

University of Massachusetts Medical School

eScholarship@UMMS

---

GSBS Dissertations and Theses

Graduate School of Biomedical Sciences

---

2008-05-02

## Functional Analysis of Ing1 and Ing4 in Cell Growth and Tumorigenesis: a Dissertation

Andrew H. Coles

*University of Massachusetts Medical School*

Let us know how access to this document benefits you.

Follow this and additional works at: [https://escholarship.umassmed.edu/gsbs\\_diss](https://escholarship.umassmed.edu/gsbs_diss)



Part of the [Amino Acids, Peptides, and Proteins Commons](#), [Animal Experimentation and Research Commons](#), [Cell Biology Commons](#), [Cells Commons](#), [Enzymes and Coenzymes Commons](#), [Genetic Phenomena Commons](#), and the [Neoplasms Commons](#)

---

### Repository Citation

Coles AH. (2008). Functional Analysis of Ing1 and Ing4 in Cell Growth and Tumorigenesis: a Dissertation. GSBS Dissertations and Theses. <https://doi.org/10.13028/pr7a-s521>. Retrieved from [https://escholarship.umassmed.edu/gsbs\\_diss/375](https://escholarship.umassmed.edu/gsbs_diss/375)

This material is brought to you by eScholarship@UMMS. It has been accepted for inclusion in GSBS Dissertations and Theses by an authorized administrator of eScholarship@UMMS. For more information, please contact [Lisa.Palmer@umassmed.edu](mailto:Lisa.Palmer@umassmed.edu).

**Functional Analysis of Ing1 and Ing4 in Cell Growth and  
Tumorigenesis**

A Dissertation Presented

By

Andrew H. Coles

Submitted to the Faculty of the  
University of Massachusetts Graduate School of Biomedical Sciences, Worcester  
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2, 2008

PROGRAM IN CELL BIOLOGY

**Functional Analysis of Ing1 and Ing4 in Cell Growth and Tumorigenesis**

A Dissertation Presented

By

Andrew H. Coles

Approved as to style and content by:

---

Anthony N. Imbalzano, Ph.D., Chair of Committee

---

Roger Davis, Ph.D., Member of Committee

---

Steven Grossman, MD. Ph.D., Member of Committee

---

Jianyuon Luo, Ph.D., Member of Committee

---

Zhi-Xiong Jim Xiao Ph.D., Member of Committee

---

Stephen N. Jones, Ph.D., Thesis Advisor

---

Anthony Carruthers, Ph.D.

Dean of the Graduate School of Biomedical Sciences

Cell Biology

May 2, 2008

## TABLE OF CONTENTS

<b>COPYRIGHT NOTICE</b>	<b>i</b>
<b>DEDICATION</b>	<b>ii</b>
<b>ACKNOWLEDGEMENTS</b>	<b>iii</b>
<b>SUMMARY</b>	<b>iv</b>
<b>LIST OF TABLES</b>	<b>vi</b>
<b>LIST OF FIGURES</b>	<b>vii</b>
<b>CHAPTER I: Introduction</b>	<b>1</b>
<b>1.1 Forward</b>	<b>2</b>
<b>1.2 Suppression of Tumorigenesis</b>	<b>3</b>
<b>1.3 Identification, Organization, and Expression of ING Genes</b>	<b>5</b>
<b>1.4 Structural Features of ING Proteins</b>	<b>8</b>
<b>1.5 ING Proteins Participate in a Variety of Cellular Processes and Pathways</b>	<b>12</b>
<b>1.5.1 ING1</b>	<b>13</b>
<b>1.5.2 ING2</b>	<b>28</b>
<b>1.5.3 ING3</b>	<b>30</b>
<b>1.5.4 ING4</b>	<b>31</b>
<b>1.5.5 ING5</b>	<b>34</b>
<b>1.6 Regulation of ING Gene Expression and Protein Function</b>	<b>35</b>
<b>1.7 ING Genes as Tumor Suppressors</b>	<b>37</b>
<b>1.8 Aims of this Dissertation</b>	<b>45</b>

<b>CHAPTER II:</b>	<b>Deletion of p37<sup>Ing1b</sup> in mice reveals a p53 independent role for Ing1 in suppression of cell proliferation, apoptosis, and tumorigenesis.</b>	<b>46</b>
	<b>FOREWORD</b>	<b>47</b>
	<b>INTRODUCTION</b>	<b>48</b>
	<b>RESULTS</b>	<b>51</b>
	<b>DISCUSSION</b>	<b>70</b>
	<b>MATERIALS AND METHODS</b>	<b>75</b>
<b>CHAPTER III:</b>	<b>p37<sup>Ing1b</sup> regulates B-cell development and cooperates with p53 in suppression of follicular lymphoma progression.</b>	<b>80</b>
	<b>FOREWORD</b>	<b>81</b>
	<b>INTRODUCTION</b>	<b>82</b>
	<b>RESULTS</b>	<b>86</b>
	<b>DISCUSSION</b>	<b>102</b>
	<b>MATERIALS AND METHODS</b>	<b>104</b>
<b>CHAPTER IV:</b>	<b>Ing4 suppresses NF-<math>\kappa</math>B signaling and innate immunity in mice.</b>	<b>109</b>
	<b>FOREWORD</b>	<b>110</b>
	<b>INTRODUCTION</b>	<b>111</b>
	<b>RESULTS</b>	<b>114</b>
	<b>DISCUSSION</b>	<b>140</b>
	<b>MATERIALS AND METHODS</b>	<b>142</b>

<b>CHAPTER V:</b>	<b>General Discussion</b>	<b>148</b>
<b>REFERENCES</b>		<b>161</b>
<b>APPENDIX I:</b>	<b>Additional Work</b>	<b>197</b>

## COPYRIGHT NOTICE

**Parts of this dissertation have been presented in the following publications:**

**Coles AH**, Marfella CG, Garelick DS, Gerstein R, Jones SN. p37<sup>Ing1b</sup> regulates B-cell development and cooperates with p53 in suppression of follicular lymphoma progression. *(Manuscript submitted)*

**Coles AH**, Liang H, Zhu Z, Marfella CG, Kang J, Imbalzano A, Jones SN. Deletion of p37<sup>Ing1</sup> in mice reveals a p53-independent role Ing1 in suppression of cell proliferation, apoptosis, and tumorigenesis. *Cancer Res*, 2007. **67**(5): p. 2054-61.

Liang H, **Coles AH**, Zayas J, Jurecic R, Kang J, Jones SN. Non-canonical Wnt signaling suppresses proliferation and promotes apoptosis in thymocyte development. *J Exp Med*. 2007 Dec 24;204(13):3077-84. Epub 2007 Dec 10.

Marfella CG, Ohkawa Y, **Coles AH**, Garlick DS, Jones SN, Imbalzano AN. Mutation of the SNF2 family member Chd2 affects mouse development and survival. *J Cell Physiol*. 2006 Oct;209(1):162-71.

Liang H, Chen Q, **Coles AH**, Anderson SJ, Pihan G, Bradley A, Gerstein R, Jurecic R, Jones SN. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cell*. 2003 Nov;4(5):349-60.

## **DEDICATION**

This dissertation is dedicated to my grandfather, Michael Comperchio, who through his example showed me how important education is.



## ACKNOWLEDGEMENTS

There are several people to whom I am grateful to for their assistance and support over the past several years. First and foremost is my thesis advisor Steve Jones for his support and guidance. Being in his lab was a wonderful learning opportunity. Many current and past Jones lab members have also assisted me in numerous ways during my graduate career. One person in particular I am exceedingly grateful to is Dr. Huiling Liang for taking me under her wing and for our countless conversations. I would also like to thank all my collaborators, Rachel Gerstein and Evelyn Kurt-Jones especially, for their guidance and for teaching me many things that I may not have learned otherwise. Additionally, I would also like to thank all my friends, both in and outside UMMS, for seeing me through this long arduous road. Finally, I would like to thank my parents, Ann and Richard Coles, and the rest of my family for putting up with me during the many low points.

## SUMMARY

The five member Inhibitor of Growth (ING) gene family has been proposed to participate in the regulation of cell growth, DNA repair, inflammation, chromatin remodeling, and tumor suppression. All ING proteins contain a PHD motif implicated in binding to methylated histones and are components of large chromatin remodeling complexes containing histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes, suggesting a role for ING proteins in regulating gene transcription. Additionally, forced overexpression studies performed *in vitro* have indicated that several ING proteins can interact with the p53 tumor suppressor protein and/or the NF- $\kappa$ B protein complex. Since these two proteins play well-established roles in numerous biological processes, several models have been proposed in the literature that ING proteins act as key regulators of cell growth and tumor suppression not only through their ability to modify gene transcription but also through their ability to alter p53 and NF- $\kappa$ B activity. However, these models have yet to be substantiated by *in vivo* experimentation.

Research described in this dissertation utilizes a genetic approach to analyze the functional role of two ING proteins, Ing1b and Ing4, in regulating cell growth, inflammation, and tumorigenesis. Loss of p37<sup>Ing1b</sup> increased proliferation and DNA damage-induced apoptosis irrespective of p53 status in primary cells and mice. However, all other p53 responses were unperturbed. Additionally, p37<sup>Ing1b</sup> suppressed the formation of spontaneous follicular B-cell lymphomas in mice.

Analysis of B-cells from these mice indicates that  $p37^{\text{Ing1b}}$  inhibits the proliferation of B cells regardless of p53 status, and loss of p53 greatly accelerates the rate of B-cell lymphomagenesis in  $p37^{\text{Ing1b}}$ -null mice, with double null mice presenting with aggressive diffuse large B-cell lymphomas (DLBL). Marker gene analysis in  $p37^{\text{Ing1b}}/p53$  null tumors indicates that these mice develop both non-germinal center and germinal center B cell-like DLBL, and also documents upregulation of NF- $\kappa$ B activity in both B-cells and tumors. Similarly, Ing4  $-/-$  mice did not have altered p53 growth arrest or apoptosis, and did not develop spontaneous tumors. However, Ing4  $-/-$  cells displayed reduced proliferation, and Ing4  $-/-$  mice and macrophages were hypersensitive to treatment with LPS and exhibited decreased *I $\kappa$ B* gene expression and increased NF- $\kappa$ B activity. These studies demonstrate that Ing proteins can function to suppress spontaneous tumorigenesis and/or inflammatory responses without altering p53 activity, and identifies NF- $\kappa$ B as a biologically-relevant *in vivo* target of Ing1 and Ing4 signaling.

**LIST OF TABLES****CHAPTER I**

<b>Table 1.1:</b>	The composition of each ING protein containing chromatin remodeling complex.	26
<b>Table 1.2:</b>	Alterations in expression and the mutations found in human cancers for each ING gene.	39

**CHAPTER III**

<b>Table 3.1:</b>	Comparison of the tumor spectrums of p53, p37 <sup>Ing1b</sup> , and p37/p53 null mice.	91
-------------------	---	----

## LIST OF FIGURES

### CHAPTER I

<b>Figure 1.1:</b>	Genomic organization of mouse and human ING family members.	7
<b>Figure 1.2:</b>	Domain structure of human ING proteins.	10
<b>Figure 1.3:</b>	Proposed model of ING1 in the p53 pathway.	19
<b>Figure 1.4:</b>	Model of a role for ING1, ING2, and ING4 in the regulation of the NF- $\kappa$ B pathway.	24

### CHAPTER II

<b>Figure 2.1:</b>	Generation of p37 <sup>Ing1b</sup> -deficient mice.	52
<b>Figure 2.2:</b>	Growth regulation by p37 <sup>Ing1</sup> .	54
<b>Figure 2.3:</b>	Immortalization of p37 <sup>Ing1</sup> -deficient cells.	55
<b>Figure 2.4:</b>	<i>Ras</i> -induced senescence in p37 <sup>Ing1</sup> -deficient cells is unchanged.	57
<b>Figure 2.5:</b>	Cell cycle arrest is normal in p37 <sup>Ing1</sup> -deficient MEFs.	59
<b>Figure 2.6:</b>	Apoptosis is elevated in p37 <sup>Ing1</sup> -deficient MEFs transduced with E1A.	62
<b>Figure 2.7:</b>	Apoptosis is increased in p37 <sup>Ing1</sup> -deficient thymocytes.	63
<b>Figure 2.8:</b>	Apoptosis is altered in p37 <sup>Ing1</sup> -deficient thymocytes.	65
<b>Figure 2.9:</b>	Quantitative real-time PCR for <i>Puma</i> and <i>Bax</i> was done using p37 <sup>Ing1</sup> -deficient tissues.	67
<b>Figure 2.10:</b>	Tumorigenesis in mice deficient for p37 <sup>Ing1</sup> .	69

### CHAPTER III

<b>Figure 3.1:</b>	Follicular (FO) B-cells in p37 <sup>Ing1</sup> deficient mice are increased.	87
<b>Figure 3.2:</b>	Analysis of follicular (FO) B-cells in p37 and p37/p53 deficient mice.	89
<b>Figure 3.3:</b>	Kaplan-Meier survival curve showing tumor formation in p37/p53 deficient mice.	91
<b>Figure 3.4:</b>	Analysis of tumors and from p37/p53 deficient mice.	93
<b>Figure 3.5:</b>	<i>Bax</i> qRT-PCR of DP sorted T-cells from non-cancerous wt or p37/p53 null mice.	94
<b>Figure 3.6:</b>	Characterization of the tumors in p37/p53 deficient mice.	97
<b>Figure 3.7:</b>	p37/p53 deficient mice develop predominately GC B-cell DLBL and have elevated <i>RelA</i> expression and activity.	99
<b>Figure 3.8:</b>	p37/p53 deficient mice have elevated NF-κB activity.	101

### CHAPTER IV

<b>Figure 4.1:</b>	Generation of Ing4-deficient mice.	115
<b>Figure 4.2:</b>	Growth regulation by Ing4.	118
<b>Figure 4.3:</b>	Classical p53 responses are not altered in Ing4 <sup>-/-</sup> cells.	121
<b>Figure 4.4:</b>	Ing4 null mice are hypersensitive when treatment with LPS.	123
<b>Figure 4.5:</b>	Ing4 null mice show elevated cytokine levels in the serum and organs following treatment with LPS.	125
<b>Figure 4.6:</b>	Ing4 null thioglycolate-elicited peritoneal macrophages also show elevated cytokine levels.	128
<b>Figure 4.7:</b>	Higher nuclear p65/RelA levels in Ing4 null macrophages.	129

<b>Figure 4.8:</b>	EMSA of nuclear extracts from Ing4 null and wt thioglycolate-elicited macrophages.	130
<b>Figure 4.9:</b>	Elevated p65/RelA to the Il-6 promoter in Ing4 null macrophages.	131
<b>Figure 4.10:</b>	Overexpression of Ing4 inhibits the expression of luciferase from a NF- $\kappa$ B reporter.	132
<b>Figure 4.11:</b>	Absence of Ing4 causes a decrease in <i>I<math>\kappa</math>B</i> transcript levels.	134
<b>Figure 4.12:</b>	Overexpression of Ing4 enhances the expression of <i>I<math>\kappa</math>B<math>\alpha</math></i> .	135
<b>Figure 4.13:</b>	Ing4 null mice show a greater drop in body weight following treatment with dextran sodium sulfate (DSS).	136
<b>Figure 4.14:</b>	Kaplan-Meier survival curve for Ing4 $-/-$ (n=8) and Wt (n=8) mice during DSS treatment.	137
<b>Figure 4.15:</b>	Ing4 null mice treated with DSS show a reduction of colon length and complete removal of the intestinal mucosa.	139

## CHAPTER V

<b>Figure 5.1:</b>	Expression of <i>I<math>\kappa</math>B<math>\alpha</math></i> in p37/53 null purified FO (IgD <sup>Hi</sup> ) B-cells and tumor samples.	155
--------------------	--	-----

## APPENDIX I

<b>Figure S1:</b>	Generation of Ing5-deficient mice.	199
<b>Figure S2:</b>	Generation of Foxo3a-deficient mice.	201

**CHAPTER I:**  
**INTRODUCTION**



## 1.1 Forward

ING1, the initial member of the Inhibitor of Growth (ING) gene family was first cloned in a subtractive hybridization screen to identify genes under-expressed in transformed versus normal human epithelium. Subsequent database searches identified a total of five members of the ING family (ING1-ING5) conserved from yeast to humans. Little is known about the role of ING proteins in regulating cell growth. Biochemical and molecular biological analysis has revealed that all ING members encode a PHD finger motif proposed to bind both methylated histones and phosphoinositol, and most ING proteins have also been found as components of large chromatin remodeling complexes that also include histone acetylase transferase (HAT) and histone deacetylase (HDAC) enzymes, suggesting a role for ING proteins in regulating gene transcription. In addition, the results of forced overexpression studies performed in tissue culture have indicated that several of the ING proteins can interact with the p53 tumor suppressor protein and/or the nuclear factor-kappa B (NF- $\kappa$ B) protein complex. As these ING-associated proteins play well-established roles in numerous cell processes, including DNA repair, cell growth and survival, inflammation, and tumor suppression, several models have been proposed in the literature that ING proteins act as key regulators of cell growth not only through their ability to modify gene transcription but also through their ability to alter p53 and NF- $\kappa$ B activity. However, these models have yet to be substantiated by *in vivo* experimentation.

## 1.2 Suppression of Tumorigenesis

Cancer is a complex genetic disease initiated by cells that have accumulated several mutations that ultimately bestow malignant characteristics. With rare exceptions cancers arise from single somatic cells and their progeny. As the neoplastic cells divide, they accumulate either genetic or epigenetic changes resulting in altered phenotypes that provide various selective advantages to the cell (172). These advantages have been grouped by Hanahan and Weinberg into six general principles of a cancer cell phenotype: 1) disregard for signals to stop proliferating, 2) disregard for signals to differentiate, 3) capacity for sustained proliferation, 4) evasion of apoptosis, 5) invasion/metastasis, and 6) angiogenesis (82). Over the past several years evidence has been accumulating that a rare fraction of cells in the tumor, termed cancer stem cells, are the actual cells that have accumulated these six selective advantages. In this model, expansion of the cancer stem cell gives rise to the bulk of the tumor and accounts for the clonal origin of cancer (171), although whether or not cancer stem cells actually exist or if a single stem cell would be sufficient to recapitulate the tumor after surgery or in a transplanted recipient remains a point of debate.

The mutations that provide these selective advantages to a cancer cell (regardless of its “stemness”) can occur in numerous genes that encode a wide variety of proteins (111, 112). Although these corresponding oncoproteins can differ greatly in their cellular functions, the genes encoding these proteins have been conventionally grouped into two classes: oncogenes and anti-oncogenes (or tumor suppressors).

Oncogenes typically encode proteins that promote cell growth or cell survival, and are usually overexpressed in cancer cells (due to promoter mutations or gene amplification) or, in some instances, the presence of an activating mutation upregulates the function of the encoded oncoprotein. Conversely, proteins encoded by tumor suppressor genes typically inhibit cell growth. These tumor suppressor proteins are usually lost in tumor cells due to deletion or mutation of the coding region of the tumor suppressor gene, or due to DNA methylation of CpG islands in the promoter regions of these genes (52).

Two classes of tumor suppressor genes have been proposed: class I genes that are mutated or deleted in cancer and class II genes that are not mutated but rather alter cellular phenotype due to alterations in their level of gene expression (178). Regardless of class, it is clear from studies of human hereditary cancer syndromes such as familial melanoma, Li- Fraumeni syndrome, retinoblastoma, and others, that loss or mutation of tumor suppressor genes is an integral part of the genetic alterations needed to give rise to cancer.

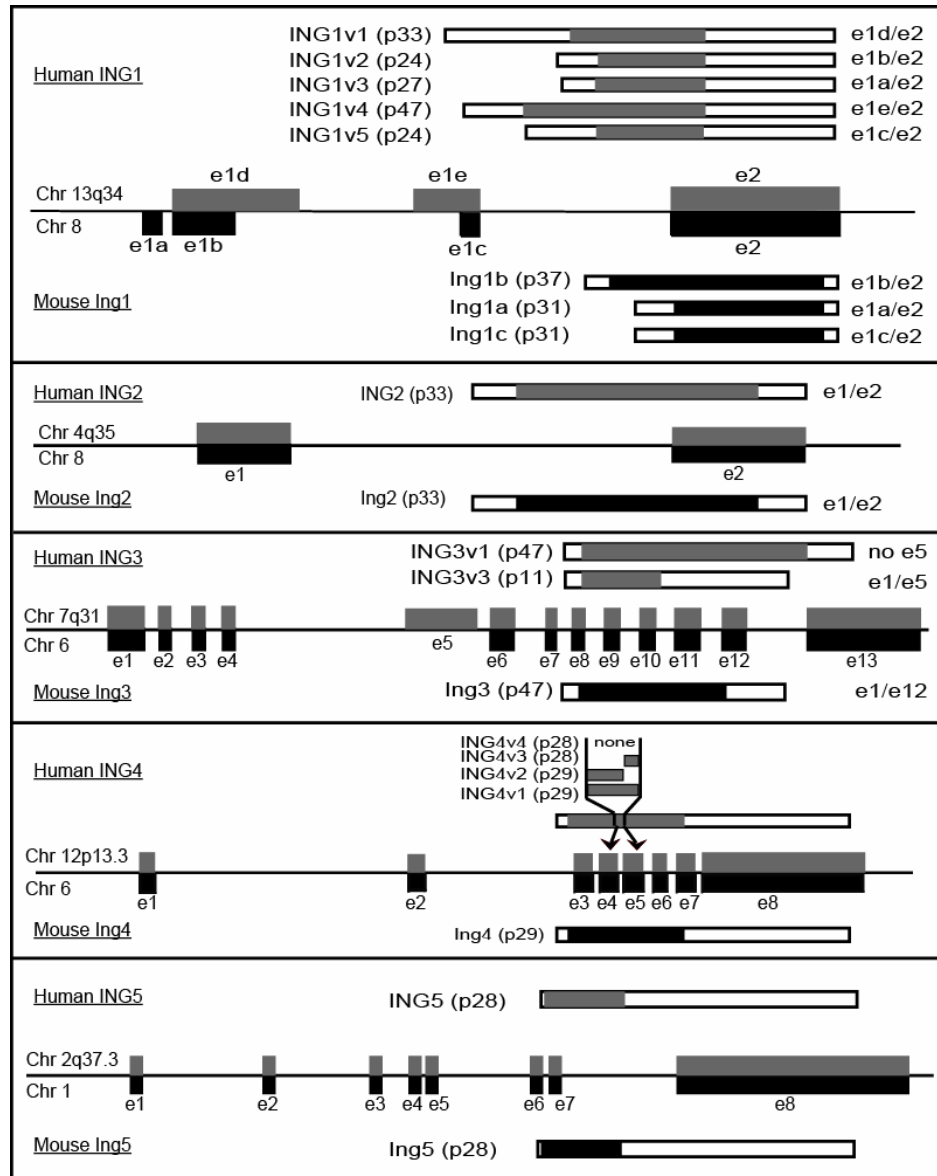
Tumor suppressor proteins have been found to regulate numerous cellular processes, including cell cycle arrest, cell senescence, DNA repair, signal transduction, and apoptosis. Reflecting this wide variety of regulatory effects, tumor suppressors include proteins that are involved in transducing external growth signals into the cell, proteins that sense or respond to genetic or metabolic insult, kinases that regulate the function of other enzymes in the nucleus or cytoplasm, proteins that can alter the cellular location or cellular levels of other regulatory proteins, and transcription factors that alter

the expression of genes involved in cell growth or survival. In addition, tumor suppressors include certain proteins that regulate chromatin remodeling and gene expression, including the Inhibitor of Growth (ING) protein family, a recently recognized group of histone binding proteins.

### **1.3 Identification, Organization, and Expression of ING Genes**

The first member of the ING family of candidate tumor suppressor proteins was discovered through a subtractive hybridization assay between normal mammary epithelium and seven breast cancer cell lines (61). Short complimentary DNA (cDNA) sequences of candidate genes identified by this screen were termed genetic suppressor elements (GSE). The antisense DNA sequence of these GSE acted effectively as an oncogene in transfection experiments to promote cellular growth and transformation by interfering with the activation of tumor suppressors. Conversely, transfection of the sense sequence of the GSE would block cellular growth and prevent transformation. Sequence analysis of a GSE from this screen and comparison to the human genome identified the ING1 gene as a putative tumor suppressor (61). Searching the database of expressed sequence tags for genes that were similar to p33ING1b yielded four other members of the gene family: ING2, ING3, ING4, and ING5, with various ING members sharing between 32% to 76% DNA sequence homology. Additionally, comparison to genomes of other organisms revealed that the ING family is conserved from yeast to humans (87). Mice were shown to possess five ING genes (Ing1- Ing5), similar to humans, whereas three ING genes were identified in yeast (Yng1, Yng2, and PHO23).

Human and mouse ING genes are dispersed throughout their respective genomes, as seen in Figure 1.1. Analysis of the genomic structure of the human ING genes revealed that most members undergo alternative splicing, with the exceptions of ING2 and ING5.



**Figure 1.1:** Genomic organization of mouse and human ING family members. Human ING genes are depicted on the top of each panel and are in grey. Mouse Ing genes are on the bottom and in black. Figure shows genomic structure of each gene and each alternatively spliced transcript with protein masses in kilodaltons. Shaded region for each transcript shows protein coding region. Currently, extensive studies of genomic organization have been conducted for human and mouse ING1 and for human ING2-5. Shown for mouse ING2-5 is the predicted genomic organization and alternative splice variants.

The number of isoforms encoded by the ING1, ING3, and ING4 gene differs between the two species. Human ING1 was found to have five alternative splice variants, each differing at the N-terminus with a conserved PHD motif at the C-terminus (Fig 1), whereas mouse Ing1 produces three splice variants, p31<sup>Ing1a</sup>, p31<sup>Ing1c</sup>, and p37<sup>Ing1b</sup>. Both human and mouse ING1 splice variants occur through alternative splicing of one of several upstream exons into a common last exon of the gene. In contrast, human ING4 encodes four splice variants, but only one Ing4 transcript has been observed in mouse. The number of splice variants encoded by mouse Ing2, Ing3, and Ing5 genes is presently unknown.

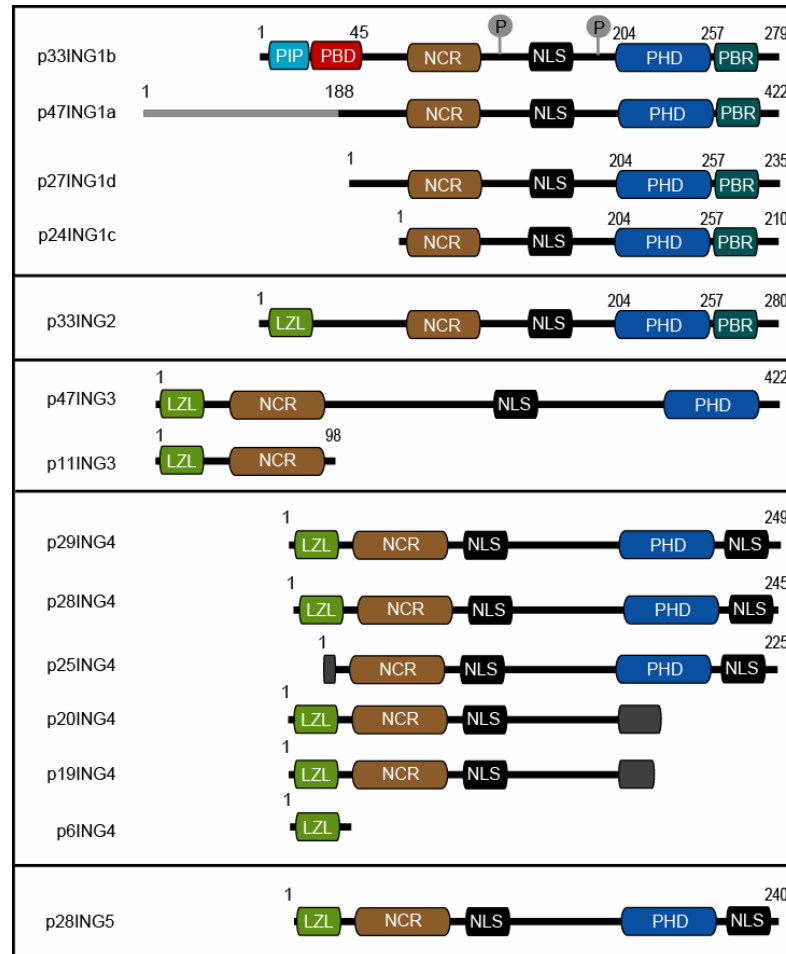
Several studies have examined the temporal and spatial pattern of human and mouse ING gene expression (79, 145, 152, 216, 222). All ING genes appear to be ubiquitously expressed in fetal and adult tissues, though the relative abundance of the expression levels of different ING genes differs between organs and developmental stages.

#### **1.4 Structural Features of ING Proteins**

All ING proteins contain a plant homeodomain (PHD) at the C-terminus, a nuclear localization signal, and a unique domain with an unknown function called the novel conserved region (Figure 1.2). The PHD motif comprises approximately 60 amino acids and displays a C4HC3 signature that typically binds two Zn<sup>2+</sup> ions (1, 14).

Approximately 150 separate PHD domain-bearing proteins have been predicted from the human genome analysis (1). Currently it is unknown how many PHD proteins actually occur in man or mouse. PHD domains closely resemble a canonical RING domain but lack the RING E2 ubiquitin ligase activity (14).





**Figure 1.2:** Domain structure of human ING proteins. The domain composition and approximate location for each domain is shown for various ING proteins. Also shown for ING1 are the two known phosphorylation sites at serine 126 and 199. 14-3-3 binds to phosphorylated serine 199 and facilitates translocation from the nucleus into the cytoplasm (68). Serine 126 is phosphorylated by CDK1 under non-stressed conditions and by CHK1 under DNA-damaging conditions. This site has also been reported to affect the half-life of ING1 and its ability to repress the expression of CyclinB1 (58). Each ING protein is predicted have three conserved regions, which are a PHD domain (Plant Homeodomain), NCR (Novel Conserved Region), and NLS (Nuclear Localization Sequence) (23). The NLS for ING4 has been reported to be the putative region which binds to p53 (242). A Luezine Zipper-Like (LZL) domain is present in ING2-5. Little is known about this domain, but it was reported to affect the ability of ING2 to function in DNA repair and apoptosis (227). ING1 also has a PBD (Partial Bromodomain) and a PIP (PCNA-Interacting Protein Motif) domain. The PBD domain appears to bind to the SAP30 subunit of the mSin3A-HDAC complex (118, 202). Both ING1 and ING2 have a PBR (Poly Basic Region) domain, which appears to be involved with phosphoinositide binding (73, 101). The domain structure for mouse Ing proteins have not been defined.

However, PHD domains have been implicated in chromatin remodeling, as they are often present in proteins that are known components of larger chromatin remodeling complexes (14). For example, PHD domains are present on the William-Beuren syndrome transcription factor (WSTF), a component of an ISWI and SWI/SNF based chromatin remodeling complexes (164), on CHD4 (Mi-2 $\beta$ ) the dermatomyositis-specific autoantigen, a component of the NuRD histone deacetylase complexes (HDAC) (46), and on the autoimmune regulator protein AIRE1, predicted to have a role in transcription control (17). Using an electrophoretic mobility shift assay with the isolated PHD finger of p300, this motif was shown to possess direct nucleosome binding activity (173), and the PHD domain of both the p300 and ACF (another protein containing a PHD domain) can cooperate with an adjacent bromodomain to constitute a functional nucleosome-binding module. Thus, the role of the PHD domain may be to consolidate or strengthen a separate chromatin-binding activity of either the same protein or of a closely associated protein, and it is interesting to note that a partial bromodomain is present in ING1 (23). Recently, the PHD finger of the ING proteins has been found to bind directly to methylated histones, specifically H3K4me2 and H3K4me3 (168, 194). This report further underscores the link between PHD domains and nucleosome binding.

Another domain common among all ING proteins is the nuclear localization signal (NLS), and some ING proteins appear to have multiple NLS. To date, the role of the NLS has been studied extensively only in ING1 (81, 184), in which deletion of the NLS results in cytoplasmic accumulation of the protein.

Localization of ING proteins to the nucleus has been proposed to be critical to their function, as is evident by the observation of loss of nuclear ING1 staining in a number of cancers (70), and because deleting the entire NLS of ING4 resulted in a protein that could no longer bind p53 in co-transfection experiments (242). Additionally, there are two copies of a nucleolar translocation signal (NTS) contained within the NLS of ING1, and translocation of ING1 to the nucleolus following exposure to UV light appears to be required for ING1-associated apoptosis following UV exposure (23). It is currently unknown if other ING genes have a similar NTS or can be detected in the nucleolar compartment.

Other protein domains found in select ING proteins include the leucine zipper-like (LZL) region present in ING2-5 (203), though the functional role of this LZL is not yet understood. The LZL consists of 4-5 conserved leucine or isoleucine residues spaced seven amino acids apart, which has the potential to form a hydrophobic face near the N-terminus of the protein. One report for the function of the LZL of ING2 suggested that this motif is required for nucleotide excision repair and induction of apoptosis (227), though these functions have yet to be substantiated by further experimentation.

### **1.5 ING Proteins Participate in a Variety of Cellular Processes and Pathways**

A review of the current literature suggests that ING proteins regulate a wide variety of cellular processes, including cell growth, apoptosis, DNA repair, senescence, and angiogenesis.

Several lines of evidence have indicated that ING proteins can influence cell cycle progression and are involved in cell cycle checkpoints (23, 203). The majority of these studies have been conducted using ING1, but the fewer studies involving ING2-5 have also identified similar roles for these proteins. In addition to the above processes, suppression of ING proteins has been shown to increase cell spreading, migration, and relieve contact inhibition (61, 109, 110, 189, 216).

The results of several cell transfection studies have suggested that most ING proteins are required for proper p53 function (23, 203). However, more recent studies have also suggested that there are p53-independent functions for the ING proteins, including regulation of the NF- $\kappa$ B (63) and hypoxia inducible factor (HIF) pathways (159) (see below). Additionally, ING proteins have been found to serve as subunits in chromatin remodeling complexes (48). These findings suggest that ING proteins act in the nucleus to regulate transcription, which could account for their multiple proposed roles in several essential biological processes (12, 23, 33, 55, 193). As dysregulation of any of these pathways might lead to neoplasia, ING proteins have also been proposed to act as tumor suppressors in human cancers. The following is a description of the most important and compelling findings for each individual ING protein:

### 1.5.1 *ING1*

**ING1 in growth regulation:** A putative role for ING genes in cell cycle regulation was initially suggested from experiments involving either overexpression or antisense

knockdown of ING1 (61, 62). Overexpression of ING1 in human diploid fibroblasts resulted in a 50% increase in the number of cells found in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle (61), indicating that ING1 might have a role in the G<sub>1</sub>-S transition. Conversely, antisense ING1 constructs in these cells resulted in abolition of this arrest and entry of the cells into S phase (61). These findings were corroborated in H1299 cells by ectopically expressing p33<sup>ING1b</sup>, which resulted in a slight increase in the doubling time with fewer cells in G<sub>1</sub>, possibly due to a delay or block in S or G<sub>2</sub>/M (215). Therefore, ING1 appears to be involved in both the G<sub>1</sub>/S and the G<sub>2</sub>/M cell cycle checkpoints. ING1 has also been found to negatively regulate the expression of cyclin B1 (206), further supporting a possible involvement of ING1 in the G<sub>2</sub>/M cell cycle checkpoint. Additionally, ING1 expression was found to be inversely related to cyclin E expression (154). However, this latter finding needs to be corroborated and its significance determined with further investigation. It was also determined that ING1 expression is cell cycle regulated, decreasing from G<sub>0</sub> to G<sub>1</sub>, increasing in late G<sub>1</sub> to become maximal during S phase, followed by a decrease in G<sub>2</sub> (64). As would be expected, ectopic expression of p33<sup>ING1b</sup> can enhance the cell cycle arrest induced by doxorubicin, a topoisomerase II inhibitor that results in double-stranded DNA breaks (215). Doxorubicin was found to decrease the population doubling time and increase the number of cells in G<sub>2</sub>/M. The enhancement of cell cycle arrest due to p33<sup>ING1b</sup> overexpression was found to be DNA damaging agent specific, with no enhanced cell cycle arrest observed with cisplatin or UV irradiation (215).

Antisense knockdown of ING1 was also found to promote the ability of anchorage independent growth in soft agar and increase foci formation, supporting a functional role for ING1 in preventing cell transformation (59, 61, 62, 206).

**ING1 in apoptosis:** Expression of ING1 increases prior to apoptosis induced by serum starvation, and ectopic ING1 expression was found to cooperate with c-myc expression to induce apoptosis in both P19 cells and rodent fibroblasts (88). Conversely, antisense knockdown of ING1 provided protection from apoptosis. Taken together, these results suggest that ING1 also has a role in regulating cell death. The ability of ING1 to induce apoptosis was found to depend upon an interaction with PCNA following UV irradiation (186) and to be cell age dependent, as only early passage fibroblasts were able to upregulate p33<sup>ING1b</sup> and undergo apoptosis following growth factor deprivation (220). This study also suggests possible ING1 isoform-specific functions in apoptosis, since ectopic expression of p33<sup>ING1b</sup>, but not p47<sup>ING1a</sup>, sensitized young, but not old, fibroblasts to UV and hydrogen peroxide-induced apoptosis (220). Ectopic expression of p33<sup>ING1b</sup> also sensitized cells to apoptosis induced by the chemotherapeutic agents etoposide, taxol, and doxorubicin (184, 206, 220). However, a more recent paper appears to contradict the previous reports that overexpression of ING1 can promote apoptosis, as down-regulation of ING1 in p53 deficient glioblastoma cells sensitized these cells to cisplatin-induced apoptosis (208). Interestingly, a very recent report found that overexpression of p33<sup>ING1b</sup> could synergize with tumor necrosis factor alpha (TNF $\alpha$ ) treatment to induce apoptosis (54).

This finding suggests an indirect role for p33<sup>ING1b</sup> in the NF- $\kappa$ B pathway, since NF- $\kappa$ B signaling protects cells from TNF $\alpha$ -induced apoptosis (217).

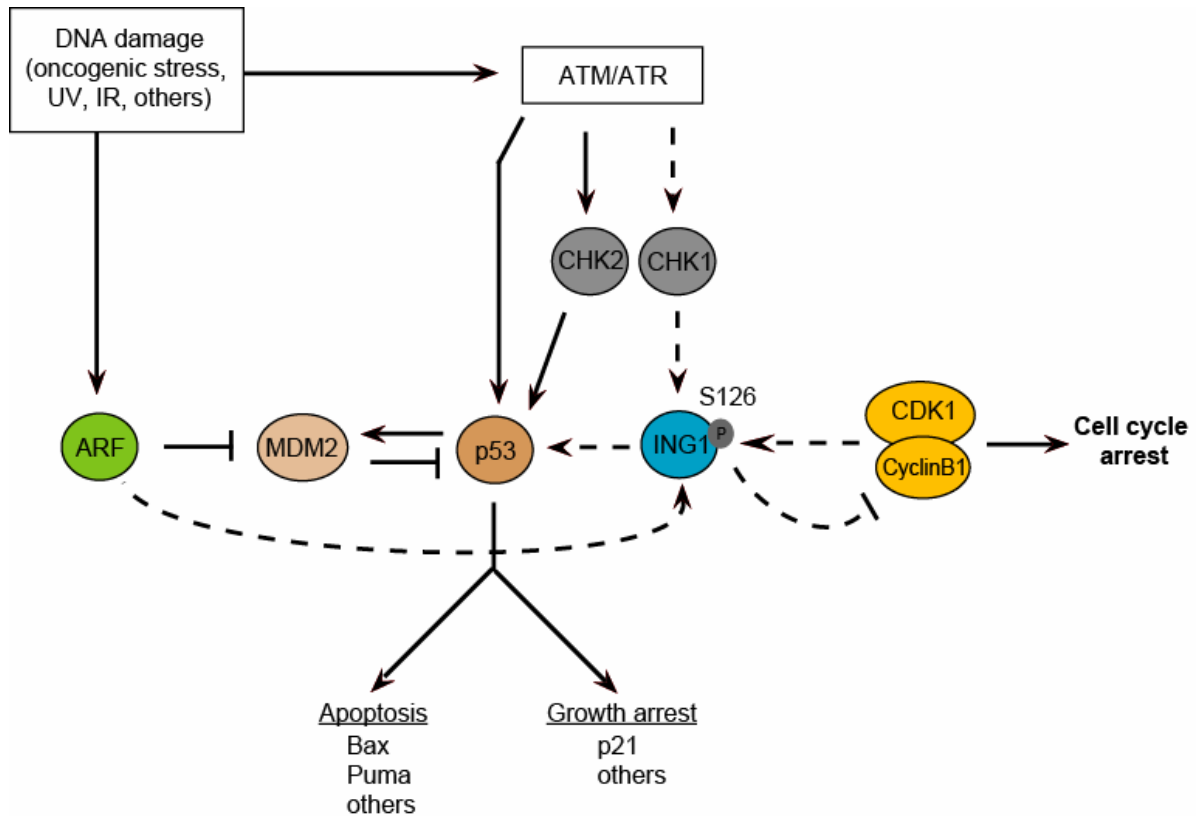
**ING1 in DNA repair:** Regulation of nucleotide excision repair (NER) following UV exposure has also been linked to ING1. Overexpression of p33<sup>ING1b</sup> can enhance nucleotide excision repair (NER) of exogenously added plasmid DNA in a host-cell- reactivation assay (34). An interaction between GADD45 and p33<sup>ING1b</sup> was also detected to support the proposed involvement of p33<sup>ING1b</sup> in NER. Mutations in the PHD domain and a region found to interact with SAP30 (Sin3A Associated Protein 30), a component of Sin3A co-repressor complexes, abrogated enhancement by p33<sup>ING1b</sup> of NER in host cell reactivation assays and radioimmunoassay (24, 121). Furthermore, the p33<sup>ING1b</sup> variant was not recruited to UV-induced DNA lesions, but enhanced NER in XPC- proficient cells possibly due to its ability to bind XPA (115). As XPC/hHR23B acts at the first step of the NER pathway by recognizing helix-distorting DNA lesions and XPA acts further downstream to stabilize the resulting open DNA structure (231), these observations suggest that p33<sup>ING1b</sup> has an essential role in the early steps of the NER pathway, possibly by facilitating access of the nucleotide excision repair machinery to chromatin.

**ING1 and p53 functions:** The ING proteins have been implicated in the regulation of the p53 pathway by several reports (60, 145, 167, 199).

p53 is a tumor suppressor that responds to disruption of DNA integrity and/or perturbation of cell division in cells exposed to various forms of stress (8). Following DNA damage, stress signals are transmitted to the p53 protein by a cascade of post-translational modifications that activate the p53 transcription factor and initiate or upregulate a wide variety of genes involved in cell cycle arrest, senescence, DNA repair, and apoptosis (8). These p53-responsive genes encode proteins that not only interact with other signal transduction pathways (Figure 1.3), but also serve to regulate the duration of the p53 response (84). One well-characterized regulator of p53 is the E3 ubiquitin ligase Mdm2. p53 upregulates the expression of Mdm2, which subsequently regulates the stability of the p53 protein by placing ubiquitin moieties onto p53 to target p53 for 26S proteosomal degradation. In addition, Mdm2 can also inhibit p53 activity by targeting p53 for export from the nucleus into the cytoplasm. Regulation of p53 by Mdm2 is governed by the p19<sup>ARF</sup> tumor suppressor. The levels of the p19<sup>ARF</sup> protein increase in response to the inappropriate expression of certain oncogenes, (190, 191), and p19<sup>ARF</sup> complexes with MDM2 and sequesters MDM2 into the nucleolus, thereby stabilizing p53 and facilitating activation of p53-dependent gene expression (192).



All ING genes except ING3 have been found to co-immunoprecipitate with p53 following ectopic co-expression of ING proteins and p53, and subsequent functional studies utilizing forced overexpression of ING genes in cultured cells have indicated that the ability of ING proteins to arrest cell proliferation and induce apoptosis is lacking in p53-deficient RKO-E6 cells, suggesting that ING proteins require functional p53 to inhibit cell growth (12, 23, 203).



**Figure 1.3:** Proposed model of ING1 in the p53 pathway. DNA damage such as oncogene overexpression or exposure to UV or  $\gamma$ -irradiation activates the ATM/ATR kinases, which can then activate CHK1/CHK2. CHK2 can phosphorylate p53, increasing its stability by preventing the binding of MDM2 and allowing p53 to translocate into the nucleus (84). Nuclear localized p53 can induce genes necessary for the cell to apoptose or enter a cell cycle arrest. Oncogenic stress can also activate the ARF protein which can inhibit the ability of MDM2 to bind with p53 and target it for degradation (139). ING proteins have been suggested to participate in at least two locations in the ARF/MDM2/p53 pathway. 1) All ING proteins except ING3 have been found to co-immunoprecipitate with p53 (23), 2) ARF has been suggested to interact with p33<sup>ING1b</sup> *in vivo* to alter the sub-cellular localization of p33<sup>ING1b</sup> from the nucleus to the nucleolus and the ability of exogenous p33<sup>ING1b</sup> to induce cell cycle arrest was impaired in ARF-deficient mouse embryonic fibroblasts (71), and 3) ING1b and MDM2 compete for the same binding site on p53 (124). In addition, ING1 has been proposed to be phosphorylated on serine 126 by CDK1 under non-stressed conditions and by CHK1 under DNA-damaging conditions (58). Phosphorylation of serine 126 has been suggested to alter the half-life of ING1 and its ability to repress the expression of CyclinB1 (58). Grey arrows designate proposed interactions and black arrows show well characterized interactions.

Conversely, expression of antisense ING1 constructs inhibited expression of a p21WAF1-CAT construct in cultured cell that were wildtype for functional p53 (59), suggesting that p53 requires the ING proteins to function as a transcriptional activator.

Additional evidence linking ING proteins and p53 function was provided by experiments utilizing adenoviral-mediated gene transfer of p33<sup>ING1</sup> and p53 into glioma cells. Increased apoptosis was observed in U251 and U-373MG glioma cell lines only when both genes were co-introduced (198), and similar results have been obtained following co-transfection of a human esophageal carcinoma cell line (196). Furthermore, expression of ING1 in transfected HepG2 (p53-wt) and Hep3B (p53-null) hepatocellular carcinoma cells repressed expression of the  $\alpha$ -fetoprotein (AFP) gene in a p53-dependent manner (61). AFP expression is normally inhibited by p53 (62), and repression of AFP by p53 was found to involve binding of ING1 to AT- motifs within the AFP promoter, with attendant acetylation of p53 (107). However, these conclusions drawn from this data are open to interpretation, as the precise role of p53-acetylation on p53 transactivation and transrepression remains a matter of some dispute (16).

