ROLE OF THE NOVEL TUMOR SUPPRESSOR P33ING2 IN CELLULAR STRESS RESPONSE TO ULTRAVIOLET IRRADIATION IN MELANOMA CELLS

By

JING WANG

M.B., Hebei Medical University, 2000

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

MASTER OF SCIENCE

IN

THE FACULTY OF GRADUATE STUDIES

(EXPERIMENTAL MEDICINE)

THE UNIVERSITY OF BRITISH COLUMBIA

April 2006

ABSTRACT

P33ING2 is a candidate tumor suppressor, which shares 58.9% homology with p33ING1b, the founding member of the inhibitor of growth (ING) family, and has been shown to be involved in the regulation of gene transcription, cell cycle arrest and apoptosis in a p53-dependent manner for maintaining the genomic stability. Previously, we demonstrated that p33ING2 promoted UV-induced apoptosis via activation of both the mitochondria/intrinsic and death-receptor/extrinsic apoptotic pathways upon high UV irradiation. Recently, studies have shown that p33ING2 may complex with acetyltransferase (FAT) or histone deacetylase (HDAC). To further reveal the role of p33ING2 in the cellular stress response to UV irradiation, we hypothesized that p33ING2 may enhance the repair of UV-damaged DNA, similar to its homologue p33ING1b, through facilitating histone acetylation and chromatin modification, which are believed to be essential for gene transcription regulation. Using luciferase reporter assays, overexpression of p33ING2 is shown to significantly enhance nucleotide excision repair of UV-induced DNA damage in melanoma cells in a p53-dependent manner. Furthermore, DNA repair is completely abolished in cells treated with p33ING2 siRNA, suggesting that a physiological level of p33ING2 is required for nucleotide excision repair. In addition, p33ING2 is found to be an essential factor for UV-induced rapid histone H4 acetylation, chromatin relaxation, and the recruitment of damage recognition protein XPA to the photolesions. These observations suggest that p33ING2 is required for initial DNA damage sensing and chromatin remodeling in the nucleotide excision repair process.

As a member of the ING family, p33ING2 contains a number of highly conserved regions with other ING members, including the plant homeodomain (PHD), leucine zipper-like domain (LZL), and potential chromatin regulatory domain (PCR). Previous studies focused on PHD region of ING proteins since it complexes with histone acetyltransferase (HAT) in its yeast homologue. However, the specific biological functions of other motifs are poorly understood. In this study, using a panel of p33ING2 deletion mutants, we performed functional analyses of different domains of p33ING2. We found that the LZL domain is critical for a functional p33ING2 in both UV-induced DNA repair and apoptosis.

In summary, the evidence presented in this thesis that p33ING2 plays an essential role in DNA repair, apoptosis and chromatin remodeling strongly supports the notion that p33ING2 acts as a tumor suppressor.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

6-4PPs	6-4 photoproducts
aa	Amino acid
Ac-H4	Acetylated histone H4
Apaf-1	Apoptotic protease activating factor-1
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia related
BCC	Basal cell carcinoma
BRMS1	Breast cancer metastasis suppressor-1
CBP	CREB binding protein
CDKs	Cyclin-dependent kinases
CPDs	Cyclobutane pyrimidine dimers
CPT	Camptothecin
DMEM	Dulbecco's modified eagle's medium
FATs	Factor acetyltransferases
FBS	Fetal bovine serum
GADD45	Growth arrest and DNA damage protein
HAT	Histone acetytransferase
HDAC	Histone deacetylase
HRP	Horseradish peroxidase
ING1	Inhibitors of growth 1
LOH	Loss of heterozygosity
LZL	Leucine zipper–like
MAPK	Mitogen-activated protein kinase
MDM2	Mouse double minute 2
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NTS	Nucleolar translocation sequences
PBS	Phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	Proliferating cell nuclear antigen
PCR	Potential chromatin regulatory domain
PDIM	Phosphorylation-dependent interacting motif
PHD	Plant homeodomain
PI	Propium iodine
PIM	Peptide-interacting motif
PIP	PCNA-interacting protein
PUMA	P53-upregulated modulator of apoptosis
PtdInsPs	Phosphoinositides
PVDF	Polyvinylidene difluoride
RT-PCR	Reverse transcriptase-polymerase chain reaction
SAP30	Sin3-associated protein 30
SCC	Squamous cell carcinoma

UV	Ultraviolet
siRNA	Small interference RNA
TNF	Tumor necrosis factor
WT	Wild-type
XP	Xeroderma pigmentosum

ACKNOWLEDGEMENTS

Here, I would like to thank my supervisor, Dr. Gang Li, who accepted and supported me for my master studies in UBC. Without his intellectual guidance and stimulation, I could not make such a big progress in my work. To Dr. Vincent Ho, Dr. Peggy Olive, Dr. Weiguo Jia, and Dr. Kevin McElwee, thank you for being my committee members. Your valuable advices and suggestions on my projects really mean a lot. To Mr. Patrick Craw, the secretary of the Experimental Medicine Program, I really appreciate your help and encouragement in my master studies. To all the Li lab members, who are not only my colleagues, but also my friends, I feel so proud of being a member of this wonderful team. To my family, your love and support always provide me the strength and confidence in my way of pursuing the success.

CHAPTER 1. INTRODUCTION

1.1 Skin Cancer

Skin cancer, which accountsfor about 45% of all human malignancies in North America annually (Landis *et al.*, 1999), is a disease in which malignant cancer cells originate from the epidermis of the skin. It is estimated that 1 out of 7 people in North America will develop some form of this cancer during their lifetime. A serious sunburn can increase the risk by as much as 50% (National Cancer Institute PDQ Statement, 1997). As the most common type of cancer in fair-skinned populations around the world, the incidence and mortality rates of skin cancers are significantly increasing and thus pose a threat to public health (Saladi *et al.*, 2005).

1.1.1 Classification and Pathology of Skin Cancer

There are two major types of skin cancer. Non-melanom skin cancer, including basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), which derives from the keratinocytes are the most common types of cancer that occur in the skin. Melanoma, which derives from the melanocytes, is a more serious type of cancer than non-melanoma skin cancer.

Basal cell carcinoma of the skin is the most common type of human cancer, responsible for almost a third of all cancer diagnoses inNorth America. Although it has been noted that most of the BCC cases is sporadic, there is a rare familial syndrome basal cell nevus syndrome (BCNS) that predisposes to the development of BCC. The etiology of BCC indicates UV exposure as a most essential risk factor for the cancer development

(Kenneth *et al.*, 2004). Recently, studies have shown that mutations in components of hedgehog pathway, TP53 or p53 family members proteins, which can induce aberrant activation of signaling, account for BCNS and sporadic BCC. For instance, inactivation of the PTCH1 gene on chromosome 9q22, a negative regulator of hedgehog signaling, is known to be one of the reasons for BCNS (Hahn *et al.*, 1996; Johnson *et al.*, 1996). In addition, mutations in TP53 occur with more than 50% frequency in sporadic BCC, which is usually along with the UV signature CC→TT and C→T transitions upon ultraviolet radiation B (UVB) exposure (Rady *et al.*, 1992; Moles *et al.*, 1993; Ziegler *et al.*, 1993; Gailani *et al.*, 1996a; D'Errico *et al.*, 1997; Zhang *et al.*, 2001). Besides, biallelic mutations and loss of heterozygosity (LOH) in PTCH1 have been observed in sporadic BCC (Gailani *et al.*, 1996b; Aszterbaum *et al.*, 1998; Evans *et al.*, 2000). Furthermore, the characteristic DNA lesions created by UV exposure occurs at neighboriingpyrimidines and account for cyclobutane pyrimidine dimers or 6-4 photoproducts. These damages can be removed by the DNA repair machinery, or 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-induce photolesions. 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 *et al.*, 1999). When these photoproducts are inappropriately repaired, they will generate mutations (Mullenders *et al.*, 1993).

SCC is another non-melanoma skin cancer. BCC is not usually derived from a precursor lesion, while SCC mostly arises from actinic keratoses (Marks *et al.*, 1988).

Changes for the development of SCC are extremely influenced by the nature and dosage of UV radiation and genetic backgrounds that regulate that interaction. As to the genetic disorder of SCC, like BCC, mutations in TP53 have also been described in actinic keratoses, in situ SCC, and invasive SCC with UV signature lesions, and a reported mutation rate is up to 45% (Brash *et al.*, 1991; Campbell *et al.*, 1993; Kubo *et al.*, 1994; Taguchi *et al.*, 1994; Ziegler *et al.*, 1994; Rehman *et al.*, 1996). In addition, a critical association between the Arg/Arg polymorphism at p53 codon 72 and non-melanoma skin cancer in renal transplant recipients has been observed (McGregor *et al.*, 2002). These phenomena are further verified by experimental observations using p53 knockout mice, in which UV irradiation gives rise to tumors in the skin resembling SCC (Ziegler *et al.*, 1994; Li *et al.*, 1998; Jiang *et al.*, 1999). In the absence of p53, there was a compromised apoptotic response normally induced by radiation indicating that p53 functions to eliminate incipient cancer cells in this situation (Ziegler *et al.*, 1994). Thus, these results demonstrated that loss of p53 function may be critical in the actinic keratoses and subsequent SCC formation. Furthermore, several studies showed that CDKN2A locus in SCC and mutations in exon 2 or DNKN2A locus with hypermethylation, which are very easy to be seen in both $p16^{INK4A}$ and $p14^{ARF}$, have been implicated in a significant proportion of SCCs (Kubo *et al.*, 1997; Soufir *et al.*, 1999). In addition, UV-induced mutations in H-RAS and K-RAS have also been characterized in both actinic keratoses and SCC (Pierceall *et al.*, 1991; Spencer *et al.*, 1995). An important pathogenetic role for RAS activation in SCC was identified by mouse models in which oncogenic H-RAS and NF-κB inhibition, leads to lesions resembling invasive SCC (Dajee *et al.*, 2003).

Cutaneous melanoma, is 2-5% of total skin cancers, which is associated with one of the highest mortality rates due to its rapid metastasis and 5-year survival rate of patients with metastatic melanoma remains less than 10% (Roses *et al.*, 1991). As in BCC and SCC, exposure to UV radiation is implicated in the melanoma development, particularly intermittent intense exposure (Gilchrest *et al.*, 1999). Recently, genetic abnormalities associated with melanoma tumorigenesis have been well established, such as germline mutations in CDKN2A, which encodes two different proteins ($p16^{FNKA}$ and $p14^{ARF}$), accounts for familial melanoma, which contributes to some 10% of all cases of melanoma. $P16^{INK4A}$ is an inhibitor of the cyclin-dependent kinases (CDK) 4 and 6 and functions through pRB-mediated regulation of the G_1-S transition of the cell cycle (Serrano *et al.*, 1993, 1995; Koh *et al.*, 1995). $P14^{ARF}$ functions by stabilizing p53 through inhibition of MDM2 activity (Lowe *et al.*, 2003). Furthermore, $p14^{ARF}$ can be upregulated by oncogenes such as MYC and RAS (Lowe *et al.*, 2003). In addition, RAS pathway has also been demonstrated to be involved in the formation of melanoma. It has been known that activating RAS mutations, especially in N-RAS (van 't Veer *et al.*, 1989; Ball *et al.*, 1994; Carr and Mackie, 1994; Platz *et al.*, 1994; Jiveskog *et al.*, 1998; Demunter *et al.*, 2001; Omholt *et al.*, 2002), are observed in melanoma. Recently, a downstream effector BRAF of the RAS pathway is found to be strongly related in human melanoma by identifying the dominant activating mutations in the *BRAF* in a majority of both melanoma samples, melanoma cell lines, and nevi (Davies *et al.*, 2002; Pollock *et al.*, 2003). Furthermore, through the genomic study of melanoma, RhoC overexpression was implicated in the transition to progressively metastatic phenotypes (Clark *et al.*, 2000), indicating another potential RAS pathway effector. The antiapoptotic factor, Bcl-

2, was also identified as a target gene of the melanocyte-specific transcription factor, microphthalmia (Mitf) (McGill *et al.*, 2002). It has been noted that increased Bcl-2 expression could rescue melanocytes after introduction of dominant-negative Mitf (McGill *et al.*, 2002). Finally, allelic loss and hypermethylation of the pro-apoptotic gene APAF1 have been seen in metastatic melanoma biopsies and melanoma cell lines (Soengas *et al.*, 2001), providing another explanation for the characteristic chemoresistance of this tumor type.

1.1.2 Etiology

1.1.2.1 UV and skin cancer

The incidence of melanoma has been rising during the past 40 years and understanding the pathogenesis of skin cancer becomes a goal for public health system. Ultraviolet radiation (UVR) is believed to be the main causative factor in melanoma development with an acute intermittent exposure being more relevant than a chronic cumulative one. Sunburns and excessive exposures cause cumulative damage, which induces immunosuppression and skin cancers. Some treatment modalities, including radiation therapy, phototherapy and psoralen and long-wave ultraviolet radiation (PUVA) can also induce skin cancers (Saladi *et al.*, 2005). UVB (280-320 nm) can directly cause DNA damage (UVB fingerprint mutations) in the cells of the epidermis, while UVA (320-400 nm) induces damage indirectly by the formation of reactive oxygen species. Since UV-associated mutations are rare in melanoma, it is speculated that UVR induces melanoma development by indirect effects, for instance, immunosuppression or stimulation of growth factors in the skin (Berking, 2005).

Other pathogenesis factor including viral infections such as the human papilloma virus can cause squamous cell carcinomas. Ionizing radiation, environmental pollutants, chemical carcinogens and work-related exposures have been associated with skin cancers. Exposure to artificial UV radiation (tanning beds and lamps), aging, skin color, diet and smoking are attributable risks (Saladi *et al.*, 2005).

Ultraviolet light can lead to the formation of several types of mutagenic DNA lesions. The cis-syn cyclobutane pyrimidine dimers (CPDs), which are formed between the 5,6 bonds of any two adjacent pyrimidine bases and the pyrimidine (6-4) pyrimidone photoproducts [(6-4)PPs], which are formed by a stable bond between positions 6 and 4 of two neighboring pyrimidines are the most frequent lesions induced by UVB or UVC radiation. Several minor photoproducts such as purine dimers and pyrimidine mono-adducts are also formed (Pfeifer *et al.*, 1997). They are formed at levels considerably lower than those of CPDs (Mitchell *et al.*, 1989; Yoon *et al.*, 2000). UVA irradiation may induce CPDs in DNA (Rochette *et al.*, 2003; Douki *et al.*, 2003) and also, through an indirect mechanism, can promote the formation of oxidized DNA bases (Cadet *et al.*, 1997; Kielbassa *et al.*, 1997; Kuluncsics *et al.*, 1999; Kvam *et al.*, 1997; Zhang *et al.*, 1997).

Mutations in cancer-relevant genes are produced by those UV photoproducts that are not repaired before DNA replication. Thus, DNA excision repair systems play an important role in preventing UV mutagenesis. Several human genetic disorders including xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are characterized by a defect in DNA repair. Cells from patients suffering from XP or CS are hypersensitive to UV light. XP is a genetically heterogeneous disease characterized by eight different

complementation groups (Friedberg *et al.*, 1993). The incidence of all types of skin cancers is increased by several orders of magnitude in XP patients (Ananthaswamy *et al.*, 1990; Hanawalt *et al.*, 1986). Although repair of (6-4)PPs in normal cells is generally faster than that of CPDs (Mitchell *et al.*, 1989), repair of both types of photoproducts is absent in many of the skin cancer-prone XP patients (Pfeifer *et al.*, 2005).

1.1.2.2 UV and melanoma

Epidemiological evidence suggests that a history of sunburn and intermittent exposure to strong sunlight, particularly during childhood, may promote the development of melanoma (Gilchrest *et al.*, 1999; Harrison *et al.*, 1994). However, the specific molecular targets in sunlight-induced melanoma remain unclear (Houghton *et al.*, 2002). Germ line mutatons of the p16/INK4A gene, has frequently been found in melanoma kindreds (Kamb *et al.*, 1994). Sporadic melanomas also carry somatic missense mutations in the *p16* gene (Pollock *et al.*, 1996). Recently developed mouse models have shown a connection between UV irradiation and melanoma induction (Jhappan *et al.*, 2003). One mouse model has shown that a single neonatal dose of UV is sufficient to induce cutaneous malignant melanoma in mice transgenic for hepatocyte growth factor/scatter factor (Noonan *et al.*, 2001). In another melanoma model, inactivation of the retinoblastoma/p16 pathway was identified as a critical event in UV mutagenesis (Kannan *et al.*, 2003).

Various epidemiological studies have addressed the relative contributions of UVB wavelengths and UVA wavelengths to photocarcinogenesis (Setlow *et al.*, 1974; Moan *et al.*, 1999; Langford *et al.*, 1998; Woodhead *et al.*, 1999; Wang *et al.*, 2001). The main

conclusions from these studies are that UVB is clearly linked to non-melanoma skin cancers and that UVA is accounting for melanoma. UVB radiation induces squamous cell carcinoma in mice (de Gruijl *et al.*, 1993) and UVA radiation is also a complete rodent skin carcinogen (de Gruijl *et al.*, 2002). Although UVB is responsible for most of the carcinogenic effect of sunlight, UVA is estimated to contribute 10–20% to the carcinogenic dose (de Laat *et al.*, 1997). UVA radiation is far more abundant in sunlight than is UVB and can produce oxidative DNA damage that is potentially mutagenic (Kielbassa *et al.*, 1997;Kvam *et al.*, 1997; Ananthaswamy *et al.*, 1990). In contrast to UVB, UVA radiation is not significantly absorbed by DNA itself. Thus, the absorption by other molecules (endogenous photosensitizers) becomes more relevant. Radicals, especially reactive oxygen species, can be generated by UVA irradiation that can damage DNA. UVA radiation is also thought to contribute substantially to the immunosuppression that prevents immunological rejection of nascent UV-induced skin cancers (Bestak *et al.*, 1996).

1.2 Tumor Suppressor Genes

1.2.1 P53

1.2.1.1 Gene structure

The p53 gene, first described in 1979, was the first tumour-suppressor gene to be identified. It was originally believed to be an oncogene. However, genetic and functional data obtained ten years after its discovery showed it to be a tumour suppressor. Moreover, it was found that the p53 protein does not function correctly in most human cancers. In

about half of these tumours, p53 is inactivated directly as a result of mutations in the p53 gene, more than any other single gene. In many other tumours, it is inactivated indirectly through binding to viral proteins, or as a result of alterations in genes whose products interact with p53 or transmit information to or from p53 (Vogelstein *et al.*, 2000). Inactivation of the p53 pathway occurs by defects in "upstream" activation of p53 or in its "downsteam" effector proteins, broadening p53's relevance to nearly all human cancers. The p53 protein acts as a transcription factor to induce target genes involved in cell cycle checkpoints, DNA repair, cell death, and cell senescence, particularly in response to DNA damage. As "guardian of the genome", p53 is responsible for ensuring that DNA repair proteins have the time and opportunity to repair damaged DNA sequence in cells under stress. In the skin these stresses include sunlight damage, drugs applied topically or systemically, even wounding or other physiological inducers of cell division and DNA conformational changes.

The mouse p53 gene contains only one promoter and transcribes only two mRNA variants with an alternative splicing of the intron 10 (Wolf *et al.* 1985). The human p53 gene contains only one promoter, and transcribes three mRNA splice variants encoding, respectively, full-length p53, p53i9 (Flaman *et al.* 1996), and Δ40p53 (Courtois *et al.* 2002; Yin *et al.* 2002; Ghosh *et al.* 2004). p53i9 encoded by alternative splicing of the intron 9 was reported as a p53 protein isoform truncated of the last 60 amino acids of p53, defective in transcriptional activity and devoid of DNA-binding activity. There is no evidence to indicate that the endogenous p53i9 isoform is expressed at the protein level. Δ40p53 (also named p47 or ΔNp53), is an N-terminally truncated p53 isoform deleted of the first 40 amino acids. Δ40p53 protein can be generated either by an alternative splicing

of intron 2 or by alternative initiation of translation. The Δ40p53 protein still contains a part of the p53 transactivation domain, and it can activate gene expression after transfection through a second transactivation domain located between amino acids 43 and 63 (Zhu *et al.* 1998, 2000; Harms and Chen 2005). Δ40p53 can also act, after transfection, in a dominant-negative manner toward wild-type p53, inhibiting both p53 transcriptional activity and p53-mediated apoptosis (Courtois *et al.* 2002; Ghosh *et al.* 2004). Δ40p53 can modify p53 cell localization and inhibits p53 degradation by MDM2 (Ghosh *et al.* 2004).

1.2.1.2 P53 signaling pathway and protein functions

The p53 signaling pathway is normally inactivated. It is activated only when cells are under stress or damage. For example, these cells contain mutations and exhibit abnormal cell-cycle control, and present a high possibility of becoming cancerous. The p53 protein shuts down the multiplication of stressed cells by inhibiting progress through the cell cycle or inducing programmed death (apoptosis) of the cells to obtain genomic stability.The p53 protein therefore plays a gatekeeper role on tumour development, explaining why it is so often mutated or inactivated in cancers. There are at least three independent pathways by which the p53 network can be activated.

The first pathway is triggered by DNA damage, such as that caused by ionizing radiation. Moreover, the activation of the signaling pathway is dependent on two protein kinases that add phosphate groups to other proteins. Two of the major kinases in question are called ATM (for ataxia telangiectasia mutated, named by a disease in which this

enzyme is mutated) and Chk2 (Carr, 2000). ATM is stimulated by double-strand breaks, and Chk2 is in turn stimulated by ATM (Vogelstein *et al.*, 2000).

The second pathway is triggered by aberrant growth signals, such as those resulting from the expression of the oncogenes Ras or Myc. In this case, activation of the p53 network in humans depends on a protein called $p14^{ARF}$ (Sherr *et al.*, 2000; Lowe *et al.*, 2000).

The third pathway is initiated by a wide range of chemotherapeutic drugs, ultraviolet light, and protein-kinase inhibitors. This pathway is distinguished from the others because it is not dependent on intact ATM, CHK2 or $p14^{ARF}$ genes, and may instead involve kinases ATR (ataxia telangiectasia related) and casein kinase II (Meek, 1999)

Taken together, all three pathways inhibit the degradation of p53 protein, thus stabilizing p53. The increased concentration of p53 allows the protein to carry out its major function: to bind to particular DNA sequences and activate the expression (transcription) of adjacent genes. These genes, directly or indirectly, lead ultimately to cell death or the inhibition of cell division (Vogelstein *et al.*, 2000).

Activated p53 has many biochemical functions, but the best established is its ability to bind to specific sequences in DNA and activate the transcription of adjacent genes (El-Deiry *et al.*, 1998). The binding regions of p53 to specific sequences and activating transcription have been well-studied in recent years. In fact, as a tumor usppressor, all naturally occurring mutations in the p53 gene have defects in transcriptional activation.

Genes that are controlled directly by p53 have been identified (El-Deiry *et al.*, 1998), and have been categorized into four groups. The first group is the genes that regulate cell-cycle inhibition. One of the first effects of p53 expression, in nearly all mammalian cell types, is cell cycle arrest to provide the time and opportunity for genomic repair. The p53 protein directly induces the expression of $p21^{WAF1/CIP1}$, an inhibitor of cyclin-dependent kinases (CDKs), which are key regulators of the cell cycle, working together with their partners — cyclins— to ensure S phase DNA replication follows properly from the cellular G1 resting phase.

Another effect of $p21^{WAF1/CIP1}$ in cell cycle arrest is that it inhibits both the G1-S and the G2-M transitions. Other genes, such as the Reprimo, can also arrest cells in G2 phase (Ohki *et al.*, 2000). In epithelial cells, p53 also stimulates the expression of protein 14-3-3σ, which blocks cyclin B1–CDK1 complexes outside the nucleus and thereby helps to maintain a G2 arrest (Chan *et al.*, 1999; Laronga *et al.*, 2000).

The second group of genes that are controlled directly by p53 is knowm to have a function in apoptosis demonstrated by some cells in which p53 is activated undergoing programmed death (Gottlieb *et al.*, 1998). P53 trigger apoptosis through several down stream targets (El-Deiry *et al.*, 1998). The Bax protein, for example, is an apoptosis-inducing protein of the Bcl-2 protein family. Transcription of the Bax gene in some human cells is directly activated by p53-binding sites in the regulatory region of the gene (Reed, 1999). However, in the mouse, there is no analogous p53-binding site in the regulatory region of the murine Bax gene (Schmidt *et al.*, 1999). Furthermore, the NOXA and p53AIP1 genes have also been discovered to be directly activated by p53 (Oda *et al*, 2000). When overexpressed in the cells, NOXA and P53AIP1 can lead to apoptosis.

Other potential mediators of p53-induced apoptosis include proteins with similarities to the classic 'death-signal' receptors, such as the TNF (tumour necrosis factor) receptor and Fas. One of the most recently discovered of these proteins is PIDD (Lin *et al.*, 2000).

In addition, the third group of genes that are regulated by p53 play a role in genetic stability. It has been known that these genes are involved in the balance of cell death or survival. For instance, defects in repair genes can lead to tumour development . Thus, the p53 protein may be important in maintaining genetic stability (Tlsty, 1997) (Wahl *et al.*, 1997) through the induction of genes that regulate nucleotide-excision repair (NER) of DNA, chromosomal recombination and chromosome segregation (Tlsty, 1997; Wahl *et al.*, 1997). Further evidence for p53's role in DNA repair comes from the induction of the ribonucleotide reductase gene by p53 after DNA damage (Tanaka *et al.*, 2000). This gene is essential for cellular responses to DNA errors in a wide range of organisms (Lozano, 2000).

