

MOLECULAR FUNCTIONS OF THE TUMOUR SUPPRESSOR p33ING2

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ABSTRACT

Since p33ING2 was cloned in 1998, there have been several studies indicating that p33ING2 is a tumour suppressor candidate as it regulates gene transcription, cell cycle arrest and apoptosis in various human carcinoma cell lines. These functions have been shown to be dependent on the activity of wild-type p53. p33ING1b, which shares 58.9% homology with p33ING2, has been shown to play a role in cellular stress response to UV irradiation. Due to the structural similarities between p33ING1b and p33ING2, we hypothesized that p33ING2 enhances UVB-induced apoptosis. To test this hypothesis, we investigated the role of p33ING2 in UV-induced apoptosis and the repair of UV-damaged DNA. We report that overexpression of p33ING2 enhances UVB-induced apoptosis in wild-type p53 MMRU melanoma cells, but not in mutant p53 MeWo melanoma cells. We demonstrate that the enhancement of UVB-induced apoptosis by p33ING2 requires the presence of functional p53. Furthermore, we found that overexpression of p33ING2 upregulates the expression of endogenous Bax and downregulates the expression of Bcl-2, resulting in an increased Bax/Bcl-2 ratio. Moreover, we also found that p33ING2 promotes Bax translocation to the mitochondria, alters the mitochondrial membrane potential, and induces cytochrome c release, thus activating caspases 9 and 3 upon UV irradiation. In addition, we show that under non-stressed conditions, p33ING2 upregulates Fas expression and activates caspase 8. These results indicate that p33ING2 cooperates with p53 to regulate apoptosis via activation of both the mitochondrial/intrinsic and death-receptor/extrinsic apoptotic pathways.

To investigate if p33ING2 is also involved in DNA repair in melanoma cells, we transfected the p33ING2 expression vector into melanoma cells. We report that overexpression of p33ING2 enhances the repair of UV-damaged DNA, and that this process requires the activity of functional p53. We also show that p33ING2 is not directly recruited to UV-induced DNA lesions, both cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), suggesting that p33ING2 is not a component of the repair core complexes. Furthermore, we found that p33ING2 enhances p53 acetylation and upregulates the expression of XPA, which is involved in DNA damage recognition. These observations suggest that p33ING2 cooperates with p53 in mediating nucleotide excision repair, and that XPA may be involved in this repair process. In conclusion, we have elucidated in this thesis, the role of p33ING2 in cellular stress response to UV irradiation, and the importance of this gene in the context of tumour suppression.

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LIST OF ABBREVIATIONS

6-4PPs	6-4 photoproducts
aa	Amino acid
AFP	α -fetoprotein
Apaf-1	Apoptotic protease activating factor-1
AS	Antisense
ATM	Ataxia telangiectasia mutated kinase
ATR	ATM-related kinase
BCC	Basal cell carcinoma
BER	Base excision repair
BH-3	Bcl-2 homology 3
CAT	Chloramphenicol acetyltransferase
CBP	CREB binding protein
Cdk	Cyclin-dependent kinase
Chk	Checkpoint kinase
CPDs	Cyclobutane pyrimidine dimers
CS	Cockayne syndrome
DDB	Damaged DNA binding protein
DISC	Death-inducing signalling complex
FADD	FAS-associated death domain
FAT	Factor acetyltransferase
FISH	Fluorescent <i>in situ</i> hybridization
GADD45	Growth arrest and DNA damage protein
HAT	Histone acetyltransferase
HDAC	Histone deacetylase complex
HRP	Horseradish peroxidase
IAPs	Inhibitors of apoptosis
ING	Inhibitor of growth
JNK	Jun amino-terminal kinase
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2
mut	Mutant
NER	Nucleotide excision repair
NLS	Nuclear localization sequence
NTS	Nucleolar translocation sequence
p53AIP1	p53-regulated apoptosis-inducing protein 1
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PHD	Plant homeodomain
PI	Propidium iodide
PIAS1	Protein inhibitor of activated STAT 1
PtdInsPs	Phosphoinositides

PTP	Permeability transition pore
PUMA	p53-upregulated modulator of apoptosis
PVDF	Polyvinylidene difluoride
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RPA	Replication protein A
SAID	SAP30-interacting domain
SAP30	Sin3-associated protein 30
SCC	Squamous cell carcinoma
Smac	Second mitochondria-derived activator of caspase
SRB	Sulforhodamine B
TH	Thyroid hormone
TPEN	N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenediamine zinc chelator
TR	Thyroid receptor
TNF	Tumour necrosis factor
UV	Ultraviolet
wt	Wild-type
XP	Xeroderma pigmentosum

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CHAPTER 1. INTRODUCTION

1.1 Skin Cancer

Skin cancer is the most common cancer among all types of cancers (Boni *et al.*, 2002). The annual incidence of skin cancer in the U.S. is estimated at about one million, accounting for about 45% of all human malignancies (Landis *et al.*, 1999). In Canada, approximately 76,000 and 4,250 new cases of non-melanoma skin cancer and melanoma respectively were diagnosed for 2003 (National Cancer Institute of Canada: Canadian Cancer Statistics 2004, Toronto, Canada, 2004).

1.1.1 Types of Skin Cancer

Skin cancers are categorized into two main types based on the cellular origin: non-melanoma skin cancer, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) that derives from keratinocytes, and melanoma that derives from melanocytes.

Non-melanoma skin cancer is the most common skin cancer, with BCC accounting for 60% of total skin cancer cases, and SCC accounting for 30-35%. Both BCC and SCC are found mainly on sun-exposed parts of body, and their incidence correlates well with cumulative sunlight exposure. Both BCC and SCC show low fatality, as BCC rarely metastasize and likewise SCC shows a very low rate of metastasis. Furthermore, surgical therapy and radiotherapy are highly effective in treating these diseases. Mutations of the p53 tumour suppressor gene have been found frequently in BCC and SCC, and the majority of these mutations are UV-specific C→T and CC→TT transitions. These mutations are also observed in BCC

precursor (basal cell nevus syndrome) and SCC precursor (actinic keratosis), indicating that p53 mutation may be an early event in the pathogenesis of these cancers (Kanjilal *et al.*, 1995; Ponten *et al.*, 1997; Sim *et al.*, 1992; McGregor *et al.*, 1992). In BCC, allelic loss of the patched gene (PTC) locus on chromosome 9q22 has been reported and may be responsible for basal cell carcinogenesis (Gailani *et al.*, 1996). Recent studies of sporadic basal cell carcinomas showed loss of heterozygosity (LOH) on chromosome 4q32-35, mapped to tumour suppressor p33/ING2 and SAP30 genes, the gene product of which is involved in transcription repression (Sironi *et al.*, 2004). In contrast to the predominance of LOH on specific chromosomes, SCC shows more widespread LOH with frequent loss of markers from chromosome 3p, 9p, 13q, 17p and 17q (Quinn *et al.*, 1994).

Melanoma contributes to 2-5% of total skin cancer cases. Melanoma development is believed to be associated with intermittent acute sunlight exposure causing severe sunburns particularly during childhood or adolescence (Holman and Armstrong, 1984; Krickler *et al.*, 1995). Melanoma is associated with one of the highest mortality rates due to its rapid metastasis, and the 5-year survival rate of patients with metastatic melanoma remains less than 10% (Roses *et al.*, 1991). There is no effective treatment for metastatic melanoma. Unlike non-melanoma skin cancers, p53 mutation is observed in only 1-5% of primary melanoma and 11-25% of metastatic melanoma (Hussein *et al.*, 2003a). Deletion of the chromosomal region 9q21, harbouring tumour suppressors p16^{INK4a} and p14^{ARF}, is among the most common known genetic alterations detected in about 50% of familial melanoma, and is also observed in 10 to 40% of sporadic melanoma (Healy *et al.*, 1996; Rizos *et al.*,

2001). However, loss of p16^{INK4a} rather than p14^{ARF} appears to be a more consistent target in familial melanoma (FitzGerald *et al.*, 1996). Methylation in the promoter region of *INK4a* that could potentially silence transcription has also been reported in 10% of melanoma cases (Gonzalogo *et al.*, 1997). In addition, the protein expression of p14^{ARF} is decreased from benign melanocytic nevi to metastatic melanoma, suggesting that inactivation of p14^{ARF} is important in melanoma progression (Dobrowolski *et al.*, 2002). Inactivation of p16^{INK4a}, an inhibitor of cyclin-dependent kinase 4 (Cdk4), leads to deregulation of the activity of Rb and E2F-1 transcription factor and thus stimulates cell cycle progression; whereas loss of function of p14^{ARF}, an inhibitor of mdm-2, disrupts the stability of p53 (de Grujil *et al.*, 2001).

Other genes, such as *Apaf-1* and *BRAF* have also been implicated in the development of melanoma. *Apaf-1* (apoptotic protease activating factor-1) is a pro-apoptotic protein that forms complexes with cytochrome c and pro-caspase 9 to mediate apoptosis. Allelic deletion of *Apaf-1* is observed in 40% of malignant melanoma cases (Hussein *et al.*, 2002a). Loss of *Apaf-1* expression is associated with resistance to chemotherapy in metastatic melanoma as cancer cells are able to escape from p53-dependent apoptosis (Soengas *et al.*, 2001). More recent findings reported that missense mutations of the *BRAF* oncogene are detected in 66% of cultured and primary melanoma cells (Davies *et al.*, 2002). The V599E mutation of *BRAF* is thought to mimic phosphorylation and activation of *BRAF*, resulting in a protein with high basal kinase activity, leading to constitutive activation of the RAS/ERK survival pathway (Mercer and Pritchard, 2003).

1.1.2 Aetiology of Skin Cancer

Sunlight is a continuous spectrum of electromagnetic radiation that can be divided into three major regions of wavelength: infrared, visible and ultraviolet (Jhappan *et al.*, 2003). The ultraviolet portion of the solar spectrum, lying in the wavelength range of 200-400 nm, is undoubtedly the major factor in skin cancer. UV radiation can be subdivided into three wavelength ranges: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm). Absorption of UV radiation by stratospheric ozone greatly attenuates these wavelengths, preventing UV radiations with a wavelength shorter than 300 nm (UVC and most UVB) from reaching the earth's surface. Although UVA and UVB light constitute a minute portion (0.0000001%) of the emitted sunlight, they are primarily responsible for the sun's pathological effects (Cleaver and Mitchell, 2001).

UVA is the predominant component of sunlight to which humans are exposed. However, its role in skin cancers, including melanoma, is not as well documented as the role of UVB. Since UVA has a longer wavelength than UVB, it can penetrate deeper into the skin, reaching melanocytes in the basal and suprabasal layers of the skin. UVA is photo-carcinogenic and is involved in photo-aging, but it is weakly absorbed in DNA and protein. UVA primarily produces reactive oxygen species and free radicals, which react with DNA to form base damages, strand breaks and DNA-protein cross-links, and ultimately mutations (Tyrrell and Keyse, 1990). An example of UVA-specific mutation, 8-hydroxyguanine, is produced through oxidation of guanine (Runger *et al.*, 1995). UVB is largely responsible for skin cancer through direct photochemical damage to DNA, which absorbs UV radiation within the wavelength

range of 245-280 nm (Tornaletti and Pfeifer, 1996). Although UVC is not supposed to be present in ambient sunlight, depletion of the ozone layer allows residual UVC radiation to reach the earth's surface. Besides, UVC is also readily produced by low-pressure mercury sterilizing lamps. Therefore its biological consequences are gaining more attention.

Both UVB and UVC preferentially cause DNA damage at the sites of neighbouring pyrimidine bases in a DNA strand, forming dimers between these bases. The two major photoproducts of such are the cyclobutane pyrimidine dimer (CPD), formed specifically between adjacent thymine (T) or cytosine (C) residues, and the 6-4 photoproduct (6-4PP), generated between adjacent pyrimidine residues. CPDs and 6-4PPs can be removed from the genome through nucleotide excision repair (NER) pathway, which is involved in the removal of bulky DNA adducts induced by UV irradiation. NER is a complex process with more than 30 gene products participating in multiple step enzymatic reaction: recognition of DNA lesion, single strand incision at both sides flanking the lesion, excision of the lesions containing 24-29 nucleotides, DNA repair synthesis to replace the excised nucleotides, and ligation of the remaining single-stranded nick (Ichihashi *et al.*, 2003; Hanawalt *et al.*, 2003). Both types of photolesions, if not removed from the genome, can cause UV-specific mutations such as C→T and CC→TT transitions, leading to cancer development. CPDs are considered to be more carcinogenic than the 6-4PPs, forming almost three times as often and being repaired less efficiently (Rosenstein and Mitchell, 1987; You *et al.*, 2001). For instance, 6-4PPs are the more rapidly repaired: 50% of 6-4PPs are removed from DNA in 2 to 6 hours, whereas

CPDs are much more slowly repaired: half of CPDs are removed from DNA in 12 to 24 hours (Freeman, 1988; Cleaver, 1989). The different rates of repair may be due to the fact that 6-4PPs cause considerably more distortion in DNA structure, and that they are preferentially located in the inter-nucleosomal regions of DNA, which are more accessible for damage recognition proteins. CPDs are distributed more randomly but are preferentially formed on DNA at the opposite side of the DNA-protein contact surface wrapped around the nucleosomes (Gold and Smerdon, 1990; Mitchell *et al.*, 1990).

1.2 p53, The Guardian of The Genome

p53 is often referred to as “the guardian of the genome” or “the cellular gatekeeper”, revealing its importance in maintaining the integrity of the genome. In response to genotoxic stresses, p53 is stabilized and activated to (1) promote cell cycle arrest, which inhibits the replication of the damaged genomes, (2) repair damaged DNA, and (3) induce apoptosis or senescence, eliminating defective cells. p53 achieves these goals via its sequence-specific transcriptional activation and repression activity, as well as via interaction with other proteins (Janus *et al.*, 1999; Hussein *et al.*, 2003a; Brooks and Gu, 2003; Ho and Benchimol, 2003). The significance of the tumour suppressive functions of p53 is reflected by the fact that p53 is the most frequently mutated gene known to date, with over 50% of human malignancies harbouring mutations and allelic deletions of p53 (Vogelstein and Kinzler, 1992; Lee *et al.*, 1994; Soussi *et al.*, 1994). Furthermore, germline mutations of p53 result in Li-Fraumeni

syndrome, a hereditary cancer susceptibility syndrome that predisposes individuals to various cancers (Malkin *et al.*, 1990; Srivistava *et al.*, 1990).

1.2.1 Gene and Protein Structures

The *p53* gene is located on chromosome 17q13.1. This gene encodes a 393-amino acid, 53 kDa nuclear phosphoprotein. The protein can be divided structurally and functionally into different domains. The N-terminus of *p53* protein contains a transcriptional activation domain, which is essential for the recruitment of transcriptional co-activators (Balint and Vousden, 2001). Following the transactivation domain is a proline-rich domain, which is required for transactivation-independent pro-apoptotic functions of *p53* and transcriptional repression (Chipuk *et al.*, 2004; Ho and Benchimol, 2003). The DNA binding domain (DBD) of *p53* spanning 200-aa residues is found after the proline-rich domain. The *p53* consensus binding site consists of two copies of the 10-bp motif, 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3', separated by 0-13 bp (El-Deiry *et al.*, 1992). The C-terminus of *p53* contains a nuclear localization signal, an oligomerization domain, which is required for the tetramerization function of *p53*, and a basic C-terminal regulatory domain.

p53 is normally a short-lived protein and is maintained at low levels within the cells under non-stressed conditions. Mdm-2, a transcriptional target of *p53*, plays a major role in this process by regulating *p53* protein stability. Mdm-2 contains an E3 ligase domain that targets both *p53* and itself for ubiquitination and degradation. Mdm-2 also inhibits the *p53* functions through binding to the N-terminal of *p53* and

thus inhibiting p53 transcriptional activity. The ubiquitin ligase activity of mdm-2 also contributes to the efficient nuclear export of p53 (Balint and Vousden, 2001; Xu, 2003). Therefore, p53-mdm-2 interaction presents a negative autoregulatory mechanism to regulate p53 stability and activity.

1.2.2 Cell Cycle Arrest

It is well documented that the involvement of p53 in cell cycle regulation depends on its transcriptional activation activities. One of the many p53 target genes, p21^{waf1}, a cyclin-dependent kinase (Cdk) inhibitor, is believed to play an important role in the induction of both G₁- and G₂-phase cell cycle arrests (Balint and Vousden, 2001). For instance, upon a DNA damage event, p53 upregulates p21^{waf1} to inactivate Cdk2/cyclin E complexes and prevent phosphorylation of Rb, resulting in the inhibition of cell cycle progression from G₁ to S phase (Harper *et al.*, 1993; El-Deiry *et al.*, 1993). Alternatively, but not mutually exclusively, p21^{waf1} binds to the proliferating cell nuclear antigen (PCNA), preventing association of PCNA with the major replicative enzyme DNA polymerase δ , thereby inhibiting DNA replication (Prosperi, 1997; Smith *et al.*, 2000). It is believed that p53 induces G₁ arrest to provide more time for the repair of DNA damage before entry into S phase for DNA replication.

Another target gene of p53, 14-3-3 σ is involved in p53-induced G₂ arrest, by binding to and sequestering cdc2-cyclin B1 complexes in the cytoplasm, and thus preventing cells from entering mitosis (Hermeking *et al.*, 1997; Muslin and Xing, 2000). 14-3-3 σ can also bind to p53 and activate its sequence-specific DNA binding, presenting a positive feedback regulation of p53 to prevent cell cycle progression in

damaged cells (Waterman *et al.*, 1998). Cdc2 can also be inhibited by other p53 downstream targets, such as p21^{waf1}, GADD45 (growth arrest and DNA damage protein) and Reprimo (Wang *et al.*, 1999; Taylor and Stark, 2001; Ohki *et al.*, 2000).

1.2.3 Apoptosis

p53 induces apoptosis when DNA damage in a cell is beyond repair. Evidence indicates that p53 can mediate apoptosis by both transcription-dependent and transcription-independent mechanisms (Prives and Hall, 1999; Chipuk *et al.*, 2004). The importance of p53 transcriptional activities in mediating apoptosis is reflected by the findings that cells in which the wt p53 gene was replaced by a transcriptionally inactive mutant showed loss of both cell cycle and apoptotic functions (Chao *et al.*, 2000; Jimenez *et al.*, 2000). p53 is capable of transactivating numerous apoptotic genes including those responsible for the mitochondrial/intrinsic pathway and death-receptor/extrinsic pathway. The first p53 apoptotic target gene identified was the *Bax* gene, a pro-apoptotic member of the Bcl-2 family (Miyashita and Reed, 1995). Other pro-apoptotic members from this family, PUMA (p53-upregulated modulator of apoptosis) and Noxa have since been identified as p53 downstream targets (Oda *et al.*, 2000a; Nakano and Vousden, 2001; Yu *et al.*, 2001). These proteins, together with another p53 target, p53-regulated apoptosis-inducing protein 1 (p53AIP1), localize to the mitochondria and promote changes of the mitochondrial membrane potential and the release of cytochrome c, which complexes with Apaf-1 to form the apoptosome (the functional apoptotic unit) and activate the caspase cascade

(Hengartner, 2000; Hussein *et al.*, 2003b). More recently, Apaf-1 has also been found to be a transcriptional target of p53 and E2F1 (Moroni *et al.*, 2001).

Besides transcriptional activation of pro-apoptotic genes, p53 transcriptional repression of anti-apoptotic genes also contributes to the apoptotic effects of p53. *Bcl-2*, *Bcl-X_L*, *Survivin*, *MAP4* and *PIK3CA* (the p110 α subunit of PI3K) are some of the genes identified as repression targets of p53 during p53-mediated apoptosis (Ho and Benchimol, 2003; Oren, 2003). There is also evidence indicating that p53 itself can localize to the mitochondria, and promote Bax translocation to the mitochondria as well as oligomerization of Bax to initiate apoptosis, presenting an additional transcriptional-independent way of mediating apoptosis (Marchenko *et al.*, 2000; Schuler *et al.*, 2000; Moll and Zaika, 2001; Chipuk *et al.*, 2004).

In addition, p53 is also involved in the death-receptor pathway by upregulating death receptors Fas/APO1 and DR5/KILLER, a death receptor ligand, FasL, and a death domain-containing protein, PIDD (Owen-Schaub *et al.*, 1995; Wu *et al.*, 1997; Lin *et al.*, 2001). The DR5 promoter is a direct target of p53, while cell surface expression of Fas is enhanced by p53 through promotion of its trafficking from the Golgi apparatus to the plasma membrane (Bennett *et al.*, 1998; Takimoto and El-Deiry, 2000). Activation of death receptors by their ligands results in trimerization of the receptors and recruitment of FAS-associated death domain (FADD), which further recruits pro-caspase 8 to form death-inducing signalling complex (DISC). Pro-caspase 8 in DISC is activated by autocatalytic cleavage and the active caspase 8 activates effector caspases such as caspase 3 to initiate apoptosis (Ashkenazi and Dixit, 1998; Igney and Krammer, 2002).