Recent findings have indicated that ING1 may also regulate several components of the Arf-Mdm2-p53 signaling axis (Fig 1.3). The p33<sup>ING1b</sup> protein has been proposed to compete with Mdm2 for the same binding site on p53 as a means of increasing the stability and activity of p53 (124).

In addition, Arf has been suggested to interact with p33<sup>ING1b</sup> *in vivo* to alter the sub-cellular localization of p33<sup>ING1b</sup> from the nucleus to the nucleolus (much like the effect of Arf on Mdm2), and the ability of exogenous p33<sup>ING1b</sup> to induce cell cycle arrest has been found to be impaired in Arf-deficient mouse embryonic fibroblasts (71).

**p53-independent functions of ING1:** There also appears to be p53-independent roles for the ING proteins (54, 67, 206, 208, 215, 226). The results of microarray analysis of cells with p33<sup>ING1b</sup> knocked down by antisense constructs indicate that the cyclin B1 gene may be regulated by p33<sup>ING1b</sup> (206). This study also reported that overexpression of p33<sup>ING1b</sup> in p53-deficient Saos2 cells caused a decrease in cyclinB1 message, revealing that regulation of cyclinB1 expression by ING1 was p53-independent. Likewise, transcriptional regulation of HSP70 was also found to be regulated by p33<sup>ING1b</sup> in a p53-independent manner (54). Several p53-independent roles for p33<sup>ING1b</sup> in growth regulation and cell cycle arrest have been proposed based upon transfection studies utilizing cell lines lacking functional p53. Overexpressing p33<sup>ING1b</sup> in H1299 lung carcinoma cells increased the doubling time of these p53-deficient cells by about 10% and enhanced a doxorubicin-induced G<sub>2</sub>/M DNA damage checkpoint (215).

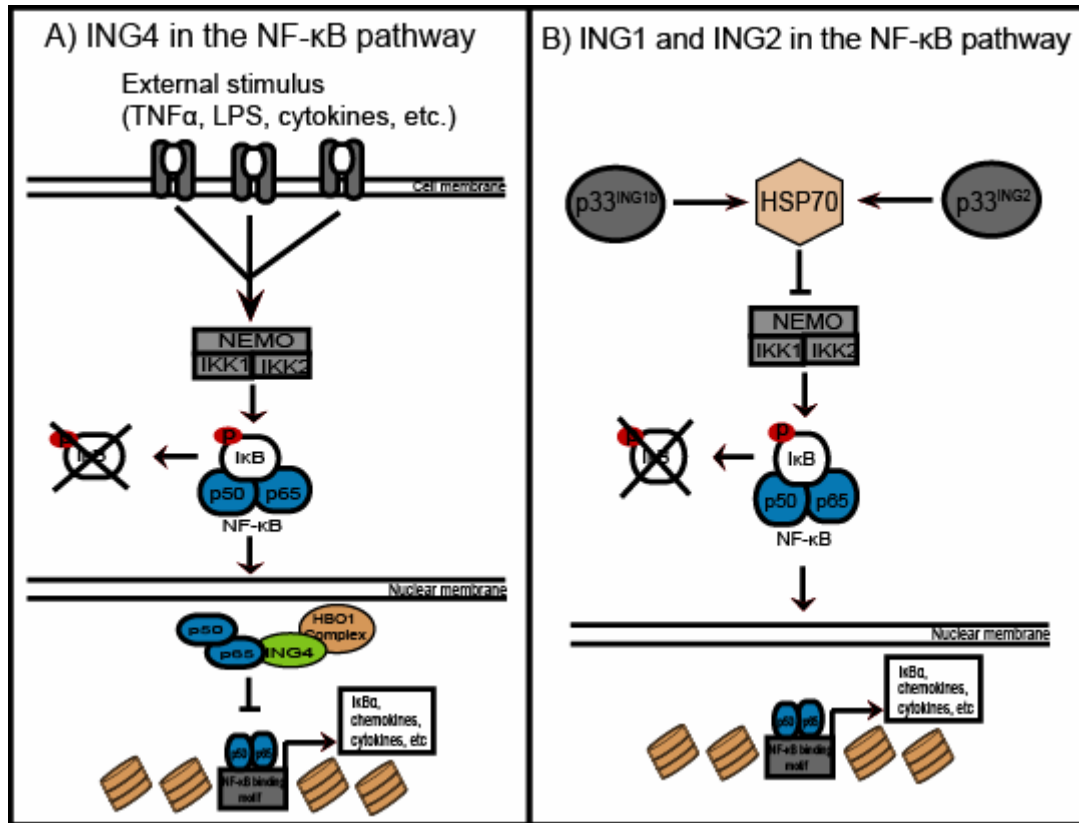
Additionally, siRNA knockdown of ING1 in LN229 glioblastoma cells caused these p53-null cells to be more sensitive to cisplatin treatment and to transition faster through the G<sub>1</sub> phase of the cell cycle (208). These findings suggest that ING1 can function to inhibit cell growth or cell death independent of p53, since overexpressing ING1 or knocking down ING1 in the absence of p53 would be otherwise expected to have no effect.

In addition, the transcriptional silencing effect of ING1 has been proposed to be p53-independent, as ectopic expression of a Gal-p33<sup>ING1b</sup> fusion protein in p53-null, H1299 cells repressed the expression of a co-transfected chloramphenicol acetyl-transferase (CAT) reporter gene that was placed under transcriptional control of a modified thymidine kinase promoter (67).

**ING1 and the NF- $\kappa$ B pathway:** ING proteins have also been proposed to regulate NF- $\kappa$ B activity. NF- $\kappa$ B can be activated by a number of stimuli, including components of microbial pathogens such as lipopolysaccharide (LPS), and by inflammatory cytokines (146). NF- $\kappa$ B is a dimeric transcription factor composed of the Rel protein family members c-Rel, RelA (p65), RelB, NF- $\kappa$ B1 (p50 and its precursor p105), and NF- $\kappa$ B2 (p52 and its precursor p100) (141). Typically, NF- $\kappa$ B exists as a hetero- or homodimer of two Rel members, the most common dimer combination being p65/p50. This heterodimer pair generates an NF- $\kappa$ B complex that functions as a transcriptional activator. In contrast, NF- $\kappa$ B composed of homodimers of p50 or p52 can repress transcription from NF- $\kappa$ B-responsive promoters (141).

Following activation of upstream cellular receptors, the NF- $\kappa$ B transcription complex positively regulates the expression of specific genes involved in the innate immune response (142). One such example involves the Toll-like receptor. Binding of cognate ligands to the Toll receptor recruits Myd88, an adaptor protein, and results in activation of the IRAK family of kinases (141, 142, 162).

Subsequent signaling steps serve to activate the downstream I $\kappa$ B kinases (IKK1 and IKK2), leading to phosphorylation and subsequent proteosomal degradation of the inhibitor I $\kappa$ B family of proteins (141) (Figure 1.4). Destabilization of I $\kappa$ B proteins cause the NF- $\kappa$ B complex to translocate into the nucleus and activate gene transcription. In addition, an alternative Myd88-independent signal transduction pathway exists which ultimately activates the IRF family of transcription factors (142, 162). NF- $\kappa$ B has been suggested to play a role in tumorigenesis by acting as a tumor promoter (75, 104, 105). Although mutations in NF- $\kappa$ B are rarely observed in tumors, alterations in upstream NF- $\kappa$ B regulators have been documented (eg. I $\kappa$ B loss of function in Hodgkin's lymphoma) that lead to constitutive activation of NF- $\kappa$ B (175). Because NF- $\kappa$ B regulates the expression of many genes proposed to govern apoptosis, angiogenesis, metastasis, proliferation, and tumor growth and survival (169), perturbation of NF- $\kappa$ B signaling has been strongly linked to tumorigenesis.



**Figure 1.4:** Model of a role for ING1, ING2, and ING4 in the regulation of the NF-κB pathway. External stimuli, such as lipopolysaccharide (LPS), cytokines, or TNFα, binds to specific receptors, in the case of LPS they are members of the Toll-like receptor family, and lead to activation of a signal transduction cascade. This cascade activates the IκB kinases (IKK1 and IKK2) which can phosphorylate IκB proteins and release the NF-κB complex. The complex can then translocate into the nucleus to induce expression of genes for cytokines and pro-survival proteins (221). A) ING4 was recently determined to directly interact with p65/RelA and negatively regulate the transactivation activity of the NF-κB complex (63). Also depicted is the possible recruitment of the HBO1 histone acetyltransferase complex by ING4. However, the exact role of the HBO1 complex in regulation of NF-κB activity is not fully understood (42). B) Recently, ING1 and ING2 were found to induce the expression of the heat shock protein HSP70 (54). HSP70 is able to bind to the IKK complex and inhibit its ability to phosphorylate and activate the NF-κB complex (174, 195). Therefore, ING1 and ING2 may have an indirect role in the NF-κB pathway.

Recent microarray data has indicated that expression of the heat shock protein HSP70 is upregulated by p33<sup>ING1b</sup> expression (54). Since HSP70 can inactivate the NF- $\kappa$ B pathway by interacting with IKK $\gamma$  and preventing degradation of I $\kappa$ B (135, 174, 195), these findings suggest a possible indirect role for p33<sup>ING1b</sup> in regulating the NF- $\kappa$ B signaling pathway.

**ING1 in chromatin remodeling and regulation of gene expression:** In addition to possible roles in p53 and NF- $\kappa$ B signaling, p33<sup>ING1b</sup> (but not p24<sup>ING1c</sup>) was found to associate with a chromatin remodeling complex (see Table 1.1). By fractionating HeLa nuclear extracts, the endogenous p33<sup>ING1b</sup> protein was found as a subunit of an approximate 1-2 MDa complex (202). This complex also contained mSin3, SAP30, histone deacetylase 1 (HDAC1), RbAp48, and additional components of the mSin3A transcriptional co-repressor complex (202), and purified p33<sup>ING1b</sup>-containing complexes were found to be active in deacetylating core histones in *in vitro* assays. Although Sin3/HDAC has been reported to act as a repressor, recent evidence suggests that it might act to positively regulate transcription as well (200). Recently, p33<sup>ING1b</sup> and ING2 have been reported to recruit SIRT1 to the Sin3/HDAC complex (15). As SIRT1 acts to negatively regulate the transcriptional repression activity of the Sin3/HDAC complex (14), this data suggests a possible role for ING proteins in transcriptional activation. Thus, p33<sup>ING1b</sup> and other ING proteins may function within the Sin3/HDAC complex to act as a global regulator of gene transcription.



Table 1.1: *ING-associated chromatin remodeling complexes*

Gene	Complex	Methods	Functions	Ref
<b>p33ING1b</b>	mSIN3A, HDAC1/2	IAP-MS, Co-IP	Deacetylation at promoters, DNA repair	(202)
<b>p33ING1b</b>	mSIN3A, BRG1, SWI/SNF components	IAP-MS, Co-IP, GST pull-down	Deacetylation at promoters, transcription, DNA repair	(118)
<b>p33ING1b</b>	CBP, p300, PCAF, TRRAP, HDAC1	Co-IP	Acetylation of histones and non-histones.	(118)
<b>ING2</b>	mSIN3A, HDAC1/2	IAP-MS	Deacetylation at promoters, DNA repair	(48)
<b>ING2</b>	mSIN3A, BRG1, SWI/SNF components	IAP-MS	Deacetylation at promoters, transcription, DNA repair	(48)
<b>ING3</b>	hNuA4/TIP60	IAP-MS	Histone H4/H2A acetylation, transcription, DNA repair, recombination	(48)
<b>ING4</b>	HBO1	IAP-MS	Global histone H4K5/8/12 acetylation, transcription, replication	(48)
<b>ING5</b>	HBO1	IAP-MS	Global histone H4K5/8/12 acetylation, transcription, replication	(48)
<b>ING5</b>	MOZ/MORF	IAP-MS	Histone H3K14 acetylation, transcription, replication	(48)

**Table 1.1:** The composition of each ING protein containing chromatin remodeling complex. Also, provided are the function of each complex and methods used to characterize the assembly of the ING protein within each complex. Abbreviations used: IAP-MS, Immune affinity purified mass spectroscopy; Co-IP, co-immunoprecipitation.

In addition, the ING proteins might contribute to other functions that have been ascribed to the Sin3/HDAC complex, including nucleosome remodeling, DNA methylation, N-acetylglucosamine transferase activity, and histone methylation (200). Interestingly, the Sin3/HDAC complex does not possess DNA-binding activity, and targeting of this complex to gene promoters likely occurs via interaction of Sin3/HDAC with DNA-binding proteins (200).

Different human ING1 isoforms have been found to associate with either HAT or HDAC activity. Overexpression of p33<sup>ING1b</sup> can induce hyperacetylation of histone H3 and histone H4 *in vitro* and in transfection assays, suggesting that ING proteins regulate HAT activity (218). Conversely, overexpression of p47<sup>ING1a</sup> induces histone deacetylation, suggesting an association between ING proteins and HDAC activity (107, 218). These effects would obviously impact transcription by altering histone-DNA interactions in the promoter region of a gene or by altering the binding ability of other regulatory proteins.

In addition, there is some evidence in the literature to suggest that ING1 might be involved in DNA methylation. The DNA methyltransferase 1-associated protein 1 (DMAP1) has been found to physically associate with p33<sup>ING1b</sup> during S-phase at sites of pericentric heterochromatin and correlate with methylated H3 lysine 9 (H3K9), histone deacetylation, and DNA methylation (233).

Additionally, the PHD domain of all human ING proteins was found to preferentially bind di- and tri-methylated H3K4 and repress gene transcription (122, 168, 194).

Finally, the p33<sup>ING1b</sup> protein has also been found to associate with Alien in GST-pull-down *in vitro* assays (53). Alien is a transcriptional co-repressor involved in gene silencing mediated by select members of nuclear hormone receptors and E2F1 (53).

### 1.5.2 *ING2*

**ING2 in cell growth regulation, apoptosis, and DNA repair:** Links between ING2, cell cycle regulation, apoptosis, and NER have also been proposed. Ectopic expression of ING2 in RKO cells inhibited colony formation and induced a G<sub>1</sub> cell cycle arrest (144). Overexpression of ING2 was also found to induce replicative senescence whereas RNAi-mediated downregulation of ING2 delayed the onset of replicative senescence (167). A recent study contradicts this observation, RNAi knockdown was found to induce a p53-independent senescence and overexpression of ING2 was found to induce a p53-dependent senescence in hTERT-immortalized human fibroblasts (114). These two studies suggest that ING2 has a role in senescence regulation; however, further work is needed to elucidate the mechanism of this regulation.

Consistent with a role for ING2 in cell proliferation, ING2 has recently been found to interact with SnoN and Smad2 to promote TGF $\beta$  dependent gene expression resulting in the inhibition of cell proliferation (182).

At present it is unknown if other ING family are potential regulators the TGF $\beta$  pathway. As TGF $\beta$  signaling is involved in cellular proliferation, cell survival, apoptosis, migration, invasion, and inflammation(140), this may be an important topic for future research. ING2 may also have a role in etoposide-induced apoptosis (223) and overexpression of ING2 enhanced apoptosis after irradiation of transfected cells with ultraviolet light (227). The BCL-2 protein was upregulated following UVB exposure of MMRU melanoma cells overexpressing ING2, which also promoted the translocation of BAX to the mitochondria followed by alterations in the mitochondrial membrane potential. Additionally, ING2 was found to regulate *Fas* expression, thus suggesting a link between ING2 and both the mitochondrial/intrinsic and death-receptor/extrinsic apoptotic pathways (35).

Truncation mutants of ING2 that lack the leucine zipper-like (LZL) domain did not display elevated apoptosis following UV exposure (227), suggesting that this ING2 domain is required for apoptosis. RNAi knockdown of ING2 was also found to abrogate the NER capacity of melanoma cells (223). Similar to p33<sup>ING1b</sup>, ING2 may be involved in the initial steps of NER by inducing chromatin relaxation and the recruitment of XPA to the photo-lesions in DNA. Interestingly, the NER ability of ING2 was found to require the LZL domain, a domain that is not present in p33<sup>ING1b</sup> (227).

**Role of ING2 in the NF- $\kappa$ B pathway and in chromatin remodeling:** Similar to p33<sup>ING1b</sup>, HSP70 expression can also be upregulated by ING2 and suggests that the ING proteins might indirectly regulate NF- $\kappa$ B activity (54). ING2, like p33<sup>ING1b</sup>, was found to associate with the co-repressor Alien through an *in vitro* GST-pull-down assay (53), to co-purify with components of the mSin3 complex, and to associate with Brg1-based SWI/SNF chromatin remodeling complexes (Table 1.1) (48). Furthermore, ING2 and p33<sup>ING1b</sup> could also interact *in vitro* and *in vivo* with the RBP1 protein, a component of the mSin3A complex, and recruit SIRT1 to the mSin3A/HDAC1 complex. The recruitment of SIRT1 to this complex was found to negatively regulate the transcriptional repression activity of the mSin3A complex (15).

### 1.5.3 ING3

**ING3 in proliferation and apoptosis:** ING3 has been linked to regulation of the cell cycle and apoptosis. However, unlike other ING proteins, an involvement of ING3 in NER and replicative senescence has not been found. Similar to ING1, ectopic expression of ING3 in RKO cells decreased colony formation, possibly by decreasing the number of cells in S phase (145, 199). ING3 overexpression also induced *Fas* expression, increased the cleavage of Bid, caspases-8, -9, and -3 and promoted apoptosis in UV treated cells (226). Similar to ING2, these findings suggest that ING3 may be involved in the death-receptor/extrinsic pathway. Unlike other ING proteins, ING3 does not appear to interact with p53 and is not thought to have a role in IR-induced cell death (145).

**ING3 and chromatin remodeling:** ING3 was found to be mainly, if not exclusively, a subunit of the NuA4/Tip60 HAT complex (Table 1.1) following biochemical purification of ING protein- containing complexes from HeLa cell nuclear extracts (50). Tip60 is an important cofactor for p53, NF- $\kappa$ B, Myc, E2F1, and nuclear-receptor dependent transcriptional activation, and is involved in the cellular response to DNA damage, apoptosis, and metastasis suppression (7, 209). Mice deficient for Tip60 die during embryogenesis prior to implantation (E4), and haplo-insufficiency for Tip60 results in accelerated lymphomagenesis in transgenic E $\mu$ -Myc mice (72). These data suggest that ING3 might also function in these processes through its association with the NuA4/Tip60 HAT complex.

#### 1.5.4 *ING4*

**Cellular functions of ING4:** Ectopic expression of ING4 in RKO cells was found to inhibit colony formation, likely due to a decrease in the percentage of cells in S phase (145, 199), and serum starvation-induced apoptosis following ING4 overexpression was increased and correlated with increased Bax expression levels (243). Thus, similar to other ING proteins, ING4 has been proposed to regulate cell cycling and apoptosis. However, ING4 appears to differ from the other ING family members in several aspects: ING4 does not appear to have a role in NER, and ING4 has been proposed to alter angiogenesis and cell migration.

**ING4 and angiogenesis and cell migration:** A relationship between ING4 and angiogenesis was uncovered by studies utilizing implantation of the glioblastoma cell line U87MG into the cranial windows of nude mice. Tumor cells in which ING4 was knocked down by siRNA grew faster than control cells and yielded tumors with higher vascular volume fractions (63). Additionally, a reduction of ING4 expression in multiple myeloma cells increased the expression of the pro-angiogenic molecules interleukin-8 (IL-8) and osteopontin (OPN). In addition to these *in vitro* observations, a correlation was noted between reduced expression of *ING4* and increased microvascular density in multiple myeloma patients (40).

ING4 has also been linked to cell migration, cell spreading, and contact inhibition. Ectopic expression of ING4 in several cell lines both decreased cell spreading and cell migration (216). Subsequent mass spectroscopy analysis of extracts from RKO cells overexpressing a FLAG-ING4 construct indicated that ING4 interacts with liprin  $\alpha 1$ , G3BP2a, COP1 $\beta$ , CaBP1, and several ribosomal proteins (189). ING4 was also found co-localized with Liprin  $\alpha 1$  in the lamellipodia of cells. Since Liprin  $\alpha 1$  may play a role in focal adhesion disassembly (187), ING4 might exert an effect upon cell spreading and migration through its interactions with Liprin  $\alpha 1$ . Interestingly, ING4 was also identified in a screen for genes that suppressed the loss of contact inhibition caused by MYCN overexpression (110). The ability of ectopically expressed ING4 to suppress contact inhibition was further supported by reports of an attenuation of the ability of T47D breast cancer cells to grow in soft agar when ING4 was overexpressed (110).

**Involvement of ING4 in the p53, NF- $\kappa$ B, and HIF-1 $\alpha$  pathways:** ING4 has also been suggested to bind to and regulate the functions of either p53, NF- $\kappa$ B, and/or HIF-1 $\alpha$ . Forced overexpression and co-immunoprecipitation experiments performed in the glioblastoma cell line U87MG indicated that ING4 physically interacts with p65 (RelA), linking ING4 to the NF- $\kappa$ B pathway (63). Further analysis of this interaction using gel mobility shift assays and siRNA knockdown of ING4 suggests that ING4 can increase the DNA-binding activity of RelA. Additionally, knockdown of ING4 could stimulate expression of a NF- $\kappa$ B-dependent luciferase reporter plasmid in transfected cells. These results suggest that ING4 interacts directly with RelA to inhibit NF- $\kappa$ B transcriptional activity.

Knockdown of ING4 by siRNA also led to elevation of the HIF-1 $\alpha$  target genes NIP3 and AK3 under hypoxic conditions (159). Nuclear levels of HIF-1 $\alpha$  were unchanged in these experiments, suggesting that ING4 was suppressing HIF-regulated genes by altering HIF-1 $\alpha$  activity, although no direct interaction between ING4 and HIF-1 $\alpha$  was observed. Subsequent experiments revealed that ING4 might regulate HIF-1 $\alpha$  activity by affecting the recruitment of chromatin remodeling enzymes (159). Additionally, in myeloma cells, ING4 has been proposed to suppress HIF-1 $\alpha$  activity by interacting with HIF prolyl hydroxylase-2 (PHD2/HPH2) (40), an oxygen sensing protein that helps to target the HIF-1 $\alpha$  protein for degradation (13).



Although suppression of ING4 increased vessel formation in RPMI-8226 cells under hypoxic conditions (40), no further corroborative studies have been published to substantiate ING4 regulation of HIF activity.

**ING4 and chromatin remodeling:** ING4 has also been reported to exist as a component of a novel HBO1- HAT complex (Table 1.1) (48). The HBO1 protein is the catalytic subunit of two different but related HAT complexes that specifically acetylate histone H4, and HBO1 has been linked to DNA replication, S-phase progression, and transcriptional regulation (7). In addition, HBO1 has been suggested to act as a co-repressor for the androgen receptor (7) and NF- $\kappa$ B (42).

Furthermore, p53 has been found to interact with HBO1 and to down-regulate HBO1 acetyltransferase activity (95). While these are intriguing findings, the ability of HBO1 to function as a regulator of transcription is controversial as the evidence is sparse and conflicting. Therefore, a role for ING4 in regulating HBO1 complex formation or contributing to its function remains uncertain.

### 1.5.5 *ING5*

**ING5 in regulation of transcription and cell growth:** Biochemically, ING5 appears similar to ING4 in that it can interact with p53 and recruit p300 to induce p53 acetylation on lysine-382 (199). Similar to ING4, ING5 was found as a member of HBO1-containing HAT complex, and ING5 was also found as a subunit of a histone H3-specific, HAT complex that included MOZ/MORF leukemic proteins (Table 1.1) (48).

However, almost nothing is known about ING5 functions to date. ING5 transfection studies have suggested that ING5 reduces colony-forming efficiency, the percentage of cells in S-phase, and induces apoptosis in a p53-dependent manner (199). Additionally, ING5 could also induce expression of the cyclin-dependent kinase inhibitor p21 (199), a p53 target gene. These results implicate ING5 in regulation of cell growth and p53 activity, but further studies are needed in order to better characterize ING5 functions.

### **1.6 Regulation of ING Gene Expression and Protein Function**

Little is known about the regulation of ING gene expression or ING protein functions. Analysis of mouse and human ING1 expression has indicated that ING expression is not regulated by p53 (31, 239). However, p53 was recently determined by chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift (EMSA) assays to bind to two regions in the promoter of ING2 (114). Studies have found that expression of ING2, but not of ING1 (144) or ING3 (145), can be induced by certain DNA damaging agents such as etoposide or neocarzinostatin, but not by other agents such as  $\gamma$ -irradiation or doxorubicin. A recent study determined that RUNX3 induces the expression of both ING1 and ING4 in MKN-1 human gastric carcinoma cell lines (143), but further information linking RUNX to ING gene expression is not available.

Subcellular localization is another mechanism utilized by cells to regulate protein activity. Localization of p33<sup>ING1b</sup> has been proposed to be regulated by 14-3-3 family members, which can tether ING1 to the cytoplasm. Binding between ING1 and 14-3-3 appears to be dependent upon the phosphorylation status of serine 199 of ING1 (68). Localization of ING2 to the plasma membrane was also reported to occur by a PHD domain-phosphoinositide interaction (73). However, clear binding preferences or consistently strong binding affinities between PHD containing proteins and specific phosphoinositides has not been observed by other groups (14), and further experiments will be needed to confirm these observations.

Proteins can also be regulated by posttranslational modifications such as phosphorylation, acetylation, and ubiquitinylation. The p33<sup>ING1b</sup> splice variant was indeed found to be phosphorylated on serine 126 by CDK1 under non-stressed conditions, and similarly by CHK1 under DNA-damaging conditions (58). As CHK1 is normally activated by ATM/ATR kinases, these findings suggest that p33<sup>ING1b</sup> might be a downstream target of ATM/ATR-CHK1 signaling following UV damage. In addition, phosphorylation of serine 126 was found to alter the half-life but not the subcellular localization of p33<sup>ING1b</sup> (58), and could alter the expression of cyclin B1 leading to altered cellular proliferation.

## 1.7 ING Genes as Tumor Suppressors

The reported involvement of the ING proteins in numerous essential biological processes predicts that ING proteins have important implications in human disease. Although analysis of ING gene mutation status, expression levels, and protein localization in a variety of primary tumors and cell lines revealed that ING genes are infrequently mutated, they often display reduced gene expression or mislocalization of proteins in cancerous cells. These findings place the ING genes in the category of type II tumor suppressors. Additionally, some reports have found a correlation between decreased expression levels of ING genes and cancer progression or a correlation between ING gene expression levels and decreased survival rates for a number of cancer types (see below). Various mechanisms have been proposed to inactivate ING gene functions in tumors, including mutations arising from gene rearrangements, loss of heterozygosity (LOH), and promoter CpG hypermethylation, and altered protein localization (23, 70, 149). A summary of reports of ING gene mutations in cancers is compiled in Table 1.2.

**ING1 in human cancers:** Several groups have looked at the expression and mutational status of ING genes in a variety of primary tumors and cell lines (see Table 1.2 for references). ING1 expression appeared to be decreased in head and neck squamous cell carcinoma (HNSCC), esophageal squamous cell carcinoma (ESCC), acute lymphoblastic lymphoma (ALL), breast cancer, transitional cell carcinoma of the bladder, hepatocarcinoma (HCC), ovarian cancer, cancers of the central nervous system,

non-small cell lung cancer (NSCLC), pancreatic cancer, and colorectal cancer. In support of a role for ING1 in tumor suppression, loss of heterozygosity (LOH) for ING1 has been occasionally seen in colorectal cancers, pancreatic cancers, HNSCC, and in breast cancers.

Table 1.2: Alterations and mutations of ING genes in human cancers

Gene	Tumor Type	Tumors	Lines	%Mutated	Mutation type	Position	Methods	Refs	
ING1	Melanoma		14	100	↑ expression, 9 silent, 1 missense N260S	N-terminus (exon 1a)	WB, RT-PCR	(21)	
	Melanoma <sup>1</sup>	46		20	2 missense: N260S, R102L	C-terminus, PHD	SSCP	(24)	
	Basal cell carcinoma	54		25	↑ expression		IHC, SSCP	(28)	
	Basal cell carcinoma			2	1 missense	PHD (exon 2)			
	HNSCC <sup>1</sup> HNSCC <sup>1</sup>	55		68	↓ expression 3 missense (C215S, D216S, A192D)	C-terminus, PHD, NLS	SSCP	(78)	
	ESCC ESCC	31		55	↓ expression 2 missense (V214I, G270V)	PHD	IHC	(29)	
	Myeloid Leukemia	10	3	0	no change			(96)	
	Melanoma	83	55	0	no change		SSCP	(204)	
	Childhood ALL <sup>3</sup>	145		78	Loss of nuclear staining		IHC	(151)	
	Mantle cell lymphoma	14			candidate gene		CGH-Array	(183)	
	Mantle cell lymphoma			5	60	↓ expression		Microarray	(176)
	T-cell ALL			11	46	↓ expression, Loss of nuclear staining		IHC	(155)
	B-cell ALL			5	80	↓ expression, Loss of nuclear staining		IHC	(155)
	Breast cancer <sup>1</sup>	86		80	80	↓ expression		IHC	(148)
	Breast cancer <sup>1</sup>	377	10	44	44	↓ expression		RT-PCR, ISH, WB	(213)
						Missense P95S 3 silent codons 188, 166, 228	N-terminus C-terminus, PHD, NLS		(213)
	Ovarian	65			71	↓ expression		qRT-PCR	(211)
	Breast cancer					↓ expression		IHC	(180)
	Bladder cancer TCC <sup>1</sup>	188	4	55	55	↓ expression		IHC	(154)
	HCC <sup>1</sup>	86		7	7	Transversion C215S	PHD	IHC, SSCP	(245)
	HCC	57		25	25	↓ expression, LOH		IHC	(188)
	Ovarian <sup>2</sup>	111				↓ expression			(207)
	CNS	100				↓ expression			(219)
	Glioblastoma <sup>1</sup>	29	6	100	100	↑ cytoplasmic staining		RT-PCR, WB, ISH, IHC	(121)
	NSCLC	88		3	42	Mutation ↓ expression	NLS	SSCP, RT-PCR	(103)
	Lung cancer	31	30	2	23	missense ↑ expression		SSCP, qRT-PCR	(103) (156)
	Colorectal cancer	35		11	11	↓ expression, LOH			(30)
	Pancreatic cancer	40		61	61	LOH			(236)
	Glioblastoma	25	25	3	3	Missense C215S	PHD	Array CGH	(236) (177)
	ING2	Lung cancer	31	30	86	86	↓ expression		SSCP, RT-PCR
Melanoma		79				↓ expression		IHC, microarray	(133)
HCC <sup>1</sup>		84		52	52	↓ expression		RT-PCR, WB	(240)
Various tumor cell lines				42	42	↓ expression		WB	(144)
ING3	HNSCC	49		13	48	↑ expression ↓ expression, LOH			(79) (79)
				6	6	Silent			(79)
	Head and Neck <sup>1</sup>	71		50	50	Missense D20G	N-terminus	qPCR	(79)
	Melanoma	114		67	67	↓ expression		Microarray, IHC	(225)
	Metastatic Melanoma	50		90	90	↓ expression		Microarray, IHC	(225)
ING4	Glioblastoma <sup>1</sup>	50		100	100	↓ expression		qRT-PCR	(63)
	HNSCC	50		76	76	↓ expression		qRT-PCR	(77)
		50		66	66	LOH		qRT-PCR	(77)
	Myeloma	50	8	100	100	↓ expression		qRT-PCR	(40)
	Metastatic Melanoma <sup>1</sup>		101			↓ expression		Microarray, IHC	(125)
Breast cancer					LOH			(110)	
ING5	Oral cancer	39		85	85	LOH, candidate gene			(27)

**Table 1.2:** Alterations in expression and the mutations found in human cancers for each ING gene. The number of samples; either primary tumors or cell lines, analyzed are depicted. Percentage is based on the number of samples harboring a mutation in an ING gene. Also shown is the position in the ING protein of the mutation and the method used to determine the alternation in the ING gene.

- 1: Associated with shortened survival times and advanced clinical stage.
- 2: MSP determined expression due to DNA methylation.
- 3: Better survival if nuclear staining for p33 is negative.

Abbreviations used: WB, western blot; IHC, Immunohistochemistry; ISH, In situ hybridization; qRT-PCR, quantitative real time polymerase chain reaction; SSCP, single stranded confirmation polymorphism PCR; and CGH-array, comparative genomic hybridization array; MSP, methylation-specific PCR; ESCC, esophageal squamous cell carcinoma; HNSCC, head and neck squamous cell carcinoma; ALL, acute lymphocytic lymphoma; TCC, transitional cell carcinoma; HCC, hepatocellular carcinoma.

Further evidence for a role for ING1 in tumor suppression was provided by reports showing that p33<sup>ING1b</sup> expression was greater in lower grade brain tumors than in high grade tumors, indicating a role for ING1 in malignant progression of astrocytomas (207). Another report on invasive carcinoma of the breast showed that reduced nuclear expression of p33<sup>ING1b</sup> correlated with larger, less differentiated and more malignant tumors that were estrogen receptor and progesterone receptor negative (148). Interestingly this study also found that most of these tumors had also lost functional p53. ING1 has also been proposed as a candidate tumor suppressor gene in mantle cell lymphoma (MCL), as expression of ING1 was decreased in 3 of 5-tested MCL cell lines (176, 183). However, these findings have not been extended to primary tumor samples.

Studies of primary ovarian tumors using methylation-specific PCR found that the p33<sup>ING1b</sup> promoter was methylated and silenced in almost a quarter of all cases (188). This was confirmed using bisulfite sequencing and statistically correlated with decreased mRNA expression. Additionally, treatment of ovarian cancer cell lines with 5'-aza-2'-deoxycytidine, a demethylating drug, caused a dosage dependent increase in ING1 expression. However, a different report utilizing cell lines and the COBRA method to detect promoter CpG methylation status found no evidence for ING1 promoter methylation in mantle cell lymphoma (176). Further analysis of primary tumors, ING expression levels, and promoter methylation is needed to determine the relevance of promoter inactivation for loss of ING function in human cancer.



In contrast, no differences in ING expression levels were observed in recent studies of myeloid leukemia (96), or of melanoma (204), contradicting an earlier report of increased p33<sup>ING1b</sup> expression in this cancer (21). However, the link between mutations in ING1 and development of melanoma is strengthened by a report identifying ING1 mutations in primary tumor samples. Two p33<sup>ING1b</sup> PHD domain mutations (R102L and N260S) were detected in 20% of tested melanomas (204), and either of these alterations proved as detrimental as deletion of the entire PHD domain to the enhancement of nucleotide excision repair mediated by p33<sup>ING1b</sup> in host-cell-reactivation assays and radioimmunoassays. Furthermore, those patients bearing an ING1 codon 102 or 260 mutation had a reduced 5-year survival rate (50% versus 82%) (24). These findings highlight the importance of the PHD domain in ING1 function and possibly in tumor suppression, as loss of NER activity would likely facilitate tumorigenesis. In agreement with this proposal, other reports have indicated the presence of mutations within the PHD motif or the NLS of ING1 in melanoma, HNSCC, ESCC, breast cancer, pancreatic cancer, and in colon cancer (245).

Surprisingly, increased expression of *ING1* was also observed during a study of glioblastomas. However, this overexpression resulted in the mislocalization (and presumed inactivation) of ING1 in these samples (219). Supporting evidence was obtained in a recent study comparing normal oral mucosa to oral squamous cell carcinoma (OSCC) finding that p33<sup>ING1b</sup> was either lost or mislocalized to the cytoplasm in the tumor cells (241).

**Other ING proteins in human cancer:** Although a majority of the analysis of ING proteins in human cancer has centered on ING1, more recent work has begun to examine the links between ING2-ING5 in tumorigenesis (see Table 1.2). Decreased ING2 expression (but not ING2 mutation) has also been observed in lung cancer, melanoma, and in colon cancer (133, 156, 197). ING2 may have a role in melanoma initiation, since reduction of nuclear ING2 expression has been reported in radial growth phase, vertical growth phase, and metastatic melanoma compared with dysplastic nevi (133). However, the reduced expression was not associated with tumor stage, subtype, or 5 year survival rate (133). In contrast, reduced ING2 expression was found to be associated with tumor progression and shortened survival time in hepatocellular carcinoma (240). These epidemiological studies suggest that ING2 loss or reduction may be important for tumor initiation and/or progression (133, 240).

Similar findings were also seen for ING3 in cutaneous melanoma, with reduced ING3 expression observed in malignant melanoma compared with dysplastic nevi (225). As with ING2, decreased nuclear ING3 was associated with poorer 5 year survival rates (225). Survival rates were 93% for strong nuclear ING3 staining and dropped to 44% for patients with negative to moderate nuclear staining. ING3 expression has also been associated with poor overall survival and tumor initiation in head and neck cancers (76), and a missense mutation in ING3 codon 20 has been observed in HNSCC (79).

ING4 also displays reduced expression levels in glioblastoma (63), HNSCC (77), and myeloma (40), and LOH of the ING4 locus has been observed in HNSCC (77) and breast cancer (110). ING4 has also been associated with tumor progression in glioblastomas (63) and myeloma (40). In both cases, decreased ING4 expression was associated with higher tumor grade and increased tumor angiogenesis. In the case of myeloma it was also associated with increased expression of interleukin-8 and osteopontin (40). Expression of ING4 was also found to be reduced in malignant melanoma compared to dysplastic nevi and was found to be an independent factor for the poor prognosis of these patients (125).

Currently the only report linking ING5 to cancer used 16 microsatellite markers to report LOH on the long arm of chromosome 2 in samples from patients with oral cancer (27). This report needs to be corroborated with further studies.

In summary, a number of epidemiologic studies have implicated alterations in ING expression to the development of a variety of cancers. However, LOH or specific point mutations within ING genes are more rarely observed, and ING mutation or loss is not proposed as the molecular basis of a familial cancer syndrome. Thus, although ING genes have been demonstrated *in vitro* and in cell culture assays to impact a number of processes that regulate cell cycling, cell transformation, and apoptosis, definitive evidence that ING proteins function *in vivo* to regulate cell growth or to suppress tumor formation is lacking.

## 1.8 Aims of this Dissertation

Multiple lines of evidence generated from *in vitro* experiments that employ transformed cell lines and overexpression or antisense knockout of the ING genes indicate that ING proteins are involved in a variety important biological processes, many of which are known to be altered in cancer. These findings have indicated that ING proteins function as tumor suppressors, and have further implicated ING proteins as key regulators of several different signaling pathways, including p53 and NF- $\kappa$ B. These considerations warrant the development of genetically modified mice bearing alterations in ING genes to further explore the role of the ING proteins in development and in the regulation of normal and neoplastic cell growth. Such mouse models might also prove useful in future preclinical analysis of diagnostic tests and to assess therapeutic options for treating cancer. Therefore, the main aims of this dissertation were to generate and characterize mice deficient for either *Ing1* or *Ing4*, to analyze the effects of *Ing* deletion on spontaneous tumorigenesis in these mice, and to explore the role of *Ing1* and *Ing4* proteins in regulating the p53 and NF- $\kappa$ B signaling pathways.

**CHAPTER II:**

**DELETION OF p37<sup>Ing1b</sup> IN MICE REVEALS A p53-INDEPENDENT  
ROLE FOR Ing1 IN SUPPRESSION OF CELL PROLIFERATION,  
APOPTOSIS, AND TUMORIGENESIS.**

## Forward

ING proteins have been proposed to alter chromatin structure and gene transcription to regulate numerous aspects of cell physiology, including cell growth, senescence, stress-response, apoptosis, and transformation. *ING1*, the founding member of the Inhibitor of Cell Growth family, encodes p37<sup>Ing1</sup>, a PHD protein that interacts with the p53 tumor suppressor protein and appears to be a critical cofactor in p53-mediated regulation of cell growth and apoptosis. In this study, we have generated and analyzed p37<sup>Ing1</sup>-deficient mice and primary cells in order to further explore the role of Ing1 in regulation of cell growth and p53 activity. The results demonstrate that endogenous levels of p37<sup>Ing1</sup> inhibit the proliferation of p53-wildtype and p53-deficient fibroblasts, and that p53 functions are unperturbed in p37<sup>Ing1</sup>-deficient cells. In addition, loss of p37<sup>Ing1</sup> induces *Bax* expression and increases DNA damage-induced apoptosis in primary cells and mice irrespective of p53 status. Finally, p37<sup>Ing1</sup> suppresses the formation of spontaneous follicular B-cell lymphomas in mice. These results indicate that p53 does not require p37<sup>Ing1</sup> in order to negatively regulate cell growth and offers genetic proof that Ing1 suppresses cell growth and tumorigenesis. Furthermore, these data reveal that p37<sup>Ing1</sup> can negatively regulate cell growth and apoptosis in a p53-independent manner.

## Introduction

ING1 was initially isolated in a screen to identify genes that were underexpressed in transformed versus normal human epithelium (62). Subsequently, ING1 was identified as a member of a 5-gene ING family that encode PHD-finger domain proteins conserved in yeast and vertebrates (22). Forced overexpression of ING1 in normal human diploid fibroblast cells was found to inhibit cell growth by inducing a G1-phase cell cycle arrest. Conversely, downregulation of ING1 in cell lines by antisense RNA increased cell proliferation and cell transformation, as assayed by colony formation in soft agar (62, 67), and inhibited Myc-induced apoptosis (89). The ability of ING1 to inhibit cell growth and promote apoptosis in these various assays indicated that ING1 might function as a tumor suppressor.

The murine *Ing1* gene produces several spliced isoforms of *Ing1* mRNA that generate two distinct *Ing1* proteins (238). The larger 37Kd protein (p37<sup>Ing1</sup>) contains all of the residues present in the smaller 31Kd (p31<sup>Ing1</sup>) protein, as well as additional sequences at the amino terminus that interact with the p53 tumor suppressor protein (238). Both mouse p37<sup>Ing1</sup> and the human ortholog p33<sup>ING1</sup> have been shown to co-immunoprecipitate with p53 in transfected cells (60, 238). Furthermore, p33<sup>ING1</sup> has been reported to stabilize p53 levels in transfected cells by binding with p53 and blocking Mdm2-p53 interactions and by inhibiting hSir2 deacetylation of p53 (60, 106, 123).

Functional data linking Ing1 with regulation of p53 activity was provided by transfection studies in which forced overexpression of ING1 in cell lines was found to both increase cell sensitivity to double strand DNA breaks in a p53-dependent manner (32), and induce the expression of certain p53 target genes such as p21 following DNA damage (106). In addition, the ability of exogenous p53 to inhibit cell growth in Balb/c 3T3 cells appears to be compromised in cells partially depleted for *ING1* by antisense RNA (60).

These cell-based studies suggest that the negative effects of Ing1 on cell growth are mediated through p53. Furthermore, mutation of ING1 has also been detected in human primary tumors and in tumor derived cell lines, further underscoring the possibility that ING1 functions as a tumor suppressor (22, 150). In support of this model, mice deleted for Ing1 via gene targeting experiments in ES cells were recently found to be smaller in size, have reduced viability following whole body irradiation, and display a slight increase in the rate of spontaneous tumor formation relative to wildtype mice, particularly in lymphomagenesis (108). However, these Ing1-deficient mouse primary fibroblasts displayed little or no differences in replicative lifespan or in sensitivity to various forms of genotoxic stress. Thus, the link between Ing1, p53 activity, and tumor suppression remains unclear.

In order to determine if Ing1 regulates p53 functions in cell growth and in tumorigenesis, we generated  $p37^{Ing1}$ -deficient mice by utilizing mouse ES cells bearing a gene trap mutation that specifically ablates  $p37^{Ing1}$ .



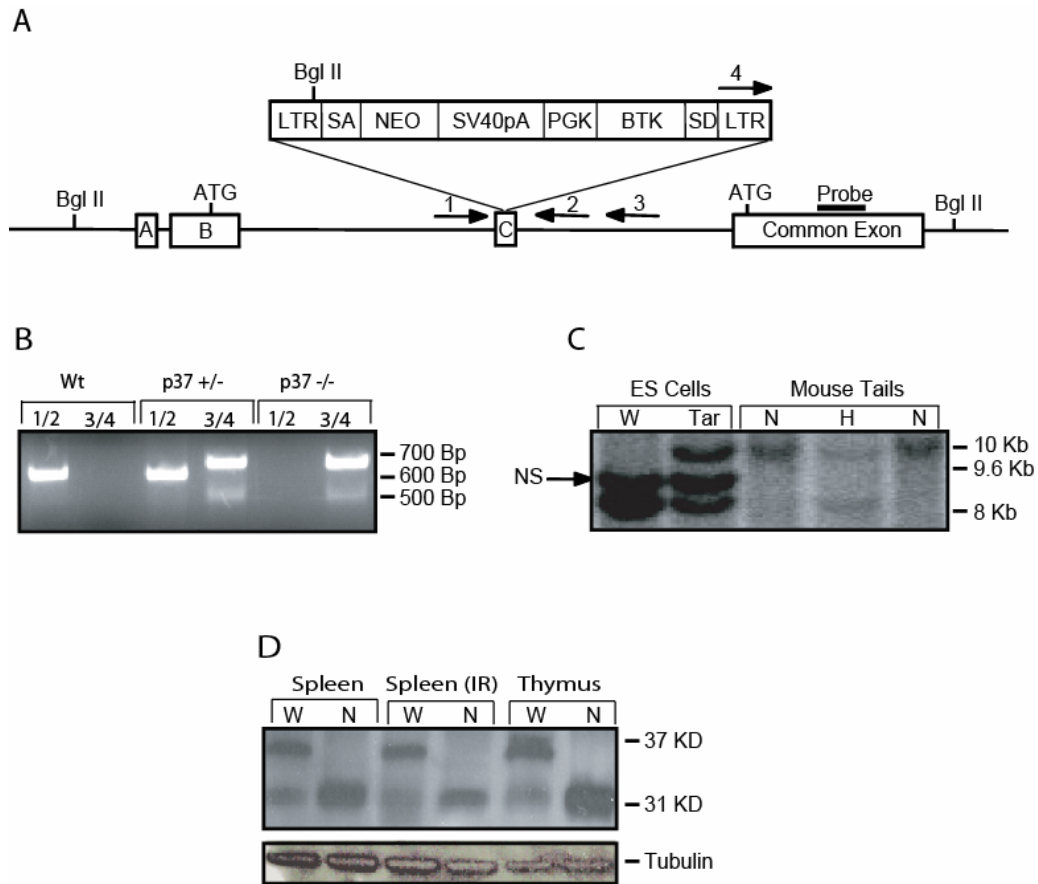
Analysis of  $p37^{\text{Ingl}}$  deficient mice and mouse embryonic fibroblasts (MEFs) revealed that loss of  $p37^{\text{Ingl}}$  increased the growth rate of MEFs, providing direct genetic evidence in primary cells that the endogenous level of *Ingl* negatively regulate cell proliferation. However, deletion of  $p37^{\text{Ingl}}$  also increased the proliferation of p53-deficient MEFs, demonstrating a p53-independent role for  $p37^{\text{Ingl}}$  in control of cell growth. Furthermore, loss of  $p37^{\text{Ingl}}$  failed to alter numerous other cell characteristics normally governed by p53, including the rate of primary cell immortalization, oncogene-induced cell senescence, or cell growth arrest following DNA damage by known inducers of p53 activity. In addition,  $p37^{\text{Ingl}}$  deletion did not affect the p53-induced embryonic lethality of *Mdm2*-null mice. These data indicate that p53 remains fully functional in cells lacking  $p37^{\text{Ingl}}$ , indicating that  $p37^{\text{Ingl}}$  downregulates cell growth in a p53-independent manner.

