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REGULATION OF MDM2 MEDIATED NFκB2 PATHWAY IN HUMAN LUNG CANCER

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REGULATION OF MDM2 MEDIATED NFκB2 PATHWAY IN HUMAN LUNG

CANCER

A Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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DEDICATION

I would like to dedicate this work to my parents who encouraged me to go ahead and pursue my doctorate in the US and my husband, Parth, who supported and motivated me throughout my PhD and has also been my strength in times of need. This work is also dedicated to my mentors, Ms. Rajeevan and Ms. Maya who developed my interest in the field of science and research.

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List of Abbreviations

ARD	Ankyrin Repeat Domain
Bcl2	B cell lymphoma-2
DMSO	Dimethyl Sulfoxide
GAPDH	Glyceraldehyde 3- phosphate dehydrogenase
IκB	Inhibitor of kappa B
IKK	Inhibitor of kappa B kinase
MAPK	Mitogen Activated Protein Kinase
MDM2	Mouse Double Minute 2
NFκB2	Nuclear factor of kappa B
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol-3-kinase
Rb	Retinoblastoma susceptibility protein
TBP	TATA Binding Protein

G. Abstract

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Overexpression of oncoprotein MDM2 and mutations of tumor suppressor p53 are frequently observed in human cancers. The NFκB pathway is one of the deregulated pathways in oncogenesis. The overall goal of the project was to study the regulation of NFκB pathway by MDM2 in lung cancer. Our first effort was to determine the frequency of MDM2 overexpression in human lung tumor samples and to identify co-occurring abnormal gene expression by studying the levels of MDM2 and members of NFκB pathway with respect to p53 status. Higher than normal levels of MDM2 were found in approximately 30% of the cancer samples harboring wild-type (WT) and mutant p53. Expression of NFκB2, a mutant p53 inducible gene showed significant statistical

correlation with MDM2 in cancer samples that harbored WT p53. A downstream target gene of NFκB2, Bcl2, showed a significant correlation to MDM2 levels, independent of p53 status. Lung cancer samples harboring mutant p53 exhibited elevated levels of NFκB2 though not statistically significant. Our next step was to corroborate findings from lung tumor samples with data from lung cancer cell line harboring WT p53-H460. Consistent with lung tumor samples, ectopic overexpression of MDM2 in H460, showed elevated expression of NFκB2 and Bcl2 with promoter upregulation of NFκB2. Silencing of MDM2 proportionally downregulated NFκB2 and Bcl2 in H460 cells. Domain analysis of MDM2 suggested that increase in the NFκB2 promoter activity was not confined to the p53 binding domain of MDM2 suggesting their interaction via p53-dependent and p53-independent mechanisms. A functional cell growth assay showed retarded cell proliferation with downregulation of MDM2. Data from human lung tumor samples and lung cancer cell line suggest that overexpression of MDM2 mediates NFκB2 upregulation to confer growth advantage, thus favoring oncogenesis.

H. Chapter1. Introduction

Enhancement of proto-oncogenes either alone or in combination with inactivation of tumor suppressor genes secondary to translocations or deletions is the most common genetic alteration underlying oncogenesis. In addition, gene amplification in which multiple extra copies of the sub-chromosomal DNA, amplicons, are observed is another mechanism of activation of proto-oncogenes. These amplicons can be integrated into chromosomes or can be present extra chromosomally. While the former can be visualized cytogenetically as homogenous stained regions of chromosomes, the latter are often present as double minute chromatin bodies. One of the most commonly amplified oncogenes is the mouse double minute (mdm2) that confers selective survival advantage by interfering with control of cell cycle and proliferation. The mdm2 gene is situated on chromosome 12q14 and encodes for a nuclear phosphoprotein [1].

Functional domains of MDM2: The mdm2 gene encodes a 491 amino acid protein. The structural domains of MDM2 include an N-terminal p53 interaction domain, a central acidic domain (residues 230-300) and a C- terminal zinc finger domain (amino acid residues 430-480), responsible for the E3 ubiquitin ligase activity. The acidic domain contains the nuclear export and import signals that are necessary for the nuclear-cytoplasmic shuttling of MDM2 [2, 3]. MDM2 has two zinc fingers which mediates its ability to bind to specific RNA sequences or structures in vitro [4]. Figure 1 show the domains and the interacting proteins of MDM2.
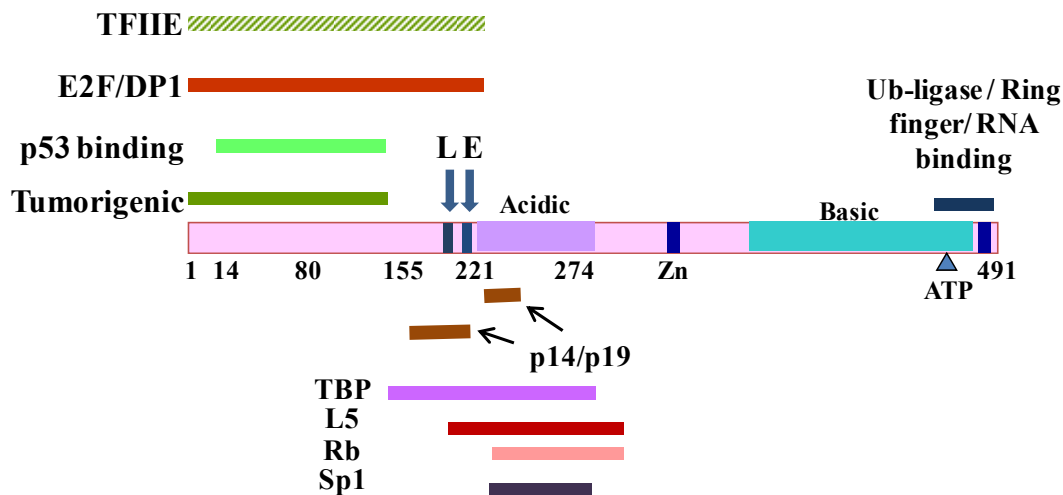
Figure 1: MDM2 domains and its various interaction proteins.

MDM2 is potentially oncogenic: The human homologue of mouse double minute-2 gene

is frequently overexpressed in many human carcinomas, soft tissue sarcomas and other

cancers [5-8], suggesting their role in oncogenesis.   Amplification of the mdm2 gene

enhances the tumorigenic potential of murine cells [1, 8]while targeted overexpression of

MDM2 in transgenic mice causes polyploidy [9]. Thus, the proposed mechanisms of

MDM2 mediated oncogenesis include amplification of the mdm2, overexpression of

mdm2 messenger RNA [6, 10-12] and enhanced translation of mRNA [13, 14].

Structure and function of p53: p53, a well studied tumor suppressor, is located on

chromosome 17p and is mutated in 50 percent of human cancers. The p53 gene encodes a

nuclear phosphoprotein (transcription factor) that functions as a tumor suppressor. The p53

gene has five highly conserved regions shown as domains I-V in the Figure 2. The

sequence specific DNA binding domain of p53 is found in the central region covering the

domains II-V, spanning the residues 100-293 [15-17]. The transactivation domain of p53 is

located within residues 1-73 [18-20] and the oligomerization domain covers the residues 300-360 [21].
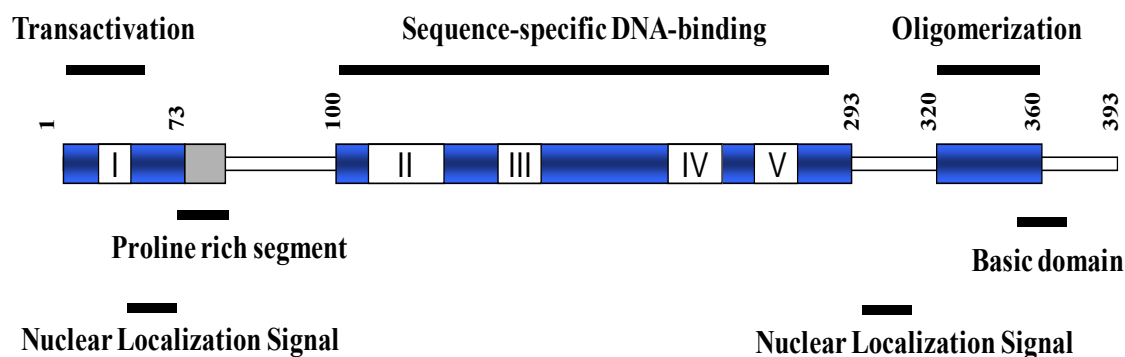
Figure 2: Structure and domains of the tumor suppressor p53.

p53 is a sequence specific DNA binding protein and can activate or repress transcription of a variety of proteins [22, 23]. Levels of p53 in the cell increase after stress signals, DNA damage and hypoxia. Elevated levels of wildtype (WT) p53 lead to apoptosis and G1/S cell cycle arrest [24]. p21/WAF-1 is an integral component of the G1/S checkpoint that is under the transcriptional control of p53 [25, 26]. Loss of this checkpoint contributes to an increase in polyploidy cells [27].

MDM2 interacts with tumor suppressor p53: MDM2 recognizes the transactivation domain of p53 and inactivates p53-mediated transcriptional activation [2, 3, 11, 28-31]. Interaction of MDM2 with p53 is necessary for inhibition of p53-mediated transactivation [2, 3]. Consistent with this, MDM2 also inhibits p53-mediated growth suppression and apoptosis in tumor-derived cells [31-34].

