since a proportion of

the $p33^{ING1b}$ protein pool is phosphorylated in untreated cells as shown by both band-shift assays and western blots on endogenous $p33^{ING1b}$ (Figure 5-1).

It is noteworthy to examine the effect of $p33^{ING1b}$ phosphorylation on p53 protein stability as a previous study has shown that $p33^{ING1b}$ promotes p53 acetylation on lysines 373 and/or 382 (Kataoka *et al.*, 2003). Carboxyl-acetylation of p53 has been proposed to affect p53 stability by preventing mdm2-dependent ubiquitination (Ito *et al.*, 2002) although some studies demonstrate contradicting results (Feng *et al.*, 2002; Nakamura *et al.*, 2000). As previously established, the short-lived p53 protein underwent rapid degradation upon cycloheximide treatment (Figure 5-7). However, the p53 turnover rate was not influenced by the expression of mutant $p33^{ING1b}$ compared to the wild-type counterpart. In both cases the half-life of p53 was of approximately 50 min.

5.2.5 Serine 126 is Phosphorylated by both Chk1 and Cdk1

Our initial attempt to identify the kinase responsible for serine 126 phosphorylation was focused on the protein kinase C (PKC) based on two observations: 1) Staurosporine, a potent PKC inhibitor caused drastic de-phosphorylation of $p33^{ING1b}$ (Figure 5-1) The peptide sequence N-S-G-K, where 'S' corresponds to amino acid 126 of $p33^{ING1b}$ matches the consensus PKC substrate sequence (X-S/T-X-R/K). However, an ING1 peptide containing serine 126 could not be phosphorylated *in vitro* by PKC, while the PKC control peptide QKRPSQRSKYL easily incorporated radiolabeled-phosphate groups (Dr. M. Garate, personal communication). The NetPhosK 1.0 prediction algorithm based on

primary protein sequence (Blom *et al.*, 2004) was used to predict candidate kinases which can phosphorylate serine 126 of $p33^{ING1b}$ (Table 5-1). NetPhosK 1.0 prediction is restricted to 17 protein kinases, and excludes the DNA-damage responsive ATR and Chk1 kinases. Kinases scoring above 0.4 (Cdk1, CaM-II and GSK3) were chosen to be screened by the commercially available KinaseProfiler™ service. Serine 126 is phosphorylated in response to genotoxic stress. Yet the most probable kinase predicted to phosphorylate serine 126 is Cdk1 and not any of the PIKKs. We therefore decided to add the ATM/ATR-downstream and Cdk1-upstream Chk1 kinase in the KinaseProfiler™ screening (ATM and ATR were unavailable). Unexpectedly, both Chk1 and Cdk1 were able to phosphorylate serine 126 *in vitro* (Figure 5-8).

To further confirm that both Chk1 and Cdk1 can phosphorylate serine 126, we immunoprecipitated Chk1 and Cdk1 proteins from untreated, doxorubicin- or UV-treated MMRU cells. Our data indicated that Cdk1 immunoprecipitated from untreated cells was able to phosphorylate $p33^{ING1b}$ peptide at serine 126, while doxorubicin and UV suppressed Cdk1 phosphorylation of $p33^{ING1b}$. In contrast, Chk1 easily phosphorylated serine 126 when immunoprecipitated from UV- or doxorubicin- treated cells but not from untreated cells. This is likely linked to Cdk1 and Chk1 degradation as little Cdk1 was immunoprecipitated from UV- or doxorubicin- treated cells, while the opposite was observed with Chk1 (Figure 5-8). While doxorubicin caused an increase in endogenous serine 126 phosphorylation, the kinase inhibitors kenpaullone and staurosporine also caused a decrease in phosphorylation levels (Figure 5-8). The Cdk1 inhibitor roscovitin and Chk1 inhibitor H-89 also attenuated the phosphorylation of $p33^{ING1b}$ peptide

(personal communication). Since Chk1 is activated by ATM upon doxorubicin treatment we further observed the effect of validated ATM RNAi on the phosphorylated of endogenous $p33^{ING1b}$. As expected, ATM directed short interfering RNA inhibited $p33^{ING1b}$ phosphorylation at serine 126 *in vivo* after doxorubicin treatment (Figure 5-8). To confirm the kinase specificity of this result we further immunoprecipitated ATM and found ATM incapable of phosphorylating serine 126 *in vitro* in kinase assays (Dr. M. Garate, personal communication). Although this experiment implicates the ATM/ATR cascade it remains however preliminary data and much more should be done to examine the role of ATM, but also ATR since ATR is mainly responsive to UV damage.

To further confirm that serine 126 phosphorylation is important for protein stability, we treated MMRU cells with the Cdk1 inhibitor roscovitin and analyzed the half-life of $p33^{ING1b}$ protein. Consistent with the finding that mutation of serine 126 to alanine greatly shortens the half-life of $p33^{ING1b}$ protein (Figure 5-7), the Cdk1 inhibitor roscovitin also drastically reduced the half-life of wild-type $p33^{ING1b}$ (Figure 5-8). This is an important observation since a reduction in the half-life of wild-type $p33^{ING1b}$ protein through the inhibition of the Cdk1 kinase, confirms that the reduction in the half-life of the S126A mutant is linked to serine phosphorylation and not necessarily to missfolding of the S126A $p33^{ING1b}$ protein.

5.2.6 P33^{ING1b} is not Degraded through the Classical Ubiquitin-proteasome Pathway

In order to verify if p33^{ING1b} could be ubiquitinated, MMRU cells were co-transfected with a construct expressing polyhistidine (His)-tagged Ub and a vector expressing either wild-type or S126A FLAG-tagged p33^{ING1b}. Cells were then treated with the proteasome inhibitor MG132 or with both MG132 and cycloheximide. Histidine has a strong natural affinity for certain metals and His-tagged proteins can therefore easily be purified through binding of a metal moiety of a metal chelator complexes such as the Ni(2+)-nitrilotriacetic acid (Ni-NTA) bound to beads. Proteins incorporating His-tagged ubiquitin were therefore isolated through immobilized metal affinity purification using Ni-NTA beads and the bound fraction analyzed by SDS-PAGE/western blotting. The p53 protein, which is degraded through the classical ubiquitin-proteasome pathway was found bound to the Ni-NTA beads; however upon inhibition of the proteasome there was more p53 bound to the Ni-NTA beads since it could still be ubiquitinated but not degraded (Figure 5-10). As expected this p53 accumulation was attenuated if cells were also treated with cycloheximide, which prevents *de novo* protein synthesis. The amount of isolated p53, which incorporated His-Ub did not change with the expression of wild-type or mutant p33^{ING1b}. An appreciable amount of exogenous p33^{ING1b} was found bound to the Ni-NTA beads, suggesting that it can be ubiquitinated. As expected, cycloheximide-treatment translated into a reduced amount of p33^{ING1b} that incorporated the His-Ub group. However, upon inhibition of the proteasome, there was no accumulation of Ni-NTA bound p33^{ING1b}, suggesting that although it may be ubiquitinated it may not be degraded by the proteasome. The same was observed

regardless of the mutational status of the $p33^{ING1b}$ protein (Figure 5-10). Ubiquitin was also detected on immunoprecipitated endogenous $p33^{ING1b}$ (Dr. M. Garate, personal communication), confirming that it can be ubiquitinated. Our assays cannot however discriminate between mono- and poly-ubiquitination (mono-ubiquitination is not necessarily involved in protein degradation).