Although loss of  $p37^{\text{Ingl}}$  did not alter the levels of the pro-apoptotic, p53 response gene *Puma*,  $p37^{\text{Ingl}}$  deletion was found to significantly increase *Bax* gene expression and promote apoptosis following DNA damage in thymocytes irrespective of p53 status, demonstrating that  $p37^{\text{Ingl}}$  also has a p53-independent, pro-survival role in apoptosis. While DNA damage-induced apoptosis was upregulated in  $p37^{\text{Ingl}}$ -deficient primary cells, mice lacking  $p37^{\text{Ingl}}$  developed spontaneous follicular B cell lymphomas, indicating that  $p37^{\text{Ingl}}$  functions as a tumor suppressor in vivo.

## Results

### *Generation of p37<sup>Ing1</sup>-deficient mice*

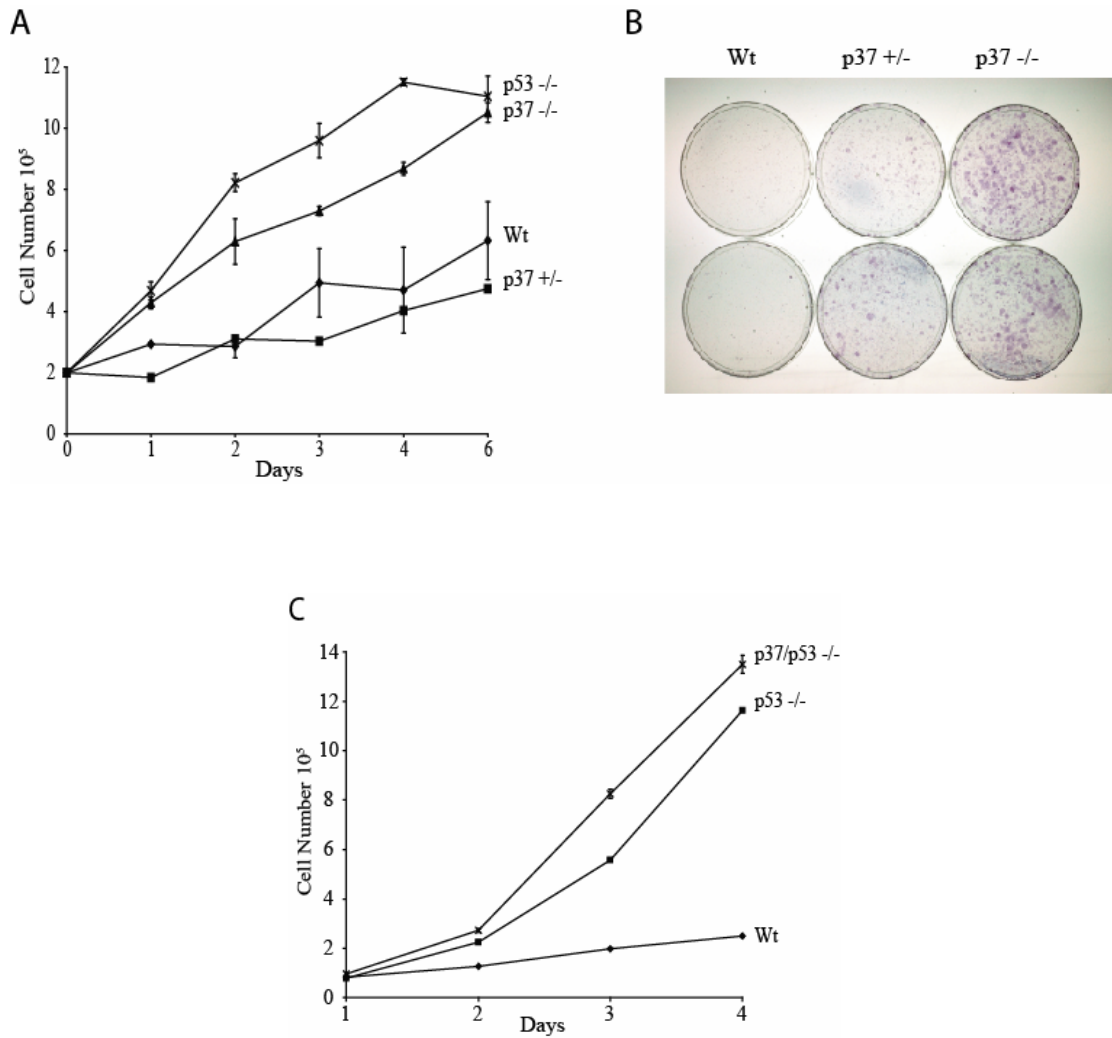
A mouse embryonic stem (ES) cell line bearing a retroviral promoter trap inserted into one allele of the *Ing1* gene was used in standard blastocyst injection experiments to generate chimeric mice (237). DNA sequence analysis of the gene-trapped locus revealed that the retrovirus inserted into exon C, the third exon of *Ing1* (238) thereby interrupting coding of the longer, *Ing1b* transcript that encodes p37<sup>Ing1</sup> (Figure 2.1A). Several high-degree chimeric mice were bred with C57Bl/6 mice to generate agouti offspring, and genomic DNA was harvested from tail biopsies and analyzed by Southern blot hybridization (Figure 2.1B) and PCR (Figure 2.1C) to identify mice that inherited the *Ing1*-targeted allele. Heterozygous F1 generation mice were intercrossed to obtain mice homozygous for the mutant *Ing1* allele, which were recovered with normal Mendelian frequency. Similar to a recent report of mice mutated at the *Ing1* locus (108), homozygous mutant mice weighed approximately 15% less at weaning but were otherwise indistinguishable from the *Ing1*-heterozygous and wildtype littermates. Western analysis of *Ing1* proteins harvested from the spleen and thymus of wildtype and homozygous mutant mice revealed that the homozygous mutant mice retained expression of the p31<sup>Ing1</sup> protein in the presence (4 Gy whole body) or absence of DNA damage, but displayed loss of p37<sup>Ing1</sup> protein (Figure 2.1D). Thus, retroviral insertion into the *Ing1* locus in the ES cells resulted in a p37<sup>Ing1</sup>-specific knockout allele.



**Figure 2.1:** Generation of p37<sup>Ing1b</sup>-deficient mice. **A)** Schematic of the gene-trapped locus showing the gene-trap inserted into exon C. The trap interrupts the Ing1b message isoform and corresponding p37 protein, but encodes the shorter Ing1c isoform and p31 protein. **B)** PCR genotyping of the ES cells and mouse tail biopsies showing the presence of the gene-trap. A 650bp fragment is generated from wildtype Ing1 using primers 1 and 2 and a 700bp fragment is generated from the targeted Ing1 allele using primers 3 and 4. **C)** A Southern blot strategy using a Bgl II digest and a probe to the common exon was used to confirm germ-line transmission of the gene-trap. The wild-type fragment is ~8kb and the mutant fragment is ~10kb in length. A non-specific (NS) band corresponding to an Ing1 pseudogene present in 129-strain mouse DNA is also observed. **D)** To confirm that the longer p37<sup>Ing1</sup> form was specifically deleted, a western blot was performed using an Ing1 antibody that recognizes sequences encoded in the common exon. The presence of the shorter form (p31) and the absence of the longer form (p37) were observed. Tubulin was used as a loading control. Abbreviations used: wt (W), heterozygous (H), null (N), kilodalton (KD), and Tar refers to the targeted ES cells.

### ***Negative regulation of cell growth by p37<sup>Ing1</sup>***

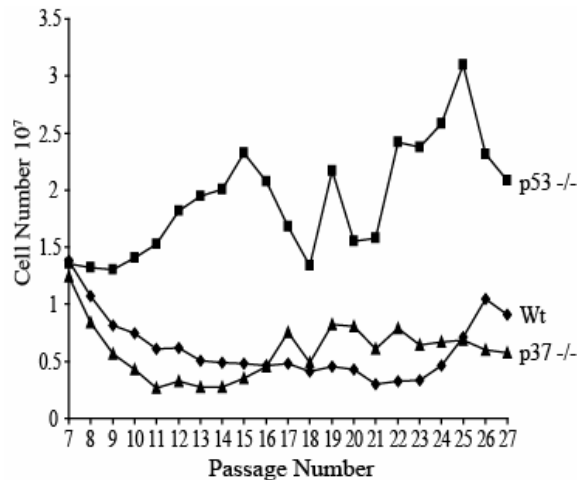
We generated primary fibroblasts from wildtype, p37<sup>Ing1</sup> heterozygous, and p37<sup>Ing1</sup> homozygous-deficient embryos to characterize and compare the growth rates of these cells with p53-null MEFs (Figure 2.2A). The results indicate that the proliferation rate of p37<sup>Ing1</sup>-heterozygous MEFs is similar to wildtype MEFs. However, p37<sup>Ing1</sup>-null MEFs proliferate significantly faster than wildtype MEFs, though not as fast as MEFs lacking p53. Although p37<sup>Ing1</sup>-null MEFs displayed wildtype plating efficiencies under normal conditions, these cells showed increased survival and growth relative to wildtype MEFs when plated at low density (Figure 2.2B), similar to results obtained with p53-null MEFs (86). In order to determine if the downregulation of cell proliferation by p37<sup>Ing1</sup> is mediated through p53, we bred the p37<sup>Ing1</sup> deficient mice with p53-null mice (47) and generated p37<sup>Ing1</sup>/p53- null MEFs. Analysis of the growth rate of these double-null cells revealed that deletion of p37<sup>Ing1</sup> enhanced the growth of p53-deficient primary fibroblasts (Figure 2.2C), indicating that the increased proliferation rate of p37<sup>Ing1</sup>-null MEFs was not due to alteration of p53 activity in these cells.



**Figure 2.2:** Growth regulation by p37<sup>Ingl1</sup>. **A)** Proliferation of p37<sup>Ingl1</sup>- deficient cells. Two independent MEF cell lines of p37<sup>Ingl1</sup>-wildtype, p37<sup>Ingl1</sup>-heterozygous, p37<sup>Ingl1</sup>-null, or p53-null genotypes were plated in triplicate in 60mm plates and the growth analyzed over the course of 7 days. Statistical analysis between Wt and p37<sup>Ingl1</sup>-heterozygous curves shows no significant difference ( $p < 1$ ), but there is statistical difference between the Wt and p37<sup>Ingl1</sup>-null curves ( $p < 0.005$ ). **B)** Growth of p37<sup>Ingl1</sup>- deficient cells at low density. Two lines of either wildtype, p37<sup>Ingl1</sup>-heterozygous, or homozygous genotype were seeded at  $10^4$  cells per 10cm plate and incubated for 8-12 days before being stained with crystal violet. **C)** Growth inhibition by p37<sup>Ingl1</sup> is independent of p53 status. Three MEF cell lines deficient for p53 or for p53 and p37<sup>Ingl1</sup> were plated in triplicate in 6-well plates and counted over a 7-day period. Wildtype MEFs were also similarly plated as a control. A statistical difference exists between the proliferation rate of p53-null and p53/p37<sup>Ingl1</sup>-double null MEFs ( $p < 0.0005$ ).

### *Immortalization of p37<sup>Ingl1</sup> null MEFs*

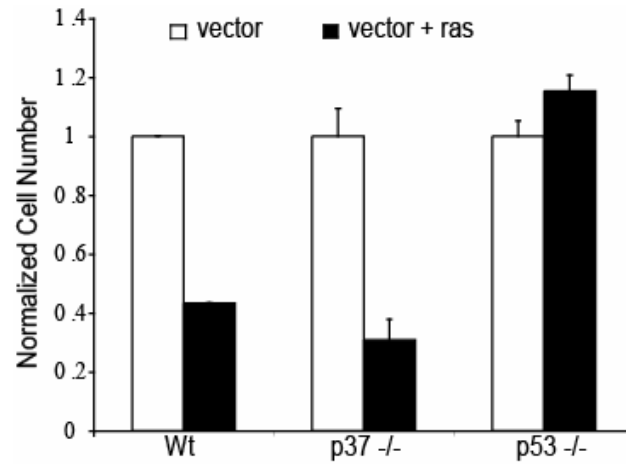
Spontaneous immortalization of MEFs is dependent, in part, on p53 functions. To analyze further the role of p37<sup>Ingl1</sup> in cell growth, we performed a modified 3T3 (3T9) cell immortalization assay. MEFs were generated from wildtype, p37<sup>Ingl1</sup>-null, or p53-null embryos and passed continuously in culture following a standard protocol (86). In agreement with our previous reports (99, 205), p53-null MEFs displayed a rapid rate of cell growth throughout the course of the assay relative to wildtype MEFs (Figure 2.3). In contrast, p37<sup>Ingl1</sup> deficient MEFs continued to divide more slowly than p53-null MEFs, and never exhibited robust induction of cell growth. Thus, unlike deletion of p53, loss of p37<sup>Ingl1</sup> does not alter the immortalization rate of primary cultured cells.



**Figure 2.3:** Immortalization of p37<sup>Ingl1</sup>- deficient cells. A 3T9 assay was performed using two independent MEF cell lines of p37<sup>Ingl1</sup>-wildtype, p37<sup>Ingl1</sup>-null, or p53-null MEFs.

***Ras-induced cell senescence in p37<sup>Ingl</sup> deficient MEFs***

Oncogene-induced stress induces a p53-dependent, replicative senescent phenotype in MEFs (57). To determine if forced expression of an activated *ras* oncogene induces senescence in p37<sup>Ingl</sup>-deficient fibroblasts, MEFs that were either wildtype, p37<sup>Ingl</sup>-null, or p53-null MEFs were infected with pBABE retrovirus (vector) or with pBABE bearing an activated H-ras gene (vector + ras). After puromycin selection for 72 hours, the transduced cells were plated at equal densities and scored for cell growth at day 6 post-infection. Vector alone (no *ras*) had little effect of the growth of MEFs regardless of genotype (Figure 2.4). As expected, exogenous *ras* suppressed the growth of wildtype MEFs whereas the growth of p53-null MEFs was not affected by activated *Ras* expression. Similar to wildtype MEFs, the growth of p37<sup>Ingl</sup>-null MEFs was suppressed following oncogene transduction. These data indicate that p53-mediated cell senescence induced by inappropriate oncogene expression in MEFs is unaffected by the presence or absence of p37<sup>Ingl</sup>.

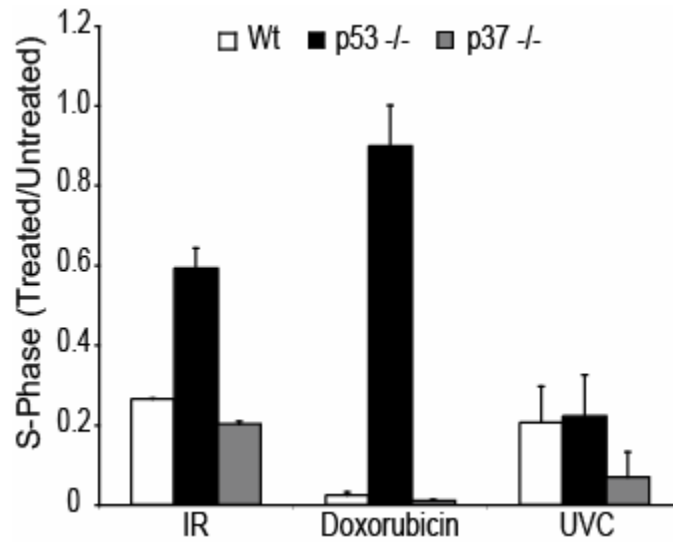


**Figure 2.4:** *Ras*-induced senescence in p37<sup>Ingl1</sup>- deficient cells is unchanged. Triplicate experiments of multiple lines each of wild-type, p37<sup>Ingl1</sup>-null, or p53-null MEFs were transduced with recombinant virus with or without H-*ras*, and cells were plated at equal densities and counted. Graph represents the ratio of cell number after 6 days in culture versus the cell number at initial plating.



***Response of p37<sup>Ingl1</sup> deficient MEFs to DNA damage***

Induction of double-stranded DNA breaks in MEFs by treatment with ionizing radiation (IR) or doxorubicin induces stabilization of p53 and subsequent inhibition of cell growth. In order to determine if loss of p37<sup>Ingl1</sup> alters the ability of p53 to arrest cells following DNA damage, wildtype, p37<sup>Ingl1</sup>-null, or p53-null MEFs were synchronized in their cycling and mock-treated or treated with either 8Gy IR or 0.3 ug/ml doxorubicin. The results of this experiment (Figure 2.5) are expressed as the ratio of treated cells in S phase to untreated cells in S phase. As expected, wildtype MEFs treated with either IR or doxorubicin had a large reduction in the numbers of cells present in the S phase of the cell cycle, whereas p53-null MEFs displayed far less cell growth inhibition following treatment with IR or doxorubicin. However, MEFs lacking p37<sup>Ingl1</sup> were indistinguishable from wildtype MEFs in their response to these DNA damaging agents, indicating that the ability of p53 to induce cell growth arrest in response to DNA double-strand breaks is unaltered in p37<sup>Ingl1</sup>-null MEFs. In addition, wildtype, p37<sup>Ingl1</sup>-null, or p53-null MEFs were treated with 60 Joules/m<sup>2</sup> UVC and assayed for a damage-induced reduction in S phase. Although UVC treatment strongly inhibits cell growth in wildtype MEFs, this effect requires functional JNK signaling and is not p53-dependent in MEFs (6, 212). Similar to wildtype and p53-deficient MEFs, cells lacking p37<sup>Ingl1</sup> were also growth-inhibited by UVC treatment.



**Figure 2.5:** Cell cycle arrest is normal in p37<sup>Ingl</sup>-deficient MEFs. Absence of p37<sup>Ingl</sup> does not alter cell cycle arrest due to DNA damaging agents. Three lines each of wt, p37<sup>Ingl</sup>-null, or p53-null MEFs were plated in duplicate and synchronized in their growth prior to mock-treatment or treatment with doxorubicin, IR, or UVC. Cells were stained with BrdU and PI, and analyzed by FACS. Results are expressed as a ratio of treated cells in S-phase versus untreated cells in S-phase.

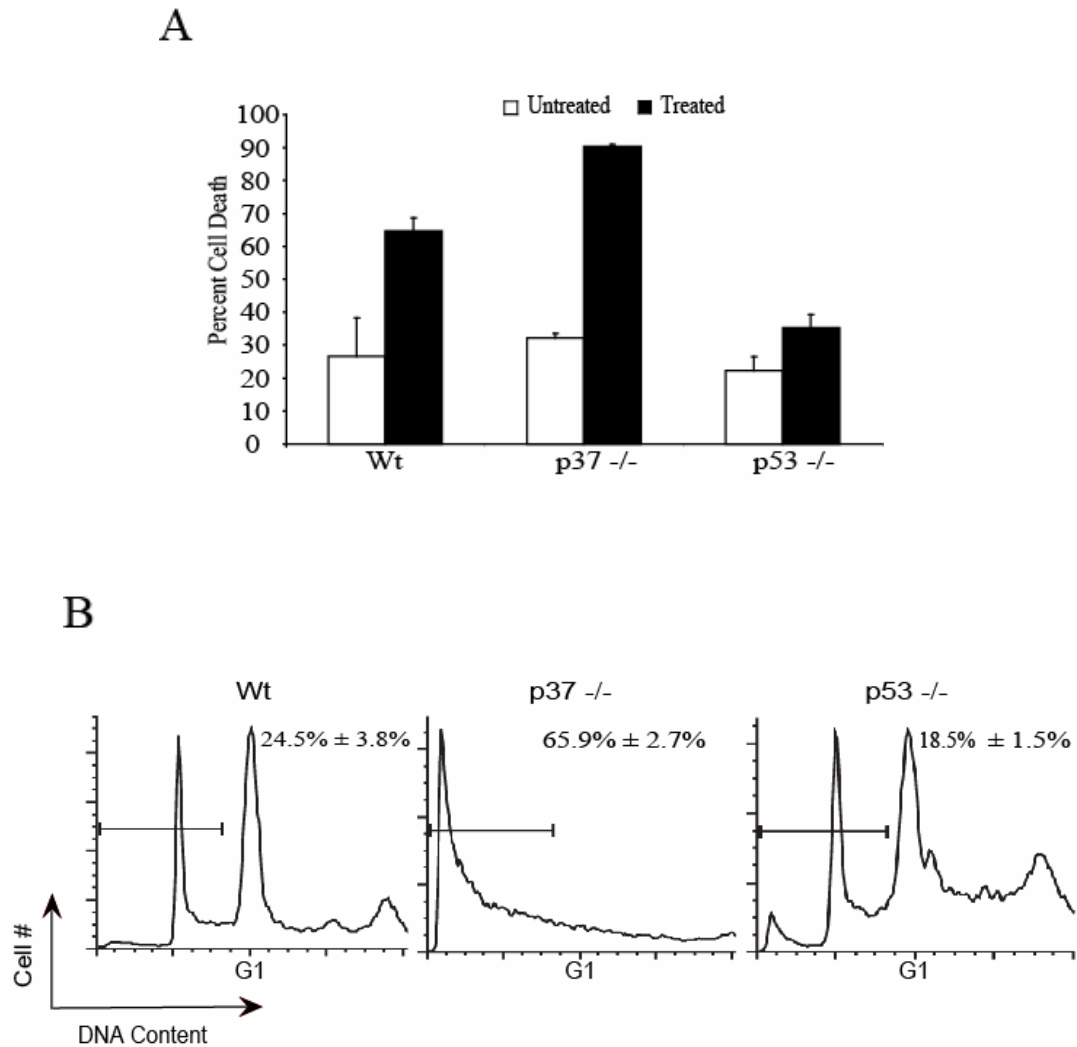
***Deletion of p37<sup>Ing1</sup> does not alter p53-induced lethality in Mdm2-null mice***

Mdm2 is a well-established regulator of p53 activity and p53 stability in cells (10, 113), and mice deleted for Mdm2 display an early embryonic lethal phenotype that is rescued by deletion of p53 (98). Recently, the amino terminus of p33<sup>ING1</sup>, the human orthologue of p37<sup>Ing1</sup>, has been found to complex with the p19-ARF tumor suppressor (119). In cells, p19-ARF regulates p53 activity by binding with Mdm2 and inhibiting Mdm2-ubiquitination of p53 (132). Interaction of p19-ARF and p33<sup>ING1</sup> suggests that Ing1 may play a role in p19-Mdm2-p53 signaling, and Ing1 has been previously proposed to alter p53 stability possibly by interfering with Mdm2-p53 interactions (123).

In order to explore whether deletion of p37<sup>Ing1</sup> alters p53 activity and Mdm2-p53 signaling during development, we bred p37<sup>Ing1</sup>-null mice with mice bearing a mutated Mdm2 allele (98). Intercrosses of the resulting Mdm2+/-, p37<sup>Ing1</sup>-null mice were performed, and DNA isolated from the offspring (n= 40) of these matings were genotyped to determine Mdm2 status. This experiment failed to generate any Mdm2-null mice (expected n= 10), indicating that the embryonic lethality induced by p53 in the absence of Mdm2 was not dependent upon p37<sup>Ing1</sup> function. This result is in keeping with our finding that p37<sup>Ing1</sup>-null MEFs are not compromised in p53-mediated cell growth arrest following DNA damage.

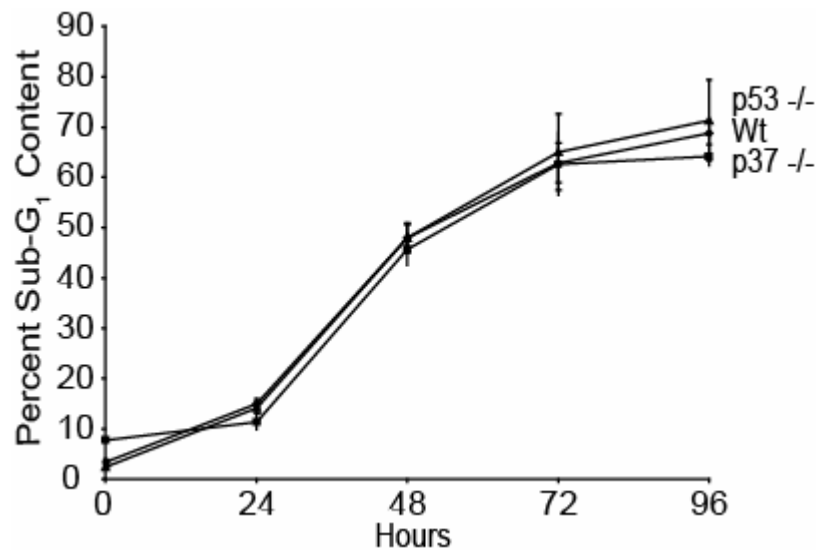
### ***Anti-apoptotic role of p37<sup>Ingl</sup> in DNA damage response***

MEFs transduced with adenovirus *E1A* undergo p53-induced apoptosis following treatment with doxorubicin (6). To determine if p53 apoptosis was compromised in p37<sup>Ingl</sup>-null cells, MEFs that were wildtype, p53-null or p37<sup>Ingl</sup>-null were infected with a recombinant retrovirus encoding both the adenovirus E1A gene and a puromycin resistance gene. Following a two-day selection in puromycin, E1A-transduced MEFs were recovered, plated in triplicate at equal cell density, and mock-treated or treated with 0.25 µg/ml doxorubicin for 24 hours. Cell viability was scored by trypan blue exclusion (Figure 2.6A). As expected, wildtype MEFs displayed reduced cell viability upon treatment with doxorubicin ( $35.3 \pm 4\%$  viable cells), whereas p53-null MEFs were much less susceptible to treatment with this DNA damaging agent ( $64.7 \pm 4\%$  cell viability). Surprisingly, p37<sup>Ingl</sup>-null MEFs showed far greater sensitivity to doxorubicin-induced death ( $9.7 \pm 1\%$  viable cells) than wildtype cells (Figure 2.6A). To confirm that cell death was due to apoptosis, the experiment was repeated and the percentage of cells undergoing apoptosis was determined by FACS analysis of the sub-G1 DNA content for each genotype (Figure 2.6B). As before, p53-null MEFs were compromised in their ability to undergo apoptosis relative to wildtype MEFs, whereas p37<sup>Ingl</sup>-null MEFs were highly sensitive to the effects of doxorubicin with most of the p37<sup>Ingl</sup>-null MEFs undergoing apoptosis.



**Figure 2.6:** Apoptosis is elevated in p37<sup>Ingl</sup>-deficient MEFs transduced with E1A. **A)** Trypan blue exclusion of wt, p37<sup>Ingl<sup>b</sup></sup> null, and p53 null E1A-transduced MEFs treated with doxorubicin. Graph shows average and standard deviation of three independent experiments. **B)** Triplicate experiments of multiple lines of E1A-transduced MEFs were mock-treated or treated with doxorubicin for 24 hours. Cell viability was determined by staining with propidium iodide (PI) before being analyzed by FACS. Graphs show a representative result and sub-G1 content (mean plus standard deviation) derived from 3 separate experiments.

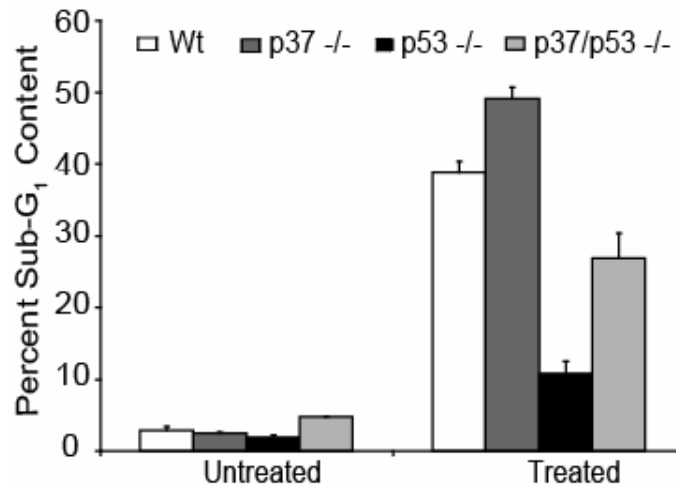
In order to further explore aberrant apoptosis in  $p37^{\text{Ingl}}$ -null mice, we isolated thymocytes from 6 week-old wildtype,  $p53$ -null or  $p37^{\text{Ingl}}$ -null mice. The total number of thymocytes recovered from the thymii of  $p37^{\text{Ingl}}$ -null mice was reduced 2-fold relative to the cell numbers recovered from wildtype mice, whereas no differences were observed in cellularity of the spleen or peripheral lymph nodes in these mice (data not shown). However, wildtype,  $p53$ -null and  $p37^{\text{Ingl}}$ -null thymocytes displayed similar kinetics of cell death when plated in culture (Figure 2.7), suggesting that the spontaneous, non-DNA-damage induced thymocyte death that occurs under these culture conditions is independent of either  $p53$  or  $p37^{\text{Ingl}}$  status.



**Figure 2.7:** Apoptosis is increased in  $p37^{\text{Ingl}}$ -deficient thymocytes. Spontaneous apoptosis is unchanged in  $p37^{\text{Ingl}}$ -deficient thymocytes. Thymocytes were isolated from two wildtype,  $p37^{\text{Ingl}}$ -null, or  $p53$ -null mice, plated in triplicate, and cultured for 4 days *ex vivo*. Cells were harvested at 24-hour intervals, fixed in 70% ethanol, and stained with PI and analyzed by FACS.

Mouse immature thymocytes (CD4/8 double positive T cells) will undergo p53-dependent apoptosis in response to ionizing radiation (38, 131). To determine if DNA-damage induced cell death is upregulated in thymocytes lacking p37<sup>Ingl</sup>, three 4-5 week old mice that were either wildtype, p53-null, p37<sup>Ingl</sup>-null, or both p53 and p37<sup>Ingl</sup>-null were whole-body treated with 10Gy ionizing radiation and thymocytes were recovered at 8 hours post-treatment from these mice and from age-matched, non-treated mice. FACS analysis of thymocyte populations stained with CD4 and CD8 antibodies revealed that  $45.5 \pm 3.1\%$  of the wildtype CD4+/8+ double positive (DP) cells survived IR treatment, whereas only  $31.4 \pm 2.1\%$  of the non-damaged levels of p37<sup>Ingl</sup>-null DP T cells were recovered after IR treatment. As expected, far more p53-null DP T cells survived IR damage ( $78.5 \pm 1.4\%$ ). However, deletion of p37<sup>Ingl</sup> also significantly reduced the survival of p53-null DP T cells ( $56.3 \pm 0.3\%$ ). These data suggest that p37<sup>Ingl</sup> plays a pro-survival role in thymocytes following DNA damage irrespective of p53 status. To confirm that p37<sup>Ingl</sup> deletion alters thymocyte apoptosis, thymocytes were harvested from wildtype, p53-null, p37<sup>Ingl</sup>-null, or both p53/p37<sup>Ingl</sup>-double null mice that were either untreated or treated with 2.5Gy of ionizing radiation. Cells were collected four hours after treatment or mock treatment and analyzed by PI staining and FACS to measure sub-G<sub>1</sub> DNA content (Figure 2.8). In agreement with the findings of the previous E1A-MEF experiments, deletion of p37<sup>Ingl</sup> led to more DNA damage induced apoptosis in wildtype thymocytes.

Furthermore, although deletion of p53 correlated with reduced thymocyte apoptosis following IR damage, loss of p37<sup>Ingl</sup> also resulted in increased apoptosis following DNA damage in p53-null cells, indicating that p37<sup>Ingl</sup> has a p53-independent, anti-apoptotic role in thymocytes.



**Figure 2.8:** Apoptosis is altered in p37<sup>Ingl</sup>-deficient thymocytes. Thymocytes were isolated from wildtype, p37<sup>Ingl</sup>-null, p53-null, or p53/p37<sup>Ingl</sup>-double null mice and either mock-treated or irradiated with 2.5 Gy of IR, fixed in 70% ethanol after 4 hours, and stained with PI for FACS. Graph shows the average of three separate experiments with standard deviations (error bars) for each genotype. Statistical analysis between wt and p37<sup>Ingl</sup>-null or p53-null and p53/p37<sup>Ingl</sup>-double null thymocytes showed that the difference in apoptosis is statistically significant ( $p < 0.005$  in both cases).

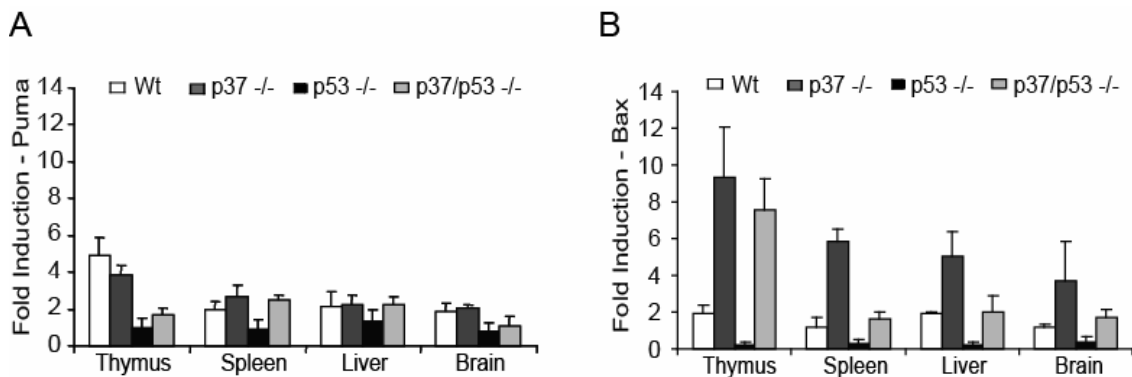
To determine if increased apoptosis in p37<sup>Ingl</sup>-null thymocytes reflected upregulated expression of the pro-apoptotic, p53-target gene *Puma*, an important regulator of DNA damage induced apoptosis in lymphocytes and in MEFs (29), the levels of *Puma* RNA transcripts were measured by quantitative PCR before and after DNA damage.



Total RNA was harvested at 4 hours post-treatment from the thymus, spleen, liver, and brain of mock-treated or IR-treated (10 Gy) mice that were either wildtype, p53-null, p37<sup>Ingl1</sup>-null, or p53/p37<sup>Ingl1</sup>-double null, and real-time PCR analysis was performed to determine the expression levels. As expected, *Puma* expression was increased in the thymus of wildtype mice in response to DNA damage, but not in mice lacking functional p53 (Figure 2.9A). However, there was no significant difference in the levels of *Puma* induction in thymus of wildtype mice and p37<sup>Ingl1</sup>-null mice, or in p53-null mice in the presence or absence of p37<sup>Ingl1</sup>.

*Bax* is also a critical inducer of apoptosis in T cells and in other cell types, but is not thought to be an important transcriptional target of p53 in the mouse (36, 37). In keeping with this model, *Bax* mRNA levels were only slightly upregulated in wildtype thymus, spleen and liver following DNA damage, and were unchanged or slightly decreased following IR treatment in p53-null samples. In contrast, *Bax* mRNA levels were induced after DNA damage approximately 8-10 fold in both p37<sup>Ingl1</sup> null thymus and in p37<sup>Ingl1</sup>/p53-double null thymus (Figure 2.9B). Induction of *Bax* expression following DNA damage was also elevated 4-6 fold in the spleen, liver, and brain of p37<sup>Ingl1</sup>-null mice, and approximately 2 fold in these tissues in p37<sup>Ingl1</sup>-null mice lacking p53. In addition, levels of *Bcl-2* RNA, an anti-apoptotic gene, were not significantly altered in these tissues by the presence or absence of either p37<sup>Ingl1</sup> or p53 (data not shown).

These data suggest that loss of  $p37^{Ingl}$  does not promote thymocyte apoptosis by altering p53-transactivation of *Puma* following DNA damage. Instead, loss of  $p37^{Ingl}$  leads to upregulated *Bax* gene expression following DNA damage of thymocytes irrespective of p53 status.

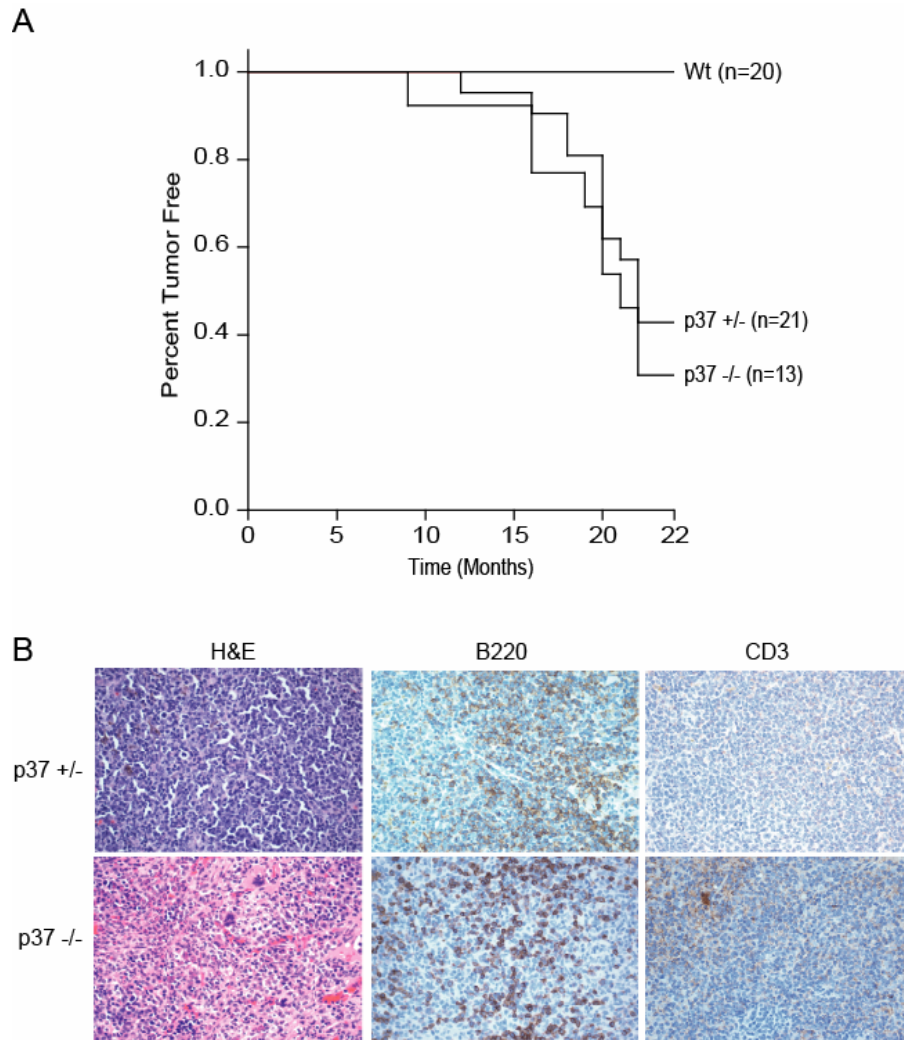


**Figure 2.9:** Quantitative real-time PCR for *Puma* and *Bax* was done using  $p37^{Ingl}$ -deficient tissues. **A)** and **B)** Quantitative real-time PCR was performed with primers to *Puma* (**A**) or *Bax* (**B**) on tissues harvested from mice that were either mock-treated or whole-body treated with 10Gy of IR and harvested 4 hours later. Graphs show the average values (relative fold induction) and standard deviations (error bars) of three mice for each genotype. The difference in *Bax* expression between wt and  $p37^{Ingl}$ -null for thymus and spleen is statistically significant ( $p < 0.05$ ), but not for brain and liver ( $p \geq 0.05$ ). Statistical analysis of the difference in *Bax* expression between p53-null and p53/ $p37^{Ingl}$ -double null tissues indicates a significant difference for thymus, spleen, and brain ( $p < 0.05$ ), but not liver ( $p \geq 0.05$ ).

### *Tumorigenesis in $p37^{Ingl}$ -deficient mice*

In order to determine if mice specifically deleted for  $p37^{Ingl}$  are tumor prone, we generated cohorts of wildtype mice,  $p37^{Ingl}$ -heterozygous ( $p37^{Ingl} +/-$ ) mice, and  $p37^{Ingl}$ -null ( $p37^{Ingl} -/-$ ) mice and monitored these mice for the development of spontaneous tumors.

Approximately 40% of the  $p37^{\text{Ingl}} +/-$  mice and half of the  $p37^{\text{Ingl}} -/-$  mice formed tumors by 22 months of age, whereas spontaneous tumorigenesis was not observed in the wildtype (C57Bl/6 x 129-SV hybrid) mice during this time (Figure 2.10A). All of the tumors arose in the spleen or peripheral lymph nodes in these mice and were classified as follicular B cell lymphomas. Staining of tumor tissues with antibody against B220 and CD3 confirmed that these tumors originated in the B cell compartment (Figure 2.10B) and not in T cells. Thus, although deletion of  $p37^{\text{Ingl}}$  does not alter p53 growth control and greatly upregulates apoptosis in response to DNA damage,  $p37^{\text{Ingl}}$  clearly functions as a tumor suppressor in B cells in mice.



**Figure 2.10:** Tumorigenesis in mice deficient for  $p37^{Ingl}$ . **A)** Kaplan-Meier survival curves of cohorts of wildtype,  $p37^{Ingl}$ -heterozygous, or  $p37^{Ingl}$ - null mice. Mice that were either moribund or reached 22 months of age were sacrificed for necropsy and fixed in 10% phosphate-buffered formalin. The rate of tumor incidence between wt and either  $p37^{Ingl}$ -heterozygous or  $p37^{Ingl}$ - null mice is statistically significant ( $p < 0.005$  or  $p < 0.05$ , respectively). **B)** Tumors arising in mice were paraffin embedded and stained for hematoxylin and eosin (H&E) for pathological analysis. Tumor sections were also stained with either B220 or CD3 antibodies to determine tissue of tumor origin.

## Discussion

ING family members are highly-conserved, plant homeodomain (PHD)-containing proteins that are linked to chromatin remodeling and transcriptional regulation through their association with histone acetyl transferase (HAT) and histone deacetylase (HDAC) complexes (22, 49, 56, 153). Previous studies have implicated Ing1 (or human ING1) as an important regulator of numerous aspects of mammalian cell physiology, including cell growth, apoptosis, senescence, and tumorigenesis (22). The Ing1 gene encodes for three alternatively spliced isoforms of Ing1 message: Ing1a and Ing1c encode a 31kDa protein and Ing1b encodes for a 37kDa protein in mice (p33 kDa in human). Forced Ing1b overexpression or antisense RNA knockdown experiments in cultured cells have indicated that p37<sup>Ing1</sup> (or p33<sup>ING</sup>) and p53 co-immunoprecipitate with one another and can function in a synergistic manner to inhibit cell growth or promote apoptosis following DNA damage (60, 106, 238). Furthermore, some ING proteins, including p33<sup>ING</sup>, have been proposed to regulate cellular p53 levels by modifying p53 interactions with components of the p19-Mdm2-p53 signaling pathway (22, 123). In addition to altering p53 activity, transfection experiments have also suggested possible p53-independent roles for ING1b in regulation of cell proliferation and apoptosis (69, 89, 214).

In this study, we generated p37<sup>Ing1</sup>-deficient mice and primary cells in order to examine the role of this p53-binding protein in the regulation of cell growth, cell death, and tumorigenesis.

Specific deletion of p37<sup>Ing1</sup> in MEFs increases the rate of cell proliferation and growth at low plating densities, confirming that physiological levels of an ING protein can function to inhibit cell growth in primary cells. However, this regulation does not appear to involve p53, as deletion of p37<sup>Ing1</sup> also increases the proliferation of MEFs lacking p53. The increase in proliferation seen in the p37<sup>Ing1</sup> null cells could be due to the observed inverse relationship between p37<sup>Ing1</sup> and *cyclin B1* expression (206), but this possibility needs to be validated. Furthermore, p37<sup>Ing1</sup>-deficient MEFs fail to exhibit perturbation of other cell growth parameters governed by p53, including spontaneous cell immortalization, oncogene-induced senescence, or cell cycle arrest following DNA damage. In addition, deletion of p37<sup>Ing1</sup> failed to rescue the p53-dependent embryonic lethality seen in Mdm2-null mice, offering further support that loss of p37<sup>Ing1</sup> does not appear to compromise p53 activities in cells or in mice.

Human ING1b (p33<sup>ING1</sup>) has been proposed previously to play a pro-apoptotic role in fibroblasts (89, 185). In contrast, analysis of apoptosis in p37<sup>Ing1</sup>-deficient MEFs indicates that physiologic levels of p37<sup>Ing1</sup> strongly inhibit apoptosis in these cells following DNA damage. To confirm a pro-survival role for p37<sup>Ing1</sup> following DNA damage, we analyzed IR-induced apoptosis in primary thymocytes and in thymus tissue *in vivo*. As expected, apoptosis was decreased in cells lacking p53 following DNA damage. However, apoptosis was increased in thymocytes both *in vitro* and *in vivo* in p37<sup>Ing1</sup>-deficient cells, in keeping with our previous results in primary fibroblasts.

Notably, apoptosis was dramatically increased in thymocytes isolated from both  $p37^{\text{Ingl}}$ -deficient mice and from  $p37^{\text{Ingl}}/p53$ -double null mice following DNA damage. Thus, in contrast to the pro-apoptotic role of p53 in these cells,  $p37^{\text{Ingl}}$  plays an anti-apoptotic role in thymocytes following DNA damage that is clearly p53-independent.