1.2.4 DNA Repair

In addition to preventing replication of cells with damaged genomes via the induction of cell cycle arrest and apoptosis, p53 also participates in the repair of damaged DNA. Evidence indicates that p53 is involved in both NER that removes UV-induced DNA lesions and chemical-induced bulky DNA adducts, and base excision repair (BER) that removes bases damaged by alkylating agents, oxygen free radicals and hydrolysis (Ford and Hanawalt, 1995; Wani *et al.*, 1999; Offer *et al.*, 2001; Zhou *et al.*, 2001). While the exact molecular mechanism by which p53 enhances NER is not fully understood, there is evidence indicating both direct and indirect participation of p53 in NER. For instance, the C-terminus of p53 directly binds to different forms of damaged DNA, including single-stranded breaks, ends of double-stranded breaks and insertion/deletion mismatches (Steinmeyer and Deppert, 1988; Bakalkin *et al.*, 1995; Lee *et al.*, 1995; Balint and Vousden, 2001). Furthermore, p53 can directly interact with several components of the repair machinery *in vitro*, including XPB, XPD, p62 subunit of TFIIH, CSB, replication protein A (RPA), and Ref-1 (Wang *et al.*, 1995; Leveillard *et al.*, 1996; Troelstra *et al.*, 1990; Janus *et al.*, 1999). p53 also possesses other biochemical activities, such as DNA reannealing and 3'-5' exonuclease activity, that might also play a role in its repair functions (Mummenbrauer *et al.*, 1996; Janus *et al.*, 1999; Albrechtsen *et al.*, 1999; McKay *et al.*, 1999; Gila *et al.*, 2003).

The indirect participation of p53 in DNA repair is mainly achieved through upregulation of p53-responsive genes that are involved in DNA repair. For instance, p53 sustains the expression of the p48 component of the damaged DNA binding

protein (DDB2) complexes and *XPC* gene, both of which are involved in damage recognition (Hwang *et al.*, 1999; Adimoolam *et al.*, 2002). Another p53 downstream target involved in DNA repair, GADD45, is shown to bind to PCNA and inhibit DNA replication, thus allowing DNA repair to proceed (Smith *et al.*, 1994). Although the exact mode of action of GADD45 in NER is not fully understood, the significance of GADD45 in NER is supported by the findings that GADD45-null fibroblasts have defects in NER similar to those seen in p53-null fibroblasts, and that GADD45-deficient mice show increased radiation carcinogenesis and genomic instability comparable to that seen in p53-deficient mice (Smith *et al.*, 2000; Hollander *et al.*, 1999). More recently, p53 has been found to mediate UV-induced global chromatin relaxation by recruiting p300 to the sites of NER and inducing histone acetylation, thus acting as a chromatin accessibility factor for NER (Rubbi and Milner, 2003).

1.2.5 Post-translational Modifications of p53

The choice of p53 whether to undergo cell cycle arrest, apoptosis, senescence or DNA repair upon genotoxic stimuli depends on multiple factors, such as cell type and magnitude of the cellular stress, post-translational modifications and conformation of p53, and the presence of co-factors in p53 transcriptional activation and repression. Accumulating evidence indicates that post-translational modifications of p53 are critical in making such choices, by modulating its protein stability, sequence-specific transcriptional activation and interaction with other proteins (Appella and Anderson, 2001; Xu, 2003; Oren, 2003; Brooks and Gu, 2003). p53 phosphorylation and acetylation are the two major post-translational modifications of p53 that have been

extensively studied for the past decade. More recently, other post-translational modifications such as sumoylation and ribosylation of p53 have been demonstrated (Wesierska-Gadek *et al.*, 1996; Gostissa *et al.*, 1999; Rodriguez *et al.*, 1999). Generally, the N-terminus of p53 is heavily phosphorylated while the C-terminus contains phosphorylation, acetylation and sumoylation sites.

p53 is phosphorylated at multiple sites by a number of kinases upon genotoxic stimuli. For instance, phosphorylation of p53 at Ser15 by ataxia telangiectasia mutated (ATM) kinase upon exposure to ionizing radiation is found to disrupt p53 interaction with mdm-2, leading to p53 stabilization (Shieh *et al.*, 1997). Additional studies suggest that phosphorylation of Ser15 prevents p53 nuclear export (Zhang and Xiong, 2001). Phosphorylation at Ser20 and Thr18 by Checkpoint kinase 1 and 2 (Chk1 and Chk2), downstream targets of ATM kinase and ATM-related (ATR) kinase, and Thr81 by Jun amino-terminal kinase (JNK) are also reported to contribute to p53 stabilization by disrupting p53-mdm-2 interaction (Chehab *et al.*, 2000; Shieh *et al.*, 2000; Hirao *et al.*, 2000; Buschmann *et al.*, 2001). UV-induced phosphorylation at Ser33 and Ser37 is important for p53 activation (Sakahgushi *et al.*, 1998). There is also evidence suggesting that phosphorylation at Ser15 or Ser33/Ser37 promotes an interaction between p53, CBP/p300 and PCAF, and this interaction is crucial for acetylation at the C-terminus (Sakahgushi *et al.*, 1998; Dumaz and Meek, 1999). Phosphorylation of Ser46, probably by p38 mitogen-activated protein kinase (MAPK), seems to be specifically important in UV-induced apoptosis through the activation of *p53AIP* (Bulavin *et al.*, 1999; Oda *et al.*, 2000b).

The initial observations that CBP/p300, possessing histone acetyltransferase (HAT) activity, is a co-activator of p53 transcriptional activity led to the discovery of a novel factor acetyltransferase (FAT) function of CBP/p300 on p53 (Gu and Rader, 1997). CBP/p300 specifically acetylates p53 at multiple lysine residues (Lys370, Lys371, Lys372, Lys381 and Lys382) at the C-terminal regulatory domain in response to DNA damage (Gu and Rader, 1997; Sakahgushi *et al.*, 1998). PCAF, a p300/CBP-associated protein, is also found to acetylate p53 at Lys320 (Liu *et al.*, 1999). Acetylation at these sites of p53 activates sequence-specific DNA binding activity of p53, recruits CBP/p300 to p53-dependent promoters, and stabilizes p53 by preventing mdm-2 ubiquitination at the regulatory domain for degradation (Gu and Rader, 1997; Balev *et al.*, 2001; Rodriguez *et al.*, 2000; Li *et al.*, 2002). p53 is also modified by conjugation of Lys386 to a small ubiquitin-like protein, SUMO-1, in response to DNA damage (Gostissa *et al.*, 1999). While the knowledge on p53 sumoylation is still limited, it is found that sumoylation of p53 activates p53 transcriptional activities, and that the protein inhibitor of activated STAT 1 (PIAS1) promotes sumoylation of p53 (Rodriguez *et al.*, 1999; Kahyo *et al.*, 2001).

1.3 The ING Tumour Suppressor Family

The ING (Inhibitor of growth) family consists of five members, ING1, p33ING2, p47ING3, p29ING4 and p28ING5. *ING1*, through alternative splicing, encodes three isoforms, namely p47ING1a, p33ING1b and p24ING1c. p33ING1b is the most abundant form of ING1, and most tumour suppressive functions have been credited to this isoform. ING proteins are found to be differentially involved in various

biological activities including the regulation of cell cycle arrest, apoptosis, gene transcription, and DNA repair. Various studies have shown that ING proteins mediate their tumour suppressive functions in a p53-dependent manner. ING proteins cooperate with p53 via at least two mechanisms, the direct association of ING proteins with p53 and ING-mediated p53 acetylation (Campos *et al.*, 2004a). For instance, p33ING1b, p29ING4 and p28ING5 are known to co-precipitate with p53, while p33ING2 and p47ING3 do not. All ING proteins, except p47ING3, are found to facilitate p53 acetylation at Lys373/382 in different cell types (Nagashima *et al.*, 2001; Nagashima *et al.*, 2003; Kataoka *et al.*, 2003; Shiseki *et al.*, 2003). Evidence also indicates that the biological functions of ING family genes may at least in part result from interactions of ING proteins with histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes, and phosphoinositides.

1.3.1 p33ING1b

ING1, the founding member of the ING tumour suppressor family, was isolated by Garkavtsev and colleagues (1996) using subtractive hybridization between cDNA from normal human mammary epithelial cells and seven breast cancer cell lines. It contains three exons, numbered as 1a, 1b and 2, and two introns, producing three alternatively spliced forms, encoding protein variants p47ING1a, p33ING1b, and p24ING1c, all of which possess a highly conserved plant homeodomain (PHD) zinc finger motif, nuclear localization sequence (NLS) and two nucleolar translocation sequences (NTS) (Saito *et al.*, 2000; Gunduz *et al.*, 2000; Cheung and Li, 2001). p33ING1b and p24ING1c have previously been reported to mediate growth arrest,

apoptosis, DNA repair, senescence, anchorage-dependent growth and chemosensitivity.

p33ING1b, the most extensively studied isoforms, are found to induce cell cycle arrest at G₁-phase, by increasing the activation of the p21^{waf1} promoter in a p53-dependent manner (Garkavtsev *et al.*, 1998). p21^{waf1}, a well-known downstream target of p53, is a cyclin-dependent kinase (Cdk) inhibitor involved in negative regulation of cell growth by inhibiting the kinase activity of Cdk2 and thus the phosphorylation of the retinoblastoma (Rb) protein (Harper *et al.*, 1993; El-Deiry *et al.*, 1993). Therefore, increased expression of p21^{waf1} in p33ING1b-overexpressing cells prevents cell proliferation. p33ING1b involvement in cell cycle arrest is further supported by the finding that the antisense construct of p33ING1b promotes cell transformation (Garkavtsev *et al.*, 1996). Evidence also indicates the involvement of p33ING1b in G₂ cell cycle arrest. It has more recently been found that cyclin B1 is a down-regulatory target of p33ING1b, and that overexpression of p33ING1b enhances adriamycin-induced G₂ arrest in a p53-dependent manner (Takahashi *et al.*, 2002; Tsang *et al.*, 2003).

In addition to its involvement in cell cycle regulation, various lines of evidence indicate that p33ING1b is also involved in apoptosis; its dependency on p53 is also well documented. Increased expression of p33ING1b enhances serum starvation-induced cell death in p19 mouse teratocarcinoma and NIH 3T3 cells (Helbing *et al.*, 1997). Co-expression of p33ING1b and c-myc dramatically enhances serum starvation-induced apoptosis, suggesting that p33ING1b-mediated cell death may be synergistic with the c-myc apoptotic pathway. p33ING1b has also been shown to

sensitize cells to chemotherapeutic agents and radiation, such as etoposide, UV irradiation and γ -irradiation, in the presence of wt p53 (Garkavtsev *et al.*, 1998). Adenoviral delivery of both p33ING1b and p53 has been shown to synergistically induce apoptosis in glioma and human oesophageal carcinoma cells (Shinoura *et al.*, 1999; Shimada *et al.*, 2002).

Involvement of p33ING1b in UV-induced response has also been demonstrated. The expression of p33ING1b is increased in a dose-dependent manner in normal human keratinocytes, keratinocyte HaCat cell line, and human melanoma MMRU cell line after UV irradiation (Cheung *et al.*, 2000; Cheung *et al.*, 2001). Overexpression of p33ING1b in MMRU cells upregulates the expression of the pro-apoptotic protein Bax, thus enhancing UV-induced apoptosis via activation of the mitochondrial apoptotic pathway, and this process requires the presence of wt p53 (Cheung and Li, 2002). Another study has demonstrated that p33ING1b physically binds to PCNA via its PCNA-interacting protein (PIP) domain following UV exposure and enhances apoptosis (Scott *et al.*, 2001a). UV irradiation induces p33ING1b translocation to nucleolus and this facilitates the apoptosis of human primary fibroblasts (Scott *et al.*, 2001b).

p33ING1b has also been shown to be involved in DNA repair. Overexpression of p33ING1b enhances NER of UVC-damaged exogenous plasmid DNA and UVB-damaged genomic DNA in a p53-dependent manner (Cheung *et al.*, 2001). More recent studies have reported that mutations at codons 102 and 260 of *p33ING1b*, which are detected in human cutaneous melanoma biopsies, as well as deletion of the PHD finger motif abolish p33ING1b enhancement in NER, suggesting the

requirement of this motif for proper DNA repair functions (Campos *et al.*, 2004b). Several findings have clarified the involvement of p33ING1b in DNA repair machinery. p33ING1b may compete with p21^{waf1} for the binding with PCNA, resulting in p21^{waf1} dissociation with PCNA, and thus favour DNA repair over DNA replication (Waga *et al.*, 1994; Scott *et al.*, 2001a). Furthermore, the observations that p33ING1b can interact with chromatin, and physically interact with p300 and PCNA, potentially link chromatin remodelling to DNA repair (Vieyra *et al.*, 2002). p33ING1b may also be involved in the repair of double strand break generated by a topoisomerase II inhibitor, adriamycin, in a p53-dependent manner (Tsang *et al.*, 2003).

1.3.2 p33ING2

p33ING2, also known as *p33ING1L* (ING1-like), was originally isolated by Shimada and colleagues (1998), by a homology search with the p33ING1b cDNA sequence from the Otsuka cDNA database containing randomly selected cDNA from human placental, aortal and foetal brain cDNA libraries. It is found to be located on chromosome 4q35.1 by R-banding FISH, confirmed with radiation-hybrid mapping (Shimada *et al.*, 1998).

xp32ING2, the first frog homologue of p33ING2, was isolated from *X. laevis* tadpole tail and brain by using a gene-specific primer from the conserved region 5' to the region encoding the PHD finger of human *ING1* (Wagner *et al.*, 2001).

1.3.2.1 Gene and Protein Structures

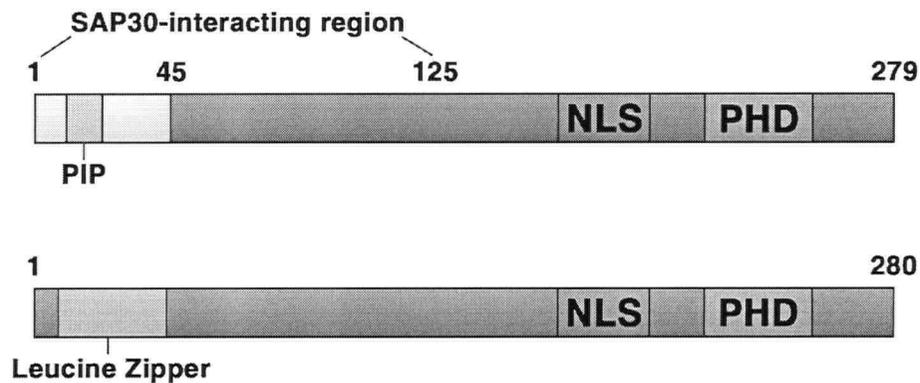
The full length of human *p33ING2* cDNA contains an open reading frame of 840 bp, encoding for a 280-amino acid polypeptide with a molecular weight of 32.8 kDa. The gene products of *p33ING2* show 58.9% homology with *p33ING1*'s, and the nucleotide sequences between these two genes shows 60% identity (Figure 1.1). The C-terminus of *p33ING2* (amino acid residues 213-260) contains a plant homeodomain (PHD) zinc-finger motif (C4HC3-type), suggesting that *p33ING2* may function as a transcription regulator and/or chromatin remodelling factor (Aasland *et al.*, 1995; Nouman *et al.*, 2003). The amino acid sequence of this motif is highly conserved between *p33ING1* and *p33ING2*, suggesting that they may be competing for the same targets (Shimada *et al.*, 1998). Like *p33ING1b*, *p33ING2* contains a nuclear localization sequence (NLS) motif, suggesting that *p33ING2* is a nuclear protein. It also contains a unique leucine zipper domain, which promotes interaction with other leucine-zipper-containing proteins and hydrophobic interaction (Hai and Hartman, 2001; Feng *et al.*, 2002) (Figure 1.2). *p33ING2* proteins are mainly localized in the nucleus (74% in the chromatin-enriched/nuclear matrix and 9% in the nucleoplasm), with a diffuse distribution in the cytosol (17%) in human fibrosarcoma cells (Gozani *et al.*, 2003).

xp32ING2, consists of 834 nucleotides, 6 nucleotides shorter than the human *p33ING2*, encoding for a 277-aa (32 kDa) protein. *xp32ING2* is 68% identical to human *p33ING2*, 67% identical to murine *p33ING2*, 31% identical to human *p47ING1a*, 54% identical to human *p33ING1b* and 58% identical to murine *p33ING1b*. The C-terminal PHD finger domain is almost completely conserved

Figure 1.1 Comparison of the amino acid sequences of p33ING1b and p33ING2. The amino acid sequences of p33ING1b and p33ING2 share 60% homology. The highly conserved plant homeodomain (PHD) zinc finger motif at the C-terminus of these proteins is indicated by shaded bar below this domain. Asterisks indicate the conserved cysteine and histidine residues in this domain.

p33ING1b	1	MLSPANGEQLHLVN-YVEDYLDSIESLPEFDLQORNVSIMR	38
p33ING2	1	MLGQQQQQLYSSAALLTGERSRLLTCYVQDYLECVEESLPHDMQORNVSILR	50
p33ING1b	12	EIDAKYQETILKELDECYERFSRETDGAQKRRLHCVQRALIRSQELGDEK	88
p33ING2	51	ELDNKYQETLKEIDDVYEKYKEDDLNQQKRLQQLLQRALINSQELGDEK	100
p33ING1b	72	IQIVSQMVELVENRTRQVDSHVELFEAQQELGDTVGNVSGKVGADRPNGDA	138
p33ING2	101	IQIVTQMLELVENRARQEMELHSQCFQDPAE-SERASDKAKMDSSQP----	145
p33ING1b	132	VAQSDKPNKRSRRQRNNENRENASSNHDHDDGASGTPKEKKAKTSKSKK	188
p33ING2	151	-----ERSRRPRRORTSESRDLCHMANGIEDCDDQPPKEKKSLSAKSKK	190
p33ING1b	192	RSKAKAEREASPADLPIDPNEPTYCLCNQVSYGEMIGCDNECPIEWFHF	238
p33ING2	201	RSKAKQEREASPVFAIDPNEPTYCLCNQVSYGEMIGCDNEQCPIEFHF	240
		* * * * *	
p33ING1b	252	SCVGLNHKPKGKWCYCPKCRGENEKTMDKALEKSKKERAYNR	279
p33ING2	251	SCVSLTYKPKGKWCYCPKCRGDNEKTMDKSTKTKKDRSR	280
		* * * *	

Figure 1.2 Schematic diagrams of the protein structural features of p33ING1b and p33ING2. Both p33ING1b and p33ING2 contain a nuclear localization sequence (NLS) and a plant homeodomain (PHD) zinc finger motif, which is a putative domain of transcription regulators and chromatin-remodelling factors. p33ING1b contains a PCNA-interacting domain (PIP) and a SAP30-interacting region at its N-terminus. p33ING2 contains a unique leucine zipper at its N-terminus.



between *xp32ING2* and human *p33ING2*. Although it is still unclear that other members of ING family, except *ING1*, express different isoforms, it is probable that the *xp32ING2* transcript can be alternatively spliced in a tissue-specific manner (Wagner *et al.*, 2001).

Jager and colleagues (1999) also identified an *ING1*-related gene by screening breast cancer cDNA library using *ING1* as a probe, designated *ING2* (GenBank accession number: AF149724). The full-length cDNA is 771 bp in size. This gene shows 76% homology to *ING1* in its 5' two-thirds of the sequence (nucleotide 1-480). It contains 203 bp of 5' untranslated region and 439 bp of 3' untranslated region, and the longest open reading frame of this gene would encode a 42-amino acid polypeptide. However, this variant is distinct from *p33ING2*, and the functional relevance of this variant between *p33ING2* and other *ING* family members is unknown as there has been no further study carried out to investigate the function of this variant.

1.3.2.2 Expression Profile

p33ING2 mRNA expression levels were found to be ubiquitous in normal human tissues in the form of two major bands (1.5 kb and 1.3 kb) by Northern blot analysis (Shimada *et al.*, 1998). Most human tissues were found to express *p33ING2* except for the spleen, and the expression levels were variable in different tissue types. For example the mRNA expression levels of *p33ING2* was high in the testis but low in the lung and kidney, in which *p33ING1b* expression was undetectable. Like human *p33ING2*, *xp32ING2* was found to be expressed in all adult *Xenopus* tissues, with

variable expression levels in different tissues. Similarly, the mRNA expression of *xp32/ING2* was abundant in the testis, but low in muscle and liver (Wagner *et al.*, 2001).