SDS-PAGE analysis of cycloheximide-treated cells collected at various points in time further support the observation that $p33^{ING1b}$ may not be degraded through the proteasome. The S126A $p33^{ING1b}$ protein displayed a higher turnover rate than its wild-type counterpart. Little S126A $p33^{ING1b}$ and p53 protein remained twelve hours after cycloheximide treatment (Figure 5-10). As expected the p53 turnover rate was greatly reduced when the cells were co-treated with cycloheximide and the MG132 proteasome inhibitor since p53 is mainly degraded by the proteasome (Figure 5-10). However, the turnover rate of the $p33^{ING1b}$ protein remained unchanged even after inhibition of the proteasome, suggesting that it is still degraded independently of the proteasome.

5.3 Discussion

The steady state level of any cellular protein is dependent on its synthesis, degradation, and/or secretion. Upon exposure to genotoxic agents, cells must quickly and efficiently regulate biosynthesis and proteolysis of proteins to fulfill the immediate requirements of a stress response. Posttranslational modifications such as protein phosphorylation, are an elegant and transient way of regulating such processes. *ING1* greatly enhances

genotoxic-stress induced-NER and is transcriptionally up-regulated within 4 h following UV irradiation in melanoma cells (Cheung *et al.*, 2001). Yet kinetic studies demonstrate that NER factors are mobilized seconds after cellular exposure to UV (Mone *et al.*, 2004). Since no regulatory mechanism has been described for any ING protein, we investigated whether the $p33^{ING1b}$ protein would be subject to posttranslational modifications upon genotoxic stress.

The identification of phospho-residues within a protein has always been a challenging task. Traditionally, studies followed the archetypal steps: 1) Radiolabeling of the protein of interest; 2) Enzymatic or chemical hydrolysis; 3) Separation by HPLC, TLC and/or electrophoresis; and 4) Identification of the phospho-amino acid through Edman sequencing or mass spectrometry. This time-consuming and demanding process is being replaced by more direct and non-radioactive enrichment techniques such IMAC phospho-peptide isolation followed by mass spectrometry. Here we demonstrate the application of a chemical procedure that uses the difference in survey modes when doing MALDI-TOF mass spectrometry, therefore allowing direct analysis of peptides without the need for phospho-peptide enrichment. Using this technique we have identified a phospho-amino acid at position 126 of the $p33^{ING1b}$ tumour suppressor (Figure 5-3).

By low-*bis* acrylamide SDS-PAGE, one of the first observations that can be made is the striking net accumulation of $p33^{ING1b}$ protein upon treatment with topoisomerase inhibitors and the overall reduction when cells are treated with kinase inhibitors (Figure 5-1). ATM, ATR and DNA-PK largely influence cellular responses to genotoxic stress

rendering $p33^{ING1b}$ a likely target of DNA-damage responsive PIKKs. Both Chk1 and Cdk1 are downstream of PIKKs and can phosphorylate serine 126 of $p33^{ING1b}$. It is therefore not surprising that the reduction of phosphorylated $p33^{ING1b}$ caused by wortmannin was limited compared to staurosporine (Figure 5-1). In the absence of genotoxic stress Chk1 would not get activated by ATM/ATR and PIKK inhibition would therefore have a limited effect. However, it is possible that other PIKK phospho-acceptor residues exist and could account for the reduction of phospho-$p33^{ING1b}$ observed upon wortmannin treatment. In fact, the prediction algorithm NetPhosK 1.0 implies high likelihood of ATM and DNA-PK phosphorylation on multiple serines. Staurosporine is recognized as a highly potent albeit non-selective inhibitor of PKC with reported IC_{50} values as low as 3 nM in cultured cells (Vegesna *et al.*, 1988). However, it has been recently reported to inhibit both Chk1 and Cdk1 within a comparable range (Zhao *et al.*, 2002), thus explaining the drastic reduction of phosphorylated $p33^{ING1b}$ as well as overall reduction of protein levels that we observed upon treatment with staurosporine (Figure 5-1). Although serine 126 of $p33^{ING1b}$ encompassed well the PKC consensus X-**S**/**T**-X-R/K recognition motif, immunoprecipitated PKC failed to phosphorylate serine 126 but did radiolabel a validated PKC substrate *in vitro*. However, PKC is also predicted to phosphorylate serine 190 and cannot yet be ruled out as a potential $p33^{ING1b}$ kinase.

Although serine 126 does not alter sub-cellular localization of the $p33^{ING1b}$ protein it is noteworthy to mention that serine 126 was found mutated in one melanoma patient (see previous chapter) further supporting the importance of this residue. Furthermore serine 126 is highly conserved in human ING proteins and is the last residue of the

undefined but highly conserved 'potential chromatin remodeling' domain. Like the PHD domain, the PCR domain is present and defines all ING proteins of all organisms. It would be interesting to investigate whether this residue can also be phosphorylated and if it can also alter the turnover rate of other ING proteins.

Site-directed mutagenesis of serine to alanine prevents phosphorylation. In $p33^{ING1b}$, S126A substitution results in a near 3-fold decrease in the half-life of the protein (Figure 5-7) confirming that the protein level changes after treatment with kinase inhibitors observed on low-*bis* gels are largely due to protein stability (Figure 5-1). This not likely a result of protein misfolding due to the S126A substitution since the use of a Cdk1 inhibitor also drastically shortens the half-life of wild-type $p33^{ING1b}$ (Figure 5-8) and also because the use of topoisomerase inhibitors and kinase inhibitors seem to have opposite effects on overall wilt-type $p33^{ING1b}$ protein levels (Figure 5-1). Unlike p53, the degradation of $p33^{ING1b}$ does not seem to occur through the proteasome. Although $p33^{ING1b}$ can be ubiquitinated our experiments could not discriminate between mono-ubiquitination, a regulatory mechanism independent of degradation, and poly-ubiquitination, which often leads to proteasomal but also lysosomal degradation. There also seems to be a natural equilibrium between non-phosphorylated and phosphorylated $p33^{ING1b}$ (regardless of whether it is endogenous or exogenous) in asynchronous cells (Figure 5-1). It would be a great interest to verify whether serine 126 phosphorylation levels correlate with Cdk1-Cyclin B activity in synchronized cells. However, upon genotoxic stress, $p33^{ING1b}$ is quickly mobilized to prolong its turnover rate as our phospho-specific antibody showed that $p33^{ING1b}$ is phosphorylated on serine 126 within a

minute after UVR (Figure 5-4). The delay in serine 126 phosphorylation upon doxorubicin treatment is likely due to the lag in which the drug incorporates into the cell nucleus and affects cellular functions. Thus, stabilized $p33^{ING1b}$ phosphorylated on serine 126 is likely more apt to mediate cellular stress responses such as cell cycle arrest and DNA repair.

The initial finding that both Chk1 and Cdk1 can phosphorylate $p33^{ING1b}$ therefore presented a paradox. On one hand Chk1 is a direct PIKK target, thus leading to the phosphorylation of $p33^{ING1b}$ but also to the inhibition of Cdc25, the phosphatase that activates Cdk1-cyclin B, required for mitotic entry. However, this very same Cdk1 protein which promotes cell division also phosphorylates $p33^{ING1b}$. Closer investigation revealed that while Cdk1 mainly phosphorylates serine 126 under normal conditions it is not responsible for the phosphorylation of serine 126 when cells are exposed to genotoxic stress (Figure 5-8). This is likely since Chk1 is activated upon DNA damage events and activated Chk1 inhibits Cdk1 activity in the cells. On the contrary, in the absence of genotoxic stress, inactive Chk1 does not phosphorylate $p33^{ING1b}$. However, genotoxic stress triggers the ATM/ATR signaling pathway and activated Chk1 can thus phosphorylate $p33^{ING1b}$. Using the anti-*p*-S126 antibody, we observed attenuated phosphorylation *in vivo* caused by the Cdk1 inhibitor kenpaullone in the absence of DNA damage and by the broad-range Chk1 inhibitor staurosporine in the presence of doxorubicin (Figure 5-8), further confirming the duality of the phospho-residue. However again, the use of the Cdk1 inhibitor roscovitin greatly reduced the half-life of wild-type $p33^{ING1b}$. Our results therefore suggest that $p33^{ING1b}$ is a downstream

component in the ATM/ATR signaling pathways in response to genotoxic events but that $p33^{ING1b}$ is also be regulated under normal conditions through the cell cycle.