*Puma* is a p53 transcriptional target validated *in vivo* as important regulator of apoptosis following DNA damage (29). In thymocytes, p53-mediated apoptosis is governed primarily through Puma activation, since mice deleted for either Puma or p53 are equally deficient in IR-induced thymocyte apoptosis. However, Puma expression was equally upregulated in wildtype and  $p37^{\text{Ingl}}$ -null thymocytes following DNA damage, further supporting a p53-independent role for  $p37^{\text{Ingl}}$  in suppression of apoptosis.

Bax, another critical regulator of mitochondrial-associated apoptosis, functions by altering permeabilization of the mitochondrial outer membrane. Deletion of  $p37^{\text{Ingl}}$  dramatically increased upregulation of Bax expression *in vivo* in both wildtype and p53-null thymocytes following DNA damage. Loss of  $p37^{\text{Ingl}}$  also upregulated Bax levels to a lesser extent in spleen, liver, and brain tissue. Increased levels of Bax induction in  $p37^{\text{Ingl}}$ -deficient mice following DNA damage occurred independent of p53 status in all tissues, confirming a p53-independent role for  $p37^{\text{Ingl}}$  in suppressing Bax expression following DNA damage.

The results of our study indicate that p37<sup>Ing1</sup> suppresses cell proliferation in the presence or absence of p53, and does not alter p53-mediated cell growth arrest following DNA damage. In addition, p37<sup>Ing1</sup> negatively regulates Bax levels *in vivo* and functions as a pro-survival molecule following DNA damage in thymocytes regardless of p53 status. These data suggest that p37<sup>Ing1</sup> might also suppress tumor formation in a p53-independent manner. Interestingly, all of the tumors that arose in the p37<sup>Ing1</sup> deficient colonies were classified as follicular B cell lymphoma. This tumor type is rarely observed in p53-null mice (47), which present mainly with thymic lymphomas and osteosarcomas, offering further support that p53 and p37<sup>Ing1</sup> may suppress tumorigenesis through different mechanistic pathways. Furthermore, while there is a significant acceleration of tumorigenesis in mice haploinsufficient or deleted for p31<sup>Ing1</sup>, the onset of tumors in p31<sup>Ing1</sup> heterozygous or homozygous null mice is similar, suggesting that any reduction in p31<sup>Ing1</sup> levels might promote spontaneous tumor formation. This would be consistent with observations from human cancers, where ING gene expression is often reduced but not completely absent (70).

Recently, mice lacking both p37<sup>Ing1</sup> and p31<sup>Ing1</sup> have been analyzed (11). The p37<sup>Ing1</sup>/p31<sup>Ing1</sup>-deficient mice undergo normal development, but have reduced body size and present with spontaneous tumors later in life. In addition, MEFs derived from p37<sup>Ing1</sup>/p31<sup>Ing1</sup>-deficient mice display little or no changes in cell cycling after treatment with taxol or other DNA damaging agents.



As these characteristics are also observed in p37<sup>Ingl</sup>-deficient mice, it is possible that loss of p37<sup>Ingl</sup> is the underlying cause of the defects observed in the p37<sup>Ingl</sup>/p31<sup>Ingl</sup>-deficient mice and cells. However, in addition to B cell lymphomas, histiocytic sarcomas and myeloid leukemias were also observed in p37<sup>Ingl</sup>/p31<sup>Ingl</sup>-deficient mice, suggesting that the p31<sup>Ingl</sup> protein may also have unique tumor suppressing properties. Interestingly, expression of p31<sup>Ingl</sup> appears slightly elevated in mice lacking the p37<sup>Ingl</sup> isoform. Therefore, it is also possible that upregulation of p31<sup>Ingl</sup> may contribute in some way to the increased proliferation and DNA damage induced apoptosis observed in p37<sup>Ingl</sup>-ablated cells and mice. However, any contribution of increased p31<sup>Ingl</sup> levels to the phenotype of p37<sup>Ingl</sup>-deficient mice and cells must also be p53-independent.

The results of this study demonstrate a p53-independent role for p37<sup>Ingl</sup> in negative regulation of cell proliferation and apoptosis and highlights a role for p37<sup>Ingl</sup> in suppression of B-cell lymphomagenesis. Furthermore, the data indicate that loss of p37<sup>Ingl</sup> correlates with increased Bax expression in thymocytes following DNA damage. Although upregulation of Bax has been found to also increase cell proliferation in some settings (18, 66), it is possible that the increased apoptosis observed in p37<sup>Ingl</sup> null thymii might prevent tumor formation in this tissue. Further work will be needed to better characterize the role of Bax in p37<sup>Ingl</sup> regulation of cell proliferation, apoptosis, and B cells lymphomagenesis.

## Materials and Methods

### Generation of p37<sup>Ing1b</sup>-deficient mice:

Mouse (129 strain) ES cells (Omnibank no. OST206270, Lexicon Genetics) containing a retroviral promoter gene trap inserted into one allele of *Ing1* were used to generate chimeric mice, which were bred to generate p37<sup>Ing1</sup>-heterozygous mice and subsequent p37<sup>Ing1</sup>-homozygous mice. Mice were genotyped by PCR analysis of genomic DNA using the following primer sequences: wildtype allele- ING1g6673SN: 5'-TCCCCCGTGGAATGTCCTTATC-3', ING1g7259ASN: 5'-CTGCCAACAGTAGTTCTGAGCAAG-3'; targeted allele- ING1gLTR: 5'-AAATGGCGTTACTTAAGCTAGCTTGC-3', INGg1874ASN 5'-CTTGTGGAGAAAAATGCC-3'. A Southern blot strategy using a Bgl II digest of genomic DNA and a probe to the p31/p37<sup>Ing1</sup>- common exon was used to confirm the PCR genotyping results. *Ing1b* null mice were crossed to *Mdm2* +/- or *p53*-null mice and genotyped as described previously (47, 98). All mice were maintained and used in accordance with both federal guidelines and the University of Massachusetts Animal Care and Use Committee.

### Cell culture and proliferation assay.

Mouse embryonic fibroblasts (MEFs) were generated from E13.5 day embryos as described previously (210). All subsequent studies were conducted using low passage embryonic fibroblasts (pass 2-pass 4), maintained in a 37°C, 5% CO<sub>2</sub> incubator in

Dulbecco's modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (MEF media). To determine the rates of cell proliferation, multiple lines of wildtype, p53-null, p37<sup>Ingl</sup>-heterozygous, p37<sup>Ingl</sup>-null, or p37<sup>Ingl</sup>/p53-double null MEFs were seeded at  $2 \times 10^5$  cells per 60mm plate or  $1 \times 10^5$  cells per well of a 6-well plate. Triplicate plates of each line were harvested and counted every 24 hours using a Z1 Coulter Particle Counter (Beckman Couture, Miami, FL). Duplicate gelatinized 10 cm plates with 10,000 cells per plate were seeded in MEF media for each genotype to assay for cell survival and growth at low plating density. At 8-12 days post-plating, the cells were fixed with methanol and stained with 0.1% crystal violet to visualize colony formation.

**Cell immortalization assay:**

A 3T9 assay was performed as described (86) to examine the rate of spontaneous immortalization of wildtype, p37<sup>Ingl</sup>-null, or p53-null MEFs. Briefly,  $3 \times 10^6$  cells were plated into MEF media on 10cm plates every three days. A total of 3 plates ( $9 \times 10^6$  cells) were maintained for two separate lines of fibroblasts for each genotype. Triplicate plates for each line were trypsinized prior to counting and replating at a density of  $3 \times 10^6$  cells per 10cm plate every three days.

**Cell senescence assay:**

MEFs were transduced with a recombinant *Ras* retrovirus as described previously (57) and plated at  $2 \times 10^5$  cells per 60 mm plate.

Triplicate wells of each of two lines of cells per genotype were harvested and counted every 48 hours following initial plating using a Z1 Coulter Particle Counter (Beckman Coutuere, Miami, FL). Data was expressed as a ratio of the cell number after six days in culture versus the cell number at initial plating.

**Cell growth arrest studies:**

MEFs from three independent lines of each genotype were seeded onto 10cm plates at a density of  $8 \times 10^5$  cells per plate in 0.1% fetal bovine serum for three days. Cells were then fed with media supplemented with 10% serum for 4 hours, and the cultures left untreated or exposed either to 8 Grays  $\gamma$ -radiation in a cesium irradiator, to 300 ng/ml doxorubicin (Sigma-Aldrich, St. Louis, MO), or to 60 Joules/cm<sup>2</sup> UVC in a UV-crosslinker (Stratagene, Cedar Creek, TX). At 15 hours post-treatment, the MEFs were pulse labeled with 60uM bromodeoxyuridine (BrdU) for 3 hours, harvested by trypsinization into phosphate-buffered saline (PBS), and fixed in 70% ethanol overnight at 4°C. Flow cytometric analysis of DNA synthesis and total DNA content was performed by the UMMS FACS Core using an anti-BrdU antibody, propidium iodide (PI) staining, and Flowjo software. Data is presented as a ratio of percentage of cells in S phase for treated versus untreated cells.

**Apoptosis studies:**

MEFs transduced with a recombinant retrovirus encoding the Adenovirus E1A gene (17) were plated at  $10^6$  cells per 10cm plate and treated with 0.3ug/mL of doxorubicin for 24 hours. Adherent and non-adherent cells were collected by trypsinization and assayed either for trypan blue exclusion or for PI staining by FACS analysis. Wildtype, p37<sup>Ingl</sup>-heterozygous, p37<sup>Ingl</sup>-null, or p53-null mice that were 4-6 weeks old were whole-body irradiated with 10Gy of ionizing radiation (IR), and thymii harvested at 12 hours post-IR. Thymii were ground between frosted glass slides to make single cell suspensions, and cells were assayed for viability by trypan blue exclusion. Approximately  $10^6$  thymocytes were stained with CD4-phycoerythrin (BD Pharmingen, diluted 1/250) and CD8-allophycocyanin (BD Pharmingen, diluted 1/100) for 30 minutes prior to FACS analysis. For spontaneous apoptosis experiments, single cell suspensions of thymocytes were plated in RPMI media with 5% fetal calf serum at  $10^6$  cells per 60 mm plate and incubated at 37°C, 5% CO<sub>2</sub> for 96 hours. Plates were harvested, fixed in 70% ethanol, PI-stained, and analyzed by FACS. *Ex vivo* thymocyte apoptosis experiments were performed by plating  $10^6$  thymocytes onto 60mm plates in RPMI media with 5% fetal calf serum and either mock-treating or irradiating the plates with 2.5 Gy of IR. After 4 hours, cells were harvested, stained with PI, and FACS analyzed.

**Quantitative real-time RT-PCR (QRT-PCR):**

Relative levels of mRNA expression were analyzed by QRT-PCR as described previously (136).

cDNA was first generated from RNA extracted from whole mouse tissue using Invitrogen Superscript first-strand synthesis system for RT-PCR. The following primer sequences (shown 5' to 3') were used in the PCR reactions: *Bcl-2*:

CTCGTCGCTACCGTCGTGACTTCG and GTGGCCCAGGTATGCACCCAG, *Puma*:

CCTGGAGGGTCATGTACAATCT and TGCTACATGGTGCAGAAAAAGT, and

*Bax*: CTGAGCTGACCTTGG and GACTCCAGCCACAAA. All samples were normalized to EF1 $\alpha$  levels present in each tissue as described previously (136).

#### **Tumor Assays:**

A tumor cohort was established for p37<sup>Ing1</sup>-heterozygous and p37<sup>Ing1</sup>-homozygous null mice and aged for 22 months. Moribund mice or those that reached 22 months of age were sacrificed for necropsy, and select tissues harvested for DNA, RNA, and protein.

A portion of each tissue was fixed in 10% phosphate-buffered formalin, paraffin embedded, and stained with eosin and hematoxylin by the UMMS Histology Core.

Tumors were classified by morphology and by immunostaining using antibodies against surface antigens B220 (BD Pharmingen, diluted 1/50) or CD-3 (DAKO, diluted 1/400).

**CHAPTER III:**

**P37<sup>ING1B</sup> REGULATES B CELL PROLIFERATION AND  
COOPERATES WITH P53 TO SUPPRESS DIFFUSE LARGE B  
CELL LYMPHOMAGENESIS.**

## Foreword

The Inhibitor of Growth (ING) gene family encodes structurally related proteins that alter chromatin to regulate gene expression and cell growth. The initial member, ING1, has also been proposed to function as a tumor suppressor in human cancer based upon its ability to suppress cell growth and transformation in vitro. Mouse Ing1 produces two proteins (p31 and p37) from differentially spliced transcripts. We have recently generated p37<sup>Ing1b</sup>-null mice and observed spontaneous follicular B cell lymphomagenesis in this model to demonstrate that ING proteins can function in vivo as tumor suppressors. In this present report, we examine the role of p37<sup>Ing1b</sup> in the regulation of B cell growth and explore the relationship between p37<sup>Ing1b</sup> and p53-mediated tumor suppression. Our results indicate that p37<sup>Ing1b</sup> inhibits the proliferation of B cells and follicular B cells regardless of p53 status, and loss of p53 greatly accelerates the rate of B-cell lymphomagenesis in p37<sup>Ing1b</sup>-null mice. However, in contrast to the highly penetrant follicular B-cell lymphomas observed in p37<sup>Ing1b</sup>-null mice, mice lacking both p37<sup>Ing1b</sup> and p53 typically present with aggressive diffuse large B-cell lymphomas (DLBL). Analysis of marker gene expression in p37<sup>Ing1b</sup>/p53 null tumors indicates that the double-null mice develop both non-germinal center and germinal center B cell-like DLBL, and also documents upregulation of NF-κB activity in p37<sup>Ing1b</sup>/p53-null B cells and B cell tumors. These results confirm that p53 mutation is an important mechanistic step in the formation of diffuse large B-cell lymphomas and reveals a p53-independent role for Ing1b in suppressing B cell tumorigenesis.



## Introduction

Follicular B-cell lymphoma (FL) and diffuse large B-cell lymphoma (DLBL) account for approximately half of all malignant, non-Hodgkin lymphomas in adults (90, 128, 147). Patients with FL typically display microscopic accumulations (follicles) of CD45R/B220+ B-cells in lymph nodes. The median survival time for FL patients is 8 to 10 years with a variable disease course that is usually protracted with multiple relapses after treatment (90, 91). Eventually, the tumor becomes resistant to chemotherapy and can undergo transformation to a more aggressive phase that is often fatal to the patient. In contrast, diffuse large B-cell lymphoma (DLBL) is a more aggressive type of lymphoma, but with a more varied clinical course (2, 93). Patients with DLBL display large, CD45R/B220+ lymphocytes in their tumor masses and a relatively high frequency of widespread organ involvement. DLBL can arise either *de novo* from mature germinal center B-cells or via transformation from a less aggressive B cell lymphoma, such as FL (5). Although the disease responds initially to chemotherapy, a durable remission occurs in fewer than half of treated DLBL patients.

Several groups have used either cDNA microarrays or immunohistochemistry to divide DLBL into three subgroups with prognostic significance; germinal center B-cell like (GCB), activated B-cell-like (ABC), or type 3 expression profile (4, 138). The expression of CD10, BCL-6, and IRF-4 (MUM1) are used to differentiate the GCB from ABC subtypes. Germinal center B-cell-like DLBL usually expresses CD10, but can also have a CD10-, BCL-6+, IRF-4- signature (83).

This subgroup has the best prognosis with 60% of patients surviving for 5 years or more (130). Activated B-cell-like DLBL can have several different expression signatures, such as CD10-, BCL-6-, or CD10-, BCL-6+, IRF-4+. ABC-DLBL has a less favorable prognosis with only about 35% of patients surviving to 5 years (130). The third subgroup of DLBL encompasses those cases that do not express genes characteristic of either the ABC or GCB types. It also has a poor prognosis similar to ABC-DLBL, but is less well studied (45).

Despite the morphologic heterogeneity of human B cell lymphomas, a number of common genetic alterations have been observed. The most prevalent is a t(14;18) translocation seen in human FL, which results in *BCL-2* overexpression and protection of cells from apoptosis. Since the t(14;18) translocation is also detected in healthy human B-cells, Bcl-2 overexpression is thought to be necessary but not sufficient to induce FL. Furthermore, mice harboring an E $\mu$  promoter-Bcl-2 transgene fail to develop spontaneous lymphoma (11), and overexpression of BCL-2 is not observed in all human FL. These data suggest that an alternative pro-survival mechanism may exist in these tumors. In support of this finding, overexpression of MCL-1 is also detected in human B cell lymphomas (137). MCL-1 is an antiapoptotic BCL-2 family member required for survival of both T and B-cells (157), and MCL-1 transgenic mice develop widely disseminated B-cell lymphomas displaying a variety of histological subtypes, including FL and DLBL (244).

In addition, BCL-6, a critical regulator of the germinal center (GC) response wherein B-cells undergo antigen-driven somatic hypermutation in order to generate high affinity antibodies, is another pro-survival BCL-2 family member often dysregulated in B cell lymphoma. Chromosomal translocations involving BCL-6 have been observed in 15-40% of human DLBL and 5-10% of FL cases (179), and transgenic mice expressing exogenous Bcl-6 in B-cells developed a GC B cell-like subtype of DLBL (26). Collectively, these findings support a role for overexpression of various BCL-2 family members in the pathogenesis of B cell lymphomas.

Mutations in the p53 tumor suppressor gene have been observed in many types of human lymphomas, and often correlate with a poor patient prognosis. Furthermore, mutations in p53 have been proposed to play a role in the transformation of FL to a more aggressive DLBL (94, 129, 181). However, recent work suggests that p53 may have a limited role in the transformation of FL to DLBL (43), and mice either deficient for p53 or bearing a transgene encoding the p53-inhibitor Mdm2 do not develop FL or DLBL. Rather, these mouse models develop CD4<sup>+</sup>/CD8<sup>+</sup> T cell lymphomas, B220<sup>+</sup> marginal zone B cell lymphomas, or mixed lineage T cell and B cell lymphomas (85, 97, 224, 228). Thus, the precise role of p53 in suppressing the formation or transformation of FL and DLBL is unclear.

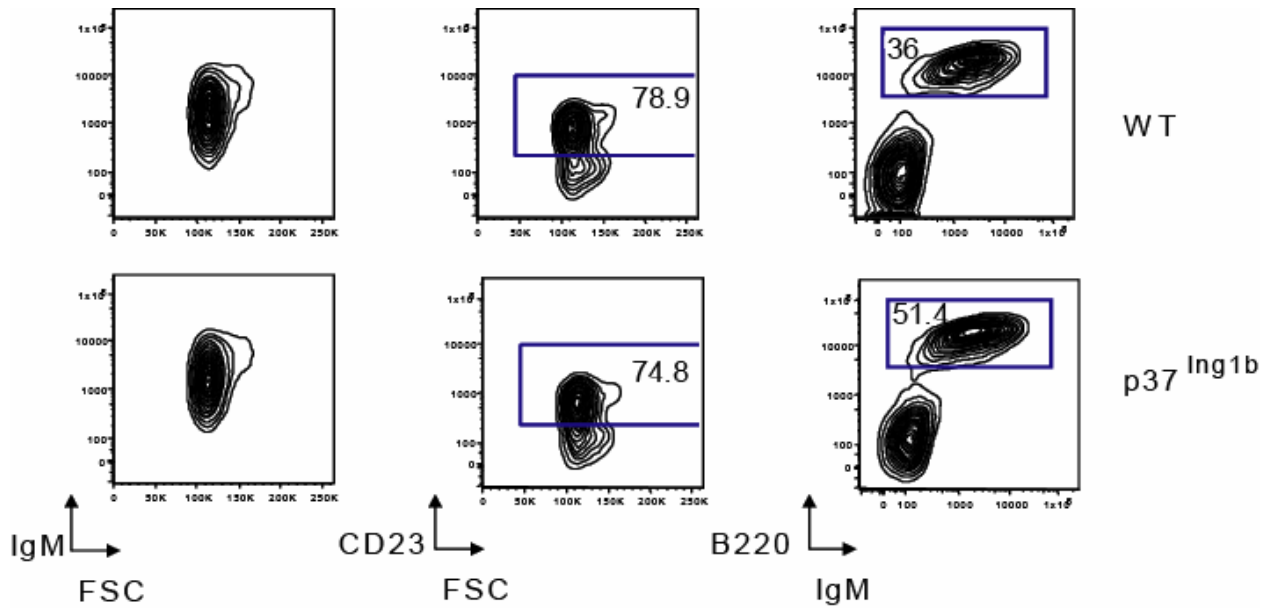
Recently, studies of *Ing1*-mutated mice have revealed a role for ING proteins in suppressing B cell lymphomagenesis (39, 108). *ING1*, a member of the Inhibitor of Growth gene family, has been proposed to regulate a number of biological processes, including nucleotide excision repair, chromatin remodeling, proliferation, apoptosis, and senescence. In addition, *ING1* has been proposed to function as a tumor suppressor by regulating p53 activity (23, 70, 149, 203). Mouse *Ing1* encodes two different proteins (p31 and p37) from differentially spliced transcripts (239). Approximately 20% of mice lacking both p31 and p37 *Ing1* proteins developed a B220+, B cell lymphoma by 23 months of age (108). A small percentage of these tumors were bi-phenotypic, expressed both B220 and CD3 surface antigens, and were classified as follicular center B-cell lymphomas. We have previously generated mice deficient solely for p37<sup>Ing1b</sup>, the most prevalent isoform of *Ing1*. These mice developed spontaneous follicular B220+ lymphomas between one and two years of age, and analysis of the growth and response of p37<sup>Ing1b</sup> deficient mouse embryonic fibroblasts (MEF) and thymocytes to DNA damaging agents revealed that p37<sup>Ing1b</sup> inhibited MEF proliferation and suppressed thymocyte apoptosis in a p53-independent manner (39). In this present study, we examine the role of *Ing1b* in B-cell growth and apoptosis, and explore the relationship between p37<sup>Ing1b</sup> and p53 in tumor suppression. Our data indicate that p37<sup>Ing1b</sup> inhibits follicular (FO) B cell proliferation regardless of p53 status and that p37<sup>Ing1b</sup> and p53 cooperate to suppress the formation of non-germinal center and germinal center B cell-like DLBL. In addition, we find that NF- $\kappa$ B activity is upregulated in the absence of p37<sup>Ing1b</sup>, supporting a role for mouse *Ing1b* in regulating NF- $\kappa$ B signaling.

These results reveal a p53-independent role for Ing1b in suppressing B cell lymphomagenesis and demonstrate that p53 deletion facilitates the formation of DLBL in a mouse model of FL.

## Results

### *Mice deficient for p37<sup>Ing1b</sup> have an expanded follicular (FO) B-cell compartment.*

To characterize the B cell compartment in p37<sup>Ing1b</sup>-deficient mice, single-cell suspensions were prepared from spleens isolated from 6 month old, asymptomatic mice and FACS analysis was performed. CD45R/B220, a B-cell marker, staining revealed a subtle expansion of splenic B cell lymphocytes in p37<sup>Ing1b</sup> null mice compared to age-matched wildtype (wt) mice (51.4% versus 36% in wt), with a majority of the B220+ cells also staining positive for both IgM and CD23, a marker of follicular (FO) cells (Fig 3.1). These data suggest that loss of p37<sup>Ing1b</sup> leads to an increase in the number of mature follicular B cells in adult mice, consistent with a role for p37<sup>Ing1b</sup> in regulating the growth of these cells. Spleens harvested from mice deleted for both p37<sup>Ing1b</sup> and p53 revealed a similar expansion of these mature B cells (data not shown).



**Figure 3.1:** Follicular (FO) B-cells in  $p37^{Ingl1}$  deficient mice are increased. FACS analysis of spleen cells from a healthy 8-month old wild-type control mouse and matched ING1 KO mouse. FSC is forward scatter (proportional to cell size). The middle and right plots depict all live spleen cells within the lymph scatter gate, whereas the left plots displays data from CD23+ B cells.

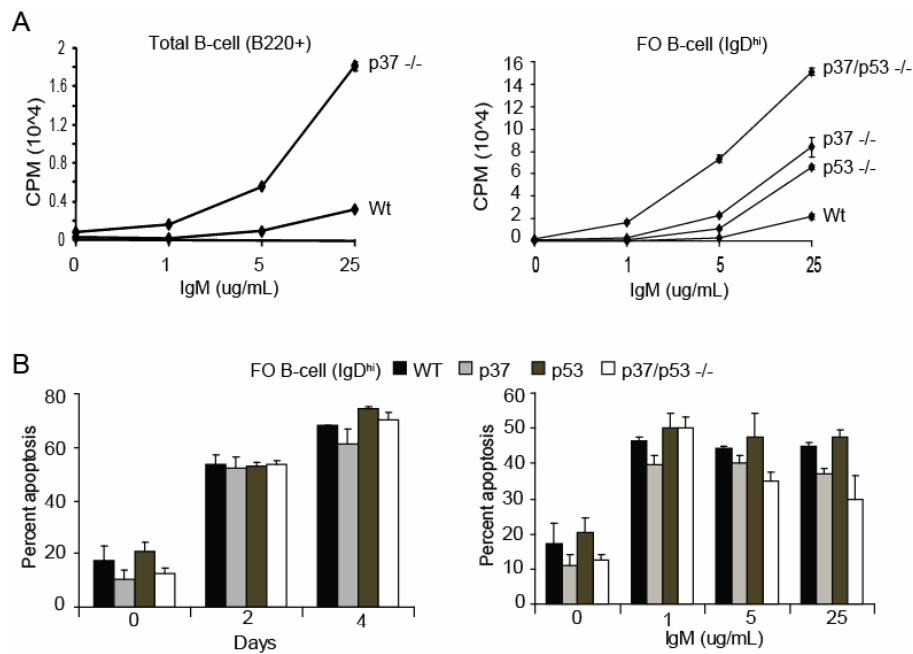
***Proliferation, but not apoptosis, is altered in  $p37^{Ingl1b}$  and  $p37/p53$  deficient B-cells.***

In order to further investigate the role of  $p37^{Ingl1b}$  in B-cell growth, we isolated either total CD45R/B220+ B-cells or follicular ( $IgD^{hi}$ ) B-cells from the spleens of adult wt,  $p37^{Ingl1b}$ -null,  $p53$ -null, and  $p37/p53$ -double null mice by FACS sorting using either anti-B220 or IgD-PE antibodies. Since anti-IgM F(ab')<sub>2</sub> antibodies activate B-cells by cross-linking of the B-cell receptor complex (BCR) to initiate cell proliferation and death, we treated either total B-cells or FO B-cells with increasing amounts of anti-IgM antibody for 16 hours and measured cell proliferation via [<sup>3</sup>H] thymidine incorporation (Fig 3.2A).

Both total B-cells (Fig 3.2A, left panel) and FO B-cells (Fig 3.2A, right panel) isolated from  $p37^{\text{Inglb}}$ -deficient mice displayed increased proliferation relative to their wt counterparts. Although deletion of p53 also increased the proliferative response of FO B-cells, co-deletion of both  $p37^{\text{Inglb}}$  and p53 further increased the proliferation of the  $\text{IgD}^{\text{hi}}$ , FO B-cells in response to BCR activation. As FO B-cells from  $p37/p53$ -double null mice displayed even greater proliferation than observed in either  $p37$ -null or  $p53$ -null cells, these data indicate that  $p37^{\text{Inglb}}$  does not require functional p53 to inhibit FO B cell growth. Rather, the  $p37$  and  $p53$  tumor suppressors co-operate in negatively regulating the proliferation of these cells.

To confirm that the increased numbers of FO B cells observed both *in vivo* in  $p37^{\text{Inglb}}$ -null mice and in the *in vitro* proliferation assays were due to increased cell proliferation and not a reflection of altered cell death, we examined apoptosis in  $p37^{\text{Inglb}}$ -null cells. FO B-cells were isolated from the spleens of adult wt,  $p37^{\text{Inglb}}$ -null,  $p53$ -null, and  $p37/p53$ -double null mice and grown in prolonged culture or treated with increasing amounts of anti-IgM. Sub-G1 content following staining of the  $\text{IgD}^{\text{hi}}$  cells with propidium iodide was used to identify the percentage of apoptotic cells in each population (Fig 3.2B). Although prolonged culture *in vitro* or activation of BCR by anti-IgM treatment increased apoptosis in FO B cells, the presence or absence of  $p37^{\text{Inglb}}$  did not alter the apoptotic response of the FO B-cells.

In addition, there was no difference in the apoptotic response of wt, p37<sup>Inglb</sup>-null, p53-null, or p37/p53-double null FO B-cells to treatment with either 2.5 Gy ionizing radiation or with 100nM dexamethasone (data not shown). Thus, neither p37<sup>Inglb</sup> nor p53 plays a role in regulating apoptosis in FO B-cells in response to these stimuli. These findings contrast with the increased apoptosis we observed previously in p37<sup>Inglb</sup>-null thymocytes (39), and confirm that the increase in FO B-cell numbers observed in adult p37<sup>Inglb</sup>-null mice is likely due to increased proliferation of these cells.



**Figure 3.2:** Analysis of follicular (FO) B-cells in p37 and p37/p53 deficient mice. **A)** [<sup>3</sup>H] thymidine uptake assay of wt, p37, p53, and p37/p53 deficient purified B-cells. Assay was done in triplicate and repeated twice. B-cells were treated with the indicated amount of anti-IgM and harvested 24 hours for [<sup>3</sup>H] thymidine uptake assay. **B)** Apoptosis assay on purified FO B-cells from wt, p37, p53, and p37/p53 deficient mice. Plots show percent of sub-G1. Assay was done in triplicate and repeated twice.

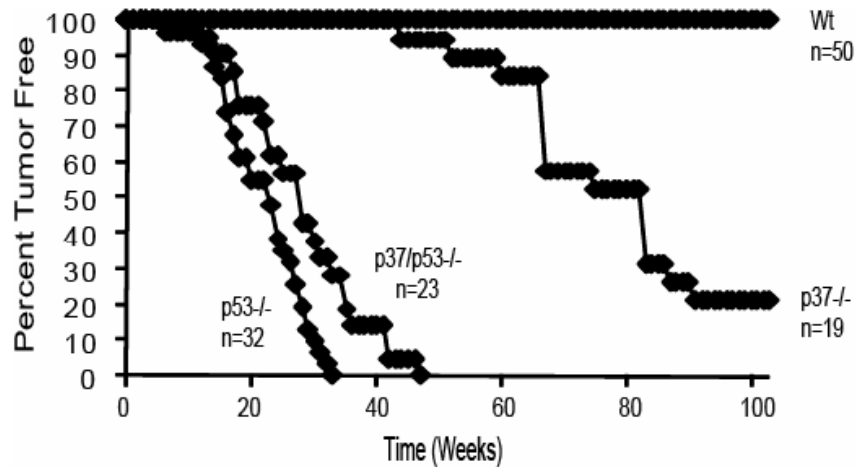


***p37/p53 double null mice primarily develop aggressive B-cell lymphomas.***

The increase in FO B cell proliferation when co-deleted for p37<sup>Inglb</sup> and p53 suggests that p37<sup>Inglb</sup> and p53 may cooperate to suppress B cell tumorigenesis. To test this hypothesis, we collected cohorts of wt mice, p37<sup>Inglb</sup>-null mice, p53-null mice, and p37/p53 double-null mice and compared the rate of spontaneous tumor formation (Figure 3.3) and the spectrum of tumor types in these mice (Table 3.1). The p37<sup>Inglb</sup>-null mice presented with FL between one and two years of age, in agreement with our previous report (39). Three quarters of the p37<sup>Inglb</sup>-null cohort developed spontaneous cancer, whereas wt mice did not develop tumors during this time. Tumors arising in p37<sup>Inglb</sup>-null mice were predominantly localized to the lymph or spleen, with little or no involvement of peripheral tissues. As expected, all p53-null mice succumbed to spontaneous tumorigenesis within 10 months of age, with 75% of the tumors classified as lymphomas and the remainder a mix of sarcomas and carcinomas.

As previously reported, two-thirds of the p53-null lymphomas formed in the thymus and were composed of CD4+CD8+ T cells, whereas the remaining third of the lymphomas formed in the spleen and were classified as marginal zone B cell lymphomas or as polymorphic tumors containing both B cells and T cells (47, 85, 97, 98, 228). Similarly, p53-null mice co-deleted for p37 rapidly formed spontaneous tumors by 45 weeks of age (Fig 3.3). However, all of the p37/p53-double null mice presented with lymphoma, with three quarters (17/23) of the p37/p53-null mice displaying enlarged spleens, discolored and enlarged livers, and lymph node involvement.

Microscopic analysis of hematoxylin and eosin stained tumor samples isolated from p37/p53-double-null mice revealed evidence of a lymphoblastic lymphoma involving spleen, liver, and/or mesenteric lymph node. In contrast to tumorigenesis in p53-null mice, only a small subset (6/23) of lymphomas arising in p37/p53-double null mice formed in the thymus.



**Figure 3.3:** Kaplan-Meier survival curve showing tumor formation in p37/p53 deficient mice. Cohorts of mice that were either wt (n=50), p53 (n=32), p37 (n=19), or p37/53 (n=23) null were monitored for 20 months.

Table 3.1: Comparison of the tumor spectrums of p53, p37<sup>Ing1b</sup>, and p37/53 null mice.

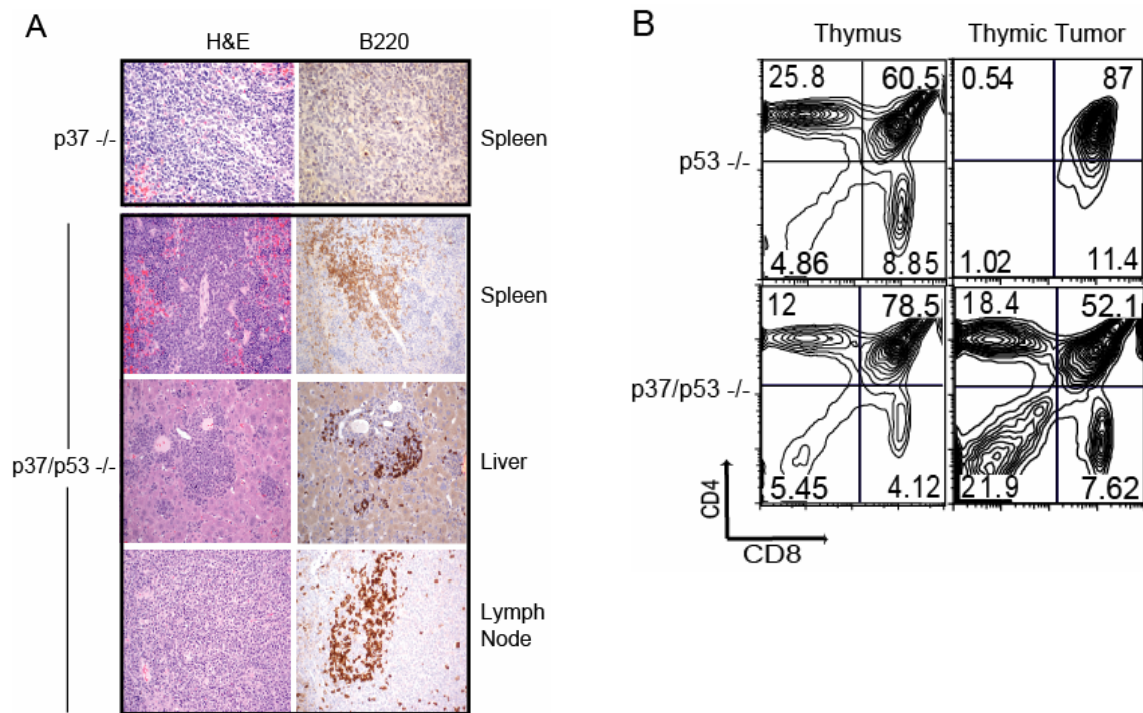
p53 null			p37 <sup>Ing1b</sup> null			p37/p53 null		
Tumor Type	%	n	Tumor Type	%	n	Tumor Type	%	n
Thymic Lymphoma	53	18	B-cell Lymphoma (FL)	100	19	DLBL	60	15
B-cell Lymphoma (MZ)	26	9				Thymic Lymphoma	12	3
Sarcomas	15	5				Both (B and T cell)	24	6
Carcinoma	6	2				Hematoma	4	1

**Table 3.1:** Comparison of the tumor spectrums of p53, p37<sup>Ing1b</sup>, and p37/p53 null mice. Both (B and T-cell) refers to mice that had two or more tumors which were classified as being of both B-cell and T-cell origin. Abbreviations used: MZL; marginal zone B-cell Lymphoma, FL; follicular lymphoma, DLBL; diffuse large B-cell lymphoma.

The non-thymic p37/p53–null tumors were characterized by a uniform population of large, malignant lymphocytes with round, vesicular nuclei with frequent membrane indentations, occasional centrally placed nucleoli, and scanty cytoplasm. Lymphoblasts completely effaced lymph node architecture, diffusely involved the splenic white pulp, and infiltrated periportal and sinusoidal regions in the liver. Immunohistochemistry performed on select sections of paraffin embedded tumors isolated from p37<sup>Ing1b</sup> mice and from p37/p53-double null mice using antibodies against CD45R/B220 and CD3 antigens confirmed that the predominant tumor type was a CD45R/B220+ lymphoma (Fig 3.4A). Additionally, histopathology suggests that the tumor cells frequently present in the different associated lymphatic tissues of p37/p53-double null mice all originate from a common tumor, suggesting that deletion of both tumor suppressor genes results in the formation of an aggressive B cell tumor.

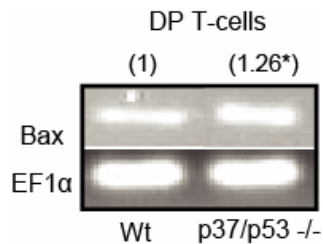
Although deletion of p53 greatly accelerated tumorigenesis in p37<sup>Ing1b</sup>–null mice ( $p < 0.05$ ), co-deletion of p37<sup>Ing1b</sup> and p53 appeared to also delay slightly tumor onset in p53-null mice (Fig 3.3), though the difference in the tumor curves was subtle ( $p < 0.1$ ). This may reflect the reduced incidence in p37/p53-double null mice of the highly aggressive thymic lymphomas typically seen in p53-null mice. Furthermore, the cellular characteristics of the thymic lymphomas arising in the p37/p53-double null mice appeared different from those arising in p53-null mice. FACS analysis of thymii isolated from p53-null and p37/p53-double null mice revealed that the CD4+CD8+ population was somewhat larger in p37/p53-double null mice relative to p53-null mice (Fig 3.4B).

Analysis of a thymic tumor originating in p37/p53-double null mice revealed an expansion of the non- CD4+CD8+ compartments. In contrast, FACS analysis of p53-null thymic tumors indicated that these tumors originated in CD4+/8+ double positive T-cell compartment, in agreement with earlier studies (97).



**Figure 3.4:** Analysis of tumors and from p37/p53 deficient mice. **A)** Representative hemotoxylin and eosin or B220 stained tumor sections from p37 or p37/p53null mice. The p37/p53 null spleen, liver, and lymph node sections are from one mouse. All pictures were taken at 20x magnification. **B)** FACS plot of CD4 and CD8 stained thymic tumors from p53 or p37/p53 null mice.

We previously reported that thymocytes lacking p37<sup>Ing1b</sup> display upregulated levels of pro-apoptotic *Bax* expression and possess an increased susceptibility to cell death induced by various stimuli (39). To confirm that the p37<sup>Ing1b</sup> null CD4+CD8+ cells co-deleted for p53 also have increased *Bax* expression, we isolated total RNA from CD4+CD8+ thymocytes harvested from p37/p53-double null mice and performed qPCR to measure *Bax* transcripts. A modest increase in *Bax* mRNA ( $1.26 \pm 0.02$ ) was observed relative to wt CD4+CD8+ *Bax* transcript levels (Fig 3.5). The increase in *Bax* expression in p37/p53-double null CD4+CD8+ cells correlates with the decrease incidence of CD4+CD8+ thymic lymphomagenesis observed in the double null mice and may account for the overall slight delay in the rate of tumorigenesis in p53-null mice co-deleted for p37<sup>Ing1b</sup>.



**Figure 3.5:** *Bax* qRT-PCR of DP sorted T-cells from non-cancerous wt or p37/p53 null mice. Experiment was done in triplicate. Shown is a representative gel picture with quantified values above (\* indicates statistically significant difference  $P \leq 0.05$ ).

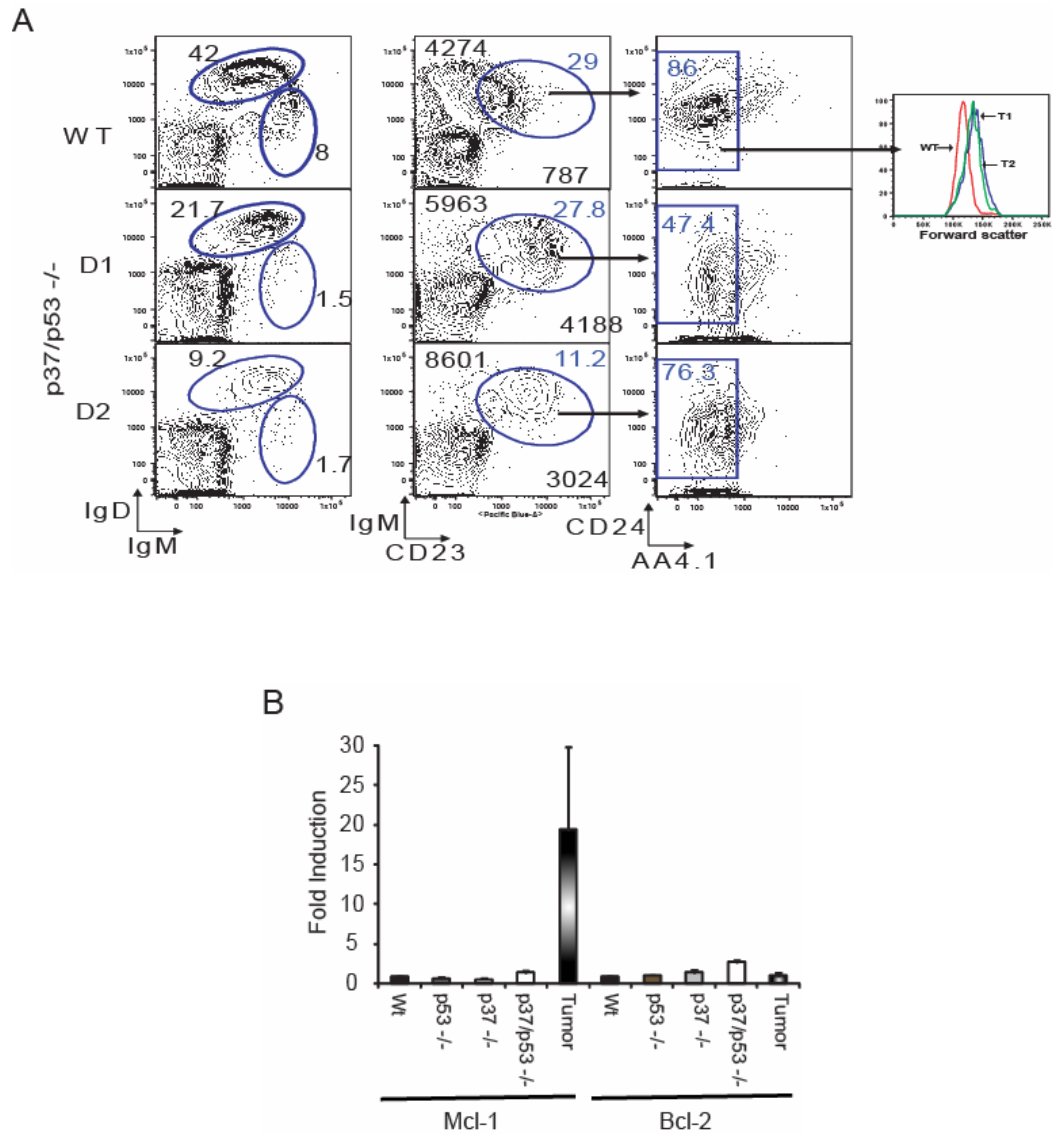
#### ***DLBL in p37/p53 double null mice***

FACS analysis was performed on the B-cell compartments of two tumor-bearing mice to further characterize the tumor phenotype of the p37/p53-double null mice (Fig 3.6).

Several distinct splenic B cell populations were readily observed in non-tumor bearing mice, including FO B cells (IgD<sup>++</sup> IgM<sup>lo</sup> CD23<sup>+</sup>), immature T1 (IgD<sup>lo</sup> IgM<sup>++</sup> CD23<sup>-</sup>) and T2 (IgD<sup>lo</sup> IgM<sup>++</sup> CD23<sup>+</sup> CD24<sup>++</sup> AA4.1<sup>++</sup>) B cells. However, the splenic B cells of tumor-bearing p37/p53-double null mice were almost exclusively composed of follicular B cells (IgD<sup>++</sup> IgM<sup>lo</sup> CD23<sup>+</sup> CD24<sup>lo</sup> AA4.1<sup>-</sup>). In addition, the forward scatter distribution of this FO B population is shifted (Fig 3.6A), indicating that the majority of the FO B cells are larger, blast stage cells rather than resting cells. The phenotype of FO B cells in p37/p53 tumorigenic mice was distinguished by elevated levels of IgM and CD23 staining, and reduced amounts of CD24 staining relative to levels observed in WT FO B cells. Similar results were obtained in a repeat of this experiment (data not shown). The difference in the staining patterns of the p37<sup>Ing1b</sup>-null tumor cells and p37/p53-double null tumor cells, coupled with the widespread organ involvement and larger size of the tumor cells arising in p37/p53-double null mice, classifies these CD45R/B220+ tumors as DLBL rather than FL.

To determine if there was increased expression of pro-survival genes in lymphomas arising in the double null mice as had been previously observed in human FL and DLBL (11, 44, 130), real-time PCR was performed on total RNA isolated from the spleens and tumors of various mice. *Mcl-1* expression appeared unaltered in wt, p37<sup>Ing1b</sup>-null, and in p53-null cells, but was slightly upregulated in p37/p53 double-null spleens (Fig 3.6B). However, *Mcl-1* expression levels were greatly increased in tumors arising in p37/p53-double null mice.

In contrast, *Bcl-2* was not elevated in splenic tissues or in tumors, regardless of the genotype of the mice. Reduced expression of *Bcl-X<sub>L</sub>* has also been seen in non-Hodgkin's lymphomas (234); however we did not determine expression of *Bcl-X<sub>L</sub>* in these tumor samples.