MDM2 is an E3 ubiquitin ligase: MDM2 binds to an ubiquitin molecule through sulfhydroxyl bond which is characteristic of ubiquitin ligase (E3)-ubiquitin binding. The

carboxyl terminus of MDM2 has the cysteine residue that is essential for the activity suggesting that MDM2 functions as ubiquitin ligase, E3 [4]. MDM2 promotes degradation p53 by targeting p53 to ubiquination [4, 35-37]. However, mutants of MDM2 lacking the E3 ubiquitin ligase activity can efficiently bind with wild-type p53 and inhibit p53-mediated transcriptional activation but not degrade it [3]. MDM2 can bind and promote degradation many other proteins including its own degradation [38], mutant p53 [39], growth suppressor p14/p19ARF [40] and the cell-fate protein "numb" [41].

MDM2-p53 interaction: An autoregulatory feedback loop: The mdm2 gene contains two transcriptional promoter elements (P1 and P2). The P1 promoter is utilized constitutively. p53 directly activates MDM2 expression by recognizing a response element situated downstream of the first exon of the oncogene inducing transcription from the P2 promoter [42-44]. On the other hand, MDM2 inhibits the functions of p53 by several mechanisms. MDM2 binds to p53 and blocks its ability of transcriptional activation: it represses basal and p53- activated transcription when p53 is recruited to a promoter. MDM2 also regulates the stability and turnover of p53 protein due to its ability to bind and degrade p53 by ubiquination. These findings suggest the existence of an autoregulatory feedback loop between MDM2 and p53 in which a higher level of p53 expression causes G1 arrest and simultaneously induces MDM2 expression, which in turn inactivates p53 [45].

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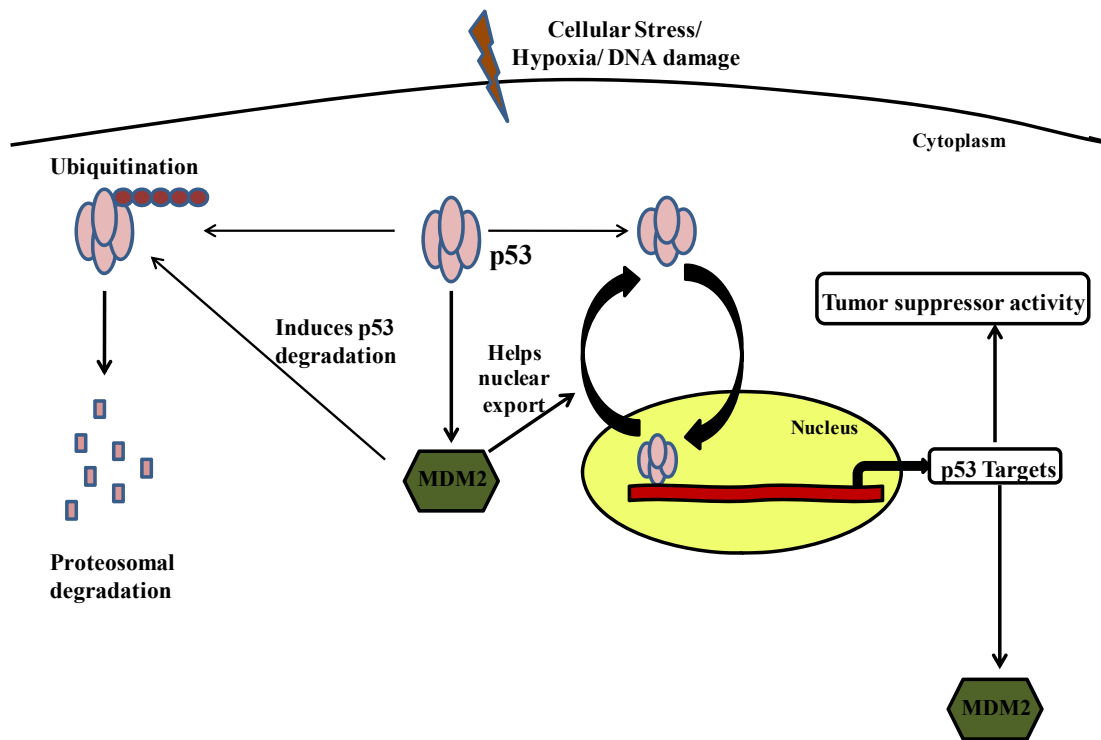
Figure 3: Interaction between MDM2 and p53: An autoregulatory loop.

MDM2 interacts with several growth suppressors and other proteins: MDM2 interacts with several growth suppressors, including p53, the retinoblastoma susceptibility gene product, Rb and p14. These interactions are perceived as possible mechanisms for oncogenic function of MDM2 [46-49].

MDM2 can function as a transcriptional regulator: MDM2 harbors distinct structural properties of a transcriptional regulator. It has an acidic activation domain, Zn finger domain and a basic region. MDM2 is not a general regulator of transcription: it however regulates transcription when recruited to a promoter [50]. MDM2 represses telomerase RNA gene promoter and upregulates NFκB p65 expression [51, 52]. Both WT and mutant p53 can modulate transcription and MDM2 interferes with their activity. MDM2

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overexpression in cancer cells therefore could alter gene expression due to its direct effect on transcription or through degradation of WT or mutant p53.

MDM2 and related proteins: MDMX is a structurally similar protein to MDM2 with a conserved p53 binding domain and a C terminal RING finger domain [53, 54]. MDMX interacts with p53 and inhibits p53 mediated transactivation. MDMX binds with MDM2 at the RING finger domain and stabilizes MDM2 [55, 56]. In contrast to MDM2, MDMX does not act as an E3 ubiquitin ligase and cannot stimulate degradation of p53 [57, 58].

NFκB pathway: Nuclear factor of κB (NF-κB) is a collection of dimeric transcription factors that control diverse biological processes. They are composed of five Rel family proteins with shared structural similarities and ability to bind related DNA motifs called κB sites. The canonical pathway consists of the Rel family proteins RelA (p65), RelB and c-Rel that are synthesized as active proteins whereas the other two members, NFκB1 (p50) and NFκB2 (p52), processed from precursor proteins, p105 and p100 respectively, belong to alternate or non-canonical pathway.
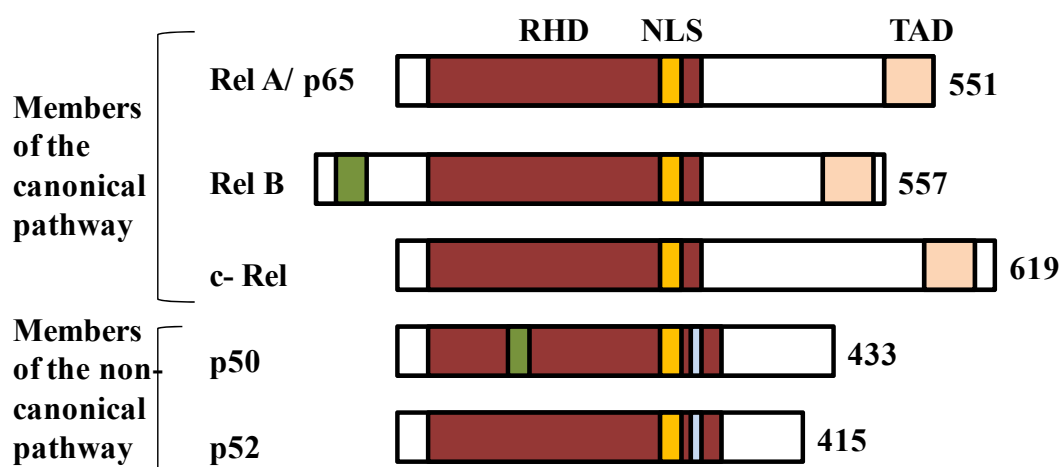
Figure 4: Members of the NFκB pathway.