The last group of p53 regulating genes are noted to be involved in the inhibition of blood-vessel formation. To enlarge beyond microscopic proportions tumours must establishnew blood vessels to supply the necessary nutrients. The normal p53 protein stimulates the expression of genes that prevent this process (Hendrix, 2000). Cells in which p53 is inactivated by mutation would therefore be more likely to form new blood vessels, providing a critical growth advantage at a late point and assists in tumour development and formation. This stage is the time when most natural p53 mutations occur. Studies of other tumour suppressors support the idea that preventing the formation of new blood vessels can be an important component of the activity of a tumour suppressor (Schwarte-Waldhoff *et al.*, 2000).

1.2.1.3 P53 modification and stabilization

The p53 is degraded by ubiquitin-mediated proteolysis. This ubiquitin chain acts as a 'flag', enabling p53 to be detected by the protein-degrading machinery. Through a feedback loop, the MDM2 protein is one of the enzymes involved in labelling p53 with ubiquitin (Momand, *et al.*, 2000).

However, the transcriptional activation of downstream targets by p53 requires conformational changes in the protein, resulting from modifications such as the addition or removal of phosphate, acetyl, glycosyl, ribose, ubiquitin or 'sumo' chemical groups ('sumo' is a ubiquitin-like polypeptide that can reversibly modify proteins) (Prives *et al.*, 1999; Giaccia *et al.*, 1998; Meek, 1999). Since the C terminus of p53 normally folds back and inhibits the DNA-binding domain located in the central part of the p53 protein the acetylation of lysine residues or phosphorylation of serine residues near the carboxy terminus of p53 can enhance the binding of p53 to DNA by interfering with this folding.

Phosphorylation of the amino terminus of p53 does not affect its DNA-binding abilities, but does affect its affinity for MDM2 and subsequent degradation. Other changes to the p53 protein and its MDM2 partner are also important in the p53 network. For instance, sumolation of MDM2 can reduce its ubiquitination and degradation (Buschmann *et al.*, 2000). This will lead to the accumulation of MDM2 to ubiquitinate p53 and promote p53 degradation.

1.2.2 The Inhibitor of Growth (ING) Family

1.2.2.1 ING family characterization

1.2.2.1.1 Gene structure and expression profile

Five different ING genes (ING1 to ING5) encoding proteins with highly conserved plant homeodomain (PHD) motifs and several splicing isoforms of the ING1 and ING2 gene have been identified after the discovery of ING1 tumor suppressors in 1996. ING sequences have been reported in human, mouse, rat, frog, fission and budding yeast, *Drosophila*, and *C. elegans*, as well as in other species; however, most of these homologs remain unrecognized and uncharacterized. Studies revealed that the ING family functions in DNA repair and apoptosis under UV stress through binding to proliferating cell nuclear antigen (PCNA); chromatin remodeling and regulation of gene expression through regulating and/or targeting histone acetyltransferase/deacetylase (HAT/HDAC) activities; binding targets of rare phosphatidylinositol phosphates (PtdInsPs) that function in DNA damage-initiated stress signaling; and regulating brain tumor angiogenesis through transcriptional repression of NF-κB responsive genes (Gordon *et al.*, 2005).

As the founding member of the ING family, ING1 was identified by the subsequent selection for sense or antisense cDNA fragments capable of promoting neoplastic transformation through subtractive hybridization between cDNAs from a normal mammary cell line and several transformed breast cancer epithelial cell lines (Garkavtsev *et al.*, 1996). The human ING1 gene has three exons that can be alternatively spliced onto a common 3' exon, thereby generating $p27^{ING1d}$, p33ING1b, and $p47^{ING1a}$ and an internal initiation at an ATG within the common exon generates $p24^{ING1c}$ (Garkavtsev *et al.*, 1999; Jager *et al.*, 1999; Gunduz *et al.*, 2000; Saito *et al.*, 2000). Expression level of ING1is significantly repressed in more than 42% of human primary breast cancers, 100% of established breast cancer cell lines (Toyama *et al.*, 1999), and many other forms

of blood and solid tumors (Ohmori *et al.*, 1999; Oki *et al.*, 1999; Shinoura *et al.*, 1999; Tokunaga *et al.*, 2000; Chen *et al.*, 2001; Krishnamurthy *et al.*, 2001; Bromidge, 2002; Gunduz *et al.*, 2002; Ito *et al.*, 2002; Noumann *et al.*, 2002; Chen *et al.*, 2003; Hara *et al.*, 2003; Garkavtsev *et al.*, 2004; Tallen *et al.*, 2004).

All ING genes share strongest homology in several highly conserved regions. The plant homeodomain (PHD) motif has been implicated in the regulation of ubiquitination (Coscoy *et al.*, 2003; Aravind *et al.*, 2003; Scheel *et al.*, 2003) and as a binding target of phosphatidylinositol phosphates (PtdInsPs) that function in DNA damage–initiated stress signaling (Gozani *et al.*, 2003). There are two intrinsic nucleolar translocation sequences (NTS) within the nuclear localization signal (NLS) in all ING family members, which target the proteins to nuclear (Scott *et al.*, 2001*b*). The PIP domain is a PCNA-interacting protein domain, through which p33ING1b binds to the proliferating cell nuclear antigen (PCNA) (Warbrick, 1998). PIP has also been found in proteins involved in growth inhibition, growth arrest after DNA damage and DNA replication and repair (Feng *et al.*, 2002). The leucine zipper–like (LZL) motif exists in ING2 consists of leucine residues spanning every seven amino acids, forming a hydrophobic patch. Sequence-based analysis has shown a similar leucine distribution at the N-terminus of ING3 to ING5 suggesting similar potential functions. The conserved LZL region from ING2-ING5, shows the potential ability of ING proteins to bind other leucine zipper–containing proteins, such as transcription factors. Initial results suggest that this is the case, making the association of particular HAT and HDAC complexes to chromatin considerably more dynamic (Gong *et al.*, personal communication). A potential chromatin regulatory (PCR) domain may also link ING proteins to HAT and HDAC complexes. In addition, The

peptide-interacting motif (PIM) at the C-terminus of ING1 and ING2 has been recently identified, which is believed to stabilize proteins with particular posttranslational modification (Feng *et al.*, 2004). In addition, interaction of ING with other proteins may be mediated through the candidate phosphorylation-dependent interacting motif (PDIM), which is relatively similar to the canonical RSXpSXP 14-3-3 binding motif. This implies that phosphorylation of this region could recruit proteins important in the modulation of such cellular processes as apoptosis, signal transduction, and cell cycle regulation (Hermeking, 2003). Most recently, the ability of ING proteins to bind to and alter the activity of histone acetyltransferases (HATs), histone deacetylases (HDACs), and factor acetyltransferases (FATs) has also been shown (Loewith *et al.*, 2000; Skowyra *et al.* 2001; Nagashima *et al.*, 2001; Vieyra *et al.*, 2002; Kuzmichev *et al.*, 2002; Feng *et al.*, 2002).

Although ING1 mutations are very rare, most of the mutations identified so far are either in the NLS or the PHD domains. More and more evidence is emerging that naturally occurring mutations in the PHD domains of different proteins lead to a variety of diseases including cancer (Gibbons *et al.*, 1997; Pascual *et al.*, 2000).

Furthermore, mislocalization of ING1 proteins has been observed in brain tumors by different groups (Tallen *et al.*, 2004; Vieyra *et al.*, 2003). In contrast, melanoma, papillary thyroid carcinoma and ductal breast carcinoma, increased levels of cytoplasmic p33ING1b were noted, concomitant with loss of nuclear localization (Nouman *et al.*, 2002). In ING3, loss and transcriptional repression are also seen for both the gene and protein level (Nagashima *et al.*, 2003). RNA levels of ING4 are also lower in low-grade gliomas (two to three times) and glioblastomas (six times) than in normal brain tissue

(Garkavtsev *et al.*, 2004) suggesting that expression of ING4 is progressively suppressed in brain tumors with increasing tumor grade and degree of malignancy.

1.2.2.1.2 Biological functions

The biochemical functions of ING proteins as histone acetyltransferases and histone deacetylase co-factors related this new tumor suppressor family to the regulation of transcription regulation, DNA repair and apoptosis. For instance, senescent human fibroblasts were shown to express 10-fold more ING1 mRNA compared to early passage fibroblasts, while antisense expression of ING1 resulted in prolonged proliferative life span of these cells (Garkavtsev *et al.*, 1997). In addition, further evidence emerged supporting the involvement of ING1 in the p53-mediated intrinsic apoptotic pathway by demonstrating that p33ING1b enhances transactivation of the proapoptotic Bcl-2 family protein Bax upon ultraviolet (UV) irradiation and consequently promotes change in mitochondrial membrane potential (Cheung *et al.*, 2002) Other ING members are also shown to be actively, but differentially involved in apoptosis. The expression of p33ING2 was found to be specifically induced by etoposide or neocarzinostatin, but not by gamma irradiation, doxorubicin, *cis*-platinum, or bleomycin in a normal lymphoblastoid cell line (Nagashima *et al.*, 2001). ING3 plays an important role in cellular response to UV irradiation by enhancing UV-induced apoptosis through the activation of Fas/caspase-8 pathway (Wang *et al.*, 2006). The interrelation between p53 and different ING members is apt to influence the apoptotic response. However, the interaction of ING proteins with the proliferating cell nuclear antigen (PCNA) and histone-modifying factors may also play a large part.

Most ING members function through p53 dependent manner. Two main mechanisms may help explain how ING proteins enhance the p53 response: association of ING members with p53 and ING-mediated acetylation of p53. P33ING1b, p29ING4 and p28ING5 are now known to co-precipitate with p53, while p33ING2 and p47ING3 do not (Nagashima *et al.*, 2003; Shiseki *et al.*, 2003; Nagashima *et al.*, 2001). Although certain ING proteins do not physically associate with p53, they may modulate p53 activity by enhancing protein stability. Overexpression of p33ING2 was found to induce increased acetylation of p53 at Lys-382 in the colorectal carcinoma cell line RKO (Nagashima *et al.*, 2003; Nagashima *et al.*, 2001). Posttranslational modifications such as acetylation and phosphorylation within the C-terminal region of p53 are believed to facilitate activation and stimulate p53 sequence-specific DNA binding activity (Gu *et al.*, 1997; Sakagushi *et al.*, 1998).

PCNA is an essential processivity factor for DNA polymerases, functioning in both DNA replication and nucleotide excision repair. It forms a sliding homotrimeric clamp encircling DNA as part of a large multiprotein complex and interacts with proteins through different faces of the homotrimer, but has no known enzymatic activity. It binds to and orientates other proteins in DNA replication and repair complexes, including the FEN1 nuclease, the CAF-1 chromatin assembly and/or remodelling factor, DNA ligase, the BASC super-complex of proteins, which includes the BRCA1 tumour suppressor, the MyD118, CR6 and GADD45 proteins, and $p21^{WAF1}$. Following exposure to UV, the p33ING1b isoform of ING1 rapidly binds to PCNA, and mutations within the PIP motif strongly inhibit binding. Co-localization of PCNA and p33ING1b increases several fold in the nucleus, and the PCNA–p33ING1b interaction can be inhibited by $p21^{WAF1}$, but not

by the p16 CDK inhibitor (Scott *et al.*, 2001). Furthermore, our group demonstrated that overexpression of the p33ING1b protein enhanced nucleotide excision repair (NER) of UVC-damaged exogenous plasmid DNA and UVB-damaged genomic DNA. These observations support the idea that ING1 binds to PCNA after DNA damage and contributes to the induction of apoptosis by altering PCNA function through the displacement of specific proteins.

ING proteins are also proposed to be involved in the repair of double-strand breaks. The chemotherapeutic agent adriamycin is a topoisomerase II inhibitor that generates double-strand DNA breaks (Tewey *et al.*, 1984). In a study by Tsang and colleagues (Tsang *et al.*, 2003), a prominent G2/M phase arrest was observed upon exposure to adriamycin when p33ING1b was expressed in p53-null H1299 cells. Interestingly, this G2/M arrest was not observed upon treatment with cisplatin and UV exposure, both of which generate intrastrand cross-links (Fichtinger-Schepman *et al.*, 1985; Cadet *et al.*, 1992). Reintroduction of p53 restored the G1 arrest after UV irradiation, suggesting that in the absence of p53, p33ING1b can respond to double-stand breaks but not to intrastrand cross-links. Further investigation should be conducted to verify whether ING proteins are involved in homologous repair or non-homologous end joining (NHEJ) repair pathways (Campos *et al.*, 2004).

Regarding to the gene transcription regulation, although how p33ING1b is able to regulate gene expression is less clear, it might bind to and activate p53 (Nagashima *et al.*, 2001; Garkavtsev *et al.*, 1998). Evidence showed that ING1 can block the entry of cells into S phase of the cell cycle (Garkavtsev *et al.*, 1996), but is less effective in blocking the growth of cells in which the activity of the p53 tumour suppressor is inhibited by

SV40 T-antigen (Garkavtsev *et al*, 1997). These findings suggested that ING1 might operate together with p53 providing a growth inhibitory effect (Garkavtsev *et al.*, 1998; Shinoura *et al.*, 1999; Garkavtsev *et al.*, 1998; Shimada *et al.*,2002). Overexpression studies confirmed that p33ING1b can activate endogenous genes, such as the $p21^{WAF1}$ CDK inhibitor and reporter constructs linked to the $p21^{WAF1}$ or Bax promoters. In addition to the $p21^{WAF1}$ and Bax genes, several additional genes have been identified that are regulated by altered levels of p33ING1b. One of the genes examined, cyclin B1, which activates CDK1 in late G2 and M phase of the cell cycle, was induced by antisense ING1 and inhibited by ectopic overexpression of p33ING1b. The effects of ING1 on cyclin B1 expression were enhanced by, while not dependent on, p53. Furthermore, the cyclin B1 gene lacks p53 binding sites, suggesting that p53 and p33ING1b contribute to the regulation of cyclin B1 by a mechanism different from sequence-specific DNA binding and its subsequent transcriptional activation. These observations suggest that ING proteins affect gene expression through more general mechanisms such as regulation of histone acetylation levels (Feng, *et al.*, 2002)

Besides ING1, ING2 (Nagashima *et al.*, 2001) and ING3 (Nagashima *et al.* in press) can also regulate gene transcription. The most probable mechanism by which ING2 regulates gene expression is through inducing acetylation of p53 on lysine 382 (Nagashima *et al.*, 2001), presumably by activating a FAT (factor acetyltransferase). While in the other hand, Sir2, which also targets the same residue of p53, can induce p53 deacetylation (Vaziri *et al.*, 2001). Although it is unknown whether ING proteins interact directly with p53, they do constitute components of larger protein complexes that affect

more general gene-regulatory mechanisms, such as chromatin structure, through residing in co-represser and co-activator complexes.

A few lines of evidence indicate that ING proteins can lead to transcriptional activation. For instance, UV treatment of HS68 cells, a stress event leading to the transcription of appropriate response genes, resulted in a clear translocation of p33ING1b protein into the nucleolus of cells where immunofluorescent imaging of high levels of transcription was evidenced by using anti-BrdU antibodies (Scott *et al.*, 2001). ING-mediated transcriptional response may also help modulate responses to endocrine factors. TH was shown to differentially affect the expression of different ING members in *Xenopus laevis* and correlate with apoptosis during development (Wagner *et al.*, 2001). Such observations suggest the possibility that ING family members may be involved in transducing signals initiated by certain endocrine factors such as TH. More recently, studies using an estrogen response element luciferase construct found that p33ING1b is able to increase estrogen receptor *a* expression in a dose-dependent manner via the AF2 domain (Toyama *et al.*, 2003; Toyama *et al.*, 2004). The modes of action of ING family members and the different stressed cellular pathways which cooperate with p53 might attribute to the variations in the effectiveness of facilitating p53 transcriptional activities.

In eukaryotes, DNA metabolism is strongly influenced by the packaging of DNA into higher order chromatin. Nucleosomes, as the fundamental repeat unit of chromatin, contain 147 bases of DNA wound around an octamer of four basic core histones, H2A, H2B, H3 and H4. It has been known that histones can be modified in several ways that can have major effects on gene transcription. Nucleosome remodeling, though acetylation, as well as other modifications including phosphorylation and methylation of

the N-terminal "tails" of core histones, affects accessibility of general transcription factors and DNA binding activators to the promoter sites, which facilitates or blocks the expression of downstream genes. Similar modifications of lysine residues in the histone tails neutralize their positive charge, thus reducing ionic interactions between DNA and the core histone of the nucleosomes. Consequently, the equilibrium between free histones and histones bound in nucleosomes shifts to favour free histones, and chromatin enters a relatively more "relaxed" state facilitating the activation of transcription. On the other hand, deacetylation of lysine residues in histone tails usually induces nucleosome formation and represses transcription. Therefore, histone acetylases and deacetylases are believed to serve as relatively general activators or repressors of gene expression with specific combinations of transcription factors providing a higher degree of gene specificity. It is clear that inappropriate regulation of gene expression is a common molecular change found in the development of cancer and subsequent disruption of regulatory pathways contributing to the malignant phenotype (Jacobson *et al.*, 1999). HATs and HDACs were initially isolated as transcriptional regulators; most HATs have been proven to be transcriptional adapters or co-activators while HDACs generally act as co-repressors. The state of histone acetylation is now known to affect gene expression significantly, and therefore has major roles in DNA repair, apoptosis, DNA recombination and cell cycle progression (Roth *et al.*, 2001).

Evidence have shown that ING1 proteins both physically and functionally associate with several factors possessing intrinsic HAT and HDAC activities, linking ING1 tumor suppressor function to chromatin remodeling. The link between ING1 and histone modification was first established in yeast (Loewith *et al.*, 2000). A yeast two-

hybrid screen implicated that all three budding yeast ING proteins, Yng1, Yng2 and Pho23, are able to associate with HAT activity. Further evidence indicated that the Yng2-associated HAT is Esa1, suggesting that Yng2 is a component of the NuA4 HAT complex (Loewith *et al.*, 2000). Human ING1 proteins also differentially associate with different HATs and HDACs. p33ING1b binds SAP30, a component of the Sin3–HDAC complex that associates with the Swi/Snf chromatin remodeling complex (Kuzmichev *et al.*, 2002) and suppression of cell growth by p33ING1b is dependent on an intact Sin3/HDAC interacting domain. Immunoprecipitation studies have shown that two major ING1 isoforms, p47ING1a and p33ING1b, can associate with both HAT and HDAC complexes in mammalian cells (Vieyra *et al.*, 2002) and different isoforms have differential affinities for HATs and HDACs. The fact that p33ING1b associates with HAT complexes containing TRRAP, CBP, p300, and PCAF and increases the level of acetylation of histones H3 and H4, whereas p47ING1a exerts the opposite effect, suggest that these two major ING1 isoforms might act in a reciprocal manner in altering chromatin structure.

1.2.2.2 P33ING2

1.2.2.2.1 Gene structure

p33ING2 (formally known as *ING1L*) was cloned through a homology search of p33ING1b cDNA sequence with the Otsuka cDNA database containing randomly selected cDNA from human placental, aortal, and fetal brain cDNA libraries. It is found to be located on chromosome 4q35.1 (Shimada, *et al.*, 1998). Full-length *ING2* cDNA contains an open reading frame of 840 bp, encoding for a 280-amino acid polypeptide

with a molecular weight of 32.8 kDa. P33ING2 shares 58.9 % homology with p33ING1b, while the nucleotide sequences between these two genes show 60% identity (Shimada *et al.*, 1998). The C-terminus of both p33ING2 (amino acid residues 213–260) and p33ING1b (amino acid residues 213–279) contains a highly conserved PHD zinc-finger motif, suggesting their roles as transcription regulators (Shimada *et. al.*, 1998). In addition, p33ING2 contains a unique leucine zipper-like domain that is thought to mediate hydrophobic protein–protein interaction or DNA binding (Feng *et al.*, 2002). ING2 proteins were mainly localized in the nucleus (74% in chromatin-enriched/nuclear matrix and 9% in nucleoplasmic), with a diffuse distribution in the cytosol (17%) (Gozani *et al.*, 2003). P33ING2 expression levels were found to be ubiquitous in normal human tissues, with varying expression levels in different tissue types. For example, the mRNA expression level of ING*2* is high in testis but low in lung and undetectable in kidney (Shimada *et al.*, 1998). Also ING2 expression level in spleen is undetectable, but less expressed in lung and brain (Nagashima et al., 2003).

1.2.2.2.2 Protein functions

P33ING2 has been shown to negatively regulate cell growth in a p53-dependent manner through induction of G_1-phase cell cycle arrest and apoptosis, by enhancing the promoter activities of $p21^{Waf1}$ and Bax in wild-type p53 colorectal carcinoma RKO cells, but not in p53-deactivated, RKO-E6 cells (Nagashima *et al.*, 2001). In contrast to upregulation of $p21^{Waf1}$ promoter, p33ING2 strongly repressed the promoter activity of human α-fetoprotein AFP (a gene often found to be aberrantly expressed in hepatocellular

carcinoma) through both p53-dependent and p53-independent pathways (Kataoka *et al.*, 2003).

Gozani and colleagues reported that p33ING2 interacted with Ptdlns(3)P and Ptdlns(5)P through its PHD finger motif, which alone is sufficient for this binding (O. Gozani *et al.*, 2003). Interaction with Ptdlns(5)Ps can modulate the subcellular localization of p33ING2 and is critical for the ability of p33ING2 to enhance p53 acetylation and p53-dependent apoptosis (O. Gozani *et al.*, 2003). Thus, it proposed that PHD finger motif is a general PtdlnsPs binding domain that Ptdlns(5)P recruits or stabilizes p33ING2 in the chromatin and interaction with Ptdlns(5)P might lead to allosteric activation of p33ING2.

Furthermore, ING2 protein was demonstrated to be associated with a very similar set of polypeptides classically found with mammalian Sin3 HDAC complexes, including the chromodomain-containing RBP1 and its paralog. RBP1 allows recruitment of mSin3 HDAC complex by retinoblastoma tumor suppressor family pocket proteins to induce cell cycle arrest by repressing E2F-dependent transcription and DNA replication origins (Lai *et al.*, 2001). Several peptides identified by mass spectrometry also corresponded to breast cancer metastasis suppressor-1 (BRMS1) and its paralog. BRMS1 suppresses metastasis of multiple human and murine cancer cells and has been found with RBP1 in mSin3 HDAC complexes (Meehan *et al.*, 2004). In addition, studies found not only the PHD finger region of ING2, but also the BRMS1 was implicated in phosphoinositide signaling (Gozani *et al.*, 2003; Campos *et al.*, 2004; DeWald *et al.*, 2005).

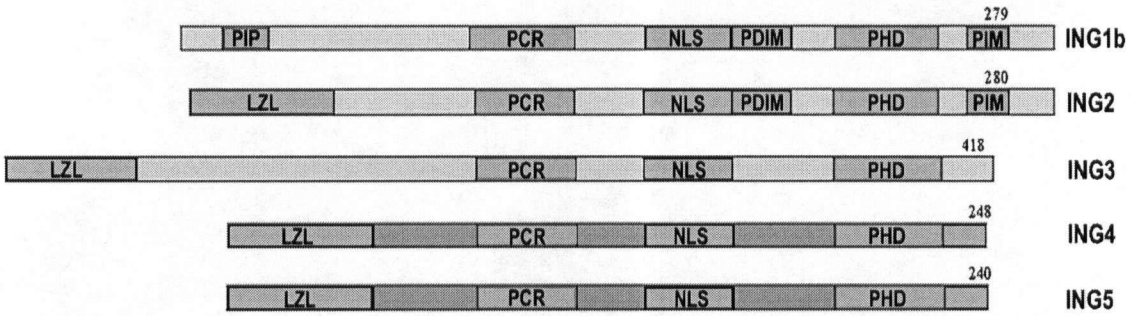
Most recently, studies have been done in the functional role of ING2 in replicative senescence by showing that ING2 and serine 15-phosphorylated p53 (termed phospho-

p53 [Ser15]) protein expression is upregulated during replicative senescence in human fibroblasts. Under these conditions, ING2 and phospho-p53 (Ser15) colocalize and physically interact with p300. This complex is located outside PML nuclear bodies. Mechanistically, ING2 physically interacts with p300 in vitro and in vivo and enhances p300-mediated p53 acetylation in vitro. ING2 also regulates the onset of replicative senescence: the overexpression of ING2 in young fibroblasts induces premature senescence in a p53-dependent manner, and downregulation of ING2 decreases p53 acetylation and delays the onset of replicative senescence. Thus, it is proposed that ING2 plays a major role in regulating the p53-dependent senescence checkpoint (Pedeux, *et al.*, 2005).

1.3 Objectives

Previously, we showed that overexpressed p33ING2 promoted UV-induced apoptosis and DNA repair in human melanoma cells. To further reveal the mechanisms of p33ING2 as cellular stress response to UV irradiation in a physiological condition, we investigated the both functional studies (Chapter 3) and a domain truncation study (Chapter 4) regarding the role of p33ING2 in linking the histone acetylation, chromatin relaxation to facilitating the UV-induced DNA damage recognition using MMRU melanoma cell lines as an experimental model system. We hypothesize that p33ING2 would be required for efficient repair of UV induced DNA damage via its effects on damaging sensing, chromatin remodeling and repair enzyme recruitment.

Figure 1.1 A diagrammatic representation of the major structural features of the ING proteins. Amino acid sequence alignment of the ING protein members, including ING1b, ING2, ING3, ING4 and ING5. Different conserved domains are indicated, including a proliferating cell nuclear antigen (PCNA)-interacting protein (PIP) domain on ING1b; a leucine zipper–like motif (LZL) on ING2, ING3, ING4, and ING5; the nuclear localization sequence (NLS) and a plant homeodomain (PHD) conserved in all members; a phosphorylation-dependent interacting motif (PDIM), and a peptide-interacting motif (PIM) only found on ING1 and ING2. A potential chromatin regulatory (PCR) domain is also shown that may link ING proteins to HAT and HDAC complexes.