It was also reported that human *p33/ING2* mRNA expression was significantly higher in colon cancers, where p53 abnormalities occur very frequently compared to the adjacent normal tissues. Importantly, its expression levels increased with tumour progression as poorly differentiated carcinoma expressed higher *p33/ING2* levels than differentiated adenocarcinoma (Shimada *et al.*, 1998). Unlike *p33/ING1b*, protein levels of *p33/ING2* in cancer cell lines were found to be highly variable, with undetectable expression in some cell lines. It is reported that the expression profile of *p33/ING2* did not correlate with the mutational status of p53. However, three out of twelve cell lines examined, expressing the most abundant *p33/ING2*, contained either null or mutant p53 (Nagashima *et al.*, 2001).

Recent studies on loss of heterozygosity in sporadic basal cell carcinomas (BCCs) reported a high frequency of LOH on chromosome 4q32-35, which is mapped to *p33/ING2* and *SAP30* (Sin3-associated protein), both of which are believed to be involved in chromatin remodelling and gene regulation (Sironi *et al.*, 2004). Since *p33/ING2* upregulates *p21^{waf1}* and Bax, LOH of *p33/ING2* could impair the proper regulation of cell cycle and apoptosis, resulting in cellular transformation and tumour progression. In fact, this is supported by previous findings that reported loss of expression of *p21^{waf1}* in BCCs (Ahmed *et al.*, 1997). These findings suggest a possible involvement of *p33/ING2* in the formation of basal cell carcinoma.

1.3.2.3 Biological Functions

So far, there are three studies reporting the biological functions of p33ING2 as a tumour suppressor. p33ING2 has been found to regulate cell cycle, apoptosis and gene transcription, and the presence of functional p53 is required in these processes (Figure 1.3) (Nagashima *et al.*, 2001; Gozani *et al.*, 2003; Kataoka *et al.*, 2003).

The first evidence indicating p33ING2 as a tumour suppressor is that p33ING2 negatively regulates cell growth of colorectal carcinoma RKO cells. Nagashima and colleagues (2001) demonstrated that overexpression of p33ING2 completely inhibits colony formation of RKO cells that carry wild-type p53, through the induction G₁-phase cell cycle arrest and apoptosis. However, this is not observed in p53-deactivated RKO-E6 cells (Nagashima *et al.*, 2001). The role of p33ING2 as a tumour suppressor is further supported by the findings that the expression of p33ING2 is specifically induced by DNA damage agents, etoposide or neocarzinostatin, but not by gamma irradiation, doxorubicin, *cis*-platinum or bleomycin in the normal lymphoblastoid C3ABR cell line (Nagashima *et al.*, 2001). This induction of p33ING2 is independent of ATM or p53 function (Nagashima *et al.*, 2001). Taken together, these observations suggest that p33ING2 negative regulation of cell growth is p53-dependent, indicating that p33ING2 is a potential regulator in p53-mediated cellular processes (Nagashima *et al.*, 2001).

In addition to its involvement in growth control, p33ING2 has been shown to play a role in the regulation of gene transcription. Like p33ING1b, p33ING2 was found to enhance the promoter activities of p21^{waf1} and Bax in wild-type p53 RKO cells, but not in RKO-E6 cells (Nagashima *et al.*, 2001; Kataoka *et al.*, 2003).

Moreover, p33ING2 was significantly more effective than p33ING1b in enhancing these p53 transcriptional-transactivation activities (Nagashima *et al.*, 2001). In contrast to upregulation of the p21^{waf1} promoter, p33ING2 strongly repressed the promoter activity of human α -fetoprotein (AFP), a tumour-specific marker of the hepatocellular carcinoma, possibly through binding to AFP's AT-motif, in p53 wild-type hepatocellular carcinoma HepG2 cells (Kataoka *et al.*, 2003). This repressive effect of p33ING2 on the AFP promoter was much weaker in the Hep3B p53-null cell line. When co-transfected with wild-type p53, the repressive effect was restored but to a lesser extent than in the HepG2 cells, suggesting plausible p53-dependent and p53-independent regulatory mechanisms. It was also shown that p33ING2 inhibited the AFP promoter by antagonizing deacetylation effects of hSir2 on p53, but p33ING2 was not detected in the co-immunoprecipitate with hSir2 (Kataoka *et al.*, 2003).

The role of ING2 in hormone-dependent transcriptional response during developmental events was studied using *Xenopus* tadpole metamorphosis as a model system, in which a single stimulus thyroid hormone (TH) initiates tissue-specific proliferation, apoptosis and remodelling simultaneously. Thyroid receptors (TRs) selectively repressed or activated gene transcription in response to absence or presence of TH. It is shown that *xp32ING2* transcript levels increased in response to TH-induced metamorphosis and during spontaneous metamorphosis in *X. laevis* tadpole tissues (Wagner *et al.*, 2001). It is interesting to note that in the tail and brain, which apoptose extensively during metamorphosis, *xp32ING2* induction followed the TRs expression pattern; whereas in the leg, which proliferate and grow,

xp32ING2 expression was not induced, similar to that of TRs. This indicates the role of xp32ING2 in hormonally regulated nuclear transcription factor-mediated apoptotic response.

1.3.2.4 Modes of Action

Although p33ING2 is involved in various biological functions, it possesses no known enzymatic activity. It is therefore reasonable to speculate that p33ING2 acts by facilitating specific protein-protein, possible protein-DNA, and even protein-phospholipid interactions. In fact, p33ING2 is known to interact with phosphoinositides and Sin3-histone deacetylase (HDAC) complexes (Gozani *et al.*, 2003; Kuzmichev *et al.*, 2002).

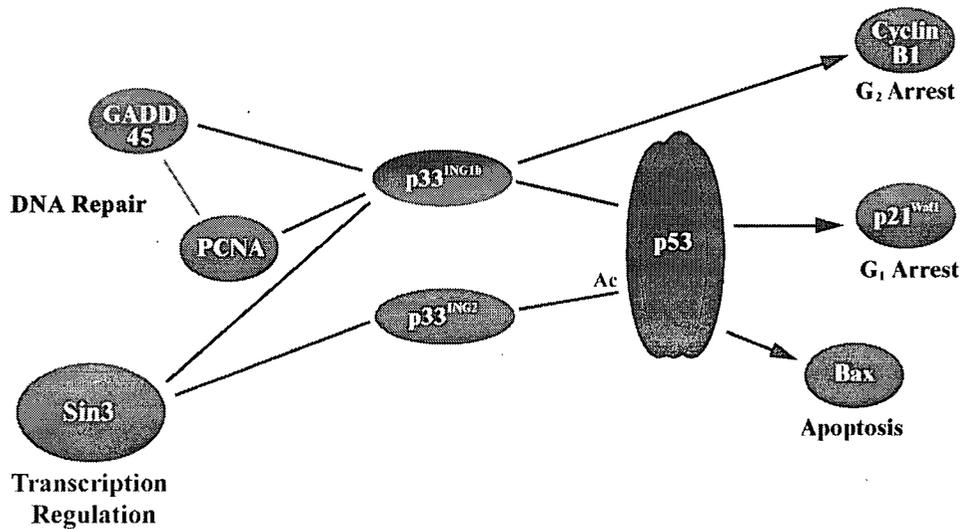
The p53 dependency of p33ING2 in exerting its tumour suppressive functions has led to investigation of the interaction between p33ING2 and p53. Interestingly, p33ING2 was not detected in a physical interaction with p53, unlike three other ING family members (p33ING1b, p29ING4 and p28ING5) (Nagashima *et al.*, 2001; Shiseki *et al.*, 2003). However, overexpression of p33ING2 led to an increased acetylation of p53 at Lys382 in colorectal carcinoma RKO cell line (Nagashima *et al.*, 2001; Nagashima *et al.*, 2003). It was reported that p53 acetylation at Lys382 in the normal lymphoblastoid C3ABR cell line after exposure to DNA-damaging agents, etoposide or neocarzinostatin, correlated with an increased p33ING2 expression levels (Nagashima *et al.*, 2001). Furthermore, It was shown that antisense-p33ING2 expression in osteosarcoma OsACL cells reduced the level of endogenous p33ING2 and acetylated p53 proportionally following exposure to etoposide (Nagashima *et al.*,

2001). However, p33ING2 expression did not alter the phosphorylation of p53 at either Ser15 or Ser392. Post-translational modifications such as acetylation and phosphorylation within the C-terminal region of p53 have been shown to facilitate p53 activation and stimulate p53 sequence-specific DNA-binding activity (Gu and Roeder, 1997; Sakagushi *et al.*, 1998). These observations lead to the speculation that p33ING2 involvement in the regulation of cell cycle, apoptosis and transcription may be the results of p33ING2-mediated p53 acetylation that stabilizes p53 and enhances p53 cellular responses to genotoxic stresses and apoptotic stimuli.

Gozani and colleagues (2003) were the first and the only group to identify p33ING2 as a phosphoinositides-binding module through a library expression screening using phosphoinositides (PtdInsP)-affinity resins. It was found that p33ING2 interacted with PtdIns(3)P and PtdIns(5)P through its PHD finger motif, which alone was sufficient for this binding. The importance of the PHD finger motif in this interaction was further evidenced by the findings that the zinc chelator TPEN [N,N,N',N'-tetrakis(2-pyridyl-methyl)ethylenediamine] and the mutations of the basic residues at the C-terminal of the PHD finger of p33ING2 greatly reduced the binding affinity of p33ING2 with PtdIns(3)P and PtdIns(5)P. In fact, it was shown that the PHD finger motif was a general PtdInsPs binding domain, and that the general structure for PtdInsPs binding was conserved. Therefore, we speculated that other ING family members might be phosphoinositide-interacting partners as well, with different specificity and binding affinity.

PtdIns(5)Ps can modulate the subcellular localization of p33ING2. This is evidenced by the finding that generation of PtdIns(5)Ps at the plasma membrane by

Figure 1.3 Biological functions of p33ING1b and p33ING2. p33ING1b physically associates with p53 while p33ING2 induces p53 acetylation. Both p33ING1b and p33ING2 induce G₁-phase cell cycle arrest, promote apoptosis and interact with Sin3, a component of transcription co-repressor complexes, to regulate gene transcription. p33ING1b downregulates cyclin B1 to induce G₂-phase cell cycle arrest and enhances DNA repair through interaction with GADD45 and PCNA.



lipid phosphatase IpgD, which dephosphorylates PtdIns(4,5)P₂ to PtdIns(5)P, recruited p33ING2 PHD finger to plasma membrane and caused loss of diffuse p33ING2 cytoplasmic distribution (Gozani *et al.*, 2003). Furthermore, reducing nuclear PtdIns(5)P levels by overexpressing PIKIIβ, which phosphorylates PtdIns(5)P to form PtdIns(4,5)P₂, reduced p33ING2-association with the chromatin/nuclear matrix and increased p33ING2 levels in the cytosol, without changing the total p33ING2 protein levels (Gozani *et al.*, 2003). In addition, mutations in the PHD finger motif that abrogated PtdIns(5)P binding disrupted the ability of p33ING2 to enhance p53 acetylation and p53-dependent apoptosis. Overexpression of a PHD zinc finger demonstrated dominant-negative effects on p33ING2 functions. Taken together, these observations suggest that PtdIns(5)P recruits or stabilizes p33ING2 in the chromatin, and/or interaction with PtdIns(5)P might lead to allosteric activation of p33ING2.

The interaction between p33ING2 and Sin3-HDAC complexes was reported by Kuzmichev and colleagues (2002), who found that immunoaffinity purification of HeLA cell extract using anti-SAP30 antibodies yielded a set of abundant polypeptides corresponding to the core HDAC complexes, composed of HDAC1, HDAC2, RbAp46, RbAp48, Sin3 and SAP30, and less abundant polypeptides that may associate indirectly with the core Sin3-HDAC complex. Mass spectrometry analysis confirmed the presence of p33ING1b and p33ING2 in this immunopurified cell extract. A unique N-terminal domain of p33ING1b, SAID (SAP30-interacting domain) was identified as a domain required for interaction with SAP30, a component of the core Sin3-HDAC complex (Kuzmichev *et al.*, 2002). A computational search

revealed that p33ING2 also possesses SAID domain, suggesting that the presence of p33ING2 in Sin3-HDAC complexes may be achieved by direct interaction with SAP30. Since Sin3-HDAC complexes are involved in transcription repression, interaction between p33ING2 and Sin3-HDAC complexes may be important for the growth inhibitory activity of p33ING2.

1.4 Objectives and Hypotheses

The primary objective of this study is to further understand the tumour suppressive role of the p33ING2 novel tumour suppressor in cellular stress response to UV irradiation, and the molecular mechanisms of p33ING2 involvement in this stress response. We hypothesized that p33ING2 enhances UVB-induced apoptosis and the repair of UV-damaged DNA in a p53-dependent manner in melanoma cells. Using melanoma cell lines as an experimental model system, we have investigated the role of p33ING2 in UV-induced apoptosis (Chapter 3) and the repair of UV-damaged DNA (Chapter 4), and the molecular mechanisms involved in these stress responses.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell lines and Cell Culture

Two melanoma cell lines with known p53 status were used. The MMRU cell line was a kind gift from Dr. H.R. Byers (Boston University, School of Medicine, Boston, MA) (Byers *et al.*, 1991); the MeWo cell line was a kind gift from Dr. A.P. Albino (Memorial Sloan-Kettering Cancer Centre, New York, NY). The p53 status in the melanoma cell lines has previously been determined by DNA sequencing: MMRU contains wild-type (wt) p53 (Li *et al.*, 1995), while MeWo contains mutant (mut) p53 (Tilgen *et al.*, 1983; Li *et al.*, 1995; Bae *et al.*, 1996). All melanoma cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS (Invitrogen, Mississauga, ON, Canada), 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂.

2.1.2 Plasmids

Plasmids used for transfection included pcDNA3-vector (Invitrogen), pcDNA3-p33/ING2, pcDNA3-antisense (AS)-p33/ING2, pCl-p33/ING1b (kind gifts from Dr. C.C. Harris, National Cancer Institute, National Institutes of Health, Bethesda, MD), p3XFLAG-vector (Sigma-Aldrich, Mississauga, ON), p3XFLAG-ING2 (a kind gift from Dr. O. Gozani and Dr. J. Yuan, Harvard Medical School, Boston, MA), pβhwtp53 which contains human wild-type p53, and pED-1 which contains a point mutation in the human p53 cDNA that changes Cys135 to Serine (Johnson *et al.*, 1991) (kind

gifts from Dr. S. Benchimol, University of Toronto, Toronto, ON, Canada), pCMV_{CAT} (a kind gift of Dr. L. Grossman, Johns Hopkins University, Baltimore, MD), and pGFP-N2 (Clontech, Windsor, ON, Canada).

2.1.3 Antibodies

Antibodies used for Western blotting were anti-p33ING2 rat monoclonal antibody (a kind gift from Dr. O. Gozani and Dr. J. Yuan, Harvard Medical School, Boston, MA), anti-CPD and anti-6-4PP primary antibodies (kind gifts from Dr. T. Matsunaga from Kanazawa University, Japan) (Mori *et al.*, 1991), anti- β -actin goat monoclonal antibody, anti-p53 (DO-1) mouse monoclonal, anti-Bax (N-20) rabbit polyclonal, anti-GADD45 mouse monoclonal, anti-XPA rabbit polyclonal, and anti-XPB rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bcl-2, anti-caspase 3, anti-caspase 8, anti-caspase 9, anti-acetylated Lys382 p53 rabbit polyclonal antibodies (Cell Signalling, Beverly, MA), anti-cytochrome c mouse monoclonal antibody (BD Pharmingen, Mississauga, ON, Canada), and anti-Fas (Ab-1) mouse monoclonal antibody (Calbiochem, San Diego, CA), secondary IgG (Santa Cruz), Cy2-conjugated goat anti-rabbit and Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA).

2.2 Methods

2.2.1 Transfection

Cells were transfected at 40-50% confluency with Effectene reagent (Qiagen, Mississauga, ON, Canada) at a ratio of 1 μg DNA to 25 μl Effectene. An empty vector was used as control DNA.

2.2.2 Determination of Transfection Efficiency

Transfection efficiency of a particular cell line was determined by transfecting the cells with GFP-containing plasmid, pGFP-N2 with Effectene. Twenty-four hours after transfection, the number of green fluorescence-emitting cells was assessed under a fluorescent microscope. Transfection efficiency was calculated by the equation number of green fluorescence-emitting cells / the total number of cells (fluorescent and non-fluorescent) X 100%. Table 1 shows the percentages of transfection efficiencies of the cell lines used in this study.

Table 1.1 Relative percentages of transfection efficiencies of cell lines used in the study. Percentages were determined by counting the number of cells emitting green fluorescence / total number of cell counted X 100%. Experiments were repeated three times.

Cell Line	Transfection Efficiency (%)
MMRU	60 - 70
MeWo	50 - 60

2.2.3 Ultraviolet Irradiation

Medium was removed and the cells (at 70% confluency) were exposed to UVB (280-320 nm) using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ). The petri-dish cover was left on to filter possible UVC emissions from the UVB bulb. Medium was replaced and cells were incubated in a 5% CO₂ incubator at 37°C for desired time periods after UVB irradiation. The intensity of the UV light was measured by the IL 700 radiometer fitted with a WN 320 filter and an A127 quartz diffuser (International Light, Newburyport, MA).

2.2.4 Light Microscopy

The morphology of MMRU cells transfected with empty vector, pcDNA3-p33/NG2 or pcDNA3-AS-p33/NG2 were subjected to microscopic assessment 24 h after UVB irradiation at 600 J/m², using an inverted microscope (Zeiss, Chester, VA). Images were taken using a cooled mono 12-bit Retiga-Ex camera (Q-imaging, Burnaby, BC, Canada).

2.2.5 SRB Cell Survival Assay

Cells were grown in 24-well plates and transfected with empty vector, pcDNA3-p33/NG2 or pcDNA3-AS-p33/NG2 at 50% confluency. They were irradiated with UVB at 600 J/m² 24 h after transfection. Twenty-four hours after UV irradiation, cell survival was determined with the sulforhodamine B (SRB) (Sigma) assay as described previously (Skehan *et al.*, 1990; Li *et al.*, 1998). SRB is a pink aminoxanthene dye with two sulfonic groups that bind to basic amino acid residues in

proteins in cells fixed with trichloroacetate (TCA). SRB provides a sensitive index of cellular protein content. Briefly, the medium was removed and the cells were fixed with 500 μ l of 10% TCA for 1 h at 4°C after treatment. The cells were then washed five times with tap water and the excess water was removed by flicking. The cells were air-dried and then stained with 500 μ l of 0.4% SRB (dissolved in 1% acetic acid) for 30 min at room temperature, washed four times with 1% acetic acid, and air-dried. The cells were then incubated with 500 μ l of 10 mM Tris (pH 10.5) on a shaker for 20 min to solubilize the bound dye. Spectrophotometric readings were then taken at 550 nm for 100 μ l aliquots.

2.2.6 Trypan Blue Exclusion Assay

MMRU cells were grown in 24-well plates to 50% confluency before transfection. Twenty-four hours after transfection, the cells were irradiated with UVB at 600 J/m². After incubation in fresh medium for 24 h, attached cells were trypsinized, pooled with the floating dead cells, and the percentage of the dead cells were determined using a hemacytometer in the presence of 0.4% of trypan blue reagent (Sigma).

2.2.7 Propidium Iodide (PI) Staining

Cells grown on coverslips in 6-well plates were transfected and then irradiated with UVB at 600 J/m² 24 h after transfection. Twenty-four hours later, cells were fixed by gently overlying the media with 6 ml of ice-cold 95% ethanol and incubated for 3 min at room temperature. The media and 95% ethanol were mixed by pipetting gently and then incubated for 3 min at room temperature before the mixture was removed.

The fixed cells were stained with the mixture of 5 mg/ml PI, 0.1% Triton X-100 and 500 µg/ml RNase A at room temperature for 2 min. Coverslips were washed twice with phosphate buffered saline (PBS) before mounted onto slides with mounting media (Fisher Scientific, Nepean, Ontario, Canada). The slides were visualized under a fluorescent microscope (Zeiss) for apoptotic bodies. Images were taken with a cooled mono 12-bit Retiga-Ex camera.

2.2.8 Flow Cytometry

Cells were grown in 6-well plates for 24 h before transfection at 50% confluency. They were irradiated with UVB at 600 J/m² 24 h after transfection. Twenty-four hours later, cells were collected by trypsinization and pelleted by centrifugation at 2,000 g for 2 min. Next, cell pellets were resuspended in 1 ml of hypotonic fluorochrome buffer (0.1% Triton X-100, 0.1% sodium citrate, 25 µg/ml RNase A, 50 µg/ml PI). After overnight incubation at 4°C, the samples were then analyzed by flow cytometry to determine the percentage of sub-diploid DNA. Samples were run on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Mississauga, ON) and analyzed with EXPO32 ADC analysis software.