A past study demonstrated that *CCNB1*, which expresses cyclin B1, is transcriptionally down-regulated by $p33^{ING1b}$ (Takahashi *et al.*, 2002). This report, together with our finding that Cdk1 can phosphorylate $p33^{ING1b}$, suggest the existence of a potential negative feedback loop where upon mitotic onset Cdk1-cyclin B1 phosphorylate serine 126, leading to $p33^{ING1b}$ accumulation and down-regulation of *CCNB1* (Figure 5-9). It would therefore be interesting to further investigate this potential negative regulation between Cdk1-Cyclin B1 and $p33^{ING1b}$ through the cell cycle in synchronous cells. However, although this idea remains to be tested, it may be supported by a few observations. Overexpression of $p33^{ING1b}$ can enhance doxorubicin-induced G_2 arrest in p53-null H1299 cells (Tsang *et al.*, 2003), while *CCNB1* is repressed in cells that are arrested in G_2 in response to genotoxic stress (Manni *et al.*, 2001). Furthermore, $p33^{ING1b}$ contains a potential cyclin-dependent kinase binding (Cy) motif (a.a. 76-79). This motif can act as a substrate recognition site that directly interacts with cyclin to promote phosphorylation by the Cdk-cyclin complex (Takeda *et al.*, 2001) and may help Cdk1-cyclin B1 phosphorylate $p33^{ING1b}$. Taken together, our data suggests that $p33^{ING1b}$ potentially participates in the regulation of mitotic cyclin B1 and is itself regulated by the Cdk1-cyclin B1 complex. Upon genotoxic stress however, ATM/ATR activate Chk1, which inhibits cdc25 and Cdk1, but also prolongs $p33^{ING1b}$ activity necessary for cell cycle arrest and repair of DNA damage (Figure 5-9).

5.4 Figures

Figure 5-1 Posttranslational modifications of the $p33^{ING1b}$ protein. (A) Low-bis SDS-PAGE analysis of FLAG-tagged $p33^{ING1b}$ in MMRU cells 24 h following exposure to 0.5 μg/ml or 1 μg/ml doxorubicin and 0.25 μg/ml or 0.5 μg/ml etoposide topoisomerase inhibitors, (B) 0.1 mM okadaic acid phosphatase inhibitor or 100 mM wortmannin or 50 nM staurosporine kinase inhibitors.

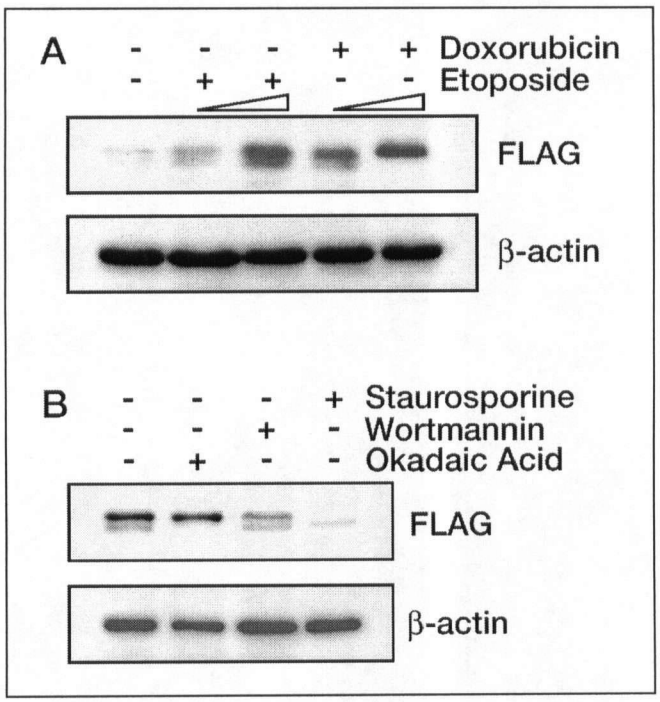


Figure 5-2 $P33^{ING1b}$ is subject to phosphorylation *in vivo* and *in vitro*. (A) FLAG epitope was used to immunoprecipitate $p33^{ING1b}$ from MMRU cells expressing FLAG-$p33^{ING1b}$ and treated with 1 μg/ml doxorubicin and mock-treated (left lane) or treated with calf intestinal alkaline phosphatase (CIP) (right lane). Immunoprecipitates were resolved by SDS-PAGE and subjected to immunoblotting using antibodies specifically raised against phospho-serine residues or against $p33^{ING1b}$. (D) Autoradiogram of [^{32}P]-metabolic labeling of GST-$p33^{ING1b}$ recombinant protein exposed to doxorubicin-treated MMRU cell extracts.

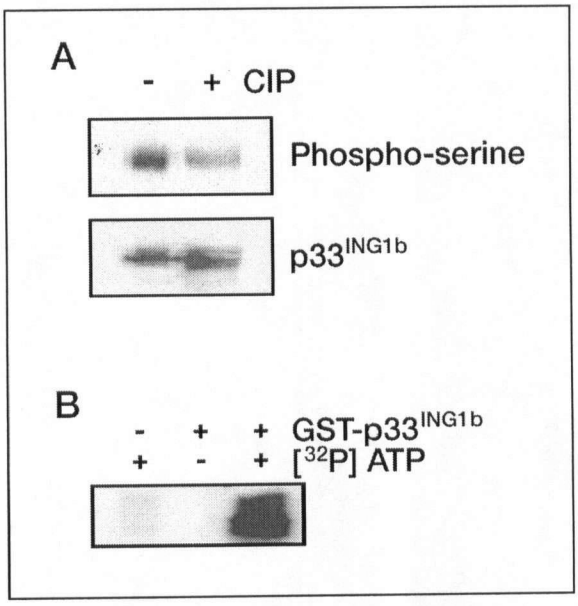


Figure 5-3 Mass spectrometry analysis of AspN-digested recombinant GST-$p33^{ING1b}$. The $p33^{ING1b}$ protein was exposed to cell extracts of untreated (A-B) or doxorubicin-treated (C-D) MMRU cells prior to digestion. The peptide DTAGNSGKAGA corresponding to amino acids 121-131 has a predicted mass of 947 in a non-phosphorylated form (black arrows) and of 1046 in its phosphorylated form (grey arrows). (B-D) CIP-treatment induced de-phosphorylation of serine 126. (D) The 1044.8 m/z peak was reduced to 964 m/z due to the loss of a 80 HPO_3 group (black arrows).