CHAPTER 2 MATERIALS AND METHODS

2.1 Cell Lines and Cell Culture

Wild-type p53 MMRU melanoma cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Ontario, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2.

2.2 Plasmids

pRL-CMV Luciferase plasmid was from Promega (Nepean Ontario, Canada). pcDNA3-p33ING2 was a kind gift from Dr. O. Gozani and Dr. J. Yuan (Harvard Medical School, Boston, MA). pcDNA3 vector (Fig. 2.1) was from Invitrogen (Burlington, Ontario, Canada). PGFP-N2 was from Clontech (Windsor, ON, Canada). 3×FLAG vector (Fig. 2.1) was from Sigma (Oakville, Ontario, Canada). 3×FLAG-p33ING2, 3×FLAG-ΔPHD, 3×FLAG-ΔLZL and 3×FLAG-ΔL+P are generated by polymerase chain reaction from a pcDNA3-p33ING2 vector (Invitrogen) and subcloned into a 3×FLAG vector (Sigma, Oakville, Ontario, Canada) using site-specific primers. All of the constructs were sequenced across the newly created junctions to confirm there are no extra nucleotides induced by polymerase chain reaction.

2.3 Antibodies

Antibodies used for Western blotting were rat anti-p33ING2 monoclonal antibody (a kind gift from Dr. O. Gozani and Dr. J. Yuan, Department of Cell Biology, Harvard Medical

School, Boston, MA 02115, USA), anti-acetylated histone H3 and H4 polyclonal antibodies (Upstate, Charlottesville, VA). anti-CPD and anti-6-4PP primary polyclonal antibodies (kind gift 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-XPA rabbit polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), secondary IgG (Santa Cruz), Cy2-conjugated goat anti-rabbit and Cy3-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratory, West Grove, PA), anti-p53 DO-1, anti-β-actin, (Santa Cruz), anti-acetylated Lys382 p53 (Cell Signaling, Beverly, MA).

2.4 Methods

2.4.1 Transfection

Cells were seeded at 40-50% confluency the day before transfection. 24 hours later the cells were transfected with Effectene reagent (Qiagen, Mississauga, ON, Canada) at a ratio of 1 μg DNA to 25 μl Effectene.

2.4.1.1 Determination of Transfection Efficiency

Transfection efficiency of a particular cell line was determined by transfecting the cells with GFP-containing palsmid, pGFP-N2 with Effectene. 24 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) × 100%. The transfection efficiency of MMRU cells is 60-70%.

2.4.1.2 siRNA Transfection

MMRU cells were transfected at 50-70% confluency with siRNA (10 nM) using SiLentFect (Bio-Rad). The sense sequence of the ING2 siRNA (Qiagen, Mississauga, ON, Canada) is r(GGGUAAAUGCAUAAGACUA)dTdT, and the antisense sequence is r(UAGUCUUAUGCAUUUACCC)dTdA. The p53 siRNA (Qiagen) sense sequence is GCAUGAACCGGAGGCCCAUdTdT and antisense sequence is AUGGGCCUCCGGUUCAUGCdTdT.

2.4.2 Ultraviolet Irradiation

For apoptosis experiments, 400J/m^2 UVB irradiation was used. 24 hours before flow cytometry, medium was removed and the cells (at 70% confluency) were exposed to UVB (280-320nm) using a bank of four unfiltered FS40 sunlamps (Westinghouse, Bloomfield, NJ). Medium was replaced and cells were incubated in a 5% CO_2 incubator at 37°C for desired time periods after UVB irradiation. For the plasmids irradiation in DNA repair assay and histone acetylation detection experiments, 200 J/m^2 UVC was used through a UVC cross-linker (Ultralum, Claremont, CA). The intensity of the UV light was measured by the IL 700 radiometer fitted with a WN 320 filter and an A 127 quartz diffuser (International Light, Newburyport, MA).

2.4.3 Light Microscopy

The morphology of MMRU cells transfected with empty 3×FLAG vector, 3×FLAG-p33ING2, 3×FLAG-ΔPHD, 3×FLAG-ΔLZL and 3×FLAG-ΔL+P were subjected to microscopic assessment 24 hours after UVB irradiation at 400 J/m^2, using an inverted microscope (Zeiss, Chester, VA). Images were taken using a cooled mono 12-bit E× camera (Q-imaging, Burnaby, BC, Canada).

2.4.4 Hoechst 33258 Staining

Cells grown on coverslips in 6-well plates were transfected with WT or mutant p33ING2 for 24 hours and then irradiated with UVB at 400 J/m^2. Twenty-four hours later, cells were fixed with fixation buffer (2% formaldehyde, 0.5% Triton X-100 in PBS, pH 7.2) and incubated for 45 min at room temperature. The fixed cells were washed with 0.1% Triton X-100/PBS for 5 min and then stained with the mixture of 2.5 μg/ml Hoechst 33258, PBS/0.1% Triton X-100 at room temperature for 5 min. Coverslips were washed twice with PBS before mounted onto slides with mounting media (Fisher Scientific, Nepean, ON, Canada). The slides were visualized under a fluorescent microscope (Zeiss, Chester, VA) for apoptotic bodies. Images were taken with a cooled mono 12-bit Retiga-Ex camera.

2.4.5 Flow Cytometry

Cells were grown in 6-well plates, transfected with wt or mutant p33ING2 at 50% confluency, and irradiated with UVB at 400 J/m^2 24 h after transfection. Twenty-four hours later, cells were collected by trypsinization and pelleted by centrifugation at 2000× *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 incubation at 4°C overnight, the samples were then analyzed by flow cytometry to determine the percentage of subdiploid DNA. Samples were run on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Mississauga, ON, Canada) and analyzed with EXPO32 ADC analysis software.

2.4.6 Western Blot Analysis

Cells were grown in 60-mm or 6-well plates, transfected with empty 3×FLAG vector, 3×FLAG-p33ING2, 3×FLAG-ΔPHD, 3×FLAG-ΔLZL, 3×FLAG-ΔL+P expression vector or p33ING2 siRNA at 50% confluency and irradiated with UVC at 20 J/m^2, 200 J/m^2 24 h after transfection. 24 h after UV irradiation , cells were washed with PBS, harvested by scraping on ice and pelleted by centrifugation at 2,000 *g* for 2 min. Cells pellets were lysed in 80 μl of triple detergent buffer (50mM Tris-Cl, (PH 8.0), 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing freshly added protease inhibitors (100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/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 μg/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 hat 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 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 defferences in the actin loading control.

2.4.7 Renilla Luciferase Assay

pRL-CMV plasmid DNA was irradiated with UVC at 200 J/m^2 and co-transfected with gene of interest using Lipofectamine^{TM} 2000 (Invitrogen). Forty hours after transfection, Luciferase assay was performed with a luciferase assay kit (Promega). The growth medium was removed from the cultured cells in the 24-wells, and a sufficient volume of PBS was gently added to wash the surface of the culture vessel. The vessel was swirled briefly to remove detached cells and residual growth medium. The rinse solution was completely removed before adding RenillaLuciferase Assay Lysis Buffer. To each culture well 100 µl of 1X RenillaLuciferase Assay Lysis Buffer was added. The culture plate was placed on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1× RenillaLuciferase Assay Lysis Buffer. The culture plates rocked at room temperature for 15 min. The lysate was transferred to a tube or vial for further handling and storage. The lysate samples were then cleared the by centrifugation for 30 seconds at top speed in a refrigerated microcentrifuge. The cleared lysates were transferred to a fresh tube prior to reporter enzyme analyses. Then the tubes were placed in luminometer for measurement.

2.4.8 Immunofluorescent Staining

MMRU cells were seeded on coverslips. After 24 h, the medium was removed, and an isopore polycarbonate filter with 5-μm pores (Millipore) was placed on top of the cell monolayer, and irradiated with UVC at 200 J/m^2. The filter was gently removed and cells were fixed with 2 ml of fixation solution (2% paraformaldehyde, 0.5% Triton X-100 in PBS) for 30 min at 4°C. After washing with PBS, the cells were incubated with 2 M HCl for 10 min to denature DNA. Then the cells were incubated with normal goat serum for 1 h, followed by incubation with anti-CPD or anti-6-4PP primary antibodies (1:30 dilution) for 1 h in a humid chamber. After washing with PBS, the cells were incubated with 1:150 dilution of Cy3-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) for 1 h and then 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, the coverslips were incubated with 1:3000 dilution of stock Hoechst 33258 (20 mM) for 10 min, and visualized under a fluorescent microscope (Zeiss).

2.4.9 Micrococcal Nuclease (MNase) Digestion

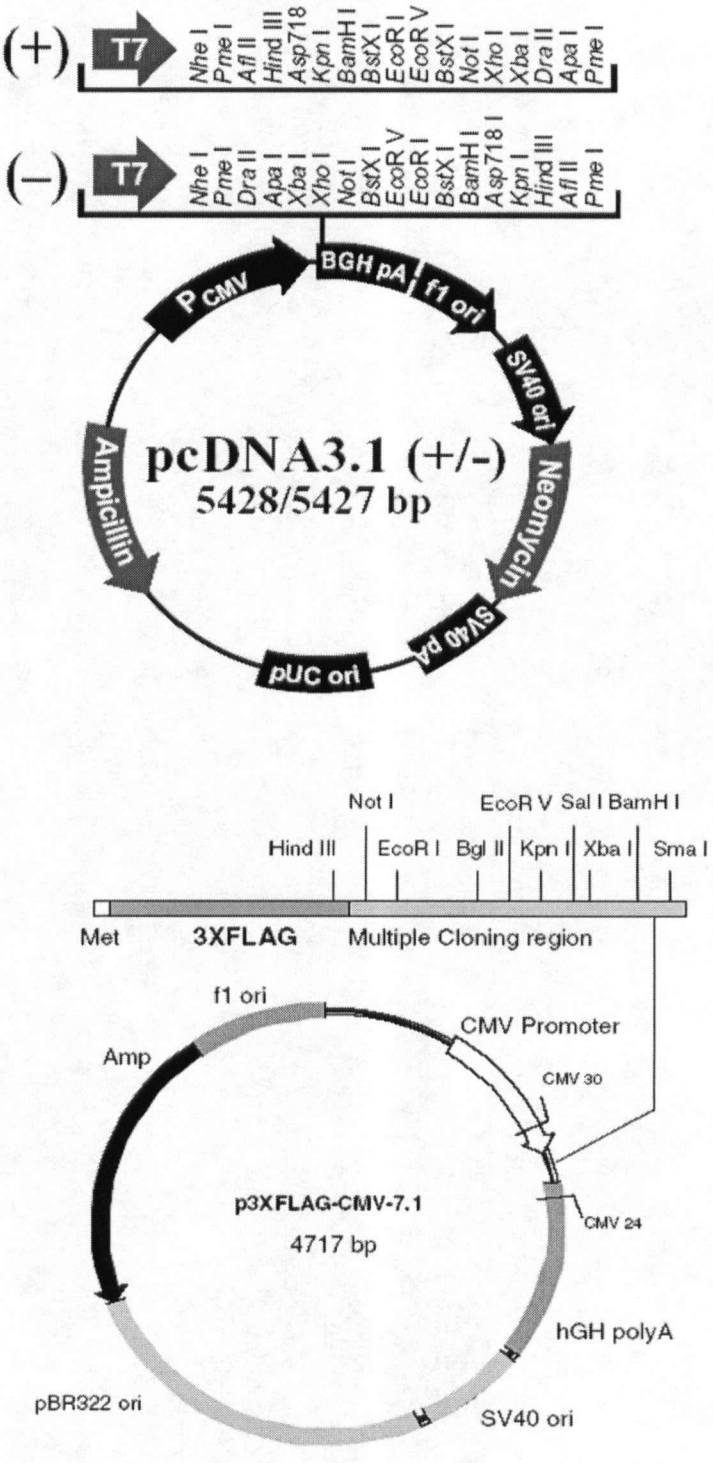
MMRU cells were irradiated with UVC (200 J/m^2) and the cells were harvested by scraping in 12 ml lysis solution (10 mM Tris/HCl [pH 8], 10 mM $MgCl_2$, 1 mM DTT) 30 min after UV irradiation. Then 0.3 ml of 2% NP-40 detergent was added to the cells. After centrifugation at 1,200× *g* for 10 min, the supernatant was removed and the nuclei pellet was resuspended in 200 μl MNase buffer (10 mM Tris/HCl [pH 8], 50 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$). MNase (Sigma) was added to the nuclei samples to

digest the DNA for 5 min at 37°C. The reaction was terminated by adding the stop solution (1% SDS, 20 mM EDTA) and vortexed briefly. After centrifugation at 14,000× *g* for 10 min, the aqueous phase was transferred to a new tube and the DNA extracted twice with phenol:chloroform.

2.4.10 Generation of Deletion Mutants

3×FLAG-p33ING2, 3×FLAG-ΔPHD, 3×FLAG-ΔLZL and 3×FLAG-ΔL+P plasmids are generated by polymerase chain reaction from a pcDNA3.1-p33ING2 vector (Invitrogen) and subcloned between EcoRI and XbaI multiple cloning region in a p3×FLAG-CMV-7.1 vector (Sigma) using site-specific primers. All of the constructs were sequenced across the newly created junctions to confirm there are no extra nucleotides induced by polymerase chain reaction. The primer pairs for 3×FLAG-p33ING2 are GGAATTCCATGTTAGGGCAGCAGCA (forward) and GCTCTAGAGCAGAATTCTACTACCTCGA (reverse). The primer pairs for 3×FLAG-ΔPHD are GGAATTCCGTACCGAGCTCGGATCCA (forward) and GCTCTAGAGCCTACACTTGGTTGCATAAGCA (reverse). The primer pairs for 3×FLAG-ΔLZL are GGAATTCCAATTGATGATGTCTACGA (forward) and GCTCTAGAGCAGAATTCTACTACCTCGA (reverse). The primer pairs for the 3×FLAG-ΔL+P are GGAATTCCGATAAAGCAAAGATGGA (forward) and GCTCTAGAGCAGAATTCTACTACCTCGA (reverse).

Figure 2.1 pcDNA3.1 (+/-) and p3×FLAG-CMV-7.1 Map



CHAPTER 3. P33ING2 IS REQUIRED FOR NUCLEOTIDE EXCISION REPAIR THROUGH HISTONE H4 ACETYLATION AND CHROMATIN RELAXATION

3.1 Rationale and Hypothesis

Cutaneous malignant melanoma (CMM) is one of the fastest increasing cancers worldwide and is associated with sun-exposure of susceptible populations (De Fabo *et al.*, 2004). The UVB component of sunlight (Veierod *et al.*, 2003) which causes suppression of immune functions is an important environmental factor contributing significantly to the growth of cutaneous malignancies. (Hanneman *et al.*, 2006). The incidence and mortality rates of melanoma have increased at annual rates of 2-3% for the last 30 years. Disseminated disease is largely refractory to cytotoxic chemotherapy and is almost universally fatal (Perlis *et al.*, 2004).

In melanomas, p53 gene mutations are late events that occur during progression to a higher grade of malignancy (Hussein *et al.*, 2003). They were found in up to approximately 25% of the melanomas. Most of these mutations are both rare events and UVR related. They occur primarily in two forms: (a) C→T or CC→TT transition-type mutations and (b) TGT to TGG transversion mutations. These mutations, generally present in exons 4-7 are either silent or result in single amino acid substitutions without profound functional changes in gene products. These mutational changes were associated with p53 protein overexpression, which was occasionally related to the site of the lesions. For instance, when compared to other sites, p53 protein expression was generally stronger and more frequent (32 vs. 6%) among lesions arising in sites chronically

exposed to sun, such as head and neck (Hussein *et al.*, 2003; Hussein *et al.*, 2002; Hussein *et al.*, 2003).

In human cells, coimmunoprecipitation studies have indicated that protein product(s) of the ING1 gene (principally p33ING1b) physically interact with the TP53 tumour suppressor gene protein product p53, whereas cotransfection studies confirmed the ability of ING1 to modulate p53 dependent transactivation of the kinase inhibitor $p21^{WAF1}$ (Nagashima *et al.*, 2001; Skowyra *et al.*, 2001; Garkavtsev *et al.*, 1998). These preliminary findings suggested that the association of competent protein forms of each member of the ING1–TP53 complex is essential for optimal expression of the transactivational activity of TP53 (Garkavtsev *et al.*, 1998; Oren *et al.*, 1998). In addition, p33ING1b directly cooperates with p53 in growth regulation by modulating the ability of p53 to act as a transcriptional activator (Garkavtsev *et al.*, 1998). Reduction of ING1 expression inhibits the growth suppressive activity of p53, suggesting that p33ING1b is essential for p53 function. The involvement of p33ING1b in the p53 signalling pathway indicates that ING1 is a potential tumour suppressor gene, the loss or inactivation of which may contribute to altered cell growth control, resistance to apoptosis, or establishment of an immortal tumour phenotype, even if wild-type p53 is retained. Therefore, if the loss of p33ING1b compromises the function of p53 only slightly, this may provide emerging cancer cells with a selective growth advantage, and even a small advantage might make cancer imminent. Moreover, optimal function of both members of the p33ING1b–p53 complex is necessary for several important cellular processes, including restriction of cell growth, proliferation, apoptosis, cellular senescence, maintenance of genomic stability, and modulation of cell cycle checkpoints (Garkavtsev

et al., 1997; Helbing *et al.*, 1997; Garkavtsev *et al.*, 1998; Garkavtsev *et al.*, 1998; Turovets *et al.*, 2000). Because p33ING1b was found to be a member of the Sin3–HDAC1 complex (Skowyra *et al.*, 2001; Kuzmichev *et al.*, 2002), it was thought that p33ING1b modulates transcription repression through members of the Sin–HDAC1 complex, which include SAP30 and pRb1. UV was found to induce the binding of p33ING1b to PCNA (Scott *et al.*, 2001). This complex is responsible for enhancing DNA repair over DNA replication steps or, if the former fails, augmenting apoptosis to eliminate damaged cells. Moreover, a physical association was detected between p33ING1b and the GADD45 protein, which is associated with DNA repair mechanisms, after damage to melanoma cell lines induced by UV (Cheung *et al.*, 2001).

Although the ING1 gene has been extensively studied, little is known about the number of transcripts encoded by the other ING gene family members or their gene structure. P33ING2 is a novel candidate tumor suppressor, which shares 58.9% homology with p33ING1b and has been shown to be involved in the regulation of gene transcription, cell cycle arrest and apoptosis in a p53-dependent manner for maintaining the genomic stability. Previously, we showed that p33ING2 promoted UV-induced apoptosis in human melanoma cells. To further reveal the role of p33ING2 in cellular stress response to UV irradiation, we hypothesized that p33ING2 is required for the initial DNA damage sensing and chromatin remodeling in the nucleotide excision repair process similar as its homologue p33ING1b.

3.2 Results

3.2.1 P33ING2 Enhances the Repair of UV-damaged DNA in Melanoma Cells

Previous experiments showed that MMRU cells overexpressing p33ING2 had a significantly higher repair rate of UV-damaged plasmid compared with the vector control (Chin, 2004). However, whether the endogenous p33ING2 is required for repair of UV-damaged DNA remains unknown. To determine whether p33ING2 is required for DNA repair under physiological condition we performed a luciferase assay was performed by gene silencing using p33ING2 siRNA. MMRU cells were cotransfected with a UV-damaged pRL-CMV luciferase plasmid that contains the reporter gene with pcDNA3-vector (ctrl), pcDNA3-p33ING2, p33ING2 siRNA, or p53 siRNA plus pcDNA3-ING2 expression vectors. The activity of this reporter gene was used as an indicator of the extent of repair. Cells treated with p33ING2 siRNA for 48 hours had an 80% reduction in p33ING2 protein expression (Fig. 3.1A). Consistent with previous results, we found that MMRU cells overexpressing p33ING2 had a significantly increased DNA repair rate (32.5%) compared with the vector control (11.2%; $P < 0.01$, t test). On the other hand, treatment with p33ING2 siRNA, which inhibited 80% p33ING2 protein expression, completely abolished nucleotide excision repair (Fig. 3.1B), indicating that a certain threshold of p33ING2 is required for the repair of UV-damaged DNA in melanoma cells. To further measure DNA repair rate in global genomic repair, we detected the levels of UV-induced CPDs and 6-4PPs in p33ING2-transfected MMRU cells compared with vector control at various time points after UV irradiation. We found that cells transfected

with p33ING2 had a much faster removal rate of 6-4PPs and CPDs compared with the vector controls (Fig. 3.1C).

3.2.2 P33ING2-enhanced DNA Repair Requires Wild-type p53

Since p33ING2 homologue, p33ING1b, requires functional p53 to enhance NER (Cheung *et al.*, 2001), we investigated whether p33ING2-medicated DNA repair also requires the presence of wild-type p53. We disrupted the endogenous wild-type p53 in MMRU cells by introducing both the p53 siRNA. Western blot analysis, using an anti-p53 antibody, confirmed that p53 siRNA silenced the expression of endogenous p53 by 90% (Fig. 3.1A). We found that p33ING2 overexpression could not enhance DNA repair in p53 siRNA-treated cells (3.2%) (Fig. 3.1B) compared with the control cells (32.5%) ($P < 0.01$, *t*-test). This is consistent with our previous results that reduced DNA repair efficiency was observed in cells co-transfected with p33ING2 and pED1 which carries a dominant negative mutant p53 gene compared with cells transfected with p33ING2 alone (Chin, 2004). These data indicate that p33ING2-enhanced repair of UV-damaged DNA does require functional p53.

3.2.3 P33ING2 Induces Hyperacetylation of Histone H4 after UV Irradiation

Only recently has the role of histone modification in cell signaling or facilitating DNA repair begun to be elucidated (Moore and Krebs, 2004). Because it has previously been shown that histones are acetylated following UV irradiation (Ramanathan and Smerdon, 1986), we hypothesized that p33ING2 may be involved in histone acetylation and chromatin relaxation to mediate the repair of UV-damaged DNA. First, we examined if

p33ING2 expression level is increased in MMRU cells after UV irradiation as a cellular stress response. Our data showed that the endogenous p33ING2 expression is not affected by UV irradiation (Fig. 3.2A). Then, we investigated if the acetylation level of histone H3 and H4 is changed in MMRU cells overexpressing p33ING2. Our results indicated that overexpression of p33ING2 can increase the acetylation of histone H4 by around 2-fold compared with the vector-transfected control cells under normal nonstress condition (Fig. 3.2B). UV irradiation alone can also induce histone H4 acetylation in control MMRU cells by around 2-fold just 1 minute after irradiation (Fig. 3.2B). Interestingly, acetylation of histone H4 was increased even more (4-fold) in p33ING2-overexpressing cells 5 minutes after UV irradiation (Fig. 3.2C). We also examined the level of acetylated histone H3 in cells overexpressing p33ING2 and found that p33ING2 did not significantly enhance UV-induced acetylation of histone H3 (data not shown). To further investigate whether endogenous p33ING2 is required for histone acetylation, we treated MMRU cells with p33ING2 siRNA and examined histone H4 acetylation level. We found that in normal nonstress condition, knockdown of endogenous p33ING2 did not significantly affect histone H4 acetylation (Fig. 3.2D). However, p33ING2 siRNA completely abrogated UVC-induced histone H4 acetylation (Fig. 3.2D), suggesting that a physiologic level of p33ING2 is required for histone H4 hyperacetylation in cellular stress response to UVC irradiation. Due to the fact that p33ING2 cooperates with p53 in nucleotide excision repair, we next investigated if p33ING2 enhancement on histone acetylation requires p53 activity by siRNA gene silencing. As expected, p53 siRNA abolished the hyperacetylation of histone H4 in p33ING2-overexpressing MMRU cells on exposure to UVC (Fig. 3.2E), which is consistent with p53 dependency of p33ING2-mediated

nucleotide excision repair (Fig. 3.1B). To further verify that p33ING2 enhances histone H4 acetylation, we did immunofluorescence staining to visualize the expression level of acetylated histone H4 in single cells. We found that MMRU cells transfected with p33ING2 have a higher histone H4 acetylation level compared with control cells 5 minutes after UVC irradiation (Fig. 3.2F). We also noticed that p33ING2 induced global histone H4 acetylation, rather than localized histone H4 acetylation, at DNA damage sites (Fig. 3.2F). In addition, we found that p33ING2 enhanced histone H4 hyperacetylation when the cells were irradiated at a lower dose of UVC (20 J/m^2; Fig. 3.2G *top panel*) p33ING2 siRNA knockdown also reduced histone H4 acetylation after 20 J/m^2 UVC irradiation (Fig. 3.3G *bottom panel*), further confirming that p33ING2 is required for histone H4 acetylation under physiologic conditions.

3.2.4 P33ING2 Induces Chromatin Relaxation

It has been shown that acetylation of histones on their lysine-rich amino-terminal tails, which protrude from nucleosomes, regulates gene expression through weakening histone-DNA contacts, leading to formation of transcriptionally competent chromatin domains (Roth *et al.*, 2001). Thus, it is likely that histone acetylation plays an important role in chromatin remodeling to facilitate nucleotide excision repair (Gontijo *et al.*, 2003). We next did a micrococcal nuclease digestion assay to look at chromatin decondensation in p33ING2-overexpressing MMRU cells. We found that chromatin is more sensitive (79.3% of the DNA fragments <600 bp) to the nuclease (1 unit treatment) in p33ING2-overexpressing cells on UVC irradiation compared with MMRU control cells (15.9%; Fig. 3.3). These results are consistent with the observation that p33ING2 enhances UV-

induced histone H4 acetylation (Fig. 3.2C), implying that p33ING2 may serve as a chromatin accessibility factor during the repair of UV-damaged DNA.