2.2.9 Western Blot Analysis

Cells were grown in 100-mm plates, transfected with p3XFLAG-vector or p3XFLAG-*ING2* expression vector at 50% confluency and irradiated with UVB at 600 J/m² 24 h after transfection. Twenty-four hours later, cells were washed with PBS, harvested by scraping on ice and pelleted by centrifugation at 2,000 g for 2 min. Cell pellets

were lysed in 80 μ l of triple detergent buffer (50 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.02% NaN₃, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing freshly added protease inhibitors (100 μ g/ml phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin A). The samples were then sonicated, incubated on ice for 20 min, and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatants were collected and a Bradford assay was performed to determine the protein concentration. Proteins (50 μ g/lane) were separated on 12% SDS-polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, ON, Canada). The PVDF was blocked with 5% skim milk for 1 h at room temperature before incubating with primary antisera prepared in 5% bovine serum albumin for 1 h at room temperature. Blots were washed three times in PBST (PBS containing 0.04% Tween-20) for 5 min each and then incubated with horseradish peroxidase (HRP)-conjugated secondary antisera for 1 h at room temperature. The signals were detected with the enhanced chemiluminescence detection kit (Amersham Bioscience, Baie d'Urfe, Quebec, Canada). Protein expressions on Western blots were quantified by densitometry using the Quantity One software (Bio-Rad). The fold-induction or reduction was corrected for differences in the actin loading control.

2.2.10 Detection of the Mitochondrial Membrane Potential

Disruption of the mitochondrial membrane potential was detected using a MitoCapture™ Apoptosis Detection Kit (Calbiochem). The assay was performed according to the manufacturer's specifications. Briefly, cells were grown on

coverslips in 6-well plates, transfected with p3XFLAG-vector or p3XFLAG-*ING2* expression vector at 50% confluency and exposed to UVB at 600 J/m² 24 h after transfection. Twenty-four hours after UV irradiation, the medium was removed and the cells were incubated with 2 ml of diluted MitoCaptureTM solution at 37°C in a 5% CO₂ incubator for 15 min. The dye solution was then removed and the cells were washed twice with 1 ml of the pre-warmed incubation buffer. The cells were then examined immediately under a fluorescent microscope. Images were taken with a cooled mono 12-bit Retiga-Ex camera.

2.2.11 Subcellular Fractionation

Cells were grown in 100-mm plates, transfected with p3XFLAG-vector or p3XFLAG-*ING2* expression vector at 50% confluency and irradiated with UVB at 600 J/m² 24 h after transfection. Twenty-four hours later, cells were washed with PBS, harvested by scraping on ice, and pelleted by centrifugation at 2,000 g for 2 min. Subcellular fractionation was performed as described previously (Kim *et al.*, 2001), with minor modifications. Briefly, cell pellets were resuspended in 200 µl of buffer A (20 mM HEPES-KOH [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose) containing a freshly added mixture of protease inhibitors (100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin A). Cell suspensions were then passed through a 26-gauge needle eight times to lyse the cells. Under a light microscope, lysed cells appear dull while intact cells have shiny rings around the nuclei. Unbroken cells, large plasma membrane pieces, and nuclei were removed by

centrifugation at 1,000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 10,000 *g* for 20 min at 4°C to obtain crude cytosolic fraction (supernatant) and the mitochondria (pellet). The pellet was washed with buffer A and then solubilized in 50 μ l of TNC buffer (10 mM Tris-acetate [pH 8.0], 0.5% NP-40, 5 mM CaCl₂) containing freshly added protease inhibitors to obtain mitochondrial fraction. The cytosolic fraction was then generated by centrifugation of the supernatant at 100,000 *g* for 1 h at 4°C. Protein concentrations were determined by the Bradford assay. Samples were then separated on 15% SDS-polyacrylamide gels, blotted onto PVDF, and probed with anti-Bax (N-20), anti-cytochrome *c* and anti- β -actin antibodies.

2.2.12 Host-cell-reactivation Assay (CAT Assay)

The pCMV_{CAT} plasmid that contains the gene encoding for chloramphenicol acetyltransferase (CAT) at 50 μ g/ml in 10 mM Tris-Cl (pH 8.5) was irradiated with UVC at 400 J/m² using an electronic ultraviolet cross-linker (Ultralum, Claremont, CA) and used for transfection. MMRU cells were grown in 6-well plates, and co-transfected with pCMV_{CAT} and pcDNA3-vector, pcDNA3-p33/ING2 or pcDNA3-AS-p33/ING2 at 40% confluency. Forty hours after transfection, CAT assays were performed as previously described (Cheung *et al.*, 2001). Briefly, cells were harvested and the cell pellet was resuspended in a 50 μ l solution of 0.25 M Tris-Cl (pH8.0) and 5 mM EDTA. Cell-free extracts of the transfected cells were obtained by three repeated freeze-thawings (liquid-nitrogen to freeze and 37°C to thaw), heated to 65°C for 10 min, centrifuged at 12,000 *g* for 10 min, and the cleared supernatant

was used for the CAT assay. The assay reaction mixture contained 7.5 μl of 5 mM of chloramphenicol, 50 μl of cell-free extract, 1 μl of 2.5 mM [^3H]acetyl-CoA, and 16.5 μl of double-distilled water (ddH₂O). The reaction mixture was incubated with the cell-free extract at 37°C for 90 min. Following incubation, 200 μl of ice-cold ethyl acetate was added, tubes were vortexed, and centrifuged at 12,000 g for 4 min. After quick-freezing the aqueous phase in liquid-nitrogen, the organic phase was removed and extracted with 200 μl of ddH₂O. The organic phase was dried to completion and radioactivity was determined in a scintillation counter (Beckman Coulter). Determinants were performed in triplicates. Controls included transfection with undamaged plasmid DNA and mock transfection without plasmid DNA.

2.2.13 Immunofluorescent Staining

MMRU cells were grown on coverslips in 6-well plates and transfected with p3XFLAG-*ING2* at 50% confluency. After 24 h, the medium was removed and an isopore polycarbonate filter with 5 μm pores (Millipore, Nepean, Ontario, Canada) was placed on top of the cell monolayer on the coverslip. Cells were irradiated with UVC at 200 J/m² using an UV cross-linker (Ultralum). The filter was gently removed and cells were cultured for desired time periods before fixation. Cells were fixed and labelled as described previously (Wang *et al.*, 2003) with slight modifications. Briefly, cells were washed twice with phosphate buffered saline (PBS) for 5 min each, fixed with 2 ml of fixation solution (2% paraformaldehyde, 0.5% Triton X-100 in PBS) for 30 min at 4°C. Cells were then washed three times with PBS for 5 min each, incubated with 2 M HCl for 10 min to denature DNA, followed by three washes with PBS for 5

min each. Cells were blocked with goat normal serum for 1 h at room temperature, followed by three washes with PBS. Coverslips containing fixed cells were then inverted onto 80 μ l of 1:30 dilution of anti-CPD or anti-6-4PP primary antibody and incubated in a humid chamber for 1 h, followed by three washes with PBS for 5 min each. Coverslips were then incubated with 100 μ l of 1:150 dilution of Cy3-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) for 1 h and washed three times with PBS for 5 min each. Cells were then double-stained with 1:30 dilution of anti-FLAG rabbit polyclonal antibody (Sigma), followed by staining with 1:150 dilution of Cy2-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch). Finally, coverslips were incubated with 1:3000 dilution of stock Hoechst 33258 (20 mM) for 10 min, washed three times with PBS for 5 min each and mounted onto slides with mounting medium (Fisher Scientific). Slides were then visualized under a fluorescent microscope. Images were taken with a cooled mono 12-bit Retiga-Ex camera.

2.2.14 Detection of p53 Acetylation

Cells were grown in 100-mm plates, transfected with p3XFLAG-vector or p3XFLAG-*ING2* expression vector at 50% confluency and irradiated with UVB at 200 J/m² 24 h after transfection. The histone deacetylase (HDAC) inhibitor trichostatin A (TSA) (Sigma) was added at a final concentration of 5 μ M 3 h prior to harvest to aid in the detection of p53 acetylation (Nagashima *et al.*, 2001; Saito *et al.*, 2002). Cells were then harvested at the indicated time-points after UV irradiation, and lysed for Western

blot analysis. MMRU cells treated with 0.2 $\mu\text{g/ml}$ of doxorubicin (Sigma) for 8 h were used as a positive control for p53 acetylation.

2.2.15 Immunoprecipitation

MMRU cells were grown in 100-mm plates for 24 h and transfected with p3XFLAG-*ING2* plasmid at 50% confluency. Twenty-four hours after transfection, cells were lysed with 1% triton-X solubilization buffer containing freshly added protease inhibitors (100 $\mu\text{g/ml}$ phenylmethylsulfonyl fluoride, 1 $\mu\text{g/ml}$ aprotinin, 1 $\mu\text{g/ml}$ leupeptin, 1 $\mu\text{g/ml}$ pepstatin A). The samples were then sonicated, incubated on ice for 20 min, and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatants were collected and a Bradford assay was performed to determine the protein concentration. Protein (500-1000 μg) was incubated with anti-FLAG mouse monoclonal antibody (Sigma) or a control normal mouse IgG (Santa Cruz) at 4°C overnight, and then with protein G sepharose (Amersham Bioscience) at 4°C for 1 h. The beads were washed three times with solubilization buffer prior to boiling for 3 min. The precipitates were then resolved by electrophoresis, followed by Western blot analysis.

CHAPTER 3. p33ING2 ENHANCES UVB-INDUCED APOPTOSIS IN HUMAN MELANOMA CELLS

3.1 Rationale and Hypothesis

Cutaneous melanoma is a type of skin cancer resulting from uncontrolled proliferation of the epidermal melanocytes. Epidemiological studies strongly implicate UV irradiation as the primary aetiological factor for melanoma (Mackie 1998; Gilchrest *et al.*, 1999). Studies on the carcinogenic effects of different UV wavelengths identified UVB as the major cause of the carcinogenic effects of sun exposure (Yuspa *et al.*, 1991). UV irradiation induces the formation of cyclobutane pyrimidine dimers and 6-4 photoproducts which may lead to mutations and carcinogenesis if the lesions are not removed from the genome promptly (Beukers and Berends, 1960; Setlow and Carrier, 1966; Alcalay *et al.*, 1990). The incidence of melanoma is increasing most rapidly in the Caucasian population among all malignancies except lung cancer in women (Howe *et al.*, 2001; Bronchez and Naeyaert, 2000). Malignant melanoma is associated with one of the highest mortality rates due to its rapid metastasis, and the 5-year survival rate of patients with metastatic melanoma remains less than 10% (Howe *et al.*, 2001; Roses *et al.*, 1991). The reason for the poor prognosis of melanoma is due to its resistance to conventional radiotherapy and chemotherapy.

It is well accepted that apoptosis is a common mode of action employed by various anticancer drugs, and that the expression of apoptotic genes mediates chemosensitivity in cancer cells. The tumour suppressor p53 is an important transcription factor of various apoptotic genes such as *Bax*, *PUMA*, and *Noxa* (Henry

et al., 2002). It is the most frequently mutated gene known to date, with the mutation rate of more than 50% in human malignancies (Hussein *et al.*, 2003a). However, mutational studies revealed that the *p53* gene is altered in only 1-5% and 11-25% of primary and metastatic melanoma, respectively (Hussein *et al.*, 2003a). In fact, protein expression profile of *p53* evidenced that *p53* is overexpressed in the late stage of melanoma tumourigenesis (Hussein *et al.*, 2003a). The lower frequency of *p53* mutation in melanoma suggests that other tumour suppressor genes and/or apoptotic genes may also play key roles in the pathogenesis of melanoma.

Several studies have provided evidence that both *p33ING1b* and *p33ING2* require the activity of *p53* in exiting tumour suppressive functions such as regulation of transcription, growth arrest and apoptosis (Campos *et al.*, 2004a). *p33ING1b* and *p33ING2* modulate *p53* activity via distinct mechanisms. *p33ING1b* physically associates with *p53* while *p33ING2* promotes *p53* acetylation, resulting in the stabilization of *p53* and thus enhancement of the *p53* cellular responses to genotoxic stresses and apoptotic stimuli. Furthermore, recent studies indicated that the interaction of *p33ING2* with phosphoinositides, *PtdIns(3)P* and *PtdIns(5)P*, through its PHD zinc finger motif is critical for its ability to enhance *p53* acetylation and mediate *p53*-dependent etoposide-induced apoptosis (Gozani *et al.*, 2003).

To date, there are a few reports indicating that *p33ING1b* plays a significant role in stress response to UV irradiation. Previously, we found that *p33ING1b* cooperates with *p53* to enhance UVB-induced apoptosis via activation of the mitochondrial apoptotic pathway in melanoma cells (Cheung and Li, 2002). Another group has demonstrated that *p33ING1b* possesses two distinct nucleolar targeting

sequences (NTS) within the nuclear localization domain, which promotes the translocation of p33ING1b to the nucleolus after UV irradiation (Scott *et al.*, 2001b). It was also reported that the PCNA-interacting-protein (PIP) domain at the N-terminus of p33ING1b binds competitively to the interdomain connector loop of PCNA upon UV irradiation (Scott *et al.*, 2001a). However, study on the role of p33ING2 in the stress response to UV irradiation is lacking. Due to the structural similarities between p33ING1b and p33ING2, we investigated the role of p33ING2 in cellular stress response to UVB irradiation and the molecular mechanisms of p33ING2 involvement in this stress response by using melanoma cells as an experimental model system. We hypothesized that p33ING2 enhances UVB-induced apoptosis in a p53-dependent manner.

3.2 Results

3.2.1 p33ING2 Enhances UVB-induced Apoptosis in Melanoma Cells

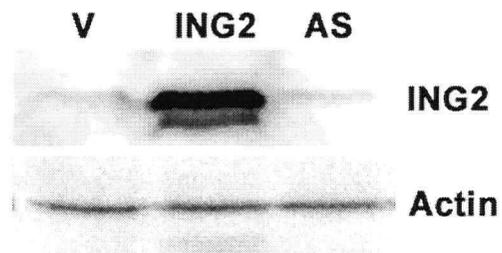
To investigate the role of p33ING2 in UVB-induced apoptosis, we transfected a melanoma cell line, MMRU, with either pcDNA3-vector, pcDNA3-p33ING2 or pcDNA3-AS-p33ING2 expression vector. Western blot analysis confirmed the expression of p33ING2, suggesting successful transfection of MMRU cells (Fig. 3.1A). To determine if p33ING2 enhances UV-induced cell death, the transfected cells were irradiated with UVB at various doses (0, 200, 400, and 600 J/m²). Cells were fixed and stained with SRB 24 h after UV irradiation. The amount of SRB dye that remains bound to the surface of the fixed cells is a determinant of cell survival. We determined the cell survival rate of UV-irradiated p33ING2-overexpressing MMRU cells in comparison to cells transfected with empty vector alone or with antisense p33ING2. We found that overexpression of p33ING2 consistently reduced cell survival at various doses of UVB (Fig. 3.1B). After UVB irradiation at 200, 400 and 600 J/m², the survival rates of MMRU cells overexpressing p33ING2 were 79.4%, 64.9% and 53.2% compared to the vector control with 94.4%, 87.9% and 78.0%, respectively ($P < 0.001$, $P < 0.0001$, and $P < 0.00001$, t-test). The microscopic images show that there were significantly more dead cells (round, shrunk, and detached) in MMRU cells overexpressing p33ING2 24 h after UVB irradiation compared to the control cells (Fig. 3.1C). To further confirm these observations, trypan blue exclusion assay was carried out to quantitate dead cells, which were stained blue due to their inability to exclude the dye by the cell membrane, while live

cells were unstained. The results in Fig. 3.1D show that MMRU cells overexpressing p33ING2 had a significantly higher rate of cell death (39.8%) after UVB irradiation at 600 J/m² compared to 16.6% in the vector control cells ($P < 0.0001$, t-test). We also observed that overexpression of p33ING2 alone, without exposure to cellular stress, induced 10.4% of cell death compared to 5.0% in the control cells ($P < 0.001$, t-test). p33ING1b was used as a positive control in this experiment as we previously reported that p33ING1b enhances UVB-induced apoptosis in melanoma cells (Cheung and Li, 2002). Consistent with the previous findings, p33ING1b significantly induced cell death after UVB irradiation ($P < 0.0001$, t-test).

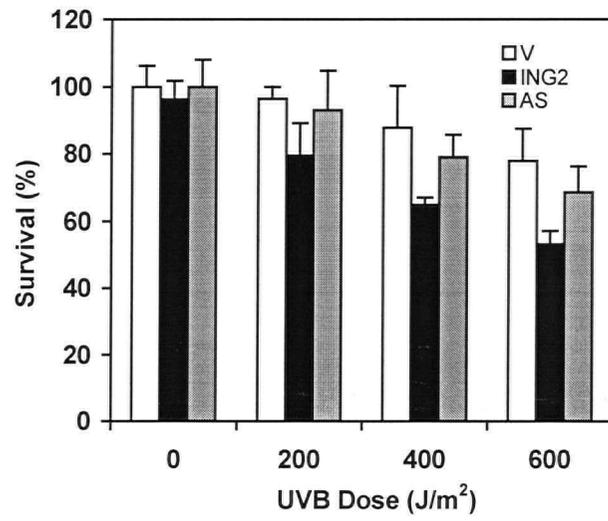
To confirm that p33ING2-mediated cell death after UVB irradiation is the result of apoptosis, not necrosis, cells were stained with propidium iodide (PI), which binds stoichiometrically to DNA. Cells undergoing apoptosis are characterized by chromatin condensation, formation of apoptotic bodies, and DNA fragmentation (Wylie, 1997; Hengartner, 2000). The representative images taken under the fluorescent microscope show that p33ING2-transfected MMRU cells underwent apoptosis after UVB irradiation (Fig. 3.1E). Quantitative data of PI staining in Fig. 3.1F indicates that there were significantly more apoptotic cells in p33ING2-overexpressing MMRU cells (43.8%) compared to the control cells (20.0%) ($P < 0.001$, t-test). Consistent with the results in Fig. 3.1D, overexpression of p33ING2 alone caused 17.1% of cells to undergo apoptosis compared to 5.1% in the control cells ($P < 0.01$, t-test). The enhancement of apoptosis induction by p33ING2 was further confirmed by flow cytometry. DNA fragmentation, a hallmark feature of apoptosis, results in a hypodiploid DNA content represented by the Sub-G₁ population in the

Figure 3.1 p33ING2 enhances UVB-induced cell death in melanoma MMRU cells. (A) Western blot analysis of p33ING2 expression in MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or pcDNA3-antisense-p33/ING2 (AS) expression vector. An anti-p33ING2 monoclonal antibody was used to detect p33ING2 and β -actin was used as a loading control. (B) Cell survival rate by SRB assay of UVB-irradiated MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), or pcDNA3-AS-p33/ING2 (AS) expression vector. Data represent means \pm SD from triplicates. The experiment was repeated twice with similar results. (C) Microscopic images of MMRU cells transfected with pcDNA3-vector (V) or pcDNA3-p33/ING2 (ING2) irradiated with UVB at 0 or 600 J/m². Photographic images were taken 24 h after UVB irradiation. Magnification, 200 \times . (D) Cell death assessment by trypan blue exclusion assay on MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or pCI-p33/ING1 (ING1b) after UVB irradiation. Data represent means \pm SD from triplicates. The experiment was repeated three times with similar results. (E) Representative PI staining images of apoptotic cells after UVB irradiation. MMRU transfected with pcDNA3-p33/ING2 were irradiated with UVB at 600 J/m² and images were taken under fluorescent microscope 24 h after UV irradiation. White arrowheads indicate cells with condensed nucleic and apoptotic bodies. (F) Quantitative data from PI staining of MMRU cells in E. Data represent means \pm SD from triplicates.