Regulation of the NFκB pathway:

Members of the NFκB pathway exist as dimers and are sequestered in the cytoplasm due to their association with the Inhibitor of κB (IκB) resulting in an inactive complex. Various stimuli such as mitogens, cytokines, and DNA damage rapidly activate the canonical pathway, leading to degradation of IκB, translocation of the dimers to the nucleus, accumulation of the active dimers in the nucleus and increased transcription of their target genes. In the non-canonical pathway, the precursor molecules p100 and p105 that function as IκB like inhibitors are proteolytically processed to form the active molecules. Processing of p100 is tightly controlled and highly inducible. The cytoplasmic processing of p100 is triggered by NF-κB inducing kinase (NIK) and the downstream IκB kinase α (IKKα) in the presence of E3 ubiquitin ligase and β-transducin repeat containing protein (β-TrCP) [59-61]. There also exists a constitutive processing of p100 regulated by its nuclear shuttling. This pathway is considered to be a pathogenic process caused by NF-κB2 gene rearrangement and loss of the C-terminal processing inhibitory domain of NF-κB2 in cancer cells [62]. Activation NFκB appears to protect tumor cells from apoptosis, through induction of anti-apoptotic genes [63]. Constitutive nuclear NFκB activity has emerged as a hallmark for human leukemias, lymphomas, and many other cancers [64]. p52 overexpression can lead to lymphocyte hyperplasia and transformation [65]. p100 is overexpressed in tumor cells compared to human mammary epithelial cells, human breast cancer cell lines as well as primary breast tumors [66, 67]. Endogenous p52 functions as a regulator of cell proliferation and can affect cell growth through modulation of p53 tumor suppressor activity [68]. Constitutive processing of p100 is associated with the

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development of various lymphomas and is known to oncogenically transform fibroblasts in vitro. Wang et al. suggests that although p100 may have apoptotic functions, p52 has growth enhancing activities that can aid in oncogenesis [69].
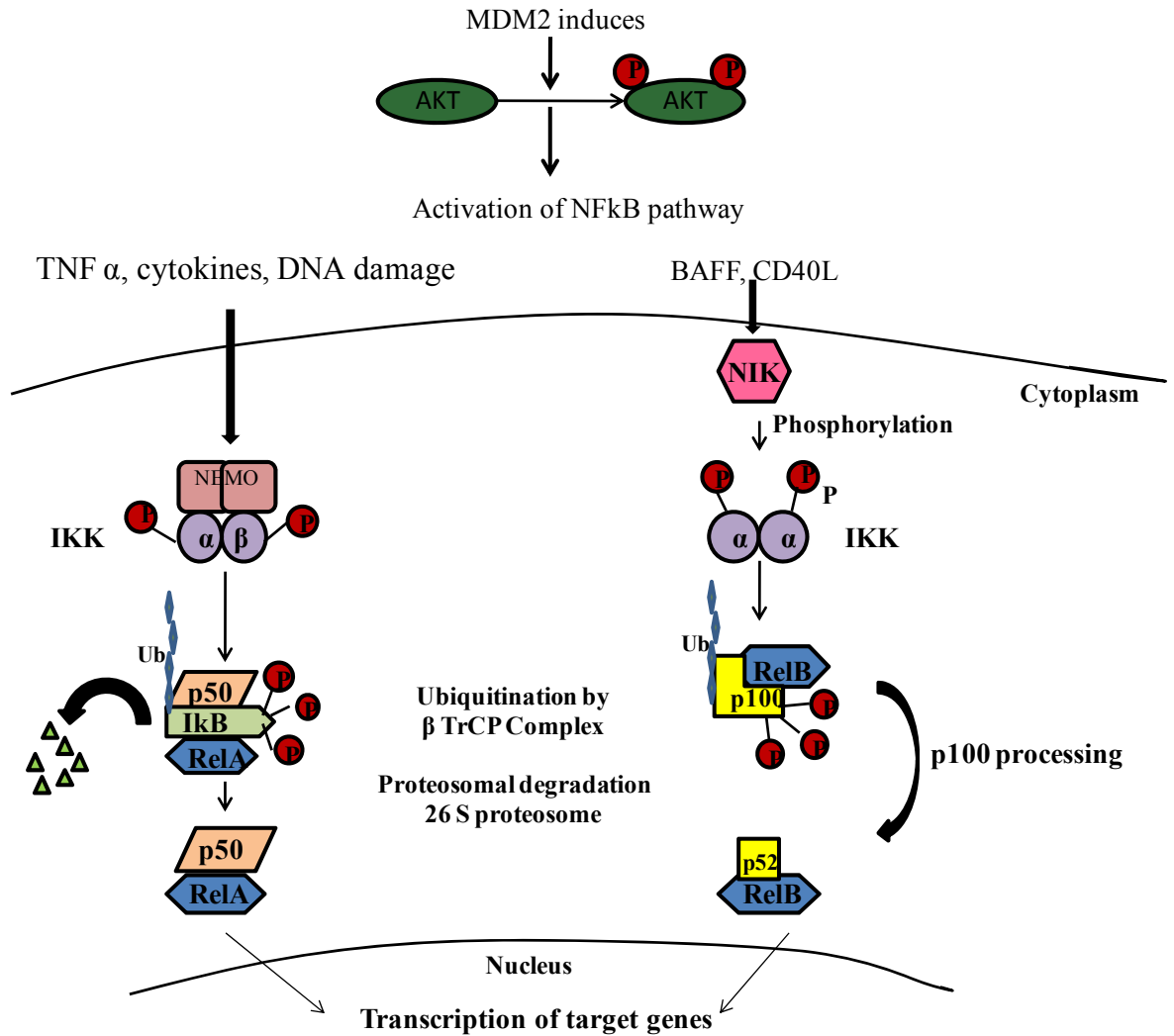
Figure 5: Canonical and Non−Canonical NFκB pathways.

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I: Chapter 2: Hypothesis

a. Major Hypothesis.

According to the WHO, lung and bronchial cancers are the single largest cause of cancer deaths in US accounting for 32% of cancer deaths in men and 25% in women. Non small cell lung cancers are a group of highly lethal and aggressive tumors that form 85% of lung cancers. Tumors are characterized by multiple genetic alterations including amplification of oncogenes and mutations of tumor suppressor genes that confer cells unique survival and proliferation advantage. Among the important genes that are implicated in cancers are oncogenes like MDM2, NFκB and c-myc and tumor suppressor genes like p53. The oncoprotein MDM2 is known to be overexpressed in various cancers. Hence, we wanted to determine the frequency and extent of MDM2 overexpression and other oncogenic markers in lung cancer. MDM2 induces Akt phosphorylation leading to activation of the Akt/PI3K pathway. In addition, the NFκB pathway, downstream of Akt/PI3K pathway, is one of the deregulated pathways in oncogenesis. NFκB2 (p100/p52), a member of NFκB family of nuclear transcription factors, induces the expression of anti-apoptotic proteins like Bcl2 and also regulates proteins of the cell cycle leading to cell proliferation. There is evidence that MDM2 upregulates NFκB/p65, a member of the canonical NFκB pathway. MDM2 binds to the transactivation domain of p53 to inactivate its tumor suppressor activity. This interaction of MDM2 and p53 increase the repertoire of genes that MDM2 influences, some of them being members of the NFκB pathway. This raised a possibility that NFκB2 may interact with MDM2 in a tumorigenic manner. Based on this, our hypothesis for Specific Aim 1 was that MDM2 overexpression correlates with members of the NFκB2

pathway including NFκB2 p100, Bcl2 and c-myc in human lung cancer. Our hypothesis for Specific Aim 2 was that MDM2 regulates NFκB2 in human lung cancer in a p53-dependent and p53- independent mechanism. Specific Aim 3 is based on the hypothesis that MDM2 influences oncogenic parameters in lung cancers and regulates the downstream target genes of the NFκB2 pathway. This leads us to the major hypothesis: MDM2 overexpression regulates NFκB2 in human lung cancers.

The specific aims are designed to better understand the interactions between the critical players, NFκB2, MDM2 and p53, in human lung cancer.

b. Specific Aims.

The specific aims are as follows: (1) To quantify the expression levels of MDM2, NFκB2, Bcl2 and other related genes by Reverse Transcriptase PCR and to determine p53 status by DNA sequencing in human lung tumor samples. (2) To decipher the mechanism by which MDM2 regulates NFκB2 upregulation in human lung cancer cell lines. (3) To examine the role of MDM2 and NFκB2 in oncogenic properties such as cell proliferation and chemoresistance in human lung cancer and to identify a downstream target of the NFκB2 pathway.

J. Chapter 3

Correlation of MDM2 with genes in the NFκB2 pathway in human lung cancer.

The manuscript for the work presented in this chapter is currently in preparation.

a. Introduction:

Lung cancer is one of the leading causes of deaths due to cancer in the United States. It can be clinically categorized into Non Small cell Lung Cancer (NSCLC) that includes adenocarcinoma, squamous cell carcinoma and large cell carcinoma. According to the SEER Cancer Statistics Review, 64% of lung cancer cases are NSCLC. Cancer is a multi step process that involves molecular and genetic alterations. mdm2 gene is frequently overexpressed in human cancers and may be one of the common causes of oncogenesis [46, 47, 70-72]. The study in this chapter involves determining the frequency of MDM2 overexpression, identification and quantification of abnormal gene expression co-occuring with MDM2 and p53 status in human non-small cell lung cancer (tumor) samples.

b. Experimental Results:

Human lung cancer samples were obtained from the Tissue and Data Acquisition Core (TDAAC) laboratory at the Virginia Commonwealth University (VCU). They consisted of 21 adenocarcinoma (cancer that originates in glandular tissue) and 9 squamous (malignant tumor of squamous epithelium) tumor samples. Histological evaluation of the frozen tumor tissues showed that all specimens studied consisted of approximately $68\pm15\%$ tumor cells. Adjacent non-neoplastic tissues were collected as control tissues for the expression studies. Advanced techniques such as the Laser Capture Microdissection can also be used in order to study a single cell.

Levels and frequency of MDM2 and p53 status in lung tumor samples: The transcript levels (mRNA) of MDM2 were measured in order to determine the levels and frequency of MDM2 overexpression in the tumor samples. RNA was extracted from the tissue samples using Trizol (phenol- choloroform) extraction followed by subsequent cDNA synthesis using Reverse Transcriptase cDNA synthesis kit (Invitrogen). cDNA for all samples were analyzed by Quantitative Polymerase Chain Reaction (QPCR) using MDM2 specific primers to quantify the mRNA levels in the samples. Primers specific for the house keeping gene, GAPDH (Glyceraldehyde 3- phosphate dehydrogenase), were used to normalize the values of MDM2 transcript levels obtained from the tumor samples.