3.2.5 P33ING2 Facilitates XPA to the DNA Damage Sites

As for every other process that requires access to DNA, efficient repair of UV-damaged DNA is dependent on the detection of bulky DNA photolesions and the recruitment of repair proteins, which require the unfolding of the chromatin into a more relaxed state (Green and Almouzni, 2003). Recruitment of the photolesion-recognition protein XPA to the DNA damage site is considered to be a rate-limiting process for nucleotide excision repair (Thoma and Vasquez, 2003). Thus, we investigated if p33ING2 is required for the recruitment of XPA by examining the colocalization of XPA and CPDs by immunofluorescence in control (Fig. 3.4A *bottom*) and p33ING2 siRNA–treated cells (Fig. 3.4A *top*). Punctate green staining of XPA at the same position with CPDs is considered to be colocalized with DNA photolesions. Our results showed that p33ING2 siRNA treatment significantly reduced the recruitment of XPA protein to DNA damage site (81%) compared with control cells (23%) 30 minutes after UV irradiation ($P < 0.0001$; Fig. 3.4B), further confirming that p33ING2, in association with p53 and possibly other factors possessing intrinsic HAT activities, acts as a DNA damage sensor to induce histone acetylation and chromatin relaxation and, therefore, enhance the recruitment of damage recognition factors to the DNA damage sites to initiate the repair process.

Discussion

Efficient NER is crucial for cells to repair UV-induced DNA damage and prevent skin carcinogenesis. Although over 30 factors have been identified to participate in different steps of the NER process including DNA damage recognition, excision of the DNA adducts, and repair synthesis, little is known on the initial cellular response for the chromatin remodeling to allow the repair proteins to access to the DNA damage site. Recently, Rubbi and Milner showed that the p53 tumor suppressor is essential to induce histone H3 acetylation and chromatin relaxation after UV irradiation (Rubbi and Milner, 2003). However, it is not clear whether other factors cooperates with p53 to induce histone acetylation and chromatin relaxation during the NER process and whether relaxed chromatin can lead to the recruitment of repair proteins to DNA damage site.

In this report, we for the first time demonstrated that the novel tumor suppressor p33ING2 significantly enhances NER of UV-damaged DNA in p53-dependent manner by inducing histone H4 acetylation and chromatin relaxation. The fact that p33ING2 enhances histone H4 acetylation as early as one minute after UV irradiation suggests that p33ING2 may act as DNA damage sensor during the NER process. Physiological levels of p33ING2 are required for UV-induced rapid histone H4 acetylation, further supporting this notion. However, it seems that there is a certain threshold of p33ING2 which is required for the repair of UV-damaged DNA. We demonstrated this by performing a luciferase reporter assay with gene silencing using p33ING2 siRNA. Similar to the CAT assay, we found that MMRU cells overexpressing p33ING2 had a significantly increased DNA repair rate (32.5%) compared with the vector control (11.2%). On the other hand,

treatment with p33ING2 siRNA, which inhibited 80% p33ING2 protein expression, completely abolished nucleotide excision repair, indicating that a certain threshold of p33ING2 is required for the repair of UV-damaged DNA in melanoma cells. It is known that UV irradiation triggers genome-wide histone acetylation on both histone H3 and H4 (Yu *et al.*, 2005). However, p33ING2 only enhanced UV-induced histone H4, but not H3, acetylation, suggesting that different factors are required for acetylation of specific subunits of histones.

Although the exact molecular mechanism of p33ING2-induced histone H4 acetylation is yet to be revealed, a few lines of evidence suggest that ING proteins may act as cofactors for histone acetyltransferases (HAT). ING1 proteins interact with various components of histone acetyltransferase such as TRRAP, PCAF, CBP, and p300 (Vieyra *et al.*, 2002). In fact, immunofluorescent analysis of cells microinjected with p33ING1b expression vector demonstrated increased staining for acetylated histones H4 and H3. On the other hand, downregulation of p33ING1b resulted in decreased histone acetylation, indicating a direct correlation between HAT activity and p33ING1b levels (Vieyra *et al.*, 2002). More recently, p47ING3 was identified as a component of a Tip60-based complex, a human equivalent to the yeast NuA4 HAT complex (Doyon *et al.*, 2004), which is responsible for acetylation of histone H4 and H2A N-terminal tails in yeast and has been implicated in DNA repair (Bird *et al.*, 2002). P29ING4 and p28ING5 have also been shown to immunoprecipitate with p300 (Shiseki *et al.*, 2003). The effect of ING proteins on histone acetylation may also be mediated by the interactions with histone deacetylases (HDAC) since p33ING1b is capable of associating with known components of the sSin3 transcriptional corepressor complex, including sSin3, HDAC1/2, RbAp48,

RbAp46 and SAP30 (Skowyra *et al.*, 2001). In addition, the deletion of SAP30-interacting domain in p33ING1b was found to be detrimental to p33ING1b-mediated cell growth regulation, implying a functional significance of the interaction between p33ING1b and HDAC. Further investigations on the interaction between p33ING2 and HAT or HDAC are required to reveal the mechanism of p33ING2-mediated histone acetylation.

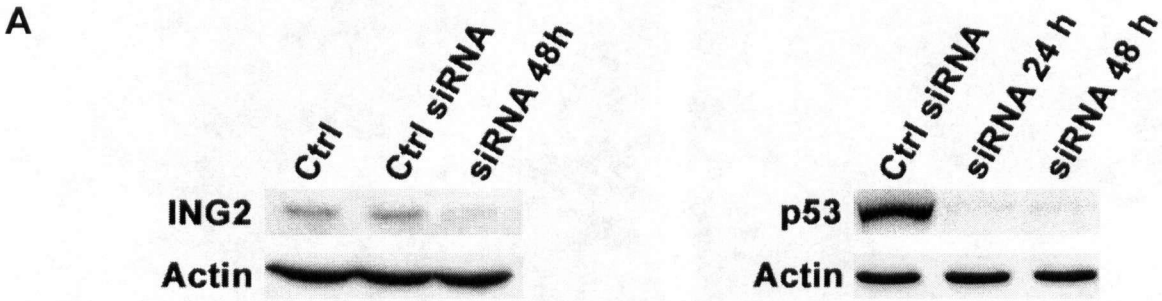
It is well known that histone acetylation can lead to chromatin relaxation. Annunziato *et al.* showed that histone acetylation by treatment with the HDAC inhibitor sodium butyrate caused moderate chromatin relaxation (Annunziato *et al.*, 1988). In our experiments, treatment with the HDAC inhibitor trichostatin A also induced chromatin relaxation. Relaxation of the chromatin is believed to be essential for repair enzymes to gain access to the DNA damage site, although this notion has not been supported with experimental evidence. Our data clearly demonstrated that cells containing normal p33ING2, which can trigger histone acetylation and chromatin relaxation, were able to recruit the DNA damage recognition factor XPA to DNA photolesions, while this recruitment was significantly impaired in cells treated with p33ING2 siRNA which abolishes histone acetylation. These results not only established a direct evidence for histone acetylation and chromatin relaxation to be the prerequisite for damage recognition factors to gain access to the photolesions, but also verified the our hypothesis that p33ING2 acts in the initial steps of chromatin remodeling in NER before the recognition of DNA damage by XPA.

The role of p53 in NER has been extensively investigated in the past decade. We and others used p53-deficient cells and demonstrated that p53 deficient cells have

reduced NER capacity (Li *et al.*, 1996; Li *et al.*, 1997; Ford and Hanawalt, 1997). However, the exact molecular mechanism for p53-mediated effects on NER have been unclear for a long time. Although p53 was able to bind with XPB and XPD proteins in vitro (Wang *et al.*, 1995), this association has never been found in vivo. In fact, immunofluorescent staining shows that p53 is not colocalized to DNA damage site (Fitch *et al.*, 2003). Only recently, p53 was found to induce histone H3 acetylation and chromatin relaxation (Rubbi and Milner, 2003), suggesting that p53 acts as a chromatin accessibility factor in NER. Our data in this report fully support this notion. In addition, it appears that both physiological levels of p33ING2 and p53 are required for proper NER as treatment with either p33ING2 or p53 siRNA inhibited UV-induced histone H4 acetylation. However, p33ING2 seems to be involved in histone H4 acetylation, while p53 is necessary for the acetylation of both histone H3 and H4. Nevertheless, other factors may also be required for p53-mediate histone H3 acetylation.

Taken together, our study demonstrated that the novel tumor suppressor p33ING2 affectsNER by enhancing histone acetylation and chromatin relaxation, and facilitating the recruitment of the indispensable repair recognition protein XPA (Liu *et al.*, 2005) to the bulky DNA lesions in a p53-dependent manner. Previous report showed that histone H3 acetylation as early as 30 minutes after UVR is dependent on wild type p53 (Rubbi and Milner, 2003). We show here that p33ING2-dependent hyperacetylation of histones H4 can be detected just one minute after UVR, suggesting that p33ING2 may act as a DNA damage sensor to initiate the NER process. The essential role of p33ING2 in NER, together with its role in cell cycle arrest and apoptosis, further indicate that p33ING2 is a tumor suppressor.

Figure 3.1 p33ING2 enhances the repair of UV-damaged DNA in melanoma cells. **(A)** Western blot analysis of p33ING2 (*left*) and p53 (*right*) expression after siRNA treatment. A scramble siRNA was used as negative control. A rat anti-p33ING2 monoclonal antibody and an anti-p53 monoclonal antibody (DO-1) were used. **(B)** Assessment of the repair rate of UV-damaged DNA by luciferase reporter assay in MMRU cells cotransfected with undamaged or damaged pRL-CMV luciferase plasmid and pcDNA3-vector (*ctrl*), pcDNA3-p33ING2, p33ING2 siRNA, or p53 siRNA plus pcDNA3-ING2 expression vectors. Forty hours after transfection, luciferase activity was measured with a luminometer. *Columns,* mean from triplicates; *bars,* SD. The experiment was repeated twice with similar results. **(C)** Slot-Western analysis of UV-induced CPDs and 6-4PPs in p33ING2-transfected or control MMRU cells at different time points after 200 J/m^2 UVC irradiation. Anti-CPDs and anti-6-4PPs polyclonal antibodies were used.



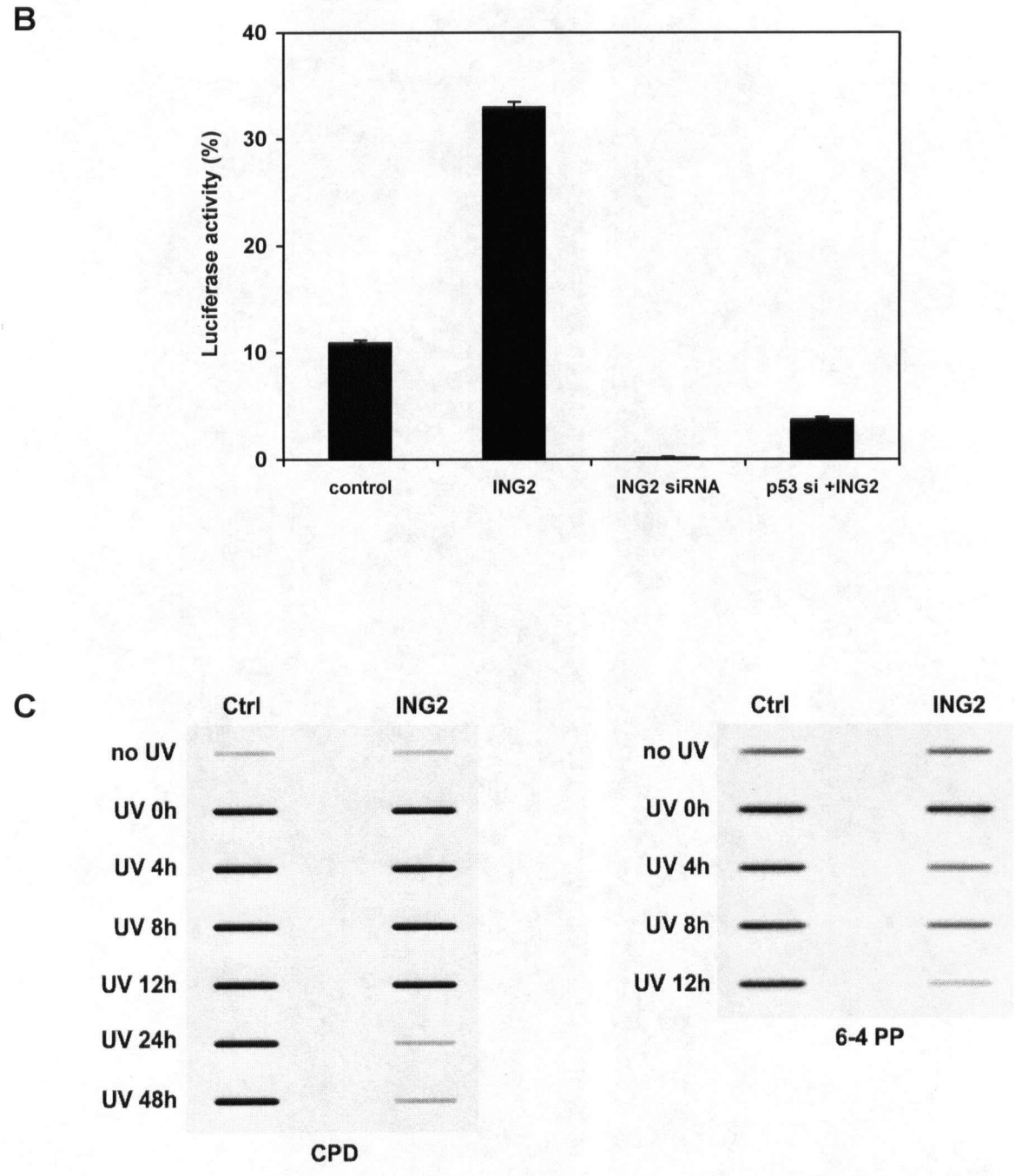
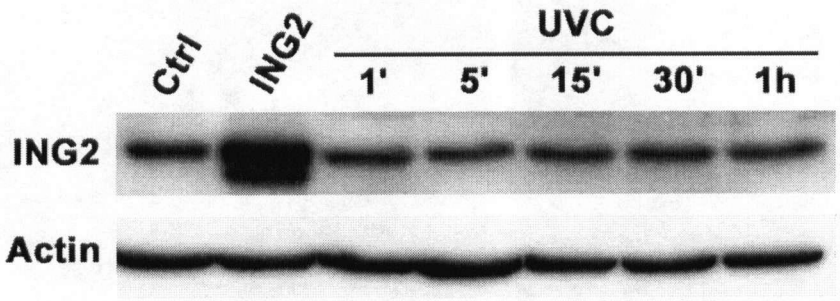
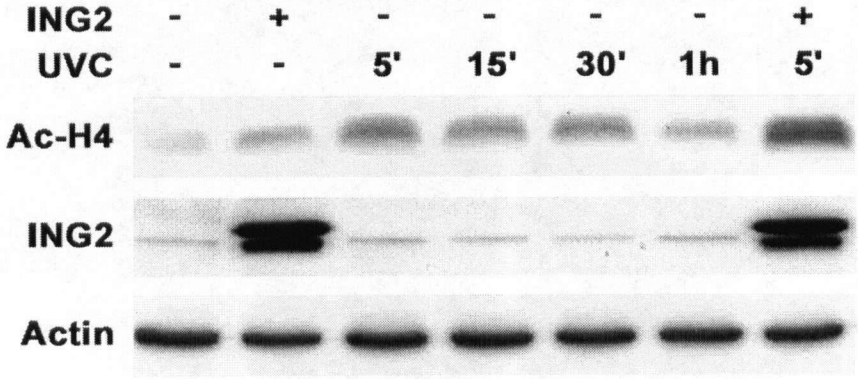

B
40
30
20
10
0
Luciferase activity (%)
control
ING2
ING2 siRNA
p53 si +ING2
C
Ctrl
ING2
no UV
UV 0h
UV 4h
UV 8h
UV 12h
UV 24h
UV 48h
CPD
Ctrl
ING2
no UV
UV 0h
UV 4h
UV 8h
UV 12h
6-4 PP

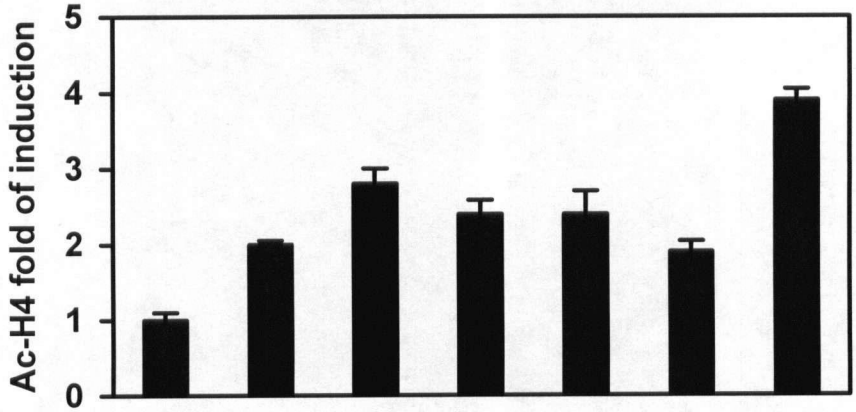
Figure 3.2 p33ING2 induces hyperacetylation of histone H4 after UV irradiation. **(A)** Western blot analysis of endogenous p33ING2 expression in MMRU cells at various time points after 200 J/m^2 UVC irradiation. MMRU cells without any treatment (*ctrl*) and MMRU cells transfected with p33ING2 (*ING2*) expression vector were used as controls. **(B,C)** Time course of acetylated histone H4 (*Ac-H4*) expression after 200 J/m^2 UVC irradiation in control (*B*) and ING2-transfected MMRU cells (*C*). Anti-Ac-H4 and anti-p33ING2 monoclonal antibodies were used. *Columns,* mean fold induction of Ac-H4 using Quantity One software (Bio-Rad) from at least three independent experiments. **(D)** Western blot analysis of histone H4 acetylation in MMRU cells treated with or without p33ING2 siRNA at 1 and 5 minutes after 200 J/m^2 UVC irradiation. **(E)** Western blot analysis of histone H4 acetylation in MMRU cells transfected with p33ING2 or cells cotransfected with p33ING2 and p53 siRNA at various time points following 200 J/m^2 UVC irradiation. The fold induction of acetylated histone H4 is shown below each blot. The experiments were repeated thrice with similar results. **(F)** Immunofluorescence staining of acetylated histone H4 in p33ING2-transfected MMRU cells and control cells 5 minutes after UVC (200 J/m^2) irradiation. Cells were irradiated through a 5-μm isopore polycarbonate filter cover, fixed at 5 minutes, and double stained for Ac-H4 (*green*) and CPDs (*red*). **(G)** P33ING2 induces histone H4 acetylation after 20 J/m^2 UVC irradiation. Top panel, time-course of acetylated histone H4 (Ac-H4) expression after 20 J/m^2 UVC irradiation in control and ING2-transfected MMRU cells. Anti-Ac-H4 and anti-p33ING2 monoclonal antibodies were used. Bottom Panel, western blot analysis of histone H4 acetylation in MMRU cells treated with or without p33ING2 siRNA at 1 min and 5 min after 20 J/m^2 UVC irradiation.

A

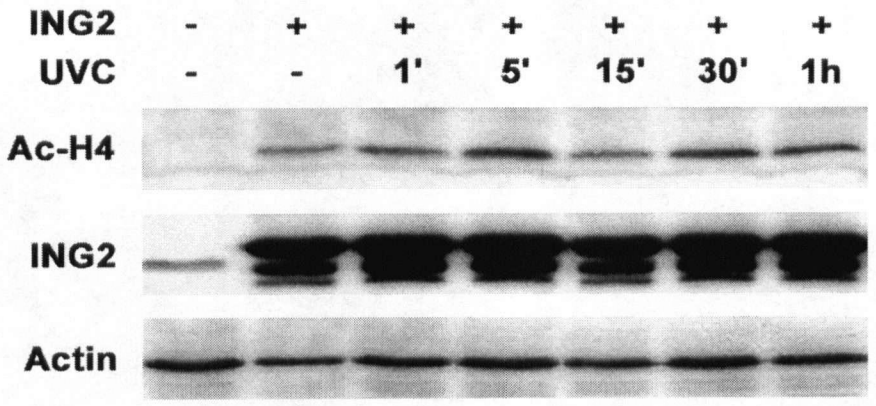

Ctrl
ING2
UVC
1'
5'
15'
30'
1h
ING2
Actin

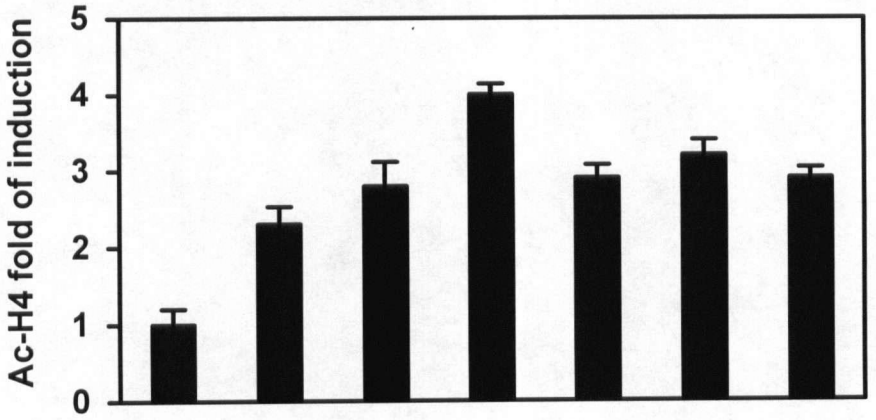
B


ING2 - + - - - - +
UVC - - 5' 15' 30' 1h 5'
Ac-H4
ING2
Actin

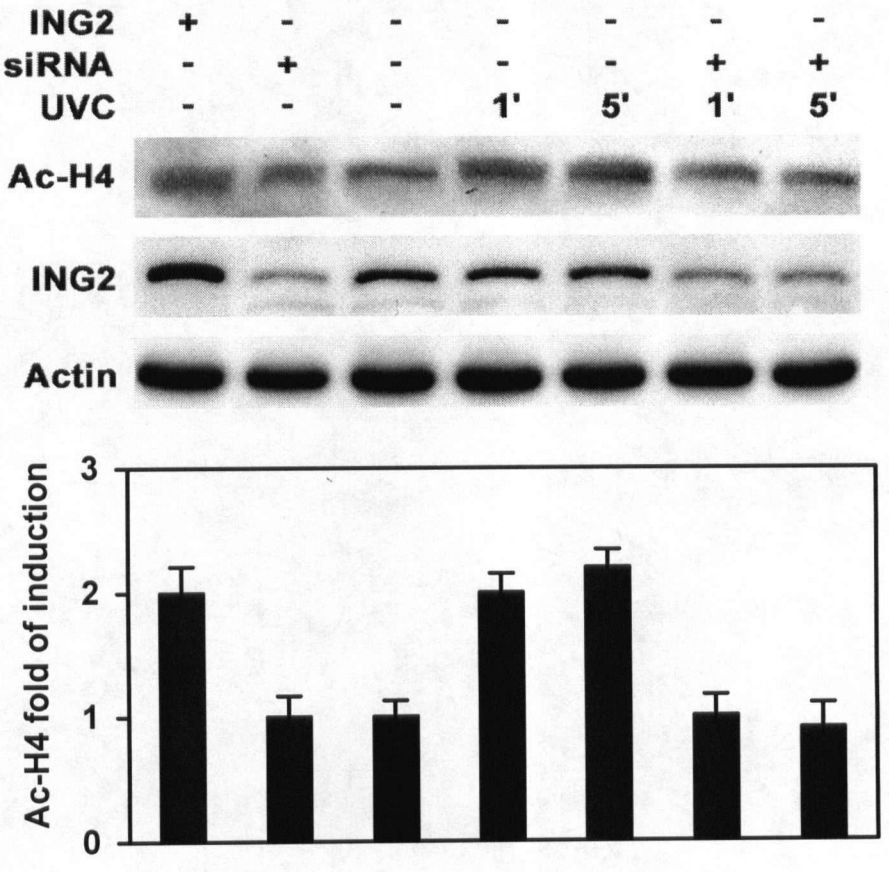

Ac-H4 fold of induction
0
1
2
3
4
5

C

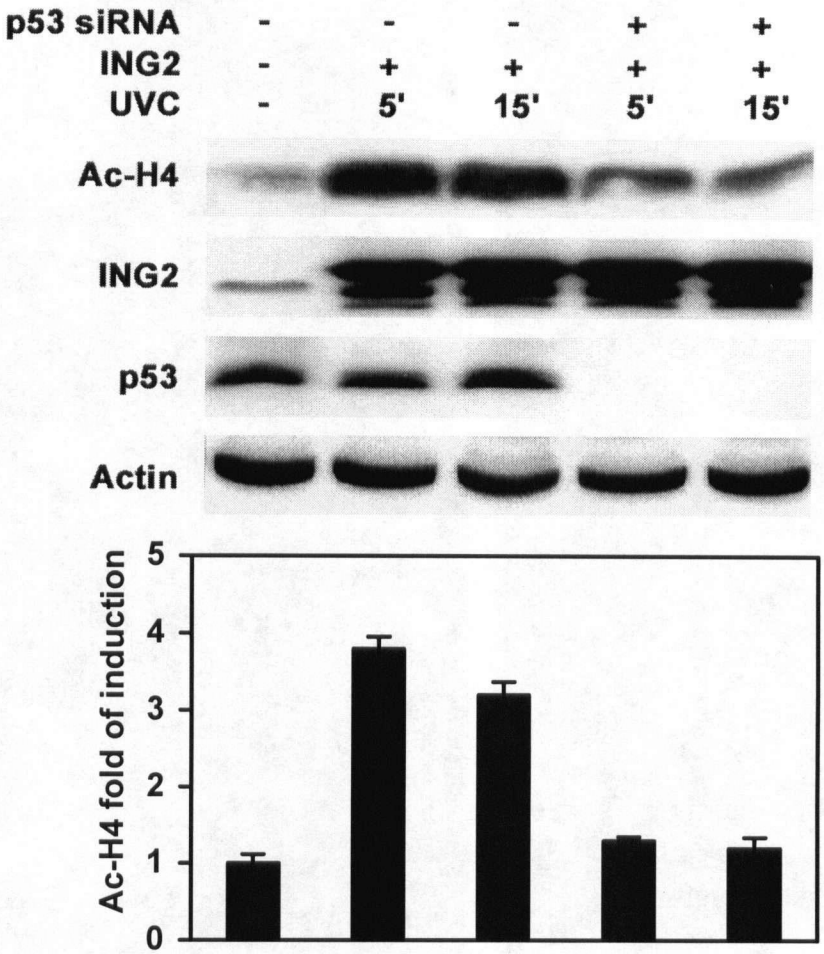

ING2 - + + + + + +
UVC - - 1' 5' 15' 30' 1h
Ac-H4
ING2
Actin


Ac-H4 fold of induction
0
1
2
3
4
5

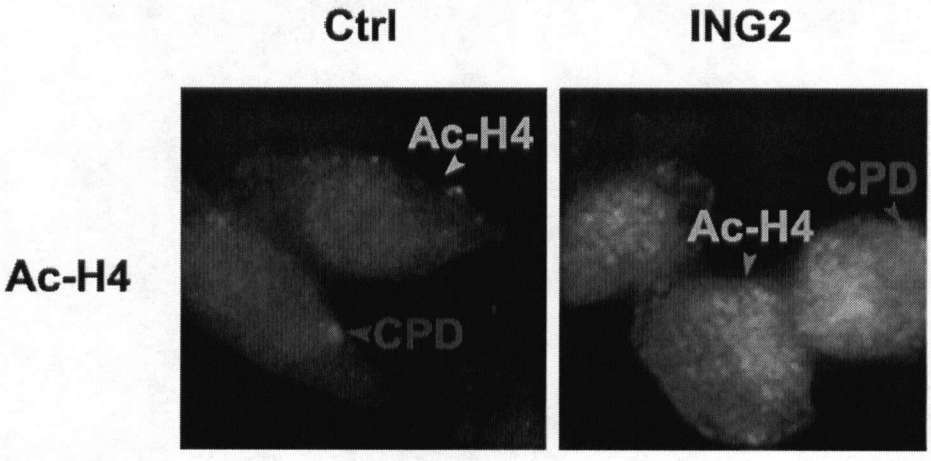
D


ING2 + - - - - - -
siRNA - + - - - + +
UVC - - - 1' 5' 1' 5'
Ac-H4
ING2
Actin
Ac-H4 fold of induction
3
2
1
0