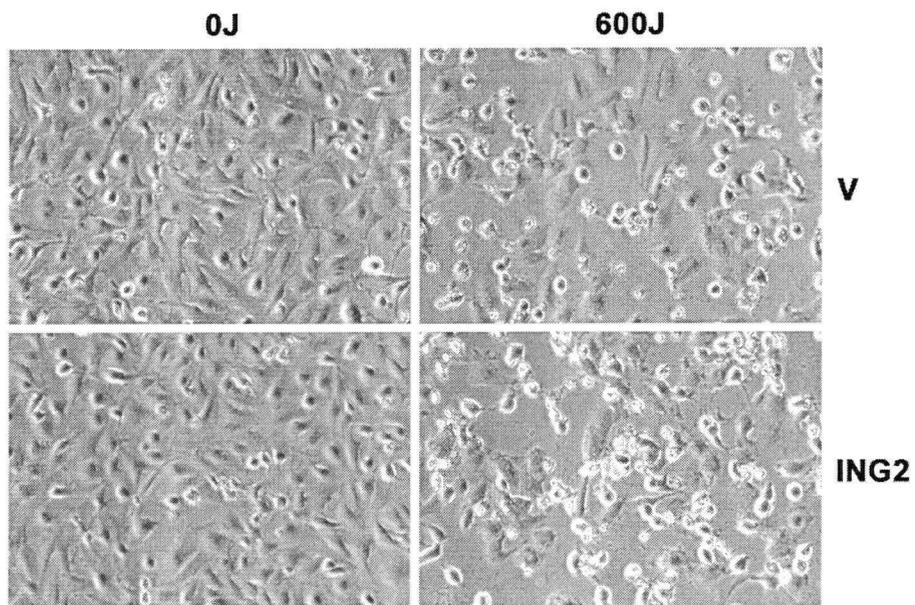
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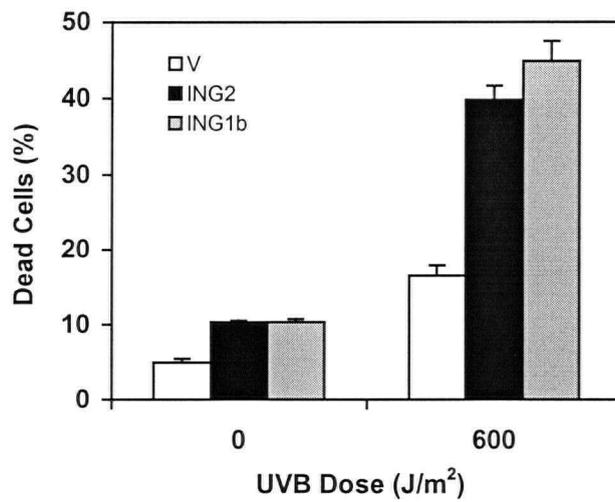
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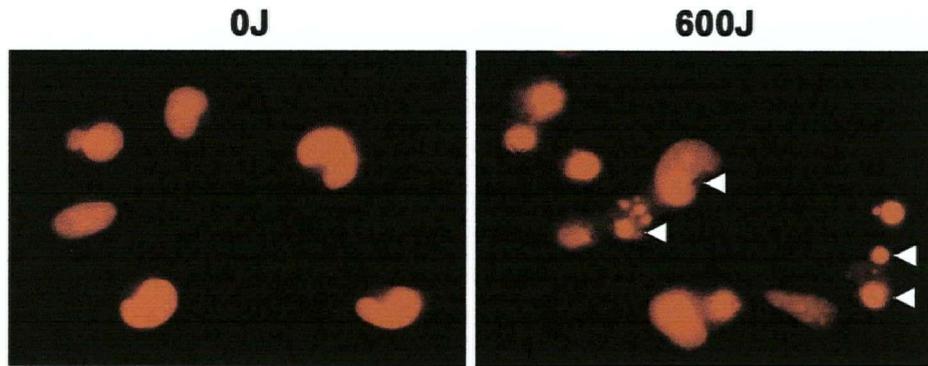
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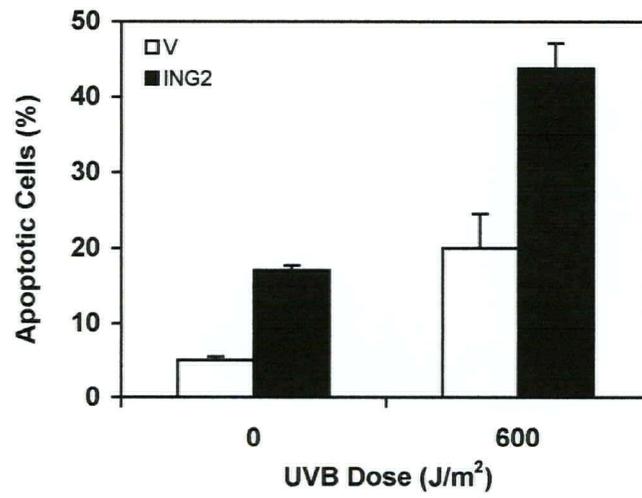
D



E



F



DNA cell cycle histogram. Similarly, without UV treatment, cells overexpressing p33ING2 had 20.7% apoptotic cells compared to 6.4% in the control cells ($P < 0.01$, t-test) (Fig. 3.2A). After UVB irradiation, p33ING2 induced apoptosis in 46.2% of cells compared to 22.3% in the control cells ($P < 0.001$, t-test) (Fig. 3.2A). Clearly, cells overexpressing p33ING2 displayed a larger Sub-G₁ population compared to the control cells under both non-UV- and UV-irradiated conditions.

The slight variation of the percentage of dead cells obtained by trypan blue exclusion assay and flow cytometry (Fig. 3.1D and Fig. 3.2A) is possibly due to variability in the technical approaches. Collectively, we demonstrate that p33ING2 enhances UVB-induced apoptosis in melanoma cells.

3.2.2 p33ING2 Enhancement of UVB-induced Apoptosis Is p53-dependent

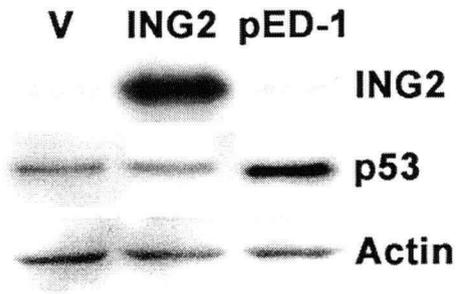
It was shown in various reports that functional p53 is required for p33ING1b in exerting its tumour suppressive functions, such as growth inhibition, apoptosis, and the repair of UV-damaged DNA (Garkavtsev *et al.*, 1996; Garkavtsev *et al.*, 1998; Shinoura *et al.*, 1999; Cheung *et al.*, 2001; Cheung and Li, 2002). Furthermore, two reports on p33ING2 have shown that p33ING2-induced growth inhibition and apoptosis are p53-dependent (Nagashima *et al.*, 2001; Gozani *et al.*, 2003). These observations led us to hypothesize that p33ING2 and p53 may work together in the enhancement of UVB-induced apoptosis in melanoma cells. To test our hypothesis, we co-transfected the wild-type p53 MMRU melanoma cells with p33ING2 and mutant p53 plasmid pED-1, and exposed the cells to UVB irradiation at 600 J/m². Western blot analysis using an anti-p53 antibody, which recognizes both wt and

mutant p53, confirmed the expression of p53 in MMRU cells, suggesting successful transfection of MMRU cells (Fig. 3.2A). Using flow cytometry, we found that co-expression of p33ING2 and mutant p53 significantly reduced the enhancement of apoptosis by p33ING2 even without UVB irradiation (9.5% in co-transfected cells compared to 20.7% in p33ING2-transfected cells; $P < 0.001$, t-test). Consistently, the enhancement of apoptosis by p33ING2 was significantly reduced by co-expression of p33ING2 and mutant p53 after UVB irradiation (34.2%) compared to 46.2% in p33ING2-transfected cells ($P < 0.001$, t-test) (Fig. 3.2B). However, co-expression of p33ING2 and mutant p53 did not completely abrogate p33ING2-mediated UVB-induced apoptosis. This may be due to the endogenous wild-type p53 not being completely inactivated.

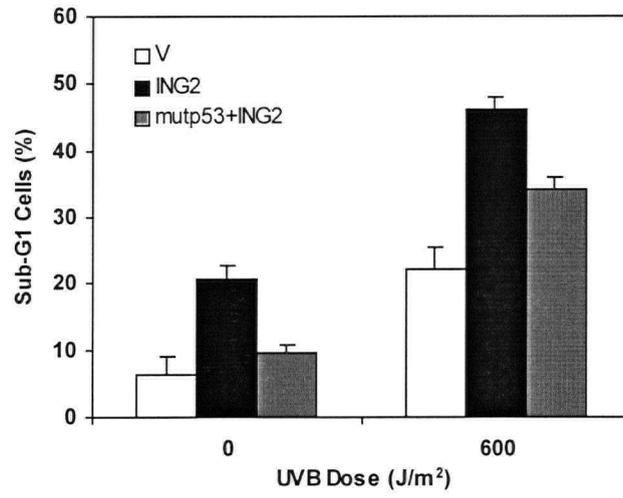
Therefore, we used MeWo melanoma cells that carry a mutant *p53* gene to further investigate the p53 dependency of p33ING2 enhancement in UVB-induced apoptosis. SRB survival assay was carried out on vector-, p33ING2- or p33ING1b-transfected MeWo cells after UVB irradiation with increasing doses. As reported previously, p33ING1b, the additional control of this experiment, did not enhance UVB-induced cell death in the absence of functional p53. Similarly, overexpression of p33ING2 did not enhance UVB-induced cell death in the absence of functional p53 at all doses examined (Fig. 3.2D). These results were also supported by the results from flow cytometry analysis showing similar percentages of Sub-G₁ apoptotic cells in MeWo cells transfected with vector or p33ING2 (Fig. 3.2E). To confirm the requirement of functional p53 in p33ING2 enhancement of UVB-induced apoptosis, we transfected MeWo cells with wild-type *p53* (p β hwtp53) or wild-type *p53* together

Figure 3.2 p33ING2 enhancement of UVB-induced apoptosis is p53-dependent. (A) Western blot analysis of p33ING2 and p53 expression in p53 wild-type MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or mutant p53 pED-1 expression vector. (B) Quantitation of apoptosis rate of MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), or pED-1 and pcDNA3-p33/ING2 (mutp53+ING2). Transfected cells were irradiated with UVB at 600 J/m² and cells were trypsinized and analyzed by flow cytometry 24 h after UVB irradiation. Data represent means ± SD from triplicates. (C) Western blot analysis of p33ING2 and p53 expression in p53 mutant MeWo cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or wild-type p53 (p53) expression vector. (D) Survival assay of MeWo cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or pCI-p33/ING1 (ING1b). Cells were irradiated with various doses of UVB and SRB survival assay was performed 24 h after UV irradiation. Data represent means ± SD from triplicates. The experiment was repeated twice with similar results. (E) Cell death assessment by flow cytometry of MeWo cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), wild-type p53 pβhwtp53 (p53), or pβhwtp53 and pcDNA3-p33/ING2 (p53+ING2). Transfected cells were irradiated with UVB at 600 J/m² and flow cytometry was performed 24 h after UV irradiation. Number in the histograms represents the percentage of Sub-G₁ population. The experiment was repeated twice with similar results.

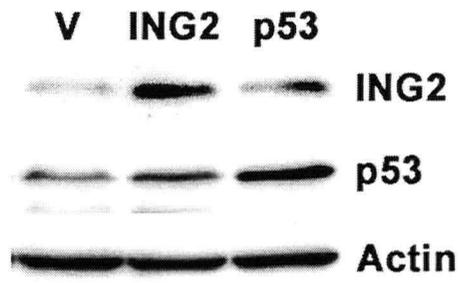
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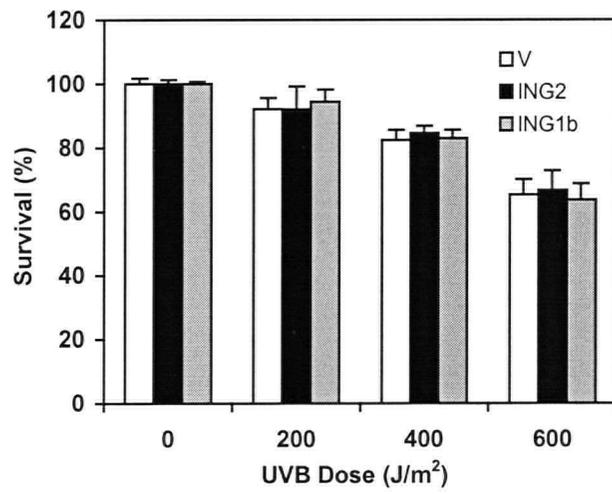
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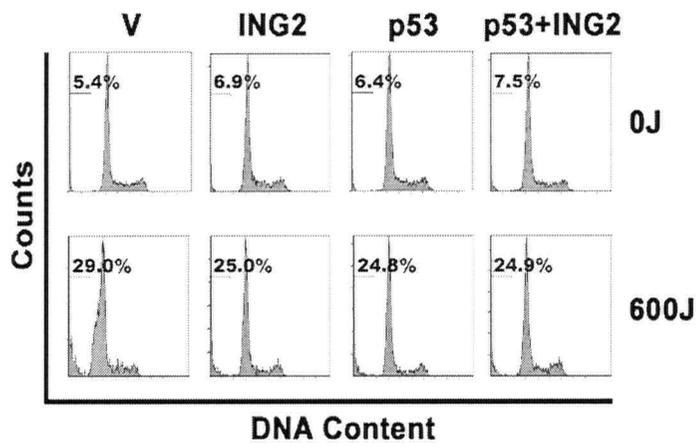
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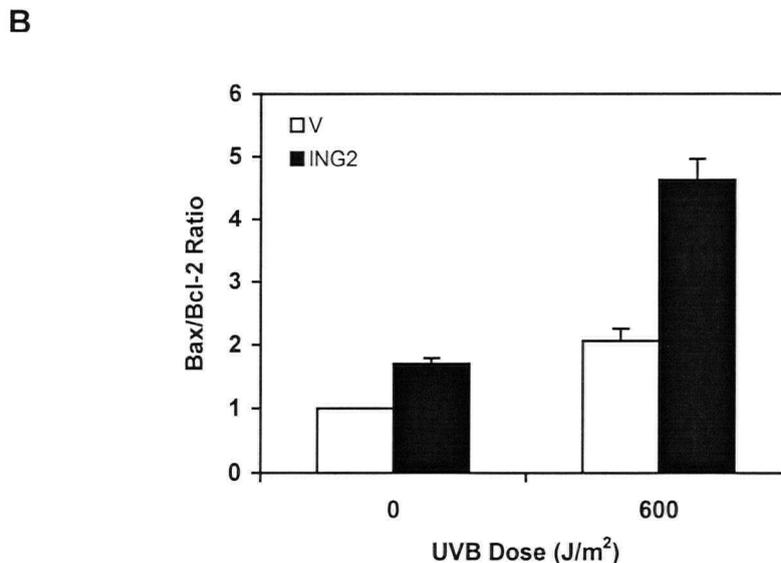
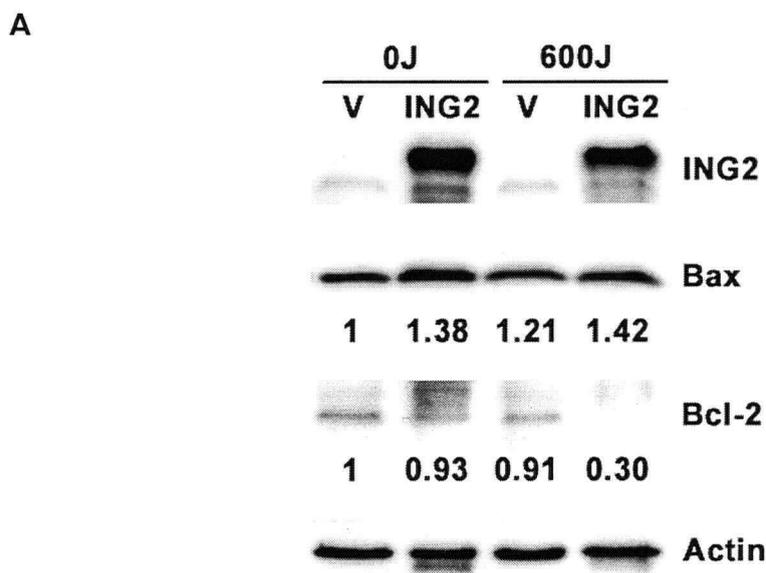


with p33/ING2, to see if re-introduction of wild-type p53 could restore the enhancement of apoptosis by p33ING2. Western blot analysis confirmed the expression of p33ING2 and p53 in MeWo cells, suggesting successful transfection of MeWo cells (Fig. 3.2C). Our data indicated that neither transfection with wild-type p53 alone nor co-transfection with p53 and p33/ING2 sensitized MeWo cells to UVB-induced apoptosis. This may be due to the presence of dominant negative p53 mutation in MeWo cells that abrogates the effect of the wild-type p53 re-introduced into the cells.

3.2.3 p33ING2 Increases the Ratio of Bax/Bcl-2 in Melanoma Cells

Recent findings of ING proteins on upregulation of Bax expression and its promoter activity (Nagashima *et al.*, 2001; Nagashima *et al.*, 2003; Cheung and Li, 2002) led us to hypothesize that p33ING2 enhances UVB-induced apoptosis by upregulating Bax expression in melanoma cells. To determine if p33ING2 upregulates endogenous Bax expression, we transfected MMRU cells with empty vector or p33/ING2 and subjected these cells to UVB irradiation at 0 or 600 J/m². Twenty-four hours after UV irradiation, proteins were extracted and Western blot analysis was performed to determine Bax expression in these cells. The levels of Bax protein were normalized with the levels of β -actin, the loading control, accordingly. The normalized Bax expression level in non-UV-irradiated vector-transfected cells was arbitrarily defined as 1, and the relative fold of Bax induction of other samples was calculated and indicated below each band in the representative figure of Western blot analysis (Fig. 3.3A). Our results demonstrate that cells overexpressing p33ING2

Figure 3.3 p33ING2 upregulates Bax and downregulates Bcl-2 expression. (A) Representative figure of Western blot analysis of Bax and Bcl-2 proteins. MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-ING2 (ING2) were irradiated with UVB at 0 or 600 J/m². Cells were harvested and lysed for Western blot analysis 24 h after UV irradiation. Antibodies against p33ING2, Bax, and Bcl-2 were used. Anti-p33ING2 antibody detected both endogenous (lower bands) and transfected p3XFLAG-ING2 (upper bands). β -actin was used as a loading control. The number below each band indicates the relative fold of Bax induction or Bcl-2 reduction by densitometry. (B) Ratio of Bax/Bcl-2 proteins in non-UV-irradiated and UV-irradiated MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-ING2 (ING2). Data represent means \pm SD from three independent experiments.



have higher Bax expression compared to the vector controls without UV irradiation. However, the induction of Bax expression by p33ING2 was not enhanced after UV irradiation (Fig. 3.3A). These observations led us to examine the expression levels of anti-apoptotic protein, Bcl-2, which was found to neutralize Bax pro-apoptotic effects by forming heterodimers with Bax (Hengartner, 2002). Interestingly, we found that p33ING2 downregulated Bcl-2 expression, with more significant reduction of Bcl-2 protein after UV irradiation (Fig. 3.3A). The ratio of Bax to Bcl-2 is thought to determine the degree of heterodimer formation, and thus the sensitivity of cells to apoptosis (Adam and Cory, 1998). Therefore, we determined the ratio of Bax to Bcl-2 in these samples. Our results revealed that p33ING2 significantly increased the ratio of Bax/Bcl-2, with more profound increment (4.6-fold) after UV irradiation ($P < 0.0001$, t-test) (Fig. 3.3B). These data strongly suggest that p33ING2 enhances UVB-induced apoptosis by increasing the overall active Bax expression levels.

3.2.4 p33ING2 Alters Mitochondrial Membrane Potential after UVB Irradiation

It has been shown that UV irradiation activates the mitochondrial apoptotic pathway, and that p33ING1b promotes UVB-induced apoptosis via this pathway (Cheung and Li, 2002; Antonsson *et al.*, 2001; Green and Reed, 1998). Overall increase of Bax induced by p33ING2 suggests that p33ING2 may also mediate UVB-induced apoptosis via the mitochondrial pathway. The mitochondrial membrane potential is decreased in the early stage of apoptosis induced by many types of stimuli (Li, *et al.*, 1999; Zhang *et al.*, 1999a; Hengartner, 2000). Therefore, we first investigated whether p33ING2 activates the mitochondrial apoptotic pathway by determining the

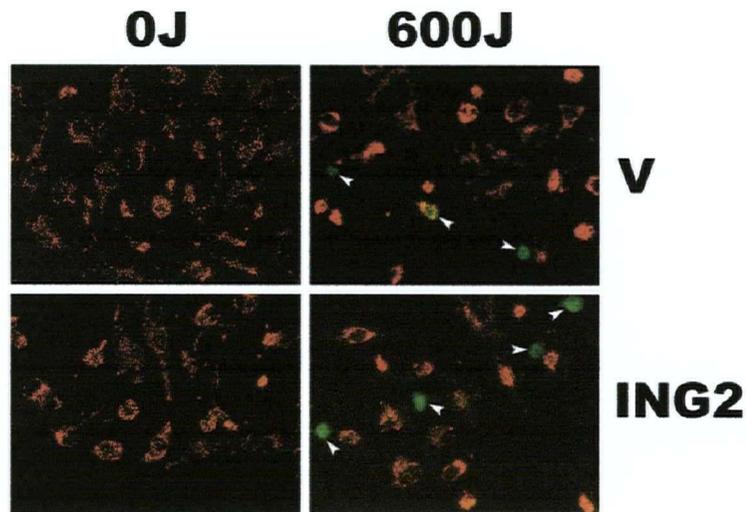
mitochondrial membrane potential changes after UVB irradiation. We transfected MMRU cells with empty vector or p33/ING2 and exposed the cells to UVB irradiation at 600 J/m². Twenty-four hours later, cells were stained with a cationic dye using the MitoCapture apoptosis detection kit. The cationic dye accumulated and aggregated in the mitochondria in healthy cells and emitted an orange-red fluorescence. In apoptotic cells, the dye remained in the cytoplasm due to alteration of the mitochondrial membrane potential and emitted green fluorescence. Stained cells were visualized under a fluorescent microscope. Fig. 3.4A shows that non-UV-irradiated control cells stained orange-red while green fluorescence staining (apoptotic cells) was observed in UVB-irradiated cells. After UV irradiation, there is a significantly higher percentage of apoptotic cells in p33/ING2-transfected cells compared to vector control (31.8% versus 7.7%; $P < 0.001$, t-test) (Fig. 3.4B). These data suggest that p33/ING2 enhances UVB-induced apoptosis via the mitochondrial apoptotic pathway by altering the mitochondrial membrane potential.