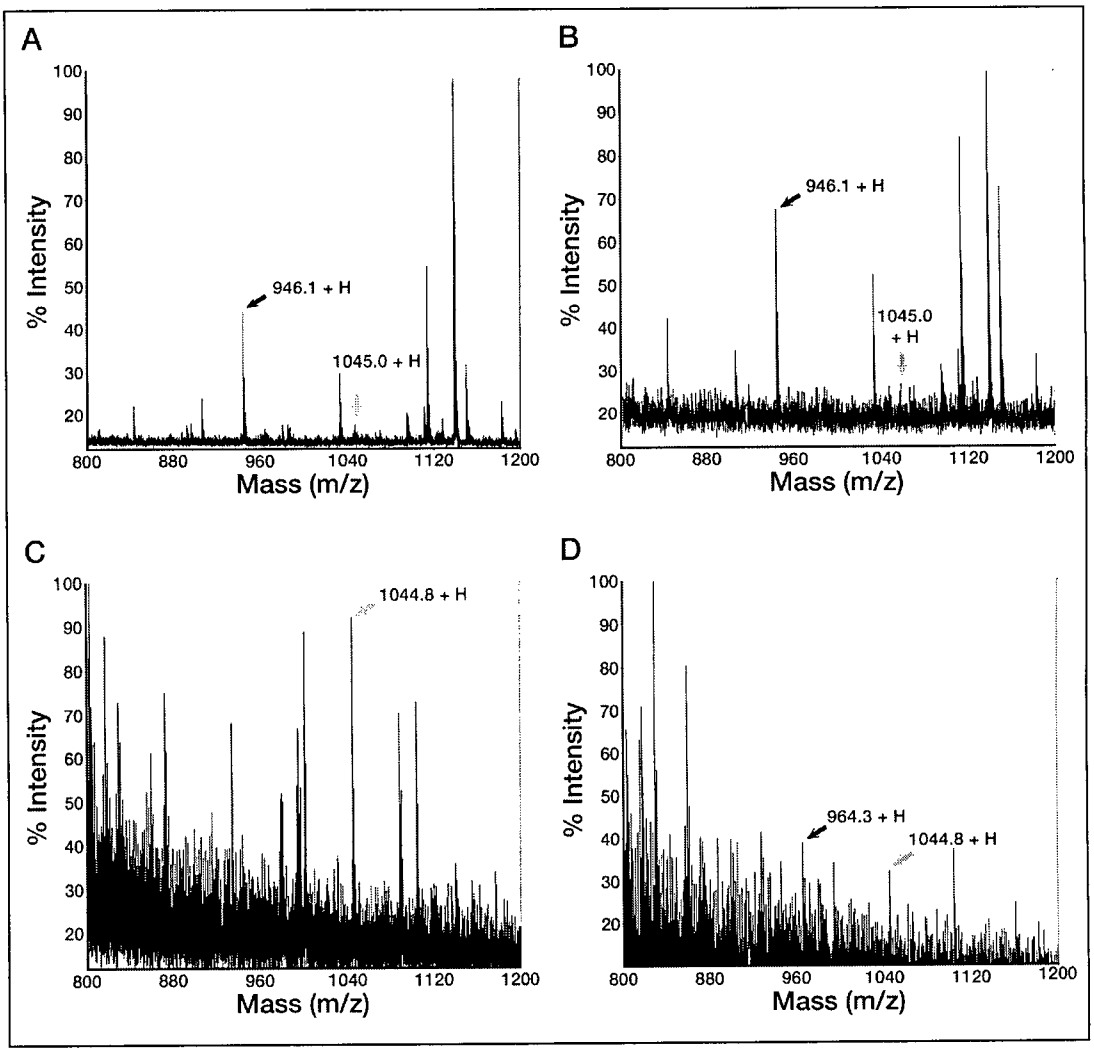


Figure 5-4 Generation of phospho-serine 126 antisera and DNA damage-induced phosphorylation of endogenous $p33^{ING1b}$. (A) Antibodies were generated and affinity purified to specifically recognize phosphorylated serine 126. ELISA of immobilized phosphorylated (black line) or non-phosphorylated (grey line) synthetic peptide containing serine 126 of $p33^{ING1b}$ incubated with increasing dilutions of the polyclonal rabbit anti *p*-S126 antiserum. (B) Western analysis of bacteria-produced recombinant $p33^{ING1b}$ and *in vitro* phosphorylated $p33^{ING1b}$ using crude cell extracts of untreated or doxorubicin-treated cells. (C-D) SDS-PAGE analysis of endogenous phosphorylation of serine 126 following exposure to combined 20 μg/ml cycloheximide and 20 mJ/cm^2 UVB (C) or 1 μg/ml doxorubicin (D). (E) SDS-PAGE analysis of endogenous phosphorylation of serine 126 upon treatment with cycloheximide alone.

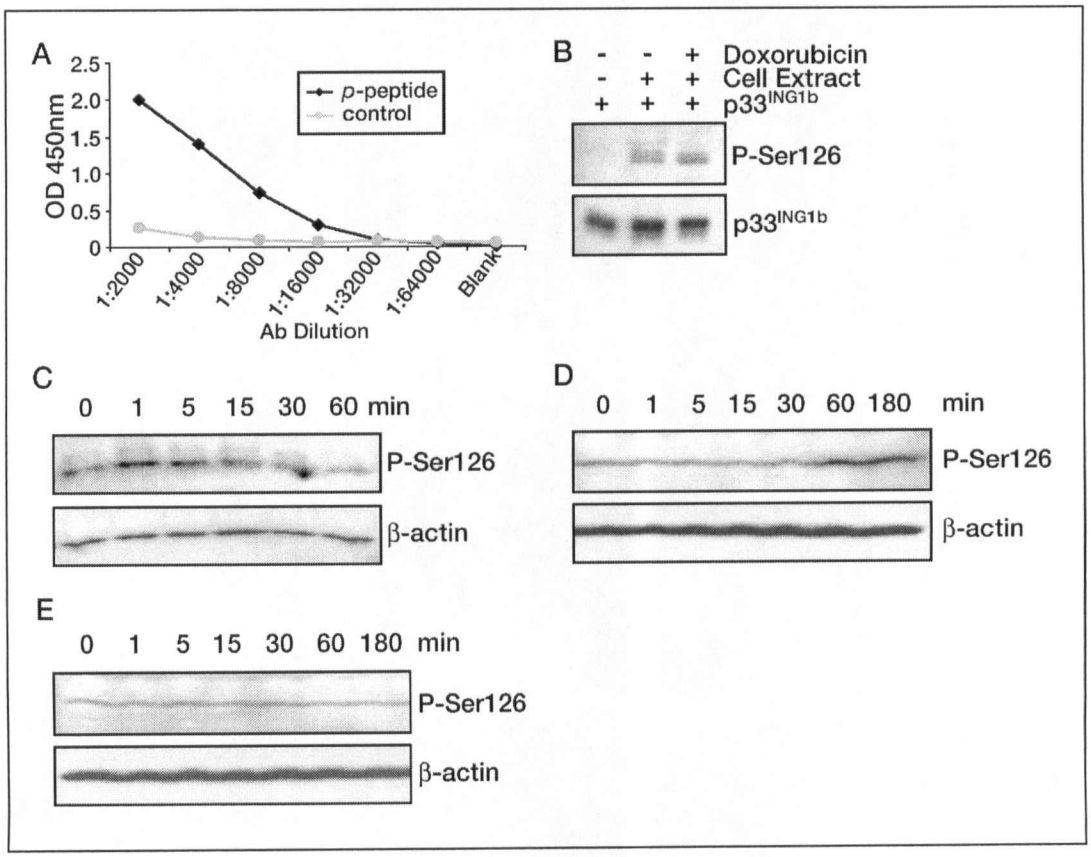


Figure 5-5 Site-directed mutagenesis of serine 126 to abolish phosphorylation. Panel below confirms the site-directed mutagenesis of serine 126 to alanine of the pCIneo-*ING1b*-FLAG plasmid by DNA sequencing.