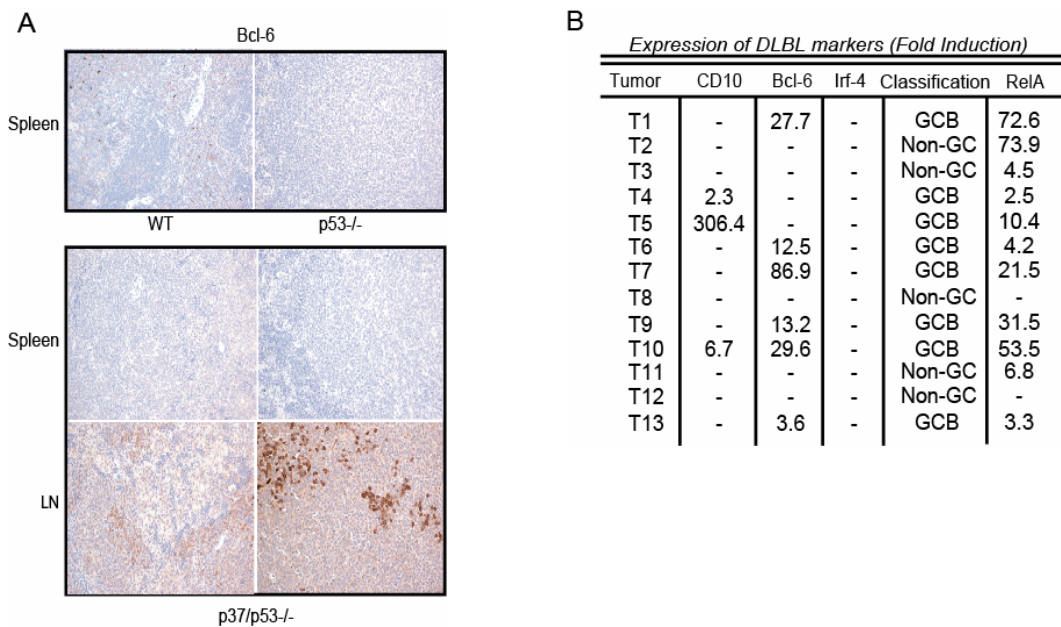


**Figure 3.6:** Characterization of the tumors in p37/p53 deficient mice. **A)** FACS analysis of spleen cells from a healthy wild-type control mouse (WT) and spleen cells from two p37/p53 null mice with tumors (D1, D2). In the middle column, the larger bold numbers report the geometric mean fluorescence intensity values for IgM (y-axis) and CD23 (x-axis) for the IgM<sup>+</sup>CD23<sup>+</sup>CD24<sup>+/-</sup>AA4.1<sup>-</sup> population. This experiment was repeated with similar results. **B)** Expression levels of Bcl-2 and Mcl-1 assayed by real-time RT-PCR in spleens from wt, p53, p37, and p37/p53 null mice or p37/p53 null splenic tumors. Real-time PCR of normal spleens was done in triplicate and the tumor samples are from three independent mouse tumors. Shown is average and standard deviations (error bars).



Approximately one-third of all NHL display translocations involving BCL-6, a transcriptional repressor required for the formation of germinal center (GC) B cells. Overexpression of BCL-6 has been proposed to block the terminal differentiation of GC B cells by inhibiting the expression of the B lymphocyte maturation protein 1 (Blimp1), resulting in prolonged exposure of the GC B cells to the mutagenic effects of the somatic hypermutation machinery (166). In addition, Bcl-6 has been proposed to suppress DNA damage-induced apoptosis caused by the hypermutation apparatus by suppressing p53 expression (170). Thus, Bcl-6 expression serves as a marker for DLBL that are derived from GC-like B cells. To determine if Bcl-6 expression was upregulated in DLBL in p37/p53-double null mice, representative tissue sections were fixed, sectioned, and stained for Bcl-6 (Figure 3.7A). Bcl-6 was not detected in wt splenic tissue, or in a B cell lymphoma arising in the spleen of a p53-null mouse (Figure 3.7A, top panels). In contrast, varying levels of Bcl-6 expression could be detected in some but not all tumor tissues harvested from p37/p53-double null mice (Figure 3.7A, bottom panels). To confirm upregulation of Bcl-6 expression in a subset of p37/p53-tumors, total RNA was harvested from thirteen p37/p53-double null DLBL and assayed for Bcl-6 transcript levels by qPCR. In addition, the expression levels of the *CD10* surface marker, *Irf-4* transcription factor, and the NF- $\kappa$ B subunit *Rela* (*p65*) were also assayed by qPCR (Figure 3.7B). *Irf-4* is a transcription co-factor expressed in plasma cells that silences BCL-6 expression and the GC transcriptional program, and is critical for GC to plasma cell differentiation (20). Bcl-6 was upregulated in 6 of 13-tested DLBL that arose in p37/p53-double null mice, marking these tumors as GC B cell-like DLBL.

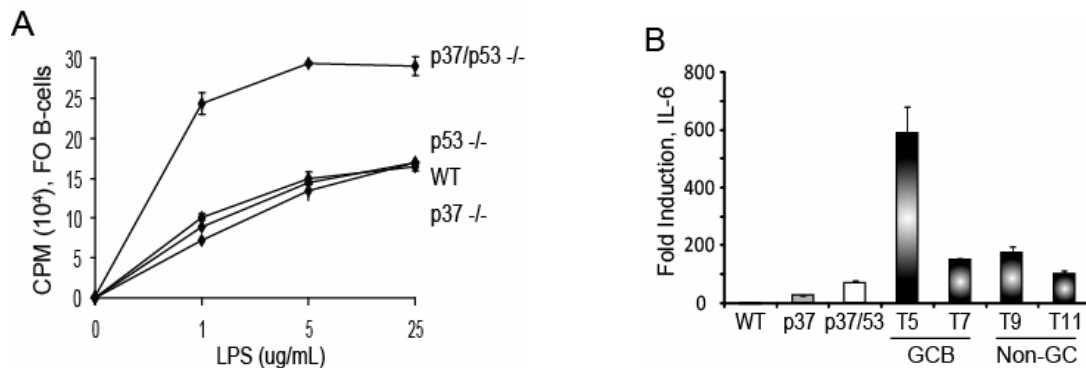
CD10, another marker for GC B cells (9), was elevated in two other tumors, indicating that 8 of the 13 tumors were GC subtypes of DLBL. The remaining 5 DLBL tumors did not display elevated levels of CD10, Bcl-6 or Irf-4. Lack of CD10, Bcl-6, or Irf-4 upregulation in the remaining 5 DLBL indicates that these are non-GC B cell-like tumors.



**Figure 3.7:** p37/p53 deficient mice develop predominately GC B-cell DLBL and have elevated *RelA* expression. **A)** Representative Bcl-6 stained p37/p53 spleen and lymph node (LN) tumors. Wt and p53 null spleen sections stained with Bcl-6 are shown as controls. All pictures were taken at 20x magnification. **B)** Table showing classification of 13 independent p37/p53 null B-cell tumors based on expression of *CD10*, *Irf-4*, and *Bcl-6* by real-time PCR. Values are averages of relative fold induction over normal p37/53 null spleens of three independent experiments for each tumor. Standard deviations were within 10% of the mean value for each sample.

NF- $\kappa$ B activity is often increased in human lymphomas due to chromosomal translocations, mutation of NF- $\kappa$ B inhibitors such as I $\kappa$ B, or through other, less well-characterized processes (92, 100). Furthermore, NF- $\kappa$ B activation is also observed in subtypes of non-GC DLBL such as in peripheral (or activated) B cell-like DLBL. Recently, ING1 has been proposed to inhibit NF- $\kappa$ B signaling in human fibroblasts by upregulating *HSP70* expression (54). To examine NF- $\kappa$ B activity in cells deleted for p37<sup>Ing1b</sup>, qPCR was performed on total RNA isolated from the 13 DLBL samples. Analysis of *RelA* expression levels in the DLBL revealed that the RelA (p65) subunit mRNA expression was elevated in nearly all of the tumors (Fig 3.7B- right column) relative to *RelA* expression in wt splenic tissue. To determine if NF- $\kappa$ B activity is upregulated in p37/p53-double null cells, FO B cells were isolated from the spleens of wt, p37<sup>Ing1b</sup>-null, p53-null, or p37/p53 double-null mice, and cell proliferation was measured in response to treatment of the cells with LPS, an established inducer of NF- $\kappa$ B activity (Fig 3.8A). Although deletion of p53 or p37<sup>Ing1b</sup> did not result in a greater proliferative response of FO B cells to LPS, co-deletion of p53 and p37<sup>Ing1b</sup> did result in increased proliferation of FO B cells to varying dosages of LPS. To confirm that the increase in RelA expression observed in the DLBL samples correlated with increased NF- $\kappa$ B activity in the tumors, qPCR was used to measure expression of the Interleukin 6 gene (IL-6), a classic target gene upregulated by NF- $\kappa$ B signaling (Figure 3.8B).

Increased IL-6 transcript levels were observed in the spleens of p37-null mice, regardless of p53 status, and very large increases (100-600 fold) in IL-6 expression levels were observed in p37/p53 double-null tumors, regardless of their classification as GC B cell-like DLBL (tumor #5 and #7) or as non-GC DLBL (tumor #9 and #11). These results confirm that loss of p37<sup>Ing1b</sup> upregulates NF- $\kappa$ B activity in p37/p53 double-null mice and in tumors, supporting a role for Ing1b in inhibiting NF- $\kappa$ B signaling in suppression of DLBL.



**Figure 3.8:** p37/p53 deficient mice have elevated NF- $\kappa$ B activity. **A)** [<sup>3</sup>H] thymidine uptake assay of wt, p37, p53, and p37/p53 deficient purified B-cells. Assay was done in triplicate and repeated twice. B-cells were treated with the indicated amount of LPS and harvested 24 hours for [<sup>3</sup>H] thymidine uptake assay. **B)** Expression of IL-6 in wt, p37 null, p37/p53 null spleens, and representative p37/p53 null GCB (n=2) and non-GC-DLBL (n=2) tumors by real-time PCR. Shown are average and standard deviations of real-time PCR performed in triplicate.

## Discussion

We have previously described the generation of mice deleted for the p37<sup>Ing1b</sup> isoform of Ing1 (39). Loss of p37<sup>Ing1b</sup> increased the proliferation of mouse embryonic fibroblasts (MEFs) regardless of p53-status, and the p53-mediated response of cells to DNA damage or to overexpression of oncogenic *Ras* was unaltered in p37<sup>Ing1b</sup> deficient MEFs. In addition, deletion of p37<sup>Ing1b</sup> upregulated *Bax* gene expression in the presence or absence of functional p53, and increased the apoptotic response of thymocytes to DNA damage. A majority of p37<sup>Ing1b</sup>-deficient mice spontaneously developed enlarged spleens between one and two years of age. Histopathology revealed a CD45R/B220+ CD3- lymphocytic hypercellularity in spleens isolated from p37<sup>Ing1b</sup>-deficient mice, and all of the tumors arising in p37<sup>Ing1b</sup>-null mice were classified as FL (39). Although our results indicated that p37<sup>Ing1b</sup> can inhibit cell growth and cell death in a p53-independent manner, in MEFs and in thymocytes, respectively, it was unclear from these data if p37<sup>Ing1b</sup> alters p53 functions to regulate tumorigenesis. In this present study, we crossed p37<sup>Ing1b</sup> mice with p53-null mice to generate p37/p53-double null mice and explored the relationship between p37<sup>Ing1b</sup> and p53 tumor suppression. Deletion of p53 greatly accelerated the rate of tumor formation in p37<sup>Ing1b</sup>-null mice, an unexpected finding if p37<sup>Ing1b</sup> and p53 were functionally redundant, or if p37<sup>Ing1b</sup> suppressed tumorigenesis by signaling through p53. Thus, co-operation observed between the two tumor suppressors in preventing B cell lymphomagenesis indicates that p37<sup>Ing1b</sup> does not suppress tumors by activating p53. Co-operation between p37<sup>Ing1b</sup> and p53 in tumor suppression is also in agreement with the increased rate of cell proliferation in FO B cells when co-deleted for p37<sup>Ing1b</sup> and p53.

Interestingly, there was a reduction in the incidence of thymic lymphomas in the p37/p53-double null mice relative to the high incidence CD4+CD8+ thymomas in p53-null mice. This reduction correlates with an increase in pro-apoptotic *Bax* expression observed in CD4+CD8+ T cells isolated from p37/p53-double null mice, in keeping with our previous finding of a dramatic increase in *Bax* mRNA in total thymus when treated with  $\gamma$ -irradiation (39). The reduced incidence of thymic lymphoma, typically an aggressive cancer resulting in rapid morbidity and mortality in p53-null mice, may account for the slight delay in the lethality of the p37/p53-double null mice. We also observed a slight decrease in expression of *Mcl-1* in thymii from p37/p53null mice (data not shown). The elevation of *Mcl-1* in p37/p53null spleens and reduction of *Mcl-1* in T-cells might also explain the shift from a predominately T-cell lymphoma in p53-null mice to a more B-cell lymphoma in p37/p53null mice.

Approximately half of the tested DLBL were found to have elevated Bcl-6 expression levels and/or increased CD10 expression, indicating a GC B cell-like subtype of DLBL. In addition, nearly all of the tumors displayed elevated RelA expression and, in some cases, increased NF- $\kappa$ B activity. It is yet unclear how Ing1b is regulating the expression of Bcl-6, CD10, or RelA in DLBL, or the expression of *Mcl-1* and *Bax* in T cells. Further experimentation is needed to determine if this effect is indirect or is a direct effect of altered factor binding at the promoters of these genes.

A few studies have examined the expression of ING1 in human primary hematopoietic malignancies and found no mutation of ING1 or alterations in ING1 expression in myeloid leukemias (108, 155, 176, 183). However, down-regulation of ING1 expression has been reported in human mantle cell lymphomas in both primary tumors and cell lines (176, 183). In addition, expression of Ing1 appears to be downregulated in gene expression profiling data performed on human DLBL samples, though the reduction in ING1 expression was not enough to highlight ING1 as a prognostic indicator for the disease in these studies (4, 138). However, the results of our analysis of Ing1b-deficient mice indicate that loss of p37<sup>Ing1b</sup> induces the highly penetrant formation of spontaneous FL and DLBL in mice, especially in mice co-deleted for p53. Thus, the role of Ing1b in suppressing human B cell lymphomas, especially DLBL, should be evaluated further.

## **Materials and Methods**

### **Mice and tumor assay**

The generation of p37<sup>Ing1b</sup>-null mice and p37/p53-double null mice was described previously (39). The p37<sup>Ing1b</sup>-null mice were backcrossed to C57Bl/6 strain for 6 generations, and cohorts of these mice and of p37/p53-double null mice, wildtype mice, and p53-null mice (47) all on a C57Bl/6 inbred background were collected and aged in order to perform a tumor assay. Mice displaying obvious tumors or signs of reduced vitality were euthanized and tissues were harvested for FACS analysis or fixed in 10% formalin for immunohistochemistry.

Samples were also flash frozen in liquid nitrogen for use in qRT-PCR analysis. Formalin fixed, paraffin embedded sections were stained with either hemotoxylin and eosin or with an antibody to CD45R/B220 (BD Pharmingen; 1:50) or to Bcl-6 (Santa Cruz; 1:50) before being analyzed by microscopy. All mice were maintained and used in accordance with both federal guidelines and those established by the Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

### **Cell sorting**

Single cell suspensions of spleens harvested from p37/p53 double null mice at 6-8 weeks of age were stained with CD45R/B220-PE (eBiosciences; 1:200) or IgD-PE (eBiosciences; 1:200) and FACS sorted. Resulting purity of either total B-cell (B220+) or follicular B-cell (IgD<sup>Hi</sup>) populations were greater than 95%. B-cells were maintained in RPMI1640 supplemented with 5% FCS, 2 mM L-glutamine, 100 ug/ml streptomycin, 100 U/ml penicillin, and 50 uM 2-mercaptoethanol (GIBCO-BRL) and cultured at 37°C and 10% CO<sub>2</sub>. Double positive (DP) thymocytes were sorted using CD4-PE (BD Pharmingen; 1:250) and CD8-APC (BD Pharmingen; 1:100) antibodies and sorted by FACS. RNA from sorted DP T-cells was purified using Trizol reagent from Invitrogen (Carisbad, CA) according to the manufacturer's instructions, and cDNA was generated using a first-strand cDNA synthesis kit from Invitrogen (Carisbad, CA).



### **Analysis of B-cell subsets**

Spleens were processed to single-cell suspension by pressing between frosted glass slides, and filtered through nylon mesh. Red blood cells were lysed using brief hypotonic shock and the remaining cells were resuspended in biotin-, flavin-, and phenol-red deficient RPMI 1640 (Invitrogen, Carlsbad, CA) staining media containing 10 mM Hepes, pH 7.2, 0.02% sodium azide, 1 mM EDTA, 3% newborn calf serum, and treated 10 min on ice with 2.4G2 Fc block (BD Biosciences, San Jose, CA). Cells were incubated for 20 min on ice with primary antibodies, washed and biotin-stained cells were incubated with streptavidin (SA)- pacific blue (Molecular Probes/Invitrogen) for 15 min on ice. After 2 washes, cells were resuspended in 1 mg/ml propidium-iodide to exclude dead cells. Primary antibodies included antibodies specific for: CD24 (clone 30-F1) FITC or PE, CD45R/B220 (RA3-6B2) APC, IgM (II/41) biotin or FITC, IgD (11-26) PE, CD93/AA4.1 APC, CD23 (B3B4) biotin. All reagents except when noted were from BD Biosciences, San Jose, CA; eBiosciences, San Diego, CA; Southern Biotechnology Associates, Inc. Birmingham, AL; or CALTAG Laboratories/Invitrogen, Burlingame, CA. Flow cytometry was performed on a 3-laser 12-color LSR II (BD Biosciences, San Jose CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

### **Proliferation and apoptosis assays**

Incorporation of [<sup>3</sup>H] thymidine was used as a measure of proliferation as described previously (127). In brief, sorted  $2 \times 10^5$  B-cells were plated into wells of a 96-well plate with 200  $\mu$ l of RPMI medium. Following 24 hr of culture, cells were pulsed with [<sup>3</sup>H] thymidine (1  $\mu$ Ci per well) for 16 hr prior to harvest. Incorporated radioactivity was quantified with a Liquid Scintillation and Luminescence Counter (Perkin Elmer Wallace Inc.). Apoptosis assays were described previously (51). In brief, B-cells were plated as before and then treated with either increasing amounts of anti-IgM F(ab')<sub>2</sub> (Jackson Immunoresearch, West Grove PA), 250 rads  $\gamma$ -irradiation, 100 nM dexamethasone (Sigma, St. Louis MO), or allowed to die by neglect. After 24 hours following IR or 100 nM dexamethasone treatment or 16 hours after IgM treatment, cells were collected, stained for propidium iodide, and analyzed by FACS.

### **qRT-PCR**

Quantitative RT-PCR was done as previously described (41). The following primers were used: *Mcl-1*: AGTCAGCACAGCTTTCCTGTCAGA and TGCCAATCCAAGAATGCCAATCCC; *Bcl-2*: CTCGTCGCTACCGTCGTGACTTCG and GTGGCCCAGGTATGCACCCAG; *Bcl-6*: GCAACGAATGTGACTGCCGTTTCT and TTTCTCACCCGTGTGGACAGTCTT; *CD10*: TTCTGTGGCCAGACTGATTCGTCA and GCAGCATTGGGTCATTTCCGGTCTT; *Irf4*: GAGCTGCAAGTGTTTGCTCACCAT and ACAGTTGTCTGGCTAGCAGAGGTT;

*RelA (p65)*: TGTGTTGATAGCTCCTGCTTCGGT and

ATCAAGGTGTACAGGAATCCGCGT; *Il-6*: AGTCACAGAAGGAGTGGCTAAGGA  
and TCTGACCACAGTGAGGAATGTCCA; and

*Efl  $\alpha$* ; AGCTTCTCTGACTACCCTCCACTT and GACCGTTCTTCCACCACTGATT.

All primers were amplified using 62<sup>o</sup>C for an annealing temperature and 24 cycles to keep the products in the linear range. PCR products were run on a 2% agarose gel with SYBR green to visualize the bands.

**CHAPTER IV:**  
**ING4 SUPPRESSES NF- $\kappa$ B SIGNALING AND INNATE IMMUNITY**  
**IN MICE.**

## Foreword

*ING4* is a member of the Inhibitor of Growth (ING) candidate tumor suppressor gene family. ING proteins serve as subunits of chromatin remodeling complexes, and have been proposed to participate in a number of biological processes, including DNA repair, apoptosis, cell cycle regulation, and tumor suppression. Recently, overexpression experiments in transformed cell lines found an interaction between ING4, the p53 tumor suppressor, and p300, which resulted in the increased acetylation of p53. Additionally, ING4 was found to bind to p65/RelA, a component of the NF- $\kappa$ B complex, and cause a reduction in the activation of a canonical NF- $\kappa$ B -responsive promoter. In this study, we have generated *Ing4*<sup>-/-</sup> mice to explore the *in vivo* role of *Ing4* in cell growth and tumor suppression. Surprisingly, deletion of *Ing4* did not alter p53-mediated growth arrest or apoptosis, and *Ing4*<sup>-/-</sup> mice failed to develop spontaneous tumors. However, *Ing4*-deficient cells displayed reduced proliferation, and mice and macrophages deleted for *Ing4* were hypersensitive to treatment with LPS and exhibited decreased I $\kappa$ B gene expression and increased nuclear p65/RelA and NF- $\kappa$ B activity. In addition, *Ing4*-deficient mice displayed an increased inflammatory response *in vivo*. These results reveal that *Ing4* suppresses NF- $\kappa$ B function and innate immunity in mice by promoting the expression of the p65/RelA inhibitory I $\kappa$ B genes.

## Introduction

ING4 is a member of the Inhibitor of Growth (ING) family, which is comprised of five evolutionarily conserved proteins (ING1-ING5) that are characterized by a conserved carboxy-terminal plant homeodomain (PHD)-like zinc finger (23). These nuclear proteins are proposed to play roles in numerous biological functions by interacting with different acetylation and deacetylation complexes involved in chromatin remodeling and gene expression (48, 203). ING4 was identified through its homology to ING1, the initial and best characterized member of the ING family. Similar to ING1, cell transfection studies have revealed that ING4 negatively regulates cell proliferation (126, 199, 216, 243). Biochemical analysis has indicated that ING4 interacts with methylated histone H3 (160, 161, 194) and with the HBO1-JADE-hEAF6 histone acetyltransferase (HAT) complex (48). This complex is responsible for most of the nucleosomal histone H4 acetylation that occurs in eukaryotes, and HBO1-ING4 complex formation is required for cells to progress properly through the DNA synthesis (S) phase of the cell cycle (7, 95). In addition to regulating cell proliferation, ING4 has been found to suppress the loss of cell contact inhibition induced by MYC or MYCN (110), to suppress cell spreading and migration by interacting with Liprin  $\alpha$ 1 (189), and to regulate the cellular response to hypoxia by interacting with hypoxia inducible factor (HIF) prolyl hydroxylase and repressing HIF activation (40, 158, 159). Collectively, these findings underscore the possibility that ING4 functions as a tumor suppressor, and several reports have implicated ING4 in the development and progression of various types of human cancers.

Reduction of ING4 message correlates with tumor grade in glioblastoma and multiple myeloma (40, 63), and decreased ING4 expression correlates with decreased overall survival of patients with malignant melanoma (19). Loss of ING4 heterozygosity has also been observed in breast cancers and in head and neck squamous cell carcinoma (HNSCC), offering further support for a role for ING4 in tumor suppression (77, 110).

Although the precise molecular basis for ING4 tumor suppression is unclear, several lines of evidence indicate that ING4 alters the tumorigenic potential of cells by altering the activity of the p53 tumor suppressor, a function that has been similarly proposed for other ING proteins (199, 203). Forced overexpression studies have indicated that ING4 can bind both to p53 and to the acetyltransferase p300 to facilitate p300-mediated acetylation of p53 (199). Furthermore, ectopic expression of ING4 in RKO cells caused diminished colony forming efficiency, a decrease in S-phase, and induction of apoptosis in a p53-dependent manner (199), and forced overexpression of ING4 in HepG2 cells decreased their rate of growth, increased the expression of the p53 target genes *p21* and *BAX*, and increased apoptosis following exposure of these cells to DNA damaging agents or to serum starvation (243). However, these transfection data were obtained using transformed cell lines engineered to either express ING4 or have reduced levels of ING4 (siRNA-knockdown of ING4), and alteration of p53 activity by ING4 has not been examined *in vivo*.

ING4 has also been proposed to regulate tumorigenesis by modulating NF- $\kappa$ B signaling. Forced overexpression and co-immunoprecipitation experiments performed in the U87MG glioblastoma cell line have revealed that ING4 can physically interact with RelA, the large subunit of nuclear factor NF- $\kappa$ B (63). Inhibition of ING4 by antisense RNA promoted tumor vascularization in transplanted SCID mice and downregulated the expression of several NF- $\kappa$ B target genes involved in angiogenesis, including interleukins IL-6 and IL-8, colony stimulating factor (CSF-3), and the prostaglandin-endoperoxide synthase (Cox-2) gene. Further analysis of ING4-p65 (RelA) interactions using gel mobility shift assays and reporter gene assays indicated that ING4-p65 binding decreased activation of a canonical NF- $\kappa$ B -responsive promoter. These findings indicate that ING4 may suppress tumorigenesis by complexing with RelA and inhibiting NF- $\kappa$ B -mediated cell survival and angiogenesis.

In this study, we have generated and characterized *Ing4*-deficient mice in order to determine if physiologic levels of ING4 regulate cell growth and development, and to further explore a role for ING4 in tumor suppression. Mouse embryonic fibroblasts (MEFs) deficient for *Ing4* proliferate slower than wildtype (wt) MEFs, indicating that *Ing4* is required for proper growth control in primary cells. However, p53 functions appear to be unaltered by deletion of *Ing4*, indicating that the p53 pathway is not compromised in *Ing4*-null mice. In keeping with this observation, *Ing4* deficient mice failed to develop spontaneous tumors.



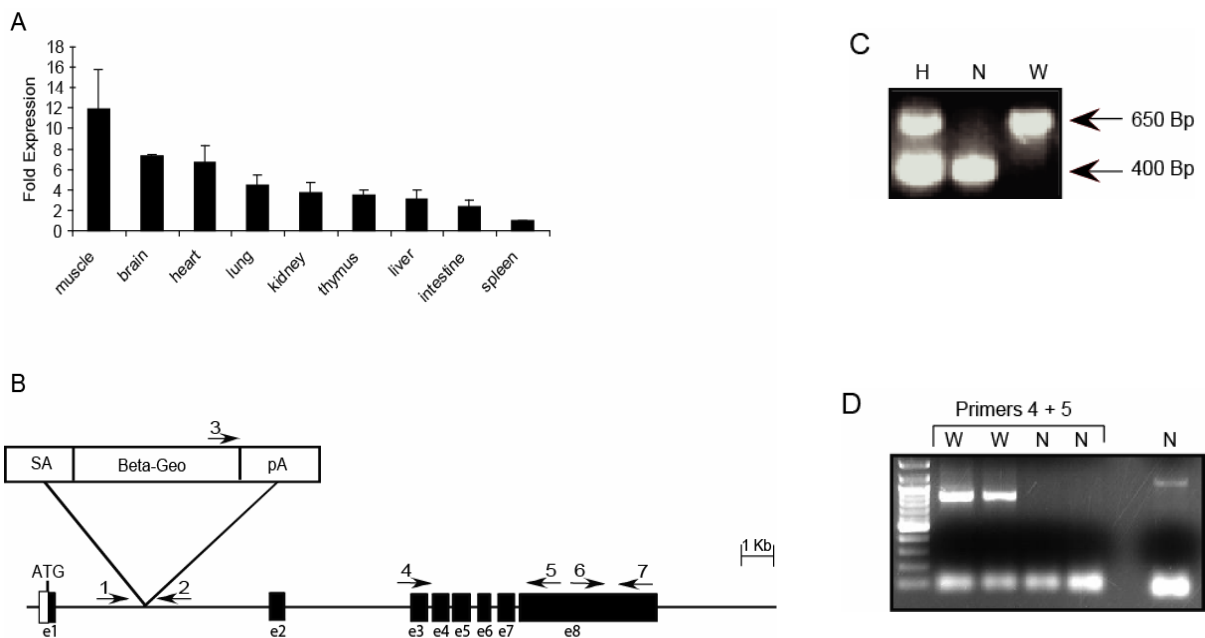
NF- $\kappa$ B signaling contributes to multiple biological processes, including tumorigenesis, angiogenesis, apoptosis, and the innate and adaptive immune response. To examine the ability of Ing4 to regulate NF- $\kappa$ B activity *in vivo*, we examined the innate immune response of Ing4<sup>-/-</sup> mice. Ing4-null mice are extremely sensitive to LPS treatment, and analysis of Ing4-null peritoneal macrophages revealed that Ing4 suppresses NF- $\kappa$ B promoter binding and NF- $\kappa$ B activation of cytokine gene expression. Upregulation of NF $\kappa$ B activity in Ing4-null mice was due to decreased expression of I $\kappa$ B genes, and Ing4-null mice display a greatly elevated inflammatory response in colorectal tissue, similar to that observed in I $\kappa$ BNS-deficient mice (117). These results establish that physiologic levels of Ing4 can regulate NF- $\kappa$ B activity *in vivo* by altering I $\kappa$ B expression, and highlights a new role for Ing4 in regulating inflammation and innate immunity in mice.

## Results

### *Generation of Ing4-deficient mice.*

To examine the physiological role of Ing4 in the mouse, we first determined the expression pattern of Ing4 in a panel of wt C57Bl/6 mouse tissues by real-time quantitative RT-PCR. *Ing4* transcript is ubiquitously expressed in the adult mouse, with the highest expression levels observed in muscle (Figure 4.1A). In contrast to the multiple spliced isoforms of ING4 present in humans, only one Ing4 transcripts has been observed in mice (203, 216, 222).

Ing4-deficient mice were generated using an Ing4-gene trapped embryonic stem (ES) cell clone that was determined to harbor a retroviral insertion within intron 1 of the Ing4 gene (Figure 4.1B). This clone was used in a standard blastocyst microinjection experiment to generate several high degree chimeric mice, and germ-line transmission of the modified Ing4 allele was obtained by breeding the chimeric mice to wt C57Bl/6 mice (Figure 4.1C).



**Figure 4.1:** Generation of Ing4-deficient mice. **A)** Real-time quantitative PCR analysis of Ing4 expression in a panel of wt C57Bl/6 mouse tissues. **B)** Schematic of the genetrapped locus showing insertion within the first intron. The trap produces a complete knockout by preventing expression of the downstream exons. **C)** PCR genotyping of the mouse tail biopsies showing the presence of the gene trap. A 650-bp fragment is generated from Wt Ing4 from primers 1 and 2 and a 400-bp fragment is generated from the targeted Ing4 allele from primers 2 and 3. Abbreviations are: Wt (W), heterozygous (H), and null (N). **D)** RT-PCR using primers 4 and 5 on mouse embryonic fibroblasts (MEFs) showing that the genetrapp causes a complete knockout of the Ing4 gene as expected. Last lane of gel shows that the Ing4<sup>-/-</sup> MEFs express an exon 1 to βGeo fusion transcript (primers not shown in B). Bottom band is primer dimer.

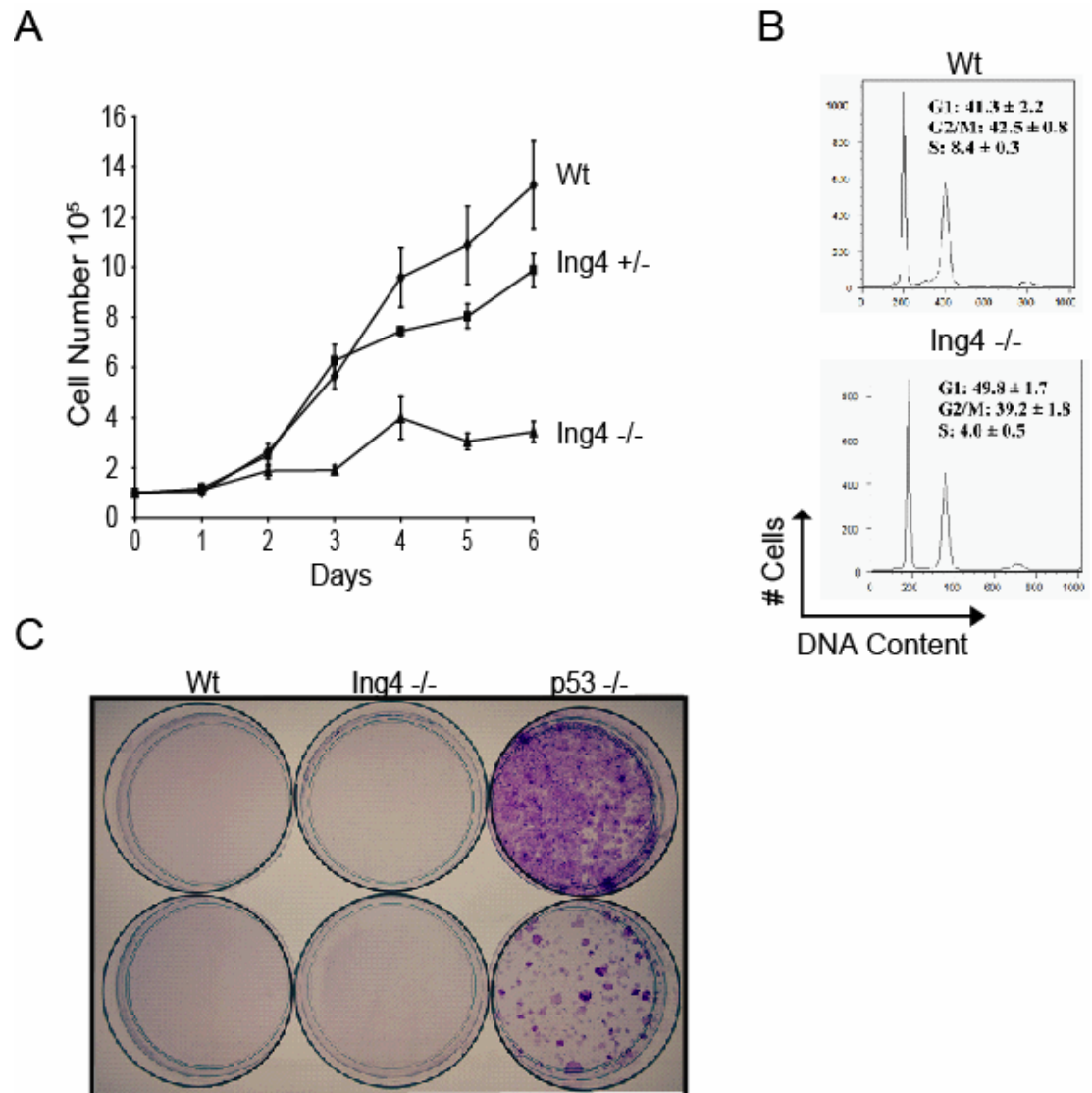
Intercrosses of Ing4-heterozygous, F1 generation mice (C57Bl/6 x 129S9) were performed to generate Ing4-homozygous mice. Ing4-heterozygous and Ing4-homozygous mice were recovered in the expected Mendelian ratios from these crosses and appeared phenotypically indistinguishable from their wt littermates. In addition, mouse embryonic fibroblasts (MEFs) were generated from embryos recovered at embryonic day 13.5 (E13.5) of gestation from female partners of Ing4-heterozygous intercrosses. RT-PCR was performed on RNA isolated from Ing4-homozygous MEFs using PCR primers spanning exons 4 to 8 (primers 4 and 5) of the Ing4 gene or using primers contained within exon 8 (primers 6 and 7) of the Ing4 gene (Figure 4.1B). In contrast to wt MEFs, no PCR products were obtained using these primers sets and RNA isolated from the Ing4-null MEFs (Figure 4.1D). These data confirm that the retroviral insertion in Ing4 locus generates an Ing4 message-minus allele, and that mice homozygous for the modified Ing4 allele fail to generate Ing4 message and are Ing4-null mice (Ing4<sup>-/-</sup>). Thus, Ing4 is not required in mice for proper embryonic development.

Fluorescence-activated cell sorting (FACS) analysis was performed on cells recovered from wt and Ing4<sup>-/-</sup> mice. An initial experiment revealed that splenic T and B cells lacking Ing4 displayed a very slight decrease in CD4<sup>+</sup> and CD4<sup>+</sup>CD8<sup>+</sup> T-cell populations, but were indistinguishable from wt mice in the formation of mature B220<sup>+</sup>CD19<sup>+</sup> B-cells, in contrast to what we had observed previously in Ing1-null mice (data not shown).

***Regulation of cell growth by Ing4.***

A standard proliferation assay was performed to assess the growth rate of Ing4<sup>-/-</sup> MEFs and Ing4<sup>+/-</sup> MEFs (Figure 4.2A). Ing4<sup>+/-</sup> cells proliferated slightly slower than wt cells, and Ing4<sup>-/-</sup> cells were much reduced in their ability to proliferate in culture. FACS analysis of synchronized wt MEFs and Ing4<sup>-/-</sup> MEFs revealed a 50% reduction in S phase in MEFs lacking Ing4 following serum stimulation (Figure 4.2B). These results are in keeping with a previous report that Ing4 is required for progression of cells through the S phase of the cell cycle (48, 199), and reveal that physiologic levels of Ing4 do not inhibit cell growth, in contrast to what we observed previously for Ing1 (39).

A number of studies have reported an association between Ing4 and p53 (199, 242, 243). To explore a possible role for Ing4 in regulating p53 activity, we performed several well-established assays for p53 function in MEFs. Ing4<sup>-/-</sup> MEFs failed to form colonies when plated at low density (Figure 4.2C). Although these results are the opposite of what we and others have observed using p53-null MEFs (86), they are consistent with the results of our Ing4<sup>-/-</sup> MEF proliferation assays.



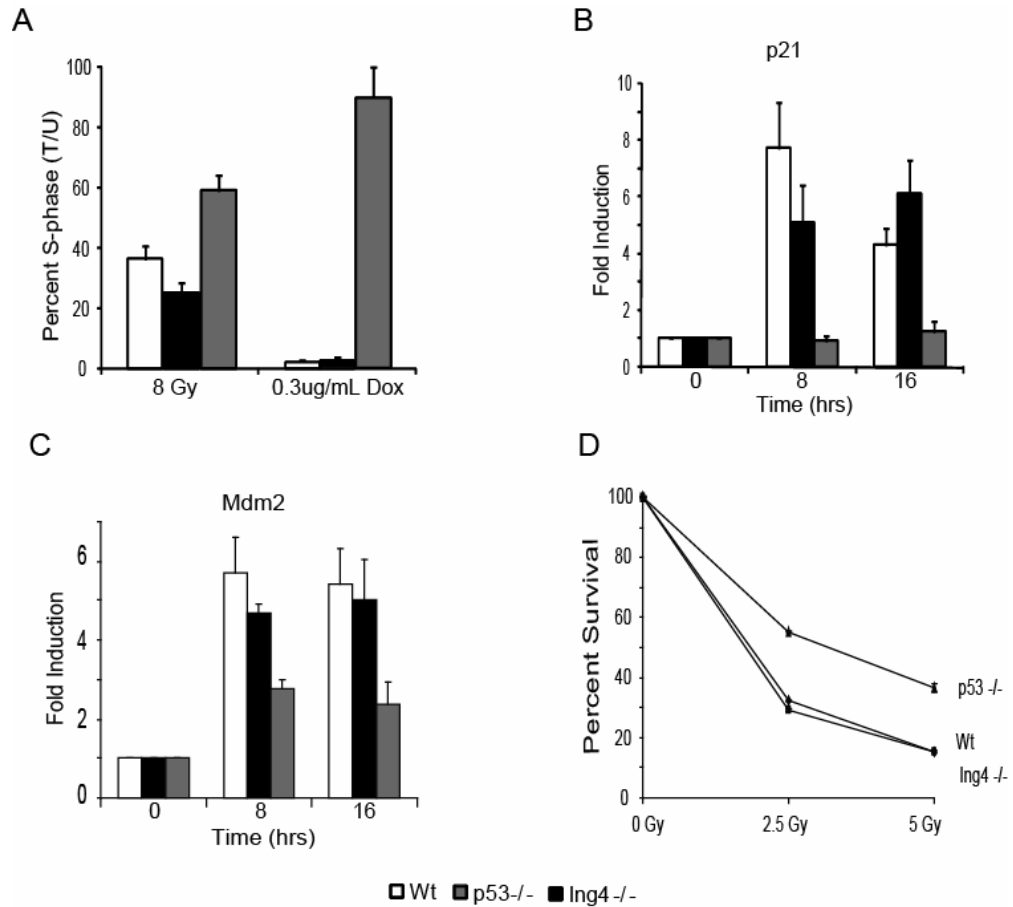
**Figure 4.2:** Growth regulation by Ing4. **A)** Proliferation of Ing4<sup>-/-</sup> cells. Two MEF cell lines for either Ing4 wildtype, heterozygous, or homozygous cells were plated in triplicate and the growth analyzed over the course of 7 days. **B)** Wt or Ing4<sup>-/-</sup> MEFs were serum starved for 3 days, following which serum was added back in order to synchronize the cells which were then harvested, fixed, and sent for FACS analysis. Experiment was repeated three times using three independent lines of cells. Shown are representative propidium iodide (PI) plots. **C)** Growth of Ing4<sup>-/-</sup> cells at low density. Two lines of wildtype, Ing4 null, or p53 null MEFs were seeded at 10<sup>4</sup> cells per 10cm plates and incubated for 8-12 days before being stained with crystal violet.

***Ing4 is not required for p53 response to DNA damage in primary cells.***

Ionizing radiation (IR) or doxorubicin treatment of MEFs induces DNA double strand breaks and a p53-mediated cell cycle arrest. Treatment of growth-synchronized wt and *Ing4*<sup>-/-</sup> MEFs with either 8 Gy IR or with 0.3 µg/mL doxorubicin (Dox) resulted in a large reduction of the percentage of cells in S phase as determined by FACS analysis (Figure 4.3A). In contrast, p53-null MEFs were compromised in their ability to undergo a DNA damage induced growth arrest. These data indicate that the ability p53 to induce cell growth arrest is not compromised in *Ing4* deficient MEFs.

To confirm that p53 activity is unaltered in *Ing4*-null cells, we treated cells with doxorubicin for various lengths of time and examined the expression of *p21* and *Mdm2*; two well-established p53-responsive genes. Quantitative (real-time) PCR was performed, and the results of this experiment (Figures 4.3B and 4.3C) were expressed as relative fold induction of p21 and Mdm2 gene expression. As expected, wt cells displayed increased expression of both genes following DNA damage, whereas p53 null MEFs were compromised in their ability to induce the expression of these p53 target genes. However, no difference was observed in the ability MEFs containing or lacking *Ing4* to induce the expression of these p53-target genes following DNA damage, again indicating that p53 activity is unaltered in *Ing4*-null cells.

Double positive (CD4+CD8+) thymocytes undergo p53-dependent apoptosis following IR exposure (38, 131). To determine if Ing4 alters p53 apoptosis in these cells, we treated wt, p53-null, and Ing4 null thymocytes with varying doses of IR. Thymocytes were collected and stained for annexin V at 4 hours post-IR, and the fraction of surviving cells was plotted versus dose of IR. Following treatment with 5Gy of IR, 20% of wt thymocytes and 20% of Ing4-null cells survived, whereas 40% of p53-null thymocytes remained viable (Figure 4.3D). This data indicates that loss of Ing4 does not alter p53-mediated apoptosis in thymocytes. These results, coupled with the MEFs analysis, indicate that Ing4 is not required for proper p53 function.



**Figure 4.3:** Classical p53 responses are not altered in Ing4<sup>-/-</sup> cells. **A)** Absence of Ing4 does not alter cell cycle arrest due to DNA damaging agents. Three lines of wt, Ing4-homozygous, or p53-null MEFs were duplicate plated and synchronized in their growth prior to mock-treatment or treatment with doxorubicin or IR. Cells were stained with BrdU and PI, and analyzed by FACS. Results are expressed as a ratio of treated cells in S-phase versus untreated cells in S-phase. Quantitative real-time RT-PCR was performed using primers to **B)** *p21* or **C)** *Mdm2* on RNA from MEFs treated with 0.3  $\mu$ g/mL of doxorubicin and harvested at the indicated time. Graphs show the average and standard deviation from three independent lines. **D)** Thymocytes were isolated from wildtype, Ing4 null, or p53-null mice and either mock-treated or irradiated, then stained with annexin V and PI. Graph shows the average percent survival (annexin V-/PI-) and standard deviation. Each time point was done in triplicate and shown is representative of two independent experiments.



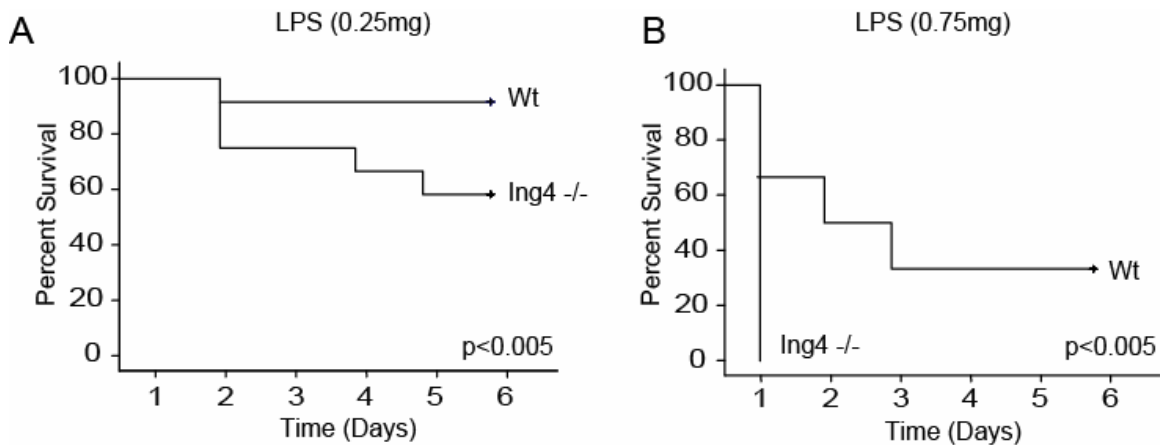
***Mice deficient for Ing4 do not develop spontaneous tumors***

A number of studies have linked ING4 loss with the development of cancer in humans (63, 70, 77). Additionally, the connection between ING4 and p53 (199) would suggest a possible tumor suppressive function for ING4. To determine if Ing4 deletion could induce the formation of spontaneous tumors in mice, we established cohorts of wt mice (n=50) and Ing4<sup>-/-</sup> mice (n=45) and aged them for 24 months. No tumors were observed in either population over the course of the study. Upon necropsy at 24 months, several Ing4 null mice presented with slightly enlarged spleens (data not shown), but this was determined to be extramedullary hematopoiesis and not cancer. Therefore, unlike deletion of p53 (47) or Ing1 (39, 108), deletion of Ing4 does not induce formation of spontaneous tumors in mice, indicating that p53-mediated suppression of spontaneous tumorigenesis does not require functional Ing4.