The tumor suppressor gene p53 is mutated in 50 percent of cancers. Studies suggest that 42 percent of NSCLCs harbor p53 mutations with significantly higher proportion of alterations in squamous cell and large cell carcinomas than adenocarcinomas [73]. To determine the p53 status of the tumor samples obtained from TDAAC, we amplified four regions of p53 transcripts spanning the entire open reading frame (ORF) by polymerase chain reaction (PCR) using overlapping primers. The amplified DNA was tested by agarose gel electrophoresis and sequenced.

MDM2 levels were overexpressed in approximately 30% of the lung tumor samples when compared with MDM2 levels in the surrounding non tumorigenic tissue samples (N3, N7, and N13) that served as a control throughout the study. Approximately 30% of the cancer samples showed p53 mutation. We identified 12 cases with single base pair substitution mutation in p53 and 18 with wildtype (WT) p53 (Table 1). All mutations were found to be in the DNA-binding domain of p53, reassuring the significance of mutation in

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this domain in oncogenesis. Studies done by other groups suggest that hotspots for p53 mutations in lung cancer are codons 158, 175, 248, 273. Amongst the 12 samples with mutant p53 we found one sample with a R158L mutation and another with R248L mutation (usually found in lung cancers). Fewer number of mutant p53 samples in our study does not let us analyze the correlation between a specific mutation and the levels of MDM2. In soft tissue sarcomas, point mutations of p53 were detected in one third of the samples but no correlation was observed between MDM2 levels and p53 mutations [74]. Analysis of non small cell lung carcinomas did not suggest a correlation between MDM2 overexpression and p53 gene alterations [75]. These clinical studies suggest that a statistical correlation has not yet been established between MDM2 and p53 mutations. Figure 6 depicts the MDM2 transcript levels in all samples and has been grouped as samples with wildtype p53 and mutant p53.

Table 1: p53 Status of Non Small Cell Lung Cancer Tissue Samples.

Sample	p53status		Sample	p53status	DNA mutation
VLU2	WT		VLU26	WT	
VLU5	WT		VLU31	WT	
VLU6-2	WT		VLU32	WT	
VLU7	WT		VLU1	F270L	TTT to TTC
VLU8	WT		VLU3	R158L	CGC to CTC
VLU10	WT		VLU4	V157F	GTC to TTC
VLU11	WT		VLU9	L111M	CTG to ATG
VLU12	WT		VLU16	H193R	CAT to CGT
VLU14a	WT		VLU22	C176W	TGC to TGG
VLU17	WT		VLU24	E285K	
VLU18	WT		VLU25	H179Y	CAT to TAT
VLU19	WT		VLU28	H179R	CAT to CGT
VLU20	WT		VLU30	Y234C	TAC to TGC
VLU21	WT		VLU33	V172G	GTT to GGT
VLU23	WT		VLU35	R248L	CGG to CTG
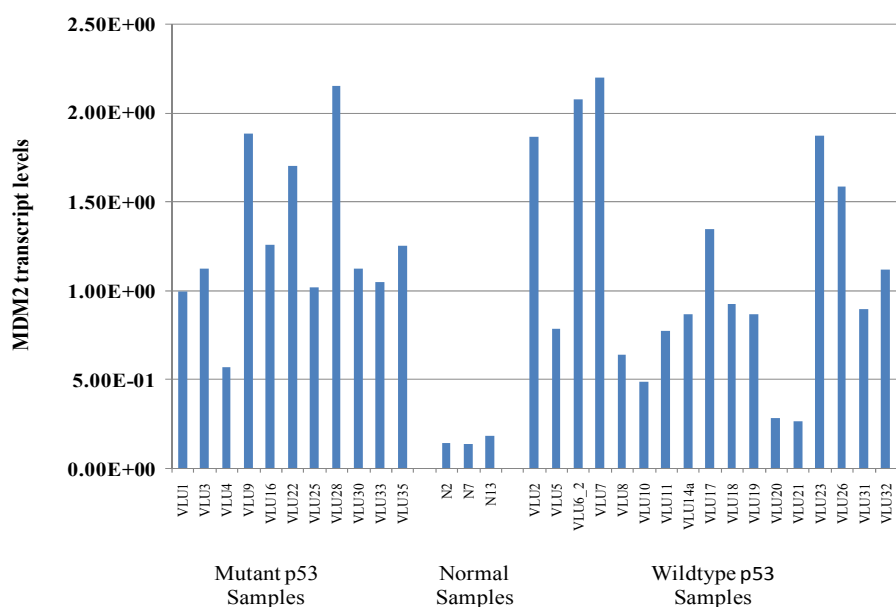
Figure 6: MDM2 overexpression in human lung tumor samples with wildtype and mutant p53.

MDM2 levels correlate with the levels of NFκB2 in lung tumor samples with wildtype p53: MDM2 overexpression in various cancers is usually accompanied by other genetic defects. Both WT and mutant p53 can modulate transcription and MDM2 interferes with their activity. Therefore, we sought to determine altered gene expression that co-occurs with MDM2 overexpression in human lung cancer samples that may explain induction of oncogenesis and allow us to identify biochemically linked group of markers that could be used for sub-typing and treatment of cancer.

MDM2 activates the Akt/PI3K pathway and NFκB pathway is downstream to the Akt pathway. Deregulation of the Nuclear factor kappa B (NFκB) pathway has been observed in various malignancies [76-79]. MDM2 has been reported to upregulate NFκB/p65 expression in leukemias [80]. A member of the non canonical NFκB pathway, NFκB2 p100/p52 has been known to have aberrant functions in human breast cancer [67].

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Endogenous p52 functions as a regulator of cell proliferation and can affect cell growth through modulation of p53 tumor suppressor activity [68]. Also, our previous study indicates that mutant p53 upregulates NFκB2 expression [81]. These observations led us to determine the pattern of NFκB2 expression in NSCLC patients. Quantitative PCR analysis was done on the lung tumor samples with primers for the NFκB2/p100 transcript. The NFκB2 transcript levels normalized to GAPDH were analyzed.
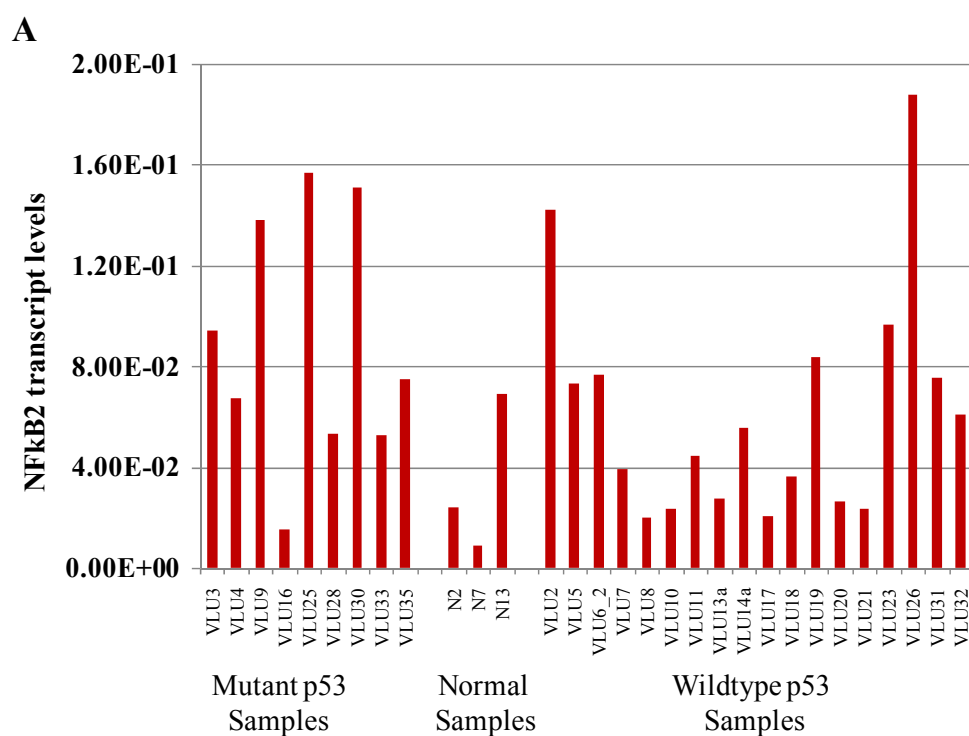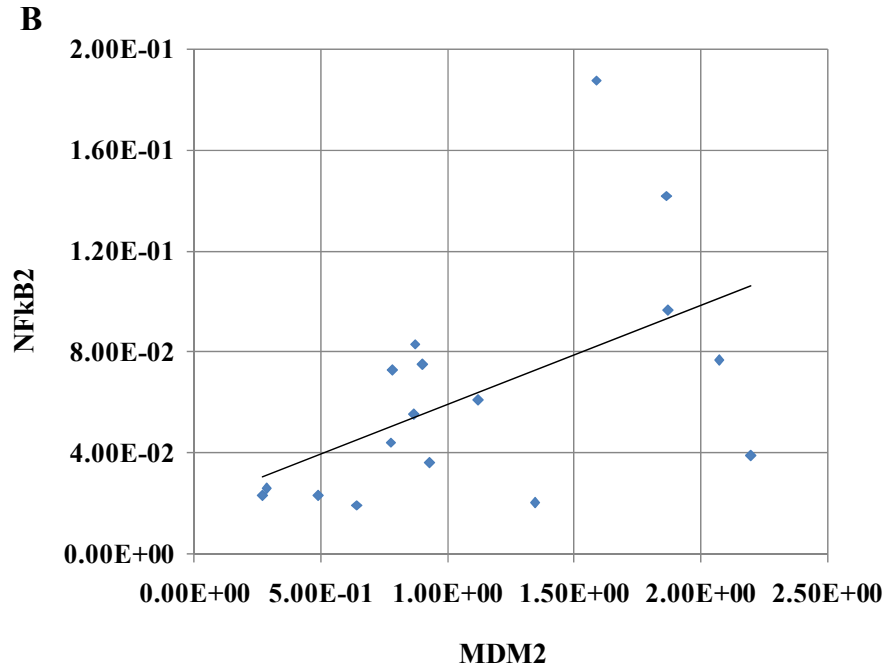
A

B

Figure 7: (A) NFκB2 transcript levels in human lung tumor samples with wildtype and mutant p53. (B) Scatter plot for MDM2 and NFκB2 transcript levels in tumor samples with wildtype p53.