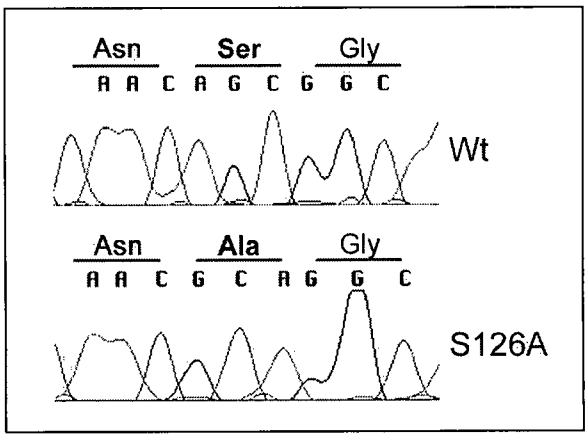


Figure 5-6 Phosphorylation of serine 126 does not affect protein sub-cellular localization. MMRU cells were transfected with pCIneo-*ING1b*-FLAG, or pCIneo-*ING1b*-S126A-FLAG, and protein sub-cellular localization was examined by immunofluorescent staining with an anti-FLAG antibody.

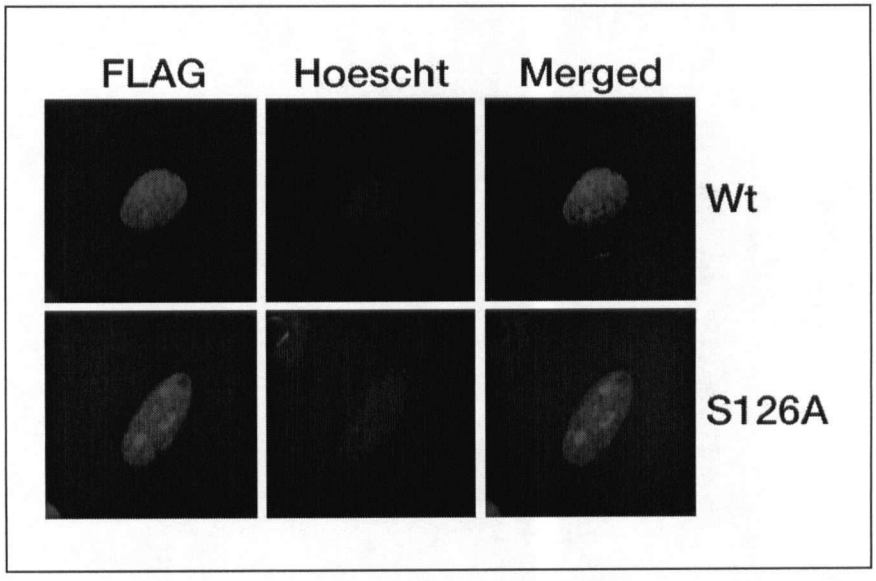


Figure 5-7 Abolishment of serine 126 phosphorylation shortens the half-life of $p33^{ING1b}$ protein. (A) MMRU cells were transfected with pCIneo-*ING1b*-FLAG or pCIneo-*ING1b*-S126A-FLAG and treated 24 h later with 20 μg/ml cycloheximide to prevent *de novo* protein synthesis. Cells were harvested at various time points after the addition of cycloheximide to the media and cell lysates were subjected to western blot analysis of wild-type or S126A mutant $p33^{ING1b}$ protein levels. (B) Densitometric analysis of $p33^{ING1b}$ protein levels upon correction for total protein loading. Wild-type $p33^{ING1b}$ was found to have a half-life of 16.8 h compared to 5.7 h for the S126A mutant. Values are based on three independent experiments. R = correlation coefficient.

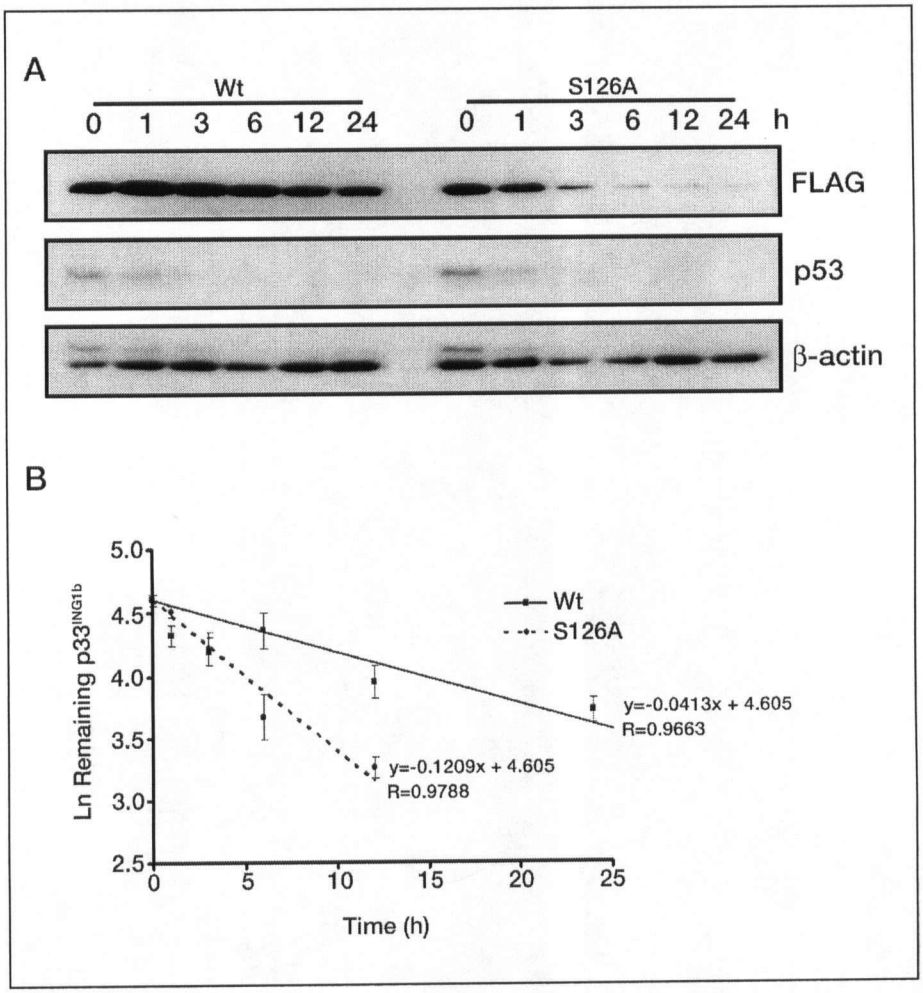


Figure 5-8 Serine 126 is phosphorylated by Chk1 upon genotoxic stress but by Cdk1 under normal conditions. (A) KinaseProfiler^{TM} kinase assay using a peptide corresponding to amino acids 118-134 of the p33^{ING1b} protein as the substrate. Results are expressed as percentage over a control reaction containing 30% phosphoric acid and based on three independent reactions. (B-C) Kinase assay of immunoprecipitated Cdk1 (B) and Chk1 (C) using a p33^{ING1b} peptide corresponding to amino acids 119-135 as substrate. Both kinases were isolated from untreated or MMRU cells exposed to 1 μg/ml doxorubicin for 24 h or 30 min following 20 mJ/cm^2 UVB. X-axis corresponds to the reaction time. (D) Immunoprecipitated Cdk1 and Chk1 from untreated, UV- or doxorubicin- treated MMRU cells used in (B) and (C). (E) Western analysis of endogenous serine 126 phosphorylation in MMRU cells exposed to 1 μg/ml doxorubicin for 24 h in the presence of the kinase inhibitors kenpaullone and staurosporine. (F) Western analysis of the endogenous serine 126 phophorylation levels in nuclear extracts from MMRU cells exposed to 1 μg/ml doxorubicin for 24 h and transfected with ATM siRNA. (G) Western analysis of the effect of the Cdk1 inhibitor roscovitin on wild-type p33^{ING1b} half-life.