E


p53 siRNA - - - + +
ING2 - + + + +
UVC - 5' 15' 5' 15'
Ac-H4
ING2
p53
Actin
Ac-H4 fold of induction
0
1
2
3
4
5

F


Ctrl
ING2
Ac-H4
Ac-H4
CPD
CPD
Ac-H4

G

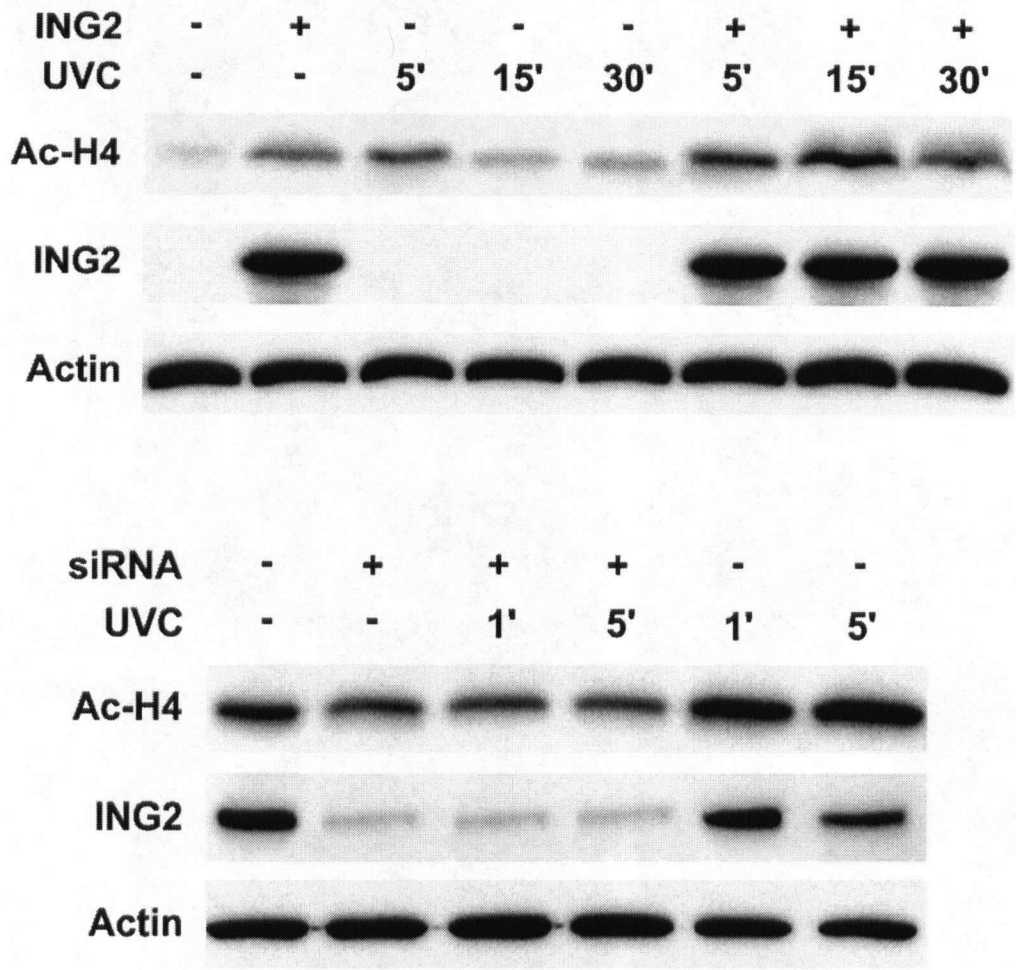

ING2 - + - - - + + +
UVC - - 5' 15' 30' 5' 15' 30'
Ac-H4
ING2
Actin
siRNA - + + + - -
UVC - - 1' 5' 1' 5'
Ac-H4
ING2
Actin

Figure 3.3 p33ING2 Induces Chromatin Relaxation. DNA was extracted from untreated, p33ING2-transfected, UVC-irradiated (200 J/m^2), or p33ING2-transfected and UVC-irradiated MMRU cells and subjected to micrococcal nuclease digestion assay with different concentrations of the nuclease (0, 0.1, or 1 unit). Cells treated with 200 ng/mL of trichostatin A (*TSA*) were used as a positive control. DNA ladder of 100 bp was used as a marker for the agarose gel. The percentage of DNA fragments <600 bp is quantified with Quantity One software. *Columns,* mean from triplicates; *bars,* SD. *Arrows,* DNA digested by micrococcal nuclease, indicating more relaxed chromatin.

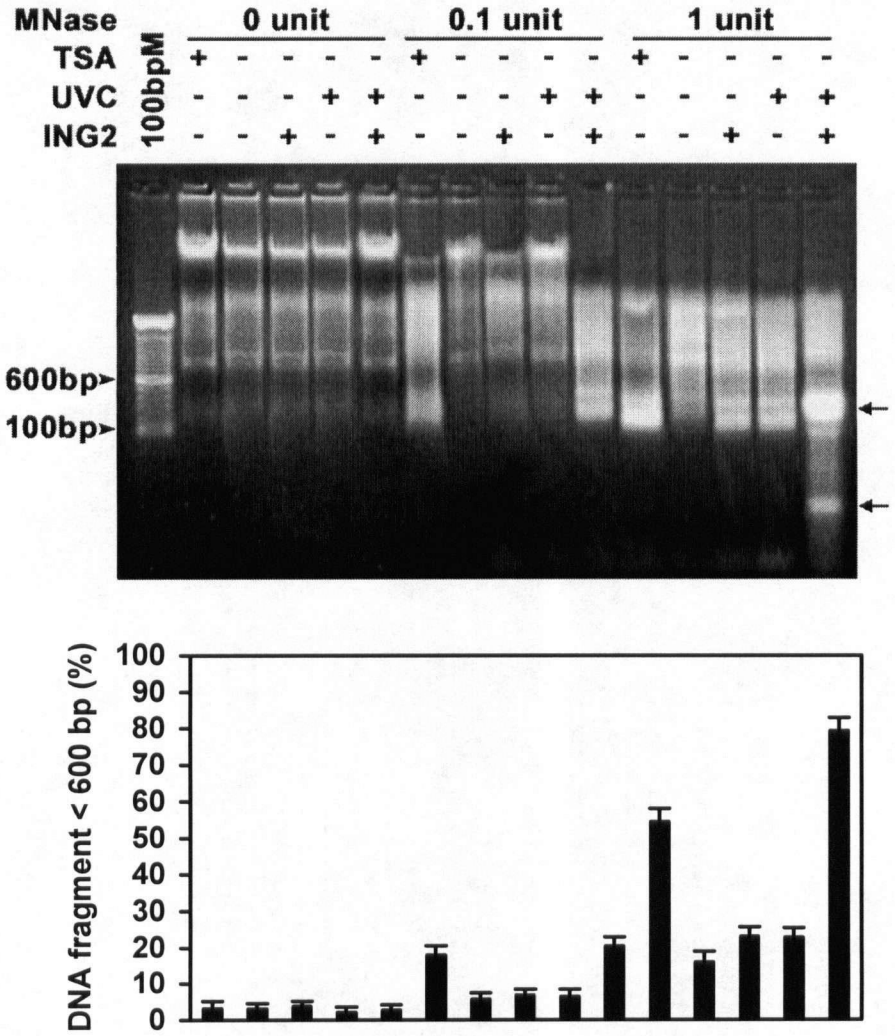
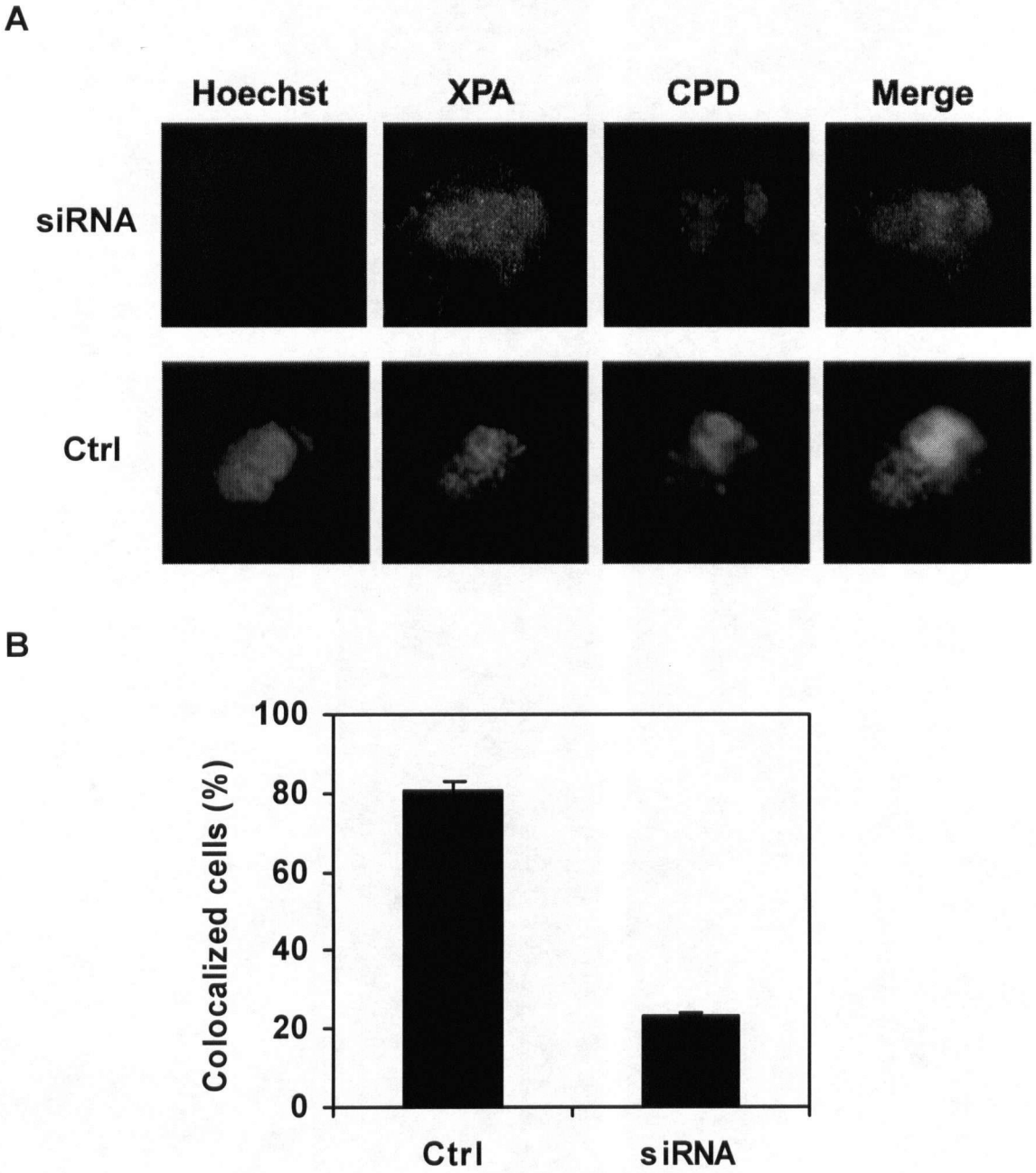


Figure 3.4 p33ING2 facilitates XPA to the DNA damage sites. (A) MMRU cells were treated with control scramble siRNA (*bottom*) or p33ING2 siRNA (*top*) for 48 hours and then irradiated with 200 J/m^2 UVC through a 5-μm isopore polycarbonate filter cover. Cells were fixed 30 minutes after UV irradiation and immunofluorescence staining of UV-induced DNA lesion CPDs and repair protein XPA was done. **(B)** The percentage of cells which have colocalization of XPA and CPDs was determined in control and p33ING2 siRNA–treated MMRU cells. Four random fields with a minimal 100 cells in each field were counted. *Columns,* mean; *bars,* SD.



CHAPTER 4. LEUCINE ZIPPER-LIKE DOMAIN IS CRITICAL FOR THE NOVEL TUMOR SUPPRESSOR P33ING2-MEDIATED NUCLEOTIDE EXCISION REPAIR AND APOPTOSIS

4.1 Rationales

The ING (inhibitor of growth) proteins consist of a family of at least seven proteins with highly conserved plant homeodomain (PHD) that is believed to be correlated with the ability of ING family proteins to induce apoptosis by serving as nuclear receptors of phosphatidylinositol phosphates (Gozani *et al.*, 2003). On the other hand, the PHD domains are also commonly found in proteins associated transcriptional regulation of specific genes. These activities suggest that mutations in the PHD domain could prevent ING proteins from inducing apoptosis and, moreover, disrupt their ability to induce or repress the expression of specific genes involved in tumorigenesis.

In addition to the PHD domain, ING family also contain two intrinsic nucleolar translocation sequences (NTS) which target the ING proteins to the nucleolus (Scott *et al.*, 2001), and several other highly conserved motifs whose functions have not been fully elucidated. p33ING1b is known to bind to the proliferating cell nuclear antigen (PCNA) through a specific sequence named the PCNA-interacting protein (PIP) domain (Warbrick *et al.*, 1998), which is found in proteins involved in growth inhibition, cell cycle arrest, DNA replication and repair (Feng *et al.*, 2002). 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.*, 2004). However, the specific biological functions of other motifs are poorly understood.

As a member of the ING family, p33ING2 contains a number of highly conserved regions with other ING members, including the plant homeodomain (PHD), leucine zipper-like domain (LZL), and potential chromatin regulatory domain (PCR). p33ING2 has been shown to negatively regulate cell growth in a p53-dependent manner through induction of G_1-phase cell cycle arrest and apoptosis (Nagashima *et al.*, 2001). Recently, functional analysis indicated that p33ING2 interacts with phosphoinositdes, Ptdlns(3)P and Ptdlns(5)P through its plant homeodomain (PHD) zinc finger motif which has been identified as binding targets of rare phosphatidylinositol phosphates (PtdInsPs) that function in DNA damage–initiated stress signaling (Gozani *et al.*, 2003). In addition, we demonstrated that p33ING2, like its homologue p33ING1b (Cheung *et al.*, 2001; Campos *et al.*, 2004), significantly enhances nucleotide excision repair (NER) in melanoma cells in a p53-dependent manner by rapidly inducing histone H4 acetylation, chromatin relaxation, and the recruitment of the damage recognition factor XPA to the DNA photolesions (Wang *et al.*, 2006). To further investigate the functions of the specific domains of p33ING2, we performed a systematic mutational analysis of the protein. To this end, we constructed a panel of p33ING2 mutants, ΔPHD, ΔLZL and ΔL+P (deletion of LZL and PCR domains), to determine which regions of p33ING2 are important for histone acetylation, chromatin assembly, DNA repair and apoptosis.

4.2 Results

4.2.1 The Leucine Zipper-Like Domain of P33ING2 Is Required for the Repair of UV-damaged DNA in Melanoma Cells

As candidate tumor suppressors, ING proteins have been implicated in playing a role in chromatin remodeling, gene regulation, DNA repair and apoptosis through binding to GADD45, the proliferating cell nuclear antigen (PCNA) (Cheung *et al.*, 2001; Scott *et al.*, 2001), histone acetyltransferase/deacetylase (HAT/HDAC) and the factor acetyltransferase complexes (FAT) (Feng *et al.*, 2002; Campos *et al.*, 2004). To further investigate the functions of subregions of p33ING2, we performed a systematic mutational analysis of the protein. As shown in Fig. 4.1A, ΔPHD, ΔLZL and ΔL+P deletion mutants were generated by subcloning strategy from the original full-length of p33ING2 plasmid. These deletion constructs are further confirmed by restriction digestion (Fig. 4.1B). In addition, constructs with similar protein expression levels were selected for following experiments (Fig. 4.1C).

Since we have previously demonstrated that p33ING2 can facilitate the removal of bulky photolesions or DNA adducts caused by UV light, we performed a luciferase reporter assay to investigate which region of p33ING2 is required for DNA repair by co-transfecting a UV-damaged pRL-CMV plasmid that contains the reporter gene with vector, wt p33ING2, ΔPHD, ΔLZL and ΔL+P into MMRU melanoma cells. The activity of this reporter gene was used as an indicator of the extent of repair. While ΔPHD construct, similar to the wt p33ING2, had a 2.8-fold enhancement in DNA repair compared with vector control ($P<0.01$, t-test), the enhancement in DNA repair was

abolished in ΔLZL and ΔL+P constructs (Fig. 4.1D), indicating that LZL domain is an important component for p33ING2 to exert its role in DNA repair in melanoma cells.

4.2.2 Leucine Zipper-Like Domain of P33ING2 Plays a Critical Role in the Process of Histone H4 Hyperacetylation upon UV Irradiation

It has been shown previously that p33ING2 has the ability to modulate acetylation levels through regulating the activity of coactivator and/or corepressor complexes (Gozani *et al.*, 2003). We recently found that p33ING2 is involved in histone acetylation and chromatin relaxation to mediate the repair of UV-damaged DNA (Wang *et al.*, 2006). With a panel of p33ING2 deletion mutants, we further explored which regions of p33ING2 are important for the hyperacetylation after UV irradiation. As shown in Fig. 4.2, cells overexpressing wt and a panel of mutant p33ING2 proteins are subjected to 20 J/m^2 of UVC irradiation and histone H4 acetylation was detected by anti-acetylated histone H4 antibody. Consistent with the DNA repair experiments, full-length p33ING2 as well as ΔPHD, enhances the histone H4 acetylation by 4-fold compared to the control cells before UV irradiation, while overexpression of ΔLZL and ΔL+P did not affect histone H4 acetylation (Fig. 4.2), suggesting that LZL domain is essential for p33ING2 in protein-protein interactions with certain HAT/HDAC to regulate histone H4 acetylation.

4.2.3 Leucine Zipper-Like Domain is Responsible for Functional P33ING2 to Enhance UV-induced Chromatin Relaxation

The activities that alter chromatin structure consist of ATP-dependent nucleosome remodeling and covalent modification of core histones, particularly acetylation or

deacetylation of lysine residues and methylation of lysine and arginine residues. These covalent modifications occur primarily within the N-terminal histone tails (Fyodorov *et al.*, 2002). Previously, we found that p33ING2 enhances histone acetylation and chromatin remodeling to facilitate nucleotide excision repair (Wang *et al.*, 2006). We further detect which region of p33ING2 is essential for the chromatin relaxation by a micrococcal nuclease digestion assay to assess chromatin decondensation in MMRU cells transfected with full-length p33ING2, ΔPHD, ΔLZL and ΔL+P. As shown in Fig. 4.3, chromatin is more sensitive to the nuclease (1 unit treatment) in cells overexpressing p33ING2 and ΔPHD mutant (86% and 85% of the DNA fragments < 2kb, respectively) upon UVC irradiation compared to MMRU cells transfected with vector control (15%), ΔLZL (22.5%) or ΔL+P (23.2%). These results are consistent with the observation that LZL domain is essential for p33ING2-enhanced histone H4 acetylation after UV irradiation (Fig. 4.2), implying that p33ING2 may bind to DNA via the LZL motif and serve as a chromatin accessibility factor during the repair of UV-damaged DNA.

4.2.4 Leucine Zipper-Like Domain of P33ING2 is Essential for the Enhancement of UV-induced Apoptosis

Recently, we showed that p33ING2 cooperates with p53 to regulate apoptosis via the activation of both the mitochondrial/intrinsic and death-receptor/extrinsic apoptotic pathways (Chin *et al.*, 2005). With the panel of the deletion constructs, we investigated which region of p33ING2 is responsible for this function. MMRU cells were transfected with vector, full-length p33ING2, ΔPHD, ΔLZL, and ΔL+P, irradiated with UVB at 400 J/m^2, and subjected to flow cytometry analysis since DNA fragmentation, a hallmark

feature of apoptosis, results in a hypodiploid DNA content represented by the Sub-G_1 population in the DNA cell cycle histogram (Fig. 4.4A). As shown in Fig. 4.4B, full-length p33ING2 and ΔPHD induced significantly higher rate of apoptosis (54.6% and 59.8%, respectively) ($P<0.001$, *t*-test) compared with cells transfected with vector control (24.4%), ΔLZL (27.2%), and ΔL+P (27.9%) ($P<0.0001$, *t*-test). To further confirm that p33ING2-mediated cell death after UVB irradiation is the result of apoptosis, not necrosis, cells were stained with Hoechst 33258, which binds to DNA. Cells undergoing apoptosis are characterized by chromatin condensation, DNA fragmentation, formation of apoptotic bodies (Hengartner *et al.*, 2000). The representative images taken under the fluorescent microscope were shown in Fig. 4.4C. Quantitation of Hoechst staining data indicates that there were significantly more apoptotic cells in p33ING2- and ΔPHD-transfected MMRU cells (50.5% and 49.6 %, respectively) after UV irradiation compared to the vector control (18.7%), ΔLZL (15.5%) and ΔL+P transfected cells (17.3%) ($P<0.001$, *t*-test) (Fig. 4.4D). This is consistent with the results from flow cytometry (Fig. 4.4A,B), suggesting that LZL domain instead of the PHD-finger motif is involved in protein-protein interactions for the assembly or activity of multi-component complexes regulating the transcriptional activation or repression of the apoptosis related proteins.

Discussion

As a member of the ING tumor suppressor proteins, p33ING2 features a characteristic domain structure of this family (Zeremski *et al.*, 1999). In addition to these common features, the ING family contains gene-specific or isoform-specific motifs that could contribute to function in various ways. These include the leucine zipper found in p33ING2, which promotes interaction with other leucine-zipper-containing proteins, including transcription factors (Hai *et al.*, 2001). However, previous research on either the founding member $p33^{ING1}$ or its homology p33ING2 has predominantly focused on the PHD finger region since it is also conserved in the yeast homolog YNG2 (Loewith *et al.*, 2000) and has been shown to be involved in chromatin remodeling and modulate p53 function as a transcription factor. Two yeast proteins, Yngl, Yng2, which have PHD-finger motifs at their C-terminal regions and have homology with human $p33^{ING1}$, are associated with a histone acetyltransferase (HAT) complex (Loewith *et al.*, 2000). So far, the function of the N-terminal region of the ING proteins remains unclear.