3.2.5 p33/ING2 Promotes Bax Translocation and Cytochrome c Release after UVB Irradiation

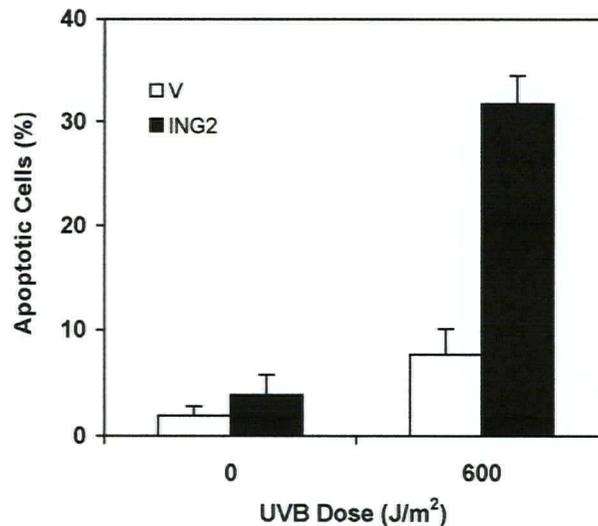
Upon activation, Bax translocates to the mitochondria where active Bax oligomerizes in the mitochondrial outer membrane to increase mitochondrial permeability, leading to cytochrome c release and apoptosis (Cory and Adam, 2002; Igney and Krammer, 2002). Since p33/ING2 upregulation of Bax is not enhanced in melanoma cells after UV irradiation (Fig. 3.3A), we hypothesized that p33/ING2 may also enhance apoptosis by promoting translocation of Bax to the mitochondria upon UV irradiation.

Figure 3.4 p33ING2 alters mitochondrial membrane potential after UVB-irradiation. MMRU cells transfected with pcDNA3-vector (V) or pcDNA3-p33/ING2 (ING2) were irradiated with UVB at 0 or 600 J/m². Disruption of mitochondrial membrane potential was detected using the MitoCapture apoptosis kit 24 h after UVB irradiation. **(A)** Representative images of MMRU control cells and cells overexpressing p33ING2 after UVB irradiation. Live cells emit orange-red fluorescence and apoptotic cells emit green fluorescence (indicated by white arrow heads). Magnification, 400 \times . **(B)** Quantitation of the percentage of apoptotic cells based on the changes in the mitochondrial membrane potential. A total of 500 cells were counted for each sample. Data represent means \pm SD from triplicates.

A



B

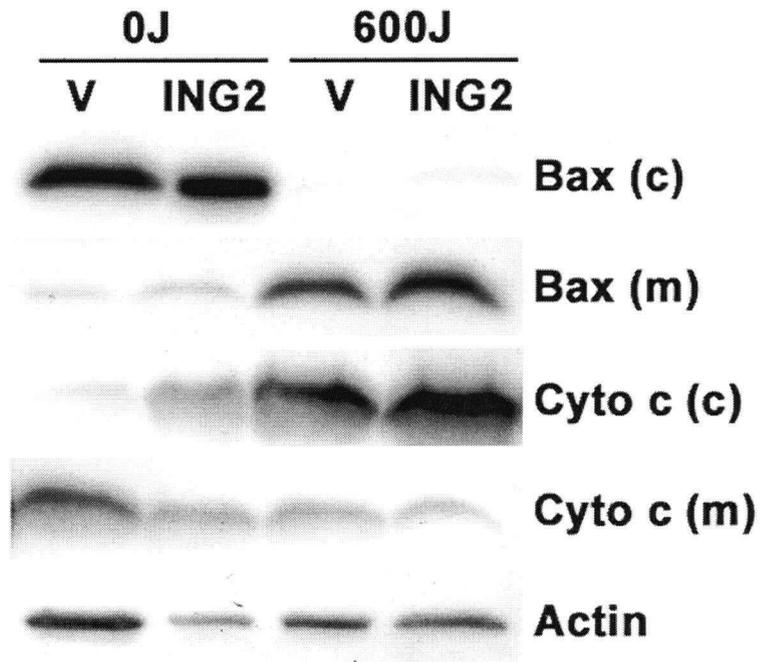


To test our hypothesis, we transfected MMRU cells with empty vector or p33ING2 and subjected these cells to UVB irradiation at 600 J/m². Cells were harvested 24 h after UV irradiation and subcellular fractionation was carried out to separate the cytosolic and mitochondrial fractions, which were then subjected to Western blot analysis. Fig. 3.5 shows that in the non-UV-irradiated cells, Bax localized in the cytosol and cytochrome *c* was mainly found in the mitochondria. Cytochrome *c* was partially released from the mitochondria in cells overexpressing p33ING2, consistent with the findings that p33ING2 alone can induce apoptosis (Fig. 3.1D, 3.1F, and 3.2B). Twenty-four hours after UV irradiation, Bax translocated from the cytosol to the mitochondria, and cytochrome *c* was released from the mitochondria into the cytosol. There is more Bax translocation and cytochrome *c* release in cells overexpressing p33ING2 compared to the control cells. These data indicate that p33ING2 promotes Bax translocation to the mitochondria, causing mitochondrial membrane potential changes and thus promotes cytochrome *c* release into the cytosol to initiate apoptosis.

3.2.6 p33ING2 Induces the Activation of Caspase 9 and Caspase 3 after UVB Irradiation

Released cytochrome *c* facilitates the interaction between Apaf-1 and pro-caspase 9, resulting in the cleavage and activation of caspase 9 and downstream effector caspases, such as caspases 3 and 7 (Li *et al.*, 1997; Cryns and Yuan, 1998). To confirm if the mitochondrial apoptotic pathway is activated in p33ING2-mediated UVB-induced apoptosis, MMRU cells transfected with empty vector or p33ING2 were

Figure 3.5 p33ING2 promotes Bax translocation and cytochrome c release. MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-ING2 (ING2) were irradiated with UVB at 0 or 600 J/m². Subcellular fractionation was performed 24 h post irradiation and Western blot analysis was carried out on the cytosolic (c) and mitochondrial (m) fractions. Antibodies against Bax and cytochrome c were used. β -actin was used as a loading control.

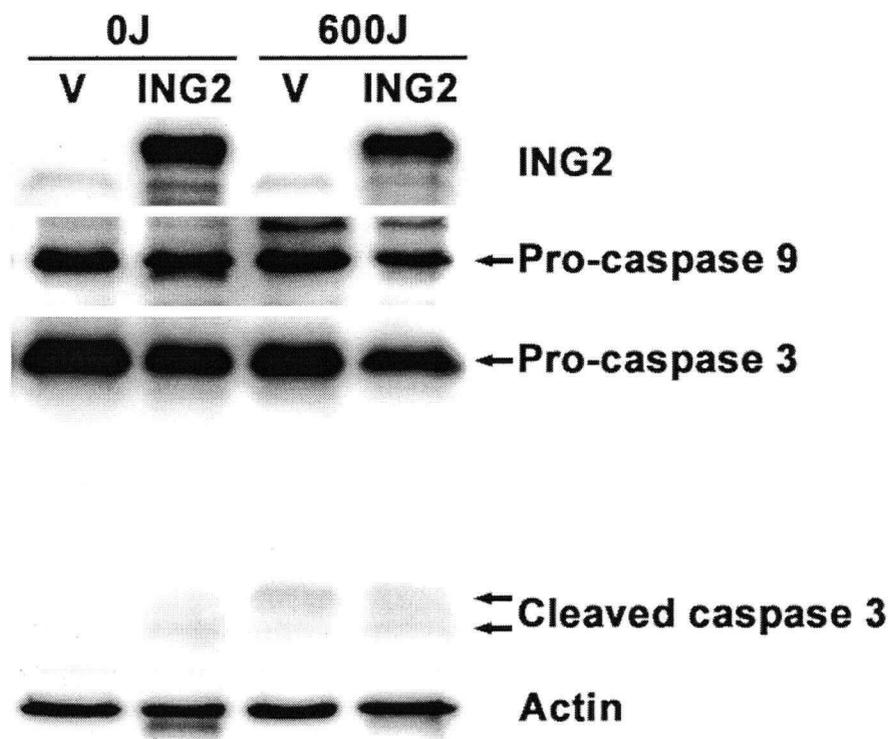


UVB-irradiated at 0 or 600 J/m². Cells were harvested 12 h after UV irradiation and lysates were subjected to Western blot analysis on the cleavage of caspase 9 and caspase 3. Fig. 3.6 showed that the pro-caspase 9 levels in p33ING2-overexpressing cells after UV irradiation were greatly reduced compared to the vector control cells and non-UV-irradiated p33ING2-overexpressing cells (0.73- and 0.69-fold respectively, $P < 0.01$, t-test). However, without UV irradiation, there was no significant cleavage of caspase 9 observed in p33ING2-overexpressing cells compared to the vector control cells. Consistently, pro-caspase 3 (35 kDa) levels were reduced and more cleaved caspase 3 fragments (19 and 17 kDa) were produced in cells overexpressing p33ING2 compared to the vector control cells. Cells overexpressing p33ING2 showed 0.69- and 0.85-fold of pro-caspase 3 reductions compared to the vector control cells, with and without exposure to UV irradiation respectively ($P < 0.01$, t-test). The cleavage was more pronounced in p33ING2-overexpressing cells after UV irradiation compared to non-UV-irradiated control cells. These observations strongly suggest that p33ING2 induces the activation of caspase 9 and caspase 3 in response to UV irradiation.

3.2.7 p33ING2 Activates the Death-receptor Apoptotic Pathway

Previous studies have shown that UV irradiation also activates the death-receptor or extrinsic apoptotic pathway by activating Fas and releasing tumour necrosis factor- α (TNF- α) (Schwarz *et al.*, 1995; Rehemtulla *et al.*, 1997; Aragane *et al.*, 1998). Therefore, we investigated if p33ING2 induces activation of this alternative pathway upon UV irradiation. The Western blot in Fig. 3.7 shows that p33ING2 upregulates

Figure 3.6 p33ING2 induces the activation of caspases 9 and 3. MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-ING2 (ING2) were irradiated with UVB at 0 or 600 J/m². Cells were harvested 24 h after UV irradiation and lysates were prepared for Western blot analysis using anti-p33ING2, anti-caspase 9 and anti-caspase 3 antibodies. Anti-caspase 9 antibody detected pro-caspase 9 (47 kDa) and anti-caspase 3 antibody detected pro-caspase 3 (35 kDa) the cleaved fragments (19 and 17 kDa). β -actin was used as a loading control.



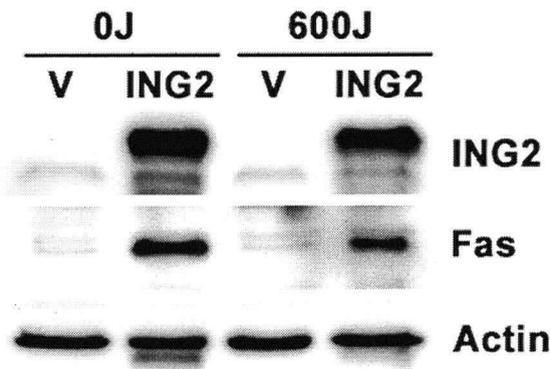
the expression of the death receptor Fas by 12.0- and 10.6-fold in non-UV- and UV-irradiated cells respectively ($P < 0.001$, t-test). These results suggest that p33ING2 enhancement of UVB-induced apoptosis may be partially attributed to the death receptor-mediated apoptosis pathway. UVC irradiation was found to upregulate both Fas in primary keratinocytes (Leverkus et al., 1997). However, we did not observe upregulation of Fas in the control cells after UVB irradiation, possibly due to different wavelengths of UV and cell types used. To confirm the involvement of p33ING2 in the death-receptor apoptotic pathway, we examined the cleavage of caspase 8, the initiator of the caspase cascade in the death receptor apoptosis pathway. The majority of the pro-caspase 8 (57 kDa) was cleaved in cells overexpressing p33ING2, producing higher levels of the cleaved fragments (43, 41 and 18 kDa) compared to that in the control cells both in non-UV- and UV-irradiated cells (Fig. 3.7). The non-UV-irradiated p33ING2-overexpressing cells showed a 0.47-fold reduction of pro-caspase 8 levels compared to the vector control cells ($P < 0.01$, t-test). However, there was not substantially more caspase 8 activation observed in UVB-irradiated p33ING2-overexpressing cells compared to the non-UV-irradiated p33ING2-overexpressing cells. These data suggest that p33ING2 activates the death-receptor apoptotic pathway regardless of UVB irradiation.

3.3 Discussion

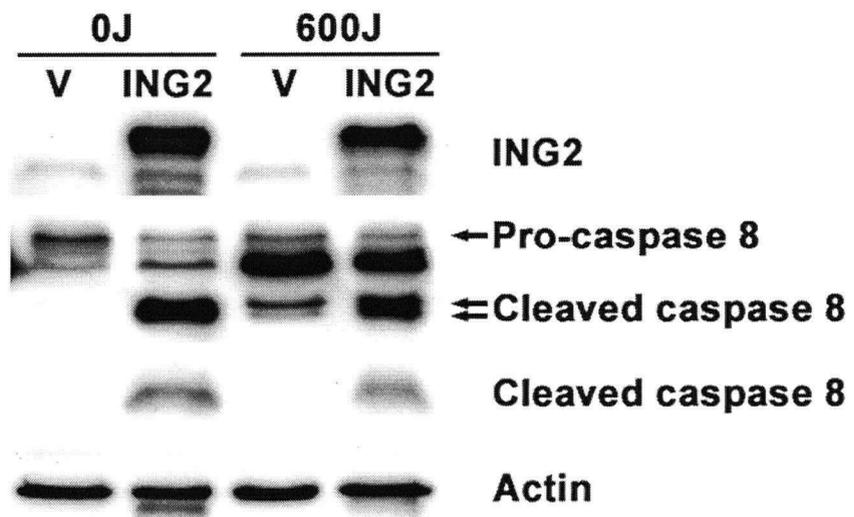
Since p33ING2 was cloned in 1998, there have only been three studies indicating the role of p33ING2 as a tumour suppressor candidate through regulation of gene transcription, induction of cell cycle arrest and apoptosis in human fibrosarcoma,

Figure 3.7 p33ING2 activates the death-receptor apoptotic pathway. MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-*ING2* (*ING2*) were irradiated with UVB at 0 or 600 J/m². Cells were harvested 24 h after UV irradiation and lysates were prepared for Western blot analysis using anti-p33ING2, anti-Fas (Ab-1), and anti-caspase 8 antibodies that detects both pro-caspase 8 (57 kDa) and the cleaved fragments (43, 41 and 18 kDa). β -actin was used as a loading control.

A



B



colorectal carcinoma, and hepatocellular carcinoma cell lines (Nagashima *et al.*, 2001; Gozani *et al.*, 2003; Kataoka *et al.*, 2003). Recent studies showed that overexpression of p33ING1b appeared to enhance DNA repair and apoptosis in melanoma cells and fibroblasts (Cheung *et al.*, 2001; Scott *et al.*, 2001a; Scott *et al.*, 2001b; Cheung and Li, 2002). Furthermore, p33ING1b has also been shown to translocate to the nucleolus and bind to PCNA after UV irradiation (Scott *et al.*, 2001a; Scott *et al.*, 2001b). It was reported that p33ING2 protein expression was specifically induced by DNA damaging agents such as etoposide in normal lymphoblastoid C3ABR cells and osteosarcoma OsA-CL cells, and that this induction of p33ING2 parallels the increase of acetylation of p53 at Lys382 (Nagashima *et al.*, 2001). However, information on the role of p33ING2 in the cellular stress response to UV irradiation is lacking.

To investigate the role of p33ING2 in UV stress response, we for the first time demonstrated that p33ING2 enhances UVB-induced apoptosis in melanoma cells, and this enhancement requires the participation of functional p53. This is consistent with the findings that its homologue p33ING1b also cooperates with functional p53 in cell growth control and apoptosis (Garkavtsev *et al.*, 1998; Shinoura *et al.*, 1999; Cheung and Li, 2002). Recent studies have shown that other ING family members, p47ING3, p29ING4 and p28ING5, like p33ING1b and p33ING2, induce growth arrest and apoptosis in a p53-dependent manner (Nagashima *et al.*, 2003; Shiseki *et al.*, 2003). p33ING1b, p29ING4, and p28ING5 are now known to co-precipitate with p53, while p33ING2 and p47ING3 do not (Nagashima *et al.*, 2001; Nagashima *et al.*, 2003; Shiseki *et al.*, 2003). In addition, p33ING1b, p33ING2, p29ING4, and p28ING5

are found to stimulate acetylation of p53 at Lys382 and/or Lys373 (Nagashima *et al.*, 2001; Kataoka *et al.*, 2003; Shiseki *et al.*, 2003), resulting in activation and stabilization of p53, and thus promote p53 sequence-specific DNA-binding and transcription activation (Gu and Roeder, 1997; Sakagushi *et al.*, 1998). Based on these findings, it is reasonable to propose that ING proteins enhance the p53 response through physical association with p53 and acetylation of p53. Furthermore, recent studies provide evidence that p33ING1b is involved in chromatin remodelling through physical association with SAP30 of the Sin3-histone deacetylase (HDAC) co-repressor complexes and with TRRAP, PCAF, and p300/CBP, which constitute histone acetyltransferase (HAT) co-activator complexes (Skowrya *et al.*, 2001; Kuzmichev *et al.*, 2002; Vieyra *et al.*, 2002). p53 has also been found to constitute HAT co-activator and HDAC co-repressor complexes (Feng *et al.*, 2002). It was reported that p53 could both induce and repress its downstream transcription targets coordinately, depending on the cell type and stress context (Weber and Zambetti, 2003). For instance, during p53-dependent apoptosis, pro-apoptotic genes *Bax*, *PUMA*, and *Noxa* are induced while anti-apoptotic genes *Map4* and *stathmin* are selectively repressed via recruitment of p53-Sin3A or p53-PID HDAC complexes (Zhang *et al.*, 1999b; Johnsen *et al.*, 2000; Hussein *et al.*, 2003a). Thus it is likely that p33ING2 cooperates with p53 in modulating the state of histone acetylation, which is known to affect gene expression (Feng *et al.*, 2002).

p33ING2 may cooperate with p53 in regulating the transcription of specific pro-apoptotic and anti-apoptotic genes in response to UV irradiation. The proper expression of pro-apoptotic and anti-apoptotic proteins dictates to the cells whether

to survive or undergo apoptosis. Bax, a downstream transcription target of p53, is a pro-apoptotic member of the Bcl-2 family that resides in the cytosol as a monomer. Upon induction of apoptosis, Bax undergoes conformational changes, translocates to the outer mitochondrial membrane and oligomerizes. This causes permeabilization of the outer mitochondrial membrane, possibly through the opening of permeability transition pore (PTP), therefore allowing efflux of apoptogenic factors such as cytochrome *c* and Smac/DIABLO (Hengartner, 2000; Cory and Adams, 2002; Hsu and Youle, 1998; Murphy *et al.*, 1999; Gross *et al.*, 1999; Shimizu *et al.*, 1999). In contrast, anti-apoptotic Bcl-2, a mitochondrial membrane protein, antagonizes the pro-apoptotic effect of Bax by forming heterodimers with Bax (Bush and Li, 2003). In this study, we found that p33ING2 upregulates the endogenous expression of Bax (Fig. 3.3A). However, this upregulation is not enhanced after UV irradiation, which does not explain the enhancement of apoptosis by p33ING2 seen in Fig. 3.1 and 3.2. Nevertheless, we also found that p33ING2 significantly downregulates Bcl-2 expression and increases the ratio of Bax/Bcl-2, most drastically after UV irradiation (Fig. 3.3B). The balance between the pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 determines the sensitivity of cells to apoptosis; cells with more pro-apoptotic proteins are sensitive to death, while cells with an excess of anti-apoptotic Bcl-2 family members are usually resistant (Hengartner, 2000). The increase of the Bax to Bcl-2 ratio by p33ING2 increases the overall active Bax expression levels, and thus enhances cell death via the mitochondrial apoptotic pathway. In addition, we found that, upon UV irradiation, p33ING2 promotes Bax translocation to the mitochondria, enables Bax oligomerization in the mitochondrial outer membrane,

enhances mitochondrial membrane potential changes, and thus induces cytochrome *c* release from the mitochondria into the cytosol (Fig. 3.4 and 3.5).