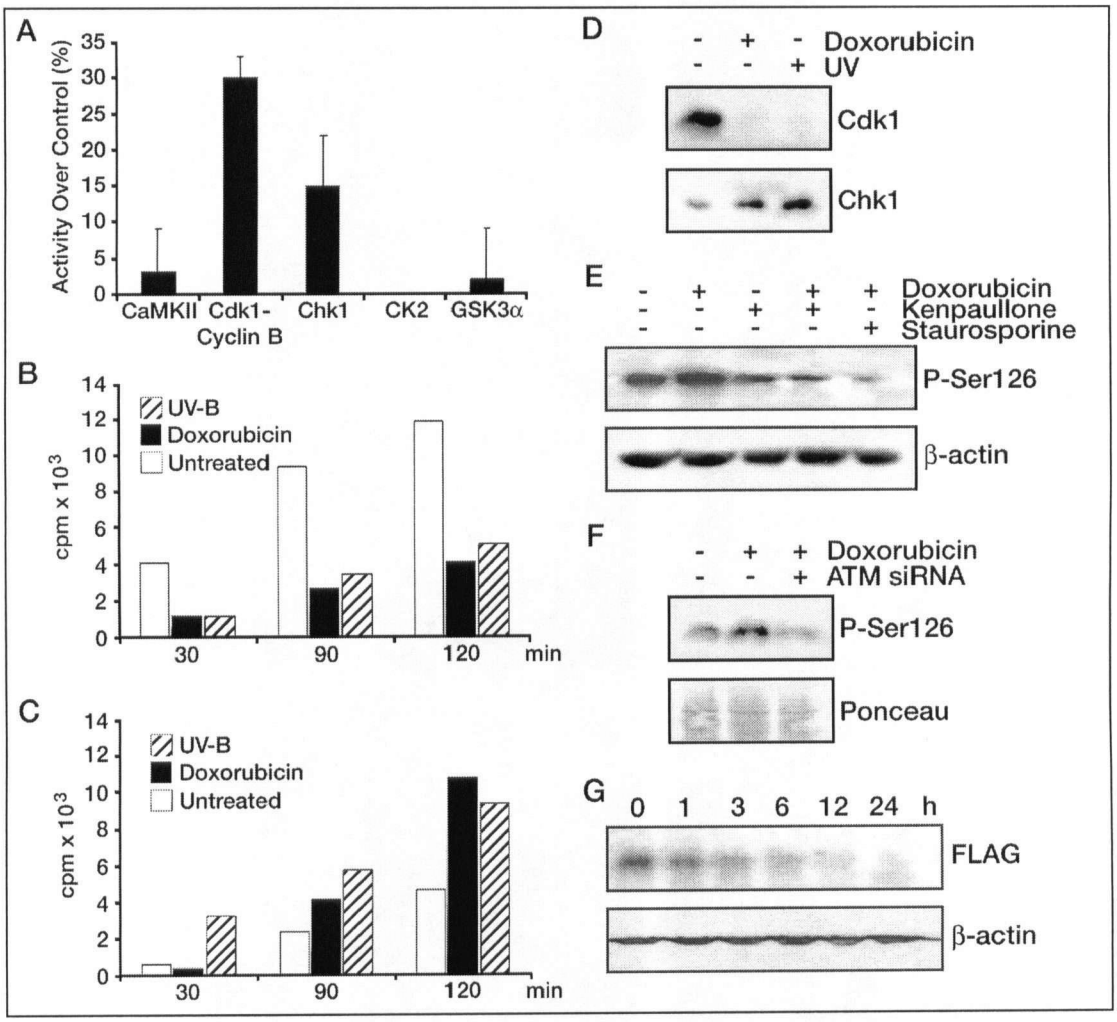

A
Activity Over Control (%)
35
30
25
20
15
10
5
0
CaMKII
Cdk1-Cyclin B
Chk1
CK2
GSK3α
B
cpm x 10³
14
12
10
8
6
4
2
0
UV-B
Doxorubicin
Untreated
30
90
120
min
C
cpm x 10³
14
12
10
8
6
4
2
0
UV-B
Doxorubicin
Untreated
30
90
120
min
D
- + - Doxorubicin
- - + UV
Cdk1
Chk1
E
- + - + + Doxorubicin
- - + + - Kenpaullone
- - - - + Staurosporine
P-Ser126
β-actin
F
- + + Doxorubicin
- - + ATM siRNA
P-Ser126
Ponceau
G
0 1 3 6 12 24 h
FLAG
β-actin

Figure 5-9 Proposed model of $p33^{ING1b}$ regulation through serine 126 phosphorylation under normal conditions (A) and following genotoxic stress (B). Under normal cell cycle progression, Chk1 is inactive during G_2 phase, thus allowing Cdc25 de-phosphorylation and activation of Cdk1. In a negative feedback loop, the Cdk1-cyclin B complex phosphorylates and stabilizes $p33^{ING1b}$, which in turn down-regulates *CCNB1* and contributes to the inactivation of Cdk1. Upon genotoxic stress ATM/ATR activate Chk1, which then inhibits Cdc25 leading to Cdk1 phosphorylation and inactivation. Activated Chk1 further phosphorylates $p33^{ING1b}$ on serine 126 to prolong its half-life, which in turn contributes to the activation of the p53 tumour suppressor and enhances the repair of DNA damage.