***Suppression of innate immunity by Ing4.***

A recent report indicates that ING4 directly binds to RelA (p65) and acts as a repressor of NF- $\kappa$ B responsive genes in glioblastoma cells (63). To determine if NF- $\kappa$ B activity was altered in mice lacking Ing4, we examined the sensitivity of Ing4-deficient mice to lipopolysaccharide (LPS) challenge, which is known to activate NF- $\kappa$ B signaling via the TLR4 receptor. Twelve wt or Ing4<sup>-/-</sup> mice were intraperitoneally (i.p.) injected with either 0.25mg (Figure 4.4A) or 0.75mg LPS and monitored over the course of one week (Figure 4.4B). Ing4<sup>-/-</sup> mice were more susceptible than wt mice to LPS at either dose.

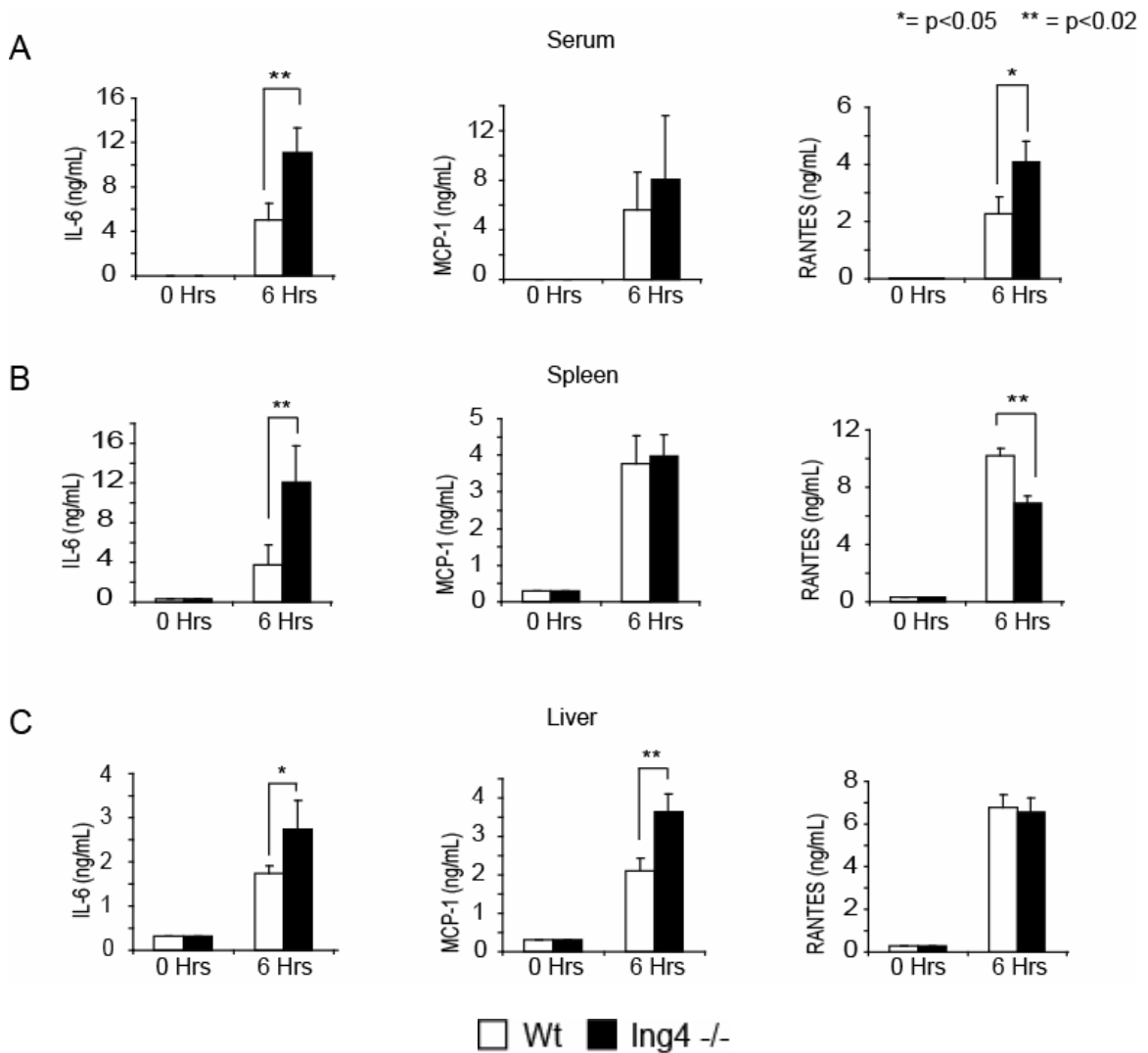
Nearly half (5/12) of the *Ing4*<sup>-/-</sup> mice exhibited morbidity at the low dose, whereas only 1/12 of the wt mice died by 6 days post-injection. An even greater difference in the response of the mice to LPS was seen at the higher dose. All 12 of the *Ing4*<sup>-/-</sup> mice died within 24 hours of LPS injection, whereas only a third (4/12) of the wt mice died at 24 hours, and one-third of the LPS treated wt mice were normal throughout the study period. These results indicate that *Ing4*-deficient mice are hypersensitive to LPS.



**Figure 4.4:** *Ing4*<sup>-/-</sup> mice are hypersensitive when treated with LPS. Age matched wt (n=12) or *Ing4*<sup>-/-</sup> (n=12) mice were injected with either 0.25mg (A) or 0.75mg (B) LPS and monitored for morbidity over the course of one week. Graphs show Kaplan-Meier survival analysis and at both doses there was a significant difference between wt and *Ing4*<sup>-/-</sup> survival ( $p \leq 0.005$ ).

We examined cytokine levels in the serum, spleen, liver, and lung of mice after LPS treatment to determine if NF- $\kappa$ B activity was elevated. Wt mice (n=8) and Ing4<sup>-/-</sup> mice (n=8) were either mock injected or injected i.p. with 0.5mg LPS, and serum and organs were collected at 6 hours post-injection. ELISAs were conducted to determine the levels of Il-6, Rantes, and Mcp-1 in these samples, as the expression of these cytokines are activated in response to LPS, either directly through MyD88-dependent NF- $\kappa$ B signaling, or through MyD88-independent upregulation of the IRF-3 transcription factor, or through a combination of both pathways (162).

Significant elevation of Il-6 was seen in the serum (Figure 4.5A), spleen (Figure 4.5B), and liver (Figure 4.5C) of Ing4<sup>-/-</sup> mice relative to wt levels following LPS injection. Rantes was also elevated in serum and in spleen of Ing4<sup>-/-</sup> mice compared to wt controls, and Mcp-1 was increased in liver (Figure 4.5C) and in lung (data not shown) in the Ing4<sup>-/-</sup> mice. The increase in cytokine levels observed in the Ing4<sup>-/-</sup> mice after LPS treatment suggests that NF- $\kappa$ B activity is upregulated in these mice.



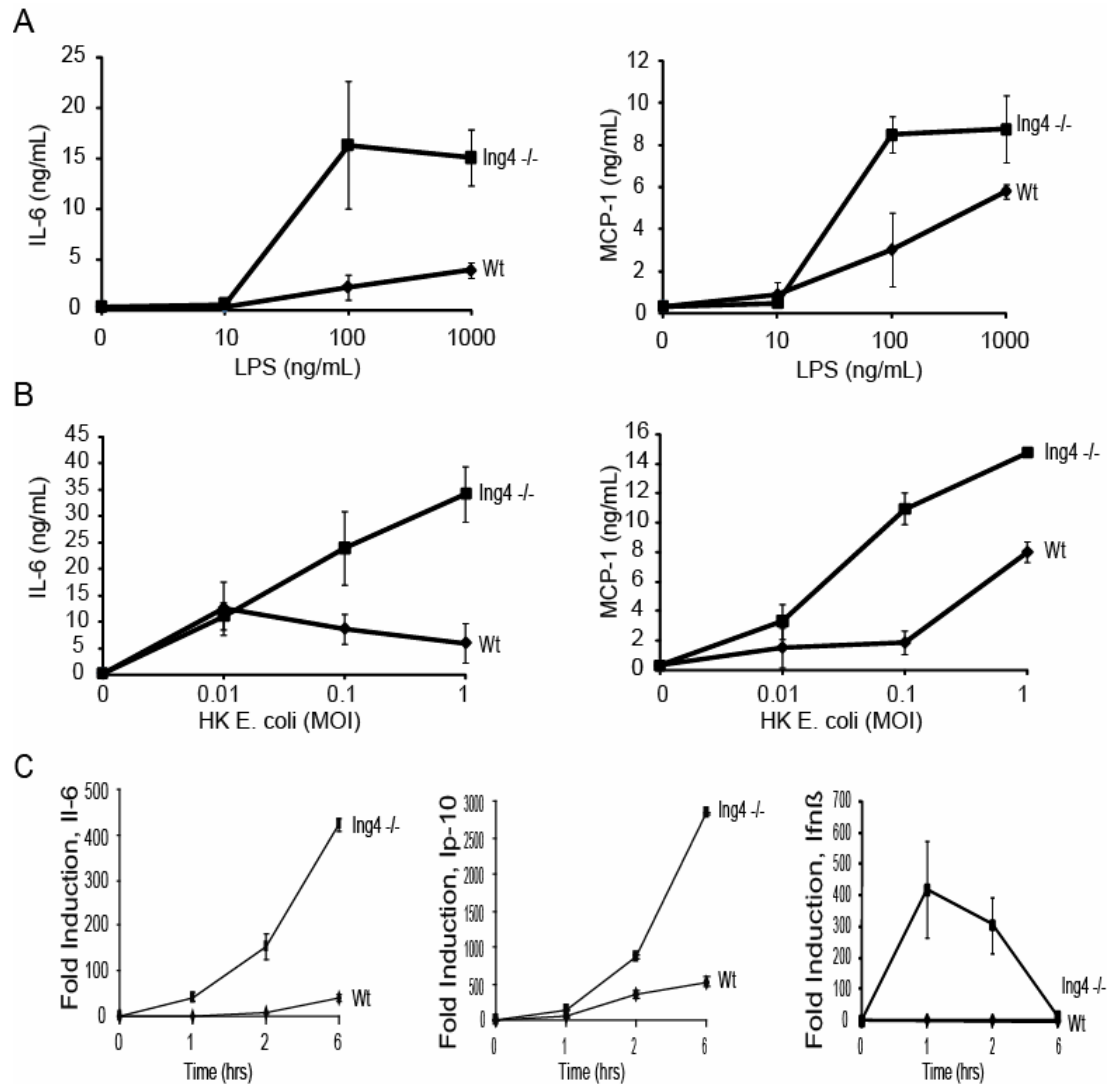
**Figure 4.5:** Ing4 null mice show elevated cytokine levels in the serum and organs following treatment with LPS. ELISAs for Il-6, Mcp-1, or Rantes were performed on serum (A), spleen (B), or liver (C) samples. Either wt (n=8) or Ing4 null (n=8) mice were injected with 0.5mg LPS and serum and organs were collected at 0 or 6 hrs after injection. Graphs show geometric mean with standard error. P-values are \* $p \leq 0.05$  and \*\* $p \leq 0.02$ .

***Ing4 null macrophages display elevated expression of NF- $\kappa$ B -responsive genes.***

Macrophages respond to bacterial infection by secreting pro-inflammatory cytokines and chemokines (235) and are essential components of innate immunity. *E. coli* and certain bacterial components such as LPS are known to activate NF- $\kappa$ B signaling in macrophages by binding to Toll-like receptors on these cells. In order to further examine the hypersensitivity of *Ing4*<sup>-/-</sup> mice to LPS, we generated thioglycolate-elicited peritoneal macrophages by injecting wt or *Ing4*<sup>-/-</sup> mice with 4% thioglycolate and collecting peritoneal exudate cells by lavage at 4 days post-induction. Macrophages were plated at a density of 10<sup>6</sup> cells per well in a 24 well plate and either mock treated or treated with either LPS or heat-attenuated *E. coli*. Macrophage supernatants were collected 24 hours after treatment, and used in ELISA assays to determine the levels of Il-6 and Mcp-1. Similar to what was observed *in vivo*, Il-6 expression was elevated in *Ing4*<sup>-/-</sup> macrophages relative to wt macrophages after exposure of cells to either LPS or to heat killed bacteria. We also detected a slight, but significant, difference in the levels of Mcp-1 in cells lacking *Ing4* following LPS or *E. coli* exposure (Figures 4.6A and 4.6B).

Quantitative real-time PCR (qPCR) was performed on RNA isolated from macrophages that were either mock-treated or treated with 1  $\mu$ g/ml LPS for 1, 2, or 6 hours to document the expression levels of various cytokines known to be activated by NF- $\kappa$ B signaling. Expression of *Il-6* and *Ip-10* was at least 4-fold higher in *Ing4*<sup>-/-</sup> cells than in wt cells (Figure 4.6C).

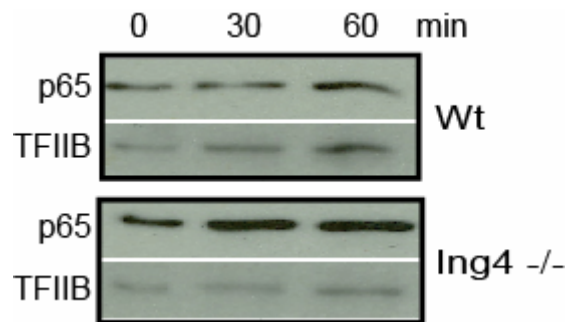
In addition, *IFN- $\beta$*  expression levels were dramatically higher in *Ing4*<sup>-/-</sup> cells at 1 or 2 hours after LPS treatment, but returned to wt levels after 6 hours (Figure 4.6C). This data indicate that *Ing4* negatively regulates the expression of NF- $\kappa$ B-responsive genes following exposure of macrophages to LPS.



**Figure 4.6:** Ing4 null thioglycolate-elicited peritoneal macrophages also show elevated cytokine levels. Macrophages from 4-5 wt or Ing4 null mice were pooled, plated at a density of  $10^6$  cells per well of a 24-well plate and either mock treated or treated with increasing amounts of LPS (A) or heat killed (HK) *E. coli* (B). ELISAs for Il-6 and Mcp-1 were performed on the supernatants 24 hours after treatment. MOI: multiplicity of infection. C) Expression of *Il-6*, *Ip-10*, and *Ifnβ* in macrophages following treatment with LPS. Macrophages from 4-5 wt or Ing4 null mice were pooled, plated at a density of  $10^6$  per 10cm plate, and treated with  $1\mu\text{g/mL}$  LPS, and then RNA was harvested at the indicated times. The qRT-PCR was done in triplicate. All graphs show average with standard deviations.

***Ing4 inhibits nuclear NF- $\kappa$ B levels and activity.***

In order to explore the molecular basis of altered NF- $\kappa$ B activity in *Ing4*<sup>-/-</sup> cells, we examined the p65/RelA levels in the nuclear compartment of macrophages. *Ing4*<sup>-/-</sup> macrophages and wt macrophages were untreated (0 minutes) or treated with 1  $\mu$ g/mL LPS and harvested at 30 or 60 minutes post-treatment. Nuclear extracts were prepared and analyzed via western blot for total p65/RelA (Figure 4.7). A clear elevation over wt levels was observed in nuclear p65/RelA levels in macrophages lacking *Ing4* following treatment with LPS.

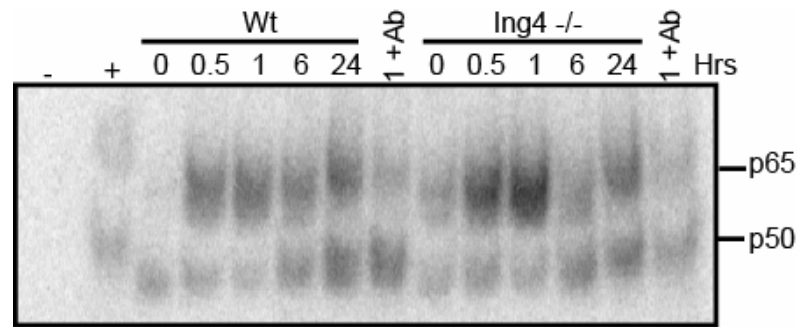


**Figure 4.7:** Higher nuclear p65/RelA levels in *Ing4* null macrophages. **A)** Nuclear extracts from wt or *Ing4* null macrophages treated with 1  $\mu$ g/mL of LPS were used in western blots to determine the amount of p65/RelA at each time point. TFIIIB was used as a loading control. Wt and *Ing4*<sup>-/-</sup> panels were from the same gel.

Electrophoresis mobility shift assays (EMSA) were performed with these nuclear extracts using a canonical RelA/p65-DNA sequence motif (163). The results revealed increased p65 binding in untreated *Ing4*<sup>-/-</sup> macrophage nuclear extracts relative to untreated wt extracts (Figure 4.8). In addition, *Ing4*<sup>-/-</sup> macrophages showed an increase in p65/RelA promoter binding at 30 or 60 minutes after LPS treatment relative to wt cells.



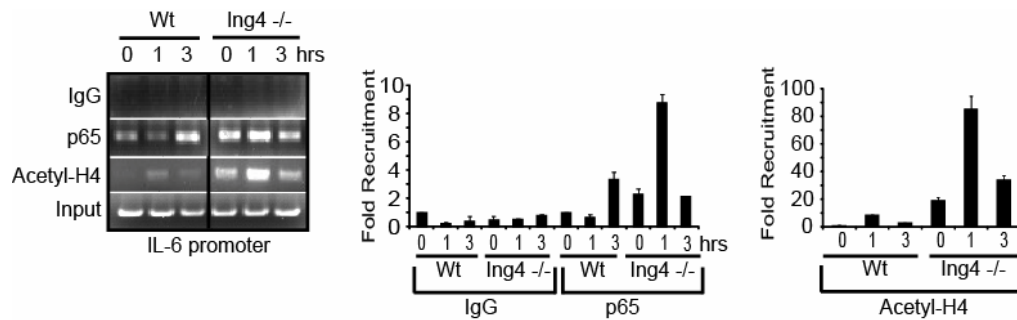
However, this difference in RelA/p65 promoter binding was lost at 6 hours post-treatment. An antibody to p65 was added to the nuclear fractions prior to the EMSA and resulted in the disappearance of the higher p65 band at 1 hour after LPS treatment, indicating that the observed binding in the EMSA assay was p65-specific (Figure 5B; 1 + Ab lanes).



**Figure 4.8:** EMSA of nuclear extracts from Ing4 null and wt thioglycolate-elicited macrophages. Macrophages were prepared as before, treated with 1  $\mu$ g/mL of LPS, and harvested at the indicated time points. Nuclear extracts were prepared and subjected to the EMSA protocol. An antibody to p65/RelA was also added to the 1 hour samples in order to determine if the observed binding was specific for p65/RelA. The first lane is a negative control (-); reaction mixture without nuclear extract, and the second lane is a positive control (+); reaction mixture with HeLa cell nuclear extract.

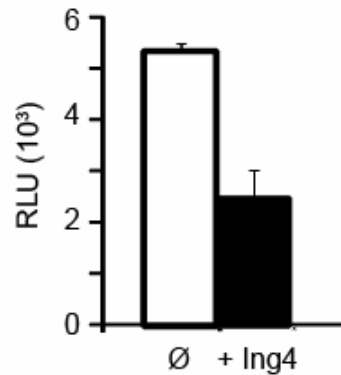
To confirm the increased RelA/p65 binding in the nuclear compartment of Ing4<sup>-/-</sup> macrophages, we performed chromatin immunoprecipitation (ChIP) experiments to examine recruitment of RelA/p65 to the endogenous Il-6 promoter. Ing4<sup>-/-</sup> or wt macrophages were either mock-treated or treated with LPS for 1 hour or 3 hours. Immunoprecipitation of crosslinked DNA was performed using either a p65/RelA antibody or an antibody to acetylated histone H4, and a qPCR to Il-6 promoter sequences was performed.

In agreement with our *in vitro* EMSA results, greater recruitment of p65/RelA to the Il-6 promoter was observed in the *Ing4*<sup>-/-</sup> macrophages at 0 hours and at 1 hour (Figure 4.9). After 3 hours of LPS treatment, less difference was seen in the amount of p65/RelA recruitment to the Il-6 promoter. This recruitment was specific, as we did not observe binding in ChIPs that employed an antibody to IgG. Furthermore, the large difference seen in p65/RelA binding to the Il-6 promoter correlated with increased levels of acetylated histone H4 at the Il-6 promoter, suggesting that binding of NF- $\kappa$ B to the Il-6 promoter is inducing transcriptional activation of the Il-6 gene. These data are in agreement with our qPCR results indicating upregulation of *Il-6* expression in *Ing4*<sup>-/-</sup> cells (Figure 4.6C), and further indicates that histone H4 can be acetylated even in the absence of *Ing4*. Human *ING4* was recently found as a component of the HBO1 HAT complex, which is responsible for the majority of global H4 acetylation (48).



**Figure 4.9:** Elevated binding of p65/RelA to the Il-6 promoter was seen in *Ing4* null macrophages. Chromatin immunoprecipitation (ChIP) experiments were performed according to the manufacturer's instructions (USB Cleveland OH). Antibodies to either p65/RelA or acetylated-H4 were used to immunoprecipitate the chromatin bound protein. Anti-IgG antibodies were used as a control for specificity. Real-time quantitative PCR using SYBR green was done in triplicate. Shown are a representative gel picture of the PCR products and a graph quantifying the results which are displayed as the average  $\pm$  standard deviation.

To confirm that mouse Ing4 can suppress NF- $\kappa$ B activity, we transfected HEK-293T cells with a luciferase reporter gene placed under transcriptional control of an NF- $\kappa$ B promoter (Figure 4.10). In agreement with previously published transfection data (63), co-transfection of Ing4 expression vector suppressed luciferase activity in this experiment, confirming that Ing4 inhibits RelA/p65 transactivation of NF- $\kappa$ B regulated gene expression.

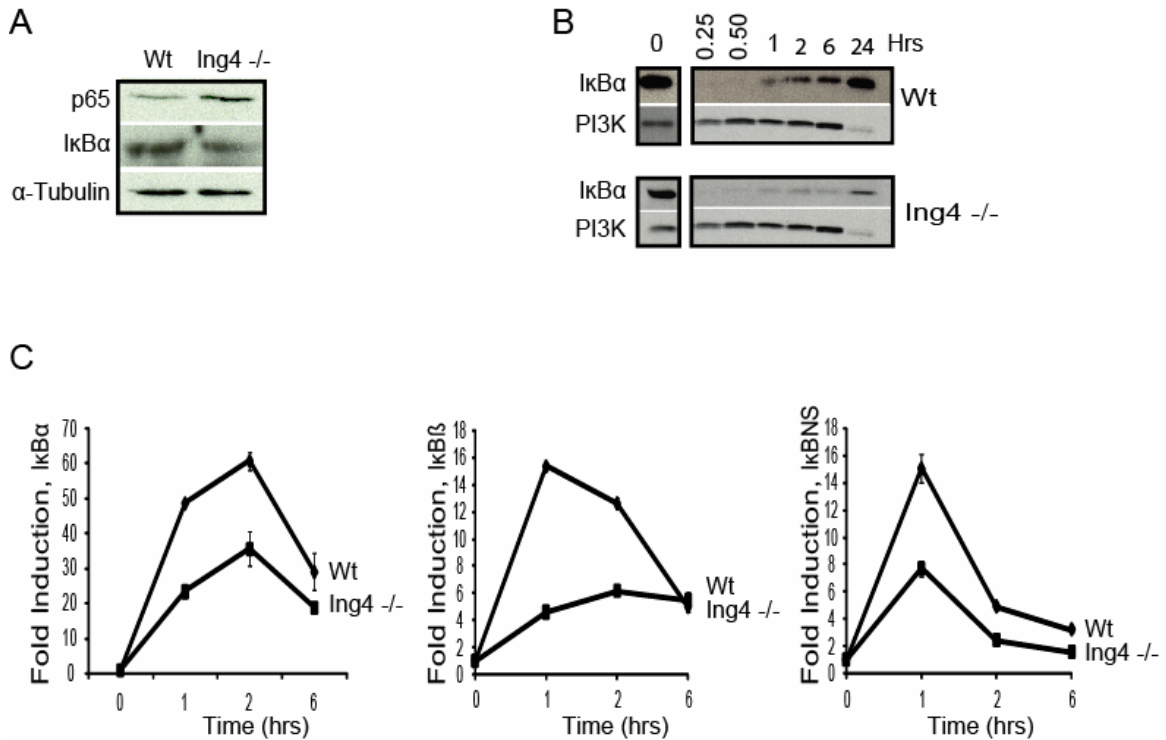


**Figure 4.10:** Overexpression of Ing4 inhibited the expression of luciferase from a NF- $\kappa$ B reporter. HEK293T cells were transfected with plasmids containing the Renilla luciferase gene, an NF- $\kappa$ B luciferase reporter construct, and the RelA cDNA. Half the transfected HEK293T cells were also transfected with a plasmid containing the Ing4 cDNA. One day after transfection, cells were treated with 1 $\mu$ g/mL of LPS for 6 hours before being harvested to perform either a luciferase assay or qRT-PCR. Graphs show the average and standard deviation of three independent experiments.

***Ing4 upregulates I $\kappa$ B expression.***

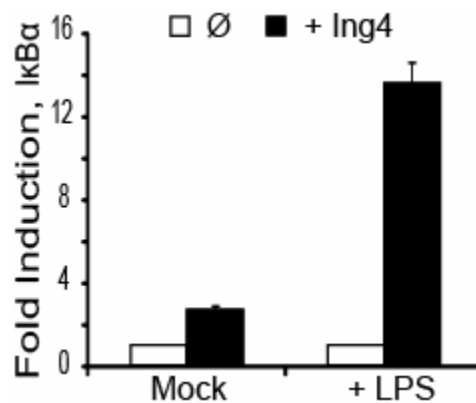
Our results indicate that nuclear p65 levels and DNA binding activity was increased in both LPS-stimulated and non-stimulated *Ing4*<sup>-/-</sup> macrophages (Figures 4.8 through 4.9). Since nuclear levels of p65/RelA and NF- $\kappa$ B promoter activation are tightly regulated by I $\kappa$ B proteins (65, 165), we examined if I $\kappa$ B levels were altered in the presence or absence of *Ing4*.

Interestingly, we observed increased levels of p65/RelA protein and decreased levels of I $\kappa$ B $\alpha$  protein in untreated whole cell extracts of *Ing4*<sup>-/-</sup> macrophages (Figure 4.11A). To confirm this observation, western blots for I $\kappa$ B $\alpha$  were performed using cytoplasmic fractions isolated from LPS-treated wt and *Ing4*<sup>-/-</sup> macrophages. In wt cells, the level of I $\kappa$ B $\alpha$  is greatly reduced 15 minutes after LPS treatment, and begins to recover by 60 minutes post-treatment (Figure 4.11B). Although *Ing4*<sup>-/-</sup> cells also display reduced I $\kappa$ B $\alpha$  levels 15 minutes after LPS treatment, the kinetics of I $\kappa$ B $\alpha$  re-synthesis appear delayed relative to wt cells, and I $\kappa$ B $\alpha$  levels remain lower in *Ing4*<sup>-/-</sup> cells than in wt cells even after 24 hours. To determine if the reduced amount of I $\kappa$ B $\alpha$  protein in these cells is reflecting a decrease in the expression of I $\kappa$ B genes, we performed qPCR on RNA isolated from LPS-treated wt and *Ing4*<sup>-/-</sup> macrophages (Figure 4.11C). These results indicate that expression levels of *I $\kappa$ B $\alpha$* , *I $\kappa$ B $\beta$* , and *I $\kappa$ BNS* are lower in *Ing4*<sup>-/-</sup> cells compared to the expression of these genes in wt cells.



**Figure 4.11:** Absence of Ing4 caused a decrease in *IκB* transcript levels. **A)** Whole cell extracts from wt and Ing4 null macrophages were run on a 10% SDS-PAGE gel and probed with antibodies to p65/RelA or IκBα. Tubulin was used as a loading control. **B)** Wt or Ing4<sup>-/-</sup> macrophages were either mock treated or treated with 1 μg/mL of LPS for the indicated times. Following treatment, cytoplasmic fractions were run on a 10% SDS-PAGE gel and probed using an antibody to IκBα. PI-3-kinase was used as a loading control. **C)** Quantitative real-time RT-PCR using primers to *IκBα*, *IκBβ*, *IκBNS* was performed on RNA from wt or Ing4 null macrophages treated with LPS (1 μg/mL) for the indicated time points. All samples were done in triplicate and shown are averages and standard deviations.

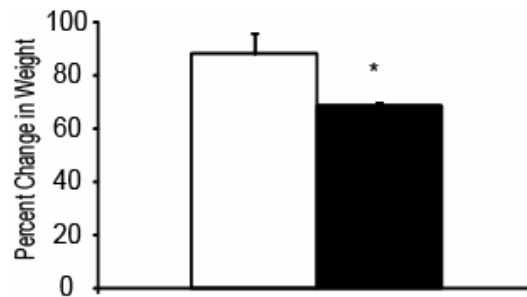
I $\kappa$ B genes are known targets of p65/RelA activation and form part of a negative feedback loop proposed to regulate RelA activity in the cell (141). Thus, inhibition of NF- $\kappa$ B activity by binding of Ing4 to RelA might be expected to inhibit the expression of the I $\kappa$ B genes. However, the expression of the I $\kappa$ B genes is upregulated by Ing4. Therefore, either Ing4 binding to RelA can have opposing effects of different RelA-responsive promoters, or Ing4 negatively regulates p65/RelA activity by positively regulating the expression of I $\kappa$ B inhibitors. To further validate this finding, we transfected an Ing4 expression vector into HEK-293T cells and measured the level of *I $\kappa$ B $\alpha$*  expression by qPCR. *I $\kappa$ B $\alpha$*  expression was elevated in both mock and LPS-treated cells overexpressing Ing4 (Figure 4.12). These results confirm that Ing4 upregulates the expression of this NF- $\kappa$ B inhibitor.



**Figure 4.12:** Overexpression of Ing4 enhances the expression of *I $\kappa$ B $\alpha$* . HEK293T cells were transfected with plasmids containing cDNA for p65/RelA and Ing4. One day after transfection, cells were treated with 1 $\mu$ g/mL of LPS for 6 hours before being harvested to for RNA in order to perform qRT-PCR. Graphs show the average and standard deviation of three independent experiments.

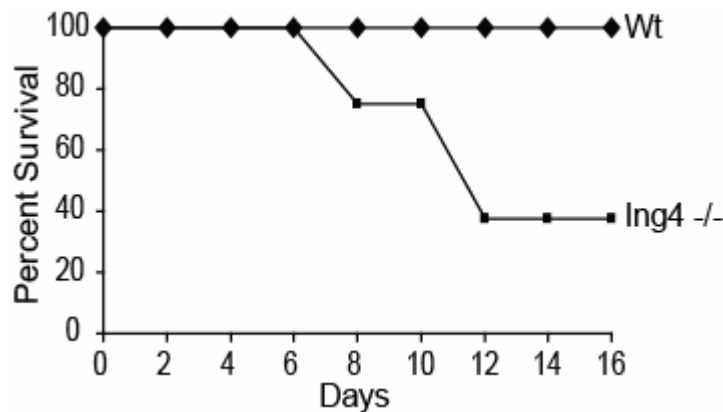
***Ing4<sup>-/-</sup> mice are hypersensitive to inflammation***

Our data indicates that Ing4 acts as a negative regulator of NF- $\kappa$ B function by upregulating the expression of *I $\kappa$ B* genes. Previous studies (75, 117) of genetically engineered mice deficient for I $\kappa$ B genes such as *I $\kappa$ BNS* have revealed that these mice have an exaggerated inflammatory response in the colon following oral administration of dextran sodium sulphate (DSS) (117). To determine if our Ing4<sup>-/-</sup> model is likewise hypersensitive to inflammation, we treated eight (6-8 week old) Ing4<sup>-/-</sup> mice and eight (6-8 week old) control wt mice with a single dose regimen of DSS (5 days on DSS in the drinking water followed by 10 days of untreated water). After DSS treatment, Ing4-null mice lost more body weight than the wt controls (Figure 4.13). Several factors can contribute to loss of body weight; one is the inflammatory response, which can cause loss of fluids or prevent nutrient absorption by the intestines.



**Figure 4.13:** Ing4 null mice show a greater drop in body weight following treatment with dextran sodium sulfate (DSS). 6-8 week old Ing4 null (n=8) and wt (n=8) mice were given 4% DSS in their drinking water for 5 days followed by 10 days of normal water. The mice were weighed initially and then every day after the exposure to DSS. The graph shows the average plus standard deviation of the percent change in weight of each group of 8 mice at day 7 of the regimen compared to the initial weighing. There is a statistically significant difference between the two groups (\* $p \leq 0.05$ ).

Two days after DSS administration, (day 7 of the assay), *Ing4*<sup>-/-</sup> mice began to display morbidity, and 60% of the *Ing4*<sup>-/-</sup> mice died within 1 week (day 12 of the assay). In contrast, none of the wt mice died during the course of this experiment (Figure 4.14).

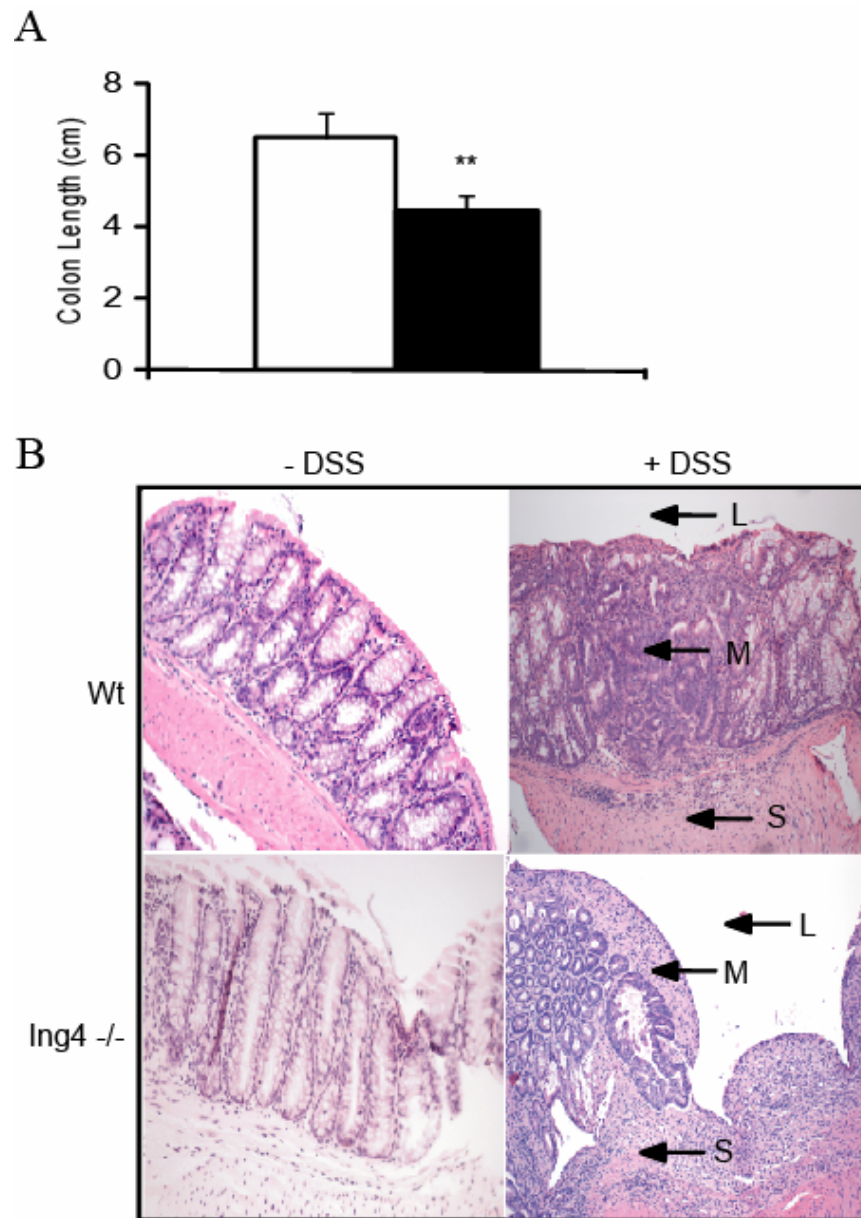


**Figure 4.14:** Kaplan-Meier survival curve for *Ing4*<sup>-/-</sup> (n=8) and Wt (n=8) mice during DSS treatment. Mice were given 4% DSS in their drinking water for 5 days followed by 10 days of untreated water.

The colons were harvested from all mice, either at their time of death or on day 16 of the experiment, and processed for histology. *Ing4*<sup>-/-</sup> colons were shrunken in length relative to wt mouse colons (Figure 4.15A) and histology revealed that *Ing4*<sup>-/-</sup> mice exhibited complete loss of the intestinal mucosal layer (Figure 4.15B). This data indicate that *Ing4*<sup>-/-</sup> mice exhibited a massive inflammatory response that destroyed the mucosal lining of the intestine. These findings are consistent with the elevated serum and macrophage cytokine levels observed in *Ing4*<sup>-/-</sup> mice following LPS treatment.



The similarity in the inflammatory response of *IkBNS*<sup>-/-</sup> mice and *Ing4*<sup>-/-</sup> mice to DSS further supports our proposal that *Ing4* acts as a negative regulator of NF- $\kappa$ B activity by upregulating expression of *I $\kappa$ B* genes.



**Figure 4.15:** Ing4 null mice treated with DSS show a reduction of colon length and complete removal of the intestinal mucosa. **A)** The graph shows the average plus standard deviation of the colon length for each group of 8 mice upon necropsy. There is a statistically significant difference between the two groups (\*\* $p \leq 0.02$ ). **B)** Colons were also fixed in 10% phosphate buffered formalin, stained with hematoxylin and eosin, and subsequently analyzed by a pathologist. Pictures were taken at the 20X magnification. Arrows point to the different layers of the intestine which are abbreviated as: serosa (S), mucosa (M), and lumen (L).

## Discussion

In this study, we generated Ing4 deficient mice and primary cells in order to explore the role of Ing4 in development, in cell growth, and in tumorigenesis. Our results reveal that Ing4 is not required during embryogenesis, yet is necessary to promote the proliferation of MEFs. This finding is consistent with previous reports that the Ing4-HB01 complex regulates a majority of histone H4 acetylation in cells and is required for proper progression of cells through S phase of the cell cycle (48, 209). Interestingly, histone H4 acetylation can be readily detected in ChIP analysis on the active IL-6 promoter in Ing4<sup>-/-</sup> macrophages, indicating that other HAT complexes are sufficient to induce chromatin remodeling in cells lacking Ing4. One mechanism proposed for Ing4 regulation of cell growth involves regulation of the p53 tumor suppressor. However, p53 functions appear to be unaltered by deletion of Ing4, as wt cells and Ing4-null cells responded similarly to DNA damage-induced growth arrest and apoptosis mediated by p53. These findings suggest that the p53 pathway is not compromised in Ing4-null mice.

ING4 has also been recently proposed to complex with the large subunit of NF- $\kappa$ B and to inhibit NF- $\kappa$ B-mediated transactivation of gene expression (63). As NF- $\kappa$ B plays a role in multiple biological processes, including angiogenesis, apoptosis, and the innate and adaptive immune response (3, 105), regulation of NF- $\kappa$ B by Ing4 could also impact tumorigenesis. To examine the ability of Ing4 to regulate NF- $\kappa$ B activity *in vivo*, we examined the innate immune response of Ing4<sup>-/-</sup> mice.

LPS treatment of Ing4-null mice were highly sensitive to LPS treatment, and contained elevated levels of IL-6 protein in serum and in various organs, indicating that Ing4 suppresses NF- $\kappa$ B signaling in mice. Peritoneal-derived, Ing4<sup>-/-</sup>macrophages displayed elevated levels of nuclear RelA/p65, increased binding of RelA/p65 to relevant NF- $\kappa$ B promoters, and increased cytokine gene expression following LPS treatment. Upregulation of NF- $\kappa$ B activity in mice deleted for Ing4 was likely due to decreased expression of the RelA inhibitors *I $\kappa$ B $\alpha$* , *I $\kappa$ B $\beta$* , and *I $\kappa$ BNS*. Furthermore, mice deficient for Ing4 displayed a similar colorectal hyperinflammatory response to DSS as reported for *I $\kappa$ BNS*-deficient mice (117), offering further genetic evidence that deletion of Ing4 results in inhibition of I $\kappa$ B activity *in vivo*.

Collectively, these results reveal that physiologic levels of Ing4 inhibit NF- $\kappa$ B activity by facilitating I $\kappa$ B gene expression in mice, and establish a role for Ing4 in regulating NF- $\kappa$ B-mediated innate immunity. Interestingly, Ing4 deficient mice failed to develop spontaneous tumors, in contrast to what we and others have reported previously for mice deficient for Ing1 proteins (21, 28). Several reports have previously linked aberrant NF- $\kappa$ B expression to the onset of various human cancers (3, 100, 102, 104, 105, 134, 169). However, the role of NF- $\kappa$ B in tumorigenesis is uncertain, and NF- $\kappa$ B may exert oncogenic and anti-tumorigenic effects in a cell context-dependent manner.

Although NF- $\kappa$ B activity is clearly altered in Ing4-null mice, further research is needed to establish Ing4 as a *bone fide* tumor suppressor and to determine if Ing4 regulates tumorigenesis in mice when cancer is induced by activated oncogene expression and/or by DNA damage.

## **Materials and Methods**

### **Generation of Ing4 knockout mice**

Mouse (129 strain) embryonic stem cells (BayGenomics clone XG370) containing a retroviral promoter gene trap inserted into one allele of Ing4 were used to generate chimeric mice, which were bred to generate Ing4-heterozygous mice and subsequent Ing4-homozygous mice. Mice were genotyped by PCR analysis of genomic DNA using the following primer sequences: ING4g1439F: 5'-CGGGCGGATTTCTAAGTTCG -3', ING4g2118R: 5'-AAAAGACAAAACAAGAGGGGGC -3', ING4c8015F: 5' – AAGGGAATAAGGGCGACACG – 3'. All mice were maintained and used in accordance with federal guidelines and those established by the University of Massachusetts Animal Care and Use Committee.

### **Cell culture**

Mouse embryonic fibroblasts (MEFs) were generated from E13.5 day embryos as described previously (39). Macrophages were generated as previously described (116). In brief, mice were injected i.p. with 4% thioglycolate and cells were harvested 4 days later. HBSS was used to flush the peritoneal cavities.

Peritoneal exudates cells were plated in RPMI1640 with 10% heat-inactivated FCS.

Thymocytes were generated by grinding thymi between frosted glass slides and cells were assayed for viability by trypan blue exclusion. Approximately  $10^6$  cells were plated per well of a 60-mm plate in RPMI1640 with 5% FCS prior to each experiment. All cells were incubated at 37°C and 5% CO<sub>2</sub>.

### **MEF experiments**

To determine the rates of cell proliferation, multiple lines of Wt, Ing4-heterozygous, or Ing4-null MEFs were seeded at  $1 \times 10^5$  per well of a six-well plate. Triplicate plates of each line were harvested and counted every 24 h using a Z1 Coulter Particle Counter (Beckman Coulter, Miami, FL). Duplicate gelatinized 10-cm plates with 10,000 cells per plate were seeded in MEF medium to assay for cell survival and growth at low plating density. At 8 to 12 days postplating, cells were fixed with methanol and stained with 0.1% crystal violet to visualize colony formation. MEFs from three independent lines of each genotype were seeded onto 10-cm plates at a density of  $8 \times 10^5$  per plate in 0.1% fetal bovine serum for 3 days. Cells were then fed with medium supplemented with 10% serum for 4 h, and the cultures left untreated or exposed to 8 Gy of  $\gamma$ -radiation in a cesium irradiator or 0.3  $\mu\text{g}/\text{mL}$  doxorubicin (Sigma-Aldrich, St. Louis, MO). At 15 h post-treatment, MEFs were pulse labeled with 60  $\mu\text{mol}/\text{L}$  bromodeoxyuridine (BrdUrd) for 3 h, harvested by trypsinization into PBS, and fixed in 70% ethanol overnight at 4°C.

Flow cytometric analysis of DNA synthesis and total DNA content was done using an anti-BrdUrd antibody, propidium iodide staining, and Flowjo software. Data are presented as a ratio of percentage of cells in S phase for treated versus untreated cells.

### **Thymocyte apoptosis**

Ex vivo thymocyte apoptosis experiments were done by plating  $10^6$  thymocytes onto 60-mm plates in RPMI medium with 5% FCS and either mock treating or irradiating the plates with 2.5, or 5-Gy ionizing radiation. After 4 h, cells were harvested and stained with annexin V and 7-AAD (BD Pharmingen, San Jose, CA) according to the manufacturer's instructions, then analyzed by FACS.

### **LPS challenge and ELISAs**

Mice were injected i.p. with either 0.25mg or 0.75 mg LPS from *Escherichia coli* serotype O111:B4 (Sigma-Aldrich, St. Louis, MO) and monitored for one week. In order to determine cytokine levels, 0.5mg LPS was injected i.p and organs or serum were harvested at 0, 6, or 24 hrs. ELISAs were performed according to the manufacturer's instructions (BD Pharmingen, San Jose, CA).