Interestingly, levels of MDM2 expressed in tumor samples harboring WT p53 significantly correlated with NFκB2 expression. Relationship between MDM2 expression (independent variable) and NFκB2 p100 expression levels (dependent variable) were determined using separate linear regression models. The relationship between MDM2 and NFκB2 in ungrouped cancer samples did not show any significance (p-value < 0.0895). Most of the samples harboring mutant p53 showed elevated levels of NFκB2 expression compared to samples with WT p53. However, the extent of increase varied with the type of p53 mutation suggesting that the transcriptional activation property of some of the observed mutants is stronger than the other. Relatively high levels of NFkB2 were observed in samples with F270L, C176W and E285K mutations in the p53 gene. However,

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when we examined the relationship by grouping the lung cancer samples into WT and mutant p53, the relationship between MDM2 and NFκB2 expression in the WT p53 group was found to be highly significant (p-value < 0.001) (Figure 7). These results suggest that in lung cancer cells harboring WT p53, MDM2 may elevate expression of NFκB2 or NFκB2 elevates expression of MDM2. Alternatively, elevated expression of MDM2 along with NFκB2 may confer a selective growth advantage in cancer cells.

MDM2 levels correlate with the levels of Bcl2, a downstream target of NFκB2: Members of the NFκB family of transcriptional regulators play a role in the activation of numerous target genes regulating cell survival, angiogenesis, metastasis and cell proliferation [64]. Our next step was to identify downstream targets of the NFκB2 pathway that could be potential candidates for MDM2 mediated regulation. Elevated expression of NFκB2/p100 was associated with high Bcl-2 expression in breast cancer and chronic lymphocytic leukemia. Bcl-2, an anti-apoptotic gene is suggested to be an in vivo target gene for NFκB2 [82]. To analyze the influence of MDM2 on Bcl2, we quantified the mRNA levels of Bcl2 in lung tumor samples. Bcl2 mRNA levels were elevated in samples with increased MDM2. A Spearman's correlation constant of MDM2 and Bcl2 normalized to GAPDH was significantly associated overall (p-value 0.02), however the Spearman's rank correlation was not significant when categorized into samples with WT p53 (rho 0.23, p value 0.3) and mutant p53 (rho 0.16, p value 0.62).
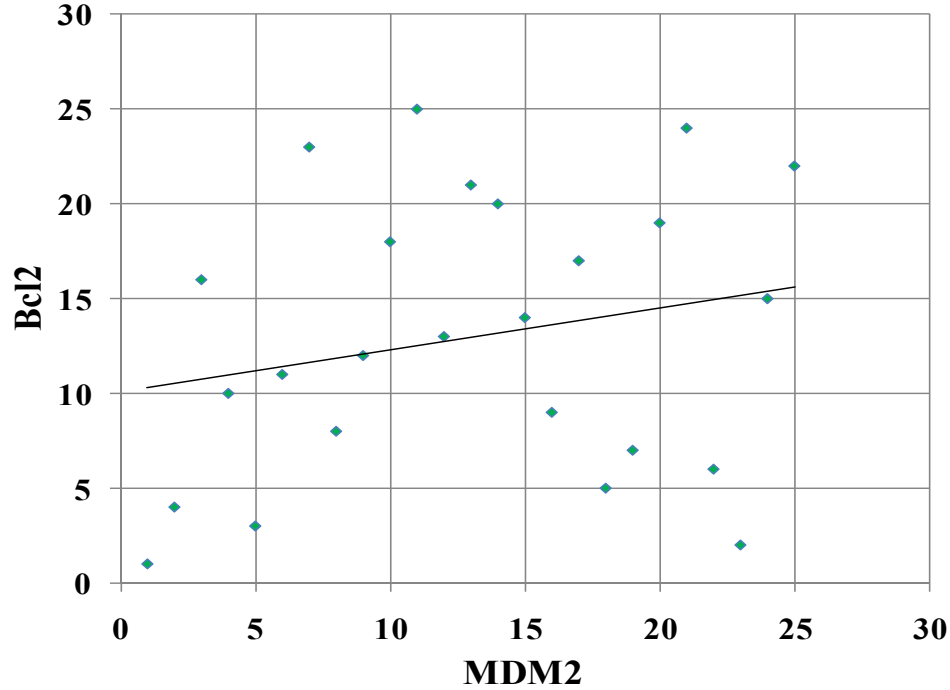
Figure 8: Scatter plot shows correlation between MDM2 and Bcl2 transcript levels in human lung tumor samples with wildtype and mutant p53.

Correlation between MDM2 and c-myc in lung tumor samples: The NFκB pathway is known to regulate c-myc transcription (30). c-myc is a protooncogene and a cell proliferation gene that is activated upon mitogenic signals [83]. In order to determine the role of MDM2 on another downstream target of NFκB pathway in lung tumor samples, c-myc transcript levels were analyzed and normalized to GAPDH. The correlation between MDM2 and c-myc were analyzed by Spearman's rank correlation. The overall correlation between the transcript levels was not significant (rho 0.23 p value 0.22). On restricting the analysis on the basis of p53 status, Spearman's rank correlation (rho) was 0.22 and the p value was 0.37 in patients with WT p53 and rho of 0.13, p value 0.69 in patients with

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mutant p53, suggesting no significance in either case. MDM2 regulates transcription of genes by recruiting itself on promoters in association with other coactivators and repressors, thereby influencing gene expression either by activating or repressing it. This suggested that MDM2 levels need not necessarily correlate with all downstream targets of the NFκB pathway.

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Figure 9: (A) c-myc transcript levels in human lung tumor samples with wildtype and mutant p53. (B) Scatter plot shows no significant correlation between MDM2 and c-myc transcript levels in human lung tumor samples.