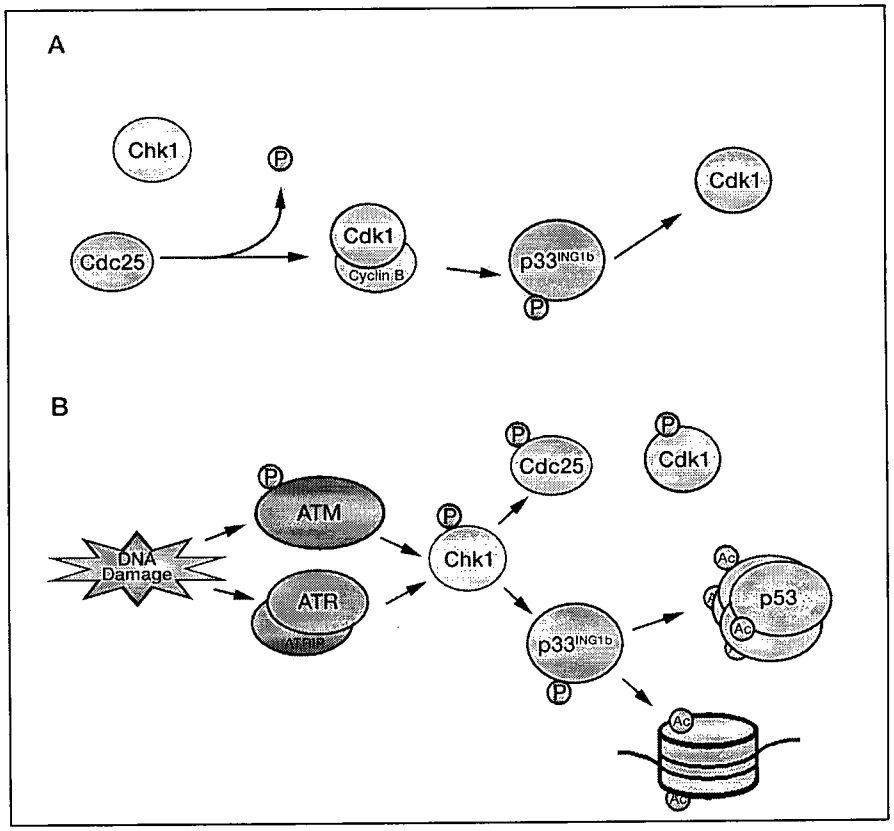


Figure 5-10 Proteasome-independent degradation of $p33^{ING1b}$. (A) Levels of S126A $p33^{ING1b}$ were followed at various points in time in cycloheximide-treated MMRU cells as previously described. (B) The levels of S126A $p33^{ING1b}$ remained unchanged in cycloheximide and proteasome inhibitor MG132-treated cells compared to cells treated with cycloheximide alone, suggesting that $p33^{ING1b}$ is not degraded by the proteasome. However, the p53 protein which is known to be degraded by the proteasome was stabilized by the proteasome inhibitor MG132. (C) Like p53, $p33^{ING1b}$ did incorporate ubiquitin moities but levels of ubiquitinated $p33^{ING1b}$ did not increase upon inhibition of the proteasome. MMRU cells were co-transfected with either empty vector or plasmids encoding wild-type or S126A $p33^{ING1b}$ and a plasmid encoding His-tagged ubiquitin. Proteins that incorporated His-Ub were affinity purified using Ni-NTA beads and resolved by SDS-PAGE. Levels of ubiquitinated p53 increased upon treatment with the proteasome inhibitor MG132 but decreased upon cycloheximide treatment. However, there was no accumulation of wild-type or S126A FLAG-tagged $p33^{ING1b}$ upon inhibition of the proteasome, suggesting that $p33^{ING1b}$ can be ubiquitinated but not degraded by the proteasome. This assay does not discriminate between mono- and poly-ubiquitination.

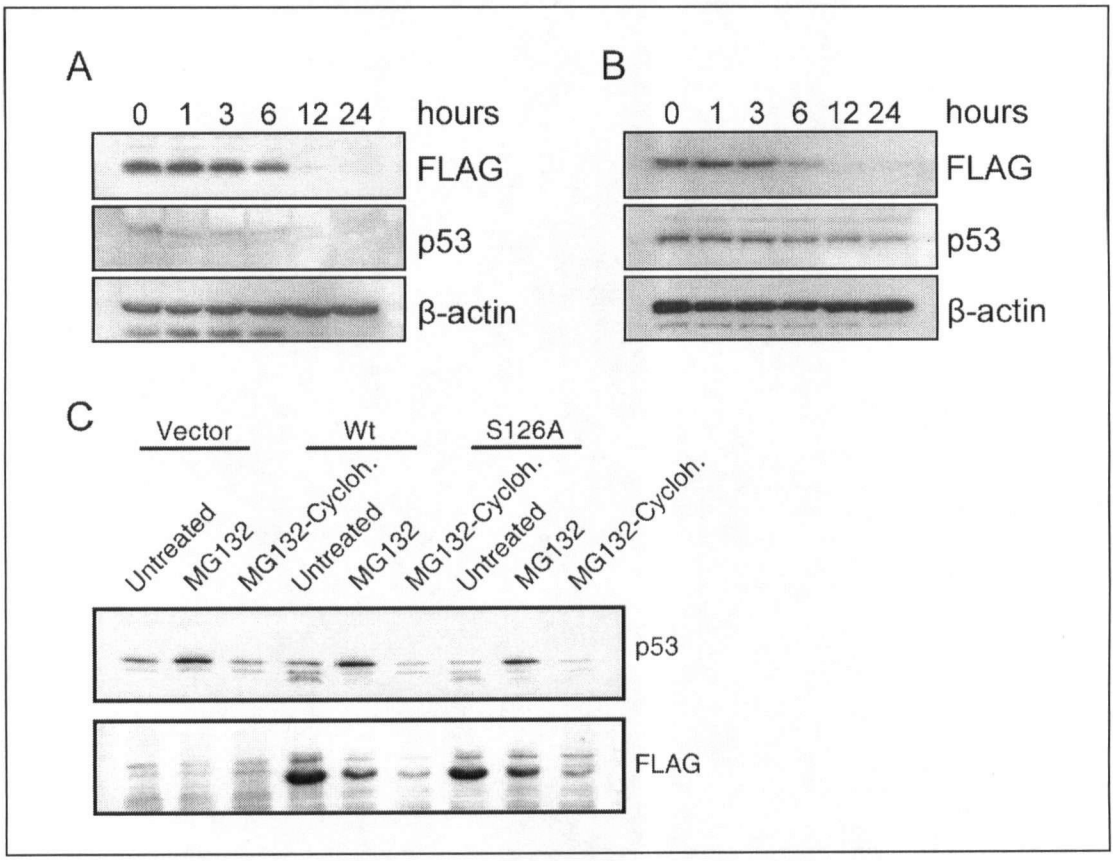

A
0 1 3 6 12 24 hours
FLAG
p53
β-actin
B
0 1 3 6 12 24 hours
FLAG
p53
β-actin
C
Vector
Wt
S126A
Untreated
MG132
MG132-Cycloh.
Untreated
MG132
MG132-Cycloh.
Untreated
MG132
MG132-Cycloh.
p53
FLAG

Table 5-1 NetPhosK 1.0 kinase prediction for $p33^{ING1b}$ serine 126.

Kinase	Score
Cdk1	0.50
CaM-II	0.44
GSK3	0.44
CKI	0.36
DNA-PK	0.35
CKII	0.34
PKG	0.32
ATM	0.31
p38MAPK	0.27
PKA	0.26
PKC	0.26
RSK	0.23
Cdk5	0.15
PKB	0.08

5.5 References

Bakkenist C.J. and Kastan M.B. Initiating cellular stress responses. 2004. Cell 118:9-17.

Blom N., Sicheritz-Ponten T., Gupta R., Gammeltoft S. and Brunak S. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. 2004. Proteomics 4:1633-49.

Campos E.I., Chin M.Y., Kuo W.H. and Li G. Biological functions of the ING family tumour suppressors. 2004. Cell Mol Life Sci 61:2597-613.

Cheung K.J. Jr, Mitchell D., Lin P. and Li G. The tumour suppressor candidate p33(ING1) mediates repair of UV-damaged DNA. 2001. Cancer Res 61:4974-7.

Feng X., Hara Y. and Riabowol K. Different HATS of the ING1 gene family. 2002. Trends Cell Biol 12:532-8.

Hirao A., Kong Y.Y., Matsuoka S., Wakeham A., Ruland J., Yoshida H., Liu D., Elledge S.J. and Mak T.W. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. 2000. Science 287:1824-7.

Hoch J.A. Two-component and phosphorelay signal transduction. 2000. Curr Opin Microbiol 3:165-70.

Ito A., Kawaguchi Y., Lai C.H., Kovacs J.J., Higashimoto Y., Appella E. and Yao T.P. MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation. 2002. Embo J 21:6236-45.

Kataoka H., Bonnefin P., Vieyra D., Feng X., Hara Y., Miura Y., Joh T., Nakabayashi H., Vaziri H., Harris C.C. and Riabowol K. ING1 represses transcription by direct DNA binding and through effects on p53. 2003. Cancer Res 63:5785-92.