### **Quantitative real-time RT-PCR (qRT-PCR)**

Relative levels of mRNA expression were analyzed by quantitative reverse transcription-PCR (RT-PCR) as previously described (136).

cDNA was generated using Invitrogen SuperScript first-strand synthesis system for RT-PCR. The following primer sequences (shown 5'-3') were used in the PCR reactions:

*IκBα*: TTCCTGCACTTGGCAATCATCCAC and  
TCAGGATCACAGCCAGCTTTCAGA,

*Ip10*: TATCGATGACGGGCCAGTGAGAAT and  
TGATCTCAACACGTGGGCAGGATA,

*Ifnβ*: TCCTGGAGCAGCTGAATGGAAAGA and  
TCTGGAGCATCTCTTGGATGGCAA,

*Ing4*: GACAGCTCTTCCAGCAAAGGCAAA and  
TGCGATCCACAGGCATATCCAACA,

*Il-6*: AGTCACAGAAGGAGTGGCTAAGGA and

TCTGACCACAGTGAGGAATGTCCA, *IκBβ*: TGATTCATCAGCATGAGCCCTTCC  
and TTTGGCCTAGGTCATTCTGCAGGT, *IκBNS*:

TTTCCCTGCCTTCCAAGAGACTGT and AGCCTTCAGAAGCAGAGAATGGGA.

Samples were normalized to *EF1α* levels present in each tissue as previously described (136).

### **Luciferase assay**

HEK293FT cells were plated at a density of  $5 \times 10^4$  cells per well of a 96-well plate. The following day they were transfected with a consensus NF-κB luciferase reporter construct, a RelA plasmid, the *Renilla* expression plasmid, and either a plasmid containing the Ing4 cDNA or plasmid backbone using Gene Juice transfection reagent.



One day post transfection the cells were stimulated with 1ug/mL LPS for 4-6 hours. Both firefly and *Renilla* luciferase were assayed using the dual luciferase reporter assay system (Promega Madison WI). Samples were read on a 96-microplate Glomax Luminometer (Promega Madison WI).

### **Antibodies, protein extracts, western blots, EMSAs**

Antibodies used for western blots were I $\kappa$ B $\alpha$  (1:1000, Cell signaling) and p65/RelA (1:4000, Santa Cruz). Cytoplasmic and nuclear protein extracts were made as previously described (232). In brief, cells were washed with phosphate-buffered saline (PBS) and pellets were resuspended in 100 $\mu$ L of hypotonic buffer A (10 mM Tris-Cl pH 7.8, 5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.1 mM EDTA [ethylenediaminetetraacetic acid], 300 mM sucrose, 5mM  $\beta$ -glycerol phosphate, 0.5 mM dithiothreitol, 0.1% NP-40, plus protease inhibitors) and incubated on ice for 10 minutes. Nuclei were pelleted and the supernatant was collected as the cytoplasmic fraction. The resulting pellet was then resuspended in 50 $\mu$ L of buffer B (20mM Tris-Cl pH 7.8, 5 mM MgCl<sub>2</sub>, 320mM KCl, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol, 5 mM  $\beta$ -glycerol phosphate, plus protease inhibitors) and incubated on ice for 15 minutes. Debris was then removed by centrifugation and the supernatant was collected as the nuclear fraction. For western blot, 50ug of wt or Ing4 null macrophage cytoplasmic or nuclear extracts were run on a 10% polyacrylamide gel. The cytoplasmic fraction was probed with anti-I $\kappa$ B $\alpha$  and the nuclear fraction was probed with anti-p65/RelA.

EMSAs were performed according to the manufacturer's instructions (Promega Madison WI) using 10ug wt or Ing4 null macrophage nuclear extracts. The antibody to p65/RelA was used for the gel shift experiments.

### **ChIPs**

ChIPs were performed according to the manufacturer's instructions (USB Cleveland OH). All PCRs were performed using SYBR Green (Qiagen Valencia CA) in an ABI real-time PCR machine. The antibodies used were acetylated-H4 (Upstate Lake Placid NY), p65/RelA (Abcam Cambridge MA), and IgG (Abcam Cambridge MA). The primer sequences for the NF- $\kappa$ B binding site in the Il-6 promoter were:

AGCTACAGACATCCCCAGTCTC and TGTGTGTCGTCTGTCATGCG.

### **DSS colitis**

Wt and Ing4<sup>-/-</sup> mice were provided *ad lib* drinking water containing 4% dextran sodium sulfate (DSS) (Miltenyi) for 5 days, followed by 11 days of non-treated water. During this experiment, mice were weighed daily and observed for rectal bleeding. Mice exhibiting any symptoms of illness or morbidity were euthanized, and colons were removed, measured, flayed and fixed in formalin. The remaining mice were euthanized and similarly processed at the end of the 16-day experiment. Colon sections were stained with hemotoxylin and eosin before being analyzed by a pathologist.

**CHAPTER V:  
GENERAL DISCUSSION**

## Forward

Research presented in this thesis details the generation and characterization of genetically engineered mouse models deleted for two ING family genes, Ing1 and Ing4. These genes are of interest as they have been proposed to be involved in p53 and NF- $\kappa$ B signaling and to have tumor suppressing capabilities. Additionally, data is present in the literature indicating that these genes are involved in several biological processes, including DNA repair, cell growth regulation, and chromatin remodeling. Chapter 2 described the characterization of a mouse deficient for p37<sup>Ing1b</sup>, the longer of two splice variants. We found that Ing1 can suppress the development of follicular lymphoma (FL), providing an *in vivo* confirmation that an ING protein can function as a tumor suppressor. However, our results also indicate that Ing1 is not required for p53 function. Chapter 3 described the characterization of mice deficient for both the p37<sup>Ing1b</sup> and p53 tumor suppressor. This report extended the observations from Chapter 2 by studying purified follicular B-cells and confirmed that Ing1 has p53-independent anti-oncogenic functions. We also observed the development of diffuse large B-cell lymphoma in these mice. Chapter 4 described the characterization of mice deficient for Ing4. Similar to Ing1, Ing4 is not required for proper development or regulation of p53 function. Instead, our data reveals that Ing4 serves as a negative regulator of the NF- $\kappa$ B pathway. In this chapter we will summarize these findings, explore their implications, and propose some unanswered questions resulting from these results.

### **Current understanding of the ING gene family**

The Inhibitor of Growth (ING) family was discovered using a subtractive hybridization of normal versus transformed human epithelial tissue. Subsequent database searches identified four additional members of this gene family, that are well-conserved from yeast to humans. Studies using overexpression or knockdown experiments in transformed cell lines indicated that the ING genes have roles in DNA repair, chromatin remodeling, and the regulation of cell growth. These studies also determined that most ING proteins interact with p53 and may be required for p53 function. Additionally, *in vitro* studies suggest that some ING proteins are also involved in the NF- $\kappa$ B pathway. Retrospective epidemiological studies revealed that ING genes are either lost or underexpressed in human primary tumors and in some cases the reduced ING gene expression is predictive of a poor prognosis. Thus, ING genes also appear to play a significant role in the development and progression of human cancers. However, the *in vivo* function of these genes had not been explored and the ever-increasing number and often conflicting results of *in vitro* studies warranted the development of genetically modified mice bearing alterations in ING genes. These mouse models were used to explore further the role of the ING proteins in development and in the regulation of normal and neoplastic cell growth. Two different Ing-deficient mouse models are described in this dissertation.

**What we have learned from Ing1 knockout mice.**

Initial studies of mouse Ing1 determined that it was ubiquitously expressed and has three splice variants generated from different promoters which are translated into two distinct protein products (239). Ectopically expressing each variant in transformed cell lines suggested that the proteins have opposing effects on p53 function. The long form, p37<sup>Ing1b</sup>, might act as a negative regulator of p53 function and the shorter forms, p31<sup>Ing1a</sup> or p31<sup>Ing1c</sup>, might act in conjunction with p53. However, it was determined that only the p37<sup>Ing1b</sup> isoform can be co-immunoprecipitated with p53, making it an open question as to how p31<sup>Ing1a</sup> or p31<sup>Ing1c</sup> might be regulating p53. Concomitant with our studies of Ing1 function in mice, Kichina et al. generated a mouse deficient for all Ing1 isoforms. These mice were viable with no obvious abnormalities (108). Mouse embryonic fibroblasts (MEFs) derived from Ing1-deficient mice display little or no changes in cell cycling after treatment with taxol or other DNA damaging agents. Surprisingly, Ing1 deficient mice were more susceptible to whole body gamma irradiation than wt littermates. A subset of these mice also developed cancer, the most prevalent being classified as follicular center B-cell lymphoma. This tumor specificity is in contrast to the various types of different tumors that spontaneously develop in p53-deficient mice. There exists the possibility that loss of Ing1 is a secondary event needed for transformation in other tumor types such as those observed in humans that have altered ING1 expression. It would be interesting and important to explore this possibility in future experiments.

One caveat of the report by Kichina et al. is that the mice were deficient for all Ing1 splice variants. Previous observations in mouse and human cells suggested that each splice variant might have different and possibly opposing functions, which could complicate the interpretation of the results. In contrast, we have generated a mouse deficient for only the long predominant Ing1 isoform, p37<sup>Ing1b</sup> (see chapter 2) (39). Our study also noted the involvement of Ing1 in the development of follicular lymphoma and further revealed that the p37<sup>Ing1b</sup> variant is responsible for tumor suppression *in vivo*. We also studied the effects of DNA damaging agents to induce apoptosis in both MEFs and thymocytes. Loss of p37<sup>Ing1b</sup> increased the susceptibility of MEFs and double positive thymocytes to both ionizing radiation (IR) and doxorubicin induced cell death. This might explain why Kichina et al observed an increase in lethality when Ing1 null mice were treated with whole body ionizing radiation. We further noted that Ing1b-deficient MEFs grew faster but had no alteration in their ability to undergo cell cycle arrest due to DNA damage.

These observations using p37<sup>Ing1b</sup> deficient MEFs and thymocytes made us question the prevailing literature that Ing1 functions in tumor suppression by altering p53 activity. To address this question, we utilized genetic approach by crossing p37<sup>Ing1b</sup> deficient mice to both Mdm2<sup>+/-</sup> and p53 null mice. We expected that p37<sup>Ing1b</sup> deficiency would rescue the embryonic lethality of Mdm2 loss and that the enhanced apoptosis seen in primary cells deficient for p37<sup>Ing1b</sup> would be rescued on a p53 null background.

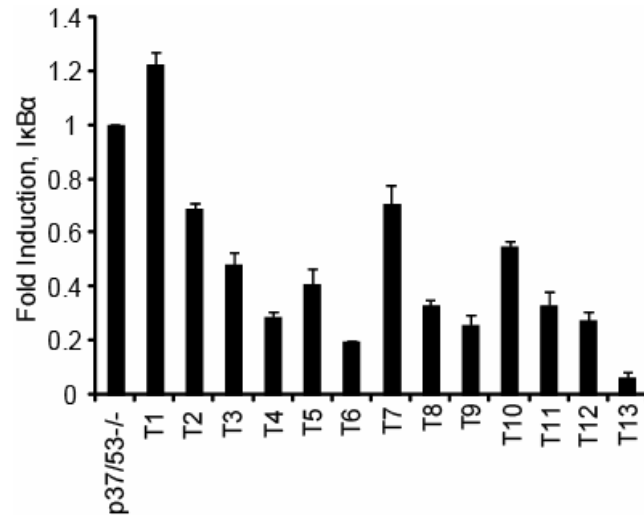
After intercrossing  $p37^{\text{Ing1b}}^{-/-}$ ,  $Mdm2^{+/-}$  mice we discovered that  $p37^{\text{Ing1b}}$  deficiency did not rescue the embryonic lethality induced by  $Mdm2$  deficiency. Additionally, mice deficient for both  $p37^{\text{Ing1b}}$  and  $p53$  ( $p37/53^{-/-}$ ) showed a greater amount of apoptosis compared to  $p53$ -null mice. We also observed that  $p37/53^{-/-}$  MEFs grew even faster than  $p53^{-/-}$  MEFs. This data clearly indicates that there are  $p53$ -independent functions for  $Ing1$ . Additionally, we observed that ectopic expression of activated Ras caused  $p37^{\text{Ing1b}}$  null MEFs to undergo premature senescence. This result also supports the conclusion that there was an intact  $Arf/Mdm2/p53$  pathway present in these cells. From these observations, we conclude that  $p37^{\text{Ing1b}}$  is not required for  $p53$  function, and  $Ing1$  may work entirely independentl of  $p53$ . We can also conclude from these findings that  $Ing1$  is indeed a tumor suppressor that can regulate primary cell growth.

One question that arises from the development of follicular B-cell lymphoma in the  $p37^{\text{Ing1b}}$  knockout mice concerns how loss of a pro-survival gene can cause mice to develop lymphoma? This was answered by analyzing the ability of purified wt or  $p37^{\text{Ing1b}}$  null total (B220+) and follicular ( $IgD^{\text{Hi}}$ ) B-cells to undergo apoptosis or to proliferate . We found that the  $p37^{\text{Ing1b}}$  null B-cells did not have compromised apoptosis following treatment with several different agents. Additionally,  $p53$  loss did not have any significant effect on the ability of  $p37^{\text{Ing1b}}$  deficient B-cells to undergo apoptosis. Interestingly, proliferation following  $IgM$  or  $LPS$  treatment was increased in  $p37^{\text{Ing1b}}$  null B-cells and was even higher in  $p37/p53$  double null B-cells confirming the results in the MEFs.



The p37/53 double null mice also developed thymomas at a slightly decreased rate compared to p53 null mice. This delay in tumor development could be due to the increased apoptosis and *Bax* expression present in the p37/53 null double positive thymocytes. In order to determine a possible mechanism for the development of DLBL in the p37/53 null mice we did quantitative real-time PCR on tumor samples from these animals for the expression of *IκBα* (Figure 5.1). The expression of the NF-κB inhibitor was dramatically lower in most tumor samples tested. This observation coupled with the increased *p65/RelA* and *IL-6* expression and hyperproliferation following LPS treatment of the p37/53 null tumors and FO B-cells suggests that the NF-κB pathway may be elevated in these mice.

In summary, these findings suggest that p37<sup>Ing1b</sup> regulates proliferation in both B-cells and MEFs, but has tissue specific effects such as differential induction of apoptosis. The cooperativity we observed between Ing1b and p53 in suppressing tumorigenesis also confirms that p37<sup>Ing1b</sup> has p53-independent functions. Additionally, our data suggests that Ing1 may be involved in the NF-κB pathway.



**Figure 5.1:** Expression of *IkBα* in p37/53 null purified FO (IgD<sup>Hi</sup>) B-cells and tumor samples. Quantitative real-time PCR using primers to *IkBα* was done on FO (IgD<sup>Hi</sup>) B-cells and tumor samples from mice deficient for both p37<sup>Ing1b</sup> and p53. Experiment was done in triplicate and the graph shows average and standard deviation.

Currently no extensive epidemiological study has been performed on ING1 expression in human FL or DLBCL, which is required before these findings can be applied to the clinic. However, it is possible that new diagnostic tests could be designed around the ING1 gene for predisposition to FL or DLBCL. Certainly loss of p53 in an Ing1-deficient background strongly shifts the tumor spectrum to a more aggressive DLBL in mice, and loss of p53 would be a key prognostic indicator of long-term outcomes in FL patients. In addition, ING1 levels might be used as a marker to determine what therapy regimen may work optimally.

In support of this possibility, ING1 has been found to greatly sensitize glioblastoma cells to DNA damaging agents (208) and we found that Ing1 loss sensitized thymocytes and MEFs to ionizing radiation and doxorubicin induced apoptosis (39). Additionally, the Ing1 knockout mice could be used to test novel chemotherapeutic regimens to treat FL and DLBCL patients, following further validation of these models. Current treatment for FL has not improved much in the past 8 to 10 years and there has been little impact on patient survival (19, 90, 91). Yet its incidence is increasing in the western world, making models of this disease very important (90). Mice are becoming more prevalent as tools for preclinical testing of novel therapies, but require additional steps to valid them so that the data obtained can inform future clinical trials (25, 80, 201, 229). It would be useful to determine how close the Ing1 knockout mice recapitulate human FL and if the mice can be treated with current therapies successfully. One caveat that is apparent is that the Ing1 deficient mice develop FL rather late, which is not overly conducive for preclinical modeling. But, this may be closer to the human disease since FL usually arises much later in life. The p37/53 knockout mice could be useful in development of treatments for DLBCL and the study of de novo and transformed DLBCL development. Treatment for DLBCL with current anthracycline based therapy is potentially curable; however, variability in response is sometimes observed (5, 93). Development of more effective treatments and diagnostic tests are needed and the p37/53 knockout mice may assist in this endeavor.

### **What we have learned from Ing4 knockout mice**

Similar to ING1, ING4 was also found via *in vitro* experiments to interact with p53 and to have potential roles in several biological processes. Additionally, ING4 was found to interact with RelA and act as a negative regulator of the NF- $\kappa$ B pathway. However, the precise *in vivo* function of ING4 is not known. Therefore, we generated mice deficient for Ing4. These mice failed to develop spontaneous tumors, but did show a hypersensitivity to lipopolysaccharide (LPS). A greater number of Ing4 null mice treated with LPS died within 24 hours and showed elevated levels of Il-6, Rantes, and Mcp-1. These elevated cytokine levels were also seen at the transcript level in macrophages and included *IFN $\beta$*  and *IP-10*. Interestingly, *I $\kappa$ B $\alpha$* , *I $\kappa$ B $\beta$* , and *I $\kappa$ BNS* transcript levels were decreased in Ing4 null macrophages. Using a dextran sodium sulfate (DSS) colitis model, we found that Ing4 null mice were more susceptible to inflammation and death following this treatment. Histological analysis following DSS treatment showed that the mucosal layer of the intestine of Ing4 null mice was completely lost. These findings support previous transfection work in glioblastoma cell lines indicating a role for Ing4 as a negative regulator of NF- $\kappa$ B. Several well-established p53 responses were also tested in the Ing4 null cells. Ing4 null MEFs grew slower and did not have defects in cell cycle arrest following treatment with doxorubicin and ionizing radiation. Thymocytes from Ing4 null mice were not compromised in their ability to undergo apoptosis following treatment with ionizing radiation. These observations suggest that the p53 pathway is not perturbed in Ing4 deficient mice.

The NF- $\kappa$ B complex is important in innate immunity and inflammation, which are important responses that protect the host from pathogens (3). In addition, alterations of NF- $\kappa$ B function have been found to lead to the development of cancer (105) and other diseases; such as inflammatory bowel disease (75), various diabetic complications (74), and atherosclerosis (120). Identifying modifiers of this important pathway could lead to better ways to manipulate deregulated NF- $\kappa$ B function during pathological conditions. We did not observe the development of tumors in the *Ing4* null mice. This observation does not rule out the possibility that *Ing4* suppresses tumorigenesis, as *Ing4* might have a role in inhibiting induced tumors. NF- $\kappa$ B has been found to act as a tumor promoter under certain conditions (104, 169), and given the function of *Ing4* to negatively regulate NF- $\kappa$ B, it is possible that loss of *Ing4* could promote tumors via this mechanism. The involvement of *Ing4* in the NF- $\kappa$ B pathway also suggests that it may have functions in other processes, such as atherosclerosis and autoimmunity. Further epidemiological studies are needed to explore these possibilities.

In summary, our data confirms that *ING4* can act as a negative regulator of the NF- $\kappa$ B pathway in vivo, that *Ing4* has a key role in regulating innate immune functions such as inflammation, and further reveals that this negative regulation of NF- $\kappa$ B activity is due to the upregulation of *I $\kappa$ B* gene expression by *Ing4*. However, the precise mechanism for the regulation of the *I $\kappa$ B* genes requires further study. Additionally, our data clearly indicates that *Ing4* does not appear to be a major participant in the p53 pathway.

### **Unanswered questions**

There remain several additional questions arising from our research into the functions of the Ing genes. Given that the phenotypes of both the Ing1 and Ing4 knockout mice are relatively subtle, it would stand to reason that the other members of the Ing family may be compensating for loss of Ing1 or Ing4. The Ing genes can be grouped based on which chromatin remodeling complex they can be found as components. Ing1 and Ing2 are part of the Sin3 HDAC complex and one Ing gene might compensate for loss of the other. Ing4 and Ing5 are part of the HBO1 complex and may compensate in the functioning of this complex. As Ing3 is in a separate complex a knockout mouse of this gene might give the most pronounced phenotype. Crossing Ing1 and Ing2 deficient mice may give a phenotype that more closely resembles what was observed in the tissue culture-based experiments, although both Ing genes were present and in these cells. Similarly, an Ing4 x Ing5 double knockout mouse may be needed before the expected tumor suppressing effects of these genes are apparent in the tumor assays. To that end, we have generated Ing5 heterozygous mice, but characterization of their phenotype is beyond the scope of this thesis. Subsequent studies should also generate and characterize development and tumorigenesis in the Ing4xIng5 mice as well. Additional questions that could be addressed would include a study to determine the extent to which the Ing4 null mice are tumor prone. Several chemical carcinogens are known to induce tumors in specific tissue types. Some of those chemicals appear to have an inflammatory requirement and therefore should be first employed in these studies.

They include treatment with diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) to induce liver cancer and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) to induce lung cancer (134, 230). Ing4 null could also be crossed to the APC<sup>Min</sup> mice or p53 null mice, as both models of mice are tumor prone. Additionally, the Ing4 null mice should be treated with a lower dose of DSS to induce IBD and colon cancer. Another experiment that could be performed is to cross the p37<sup>Ing1b</sup> null mice to E $\mu$ -myc mice in order to further explore the involvement of this gene in B-cell derived lymphomas, B-cell function, and development.

## Conclusions

In conclusion, Ing1 and Ing4 null mice have revealed that these genes have p53-independent roles in tumorigenesis but that both may regulate the NF- $\kappa$ B signaling pathway. Ing1 is a tumor suppressor gene and loss of this gene leads to the development of FL and DLBL. Additionally, Ing1 loss sensitizes DP T-cells and MEFs to apoptosis induced by DNA damaging agents. Ing4 null mice have revealed that this gene has a significant role in the regulation of the NF- $\kappa$ B pathway due to its ability to regulate the expression of *I $\kappa$ B* genes. This finding also suggests that Ing4 may have a role in several diseases processes, including infection and cancer. Thus, further functional studies of the role of Ing1 and Ing4 in development and in disease are warranted.

## REFERENCES

1. Aasland, R., T. J. Gibson, and A. F. Stewart. 1995. The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci* 20:56-9.
2. Abramson, J. S., and M. A. Shipp. 2005. Advances in the biology and therapy of diffuse large B-cell lymphoma: moving toward a molecularly targeted approach. *Blood* 106:1164-74.
3. Ahn, K. S., G. Sethi, and B. B. Aggarwal. 2007. Nuclear factor-kappa B: from clone to clinic. *Curr Mol Med* 7:619-37.
4. Alizadeh, A. A., M. B. Eisen, R. E. Davis, C. Ma, I. S. Lossos, A. Rosenwald, J. C. Boldrick, H. Sabet, T. Tran, X. Yu, J. I. Powell, L. Yang, G. E. Marti, T. Moore, J. Hudson, Jr., L. Lu, D. B. Lewis, R. Tibshirani, G. Sherlock, W. C. Chan, T. C. Greiner, D. D. Weisenburger, J. O. Armitage, R. Warnke, R. Levy, W. Wilson, M. R. Grever, J. C. Byrd, D. Botstein, P. O. Brown, and L. M. Staudt. 2000. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* 403:503-11.
5. Armitage, J. O. 2007. How I treat patients with diffuse large B-cell lymphoma. *Blood* 110:29-36.
6. Attardi, L. D., A. de Vries, and T. Jacks. 2004. Activation of the p53-dependent G1 checkpoint response in mouse embryo fibroblasts depends on the specific DNA damage inducer. *Oncogene* 23:973-80.



7. Avvakumov, N., and J. Cote. 2007. The MYST family of histone acetyltransferases and their intimate links to cancer. *Oncogene* 26:5395-407.
8. Aylon, Y., and M. Oren. 2007. Living with p53, dying of p53. *Cell* 130:597-600.
9. Bai, M., A. Skyras, N. J. Agnantis, S. Kamina, A. Papoudou-Bai, P. Kitsoulis, and P. Kanavaros. 2005. B-cell differentiation, apoptosis and proliferation in diffuse large B-cell lymphomas. *Anticancer Res* 25:347-62.
10. Balint, E. E., and K. H. Vousden. 2001. Activation and activities of the p53 tumour suppressor protein. *British Journal of Cancer* 85:1813-23.
11. Bende, R. J., L. A. Smit, and C. J. van Noesel. 2007. Molecular pathways in follicular lymphoma. *Leukemia* 21:18-29.
12. Berardi, P., M. Russell, A. El-Osta, and K. Riabowol. 2004. Functional links between transcription, DNA repair and apoptosis. *Cell Mol Life Sci* 61:2173-80.
13. Berra, E., A. Ginouves, and J. Pouyssegur. 2006. The hypoxia-inducible-factor hydroxylases bring fresh air into hypoxia signalling. *EMBO Rep* 7:41-5.
14. Bienz, M. 2006. The PHD finger, a nuclear protein-interaction domain. *Trends Biochem Sci* 31:35-40.
15. Binda, O., C. Nassif, and P. E. Branton. 2008. SIRT1 negatively regulates HDAC1-dependent transcriptional repression by the RBP1 family of proteins. *Oncogene*. 27:3384-92.
16. Bode AM, D. Z. 2004. Post-translational modification of p53 in tumorigenesis. *Nat Rev Cancer* 4:793-805.

17. Bottomley, M. J., G. Stier, D. Pennacchini, G. Legube, B. Simon, A. Akhtar, M. Sattler, and G. Musco. 2005. NMR structure of the first PHD finger of autoimmune regulator protein (AIRE1). Insights into autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) disease. *J Biol Chem* 280:11505-12.
18. Brady, H. J., G. Gil-Gomez, J. Kirberg, and A. J. Berns. 1996. Bax alpha perturbs T cell development and affects cell cycle entry of T cells. *EMBO Journal* 15:6991-7001.
19. Buske, C., O. Weigert, M. Dreyling, M. Unterhalt, and W. Hiddemann. 2006. Current status and perspective of antibody therapy in follicular lymphoma. *Haematologica* 91:104-12.
20. Busslinger, M. 2004. Transcriptional control of early B cell development. *Annu Rev Immunol* 22:55-79.
21. Campos, E. I., K. J. Cheung, Jr., A. Murray, S. Li, and G. Li. 2002. The novel tumour suppressor gene ING1 is overexpressed in human melanoma cell lines. *Br J Dermatol* 146:574-80.
22. Campos, E. I., M. Y. Chin, W. H. Kuo, and G. Li. 2004. Biological functions of the ING family tumor suppressors. *Cellular & Molecular Life Sciences* 61:2597-613.
23. Campos, E. I., M. Y. Chin, W. H. Kuo, and G. Li. 2004. Biological functions of the ING family tumor suppressors. *Cell Mol Life Sci* 61:2597-613.

24. Campos, E. I., M. Martinka, D. L. Mitchell, D. L. Dai, and G. Li. 2004. Mutations of the ING1 tumor suppressor gene detected in human melanoma abrogate nucleotide excision repair. *Int J Oncol* 25:73-80.
25. Carver, B. S., and P. P. Pandolfi. 2006. Mouse modeling in oncologic preclinical and translational research. *Clin Cancer Res* 12:5305-11.
26. Cattoretti, G., L. Pasqualucci, G. Ballon, W. Tam, S. V. Nandula, Q. Shen, T. Mo, V. V. Murty, and R. Dalla-Favera. 2005. Deregulated BCL6 expression recapitulates the pathogenesis of human diffuse large B cell lymphomas in mice. *Cancer Cell* 7:445-55.
27. Cengiz, B., M. Gunduz, H. Nagatsuka, L. Beder, E. Gunduz, R. Tamamura, N. Mahmut, K. Fukushima, M. A. Ali, Y. Naomoto, K. Shimizu, and N. Nagai. 2007. Fine deletion mapping of chromosome 2q21-37 shows three preferentially deleted regions in oral cancer. *Oral Oncol* 43:241-7.
28. Chen, B., E. I. Campos, R. Crawford, M. Martinka, and G. Li. 2003. Analyses of the tumour suppressor ING1 expression and gene mutation in human basal cell carcinoma. *Int J Oncol* 22:927-31.
29. Chen, L., N. Matsubara, T. Yoshino, T. Nagasaka, N. Hoshizima, Y. Shirakawa, Y. Naomoto, H. Isozaki, K. Riabowol, and N. Tanaka. 2001. Genetic alterations of candidate tumor suppressor ING1 in human esophageal squamous cell cancer. *Cancer Res* 61:4345-9.

30. Chen, L. S., J. B. Wei, Y. C. Zhou, S. Zhang, J. L. Liang, Y. F. Cao, Z. J. Tang, X. L. Zhang, and F. Gao. 2005. Genetic alterations and expression of inhibitor of growth 1 in human sporadic colorectal cancer. *World J Gastroenterol* 11:6120-4.
31. Cheung, K. J., Jr., J. A. Bush, W. Jia, and G. Li. 2000. Expression of the novel tumour suppressor p33(ING1) is independent of p53. *Br J Cancer* 83:1468-72.
32. Cheung, K. J., Jr., and G. Li. 2002. p33(ING1) enhances UVB-induced apoptosis in melanoma cells. *Experimental Cell Research* 279:291-8.
33. Cheung, K. J., Jr., and G. Li. 2001. The tumor suppressor ING1: structure and function. *Exp Cell Res* 268:1-6.
34. Cheung, K. J., Jr., D. Mitchell, P. Lin, and G. Li. 2001. The tumor suppressor candidate p33(ING1) mediates repair of UV-damaged DNA. *Cancer Res* 61:4974-7.
35. Chin, M. Y., K. C. Ng, and G. Li. 2005. The novel tumor suppressor p33ING2 enhances UVB-induced apoptosis in human melanoma cells. *Exp Cell Res* 304:531-43.
36. Chipuk, J. E., T. Kuwana, L. Bouchier-Hayes, N. M. Droin, D. D. Newmeyer, M. Schuler, and D. R. Green. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* 303:1010-4.
37. Chipuk, J. E., U. Maurer, D. R. Green, and M. Schuler. 2003. Pharmacologic activation of p53 elicits Bax-dependent apoptosis in the absence of transcription. *Cancer Cell* 4:371-81.

38. Clarke, A. R., C. A. Purdie, D. J. Harrison, R. G. Morris, C. C. Bird, M. L. Hooper, and A. H. Wyllie. 1993. Thymocyte apoptosis induced by p53-dependent and independent pathways.[see comment]. *Nature* 362:849-52.
39. Coles, A. H., H. Liang, Z. Zhu, C. G. Marfella, J. Kang, A. N. Imbalzano, and S. N. Jones. 2007. Deletion of p37<sup>Ingl</sup> in mice reveals a p53-independent role for *Ingl* in the suppression of cell proliferation, apoptosis, and tumorigenesis. *Cancer Res* 67:2054-61.
40. Colla, S., S. Tagliaferri, F. Morandi, P. Lunghi, G. Donofrio, D. Martorana, C. Mancini, M. Lazzaretti, L. Mazzera, L. Ravanetti, S. Bonomini, L. Ferrari, C. Miranda, M. Ladetto, T. M. Neri, A. Neri, A. Greco, M. Mangoni, A. Bonati, V. Rizzoli, and N. Giuliani. 2007. The new tumor suppressor gene inhibitor of growth family member 4 (*ING4*) regulates the production of pro-angiogenic molecules by myeloma cells and suppresses hypoxia inducible factor (*HIF*)-1 $\alpha$  activity being involved in myeloma-induced angiogenesis. *Blood* 110:4464-75.
41. Concetta G.A. Marfella, Y. O., Andrew H. Coles, David S. Garlick, Stephen N. Jones, Anthony N. Imbalzano,. 2006. Mutation of the SNF2 family member *Chd2* affects mouse development and survival. *Journal of Cellular Physiology* 209:162-171.
42. Contzler, R., A. Regamey, B. Favre, T. Roger, D. Hohl, and M. Huber. 2006. Histone acetyltransferase *HBO1* inhibits *NF-kappaB* activity by coactivator sequestration. *Biochem Biophys Res Commun* 350:208-13.

43. Davies, A. J., A. M. Lee, C. Taylor, A. J. Clear, L. K. Goff, S. Iqbal, D. Cuthbert-Heavens, M. Calaminici, A. J. Norton, T. A. Lister, and J. Fitzgibbon. 2005. A limited role for TP53 mutation in the transformation of follicular lymphoma to diffuse large B-cell lymphoma. *Leukemia* 19:1459-65.
44. de Jong, D. 2005. Molecular pathogenesis of follicular lymphoma: a cross talk of genetic and immunologic factors. *J Clin Oncol* 23:6358-63.
45. De Paepe, P., and C. De Wolf-Peeters. 2007. Diffuse large B-cell lymphoma: a heterogeneous group of non-Hodgkin lymphomas comprising several distinct clinicopathological entities. *Leukemia* 21:37-43.
46. Denslow, S. A., and P. A. Wade. 2007. The human Mi-2/NuRD complex and gene regulation. *Oncogene* 26:5433-8.
47. Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, J. S. Butel, and B. Allan. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356:215-221.
48. Doyon, Y., C. Cayrou, M. Ullah, A. J. Landry, V. Cote, W. Selleck, W. S. Lane, S. Tan, X. J. Yang, and J. Cote. 2006. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Mol Cell* 21:51-64.
49. Doyon, Y., C. Cayrou, M. Ullah, A. J. Landry, V. Cote, W. Selleck, W. S. Lane, S. Tan, X. J. Yang, and J. Cote. 2006. ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Molecular Cell* 21:51-64.

50. Doyon, Y., W. Selleck, W. S. Lane, S. Tan, and J. Cote. 2004. Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans. *Mol Cell Biol* 24:1884-96.
51. Egle, A., A. W. Harris, P. Bouillet, and S. Cory. 2004. Bim is a suppressor of Myc-induced mouse B cell leukemia. *Proc Natl Acad Sci U S A* 101:6164-9.
52. Esteller, M. 2007. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 8:286-98.
53. Fegers, I., R. Kob, M. Eckey, O. Schmidt, F. Goeman, M. Papaioannou, N. Escher, F. von Eggeling, C. Melle, and A. Baniahmad. 2007. The tumor suppressors p33ING1 and p33ING2 interact with alien in vivo and enhance alien-mediated gene silencing. *J Proteome Res* 6:4182-8.
54. Feng, X., S. Bonni, and K. Riabowol. 2006. HSP70 induction by ING proteins sensitizes cells to tumor necrosis factor alpha receptor-mediated apoptosis. *Mol Cell Biol* 26:9244-55.
55. Feng, X., Y. Hara, and K. Riabowol. 2002. Different HATS of the ING1 gene family. *Trends Cell Biol* 12:532-8.
56. Feng, X., Y. Hara, and K. Riabowol. 2002. Different HATS of the ING1 gene family. *Trends in Cell Biology* 12:532-8.
57. Ferbeyre, G., E. de Stanchina, A. W. Lin, E. Querido, M. E. McCurrach, G. J. Hannon, and S. W. Lowe. 2002. Oncogenic ras and p53 Cooperate To Induce Cellular Senescence. *Mol. Cell. Biol.* 22:3497-3508.

58. Garate, M., E. I. Campos, J. A. Bush, H. Xiao, and G. Li. 2007. Phosphorylation of the tumor suppressor p33(ING1b) at Ser-126 influences its protein stability and proliferation of melanoma cells. *FASEB J* 21:3705-16.
59. Garkavtsev, I., I. A. Grigorian, V. S. Ossovskaya, M. V. Chernov, P. M. Chumakov, and A. V. Gudkov. 1998. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control. *Nature* 391:295-8.
60. Garkavtsev, I., I. A. Grigorian, V. S. Ossovskaya, M. V. Chernov, P. M. Chumakov, and A. V. Gudkov. 1998. The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control.[see comment]. *Nature* 391:295-8.
61. Garkavtsev, I., A. Kazarov, A. Gudkov, and K. Riabowol. 1996. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation. *Nat Genet* 14:415-20.
62. Garkavtsev, I., A. Kazarov, A. Gudkov, and K. Riabowol. 1996. Suppression of the novel growth inhibitor p33ING1 promotes neoplastic transformation.[erratum appears in *Nat Genet* 1999 Nov;23(3):373]. *Nature Genetics* 14:415-20.
63. Garkavtsev, I., S. V. Kozin, O. Chernova, L. Xu, F. Winkler, E. Brown, G. H. Barnett, and R. K. Jain. 2004. The candidate tumour suppressor protein ING4 regulates brain tumour growth and angiogenesis. *Nature* 428:328-32.
64. Garkavtsev, I., and K. Riabowol. 1997. Extension of the replicative life span of human diploid fibroblasts by inhibition of the p33ING1 candidate tumor suppressor. *Mol Cell Biol* 17:2014-9.



65. Gerondakis, S., R. Grumont, R. Gugasyan, L. Wong, I. Isomura, W. Ho, and A. Banerjee. 2006. Unravelling the complexities of the NF-kappaB signalling pathway using mouse knockout and transgenic models. *Oncogene* 25:6781-99.
66. Gil-Gomez, G., A. Berns, and H. J. Brady. 1998. A link between cell cycle and cell death: Bax and Bcl-2 modulate Cdk2 activation during thymocyte apoptosis. *EMBO Journal* 17:7209-18.
67. Goeman, F., D. Thormeyer, M. Abad, M. Serrano, O. Schmidt, I. Palmero, and A. Baniahmad. 2005. Growth inhibition by the tumor suppressor p33ING1 in immortalized and primary cells: involvement of two silencing domains and effect of Ras. *Mol Cell Biol* 25:422-31.
68. Gong, W., M. Russell, K. Suzuki, and K. Riabowol. 2006. Subcellular targeting of p33ING1b by phosphorylation-dependent 14-3-3 binding regulates p21WAF1 expression. *Mol Cell Biol* 26:2947-54.
69. Gong, W., M. Russell, K. Suzuki, and K. Riabowol. 2006. Subcellular targeting of p33ING1b by phosphorylation-dependent 14-3-3 binding regulates p21WAF1 expression. *Molecular & Cellular Biology* 26:2947-54.
70. Gong, W., K. Suzuki, M. Russell, and K. Riabowol. 2005. Function of the ING family of PHD proteins in cancer. *Int J Biochem Cell Biol* 37:1054-65.
71. Gonzalez, L., J. M. Freije, S. Cal, C. Lopez-Otin, M. Serrano, and I. Palmero. 2006. A functional link between the tumour suppressors ARF and p33ING1. *Oncogene* 25:5173-9.

72. Gorrini, C., M. Squatrito, C. Luise, N. Syed, D. Perna, L. Wark, F. Martinato, D. Sardella, A. Verrecchia, S. Bennett, S. Confalonieri, M. Cesaroni, F. Marchesi, M. Gasco, E. Scanziani, M. Capra, S. Mai, P. Nuciforo, T. Crook, J. Lough, and B. Amati. 2007. Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. *Nature* 448:1063-7.
73. Gozani, O., P. Karuman, D. R. Jones, D. Ivanov, J. Cha, A. A. Lugovskoy, C. L. Baird, H. Zhu, S. J. Field, S. L. Lessnick, J. Villasenor, B. Mehrotra, J. Chen, V. R. Rao, J. S. Brugge, C. G. Ferguson, B. Payrastra, D. G. Myszka, L. C. Cantley, G. Wagner, N. Divecha, G. D. Prestwich, and J. Yuan. 2003. The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. *Cell* 114:99-111.
74. Graves, D. T., and R. A. Kayal. 2008. Diabetic complications and dysregulated innate immunity. *Front Biosci* 13:1227-39.
75. Greten, F. R., L. Eckmann, T. F. Greten, J. M. Park, Z. W. Li, L. J. Egan, M. F. Kagnoff, and M. Karin. 2004. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 118:285-96.
76. Gunduz, M., L. B. Beder, E. Gunduz, H. Nagatsuka, K. Fukushima, D. Pehlivan, E. Cetin, N. Yamanaka, K. Nishizaki, K. Shimizu, and N. Nagai. 2007. Downregulation of ING3 mRNA expression predicts poor prognosis in head and neck cancer. *Cancer Sci* 99:531-8.
77. Gunduz, M., H. Nagatsuka, K. Demircan, E. Gunduz, B. Cengiz, M. Ouchida, H. Tsujigiwa, E. Yamachika, K. Fukushima, L. Beder, S. Hirohata, Y. Ninomiya, K.

- Nishizaki, K. Shimizu, and N. Nagai. 2005. Frequent deletion and down-regulation of ING4, a candidate tumor suppressor gene at 12p13, in head and neck squamous cell carcinomas. *Gene* 356:109-17.
78. Gunduz, M., M. Ouchida, K. Fukushima, H. Hanafusa, T. Etani, S. Nishioka, K. Nishizaki, and K. Shimizu. 2000. Genomic structure of the human ING1 gene and tumor-specific mutations detected in head and neck squamous cell carcinomas. *Cancer Res* 60:3143-6.
79. Gunduz, M., M. Ouchida, K. Fukushima, S. Ito, Y. Jitsumori, T. Nakashima, N. Nagai, K. Nishizaki, and K. Shimizu. 2002. Allelic loss and reduced expression of the ING3, a candidate tumor suppressor gene at 7q31, in human head and neck cancers. *Oncogene* 21:4462-70.
80. Gutmann, D. H., K. Hunter-Schaedle, and K. M. Shannon. 2006. Harnessing preclinical mouse models to inform human clinical cancer trials. *J Clin Invest* 116:847-52.
81. Ha, S., S. Park, C. H. Yun, and Y. Choi. 2002. Characterization of nuclear localization signal in mouse ING1 homolog protein. *Biochem Biophys Res Commun* 293:163-6.
82. Hanahan, D., and R. A. Weinberg. 2000. The hallmarks of cancer. *Cell* 100:57-70.
83. Hans, C. P., D. D. Weisenburger, T. C. Greiner, R. D. Gascoyne, J. Delabie, G. Ott, H. K. Muller-Hermelink, E. Campo, R. M. Braziel, E. S. Jaffe, Z. Pan, P. Farinha, L. M. Smith, B. Falini, A. H. Banham, A. Rosenwald, L. M. Staudt, J. M.

- Connors, J. O. Armitage, and W. C. Chan. 2004. Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using a tissue microarray. *Blood* 103:275-82.
84. Harris, S. L., and A. J. Levine. 2005. The p53 pathway: positive and negative feedback loops. *Oncogene* 24:2899-908.
85. Harvey, M., M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, A. Bradley, and L. A. Donehower. 1993. Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat Genet* 5:225-9.
86. Harvey, M., A. T. Sands, R. S. Weiss, M. E. Hegi, R. W. Wiseman, P. Pantazis, B. C. Giovanella, M. A. Tainsky, A. Bradley, and L. A. Donehower. 1993. In vitro growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* 8:2457-67.
87. He, G. H., C. C. Helbing, M. J. Wagner, C. W. Sensen, and K. Riabowol. 2005. Phylogenetic analysis of the ING family of PHD finger proteins. *Mol Biol Evol* 22:104-16.
88. Helbing, C. C., C. Veillette, K. Riabowol, R. N. Johnston, and I. Garkavtsev. 1997. A novel candidate tumor suppressor, ING1, is involved in the regulation of apoptosis. *Cancer Res* 57:1255-8.
89. Helbing, C. C., C. Veillette, K. Riabowol, R. N. Johnston, and I. Garkavtsev. 1997. A novel candidate tumor suppressor, ING1, is involved in the regulation of apoptosis. *Cancer Research* 57:1255-8.

90. Hiddemann, W., C. Buske, M. Dreyling, O. Weigert, G. Lenz, and M. Unterhalt. 2007. Current management of follicular lymphomas. *Br J Haematol* 136:191-202.
91. Hiddemann, W., and M. Unterhalt. 2006. Current treatment strategies in follicular lymphomas. *Ann Oncol* 17 Suppl 10:x155-9.
92. Houldsworth, J., A. B. Olshen, G. Cattoretti, G. B. Donnelly, J. Teruya-Feldstein, J. Qin, N. Palanisamy, Y. Shen, K. Dyomina, M. Petlakh, Q. Pan, A. D. Zelenetz, R. Dalla-Favera, and R. S. Chaganti. 2004. Relationship between REL amplification, REL function, and clinical and biologic features in diffuse large B-cell lymphomas. *Blood* 103:1862-8.
93. Hunt, K. E., and K. K. Reichard. 2008. Diffuse large B-cell lymphoma. *Arch Pathol Lab Med* 132:118-24.
94. Ichikawa, A., T. Kinoshita, T. Watanabe, H. Kato, H. Nagai, K. Tsushita, H. Saito, and T. Hotta. 1997. Mutations of the p53 gene as a prognostic factor in aggressive B-cell lymphoma. *N Engl J Med* 337:529-34.
95. Iizuka, M., O. F. Sarmiento, T. Sekiya, H. Scoble, C. D. Allis, and M. M. Smith. 2007. Hbo1 Links p53-Dependent Stress Signaling to DNA Replication Licensing. *Mol Cell Biol* 28:140-53.
96. Ito, K., K. Kinjo, T. Nakazato, Y. Ikeda, and M. Kizaki. 2002. Expression and sequence analyses of p33(ING1) gene in myeloid leukemia. *Am J Hematol* 69:141-3.

97. Jacks, T., L. Remington, B. O. Williams, E. M. Schmitt, S. Halachmi, R. T. Bronson, and R. A. Weinberg. 1994. Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 4:1-7.
98. Jones, S. N., A. E. Roe, L. A. Donehower, and A. Bradley. 1995. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature* 378:206-8.
99. Jones, S. N., A. T. Sands, A. R. Hancock, H. Vogel, L. A. Donehower, S. P. Linke, G. M. Wahl, and A. Bradley. 1996. The tumorigenic potential and cell growth characteristics of p53-deficient cells are equivalent in the presence or absence of Mdm2. *Proceedings of the National Academy of Sciences of the United States of America* 93:14106-11.
100. Jost, P. J., and J. Ruland. 2007. Aberrant NF-kappaB signaling in lymphoma: mechanisms, consequences, and therapeutic implications. *Blood* 109:2700-7.
101. Kaadige, M. R., and D. E. Ayer. 2006. The polybasic region that follows the plant homeodomain zinc finger 1 of Pfl is necessary and sufficient for specific phosphoinositide binding. *J Biol Chem* 281:28831-6.
102. Kalaitzidis, D., R. E. Davis, A. Rosenwald, L. M. Staudt, and T. D. Gilmore. 2002. The human B-cell lymphoma cell line RC-K8 has multiple genetic alterations that dysregulate the Rel/NF-kappaB signal transduction pathway. *Oncogene* 21:8759-68.
103. Kameyama, K., C. L. Huang, D. Liu, D. Masuya, T. Nakashima, S. Sumitomo, Y. Takami, M. Kinoshita, and H. Yokomise. 2003. Reduced ING1b gene expression

plays an important role in carcinogenesis of non-small cell lung cancer patients.  
*Clin Cancer Res* 9:4926-34.