In this study, we investigate the functions of subregions of p33ING2 and our results revealed that the LZL motif plays a critical role for p33ING2-mediated histone H4 acetylation, chromatin decondensation, and nucleotide excision repair. Previously, we showed that PHD region of p33ING1b is important in nucleotide excision repair as the PHD deletion mutant is deficient in repair of UV-damaged DNA (Campos *et al.*, 2004). Due to the structural similarity between p33ING1b and p33ING2, we sought to investigate whether PHD of p33ING2 is also involved in the DNA repair process. Our data indicated that unlike p33ING1b, the PHD region of p33ING2 is dispensable, while the LZL domain is critical for nucleotide excision repair. The reason for different

domains of ING family members required for the DNA repair process is unclear. However, it is possible that distinct domain of each ING members is required for the interactions with specific HAT and/or HDAC complexes to induce histone acetylation and chromatin relaxation.

We also observed that LZL motif is essential for p33ING2 to enhance UV-medicated apoptosis in melanoma cells since MMRU cells transfected with p33ING2 lacking the LZL domain cannot undergo apoptosis or show less apoptosis than cells transfected with the full-length gene after UV irradiation. Gozani *et al.* previously showed that mutation in the PHD region of p33ING2 can abolish p33ING2-induced apoptosis under non-stress conditions (Gozani *et al.*, 2003). However, the role of these mutations in stress-induced apoptosis is unclear. As we recently demonstrated that p33ING2 activates the Fas pathway to induce apoptosis under non-stress conditions, but enhances UV-induced apoptosis by promoting Bax translocation to mitochondria and downregulate the expression of Bcl-2 (Chin *et al.*, 2005), thus, LZL domain may be required for the regulation of Bcl-2 family proteins under stress conditions.

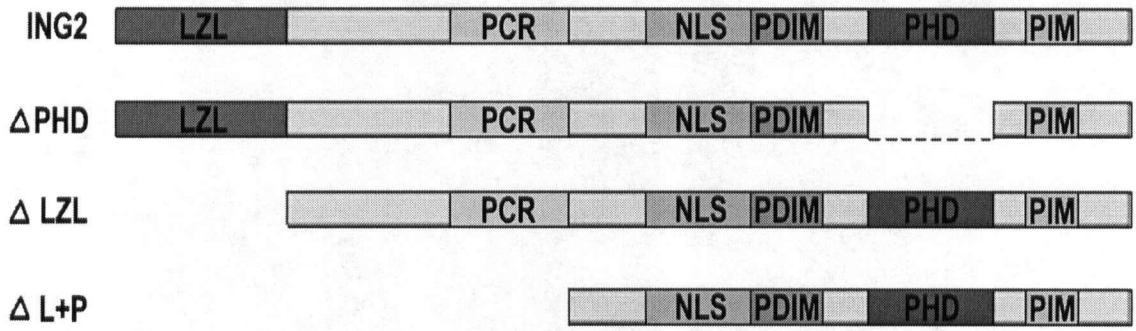
A couple of conserved motifs have been shown to be essential for the functions and activities of ING proteins. For example, the PIP domain targets p33ING1b to PCNA after DNA damage (Scott *et al.*, 2001). The nuclear localization sequence (NLS) and nucleolar targeting sequence (NTS) translocate p33ING1b and likely other INGs to the nucleus and nucleolus in response to UV-induced DNA damage (Scott *et al.*, 2001). The PCR domain is shown to link ING proteins to the Sin3/HDAC complex through direct interaction with SAP30 under non-stressed conditions (Kuzmichev *et al.*, 2002). The results from this study demonstrated that there is no significant difference in the

biological functions between the cells transfected with ΔLZL and ΔL+P constructs, suggesting LZL alone is sufficient for p33ING2 to mediate UV-induced DNA repair and apoptosis process probably distinct from complex with Sin3/HDAC pathway.

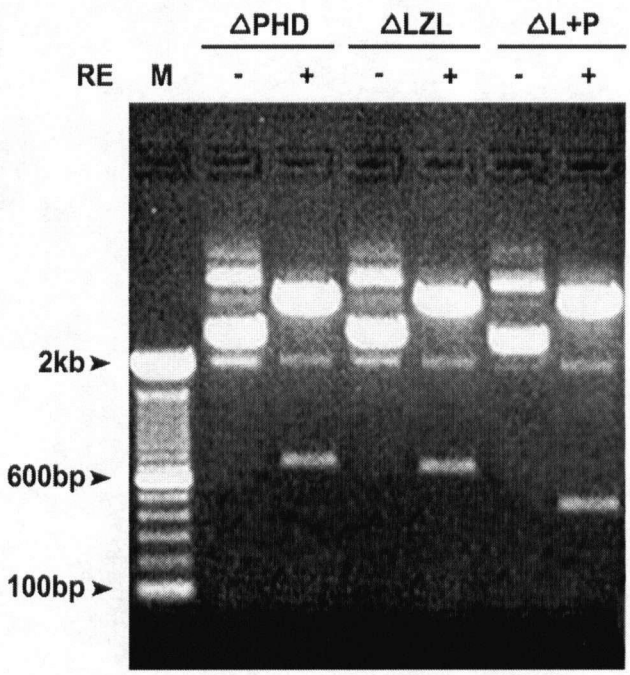
Taken together, LZL domain, which consists of leucine residues spanning every seven amino acids, forming a hydrophobic patch, is a key component for p33ING2 to exert its function in DNA repair and apoptosis, possibly by direct DNA binding or protein-protein interactions to regulate gene transcription and/or target particular HAT and HDAC complexes to chromatin. Future analyses comparing the full-length p33ING2 with its truncated mutants may provide new insights into the biological functions of p33ING2 domains.

Figure 4.1 The leucine lipper-like domain of p33ING2 is required for the repair of UV-damaged DNA in melanoma cells. (A) Schematic graph for the full-length p33ING2 (ING2), p33ING2 with PHD domain deletion (ΔPHD), LZL domain deletion (ΔLZL) and LZL plus PCR domain deletion (ΔL+P). **(B)** PCR products of different p33ING2 constructs were digested with restriction enzymes and analyzed in a 0.8% agarose gel. **(C)** Protein expression level of different p33ING2 construct in MMRU cells. MMRU cells were transfected with full-length p33ING2, ΔPHD, ΔLZL and ΔL+P constructs for 24 hours and protein expression level was detected by western blot analysis. A rat anti-p33ING2 monoclonal antibody was used to detect various p33ING2 proteins. **(D)** Effect of p33ING2 domains on nucleotide excision repair. MMRU cells were co-transfected with undamaged or damaged pRL-CMV and 3×FLAG vector, 3×FLAG-p33ING2, 3×FLAG-ΔPHD, 3×FLAG-ΔLZL, 3×FLAG-ΔL+P expression vector and luciferase activity was determined 40 hours after transfection. Data represent means ± SD from triplicates. The experiment was repeated thrice with similar results.

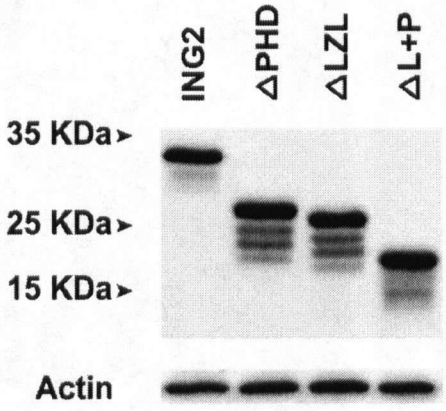
A



B


ΔPHD
ΔLZL
ΔL+P
RE
M
-
+
-
+
-
+
2kb
600bp
100bp

C


ING2
ΔPHD
ΔLZL
ΔL+P
35 KDa
25 KDa
15 KDa
Actin

D

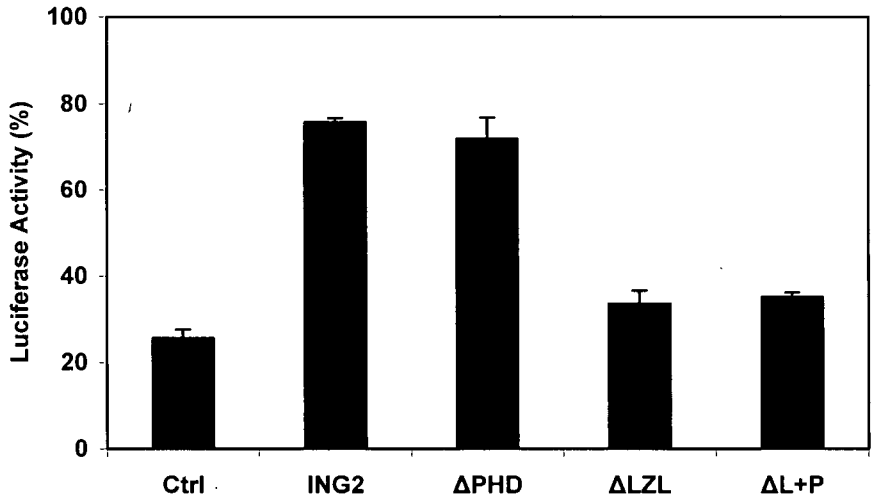


Figure 4.2 Leucine zipper-like domain of p33ING2 plays a critical role in the process of histone H4 hyperacetylation upon UV irradiation. Western blot analysis of acetylated histone H4 (Ac-H4) expression 5 min after 20 J/m^2 UVC irradiation in control (3×FLAG vector) and MMRU cells transfected with various p33ING2 constructs (upper panel). Anti-Ac-H4 and anti-p33ING2 monoclonal antibodies were used. The fold induction of acetylated histone H4 is showed in lower panel. The experiments were repeated three times and the standard deviation is shown.

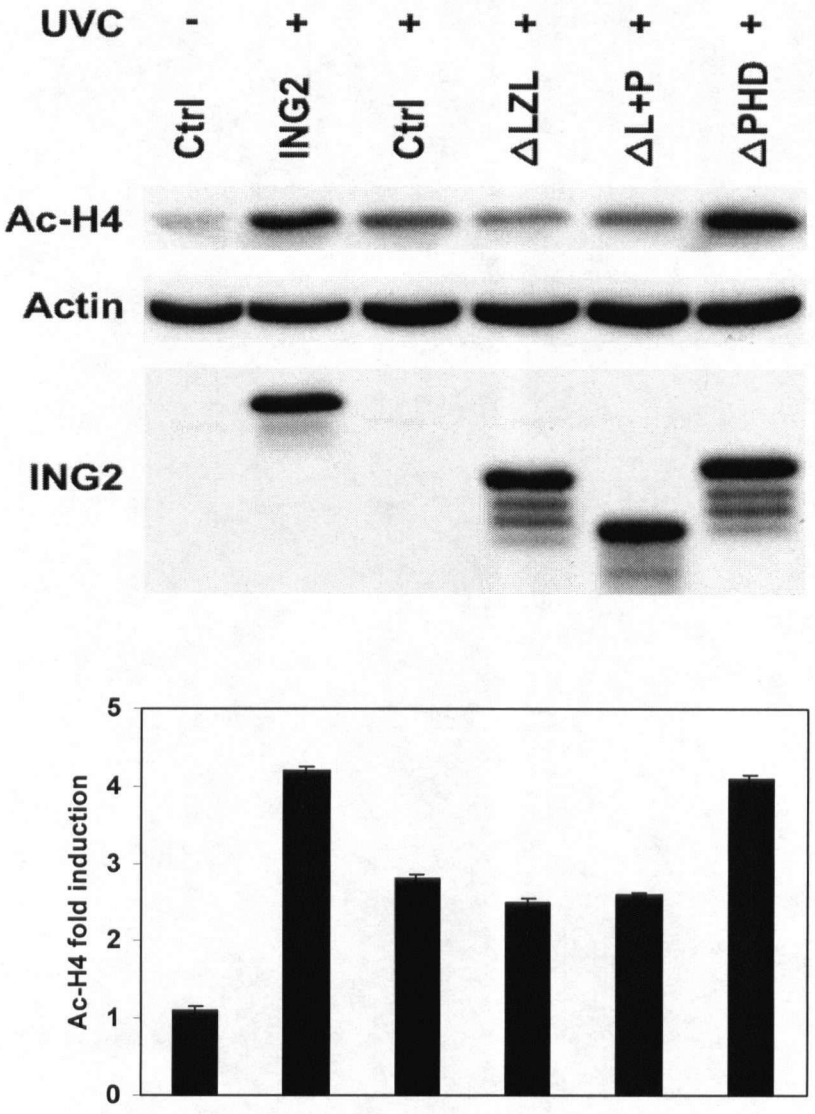


Figure 4.3 Leucine zipper-like domain is responsible for functional p33ING2 to enhance UV-induced chromatin relaxation. DNA was extracted from control MMRU cells or MMRU cells transfected with various p33ING2 constructs with or without UVC irradiation (20 J/m^2), and subjected to micrococcal nuclease digestion with different concentration of the nuclease (0, 0.1, or 1 unit). The percentage of DNA fragments below 2kb is quantified with Quantity One software. Data represent means ± SD from triplicates.

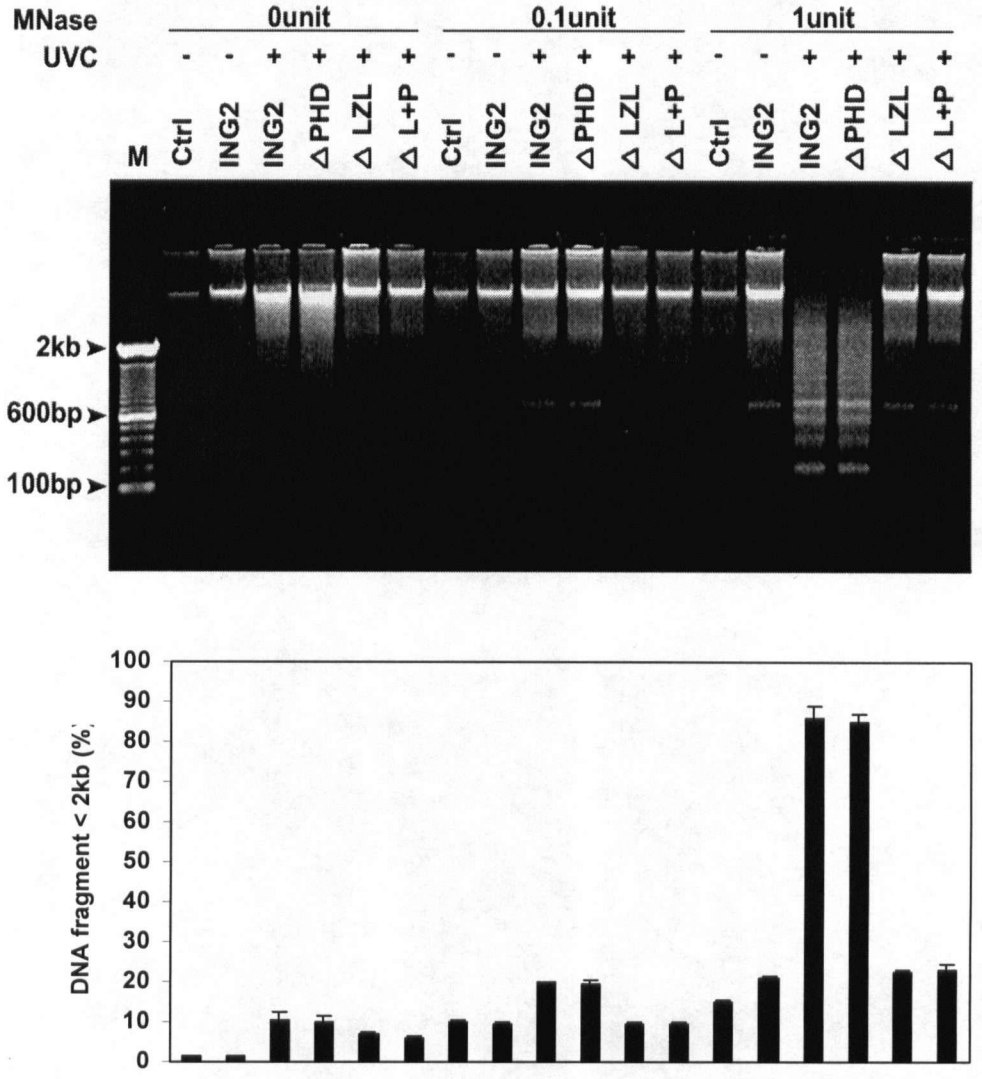
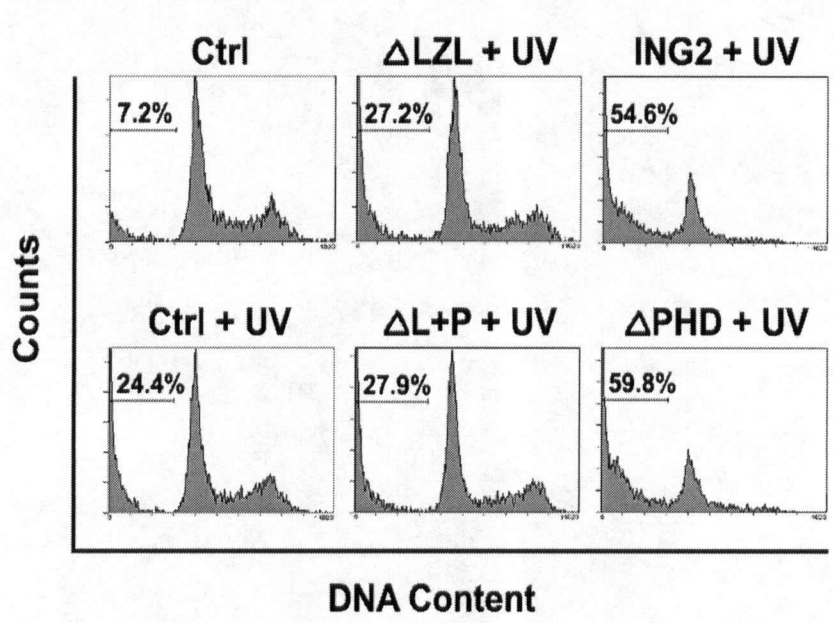
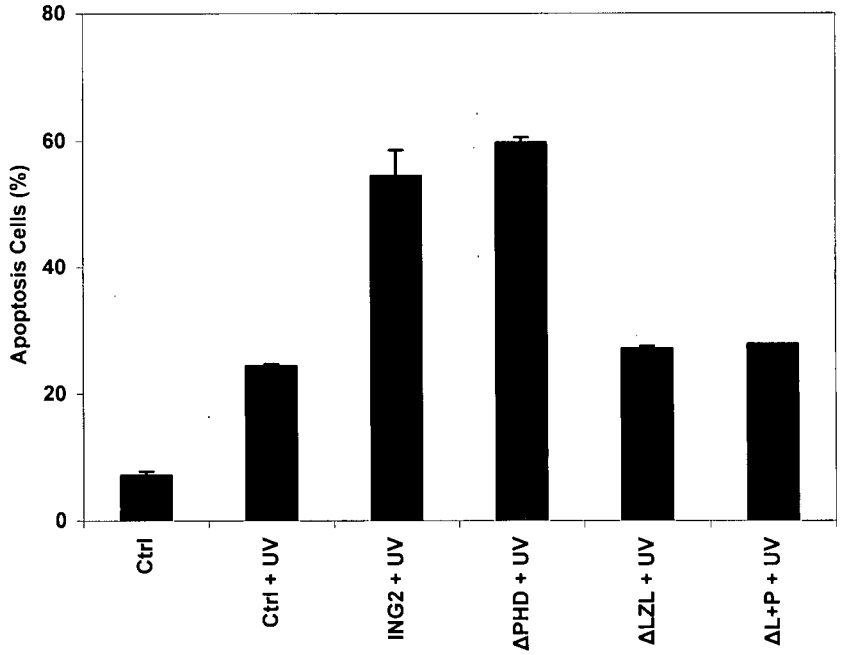


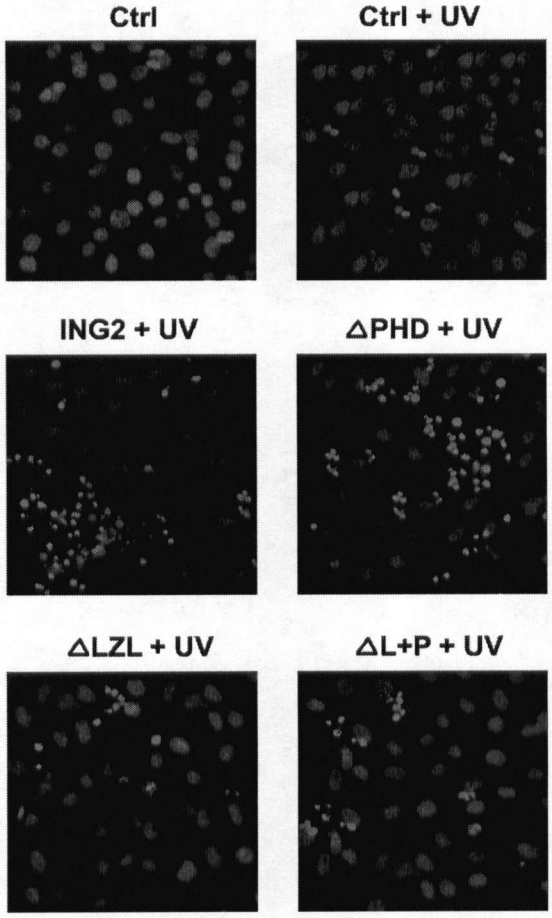
Figure 4.4 Leucine zipper-like domain of p33ING2 is essential for the enhancement of UV-induced apoptosis.. (A) MMRU cells were transfected with vector, p33ING2, or deletion mutants and irradiated with 400 J/m^2 UVB, and subjected to propidium iodide staining and flow cytometry analysis. The sub-G_1 population in the DNA cell cycle histogram represents apoptotic cells. Data represent means ± SD from triplicates. **(B)** Quantification of FACS analysis from (A). **(C)** Hoechst staining of apoptotic bodies in control MMRU cells and MMRU cells expressing different p33ING2 constructs after 400 J/m^2 UVB irradiation. Data represent means ± SD from triplicates. **(D)** Quantification of apoptotic bodies from (C).



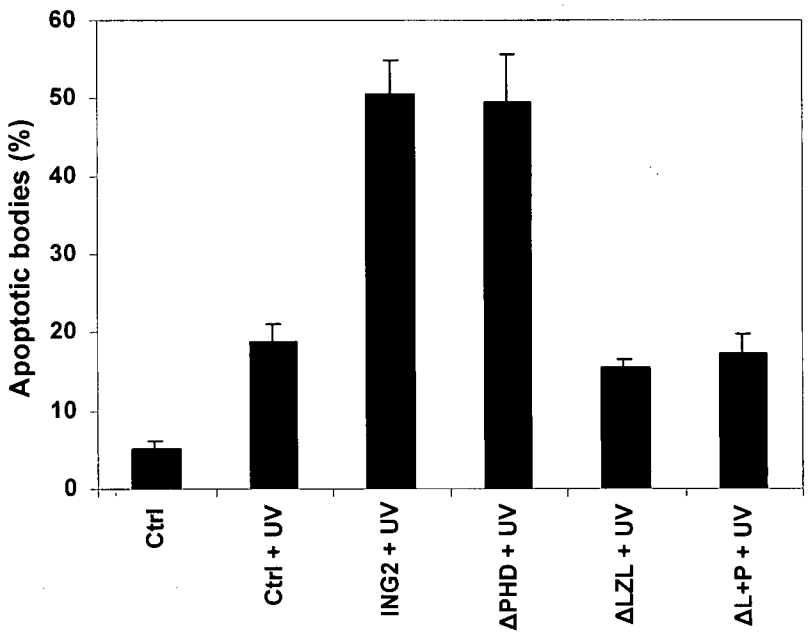
B


80
60
40
20
0
Apoptosis Cells (%)
Ctrl
Ctrl + UV
ING2 + UV
ΔPHD + UV
ΔLZL + UV
ΔL+P + UV

C



D


Apoptotic bodies (%)
60
50
40
30
20
10
0
Ctrl
Ctrl + UV
ING2 + UV
ΔPHD + UV
ΔLZL + UV
ΔL+P + UV

CHAPTER 5. GENERAL CONCLUSIONS

5.1 Summary

P33ING2 is a novel candidate tumor suppressor, which has been shown to be involved in the regulation of gene transcription, cell cycle arrest and apoptosis in a p53-dependent manner for maintaining the genomic stability. Previously, it was shownthat overexpressed p33ING2 promoted UV-induced DNA repair and apoptosis in human melanoma cells. However, the precise manner in which the INGs associate with p53 and regulate apoptosis or DNA repair remains unclear. Given those recent studies suggesting that altering the levels of ING1 protein affects the expression of certain genes and regulate gene expression through localized chromatin modification, to further reveal the role of p33ING2 in cellular stress response to UV irradiation, we hypothesized that in a physiological condition, p33ING2 may enhance the repair of UV-damaged DNA through chromatin remodeling, similar as its homologue p33ING1b. Using luciferase reporter assays, we confirmed that overexpressed p33ING2 significantly enhances nucleotide excision repair of UV-induced DNA damage in melanoma cells in a p53-dependent manner. Furthermore, DNA repair is completely abolished in cells treated with p33ING2 siRNA, suggesting that physiological level of p33ING2 is required for nucleotide excision repair. In addition, we found that a physiological p33ING2 level is an essential factor for UV-induced rapid histone H4 acetylation, chromatin relaxation, and the recruitment of damage recognition protein XPA to the photolesions. These observations suggest that p33ING2 is required for the initial DNA damage sensing and chromatin remodeling in the nucleotide excision repair process in melanoma.