MDM2 levels correlate significantly with MDMX in human lung tumor

samples: MDM2 is one of the key regulators of the tumor suppressor activity of p53. MDMX, another protein structurally related to MDM2 also has the ability to inhibit p53 induced transcription on overexpression [84]. Elevated levels of MDMX have been observed in various cancers such as head and neck squamous carcinomas, primary breast tumors and in a subset of gliomas and correlates with the presence of wildtype p53 [85-87]. MDMX stabilizes MDM2 by interfering with MDM2 auto-ubiquination by interacting with its RING domain [57]. These observations indicate that overexpression of MDMX may play a role in oncogenesis in order to inactivate p53. In order to verify this, MDMX transcript levels were quantified with MDMX specific primers and normalized to GAPDH. MDMX was elevated in 30 percent of the lung tumor samples on comparison with the normal samples (N2, N7, and N13). Statistical analysis done suggested no significant difference between MDMX levels in samples harboring wildtype and mutant p53 (p=0.33). However, Spearman's correlation for MDMX and MDM2 levels showed a significant overall correlation (rho=0.49, p=0.005). There is no significant correlation between MDM2 and MDMX when the samples were grouped into wildtype (rho 0.56, p=0.02) and mutant p53 (rho 0.23, p=0.47) samples. This suggests a possibility that MDMX stabilizes MDM2 and in turn influences NFκB2 levels or may directly regulate NFκB2 levels. However, additional studies would be necessary to understand more about the interaction between MDM2, MDMX, p53 and NFκB2.
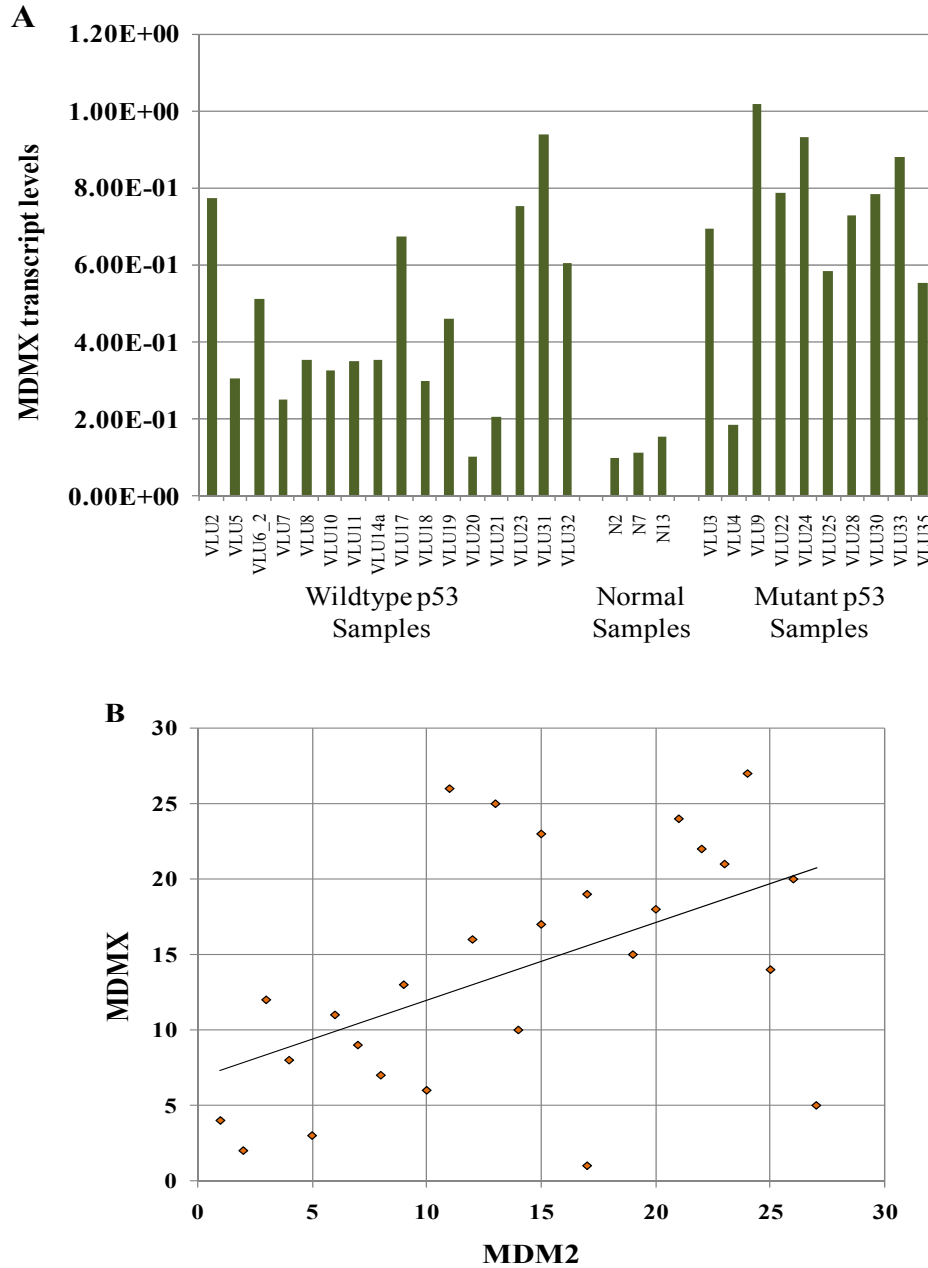
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Figure 10: (A) MDMX transcript levels in human lung tumor samples with wildtype and mutant p53. (B) Scatter plot shows a correlation between MDM2 and MDMX transcript levels in human lung tumor samples.

Correlation between Delta Np53 and MDM2 in human lung tumor samples: The tumor suppressor gene p53 has different isoforms resulting from alternative splicing. Isoforms with a deletion in the N-terminus (Delta N-p53) lack the transactivation domain, thereby acting as dominant negatives [88]. Delta N-p53, produced by the internal initiation of translation at an AUG codon at position 40 has deregulated transcriptional activation capacity. This isoform oligomerizes with full length, wildtype p53 and downregulates its growth suppressive activities. Due to the lack of the N- terminal domain, this isoform does not complex with MDM2. This led us to determine the transcript levels of Delta N-p53 in the lung tumor samples that may justify the correlation we observe in the samples with wildtype p53 [89]. No correlation was observed in the levels of MDM2 and Delta N-p53 in the samples based on the Spearman's correlation rank (rho=0.24, p=0.22). Grouping the samples on the basis of wildtype and mutant p53 did not show any significant correlation between the Delta N-p53 and MDM2, with p values of 0.78 and 0.27 respectively. This suggests that though Delta N-p53 does not correlate with MDM2, it may play a role in inactivating the functions of wildtype p53 in the samples.
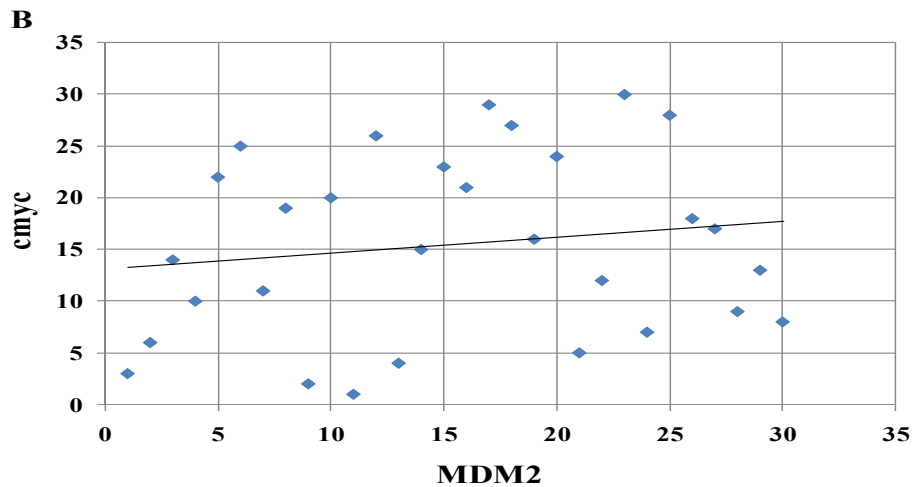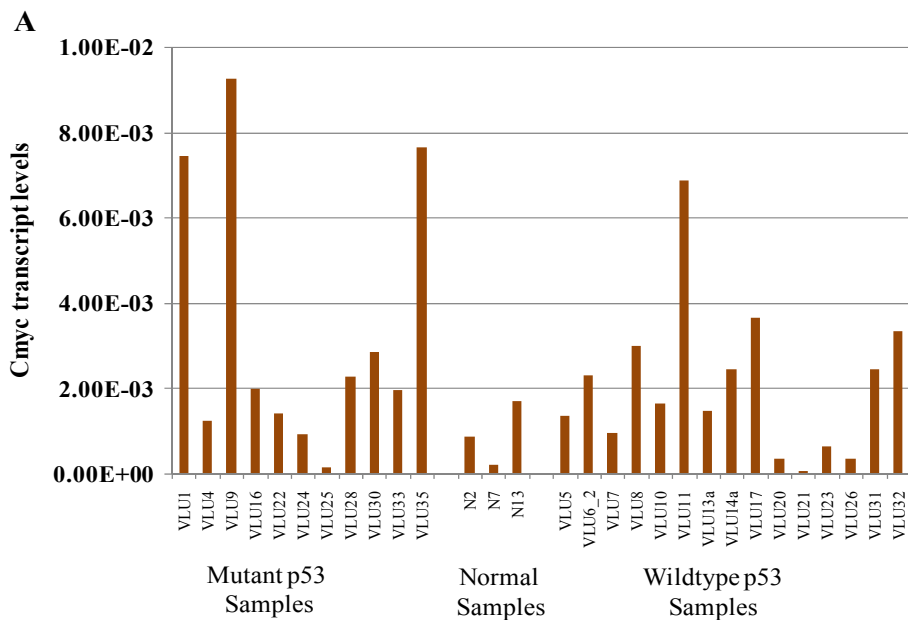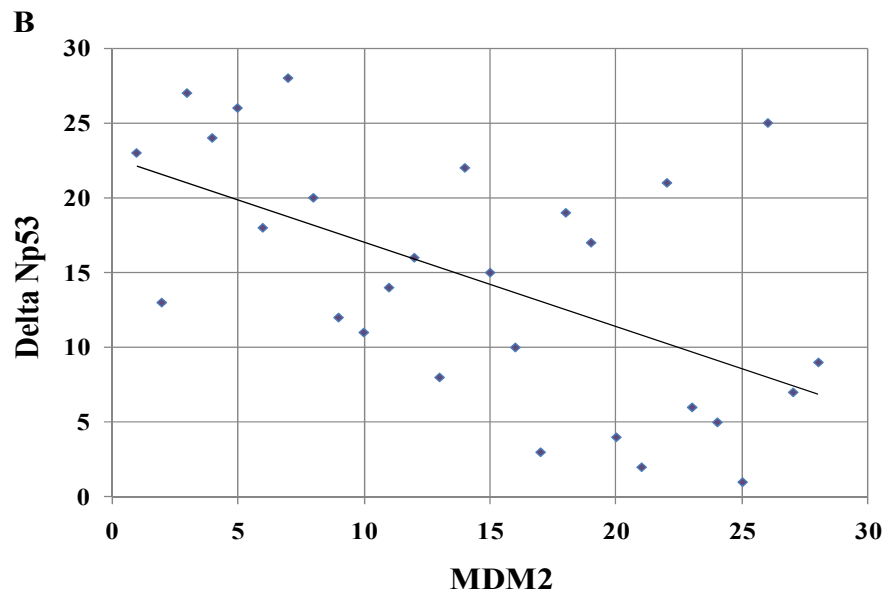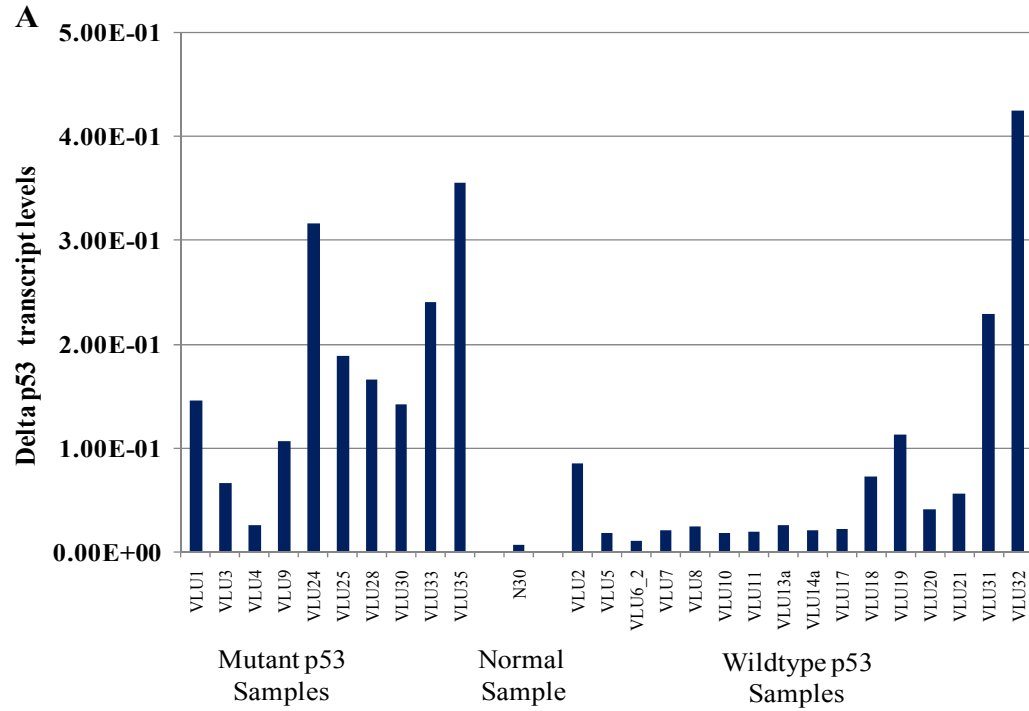
22