Once released, cytochrome *c* binds to the cytoplasmic scaffolding protein Apaf-1, causing ATP-dependent conformational changes that allow Apaf-1 to bind to the prodomain of an inactive initiator caspase, pro-caspase 9, to form a heptameric apoptosome (Hersey and Zhang, 2001; Igney and Krammer, 2002). Pro-caspase 9 in the apoptosome is activated by an allosteric change on Apaf-1 in the apoptosome (Li *et al.*, 1997; Stennicke *et al.*, 1990). The active caspase 9 then initiates a cascade of proteolytic activation of other effector/executioner caspases, first by cleaving pro-caspase 3 and 7, followed by cleavage of pro-caspase 6 by activated caspase 3. This parallels our observations that both caspase 9 and caspase 3 are cleaved after UV irradiation, and p33ING2 enhances the cleavage and thus activates both the initiator and effector caspases (Fig. 3.6). Surprisingly, caspase 8, an initiator caspase of the death-receptor pathway, is also found to be activated by p33ING2 (Fig. 3.7). This may have resulted from the upregulation of the death receptor Fas expression by p33ING2 (Fig. 3.7). The observations that p33ING2 alone, without UVB irradiation, can induce apoptosis (Fig. 3.2A) may be explained by the activation of Fas/caspase 8 pathway as a comparable amount of Fas and caspase 8 are activated in p33ING2-transfected cells with or without UVB irradiation (Fig. 7). Since Fas is also a transcription target of p53 (Johnstone *et al.*, 2002), it is possible that p33ING2 cooperates with p53 in upregulating Fas transcription. When activated, Fas homotrimerizes, leading to the recruitment of FAS-associated death domain (FADD) which further recruits pro-caspase 8 to form death-inducing signalling complex

(DISC). Pro-caspase 8 in DISC is activated by autocatalytic cleavage and the active caspase 8 activates effector caspases such as caspase 3, which explains the increased caspase 3 cleavage in p33ING2-overexpressing cells compared to vector control without UV irradiation (Fig. 3.6, lanes 1 and 2). The active effector caspases then cleave a number of cellular substrates, which lead to characteristic biochemical and morphological changes observed in Fig. 3.1C and E (Igney and Krammer, 2002). Since Bid is a transcription target of p53, and cleavage and activation of Bid by active caspase 8 can activate the mitochondrial pathway after apoptosis induction through death receptors (Luo *et al.*, 1998; Sax *et al.*, 2002), it would be of interest to investigate the role of Bid in p33ING2-mediated apoptosis and the cross-talk between the intrinsic and extrinsic apoptosis pathways.

In summary, this study demonstrates that p33ING2 cooperates with p53 to regulate apoptosis via activation of both the mitochondrial/intrinsic and death-receptor/extrinsic apoptotic pathways. Under non-stressed conditions, p33ING2 upregulates Fas expression and activates caspase 8. Upon UVB irradiation, p33ING2 significantly downregulates Bcl-2 expression and increases the Bax/Bcl-2 ratio, promotes Bax translocation to mitochondria, alters mitochondrial membrane potential, induces cytochrome c release and activation of caspase 9, and therefore enhances UVB-induced apoptosis. These findings provide a better understanding of the role of the tumour suppressor candidate p33ING2 in cellular stress response to UV irradiation, which may lead to new insights into more effective prevention and treatment regimes for human cutaneous melanoma.

CHAPTER 4. p33ING2 ENHANCES THE REPAIR OF UV-DAMAGED DNA IN HUMAN MELANOMA CELLS

4.1 Rationale and Hypothesis

DNA repair and apoptosis are two essential mechanisms by which cells maintain their genomic integrity. DNA damages accumulate due to replication errors, endogenous and environmental DNA-damaging agents such as reactive oxygen species (ROS) and ultraviolet (UV) rays. These damages can be removed by the DNA repair network, or otherwise senescence and apoptosis if the damages are beyond repair (Mitchell *et al.*, 2003). One of the major DNA repair pathways is nucleotide excision repair (NER), which eliminates a variety of DNA damage including UV-induced photolesions, cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs) (Tanaka *et al.*, 2001). The complete NER reaction involves a sequential assembly of repair proteins to the damage sites to mediate damage recognition, dual incision and gap-filling DNA synthesis (Petit and Sancar, 1999). Defects in NER lead to increased cancer incidence as observed in xeroderma pigmentosum (XP), developmental and neurological abnormalities as seen in Cockayne syndromes (CS) and trichothiodystrophy; all of these are characterized by a high sensitivity to sunlight (Mitchell *et al.*, 2003; Tanaka *et al.*, 2001).

Several studies have reported that the expression of wild-type p53 is required for efficient NER of UV-induced DNA damage. For instance, Li-Fraumeni fibroblasts homozygous for p53 mutation were found to be deficient in the rate and extent of NER of the genomic DNA (Ford and Hanawalt, 1995; 1997). Furthermore, reduced

NER was also observed when the wild-type p53 function was disrupted by the human papillomavirus E6 oncogene that targets p53 for degradation, or expression of a dominant-negative mutant p53 (Smith *et al.*, 1995). Recent studies have shown that p53 is involved both directly and indirectly in NER of UV-induced photolesions. The direct participation of p53 in this process is demonstrated by p53 binding to XPB and XPD helicases via its C-terminus (Wang *et al.*, 1995). p53 has also been shown to regulate both the basal and UV-inducible expression levels of p48, a subunit of damaged DNA binding protein (DDB), as well as the XPC gene (Hwang *et al.*, 1999; Adimoolam *et al.*, 2002). More recently, Rubbi and Milner (2003) have demonstrated that p53 can act as a chromatin accessibility factor that mediates UV-induced global chromatin relaxation by recruiting p300 to the sites of NER and inducing histone acetylation, suggesting indirect participation of p53 in NER.

p53 dependency of the tumour suppressive functions of p33ING2, as well as other members of the ING tumour suppressor family, has been well documented (Garkavtsev *et al.*, 1998; Shinoura *et al.*, 1999; Cheung and Li, 2002; Nagashima *et al.*, 2001; Nagashima *et al.*, 2003; Shiseki *et al.*, 2003). Two main mechanisms may help explain how ING proteins enhance p53 responses: the association of ING members with p53 and ING-mediated acetylation of p53 (Campos *et al.*, 2004a). Although p33ING2 does not physically associate with p53, it modulates the activity of p53 by enhancing the stability of p53 through acetylation at Lys382 and/or Lys373 (Nagashima *et al.*, 2001; Kataoka *et al.*, 2003). Recent studies indicate that the interaction of p33ING2 with phosphoinositides, PtdIns(3)P and PtdIns(5)P, through its plant homeodomain (PHD) zinc finger motif at the C-terminus is critical for its ability

to enhance p53 acetylation and mediate p53-dependent apoptosis (Gozani *et al.*, 2003). Previously, we found that overexpression of p33ING1b enhances the repair of UV-damaged DNA in melanoma cells, and that this process requires the presence of functional p53. In addition, the physical association between p33ING1b and GADD45 was detected, suggesting the involvement of p33ING1b in GADD45-mediated NER pathway (Cheung *et al.*, 2001b). More recently, we demonstrated that mutations at codon 102 or 260 as well as deletion of the PHD finger motif abolished p33ING1b enhancement in NER, suggesting that PHD zinc finger motif is required for proper DNA repair functions (Campos *et al.*, 2004b). In light of the structural and functional similarities between p33ING2 and p33ING1b (Feng *et al.*, 2002; Campos *et al.*, 2004a), we hypothesized that p33ING2 may also enhance the repair of UV-damaged DNA in a p53-dependent manner.

4.2 Results

4.2.1 p33ING2 Enhances the Repair of UV-damaged DNA

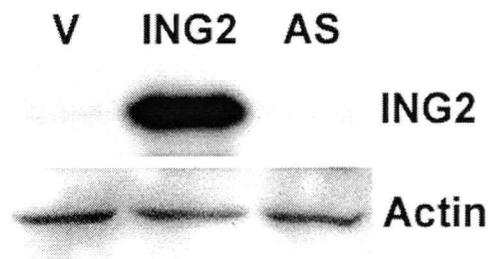
To investigate the role of p33ING2 in repairing UV-damaged DNA, we performed host-cell-reactivation assay by co-transfecting a UV-damaged pCMV_{CAT} plasmid that contains the chloramphenicol acetyltransferase reporter gene with either pcDNA3-vector, pcDNA3-p33ING2 or pcDNA3-AS-p33ING2 into melanoma MMRU cells. Western blotting confirmed the expression of these plasmids, suggesting successful transfection of MMRU cells (Fig. 4.1A). The activity of this reporter gene was used as an indicator of the extent of repair. We found that MMRU cells overexpressing p33ING2 had a significantly higher rate of repair of the UV-damaged plasmid (59.1%) compared to the vector (24.1%; $P < 0.01$, t-test) or antisense control (26.0%; $P < 0.01$, t-test) (Fig. 4.1B). These results indicate that p33ING2 enhances the repair of UV-damaged DNA in melanoma cells.

4.2.2 p33ING2 Does not Colocalize with UV-induced DNA Lesions

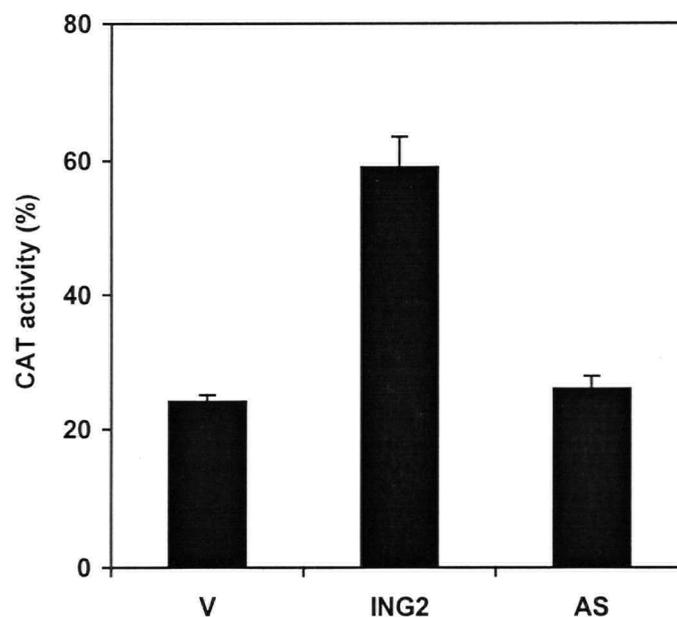
To study if p33ING2 is a component of the NER complex, MMRU cells transiently overexpressing p33ING2 were irradiated with UVC at 200 J/m² with an isopore polycarbonate filter containing 5 μm pores overlaid on the cells. Thus, only part of the nucleus was UV-irradiated through the microfilter, while remaining parts of the nucleus covered by the filter were blocked from the radiation. Therefore, this technique is useful in exploring protein colocalization with UV-induced DNA lesions. The intracellular localization of p33ING2 with two major UV-induced DNA lesions,

Figure 4.1 p33ING2 enhances the repair of UV-damaged DNA. (A) Western blot analysis of p33ING2 expression in MMRU cells transfected with pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2) or pcDNA3-antisense-p33/ING2 (AS) expression vector. An anti-p33ING2 monoclonal antibody was used to detect p33ING2 and β -actin was used as a loading control. (B) Assessment of the repair rate of UV-damaged DNA by host-cell-reactivation assay in MMRU cells co-transfected with undamaged or damaged pCMV_{CAT} and pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), or pcDNA3-AS-p33/ING2 (AS) expression vector. CAT activity was determined by scintillation counting and expressed as net dpm damaged dose/net dpm zero dose. Data represent means \pm SD from triplicates. The experiment was repeated thrice with similar results.

A



B

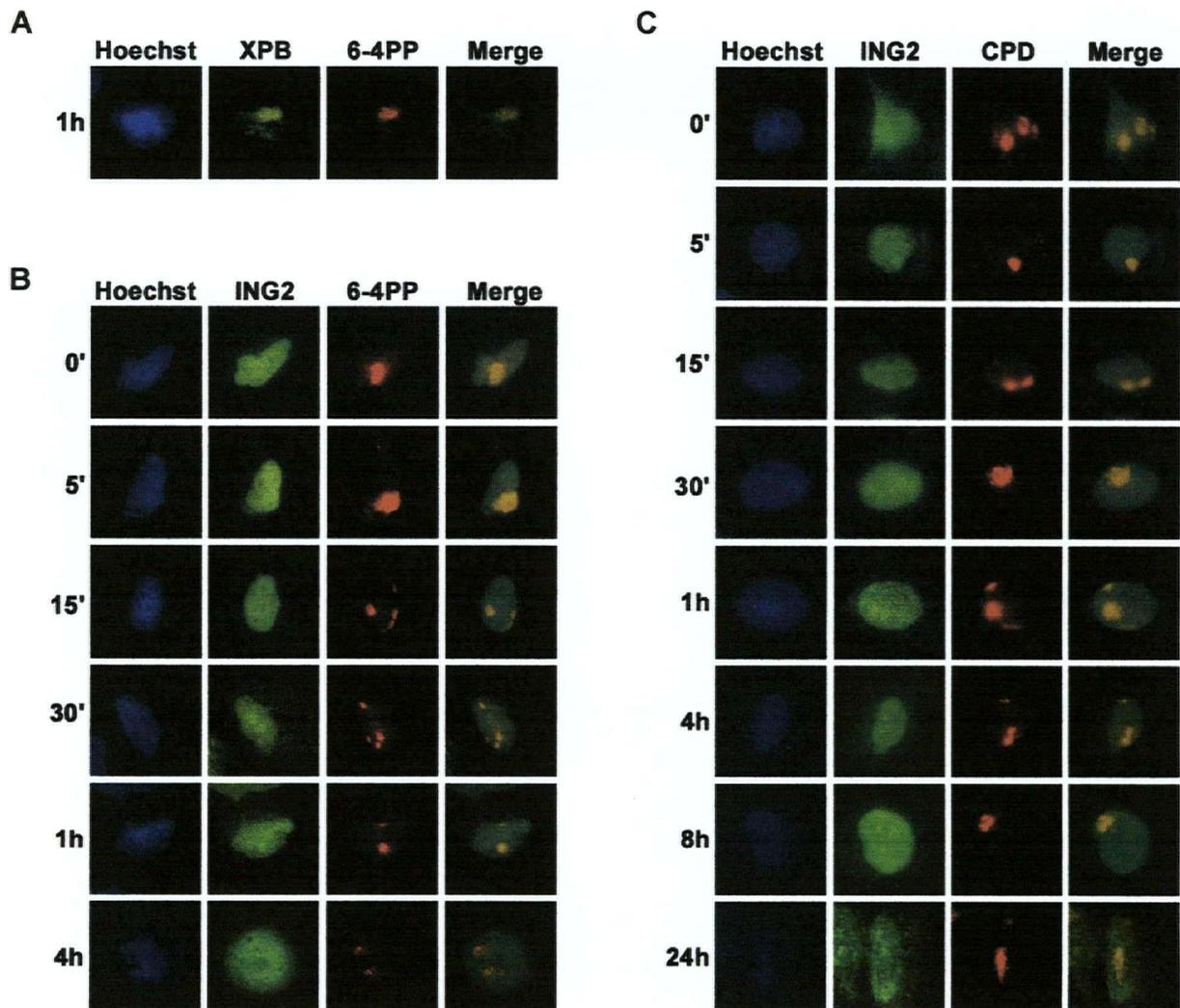


cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), at different time-points after UV irradiation were examined using immunofluorescent staining. Cells double-labelled for XPB and 6-4PPs were used as a positive control as it was previously shown that XPB colocalized with UV-induced DNA lesions (Wang *et al.*, 2003). Fig. 4.2A shows that the majority of XPB (green) colocalized with the 6-4PP sites (red) 1 h after UV irradiation. Consistent with a previous report (Gozani *et al.*, 2003), the majority of p33ING2, labelled with green fluorescence, was detected in the nucleus with a weak diffuse distribution in the cytoplasm (Fig 4.2B and C). The photolesions, 6-4PPs and CPDs were labelled with red fluorescence. The fluorescent intensity of 6-4PPs faded at a quicker rate than that of CPDs. It became very weak 4 h after UV irradiation and was undetectable after that, whereas the fluorescent intensity of CPDs remained strong in all time-points examined. Our results demonstrated that p33ING2 does not colocalize with either DNA lesions, 6-4PPs or CPDs at all time-points examined (Fig. 4.2B and C), suggesting that p33ING2 is not a component of the repair core complexes which are recruited to UV-induced DNA lesions.

4.2.3 p33ING2 Enhancement of DNA Repair Is p53-dependent

Since p33ING2 may be involved in the repair of UV-damaged DNA via indirect mechanisms, it is likely that p33ING2 cooperates with other factors in performing this function. Supported by the finding that its homologue, p33ING1b, requires functional p53 to enhance NER (Cheung *et al.*, 2001), we investigated whether p33ING2 enhancement of DNA repair requires the presence of p53. We disrupted the

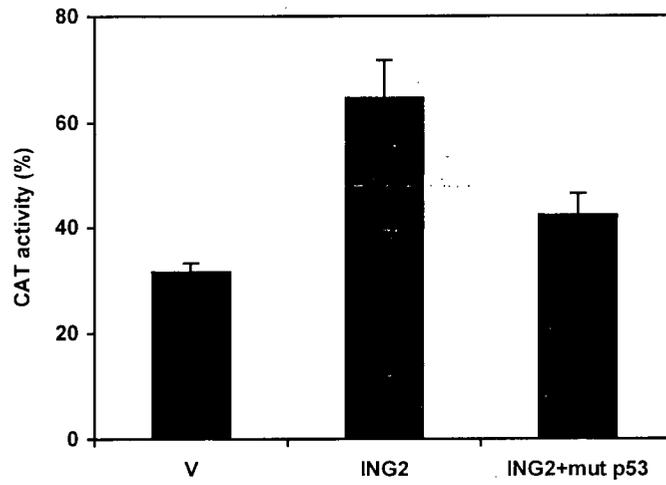
Figure 4.2 p33ING2 does not colocalize with UV-induced DNA lesions. (A) MMRU cells were fixed 1 h after exposure to UVC at 200 J/m² through a 5 μm isopore polycarbonate filter cover. Cells were double-labelled for XPB (green) and 6-4PPs (red). (B) and (C) MMRU cells transfected with p3XFLAG-ING2 were exposed to UVC at 200 J/m² through a 5 μm isopore polycarbonate filter cover. Cells were fixed at 0, 5, 15, 30 min and 1, 4, 8 and 24 h, and double-stained for p33ING2 (green) and 2 major UV photoproducts CPDs and 6-4PPs (red). Representative immunofluorescent staining images of 6-4PPs (B) and CPDs (C). Nuclei were stained blue with Hoechst stain. Magnification, 400×.