As a member of the ING family, p33ING2 contains a number of highly conserved regions with other ING members, including the plant homeodomain (PHD) in the C terminal, leucine zipper-like domain (LZL) in the N terminal, and potential chromatin regulatory domain (PCR). Studies in both yeast and human cells have implicated ING proteins in chromatin modification and in the regulation of gene expression through binding to and altering of the activity of protein complexes containing histone acetyltransferase (HAT), histone deacetylase (HDAC) and factor acetyltransfease (FAT) activities. Previous studies were mainly focused on the PHD domain since it was demonstrated to be involved in chromatin mediated transcriptional regulation. However, the specific biological functions of other motifs are poorly understood. In this study, we developed a panel of p33ING2 deletion mutants to better understand the impact of p33ING2 on multiple biological processes. Further analysis revealed that LZL domain is essential for the ability of p33ING2 in facilitating histone acetylation, chromatin relaxation and thus promote nucleotide excision or apoptosis when there is a severe damage in melanoma cells. This finding confers a novel function to leucine zipper like domain containing ING family proteins and provides additional insight into the mechanism as to how ING family proteins regulate chromatin modification and furthermore, suggests a conserved role for the ING LZL domain in this capacity.

Taken together, we demonstrated in this thesis the mechanisms of novel tumor suppressor p33ING2 in DNA repair and apoptosis, consistent with the notion that the ING family exerts diverse biological effects through altering gene expression by targeting and regulating local acetylation and/or deacetylation activities These results provided

new insights intothe functional significance of the reduced ING expression observed in melanoma.

5.2 Future Directions

Human ING proteins are involved in chromatin remodeling functions through physical association with protein complexes that have HAT and HDAC activity. Endogenous human ING1 proteins co-precipitate (Boland *et al.*, 2000) with TRRAP, the mammalian counterpart of yeast Tra1, and with PCAF, CBP and p300, all of which are either HATs themselves, or are in complexes containing HAT activity. Furthermore, a recent report demonstrating a link between p300 HAT and PCNA suggested that high levels of p33ING1b interfered with association of p300 and PCNA independently (Vieyra *et al.*, 2002). Due to the similarities between p33ING2 and p33ING1b, it would be of interest to check by immunoprecipitation analysis if p33ING2 is complexed with p300, PCNA or other proteins that have HAT activity and serve as a bridge in chromatin modification and gene transcription regulation context.

Proteins containing leucine zipper region have been found to be involved in the gene transcription activation or repression by interacting with other leucine zipper containing proteins or transcription factors (Hai *et al.*, 2001). Consistent with this result, we found that p33ING2 deleted LZL domain is deficient in histone acetylation, chromatin modification and apoptosis. However, it is of interest to investigate if the LZL domain itself can repair the photolesions, induce histone H4 acetylation, chromatin relaxation and apoptosis after UV irradiation. In addition, if we further delete PCR region, which has been known as a potential chromatin regulatory domain binding with

Sin3 HDAC complex, does not have a synergistic effect on the whole histone acetylaiton or chromatin relaxation level, indicating that LZL domain induce chromatin modification through a distinct pathway instead of interacting with HDAC. However, it is possible that other regions can also contribute to the histone acetylation together with LZL. For instance, the PIM found at the C-terminal end of ING1 and ING2 has been recently identified and found to stabilize proteins with particular posttranslational modification (Feng *et al.*, 2004). In addition, interaction of ING with other proteins may be mediated through the candidate phosphorylation-dependent interacting motif (PDIM), whose phosphorylation could recruit proteins important in the modulation of such cellular processes as apoptosis, signal transduction, and cell cycle regulation.

REFERENCES

Albino AP, Sozzi G, Nanus DM, Jhanwar SC, Houghton AN. Malignant transformation of human melanocytes: Induction of a complete melanoma phenotype and genotype. *Oncogene* 1992; **7**:2315-2321.

Ananthaswamy HN, Pierceall WE. Molecular mechanisms of ultraviolet radiation carcinogenesis. *Photochem Photobiol* 1990; **52**:1119–1136.

Annunziato AT, Frado LL, Seale RL, Woodcock CL. *Chromosoma* 1988; **96**:132-138.

Aravind L, Iyer LM, Koonin EV. Scores of RINGS but no PHDs in ubiquitin signaling. *Cell Cycle* 2003; **2**:123–126.

Aszterbaum M, Rothman A, Johnson RL, Fisher M, Xie J, Bonifas JM, Zhang X, Scott MP, Epstein EH Jr. Identification of mutations in the human *PATCHED* gene in sporadic basal cell carcinomas and in patients with the basal cell nevus syndrome. *J Invest Dermatol* 1998; **110**: 885-888.

Autier P, Dore JF, Lejeune F, Koelmel KF, Geffeler O, Hille P, Cesarini JP, Lienard D, Liabeuf A, Joarlette M. Cutaneous malignant melanoma and exposure to sunlamps or sunbeds: an EORTC multicenter case-control study in Belgium, France and Germany. EORTC Melanoma Cooperative Group. *Int J Cancer* 1994; **58**: 809–813.

Ball NJ, Yohn JJ, Morelli JG, Norris DA, Golitz LE, Hoeffler JP. Ras mutations in human melanoma: A marker of malignant progression. *J Invest Dermatol* 1994; **102**: 285-290.

Baxter, BK, Smerdon, MJ. Nucleosome unfolding during DNA repair in normal and xeroderma pigmentosum (group C) human cells. *J Biol Chem* 1998; **273**:17517-17524.

Berking C. The role of ultraviolet irradiation in malignant melanoma. *Hautarzt* 2005; **56**:687-696; quiz 697.

Bert V, David L, Arnold JL. Surfing the p53 network. *Nature* 2000; **408**:307-310.

Bestak R, Halliday GM. Chronic low-dose UVA irradiation induces local suppression of contact hypersensitivity, Langerhans cell depletion and suppressor cell activation in C3H/HeJ mice, *Photochem Photobiol* 1996; **64**:969–974.

Bird AW, Yu DY, Pray-Grant MG, Qiu Q, Harmon KE, Megee PC, Grant PA, Smith MM, Christman MF. Acetylation of histone H4 by Esa1 is required for DNA double-strand break repair. *Nature* 2002; **419**:411-415.

Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, Halperin AJ, Ponten J. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. *Proc Natl Acad Sci USA*1991; **88**: 10124-10128.

Bromidge T, Lynas C. Relative levels of alternative transcripts of the ING1 gene and lack of mutations of p33/ING1 in haematological malignanceies. *Leuk Res* 2002; **26**:631–635.

Buschmann T, Fuchs SY, Lee CG, Pan ZQ, Ronai Z. SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell* 2000; **101**:753– 762.

Cadet J, Anselmino C, Douki T, Voituriez L. Photochemistry of nucleic acids in cells. *J Photochem Photobiol B* 1992; **15**:277–298.

Cadet J, Berger M, Douki T, Morin B, Raoul S, Ravanat JL, Spinelli S. Effects of UV and visible radiation on DNA-final base damage. *Biol Chem* 1997; **378**:1275–1286.

Campbell C, Quinn AG, Ro YS, Angus B, Rees JL. p53 mutations are common and early events that precede tumor invasion in squamous cell neoplasia of the skin. *J Invest Dermatol* 1993; **100**:746-748.

Campos EI, Chin MY, Kuo WH, Li G. Biological functions of the ING family tumor suppressors. *Cell Mol Life Sci* 2004; **61**:2597-2613.

Campos EI, Martinka M, Mitchell DL, Dai DL, Li G. Mutations of the ING1 tumor suppressor gene detected in human melanoma abrogate nucleotide excision repair. *Int J Oncol* 2004; **25**:73-80.

Carr AM. Piecing together the p53 puzzle. *Science* 2000; **287**:1765–1766.

Carr J, Mackie RM. Point mutations in the N-ras oncogene in malignant melanoma and congenital naevi. *Br J Dermatol* 1994; **131**:72-77.

Chan TA, Hermeking H, Lengauer C, Kinzler KW, Vogelstein B. 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 1999; **40**:616–620.

Chen B, Campos EI, Crawford R, Martinka M, Li G. Analyses of the tumor suppressor ING1 expression and gene mutation in human basal cell carcinoma. *Int J Oncol* 2003; *22*:927–931.

Chen L, Matsubara N, Yoshino T, Nagasaka T, Hoshizima N, Shirakawa Y, Naomoto Y, Isozaki H, Riabowol K, Tanaka N. Genetic alterations of candidate tumor suppressor ING1 in human esophageal squamous cell cancer. *Cancer Res* 2001; **61**:4345–4349.

Cheung KJ, Li G. p33(ING1) enhances UVB-induced apoptosis in melanoma cells. *Exp Cell Res* 2002; **279:**291-298.

Cheung KJ, Mitchell D, Lin P, Li G. The tumor suppressor candidate p33(ING1) mediates repair of UV-damaged DNA. *Cancer Res* 2001; **61**:4974-4977.

Cheung KJ, Li G. p33(ING1) enhances UVBinduced apoptosis in melanoma cells. *Exp Cell Res* 2002; **279**:291–298.

Chin MY, Ng KC, Li G. The novel tumor suppressor p33ING2 enhances UVB-induced apoptosis in human melanoma cells. *Exp Cell Res* 2005; **304**:531-543.

Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* 2000; **406**:532-535.

Coscoy L, Ganem D. PHD domains and E3 ubiquitin ligases: viruses make the connection. *Trends Cell Biol* 2003; **13**:7–12.

Courtois S, Verhaegh G, North S, Luciani MG, Lassus P, Hibner U, Oren M, Hainaut P. ΔN-p53, a natural isoform of p53 lacking the first transactivation domain, counteracts growth suppression by wild-type p53. *Oncogene* 2002; **21**:6722-6728.

Dajee M, Lazarov M, Zhang JY, Cai T, Green CL, Russell AJ, Marinkovich MP, Tao S, Lin Q, Kubo Y, Khavari PA. NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature* 2003; **421**:639-643.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W, Davis N, Dicks E, Ewing R, Floyd Y, Gray K, Hall S, Hawes R, Hughes J, Kosmidou V, Menzies A, Mould C, Parker A, Stevens C, Watt S, Hooper S, Wilson R, Jayatilake H, Gusterson BA, Cooper C, Shipley J, Hargrave D, Pritchard-Jones K, Maitland N, Chenevix-Trench G, Riggins GJ, Bigner DD, Palmieri G, Cossu A, Flanagan A, Nicholson A, Ho JW, Leung SY, Yuen ST, Weber BL, Seigler HF, Darrow TL, Paterson H, Marais R, Marshall CJ, Wooster R, Stratton MR, Futreal PA. Mutations of the *BRAF* gene in human cancer. *Nature* 2002; **417**:949-954.

Daya-Grosjean L, Sarasin A. The role of UV induced lesions in skin carcinogenesis: an overview of oncogene and tumor suppressor gene modifications in xeroderma pigmentosum skin tumors. *Mutat Res* 2005; **571**:43-56.

Dellambra E. Downregulation of 14-3-3sigma prevents clonal evolution and leads to immortalization of primary human keratinocytes. *J Cell Biol* 2000; **149**:1117– 1130.

Demunter A, Stas M, Degreef H, De Wolf-Peeters C, van den Oord JJ. Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. *J Invest Dermatol* 2001; **117**:1483-1489.

D'Errico M, Calcagnile AS, Corona R, Fucci M, Annessi G, Baliva G, Tosti ME, Pasquini P, Dogliotti E. p53 mutations and chromosome instability in basal cell carcinomas developed at an early or late age. *Cancer Res* 1997; **57**:747-752.

Douki T, Reynaud-Angelin A, Cadet J, Sage E. Bipyrimidine photoproducts rather than oxidative lesions are the main type of DNA damage involved in the genotoxic effect of solar UVA radiation, *Biochemistry* 2003; **42**:9221–9226.

Doyon Y, Selleck W, Lane WS, Tan S, Cote J. Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. *Mol Cell Biol* 2004; **24**:1884-96.

El-Deiry WS. Regulation of p53 downstream genes. *Semin. Cancer Biol* 1998; **8**:345–357.

Evans T, Boonchai W, Shanley S, Smyth I, Gillies S, Georgas K, Wainwright B, Chenevix-Trench G, Wicking C. The spectrum of patched mutations in a collection of Australian basal cell carcinomas. *Hum Mutat* 2000; **16**:43-48.

Fabo EC, De FP, Noonan T, Fears, Merlino G. Ultraviolet B but not ultraviolet A radiation initiates melanoma. *Cancer Res* 2004; **64**:6372–6376.

Feng X, Hara Y, Riabowol K. Different HATS of the ING1 gene family. *Trends Cell Biol* 2002; **12**:532–538.

Fichtinger-Schepman AM, van der Veer JL, den Hartog JH, Lohman PH, Reedijk J. Adducts of the antitumor drug cis-diamminedichloroplatinum (II) with DNA: formation, identification and quantitation. *Biochemistry* 1985; **24**:707–713.

Fitch ME, Cross IV, Ford JM. p53 responsive nucleotide excision repair gene products p48 and XPC, but not p53, localize to sites of UV-irradiation-induced DNA damage, in vivo. *Carcinogenesis* 2003; **24**:843-850.

Flaman JM, Waridel F, Estreicher A, Vannier A, Limacher JM., Gilbert D, Iggo R, Frebourg T. The human tumor suppressor gene p53 is alternatively spliced in normal cells. *Oncogene* 1996; **12:**813-818.

Ford, JM, Hanawalt PC. Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. *J Biol Chem* 1997; **272**:28073-28080.

Friedberg EC, Henning KA. The conundrum of xeroderma pigmentosum-a rare disease with frequent complexities. *Mutat Res* 1993; **289:**47–53.

Fyodorov DV, Kadonaga JT. Binding of Acf1 to DNA involves a WAC motif and is important for ACF-mediated chromatin assembly. *Mol Cell Biol* 2002; **22**:6344-6353.

Gailani MR, Stahle-Backdahl M, Leffell DJ, Glynn M, Zaphiropoulos PG, Pressman C, Unden AB, Dean M, Brash DE, Bale AE, Toftgard R. The role of the human homologue of *Drosophila* patched in sporadic basal cell carcinomas. *Nat Genet* 1996; **14**:78-81.

Gailani MR, Leffell DJ, Ziegler A, Gross EG, Brash DE, Bale AE. Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma. *J Natl Cancer Inst* 1996; **88**:349-354.

Garkavtsev I, Riabowol K. Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. *Mol Cell Biol* 1997; **17**:2014–2019.

Garkavtsev I, Kazarov A, Gudkov A, Riabowol K. Correction and addition to ING1 isoform sequences. *Nat Genet* 199; **23**:373.

Garkavtsev I, Kazarov A, Gudkov A, Riabowol K. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. *Nat Genet*1996; **14**:415–420.

Garkavtsev I, Demetrick D, Riabowol K. Cellular localization and chromosome mapping of a novel candidate tumor suppressor gene (ING1). *Cytogenet Cell Genet* 1997; **76**:176–178.

Garkavtsev I, Grigorian IA, Ossovskaya VS, Chernov MV, Chumakov PM, Gudkov AV. The candidate tumour suppressor p33(ING1) cooperates with p53 in cell growth control. *Nature* 1998; **391**:295–298.

Garkavtsev I, Hull C, Riabowol K. Molecular aspects of the relationship between cancer and aging: tumor suppressor activity during cellular senescence. *Exp Gerontol* 1998; **33**:81–94.

Garkavtsev I, Kozin SV, Chernova O, Xu L, Winkler F, Brown E, Barnett GH, Jain RK. The candidate tumor suppressor protein ING4 regulates brain tumor growth and angiogenesis. *Nature* 2004; **428**:328–332.

Ghosh A, Stewart D, Matlashewski G. Regulation of human p53 activity and cell localization by alternative splicing. *Mol Cell Biol* 2004; **24**:7987-7997.

Giaccia AJ, Kastan MB. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev*1998; **12**:2973–2983.

Gibbons RJ, Bachoo S, Picketts DJ, Aftimos S, Asenbauer B, Bergoffen J, Berry SA, Dahl N, Fryer A, Keppler K, Kurosawa K, Levin ML, Masuno M, Neri G, Pierpont ME, Slaney SF, Higgs DR. Mutations in transcriptional regulator ATRX establish the functional significance of PHD-like domains, *Nat Genet* 1997; **17**:146–148.

Gilchrest BA, Eller MS, Geller AC, Yaar M. The pathogenesis of melanoma induced by ultraviolet radiation. *N Engl J Med* 1999; **340**:1341-1348.

Gong W, Suzuki K, Russell M, Riabowol K. Function of the ING family of PHD proteins in cancer. *Int J Biochem Cell Biol* 2005; **37**:1054-1065.

Gontijo AM, Green CM, Almouzni G. Repairing DNA damage in chromatin. *Biochimie* 2003; **85**:1133–1147.

Gordon HY, H CC, Helbing MJ, Wagner CW, Sensen, Karl R. Phylogenetic Analysis of the ING Family of PHD Finger Proteins. *Mol Biol Evol* 2005; **22**:104-116.

Gottlieb TM, Oren M. p53 and apoptosis. *Cancer Biol* 1998; **8:**359–368.

Gozani O, Karuman P, Jones DR, Ivanov D, Cha J, Lugovskoy AA, Baird CL, Zhu H, Field SJ, Lessnick SL, Villasenor J, Mehrotra B, Chen J, Rao VR, Brugge JS, Ferguson CG, Payrastre B, Myszka DG, Cantley LC, Wagner G, Divecha N, Prestwich GD, Yuan J. The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* 2003; **114**:99-111.

Green CM, Almouzni G. When repair meets chromatin. First in series on chromatin dynamics. *EMBO Rep* 2002; **3**:28–33.

Gruijl FRde, Sterenborg HJ, Forbes PD, Davies RE, Cole C, Kelfkens G, Weelden Hvan, Slaper H, van der Leun JC, Wavelength dependence of skin cancer induction by ultraviolet irradiation of albino hairless mice, *Cancer Res* 1993; **53**:53–60.

Gruijl Frde. Photocarcinogenesis: UVA vs. UVB radiation. *Skin Pharmacol Appl Skin Physiol* 2002; **15**:316–320.

Gu W, Roeder RG. Activation of p53 sequencespecific DNA binding by acetylation of the p53 C-terminal domain. *Cell* 1997; **90**:595–606.

Gunduz M, Ouchida M, Fukushima K, Hanafusa H, Etani T, Nishioka S, Nishizaki K, Shimizu K. Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. *Cancer Res* 2000; **60**:3143–3146.

Gunduz M, Ouchida M, Fukushima K, Ito S, Jitsumori Y, Nakashima T, Nagai N, Nishizaki K, Shimizu K. Allelic loss and reduced expression of the ING3, a candidate tumor suppressor gene at 7q31, in human head and neck cancers. *Oncogene* 2002; **21**:4462–4470.

Hahn H, Wicking C, Zaphiropoulous PG, Gailani MR, Shanley S, Chidambaram A, Vorechovsky I, Holmberg E, Unden AB, Gillies S, Negus K, Smyth I, Pressman C, Leffell DJ, Gerrard B, Goldstein AM, Dean M, Toftgard R, Chenevix-Trench G,

Wainwright B, Bale AE. Mutations of the human homolog of *Drosophila* patched in the nevoid basal cell carcinoma syndrome. *Cell* 1996; **85**:841-851.

Hai T, Hartman MG. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis. *Gene* 2001; **273**:1–11.

Haluska FG, Hodi FS. Molecular genetics of familial cutaneous melanoma. *J Clin Oncol* 1998; **16**:670-682.

Hanneman KK, Cooper KD, Baron ED. Ultraviolet immunosuppression: mechanisms and consequences. *Dermatol Clin* 2006; 24:19-25.

Hara Y, Zheng Z, Evans SC, Malatjalian D, Riddell DC, Guernsey DL, Wang LD, Riabowol K, Casson AG. Ing1 and dp53 tumor suppressor gene alterations in adenocarcinomas of the esophagogastric junction. *Cancer Lett*2003; **192**:109–116.

Harms KL, Chen X. The C terminus of p53 family proteins is a cell fate determinant. *Mol Cell Biol* 2005; **25**:2014-2030.

Harrison SL, MacLennan R, Speare R, Wronski I. Sun exposure and melanocytic naevi in young Australian children, *Lancet* 1994; **344**:1529–1532.

Helbing CC, Veillette C, Riabowol K, Johnston RN, Garkavtsev I. A novel candidate tumor suppressor, ING1, is involved in the regulation of apoptosis. *Cancer Res* 1997; **57**: 1255–1258.

Hanawalt PC, Sarasin A. Cancer-prone hereditary diseases with DNA processing abnormalities, *Trends Gene* 1986; **2**:124–129.

Hendrix MJ. De-mystifying the mechanism(s) of maspin. *Nat Med* 2000**; 6**:374–376.

Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; **407**:770-6.

Hermeking H. The 14-3-3 cancer connection. *Nat Rev Cancer* 2003; **3**:931–943.

Houghton AN, Polsky D. Focus on melanoma. *Cancer Cell* 2002; **2**:275–278.

Hussein MR, Haemel AK, Wood GS. Apoptosis and melanoma: molecular mechanisms. *J Pathol* 2003; **199**:275.

Hussein MR, Haemel AK, Wood GS. p53-related pathways and the molecular pathogenesis of melanoma. *Eur J Cancer Prev* 2003; **12**:93.

Hussein MR, Wood GS. Molecular aspects of melanocytic dysplastic nevi. *J Mol Diagn* 2002; **4**: 71.

Ito K, Kinjo K, Nakazato T, Ikeda Y, Kizaki M. Expression and sequence analyses of p33(ING1) gene in myeloid leukemia. *Am J Hematol* 2002; **69**:141–143.

Jacobson S, Pillus L. Modifying chromatin and concepts of cancer, *Curr Opin Genet Dev* 1999; **9**:75–184.

Jager D, Stockert E, Scanlan MJ, Gure AO, Jager E, Knuth A, Old LJ, Chen YT. Cancer—testis antigens and ING1 tumor suppressor gene product are breast cancer antigens: characterization of tissue-specific ING1 transcripts and a homolog gene. *Cancer Res* 1999; **59**:6197–6204.

Jhappan C, Noonan FP, Merlino G. Ultraviolet radiation and cutaneous malignant melanoma, *Oncogene* 2003; **22**:3099–3112.

Jiang W, Ananthaswamy HN, Muller HK, Kripke ML. p53 protects against skin cancer induction by UV-B radiation. *Oncogene* 1999; **18**:4247-4253.

Jiveskog S, Ragnarsson-Olding B, Platz A, Ringborg U. N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. *J Invest Dermatol* 1998; **111**:757-761.

Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas JM, Quinn AG, Myers RM, Cox DR, Epstein EH Jr, Scott MP. Human homolog of *patched*, a candidate gene for the basal cell nevus syndrome. *Science* 1996; **272**:1668-1671.

Kamb A., D. Shattuck-Eidens, R. Eeles, Q. Liu, N.A. Gruis, W. Ding, C. Hussey, T. Tran, Y. Miki and J. Weaver-Feldhaus. Analysis of the p16 gene (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 1994; **8**:23–26.

Kameyama K, Huang CL, Liu D, Masuya D, Nakashima T, Sumitomo S. Reduced ING1b gene expression plays an important role in carcinogenesis of non-small cell lung cancer patients, *Clin Cancer Res* 2003; **9**:4926–4934.

Kannan K, Sharpless NE, Xu J, O'Hagan RC, Bosenberg M, Chin L. Components of the Rb pathway are critical targets of UV mutagenesis in a murine melanoma model. *Proc Natl Acad Sci USA* 2003; **100**:1221–1225.

Kataoka H, Bonnefin P, Vieyra D, Feng X, Hara Y, Miura Y, Joh T, Nakabayashi H, Vaziri H, Harris CC, Riabowol K. ING1 represses transcription by direct DNA binding and through effects on p53, *Cancer Res* 2003; **63**:5787–5792.

Kenneth YT, Hensin T. The genetics of skin cancer. *American Journal of Medical Genetics Part C: Sem Med Genet.* 2000; **131C**: 82-92.

Kielbassa C, Roza L, Epe B. Wavelength dependence of oxidative DNA damage induced by UV and visible light. *Carcinogenesis* 1997; **18**:811–816.

Boland D. A panel of Cab antibodies recognize endogenous and ectopically expressed ING1 protein. *Hybridoma* 2000; **19**:161–166.

Koh J, Enders GH, Dynlacht BD, Harlow E. Tumour-derived p16 alleles encoding proteins defective in cell-cycle inhibition. *Nature* 1995; **375**:506-510.

Krishnamurthy J, Kannan K, Feng J, Mohanprasad BK, Tsuchida N, Shanmugam G. Mutational analysis of the candidate tumor suppressor gene ING1 in Indian oral squamous cell carcinoma. *Oral Oncol* 2001; **37**:222–224.

Kubo Y, Urano Y, Matsumoto K, Ahsan K, Arase S. Mutations of the INK4a locus in squamous cell carcinomas of human skin. *Biochem Biophys Res Commun* 1997; **232**:38-41.

Kubo Y, Urano Y, Yoshimoto K, Iwahana H, Fukuhara K, Arase S, Itakura M. p53 gene mutations in human skin cancers and precancerous lesions: Comparison with immunohistochemical analysis. *J Invest Dermatol* 1994; **102**:440-444.

Kuluncsics Z, Perdiz D, Brulay E, Muel B, Sage E. Wavelength dependence of ultraviolet-induced DNA damage distribution: involvement of direct or indirect mechanisms and possible artefacts, *J. Photochem. Photobiol* 1999; **49**:71–80.

Kuzmichev A, Zhang Y, Erdjument-Bromage H, Tempst P, Reinberg D. Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). *Mol Cell Biol* 2002; **22**:835–848.

Kvam E, Tyrell RM. Induction of oxidative DNA base damage in human skin cells by UV and near visible radiation. *Carcinogenesis* 1997; **18**:2379–2384.

Landis SH, Murray T, Bolden S, Wingo PA. Cancer statistics. *CA Cancer J Clin* 1999, 49: 8-31.