Figure 11: (A) Delta Np53 transcript levels in human lung tumor samples with wildtype and mutant p53. (B) Scatter plot shows a negative correlation between MDM2 and Delta Np53 transcript levels in human lung tumor samples.

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Correlation between MDM2 and p21 in human lung tumor samples: In response to oncogene activation, DNA damage or hypoxia, p53 regulates cell cycle checkpoints like the expression of the Cyclin Dependent Kinase (CDK) inhibitor, p21. The only CDK inhibitor that p53 directly controls is p21 and it does so through the DNA binding response elements situated within the p21 promoter. It is a potent cell cycle inhibitor and is required for various cell cycle transitions [90, 91]. p53 induces both MDM2 and p21 [92]. Literature suggests that levels of p21 are inversely proportional to the levels of MDM2. MDM2 is also known to regulate the proteosomal turnover of p21 in cells [93]. Since the human lung tumor samples consisted of both wildtype and mutant p53 with MDM2 overexpression, samples were analyzed by QPCR for their p21 transcript levels.
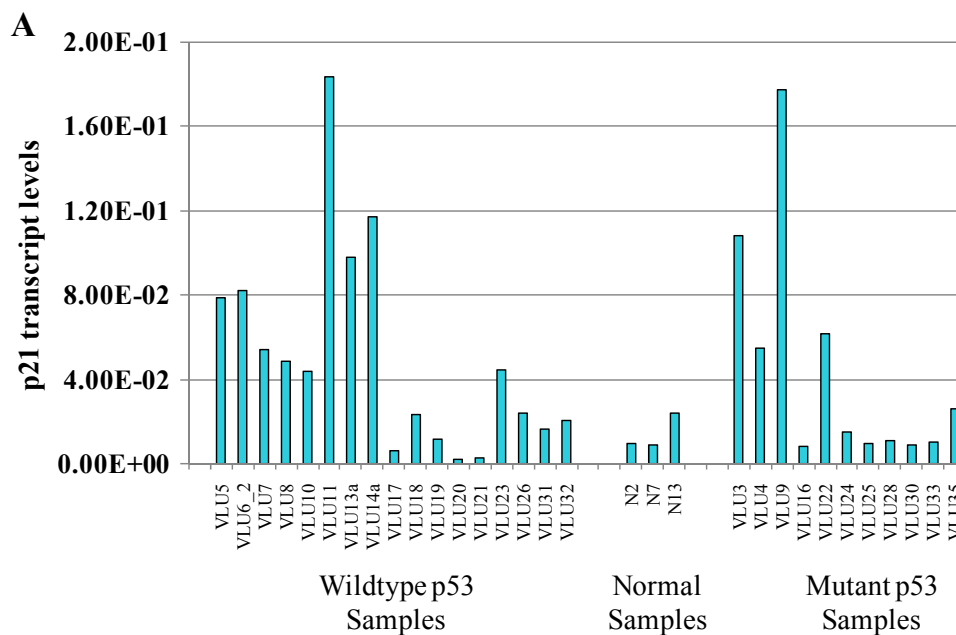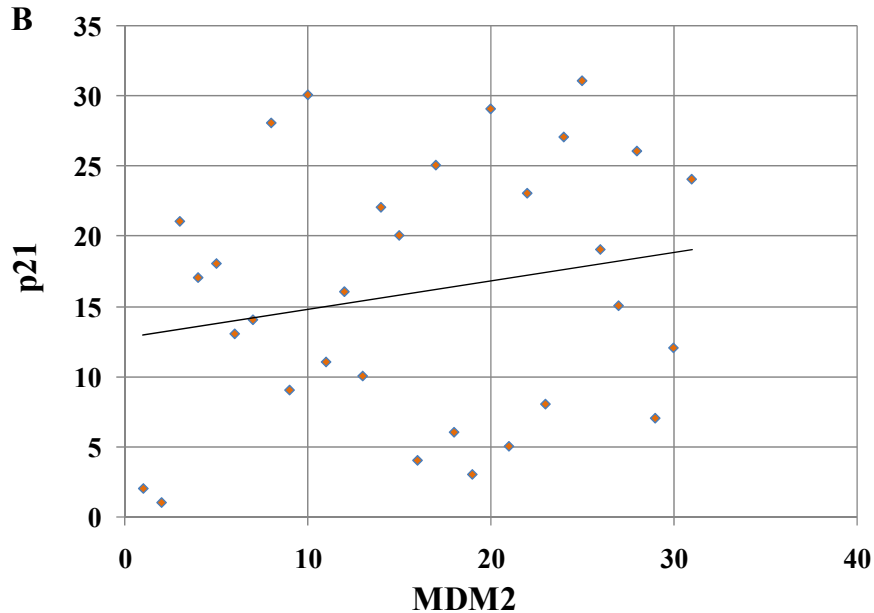
B

Figure 12: (A) p21 transcript levels in human lung tumor samples with wildtype and mutant p53. (B) Scatter plot does not show a correlation between MDM2 and p21 transcript levels in human lung tumor samples.

Statistical analysis to compare p21 transcript levels between two groups: samples with wildtype p53 versus mutant p53 showed no significant difference (p value 0.95) between the two. A Spearman's correlation analysis done to determine the correlation between p21 and MDM2 transcript values showed no significant association overall (rho=0.20, p=0.28) as well as when compared restricting to samples with wildtype (rho=0.43, p=0.08) or mutant p53 (rho=0.03, p=0.91). Our results indicates no relation between MDM2 and p21, suggesting that p21 levels could also be through p53-independent mechanisms as well.

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c. Chapter Summary:

Since MDM2 is known to be overexpressed in various cancers, our focus in this chapter was to study the frequency and level of MDM2 upregulation in lung cancer patients. Comparison with normal samples (tissue from surrounding, non tumorigenic region of patients) showed MDM2 overexpression in more than 30% of the samples. p53 sequencing of the lung tumor samples determined the p53 status categorizing the samples into patients harboring wildtype and mutant p53. Mutant p53 was detected in 12 lung tumor samples and 18 samples had wildtype p53.