Manni I., Mazzaro G., Gurtner A., Mantovani R., Haugwitz U., Krause K., Engeland K., Sacchi A., Soddu S. and Piaggio G. NF-Y mediates the transcriptional inhibition of the cyclin B1, cyclin B2, and cdc25C promoters upon induced G2 arrest. 2001. J Biol Chem 276:5570-6.

Mone M.J., Bernas T., Dinant C., Goedvree F.A., Manders E.M., Volker M., Houtsmuller A.B., Hoeijmakers J.H., Vermeulen W. and van Driel R. In vivo dynamics of chromatin-associated complex formation in mammalian nucleotide excision repair. 2004. Proc Natl Acad Sci USA 101:15933-7.

Nakamura S., Roth J.A. and Mukhopadhyay T. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. 2000. Mol Cell Biol 20:9391-8.

Shieh S.Y., Ahn J., Tamai K., Taya Y. and Prives C. The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. 2000. Genes Dev 14:289-300.

Takahashi M., Seki N., Ozaki T., Kato M., Kuno T., Nakagawa T., Watanabe K., Miyazaki K., Ohira M., Hayashi S., Hosoda M., Tokita H., Mizuguchi H., Hayakawa T., Todo S. and Nakagawara A. Identification of the p33(ING1)-regulated genes that include cyclin B1 and proto-oncogene DEK by using cDNA microarray in a mouse mammary epithelial cell line NMuMG. 2002. Cancer Res 62:2203-9.

Takeda D.Y., Wohlschlegel J.A. and Dutta A. A bipartite substrate recognition motif for cyclin-dependent kinases. 2001. J Biol Chem 276:1993-7.

Tsang F.C., Po L.S., Leung K.M., Lau A., Siu W.Y. and Poon R.Y. ING1b decreases cell proliferation through p53-dependent and -independent mechanisms. 2003. FEBS Lett 553:277-85.

Vegesna R.V., Wu H.L., Mong S. and Crooke S.T. Staurosporine inhibits protein kinase C and prevents phorbol ester-mediated leukotriene D4 receptor desensitization in RBL-1 cells. 1988. Mol Pharmacol 33:537-42.

Xu C.F., Lu Y., Ma J., Mohammadi M., and Neubert T.A. Identification of phosphopeptides by MALDI Q-TOF MS in positive and negative ion modes after methyl esterification. 2005 Mol Cell Proteomics 4:809-18.

Zhao B., Bower M.J., McDevitt P.J., Zhao H., Davis S.T., Johanson K.O., Green S.M., Concha N.O. and Zhou B.B. Structural basis for Chk1 inhibition by UCN-01. 2002. J Biol Chem 277:46609-15.

6 CONCLUDING REMARKS

The ING family of acetyltransferase and deacetylase co-factors, are increasingly capturing the attention of the scientific community. Various lines of evidence suggest that these proteins enable HAT and HDAC activity onto nucleosomal histones, crucial events to chromatin remodeling leading to the regulation of transcription, repair, cell cycle and apoptosis. The founding member, the $p33^{ING1b}$ protein is no exception. This is an exceptional protein that demonstrates versatility as it can associate with both the p300 HAT and the Sin3 HDAC complex. $P33^{ING1b}$ further plays a critical role in the p53 pathway. This work described the development of a critical tool to the study of the $p33^{ING1b}$ protein. Antibodies are used to detect, label and purify specific targets; technicalities that would prove difficult without their use. The development of polyclonal antisera specific for the $p33^{ING1b}$ protein was the first step in a series of experiments that describe its role in normal and diseased conditions.

Human malignant melanoma can become a devastating disease if it is not detected in its early stage soon after development. This disease is also increasingly found in young adults. Unfortunately melanoma tumours tend to display highly heterogeneous phenotypes and, in many cases, are also chemo-refractory. Hence efficient treatments need to be developed to treat this disease. For this reason it is important to fully understand how and why melanoma arises and identify the cellular components that are involved. It is currently believed that melanocytes can undergo neoplastic transformation upon the inactivation of pathways that activate the retinoblastoma protein and the

activation MAPK and/or disruption of MAPK-interacting proteins. Although a high proportion of melanoma cases were reported to harbor constitutively active B-Raf or N-Ras a major target leading to Rb inactivation has yet to be identified. In contract to most human cancers, the most obvious candidate, the p53 tumour suppressor protein is rarely inactivated in human melanoma. For this reason this thesis investigates the status of the *ING1* gene in human melanoma. After all $p33^{ING1b}$ physically cooperates with p53 and through up-regulation of $p21^{Waf1}$, contributes to the activation of the Rb cell cycle regulator. In this study two major findings are discussed. First $p33^{ING1b}$ is often located in an altered sub-cellular compartment in melanoma cells. Since $p33^{ING1b}$ associated with HATs and HDACs to activate and repress nucleosomal DNA, this could surely contribute to the inactivation of this tumour suppressor. This phenomenon is not however restricted to melanoma as it is also reported in various human malignancies. The factors contributing to this phenomenon remain however obscure and would definitely be of interest to the understanding of cancer biology. The second observation is that *ING1* is mutated in nearly a fifth of the melanoma biopsies studied. Two mutational 'hot spots' were further found to be detrimental to the DNA repair functions associated with the $p33^{ING1b}$ protein. Of particular interest, half of the melanoma-stricken individuals that did harbor *ING1* mutations seemed much more susceptible to the disease than those with the wild-type counterpart. It would be interesting, albeit laborious to obtain a large number of biopsies to statistically confirm this observation.

In the later part of the thesis $p33^{ING1b}$ was found to be a phospho-protein and serine 126 phosphorylation responsive to genotoxic stress. This is the first critical observation,

which describes a regulatory mechanism for the $p33^{ING1b}$ protein. Although serine 126 phosphorylation increases upon cellular exposure to UV or doxorubicin, likely through Chk1, further studies are needed, and are currently underway, to help understand the role of serine 126 phosphorylation by Cdk1-cyclin B. The observations presented in this thesis are based on asynchronous cells and we are currently pursuing this study in synchronized cells through cell cycle progression. Since $p33^{ING1b}$ is reported to repress cyclin B1 expression, chapter 5 describes a potential model in which at the G_2-M phase boundary, upon activation of Cdk1 by cyclin B1 the Cdk1-cyclin B1 complex promotes $p33^{ING1b}$ activity by phosphorylating serine 126 and therefore prolonging the protein half-life. This may therefore translate in a negative feedback loop through which $p33^{ING1b}$ may contribute to destabilize the Cdk1-cyclin B1 complex for further entry into G_1/G_0. Alternatively, but not necessarily mutually exclusively, phosphorylated $p33^{ING1b}$ may also contribute to the chromatin remodeling events required for mitosis if the half-life of the protein is increased at the G_2/M boundary. Regardless of the outcome, future results on this matter will prove invaluable to the understanding of serine 126 phosphorylation and to the role of $p33^{ING1b}$ in the cell cycle. It is also interesting to note that mass spectrometric analysis of $p33^{ING1b}$ detected potential acetylated residues, giving plenty to study in the future.

In closing, this work significantly contributes to the knowledge and understanding of the $p33^{ING1b}$ protein, particularly in melanoma. The presented results are exciting and promising, but further raise important questions and new venues that further need to be explored. There are now over 110 hits on an 'ING1' search on the PubMed search

engine where there were less than 25 publications on the protein when I first started this work. Histone acetylation is essential to cellular viability and is thought to be a basic and fundamental chromatin regulatory mechanism. Future studies on $p33^{ING1b}$ and other ING proteins will surely prove fantastically complex but at the time promising and exciting.