Laat Ade, van der Leun JC, de Gruijl FR. Carcinogenesis induced by UVA (365-nm) radiation: the dose-time dependence of tumor formation in hairless mice. *Carcinogenesis*1997; **18**:1013–1020.

Laat de WL, Jaspers NG, Hoeijmakers JH. Molecular mechanism of nucleotide excision repair. *Genes Dev* 1999; **13**:768-85.

Langford IH, Bentham G, McDonald AL. Multi-level modelling of geographically aggregated health data: a case study on malignant melanoma mortality and UV exposure in the European Community. *Stat* Med 1998; **17**:41–57.

Laronga C. Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol* Chem 2000; **275**:23106– 23112.

Li G, Bush JA, Ho VC. p53-dependent apoptosis in melanoma cells after treatment with camptothecin. *J Invest Dermatol* 2000; **114**:514-519.

Li G, Ho VC, Mitchell DL, Trotter MJ, Tron VA. Differentiation-dependent p53 regulation of nucleotide excision repair in keratinocytes. *Am J Pathol* 1997; **150**:1457-1464.

Li G, Mitchell DL, Ho VC, Reed JC, Tron VA. Decreased DNA repair but normal apoptosis in ultraviolet-irradiated skin of p53-transgenic mice. *Am J Pathol* 1996; **148**:1113-1123.

Li G, Tron V, Ho V. Induction of squamous cell carcinoma in p53-deficient mice after ultraviolet irradiation. *J Invest Dermatol* 1998; **110**:72-75.

Licht CL, Stevnsner T, Bohr VA. Cockayne syndrome group B cellular and biochemical functions. *Am J Hum Genet* 2003; **73**:1217-1239.

Lin Y, Ma W, Benchimol S. Pidd, a new death-domain-containing protein, is induced by p53 and promotes apoptosis. *Nat Genet* 2000; **26**:122– 127.

Liu Y, Yang Z, Utzat C, Wang G, Basu AK, Zou Y. *Biochemistry* 2005; **44**:7361-7368.

Loewith R, Meijer M, Lees-Miller S, Riabowol K, Young D. Three yeast proteins related to the human candidate tumor suppressor p33ING1 are associated with histone acetyltransferase activities. *Mol Cell Biol* 2000; **20**:3807–3816.

Lowe SW, Lin AW. Apoptosis in Cancer. *Carcinogenesis* 2000; **21**:485–495.

Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: Progress and puzzles. *Curr Opin Genet Dev* 2003; **13**:77-83.

Lozano G, Elledge SJ. p53 sends nucleotides to repair DNA. *Nature* 2000; **404**:24–25.

Marks R, Rennie G, Selwood TS. Malignant transformation of solar keratoses to squamous cell carcinoma. *Lancet* 1988; **1**:795-797.

McGill GG, Horstmann M, Widlund HR, Du J, Motyckova G, Nishimura EK, Lin YL, Ramaswamy S, Avery W, Ding HF, Jordan SA, Jackson IJ, Korsmeyer SJ, Golub TR, Fisher DE. Bcl2 regulation by the melanocyte master regulator Mitf modulates lineage survival and melanoma cell viability. *Cell* 2002; **109**:707-718.

McGregor JM, Harwood CA, Brooks L, Fisher SA, Kelly DA, O'Nions J, Young AR, Surentheran T, Breuer J, Millard TP, Lewis CM, Leigh IM, Storey A, Crook T.

Relationship between p53 codon 72 polymorphism and susceptibility to sunburn and skin cancer. *J Invest Dermatol* 2002; **119**:84-90.

Meek DW. Mechanisms of switching on p53: a role for covalent modification? *Oncogene* 1999; **18**:7666–7675.

Mitchell DI, Nguyen TD, Cleaver JE. Nonrandom induction of pyrimidine-pyrimidone (6-4) photoproducts in ultraviolet-irradiated human chromatin. *J Biol Chem* 1990, 265: 5353-5356.

Mitchell DL, Nairn RS. The biology of the (6-4) photoproduct, *Photochem. Photobiol* 1989; **49**:805–819.

Moan J, Dahlback A, Setlow RB. Epidemiological support for an hypothesis for melanoma induction indicating a role for UVA radiation, *Photochem. Photobiol* 1999; **70**:243–247.

Moles JP, Moyret C, Guillot B, Jeanteur P, Guilhou JJ, Theillet C, Basset-Seguin N. p53 gene mutations in human epithelial skin cancers. *Oncogene* 1993; **8**:583-588.

Momand J, Wu HH, Dasgupta G. MDM2--master regulator of the p53 tumor suppressor protein. *Gene* 2000; **242**:15– 29.

Moore JD, Krebs JE. Histone modifications and DNA double-strand break repair. *Biochem Cell Biol* 2004; **82**:446-52.

Mori Y, Hosono M, Murakami K, Katoh H, Yoshikawa Y, Kuribayashi K, Kannagi R, Sakai M, Okuma M, Masuda T. *Clin Exp Immunol* 1991; **84**:145-52.

Mortier L, Marchetti P, Delaporte E, Martin de Lassalle E, Thomas P, Piette F, Formstecher P, Polakowska R, Danze PM. Progression of actinic keratosis to squamous cell carcinoma of the skin correlates with deletion of the 9p21 region encoding the p16(INK4a) tumor suppressor. *Cancer Lett* 2002; **176**:205-214.

Mullenders LH, Hazekamp-van Dokkum AM, Kalle WH, Vrieling H, Zdzienicka MZ, van Zeeland AA. UV-induced photolesions, their repair and mutations. *Mutat Res* 1993; **299**:271-276.

Nagashima M, Shiseki M, Miura K, Hagiwara K, Linke SP, Pedeux R, Wang XW, Yokota J, Riabowol K, Harris CC. DNA damage-inducible gene *p33ING2* negatively regulates cell proliferation through acetylation of p53. *Proc Natl Acad Sci USA* 2001; **98**:9671– 9676.

Nagashima M, Shiseki M, Pedeux RM, Okamura S, Kitahama-Shiseki M, Miura K, Yokota J, Harris CC. A novel PHD-finger motif protein, $p47^{ING3}$, modulates p53-mediated transcription, cell cycle control and apoptosis. *Oncogene* 2003; **22**:343-250.

Noonan FP, Recio JA, Takayama H, Duray P, Anver MR, Rush WL, De Fabo EC, Merlino G. Neonatal sunburn and melanoma in mice. *Nature* 2001; **413**:271–272.

Norgauer J, Idzko M, Panther E, Hellstern O, Herouy Y. Xeroderma pigmentosum. *Eur J Dermatol* 2003; **13**:4-9.

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

Noumann GS, Anderson JJ, Wook KM, Lunec J, Hall AG, Reid MM, Angus B. Loss of nuclear expression of the p33(ING1b) inhibitor of growth protein in childhood acute lymphoblastic leukaemia. *J Clin Pathol* 2002; **55**:596–601.

Oda E, Ohki R, Murasawa H, Nemoto J, Shibue T, Yamashita T, Tokino T, Taniguchi T, Tanaka N. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* 2000; **288**:1053–1058.

Oda K, Arakawa H, Tanaka T, Matsuda K, Tanikawa C, Mori T, Nishimori H, Tamai K, Tokino T, Nakamura Y, Taya Y. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell* 2000; **102**:849–862.

Ohki R, Nemoto J, Murasawa H, Oda E, Inazawa J, Tanaka N, Taniguchi T. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. *J Biol Chem* 2000; **275**:22627– 22630.

Ohmori M, Nagai M, Tasaka T, Koeffler HP, Toyama T, Riabowol K, Takahara J. Decreased expression of p33ING1b mRNA in lymphoid malignancies. *Am J Hematol* 1999; **62**:118–119.

Oki E, Maehara Y, Tokunaga E, Kakeji Y, Sugimachi K. Reduced expression of p33(ING1) and the relationship with p53 expression in human gastric cancer. *Cancer Lett* 1999; **147**:157–162.

Omholt K, Karsberg S, Platz A, Kanter L, Ringborg U, Hansson J. Screening of N-ras Codon 61 mutations in paired primary and metastatic cutaneous melanomas: Mutations occur early and persist throughout tumor progression. *Clin Cancer Res* 2002; **8**:3468-3474.

Oren M. Tumor suppressors. Teaming up to restrain cancer. *Nature* 1998; **391**:233–234.

Pascual J, Martinez-Yamout M, Dyson HJ, Wright PE. Structure of the PHD zinc finger from human Williams–Beuren syndrome transcription factor, *J Mol Biol* 2000; **304**:723–729.

Perlis C, Herlyn M. Recent advances in melanoma biology. *Oncologist* 2004; **9**:182-187.

Petit C, Sancar A. Nucleotide excision repair: from *E. coli* to man. *Biochimie* 1999, **81**: 15-25.

Pfeifer GP. Formation and processing of UV photoproducts: effects of DNA sequence and chromatin environment, *Photochem Photobiol* 1997; **65**:270–283.

Pfeifer GP, You YH, Besaratinia A. Mutations induced by ultraviolet light. *Mutat Res* 2005; **571**:19-31.

Pierceall WE, Goldberg LH, Tainsky MA, Mukhopadhyay T, Ananthaswamy HN. Ras gene mutation and amplification in human nonmelanoma skin cancers. *Mol Carcinog* 1991; **4**:196-202.

Platz A, Ringborg U, Brahme EM, Lagerlof B. Melanoma metastases from patients with hereditary cutaneous malignant melanoma contain a high frequency of N-ras activating mutations. *Melanoma Res* 1994; **4**:169-177.

Pollock PM, Yu F, Qiu L, Parsons PG, Hayward NK. Evidence for UV induction of CDKN2 mutations in melanoma cell lines, *Oncogene* 1995; **11**:663–668.

Pollock PM, Harper UL, Hansen KS, Yudt LM, Stark M, Robbins CM, Moses TY, Hostetter G, Wagner U, Kakareka J, Salem G, Pohida T, Heenan P, Duray P, Kallioniemi O, Hayward NK, Trent JM, Meltzer PS. High frequency of BRAF mutations in nevi. *Nat Genet* 2003; **33**:19-20.

Prives C, Hall PA. The p53 pathway. *J Pathol* 1999; **187**:112–126.

Rady P, Scinicariello F, Wagner RF Jr, Tyring SK. p53 mutations in basal cell carcinomas. *Cancer Res* 1992; **52**:3804-3806.

Ramanathan B, Smerdon MJ. Changes in nuclear protein acetylation in u.v.-damaged human cells. *Carcinogenesis* 1986; 7:1087–1094.

Reed JC. Dysregulation of apoptosis in cancer. *J Clin Oncol* 1999; **17**:2941–2953.

Rehman I, Takata M, Wu YY, Rees JL. Genetic change in actinic keratoses. *Oncogene* 1996; **12**:2483-2490.

Remy P, Sagar S, Jiang CS, Oleg ND, Shin'ichi S, Hitoshi O, Kensuke K, Stephen W, Susan HG, Mary M, Makoto N, Steven RG, Ettore A, Curtis CH. ING2 Regulates the Onset of Replicative Senescence by Induction of p300-Dependent p53 Acetylation. *Mol Cell Biol* 2005; **25**:6639-6648.

Rochette PJ, Therrien JP, Drouin R, Perdiz D, Bastien N, Drobetsky EA, Sage E. UVA-induced cyclobutane pyrimidine dimers form predominantly at thymine–thymine dipyrimidines and correlate with the mutation spectrum in rodent cells, *Nuc Acids Res* 2003; **31**:2786–2794.

Roses DF, Karp NS, Oratz T, Dubin N, Harris MN, Speyer J, Byod A, Golmb FM, Ransohoff J, Dugan M, Valentine FT, Cooper J, Bystryn JC. Survival with regional and distant metastases from cutaneous melanoma. *Surg Gynecol Obstet* 1991; 17:2262-2268.

Roth SY, Denu JM, Allis CD. Histone acetyltransferases. *Annu Rev Biochem* 2001; 70:81–120.

Ruas M, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives, *Biochim Biophys Acta* 1998; **1378**:F115–F177.

Rubbi CP, Milner J. Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J* 2003; **22**:975-86.

Saito A, Furukawa T, Fukushige S, Koyama S, Hoshi M, Hayashi Y, Horii A. p24/ING1-ALT1 an dp47/ING1-ALT2, distinct alternative transcripts of p33ING1. *J Hum Genet* 2000; **45**:177–181.

Sakaguchi K, Herrera JE, Saito S, Miki T, Bustin M, Vassilev A, Anderson CW, Appella E. DNA damage activates p53 through a phosphorylation-acetylation cascade. *Gene Dev* 1998; **12**:2831–2841.

Saladi RN, Persaud AN. The causes of skin cancer: a comprehensive review. *Drugs Today (Barc)* 2005; **41**:37-53.

Scheel H, Hofmann K. No evidence for PHD fingers as ubiquitin ligases. *Trends Cell Biol* 2003; **13**:285–287.

Schmidt T, Korner K, Karsunky H, Korsmeyer S, Muller R, Moroy T. The activity of the murine Bax promoter is regulated by Sp1/3 and E-box binding proteins but not by p53. *Cell Death Differ* 1999; **6**:873– 882.

Schwarte-Waldhoff I, Volpert OV, Bouck NP, Sipos B, Hahn SA, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, Schmiegel W. Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 2000; **97**:9624– 9629.

Scott M, Boisvert FM, Vieyra D, Johnston RN, Bazett-Jones DP, Riabowol K. UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. *Nuc Acids Res* 2001; **29**:2052-2058.

Scott M, Bonnefin P, Vieyra D, Boisvert FM, Young D, Bazett-Jones DP, Riabowol K. UV-induced binding of ING1 to PCNA regulates the induction of apoptosis. *J Cell Sci* 2001; **114**:3455-3462.

Serrano M, Gomez-Lahoz E, DePinho RA, Beach D, Bar-Sagi D. Inhibition of ras-induced proliferation and cellular transformation by p16INK4. *Science* 1995; **267**:249-252.

Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993; **366**:704-707.

Setlow RB. The wavelengths in sunlight effective in producing skin cancer: a theoretical analysis, *Proc Natl Acad Sci USA* 1974; **71**:3363–3366.

Sherr CJ, Weber JD. The ARF/p53 pathway. *Curr Opin Genet Dev* 2000; **10**:94–99.

Shimada H, Liu TL, Ochiai T, Shimizu T, Haupt Y, Hamada H, Abe T, Oka M, Takiguchi M, Hiwasa T. Facilitation of adenoviral wild-type p53-induced apoptotic cell death by overexpression of p33(ING1) in human esophageal carcinoma cells. *Oncogene* 2002; **21**:1208–1216.

Shimada Y, Saito A, Suzuki M, Takahashi E, Horie M. Cloning of a novel gene (ING1L) homologous tumorsuppressor. *Cytogenet Cell Genet* 1998; **83**:232–235.

Shinoura N, Muramatsu Y, Nishimura M, Yoshida Y, Saito A, Yokoyama T, Furukawa T, Horii A, Hashimoto M, Asai A, Kirino T, Hamada H. Adenovirus-mediated transfer of p33ING1 with p53 drastically augments apoptosis in gliomas. *Cancer Res* 1999; **59**:5521–5528.

Shiseki M, Nagashima M, Pedeux RM, Kitahama-Shiseki M, Miura K, Okamura S, Onogi H, Higashimoto Y, Appella E, Yokota J, Harris CC. p29ING4 and p28ING5 bind to p53, and enhance p53 activity. *Cancer Res* 2003; **63**:2373–2378.

Skowyra D, Zeremski M, Neznanov N, Li M, Choi Y, Uesugi M, Hauser CA, Gu W, Gudkov AV, Qin J. Differential association of products of alternative transcripts of the candidate tumor suppressor ING1 with the mSin3/HDAC transcriptional corepressor complex. *J Biol Chem* 2001; **276**:8734–8739.

Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordon-Cardo C, Lowe SW. Inactivation of the apoptosis effector Apaf-1 in malignant melanoma. *Nature* 2001; **409**:207-211.

Soufir N, Moles JP, Vilmer C, Moch C, Verola O, Rivet J, Tesniere A, Dubertret L, Basset-Seguin N. P16 UV mutations in human skin epithelial tumors. *Oncogene* 1999. **18**: 5477-5481.

Spencer JM, Kahn SM, Jiang W, DeLeo VA, Weinstein IB. Activated ras genes occur in human actinic keratoses, premalignant precursors to squamous cell carcinomas. *Arch Dermatol* 1995; **131**:796-800.

Taguchi M, Watanabe S, Yashima K, Murakami Y, Sekiya T, Ikeda S. Aberrations of the tumor suppressor *p53* gene and p53 protein in solar keratosis in human skin. *J Invest Dermatol* 1994; **103**:500-503.

Tallen G, Kaiser I, Krabbe S, Lass U, Hartmann C, Henze G, Riabowol K, vonDeimling A. No ING1 mutations in human brain tumors but reduced expression in high malignancy grades of astrocytoma. *Int J Cancer* 2004; **109**:476–479.

Tanaka H, Arakawa H, Yamaguchi T, Shiraishi K, Fukuda S, Matsui K, Takei Y, Nakamura Y. A ribonucleotide reductase gene involved in a p53-dependent cell-cycle checkpoint for DNA damage. *Nature* 2000; **404**:42–49.

Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF. Adriamycin-induced DNA damage mediated by mammalian DNA topoisomerase II. *Science* 1984; **226:**466–468.

Thoma BS, Vasquez KM. Critical DNA damage recognition functions of XPC-hHR23B and XPA-RPA in nucleotide excision repair. *Mol Carcinog* 2003; 38:1–13.

Tijsterman M, de Pril R, Tasseron-de Jong JG, Brouwer J. RNA polymerase II transcription suppresses nucleosomal modulation of UV-induced (6-4) photoproduct and cyclobutane pyrimidine dimer repair in yeast. *Mol Cell Biol* 1999; **19**:934-40.

Tlsty TD. Genomic instability and its role in neoplasia. *Curr Top Microbiol Immunol* 1997; **221**:37– 46.

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

Toyama T, Iwase H. p33ING1b and estrogen receptor (ER) alpha. *Breast Cancer* 2004; **11:** 33–37.

Toyama T, Iwase H, Watson P, Muzik H, Saettler E, Magliocco A, DiFrancesco L, Forsyth P, Garkavtsev I, Kobayashi S, Riabowol K. Suppression of ING1 expession in sporadic breast cancer. *Oncogene* 1999; **18**:5187–5193.

Toyama T, Iwase H, Yamashita H, Hara Y, Sugiura H, Zhang Z, Fukai I, Miura Y, Riabowol K, Fujii Y. p33(ING1b) stimulates the transcriptional activity of the estrogen receptor alpha via its activation function (AF) 2 domain. *J Steroid Biochem Mol Biol* 2003; **87**:57–63 88.

Tsai KY, Tsao H.The genetics of skin cancer. *Am J Med Genet C Semin Med Genet* 2004; **131C**:82-92.

Tsang FC, Po LS, Leung KM, Lau A, Siu WY, Poon RY. ING1b decreases cell proliferation through p53- dependent and -independent mechanisms. *FEBS Lett* 2003; **553:**277–285.

Turovets NA, Agapova LS, Kopnin PB, Tulina NM, Chumakov PM, Kopnin BP. Inactivation of p33(ING1) tumor suppressor affects the function of the cell cycle "checkpoints" and stability of the genome. *Russian J Genet* 2000; **36**:305–12.

Van 't Veer LJ, Burgering BM, Versteeg R, Boot AJ, Ruiter DJ, Osanto S, Schrier PI, Bos JL. N-ras mutations in human cutaneous melanoma from sun-exposed body sites. *Mol Cell Biol* 1989; **9**:3114-3116.

Vaziri H, Dessain SK, Ng Eaton E, Imai SI, Frye RA, Pandita TK, Guarente L, Weinberg RA. hSIR2(SIRT1) functions as an NAD-dependent p53 deacetylase. *Cell* 2001; **107**:149–159.

Veierod MB, Weiderpass E, Thorn M, Hansson J, Lund E, Armstrong B, Adami HO. A prospective study of pigmentation, sun exposure, and risk of cutaneous malignant melanoma in women. *J Natl. Cancer Inst* 2003; **95**:1530–1538.

Vieyra D, Senger DL, Toyama T, Muzik H, Brasher PM, Johnston RN, Riabowol K, Forsyth PA. Altered subcellular localization and low frequency of mutations of ING1 in human brain tumors, *Clin Cancer Res* 2003; **9**:5952–5961.

Vieyra D, Loewith R, Scott M, Bonnefin P, Boisvert FM, Cheema P, Pastyryeva S, Meijer M, Johnston RN, Bazett-Jones DP, McMahon S, Cole MD, Young D, Riabowol K. Human ING1 proteins differentially regulate histone acetylation. *J Biol Chem* 2002; **277**:29832-29839.

Wagner MJ, Gogela-Spehar M, Skirrow RC, Johnston RN, Riabowol K, Helhbing CC. Expression of novel ING variants is regulated by thyroid hormone in the *Xenopus laevis* tadpole. *J Biol Chem* 2001; **276**:47013–47020.

Wahl GM, Linke SP, Paulson TG, Huang LC. Maintaining genetic stability through TP53 mediated checkpoint control. *Cancer Surv* 1997; **29**:183– 219.

Wang J, Chin MY, Li G. The novel tumor suppressor p33ING2 enhances nucleotide excision repair via inducement of histone H4 acetylation and chromatin relaxation. *Cancer Res* 2006; **66**:1906-1911.

Wang QE, Zhu Q, Wani MA, Wani G, Chen J, Wani AA. Tumor suppressor p53 dependent recruitment of nucleotide excision repair factors XPC and TFIIH to DNA damage. *DNA Repair (Amst)* 2003; **2**: 483-499.

Wang SQ, Setlow R, Berwick M, Polsky D, Marghoob AA, Kopf AW, Bart RS. Ultraviolet A and melanoma: a review. *J Am Acad Dermatol* 2001; **44**:837–846.

Wang XW, Yeh H, Schaeffer L, Roy R, Moncollin V, Egly JM, Wang Z, Freidberg EC, Evans MK, Taffe BG. *Nat Genet* 1995; **10**:188-195.

Wang Y, Li G. ING3 promotes UV-induced apoptosis via fas/caspase-8 pathway in melanoma cells. *J Biol Chem* 2006; [Epub ahead of print].

Warbrick E. PCNA binding through a conserved motif. *BioEssays* 1998; **20**:195–199.

Wolf D, Harris N, Goldfinger N, Rotter V. Isolation of a full-length mouse cDNA clone coding for an immunologically distinct p53 molecule. *Mol Cell Biol* 1985; **5**:127-132.

Woodhead AD, Setlow RB, Tanaka M. Environmental factors in nonmelanoma and melanoma skin cancer, *J Epidemiol* 1999; **9**:S102–S114.

Xiaolan F, Yasuo H, Karl T. Riabowol. Different HATS of the ING1 gene family. *Trends Cell Biol* 2002; **12**:532-538.

YinY, Stephen CW, Luciani MG, Fahraeus R. p53 Stability and activity is regulated by Mdm2-mediated induction of alternative p53 translation products. *Nat Cell Biol* 2002; **4**: 462-467.

Yoon JH, Lee CS, Connor TO, Yasui A, Pfeifer GP. The DNA damage spectrum produced by simulated sunlight, *J Mol Biol* 2000; **299**:681–693.

Yu J, Zhang L, Hwang PM, Rago C, Kinzler KW, Vogelstein B. Identification and classification of p53-regulated genes. *Proc Natl Acad Sci USA* 1999; **96**:14517– 14522.

Yu Y, Teng Y, Liu H, Reed SH, Waters R. UV irradiation stimulates histone acetylation and chromatin remodeling at a repressed yeast locus. *Proc Natl Acad Sci USA* 2005; **102:**8650-8655.

Zeremski M, Hill JE, Kwek SS, Grigorian IA, Gurova KV, Garkavtsev IV, Diatchenko L, Koonin EV, Gudkov AV. Structure and regulation of the mouse ing1 gene. Three alternative transcripts encode two phd finger proteins that have opposite effects on p53 function. *J Biol Chem* 1999; **274**:32172-32181.

Zhang H, Ping XL, Lee PK, Wu XL, Yao YJ, Zhang MJ, Silvers DN, Ratner D, Malhotra R, Peacocke M, Tsou HC. Role of *PTCH* and *p53* genes in early-onset basal cell carcinoma. *Am J Pathol* 2001; **158**:381-385.

Zhang X, Rosenstein BS, Wang Y, Lebwohl M, Mitchell DM, Wei H. Induction of 8-oxo-7,8-dihydro-2′-deoxyguanosine by ultraviolet radiation in calf thymus DNA and HeLa cells. *Photochem Photobiol* 1997; **65**:119–124.

Zhu J, Zhang S, Jiang J, Chen X. Definition of the p53 functional domains necessary for inducing apoptosis. *J Biol Chem* 2000; **275**:39927-39934.

Zhu J, Zhou W, Jiang J, Chen X. Identification of a novel p53 functional domain that is necessary for mediating apoptosis. *J Biol* Chem 1998; **273**:13030-13036.

Ziegler A, Jonason AS, Leffell DJ, Simon JA, Sharma HW, Kimmelman J, Remington L, Jacks T, Brash DE. Sunburn and p53 in the onset of skin cancer. *Nature* 1994; **372**:773-776.

Ziegler A, Leffell DJ, Kunala S, Sharma HW, Gailani M, Simon JA, Halperin AJ, Baden HP, Shapiro PE, Bale AE. Mutation hotspots due to sunlight in the *p53* gene of nonmelanoma skin cancers. *Proc Natl Acad Sci USA* 1993; **90**:4216-4220.