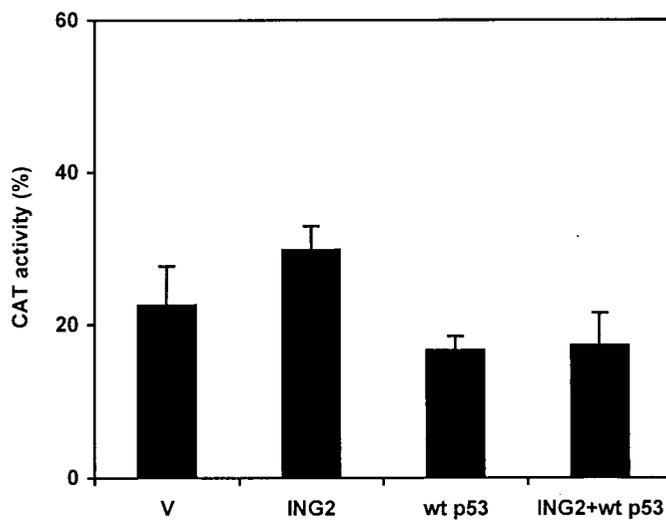
endogenous wt p53 in MMRU cells by introducing a dominant-negative mutant p53 pED-1 plasmid (Cheung *et al.*, 2001). Western blot analysis confirmed the expression of pED-1 (refer to Section 3.2.2, Fig 3.2A). Using host-cell-reactivation assay, we found that mutant p53 significantly reduced the enhancement of DNA repair by p33ING2 (42.4% in cells co-transfected with p33ING2 and pED-1 vs 64.7% in p33ING2-transfected cells; $P < 0.01$, t-test) (Fig. 4.3A). However, mutant p53 did not completely abrogate p33ING2-mediated UV-damaged DNA repair. This may be due to the endogenous wt p53 not being completely inactivated. It has been shown that the expression levels of p33ING2 in cells transfected with pED-1 were similar to those in the vector control (refer to Section 3.2.2, Fig 3.2A), eliminating the possibility that the reduced rate of DNA repair seen in pED-1-transfected cells was due to downregulated p33ING2 expression by mutant p53. Next, we performed host-cell-reactivation assay using MeWo cells that carry a mutant p53 gene to further validate the p53 dependence of p33ING2 enhancement in NER. Our results showed that p33ING2 did not enhance the repair of UV-damaged DNA in the absence of functional p53 (Fig. 4.3B). We also transfected MeWo cells with wt p53 (p β hwtp53) or wt p53 together with p33ING2, to see if re-introduction of wt p53 could restore the enhancement of NER by p33ING2. The expression of p33ING2 and wt p53 (p β hwtp53) in MeWo cells was confirmed using Western blot analysis (refer to Section 3.2.2, Fig 3.2C). Our data in Fig. 4.3B showed that neither transfection with wt p53 alone nor co-transfection with p53 and p33ING2 increased the DNA repair capacity in MeWo cells. This may be due to the presence of dominant negative p53 mutation in MeWo cells that abrogates the effect of the wt p53 re-introduced into the

Figure 4.3 p33ING2 enhancement of DNA repair is p53-dependent. (A) Host-cell-reactivation assay was performed in MMRU cells co-transfected with UV-damaged pCMV_{CAT} and pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), or pcDNA3-p33/ING2 and mutant p53 pED-1 (ING2+mut p53). (B) Host-cell-reactivation assay was performed in MeWo cells co-transfected with UV-damaged pCMV_{CAT} and pcDNA3-vector (V), pcDNA3-p33/ING2 (ING2), wild-type p53 (wt p53) alone or pcDNA3-p33/ING2 and wild-type p53 (ING2+wt p53). Data represent means \pm SD from triplicates. The experiment was repeated twice with similar results.

A



B



cells. These observations suggest that functional p53 is required for p33ING2 enhancement of DNA repair.

4.2.4 p33ING2 Enhances p53 Acetylation upon UV Irradiation

Recent findings that p33ING2 and other ING proteins (p33ING1b, p29ING4 and p28ING5) (Campos *et al.*, 2004a) induced p53 acetylation in various cell lines prompted us to examine whether p33ING2 enhances p53 acetylation after UV irradiation. We exposed MMRU cells overexpressing p33ING2 and control cells to UVB irradiation at 200 J/m², and examined the expression levels of acetylated p53 at different time-points (Fig. 4.4A). MMRU cells treated with 0.2 µg/ml of doxorubicin for 8 h were used as a positive control for p53 acetylation. p33ING2 induced p53 acetylation in both non-UV- and UV-irradiated cells compared to the vector control. Induction of p53 acetylation was associated with a maximal accumulation of p53 protein in cells overexpressing p33ING2 4 h after UV irradiation (Fig. 4.4A).

4.2.5 p33ING2 Upregulates XPA

Induction of p53 acetylation by p33ING2 after UV irradiation led us to hypothesize that p33ING2 may upregulate the p53 downstream target GADD45, which has been shown to be essential in repairing UV-damaged DNA (Smith *et al.*, 2000). We irradiated MMRU cells transfected with empty vector or p33ING2 with UVB at 200 J/m² and performed Western blot analysis 24 h later. We found that p33ING2 did not significantly increase GADD45 expression in both non-UV- and UV-irradiated cells (Fig. 4.4B). To further study the pathways involved in p33ING2-mediated DNA

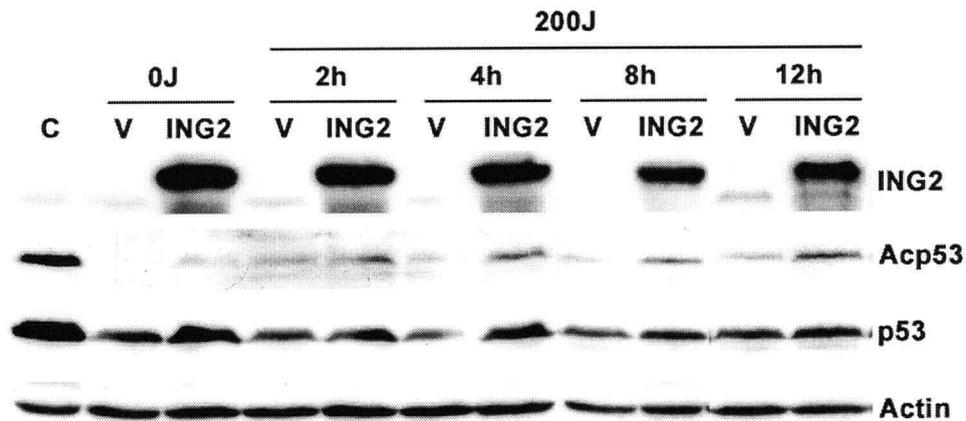
repair, we examined whether p33ING2 regulates the expression of XPA and XPB, which are part of the core complexes involved in NER. Our results revealed that p33ING2 significantly upregulated XPA expression. Under non-UV-irradiation conditions, the expression level of XPA in cells overexpressing p33ING2 was 2-fold higher than that in the vector control ($P < 0.01$, t-test); whereas after UV irradiation, cells overexpressing p33ING2 showed 5-fold induction of XPA compared to 1.4-fold in the vector control ($P < 0.001$, t-test) (Fig. 4.4B). However, p33ING2 did not upregulate the expression levels of XPB. Since p33ING1b has been shown to bind to GADD45 (Cheung *et al.*, 2001), we investigated to see if p33ING2 physically binds to GADD45, XPA, or XPB. We performed immunoprecipitation with an anti-FLAG mouse monoclonal antibody and found that p33ING2 did not physically interact with GADD45, XPA or XPB (Fig. 4.4C). These results suggest that XPA may be a crucial component in p33ING2-mediated repair of UV-damaged DNA.

4.3 Discussion

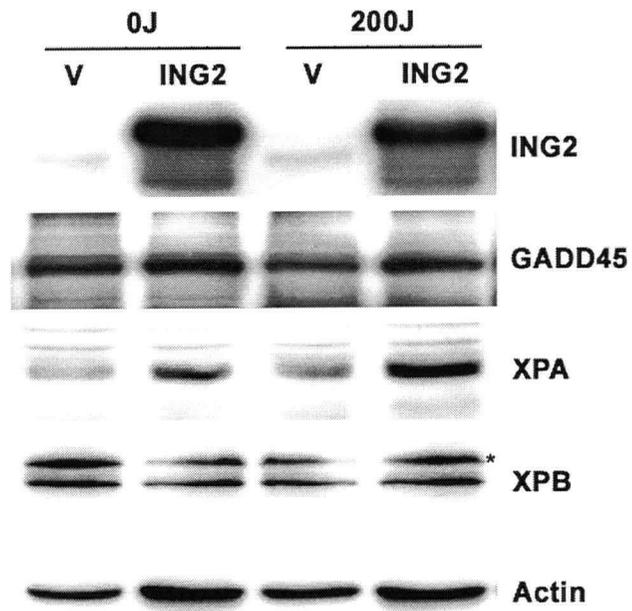
Previous studies have shown that p33ING2 is involved in the regulation of gene transcription, cell cycle progression, and apoptosis in various cancer cell lines including colorectal carcinoma, hepatocellular carcinoma and human fibrosarcoma cells, and that it requires the presence of wt p53 to exert these tumour suppressive functions (Nagashima *et al.*, 2001; Kataoka *et al.*, 2003; Gozani *et al.*, 2003). In this study, we for the first time demonstrated that overexpression of p33ING2 enhances the repair of UV-damaged DNA, and that this enhancement requires the participation of functional p53 (Fig. 4.1 and 4.3). We also showed that p33ING2 participates

Figure 4.4 p33ING2 enhances p53 acetylation and upregulates XPA. (A) Western blot analysis of acetylated p53 levels in MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-*ING2* (ING2). Cells were irradiated with UVB at 0 or 200 J/m² and harvested 2, 4, 8 and 12 h after UV irradiation. Antibodies against p33ING2, acetylated p53 at Lys382, and total p53 were used. Anti-p33ING2 antibody detected both endogenous (lower bands) and transfected p3XFLAG-*ING2* (upper bands). MMRU cells treated with 0.2 μg/ml of doxorubicin for 8 h were used as a positive control (C). (B) Western blot analysis of GADD45, XPA and XPB expression levels in MMRU cells transfected with p3XFLAG-vector (V) or p3XFLAG-*ING2* (ING2) 24 h after UVB irradiation at 0 or 200 J/m². Antibodies against p33ING2, GADD45, XPA and XPB were used. β-actin was used as a loading control. * indicates non-specific bands. (C) Densitometry of XPA induction in (B). (D) Co-immunoprecipitation (IP) of p33ING2 with GADD45, XPA and XPB. Total cell lysates of MMRU transfected with p3XFLAG-*ING2* were immunoprecipitated with a control antibody (Lane 2) or with anti-FLAG antibody (Lane 3). Ten percent of lysate input for immunoprecipitation (Lane 1) was used as a control and the supernatant of control of p33ING2 IP (Lane 4) was used to verify the IP. Antibodies against p33ING2, GADD45, XPA and XPB were used in Western blot analysis.

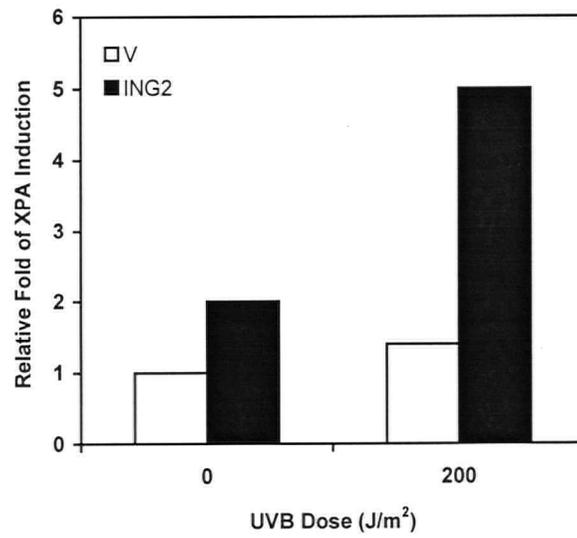
A



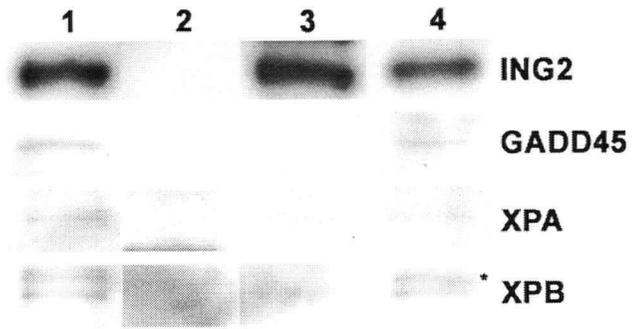
B



C



D



indirectly in the repair of UV-damaged DNA as p33ING2 does not colocalize with either of the two major UV-induced photolesions, CPDs and 6-4PPs (Fig. 4.2). These results suggest that p33ING2 is not directly recruited to the DNA damage sites with other repair core complexes required for damage recognition (XPC-hHR23B, XPE-DDB1 and XPA-RPA), excision of the lesions (TFIIH/XPB-XPB, XPG and XPF-ERCC1), gap-filling DNA synthesis and ligation (DNA polymerase δ or ϵ , DNA ligase I) (Wang *et al.*, 2003; Mitchell *et al.*, 2003). Although p53 was found to bind XPB and XPD *in vitro* (Wang *et al.*, 1995), this association was not validated *in vivo* and p53 too did not colocalize to the DNA damage sites in normal human fibroblasts following UV irradiation (Fitch *et al.*, 2003). The p53-dependence of p33ING2 enhancement of UV-damaged DNA repair suggests that p33ING2 may enhance NER by regulating the p53 DNA repair pathway.

A few lines of evidence indicate that p53 is required for efficient NER of UV-induced DNA lesions and chemical-induced bulky DNA adducts. Thus, it plays an important role in protecting cells from tumourigenesis (Rubbi and Milner, 2003). p53 has been shown to bind and modulate the activities of the NER-associated helicases XPB and XPD (Wang *et al.*, 1995). Furthermore, p53 can sustain the expression of the p48 component of the DDB2 complexes and *XPC* gene, thus participating at least indirectly in NER (Hwang *et al.*, 1999; Adimoolam *et al.*, 2002). More recently, it has been reported that p53 acts as a chromatin accessibility factor, mediating UV-induced global chromatin relaxation by recruiting p300 to the sites of NER and inducing histone acetylation (Rubbi and Milner, 2003). However, the exact molecular mechanism by which p53 enhances NER is yet to be determined.

We found that overexpression of p33ING2 induces p53 acetylation in melanoma cells after UV irradiation (Fig. 4.4A). This is in agreement with the finding that p53 acetylation at Lys382 in the normal lymphoblastoid C3ABR cell line after exposure to DNA-damaging agents, etoposide or neocarzinostatin, correlated with an increased expression level of p33ING2 (Nagashima *et al.*, 2001). Therefore, it is reasonable to conclude that p33ING2 enhances p53 acetylation, at least at residue Lys382, in response to DNA damage. It is well known that post-translational modifications such as acetylation and phosphorylation of p53 play a crucial role in the stabilization and activation of p53 as well as in protein-protein interactions (Xu, 2003). Several studies have shown that p53 acetylation within the C-terminal region regulates p53 stability by preventing ubiquitination by mdm-2 (Xu, 2003). There is also evidence to suggest that acetylation of p53 within the C-terminal region results in a conformational change that stimulates p53 sequence-specific DNA-binding activity (Gu and Roeder, 1997; Sakagushi *et al.*, 1998), and recruits CBP/p300 transcription co-activators to p53-dependent promoters (Xu, 2003), thus activating the transcriptional activity of p53. Our observations support the notion that p33ING2 enhances p53 acetylation, thus increasing the stability of p53 protein, as there was more p53 accumulated in cells overexpressing p33ING2 compared to the vector control up to 4 h after UV irradiation (Fig. 4.4A). The increased p53 acetylation by p33ING2 may account for the p53-dependence of p33ING2 enhancement in NER.

The p33ING2 enhancement in NER may also be achieved in part by upregulating the expression of the repair recognition protein XPA. We demonstrated that p33ING2 upregulates the expression of the *XPA* gene, but not the *XPB* gene, in

both UV- and non-UV-irradiated cells, with more pronounced upregulation of *XPA* after UV irradiation (Fig. 4.4B). Since DNA damage recognition is the rate-limiting step in repair and XPA is a component of the XPA-RPA DNA damage recognition complex (Thoma *et al.*, 2003), upregulation of XPA by p33ING2 may significantly enhance the DNA repair process. Previously, we found that p33ING1b physically interacts with GADD45 but does not transcriptionally regulate *GADD45* (Cheung *et al.*, 2001). In this study, we found that p33ING2 neither physically interacts with nor upregulates the expression of GADD45. These findings may reflect the distinct molecular pathways in which p33ING1b and p33ING2 are involved in mediating DNA repair. In summary, we demonstrate that p33ING2 enhances the repair of UV-damaged DNA in a p53-dependent manner. It appears that p33ING2 is not directly recruited to UV-induced DNA damage sites. The p33ING2-mediated enhancement in NER is mostly likely achieved by increasing p53 acetylation and upregulating the expression of XPA.

CHAPTER 5. GENERAL CONCLUSIONS

5.1 Summary

p33ING2 has been shown to share many biological functions with its homologue p33ING1b in regulating cell cycle, apoptosis and gene transcription. These functions have been shown to require the presence of functional p53. Despite their functional similarities, p33ING1b and p33ING2 modulate the activity of p53 via distinct modes of action. While p33ING1b has been shown to physically associate with p53, p33ING2 has been found to interact with phosphoinositides and induce p53 acetylation in response to cellular stress. Several studies have provided evidence that p33ING1b is involved in UV-induced responses. However, study of the role of p33ING2 in cellular stress response to UV irradiation is lacking. In this thesis, we have sought to investigate the role of p33ING2 in cellular stress responses to UV irradiation and the molecular mechanisms involved.

We first investigated to see if p33ING2 is involved in UVB-induced apoptosis, using melanoma cells as an experimental model system. We found that overexpression of p33ING2 enhanced UVB-induced apoptosis. We also found that the p33ING2 enhancement of UVB-induced apoptosis required the presence of wild-type p53. Moreover, we have demonstrated that p33ING2 significantly downregulated Bcl-2 expression and increased the Bax/Bcl-2 ratio, promoted Bax translocation to mitochondria, altered mitochondrial membrane potential, induced cytochrome c release and activation of caspase 9, therefore enhancing UVB-induced apoptosis. It is interesting, however, under non-stressed conditions, p33ING2

upregulated Fas expression and activated caspase 8 to induce apoptosis. Based on our findings, we therefore concluded that p33ING2 cooperates with p53 to regulate apoptosis via activation of both the mitochondrial/intrinsic and death-receptor/extrinsic apoptotic pathways.

Despite the distinct molecular mechanisms involved, similarities in the role of p33ING2 and p33ING1b in UVB-induced apoptosis prompted us to investigate whether ING2 plays a role in the repair of UV-damaged DNA. We found that overexpression of p33ING2 enhanced the repair of UV-damaged DNA, and that p53 was required for this process in melanoma cells. We also found that p33ING2 was not directly recruited to UV-induced DNA damage sites. Nevertheless, we demonstrated that overexpression of p33ING2 enhanced p53 acetylation and the expression of XPA, with more profound enhancement after UVB irradiation. These observations suggest that p33ING2 enhances the repair of UV-damaged DNA, and that this enhancement is most likely achieved by increasing p53 acetylation and upregulating the expression of XPA.

Taken together, we have elucidated in this thesis the novel functions of p33ING2 in cellular stress responses to UV irradiation and the significance of this gene in the context of tumour suppression. With further exploration, our knowledge of this gene may lead to new insights into more effective prevention and treatment regimes for human cutaneous melanoma.

5.2 Future Directions

Activation of the Fas death-receptor pathway leads to cleavage and activation of Bid by caspase 8, promoting oligomerization of Bax and Bak, resulting in cytochrome *c* release (Wei *et al.*, 2000; Luo *et al.*, 1998), thus providing a link between the death-receptor and the mitochondrial pathway. Furthermore, Bid is a transcriptional target of p53 (Sax *et al.*, 2002). It would be interesting to see if Bid is upregulated and/or activated in p33ING2-mediated apoptosis and if cross-talk between these two apoptotic pathways is involved. Recent studies reported that Smac (second mitochondria-derived activator of caspase) is one of the factors released from the mitochondria during apoptosis (Martinou and Green, 2001; van Loo *et al.*, 2002). Smac promotes apoptosis by releasing caspase 3 from the inhibition by inhibitors of apoptosis (IAPs) and inducing cleavage of caspase 3 (Wu *et al.*, 2000; Chai *et al.*, 2000). Furthermore, Smac peptides were shown to sensitize lymphoma cells to TRAIL-induced apoptosis (Gua *et al.*, 2002). It would be of interest to determine if Smac has a role in p33ING2-mediated apoptosis.

In this thesis, we clearly show that p33ING2 upregulates the expression of XPA. It is crucial to determine if p33ING2 transcriptionally upregulates XPA or stabilizes the XPA protein, and if induction of XPA by p33ING2 is p53-dependent. It is reported that p53 recruits p300 to NER sites and induces histone acetylation to relax the chromatin to allow the accessibility of other repair proteins (Rubbi and Milner, 2003). Since p33ING2 enhancement of NER is p53-dependent and p33ING2 is not recruited to NER sites, it will be interesting to determine if p33ING2 is required

to recruit p300 to the NER sites and cooperates with p53 in acetylating histones upon UV irradiation.

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