104. Karin, M., Y. Cao, F. R. Greten, and Z. W. Li. 2002. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer* 2:301-10.
105. Karin, M., and F. R. Greten. 2005. NF-kappaB: linking inflammation and immunity to cancer development and progression. *Nat Rev Immunol* 5:749-59.
106. Kataoka, H., P. Bonnefin, D. Vieyra, X. Feng, Y. Hara, Y. Miura, T. Joh, H. Nakabayashi, H. Vaziri, C. C. Harris, and K. Riabowol. 2003. ING1 represses transcription by direct DNA binding and through effects on p53. *Cancer Research* 63:5785-92.
107. Kataoka, H., P. Bonnefin, D. Vieyra, X. Feng, Y. Hara, Y. Miura, T. Joh, H. Nakabayashi, H. Vaziri, C. C. Harris, and K. Riabowol. 2003. ING1 represses transcription by direct DNA binding and through effects on p53. *Cancer Res* 63:5785-92.
108. Kichina, J. V., M. Zeremski, L. Aris, K. V. Gurova, E. Walker, R. Franks, A. Y. Nikitin, H. Kiyokawa, and A. V. Gudkov. 2006. Targeted disruption of the mouse *ing1* locus results in reduced body size, hypersensitivity to radiation and elevated incidence of lymphomas. *Oncogene* 25:857-66.
109. Kim, S. 2005. HuntING4 new tumor suppressors. *Cell Cycle* 4:516-7.
110. Kim, S., K. Chin, J. W. Gray, and J. M. Bishop. 2004. A screen for genes that suppress loss of contact inhibition: identification of ING4 as a candidate tumor suppressor gene in human cancer. *Proc Natl Acad Sci U S A* 101:16251-6.

111. Knudson, A. G. 2001. Two genetic hits (more or less) to cancer. *Nat Rev Cancer* 1:157-62.
112. Knudson, A. G., Jr. 1971. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68:820-3.
113. Kubbutat, M. H., S. N. Jones, and K. H. Vousden. 1997. Regulation of p53 stability by Mdm2. *Nature* 387:299-303.
114. Kumamoto, K., E. A. Spillare, K. Fujita, I. Horikawa, T. Yamashita, E. Appella, M. Nagashima, S. Takenoshita, J. Yokota, and C. C. Harris. 2008. Nutlin-3a activates p53 to both down-regulate inhibitor of growth 2 and up-regulate mir-34a, mir-34b, and mir-34c expression, and induce senescence. *Cancer Res* 68:3193-203.
115. Kuo, W. H., Y. Wang, R. P. Wong, E. I. Campos, and G. Li. 2007. The ING1b tumor suppressor facilitates nucleotide excision repair by promoting chromatin accessibility to XPA. *Exp Cell Res* 313:1628-38.
116. Kurt-Jones, E. A., L. Cao, F. Sandor, A. B. Rogers, M. T. Whary, P. R. Nambiar, A. Cerny, G. Bowen, J. Yan, S. Takaishi, A. L. Chi, G. Reed, J. Houghton, J. G. Fox, and T. C. Wang. 2007. Trefoil family factor 2 is expressed in murine gastric and immune cells and controls both gastrointestinal inflammation and systemic immune responses. *Infect Immun* 75:471-80.
117. Kuwata, H., M. Matsumoto, K. Atarashi, H. Morishita, T. Hirotsu, R. Koga, and K. Takeda. 2006. IkappaBNS inhibits induction of a subset of Toll-like receptor-dependent genes and limits inflammation. *Immunity* 24:41-51.



118. Kuzmichev, A., Y. Zhang, H. Erdjument-Bromage, P. Tempst, and D. Reinberg. 2002. Role of the Sin3-histone deacetylase complex in growth regulation by the candidate tumor suppressor p33(ING1). *Mol Cell Biol* 22:835-48.
119. L González, J. M. P. F., S Cal, C López-Otín, M Serrano and I Palmero. 2006. A functional link between the tumour suppressors ARF and p33ING1. *Oncogene*.
120. Laberge, M. A., K. J. Moore, and M. W. Freeman. 2005. Atherosclerosis and innate immune signaling. *Ann Med* 37:130-40.
121. Laherty, C. D., A. N. Billin, R. M. Lavinsky, G. S. Yochum, A. C. Bush, J. M. Sun, T. M. Mullen, J. R. Davie, D. W. Rose, C. K. Glass, M. G. Rosenfeld, D. E. Ayer, and R. N. Eisenman. 1998. SAP30, a component of the mSin3 corepressor complex involved in N-CoR-mediated repression by specific transcription factors. *Mol Cell* 2:33-42.
122. Lan, F., R. E. Collins, R. De Cegli, R. Alpatov, J. R. Horton, X. Shi, O. Gozani, X. Cheng, and Y. Shi. 2007. Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. *Nature* 448:718-22.
123. Leung, K. M., L. S. Po, F. C. Tsang, W. Y. Siu, A. Lau, H. T. Ho, and R. Y. Poon. 2002. The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. *Cancer Research* 62:4890-3.
124. Leung, K. M., L. S. Po, F. C. Tsang, W. Y. Siu, A. Lau, H. T. Ho, and R. Y. Poon. 2002. The candidate tumor suppressor ING1b can stabilize p53 by disrupting the regulation of p53 by MDM2. *Cancer Res* 62:4890-3.

125. Li, J., M. Martinka, and G. Li. 2008. Role of ING4 in human melanoma cell migration, invasion, and patient survival. *Carcinogenesis*.
126. Li, X., L. Cai, M. Liang, Y. Wang, J. Yang, and Y. Zhao. 2008. Ing4 induces Cell Growth Inhibition in Human Lung Adenocarcinoma A549 Cells by Means of Wnt-1/beta-Catenin Signaling Pathway. *Anat Rec (Hoboken)* 291:593-600.
127. Liang, H., Q. Chen, A. H. Coles, S. J. Anderson, G. Pihan, A. Bradley, R. Gerstein, R. Jurecic, and S. N. Jones. 2003. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cell* 4:349-60.
128. Lichtman, M. A. 2008. Battling the hematological malignancies: the 200 years' war. *Oncologist* 13:126-38.
129. Lo Coco, F., G. Gaidano, D. C. Louie, K. Offit, R. S. Chaganti, and R. Dalla-Favera. 1993. p53 mutations are associated with histologic transformation of follicular lymphoma. *Blood* 82:2289-95.
130. Lossos, I. S., and D. Morgensztern. 2006. Prognostic biomarkers in diffuse large B-cell lymphoma. *J Clin Oncol* 24:995-1007.
131. Lowe, S. W., E. M. Schmitt, S. W. Smith, B. A. Osborne, and T. Jacks. 1993. p53 is required for radiation-induced apoptosis in mouse thymocytes.[see comment]. *Nature* 362:847-9.
132. Lowe, S. W., and C. J. Sherr. 2003. Tumor suppression by Ink4a-Arf: progress and puzzles. *Current Opinion in Genetics & Development* 13:77-83.

133. Lu, F., D. L. Dai, M. Martinka, V. Ho, and G. Li. 2006. Nuclear ING2 expression is reduced in human cutaneous melanomas. *Br J Cancer* 95:80-6.
134. Maeda, S., H. Kamata, J. L. Luo, H. Leffert, and M. Karin. 2005. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. *Cell* 121:977-90.
135. Malhotra, V., and H. R. Wong. 2002. Interactions between the heat shock response and the nuclear factor-kappaB signaling pathway. *Crit Care Med* 30:S89-S95.
136. Marfella, C. G., Y. Ohkawa, A. H. Coles, D. S. Garlick, S. N. Jones, and A. N. Imbalzano. 2006. Mutation of the SNF2 family member Chd2 affects mouse development and survival. *J Cell Physiol* 209:162-71.
137. Michels, J., V. Foria, B. Mead, G. Jackson, M. Mullee, P. W. Johnson, and G. Packham. 2006. Immunohistochemical analysis of the antiapoptotic Mcl-1 and Bcl-2 proteins in follicular lymphoma. *Br J Haematol* 132:743-6.
138. Monti, S., K. J. Savage, J. L. Kutok, F. Feuerhake, P. Kurtin, M. Mihm, B. Wu, L. Pasqualucci, D. Neuberg, R. C. Aguiar, P. Dal Cin, C. Ladd, G. S. Pinkus, G. Salles, N. L. Harris, R. Dalla-Favera, T. M. Habermann, J. C. Aster, T. R. Golub, and M. A. Shipp. 2005. Molecular profiling of diffuse large B-cell lymphoma identifies robust subtypes including one characterized by host inflammatory response. *Blood* 105:1851-61.

139. Moore, L., S. Venkatachalam, H. Vogel, J. C. Watt, C. L. Wu, H. Steinman, S. N. Jones, and L. A. Donehower. 2003. Cooperativity of p19ARF, Mdm2, and p53 in murine tumorigenesis. *Oncogene* 22:7831-7.
140. Mourskaia, A. A., J. J. Northey, and P. M. Siegel. 2007. Targeting aberrant TGF-beta signaling in pre-clinical models of cancer. *Anticancer Agents Med Chem* 7:504-14.
141. Moynagh, P. N. 2005. The NF-kappaB pathway. *J Cell Sci* 118:4589-92.
142. Moynagh, P. N. 2005. TLR signalling and activation of IRFs: revisiting old friends from the NF-kappaB pathway. *Trends Immunol* 26:469-76.
143. Nagahama, Y., M. Ishimaru, M. Osaki, T. Inoue, A. Maeda, C. Nakada, M. Moriyama, K. Sato, M. Oshimura, and H. Ito. 2007. Apoptotic pathway induced by transduction of RUNX3 in the human gastric carcinoma cell line MKN-1. *Cancer Sci* 99:23-30.
144. Nagashima, M., M. Shiseki, K. Miura, K. Hagiwara, S. P. Linke, R. Pedoux, X. W. Wang, J. Yokota, K. Riabowol, and C. C. Harris. 2001. DNA damage-inducible gene p33ING2 negatively regulates cell proliferation through acetylation of p53. *Proc Natl Acad Sci U S A* 98:9671-6.
145. Nagashima, M., M. Shiseki, R. M. Pedoux, S. Okamura, M. Kitahama-Shiseki, K. Miura, J. Yokota, and C. C. Harris. 2003. A novel PHD-finger motif protein, p47ING3, modulates p53-mediated transcription, cell cycle control, and apoptosis. *Oncogene* 22:343-50.

146. Neumann, M., and M. Naumann. 2007. Beyond IkappaBs: alternative regulation of NF-kappaB activity. *FASEB J* 21:2642-54.
147. Ng, A. K. 2007. Diffuse large B-cell lymphoma. *Semin Radiat Oncol* 17:169-75.
148. Nouman, G. S., J. J. Anderson, S. Crosier, J. Shrimankar, J. Lunec, and B. Angus. 2003. Downregulation of nuclear expression of the p33(ING1b) inhibitor of growth protein in invasive carcinoma of the breast. *J Clin Pathol* 56:507-11.
149. Nouman, G. S., J. J. Anderson, J. Lunec, and B. Angus. 2003. The role of the tumour suppressor p33 ING1b in human neoplasia. *J Clin Pathol* 56:491-6.
150. Nouman, G. S., J. J. Anderson, J. Lunec, and B. Angus. 2003. The role of the tumour suppressor p33 ING1b in human neoplasia. *Journal of Clinical Pathology* 56:491-6.
151. Nouman, G. S., J. J. Anderson, K. M. Wood, J. Lunec, A. G. Hall, M. M. Reid, and B. Angus. 2002. Loss of nuclear expression of the p33(ING1b) inhibitor of growth protein in childhood acute lymphoblastic leukaemia. *J Clin Pathol* 55:596-601.
152. Nouman, G. S., B. Angus, J. Lunec, S. Crosier, A. Lodge, and J. J. Anderson. 2002. Comparative assessment expression of the inhibitor of growth 1 gene (ING1) in normal and neoplastic tissues. *Hybrid Hybridomics* 21:1-10.
153. Nourani, A., Y. Doyon, R. T. Utley, S. Allard, W. S. Lane, and J. Cote. 2001. Role of an ING1 growth regulator in transcriptional activation and targeted histone acetylation by the NuA4 complex. *Molecular & Cellular Biology* 21:7629-40.

154. Ohgi, T., T. Masaki, S. Nakai, A. Morishita, S. Yukimasa, M. Nagai, Y. Miyauchi, T. Funaki, K. Kurokohchi, S. Watanabe, and S. Kuriyama. 2002. Expression of p33(ING1) in hepatocellular carcinoma: relationships to tumour differentiation and cyclin E kinase activity. *Scand J Gastroenterol* 37:1440-8.
155. Ohmori, M., M. Nagai, T. Tasaka, H. P. Koeffler, T. Toyama, K. Riabowol, and J. Takahara. 1999. Decreased expression of p33ING1 mRNA in lymphoid malignancies. *Am J Hematol* 62:118-9.
156. Okano, T., A. Gemma, Y. Hosoya, Y. Hosomi, M. Nara, Y. Kokubo, A. Yoshimura, M. Shibuya, M. Nagashima, C. C. Harris, and S. Kudoh. 2006. Alterations in novel candidate tumor suppressor genes, ING1 and ING2 in human lung cancer. *Oncol Rep* 15:545-9.
157. Opferman, J. T., A. Letai, C. Beard, M. D. Sorcinelli, C. C. Ong, and S. J. Korsmeyer. 2003. Development and maintenance of B and T lymphocytes requires antiapoptotic MCL-1. *Nature* 426:671-6.
158. Ozer, A., and R. K. Bruick. 2005. Regulation of HIF by prolyl hydroxylases: recruitment of the candidate tumor suppressor protein ING4. *Cell Cycle* 4:1153-6.
159. Ozer, A., L. C. Wu, and R. K. Bruick. 2005. The candidate tumor suppressor ING4 represses activation of the hypoxia inducible factor (HIF). *Proc Natl Acad Sci U S A* 102:7481-6.
160. Palacios, A., P. Garcia, D. Padro, E. Lopez-Hernandez, I. Martin, and F. J. Blanco. 2006. Solution structure and NMR characterization of the binding to

methylated histone tails of the plant homeodomain finger of the tumour suppressor ING4. *FEBS Lett* 580:6903-8.

161. Palacios, A., I. G. Munoz, D. Pantoja-Uceda, M. J. Marcaida, D. Torres, J. M. Martin-Garcia, I. Luque, G. Montoya, and F. J. Blanco. 2008. Molecular basis of histone H3K4ME3 recognition by ING4. *J Biol Chem*.
162. Palsson-McDermott, E. M., and L. A. O'Neill. 2004. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* 113:153-62.
163. Park, J., J. H. Lee, M. La, M. J. Jang, G. W. Chae, S. B. Kim, H. Tak, Y. Jung, B. Byun, J. K. Ahn, and C. O. Joe. 2007. Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx. *J Mol Biol* 368:388-97.
164. Pascual, J., M. Martinez-Yamout, H. J. Dyson, and P. E. Wright. 2000. Structure of the PHD zinc finger from human Williams-Beuren syndrome transcription factor. *J Mol Biol* 304:723-9.
165. Pasparakis, M., T. Luedde, and M. Schmidt-Supprian. 2006. Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice. *Cell Death Differ* 13:861-72.
166. Pasqualucci, L., P. Neumeister, T. Goossens, G. Nanjangud, R. S. Chaganti, R. Kuppers, and R. Dalla-Favera. 2001. Hypermutation of multiple proto-oncogenes in B-cell diffuse large-cell lymphomas. *Nature* 412:341-6.
167. Pedoux, R., S. Sengupta, J. C. Shen, O. N. Demidov, S. Saito, H. Onogi, K. Kumamoto, S. Wincovitch, S. H. Garfield, M. McMenamin, M. Nagashima, S. R. Grossman, E. Appella, and C. C. Harris. 2005. ING2 regulates the onset of

- replicative senescence by induction of p300-dependent p53 acetylation. *Mol Cell Biol* 25:6639-48.
168. Pena, P. V., F. Davrazou, X. Shi, K. L. Walter, V. V. Verkhusha, O. Gozani, R. Zhao, and T. G. Kutateladze. 2006. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* 442:100-3.
169. Perkins, N. D. 2004. NF-kappaB: tumor promoter or suppressor? *Trends Cell Biol* 14:64-9.
170. Phan, R. T., and R. Dalla-Favera. 2004. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* 432:635-9.
171. Polyak, K., and W. C. Hahn. 2006. Roots and stems: stem cells in cancer. *Nat Med* 12:296-300.
172. Ponder, B. A. 2001. Cancer genetics. *Nature* 411:336-41.
173. Ragvin, A., H. Valvatne, S. Erdal, V. Arskog, K. R. Tufteland, K. Breen, O. Y. AM, A. Eberharter, T. J. Gibson, P. B. Becker, and R. Aasland. 2004. Nucleosome binding by the bromodomain and PHD finger of the transcriptional cofactor p300. *J Mol Biol* 337:773-88.
174. Ran, R., A. Lu, L. Zhang, Y. Tang, H. Zhu, H. Xu, Y. Feng, C. Han, G. Zhou, A. C. Rigby, and F. R. Sharp. 2004. Hsp70 promotes TNF-mediated apoptosis by binding IKK gamma and impairing NF-kappa B survival signaling. *Genes Dev* 18:1466-81.
175. Rayet, B., and C. Gelinas. 1999. Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 18:6938-47.



176. Ripperger, T., N. von Neuhoff, K. Kamphues, M. Emura, U. Lehmann, M. Tauscher, M. Schraders, P. Groenen, B. Skawran, C. Rudolph, E. Callet-Bauchu, J. H. van Krieken, B. Schlegelberger, and D. Steinemann. 2007. Promoter methylation of PARG1, a novel candidate tumor suppressor gene in mantle-cell lymphomas. *Haematologica* 92:460-8.
177. Roversi, G., R. Pfundt, R. F. Moroni, I. Magnani, S. van Reijmersdal, B. Pollo, H. Straatman, L. Larizza, and E. F. Schoenmakers. 2006. Identification of novel genomic markers related to progression to glioblastoma through genomic profiling of 25 primary glioma cell lines. *Oncogene* 25:1571-83.
178. Sager, R. 1997. Expression genetics in cancer: shifting the focus from DNA to RNA. *Proc Natl Acad Sci U S A* 94:952-5.
179. Saito, M., J. Gao, K. Basso, Y. Kitagawa, P. M. Smith, G. Bhagat, A. Pernis, L. Pasqualucci, and R. Dalla-Favera. 2007. A signaling pathway mediating downregulation of BCL6 in germinal center B cells is blocked by BCL6 gene alterations in B cell lymphoma. *Cancer Cell* 12:280-92.
180. Sanchez-Carbayo, M., N. D. Socci, J. J. Lozano, W. Li, E. Charytonowicz, T. J. Belbin, M. B. Prystowsky, A. R. Ortiz, G. Childs, and C. Cordon-Cardo. 2003. Gene discovery in bladder cancer progression using cDNA microarrays. *Am J Pathol* 163:505-16.
181. Sander, C. A., T. Yano, H. M. Clark, C. Harris, D. L. Longo, E. S. Jaffe, and M. Raffeld. 1993. p53 mutation is associated with progression in follicular lymphomas. *Blood* 82:1994-2004.

182. Sarker, K. P., H. Kataoka, A. Chan, S. J. Netherton, I. Pot, M. A. Huynh, X. Feng, A. Bonni, K. Riabowol, and S. Bonni. 2008. ING2 as a novel mediator of TGF-beta -dependent responses in epithelial cells. *J Biol Chem.* 283:13269-79.
183. Schraders, M., R. Pfundt, H. M. Straatman, I. M. Janssen, A. G. van Kessel, E. F. Schoenmakers, J. H. van Krieken, and P. J. Groenen. 2005. Novel chromosomal imbalances in mantle cell lymphoma detected by genome-wide array-based comparative genomic hybridization. *Blood* 105:1686-93.
184. Scott, M., F. M. Boisvert, D. Vieyra, R. N. Johnston, D. P. Bazett-Jones, and K. Riabowol. 2001. UV induces nucleolar translocation of ING1 through two distinct nucleolar targeting sequences. *Nucleic Acids Res* 29:2052-8.
185. Scott, M., P. Bonnefin, D. Vieyra, F. M. Boisvert, D. Young, D. P. Bazett-Jones, and K. Riabowol. 2001. UV-induced binding of ING1 to PCNA regulates the induction of apoptosis. *Journal of Cell Science* 114:3455-62.
186. Scott, M., P. Bonnefin, D. Vieyra, F. M. Boisvert, D. Young, D. P. Bazett-Jones, and K. Riabowol. 2001. UV-induced binding of ING1 to PCNA regulates the induction of apoptosis. *J Cell Sci* 114:3455-62.
187. Serra-Pages, C., N. L. Kedersha, L. Fazikas, Q. Medley, A. Debant, and M. Streuli. 1995. The LAR transmembrane protein tyrosine phosphatase and a coiled-coil LAR-interacting protein co-localize at focal adhesions. *EMBO J* 14:2827-38.
188. Shen, D. H., K. Y. Chan, U. S. Khoo, H. Y. Ngan, W. C. Xue, P. M. Chiu, P. Ip, and A. N. Cheung. 2005. Epigenetic and genetic alterations of p33ING1b in ovarian cancer. *Carcinogenesis* 26:855-63.

189. Shen, J. C., M. Unoki, D. Ythier, A. Duperray, L. Varticovski, K. Kumamoto, R. Pedeux, and C. C. Harris. 2007. Inhibitor of growth 4 suppresses cell spreading and cell migration by interacting with a novel binding partner, liprin alpha 1. *Cancer Res* 67:2552-8.
190. Sherr, C. J. 2006. Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer* 6:663-73.
191. Sherr, C. J. 1998. Tumor surveillance via the ARF-p53 pathway. *Genes Dev* 12:2984-91.
192. Sherr, C. J., and J. D. Weber. 2000. The ARF/p53 pathway. *Curr Opin Genet Dev* 10:94-9.
193. Shi, X., and O. Gozani. 2005. The fellowships of the ING2s. *J Cell Biochem* 96:1127-36.
194. Shi, X., T. Hong, K. L. Walter, M. Ewalt, E. Michishita, T. Hung, D. Carney, P. Pena, F. Lan, M. R. Kaadige, N. Lacoste, C. Cayrou, F. Davrazou, A. Saha, B. R. Cairns, D. E. Ayer, T. G. Kutateladze, Y. Shi, J. Cote, K. F. Chua, and O. Gozani. 2006. ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* 442:96-9.
195. Shi, Y., Z. Tu, D. Tang, H. Zhang, M. Liu, K. Wang, S. K. Calderwood, and X. Xiao. 2006. The inhibition of LPS-induced production of inflammatory cytokines by HSP70 involves inactivation of the NF-kappaB pathway but not the MAPK pathways. *Shock* 26:277-84.

196. Shimada, H., T. L. Liu, T. Ochiai, T. Shimizu, Y. Haupt, H. Hamada, T. Abe, M. Oka, M. Takiguchi, and T. Hiwasa. 2002. Facilitation of adenoviral wild-type p53-induced apoptotic cell death by overexpression of p33(ING1) in T.Tn human esophageal carcinoma cells. *Oncogene* 21:1208-16.
197. Shimada, Y., A. Saito, M. Suzuki, E. Takahashi, and M. Horie. 1998. Cloning of a novel gene (ING1L) homologous to ING1, a candidate tumor suppressor. *Cytogenet Cell Genet* 83:232-5.
198. Shinoura, N., Y. Muramatsu, M. Nishimura, Y. Yoshida, A. Saito, T. Yokoyama, T. Furukawa, A. Horii, M. Hashimoto, A. Asai, T. Kirino, and H. Hamada. 1999. Adenovirus-mediated transfer of p33ING1 with p53 drastically augments apoptosis in gliomas. *Cancer Res* 59:5521-8.
199. Shiseki, M., M. Nagashima, R. M. Pedoux, M. Kitahama-Shiseki, K. Miura, S. Okamura, H. Onogi, Y. Higashimoto, E. Appella, J. Yokota, and C. C. Harris. 2003. p29ING4 and p28ING5 bind to p53 and p300, and enhance p53 activity. *Cancer Res* 63:2373-8.
200. Silverstein, R. A., and K. Ekwall. 2005. Sin3: a flexible regulator of global gene expression and genome stability. *Curr Genet* 47:1-17.
201. Singh, M., and L. Johnson. 2006. Using genetically engineered mouse models of cancer to aid drug development: an industry perspective. *Clin Cancer Res* 12:5312-28.
202. Skowyra, D., M. Zeremski, N. Neznanov, M. Li, Y. Choi, M. Uesugi, C. A. Hauser, W. Gu, A. V. Gudkov, and J. Qin. 2001. Differential association of

- products of alternative transcripts of the candidate tumor suppressor ING1 with the mSin3/HDAC1 transcriptional corepressor complex. *J Biol Chem* 276:8734-9.
203. Soliman, M. A., and K. Riabowol. 2007. After a decade of study-ING, a PHD for a versatile family of proteins. *Trends Biochem Sci* 32:509-19.
204. Stark, M., J. A. Puig-Butille, G. Walker, C. Badenas, J. Malvehy, N. Hayward, and S. Puig. 2006. Mutation of the tumour suppressor p33ING1b is rare in melanoma. *Br J Dermatol* 155:94-9.
205. Steinman, H. A., K. M. Hoover, M. L. Keeler, A. T. Sands, and S. N. Jones. 2005. Rescue of Mdm4-deficient mice by Mdm2 reveals functional overlap of Mdm2 and Mdm4 in development. *Oncogene* 24:7935-40.
206. Takahashi, M., N. Seki, T. Ozaki, M. Kato, T. Kuno, T. Nakagawa, K. Watanabe, K. Miyazaki, M. Ohira, S. Hayashi, M. Hosoda, H. Tokita, H. Mizuguchi, T. Hayakawa, S. Todo, and A. Nakagawara. 2002. Identification of the p33(ING1)-regulated genes that include cyclin B1 and proto-oncogene DEK by using cDNA microarray in a mouse mammary epithelial cell line NMuMG. *Cancer Res* 62:2203-9.
207. Tallen, G., I. Kaiser, S. Krabbe, U. Lass, C. Hartmann, G. Henze, K. Riabowol, and A. von Deimling. 2004. No ING1 mutations in human brain tumours but reduced expression in high malignancy grades of astrocytoma. *Int J Cancer* 109:476-9.
208. Tallen, U. G., M. Truss, F. Kunitz, S. Wellmann, B. Unryn, B. Sinn, U. Lass, S. Krabbe, N. Holtkamp, C. Hagemeyer, R. Wurm, G. Henze, K. T. Riabowol, and

- A. von Deimling. 2007. Down-regulation of the inhibitor of growth 1 (ING1) tumor suppressor sensitizes p53-deficient glioblastoma cells to cisplatin-induced cell death. *J Neurooncol*.
209. Thomas, T., and A. K. Voss. 2007. The diverse biological roles of MYST histone acetyltransferase family proteins. *Cell Cycle* 6:696-704.
210. Todaro, G. J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J. Cell Biol.* 17:299-313.
211. Tokunaga, E., Y. Maehara, E. Oki, K. Kitamura, Y. Kakeji, S. Ohno, and K. Sugimachi. 2000. Diminished expression of ING1 mRNA and the correlation with p53 expression in breast cancers. *Cancer Lett* 152:15-22.
212. Tournier, C., P. Hess, D. D. Yang, J. Xu, T. K. Turner, A. Nimnual, D. Bar-Sagi, S. N. Jones, R. A. Flavell, and R. J. Davis. 2000. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 288:870-4.
213. Toyama, T., H. Iwase, P. Watson, H. Muzik, E. Saettler, A. Magliocco, L. DiFrancesco, P. Forsyth, I. Garkavtsev, S. Kobayashi, and K. Riabowol. 1999. Suppression of ING1 expression in sporadic breast cancer. *Oncogene* 18:5187-93.
214. Tsang, F. C., L. S. Po, K. M. Leung, A. Lau, W. Y. Siu, and R. Y. Poon. 2003. ING1b decreases cell proliferation through p53-dependent and -independent mechanisms. *FEBS Letters* 553:277-85.

215. Tsang, F. C., L. S. Po, K. M. Leung, A. Lau, W. Y. Siu, and R. Y. Poon. 2003. ING1b decreases cell proliferation through p53-dependent and -independent mechanisms. *FEBS Lett* 553:277-85.
216. Unoki, M., J. C. Shen, Z. M. Zheng, and C. C. Harris. 2006. Novel splice variants of ING4 and their possible roles in the regulation of cell growth and motility. *J Biol Chem* 281:34677-86.
217. Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma. 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappaB. *Science* 274:787-9.
218. Vieyra, D., R. Loewith, M. Scott, P. Bonnefin, F. M. Boisvert, P. Cheema, S. Pastyryeva, M. Meijer, R. N. Johnston, D. P. Bazett-Jones, S. McMahon, M. D. Cole, D. Young, and K. Riabowol. 2002. Human ING1 proteins differentially regulate histone acetylation. *J Biol Chem* 277:29832-9.
219. Vieyra, D., D. L. Senger, T. Toyama, H. Muzik, P. M. Brasher, R. N. Johnston, K. Riabowol, and P. A. Forsyth. 2003. Altered subcellular localization and low frequency of mutations of ING1 in human brain tumors. *Clin Cancer Res* 9:5952-61.
220. Vieyra, D., T. Toyama, Y. Hara, D. Boland, R. Johnston, and K. Riabowol. 2002. ING1 isoforms differentially affect apoptosis in a cell age-dependent manner. *Cancer Res* 62:4445-52.
221. Vogelstein, B., and K. W. Kinzler. 2004. Cancer genes and the pathways they control. *Nat Med* 10:789-99.

222. Walzak, A. A., N. Veldhoen, X. Feng, K. Riabowol, and C. C. Helbing. 2007. Expression profiles of mRNA transcript variants encoding the human inhibitor of growth tumor suppressor gene family in normal and neoplastic tissues. *Exp Cell Res*.
223. Wang, J., M. Y. Chin, and G. Li. 2006. The novel tumor suppressor p33ING2 enhances nucleotide excision repair via inducement of histone H4 acetylation and chromatin relaxation. *Cancer Res* 66:1906-11.
224. Wang, P., T. Lushnikova, J. Odvody, T. C. Greiner, S. N. Jones, and C. M. Eischen. 2007. Elevated Mdm2 expression induces chromosomal instability and confers a survival and growth advantage to B cells. *Oncogene* 27:1590-8.
225. Wang, Y., D. L. Dai, M. Martinka, and G. Li. 2007. Prognostic significance of nuclear ING3 expression in human cutaneous melanoma. *Clin Cancer Res* 13:4111-6.
226. Wang, Y., and G. Li. 2006. ING3 promotes UV-induced apoptosis via Fas/caspase-8 pathway in melanoma cells. *J Biol Chem* 281:11887-93.
227. Wang, Y., J. Wang, and G. Li. 2006. Leucine zipper-like domain is required for tumor suppressor ING2-mediated nucleotide excision repair and apoptosis. *FEBS Lett* 580:3787-93.
228. Ward, J. M., L. Tadesse-Heath, S. N. Perkins, S. K. Chattopadhyay, S. D. Hursting, and H. C. Morse, 3rd. 1999. Splenic marginal zone B-cell and thymic T-cell lymphomas in p53-deficient mice. *Lab Invest* 79:3-14.



229. Weiss, B., and K. Shannon. 2003. Mouse cancer models as a platform for performing preclinical therapeutic trials. *Curr Opin Genet Dev* 13:84-9.
230. Weng, Y., C. Fang, R. J. Turesky, M. Behr, L. S. Kaminsky, and X. Ding. 2007. Determination of the role of target tissue metabolism in lung carcinogenesis using conditional cytochrome P450 reductase-null mice. *Cancer Res* 67:7825-32.
231. Wijnhoven, S. W., E. M. Hoogervorst, H. de Waard, G. T. van der Horst, and H. van Steeg. 2007. Tissue specific mutagenic and carcinogenic responses in NER defective mouse models. *Mutat Res* 614:77-94.
232. Witcher, M., D. T. Ross, C. Rousseau, L. Deluca, and W. H. Miller, Jr. 2003. Synergy between all-trans retinoic acid and tumor necrosis factor pathways in acute leukemia cells. *Blood* 102:237-45.
233. Xin, H., H. G. Yoon, P. B. Singh, J. Wong, and J. Qin. 2004. Components of a pathway maintaining histone modification and heterochromatin protein 1 binding at the pericentric heterochromatin in Mammalian cells. *J Biol Chem* 279:9539-46.
234. Yamaguchi, H., K. Inokuchi, M. Tarusawa, and K. Dan. 2002. Mutation of bcl-x gene in non-Hodgkin's lymphoma. *Am J Hematol* 69:74-6.
235. Yan, Z. Q., and G. K. Hansson. 2007. Innate immunity, macrophage activation, and atherosclerosis. *Immunol Rev* 219:187-203.
236. Yu, G. Z., M. H. Zhu, Z. Zhu, C. R. Ni, J. M. Zheng, and F. M. Li. 2004. Genetic alterations and reduced expression of tumor suppressor p33(ING1b) in human exocrine pancreatic carcinoma. *World J Gastroenterol* 10:3597-601.

237. Zambrowicz, B. P., G. A. Friedrich, E. C. Buxton, S. L. Lilleberg, C. Person, and A. T. Sands. 1998. Disruption and sequence identification of 2,000 genes in mouse embryonic stem cells. *Nature* 392:608-11.
238. Zeremski, M., J. E. Hill, S. S. Kwek, I. A. Grigorian, K. V. Gurova, I. V. Garkavtsev, L. Diatchenko, E. V. Koonin, and A. V. Gudkov. 1999. Structure and regulation of the mouse *ing1* gene. Three alternative transcripts encode two phd finger proteins that have opposite effects on p53 function. *Journal of Biological Chemistry* 274:32172-81.
239. Zeremski, M., J. E. Hill, S. S. Kwek, I. A. Grigorian, K. V. Gurova, I. V. Garkavtsev, L. Diatchenko, E. V. Koonin, and A. V. Gudkov. 1999. 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* 274:32172-81.
240. Zhang, H. K., K. Pan, H. Wang, D. S. Weng, H. F. Song, J. Zhou, W. Huang, J. J. Li, M. S. Chen, and J. C. Xia. 2007. Decreased expression of *ING2* gene and its clinicopathological significance in hepatocellular carcinoma. *Cancer Lett* 261:183-92.
241. Zhang, J. T., D. W. Wang, Q. X. Li, Z. L. Zhu, M. W. Wang, D. S. Cui, Y. H. Yang, Y. X. Gu, and X. F. Sun. 2008. Nuclear to cytoplasmic shift of p33(ING1b) protein from normal oral mucosa to oral squamous cell carcinoma in relation to clinicopathological variables. *J Cancer Res Clin Oncol* 134:421-6.

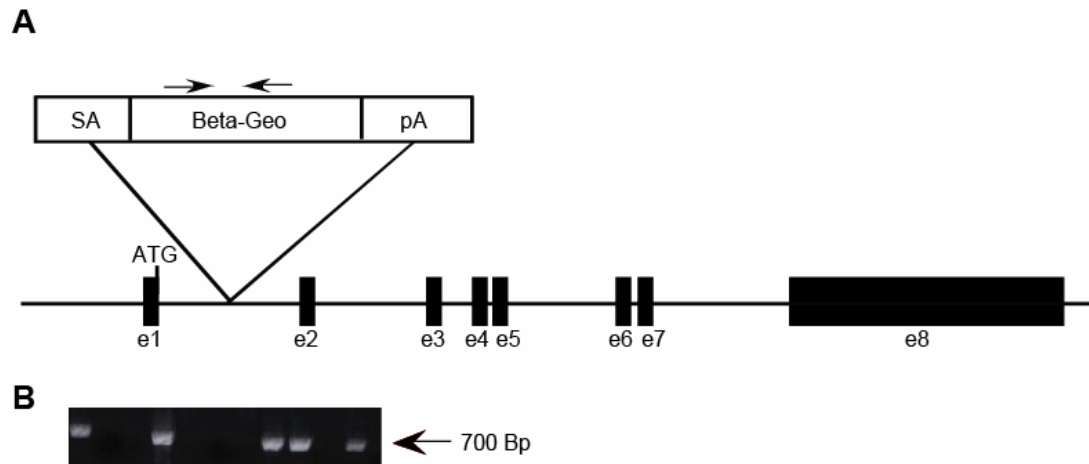
242. Zhang, X., K. S. Wang, Z. Q. Wang, L. S. Xu, Q. W. Wang, F. Chen, D. Z. Wei, and Z. G. Han. 2005. Nuclear localization signal of ING4 plays a key role in its binding to p53. *Biochem Biophys Res Commun* 331:1032-8.
243. Zhang, X., L. S. Xu, Z. Q. Wang, K. S. Wang, N. Li, Z. H. Cheng, S. Z. Huang, D. Z. Wei, and Z. G. Han. 2004. ING4 induces G2/M cell cycle arrest and enhances the chemosensitivity to DNA-damage agents in HepG2 cells. *FEBS Lett* 570:7-12.
244. Zhou, P., N. B. Levy, H. Xie, L. Qian, C. Y. Lee, R. D. Gascoyne, and R. W. Craig. 2001. MCL1 transgenic mice exhibit a high incidence of B-cell lymphoma manifested as a spectrum of histologic subtypes. *Blood* 97:3902-9.
245. Zhu, Z., J. Lin, J. H. Qu, M. A. Feitelson, C. R. Ni, F. M. Li, and M. H. Zhu. 2005. Inhibitory effect of tumor suppressor p33(ING1b) and its synergy with p53 gene in hepatocellular carcinoma. *World J Gastroenterol* 11:1903-9.

**APPENDIX I:**  
**ADDITIONAL WORK**

### **Additional work on Ing genes:**

#### **I. Generation of Ing5 knockout mice:**

We obtained a mouse ES cell line (RRC086) from Baygenomics bearing a retroviral promoter trap inserted into one allele of the Ing5 gene was used in standard blastocyst injection experiments to generate chimeric mice. DNA sequence analysis of the gene-trapped locus revealed that the retrovirus inserted between exon 1 and exon 2 thereby possibly interrupting the entire coding region for Ing5 (Figure S1A). Several high-degree chimeric mice were bred with C57Bl/6 mice to generate agouti offspring, and genomic DNA was harvested from tail biopsies and analyzed by PCR (Figure S1B) for the presence of the  $\beta$ Geo cassette to identify mice that inherited the Ing5-targeted allele. This allowed for the identification of heterozygous animals, but did not differentiate het from null mice. Circular PCR or sequencing the product from primers between either exon 1 and the 5' region of the genetrapped or exon 2 and the 3' region of the genetrapped will be needed in order to design a better genotyping strategy. No further work was done on these mice, which were subsequently cryopreserved.



**Figure S1:** Generation of *Ing5*-deficient mice. **A)** Schematic of the gene-trapped locus showing the gene-trap inserted between exon 1 and exon 2. **B)** PCR genotyping of the mouse tail biopsies showing the presence of the  $\beta$ Geo cassette. An approximately 700bp fragment is generated from the  $\beta$ Geo primers (5'-GGTCAATCCGCCGTTTGTTC-3' and 5'-CAGTTCAACCACCGCACGATAG-3').

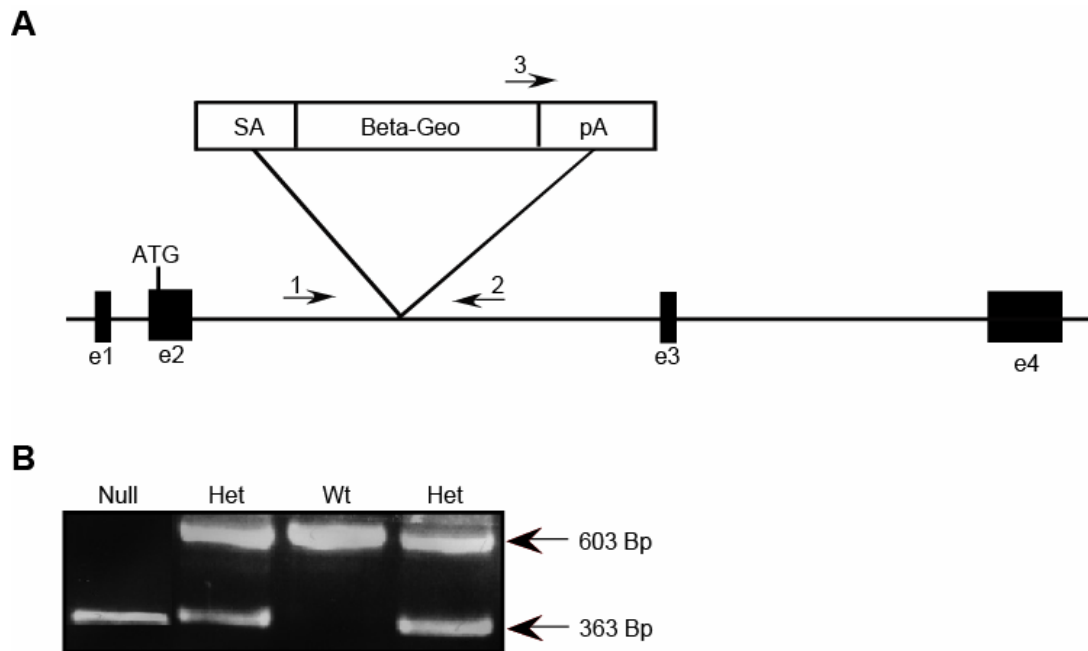
## II. Generation of *Ing1/Ing4* double null mice:

In order to explore the possibility that *Ing* genes compensate for each other, we crossed  $p37^{Ing1b}$  null mice to *Ing4* null mice. These *Ing1/Ing4* heterozygous animals were intercrossed until they were null for both alleles. We also derived *Ing1/Ing4* null mouse embryonic fibroblasts (MEFs). The resulting *Ing1/Ing4* double null animals were found to be viable, but appeared to be more prone to develop seizures than wt controls. This behavior needs further investigation. Additionally, these mice could have an even more exacerbated inflammatory response given the elevated NF- $\kappa$ B activity in *Ing1* (see Chapter 3) and *Ing4* null (see Chapter 4) animals.

### **Generation of Foxo3a knockout mice:**

FOXO3a was suggested to function at the G<sub>2</sub>/M checkpoint and to participate in DNA repair pathways (5). These findings suggested that FOXO3A may be acting as a tumor suppressor. In order to explore this possibility we obtained a mouse ES cell line (XA026) from Baygenomics bearing a retroviral promoter trap inserted into one allele of the Foxo3A gene. This ES cell line was used in standard blastocyst injection experiments to generate chimeric mice. DNA sequence analysis of the gene-trapped locus revealed that the retrovirus inserted between exon 1 and exon 2 thereby possibly interrupting the entire coding region for Foxo3A (Figure S2A). Several high-degree chimeric mice were bred with C57Bl/6 mice to generate agouti offspring, and genomic DNA was harvested from tail biopsies and analyzed by PCR (Figure S2B). PCR genotyping indicated that Foxo3a null mice were viable and appeared similar to wt littermates. During the course of this work the DePinho group published the characterization of Foxo3a deficient mice in Science (1). They discovered that female mice deficient for Foxo3a showed global follicular activation resulting in oocyte death and depletion of functional ovarian follicles. The functional assays that were conducted on MEFs from these animals did not show any significant defects. Also, Foxo3a null mice were not tumor prone. They did, however, show some physiologic defects, such as a mild compensated anemia and decreased rate of glucose uptake following an overnight fast (1). No subsequent work was conducted on these animals which were cryopreserved.

Subsequent literature searches did find papers suggesting an interaction between p53 and Foxo3a (6). Also, a paper by Lin et al. using the same Baygenomics ES cell line that we have found that Foxo3a can regulate NF- $\kappa$ B activity and also thymocyte activation (4). This group also determined by western blot that the XA026 ES cell contains a complete knockout of Foxo3a.



**Figure S2:** Generation of Foxo3a-deficient mice. **A)** Schematic of the gene-trapped locus showing the gene-trap insertion site at position 15948. **B)** PCR genotyping of the mouse tail biopsies. Primers 1 (5'-CCTCCTAAAAATAAGCGTGCTACG-3') and 2 (5'-CCTACTGTGTGACAACCTCTGCTG-3') produced a 603bp Wt product. The 363bp mutant product was generated using primers 2 and 3 (5'-GCACAGATGCGTAAGGAGAAAATACC-3').



**Work on Wnt5a null mice:**

Work in collaboration with Huiling Liang on Wnt5a deficient mice resulted in two publications. These papers found that Wnt5a null mice develop B-cell lymphomas due to its ability to negatively regulate B-cell proliferation. Wnt5a was found to signal through the non-canonical Wnt/Ca<sup>2+</sup> pathway to suppression Cyclin D1 expression (2). Therefore, Wnt5a is acting as a tumor suppressor gene in B-cell lymphomas. Wnt5a was also found to alter survival of  $\alpha\beta$ -T-cells by regulating Bax and Bcl-2 expression (3). These results suggest that Wnt5a signals through the non-canonical Wnt/Ca<sup>2+</sup> pathway, but can alter the canonical Wnt pathway. They also suggest that Wnt5a is critical for normal T cell development. My efforts contributed to these studies by assisting Huiling with several experiments including Southern blots, *in vitro* kinase assays, and fetal thymic organ culture experiments.

## REFERENCES

1. Castrillon, D. H., L. Miao, R. Kollipara, J. W. Horner, and R. A. DePinho. 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. *Science* 301:215-8.
2. Liang, H., Q. Chen, A. H. Coles, S. J. Anderson, G. Pihan, A. Bradley, R. Gerstein, R. Jurecic, and S. N. Jones. 2003. Wnt5a inhibits B cell proliferation and functions as a tumor suppressor in hematopoietic tissue. *Cancer Cell* 4:349-60.
3. Liang, H., A. H. Coles, Z. Zhu, J. Zayas, R. Jurecic, J. Kang, and S. N. Jones. 2007. Noncanonical Wnt signaling promotes apoptosis in thymocyte development. *J Exp Med* 204:3077-84.
4. Lin, L., J. D. Hron, and S. L. Peng. 2004. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity* 21:203-13.
5. Tran, H., A. Brunet, J. M. Grenier, S. R. Datta, A. J. Fornace, Jr., P. S. DiStefano, L. W. Chiang, and M. E. Greenberg. 2002. DNA repair pathway stimulated by the forkhead transcription factor FOXO3a through the Gadd45 protein. *Science* 296:530-4.
6. You, H., K. Yamamoto, and T. W. Mak. 2006. Regulation of transactivation-independent proapoptotic activity of p53 by FOXO3a. *Proc Natl Acad Sci U S A* 103:9051-6.