In an attempt to identify biochemically linked markers co-occurring with MDM2 in lung cancer, based on literature, we analyzed the transcript levels of genes in the NFκB pathway. We quantified the levels of NFκB2 p100, a member of the non- canonical NFκB pathway. Statistical analysis suggested a correlation in the levels of MDM2 and NFκB2, with a highly significant correlation in samples with wildtype p53. In order to study the role of MDM2 on downstream targets of the NFκB pathway, an anti apoptotic gene and a downstream target of NFκB2, Bcl2 and c-myc, a proto oncogene involved in cell proliferation were analyzed for their transcript levels. The mRNA levels of Bcl2 and c-myc were overexpressed in lung tumor samples. Bcl2 levels showed an overall significant correlation with MDM2, however no correlation was observed between MDM2 and c-myc. We also determined the levels of MDMX, a protein structurally related to MDM2 that is being studied as a potential gene that promotes oncogenesis. It was interesting to determine that MDMX was also upregulated in lung tumor samples and also showed a very significant correlation with levels of MDM2. p53, the well known tumor suppressor that is

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known to be mutated in cancers also exists as isoforms. The Delta N p53 isoform is known to downregulate the levels of wildtype p53 by oligomerization. We quantified the levels of Delta N p53 transcripts to determine if there is any relation between the isoform and the levels of MDM2 based on the status of p53 in the lung samples, however statistical analysis did not show any such correlation. p21 being a direct downstream target of p53 was analyzed by QPCR. This analysis did not show any correlation suggesting that p21 levels can also be regulated by a p53 independent mechanism [94]. Various cytokines and growth factors are involved in regulating the levels of p21. Interleukin-6 (IL-6) causes induction of p21 levels associated with binding of both STAT3 and STAT1 to the p21 promoter [95]. Also, growth factors such as platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) induce p21 levels in p53-deficient cells [96].

K. Chapter 4

Mechanism of MDM2 mediated NFκB2 upregulation in human lung cancer cells

The manuscript for the work presented in this chapter is currently in preparation.

a. Introduction:

MDM2 is a potent oncogene and is amplified in various cancers. Cancer cells with elevated levels of MDM2 also harbor other genetic abnormalities. MDM2 plays a role in anti apoptotic functions and has been implicated in survival signaling [97]. Various growth factors, cytokines and oncogenes influence two significant pathways: the Mitogen Activated Protein Kinase (MAPK) and the PI3K pathway. The NFκB pathway is activated via the PI3K/Akt pathway. Data from quantitative PCR of the human lung tumor samples suggested a significant correlation between MDM2 and NFκB2 levels in samples harboring wildtype p53. To further understand this observation and decipher the role and mechanism of MDM2 in the NFκB2 pathway, in vitro experiments were performed.

b. Experimental Results:

H460 is a human non-small cell lung cancer cell line harboring wildtype p53. This is our cell line of choice as it fulfills both requirements of our study: a non small cell lung cancer cell line and the presence of wildtype p53.

MDM2 elevates NFκB2 expression in H460: To determine if MDM2 can elevate NFκB2 levels in cultured lung cancer cells harboring WT p53, we nucleofected full length MDM2 expression plasmid (or vector plasmid) in H460 cell line using Amaxa nucleofection kits. The cells were harvested 16 hours post transfection in 1X Promega lysis buffer with protease inhibitors to analyze the protein levels by western blotting. The blot

was probed with antibodies against MDM2, NFκB2 and Actin (loading control) and was quantified by densitometry. Western blot analysis of cell extracts showed increase in the NFκB2 p100 and p52 levels in cells transfected with the MDM2 expression plasmid. The experiment was done more than three times and a representative experiment has been shown in Figure 13A. Densitometric analysis indicated that MDM2 overexpression elevated p100 and p52 expression approximately 2.2- and 3.5-fold respectively (Figure 13B).
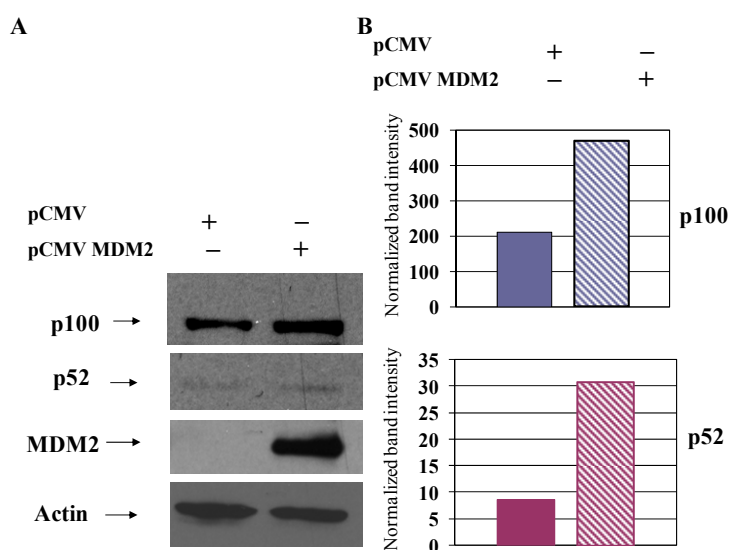
Figure 13: MDM2 overexpression in lung cancer H460 cell line elevates NFκB2 gene expression

MDM2 elevates NFκB2 transcript levels in H460: Since the previous experiment looked at the protein levels of MDM2 and NFκB2 we next analyzed the transcript levels for our genes of interest. H460 cells were similarly transfected with a full length MDM2 expression plasmid (or vector plasmid) using Amaxa nucleofection kits and harvested for RNA in trizol (Invitrogen) using phenol - chloroform extraction, 16 hours after

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transfection. cDNA was synthesized from the extracted RNA using the Invitrogen Reverse

Transcriptase Thermoscript cDNA synthesis kit and the cDNA was subjected to QPCR for

quantifying the levels of MDM2 and NFκB2. Consistent with the Western blot analysis,

the QPCR results (Figure 14) showed that levels of NFκB2 transcripts normalized to

GAPDH was higher (2.2- fold) in the H460 cells transfected with MDM2 expression

plasmid over control cells nucleofected with the vector plasmid. The QPCR analysis was

done in triplicates and also the results were reproduced by three independent experiments.

The error bars shown correspond to the standard deviation within the triplicates.
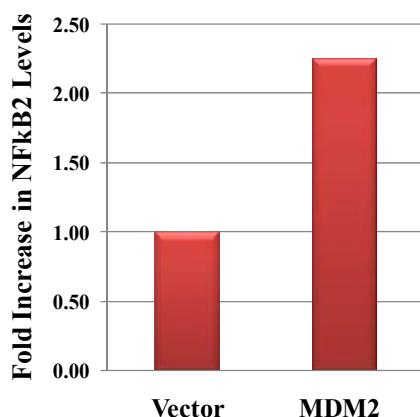
Figure 14: MDM2 overexpression in lung cancer H460 cell line elevates NFκB2 p100 transcripts.

Silencing MDM2 downregulates NFκB2 expression: Based on results from

overexpressing MDM2 in the cells, we next moved on to determine if downregulation of

MDM2 expression diminishes the levels of NFκB2 expression in H460 cells.

a) Using short hairpin RNA (shRNA) against MDM2: H460 cells were transfected with

shRNA targeted to silence MDM2 expression. H460 cells transfected with shRNA

against non endogenous luciferase gene served as a control. Cells were harvested 48

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hours after transfection and RNA was extracted. cDNA was subsequently prepared and levels of MDM2 and NFκB2 transcripts were determined by QPCR.
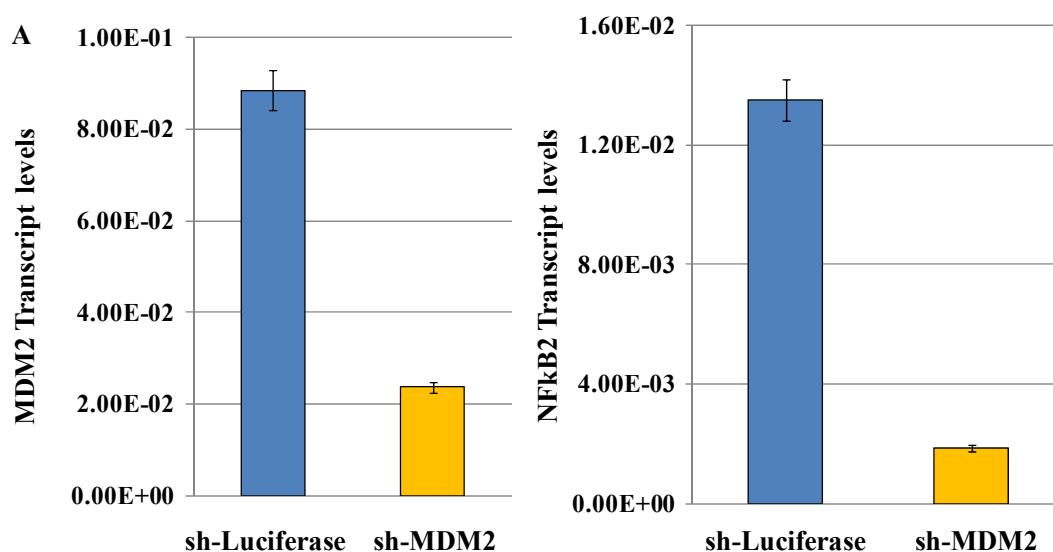
Figure 15: (A) Downregulation of MDM2 expression using shRNA against MDM2 downregulates NFκB2 expression in H460 cells.

b) *Using lentivirus containing shMDM2:* H460 cells were infected with the lentivirus containing shMDM2 or lentivirus against the non endogenous luciferase gene as a control. The virus was removed and replaced with new media after 48 hours. The infected cells were then harvested for RNA extraction after 48 hours. cDNA prepared from lentiviral infected H460 cells were analyzed for their MDM2 and NFκB2 transcript levels.

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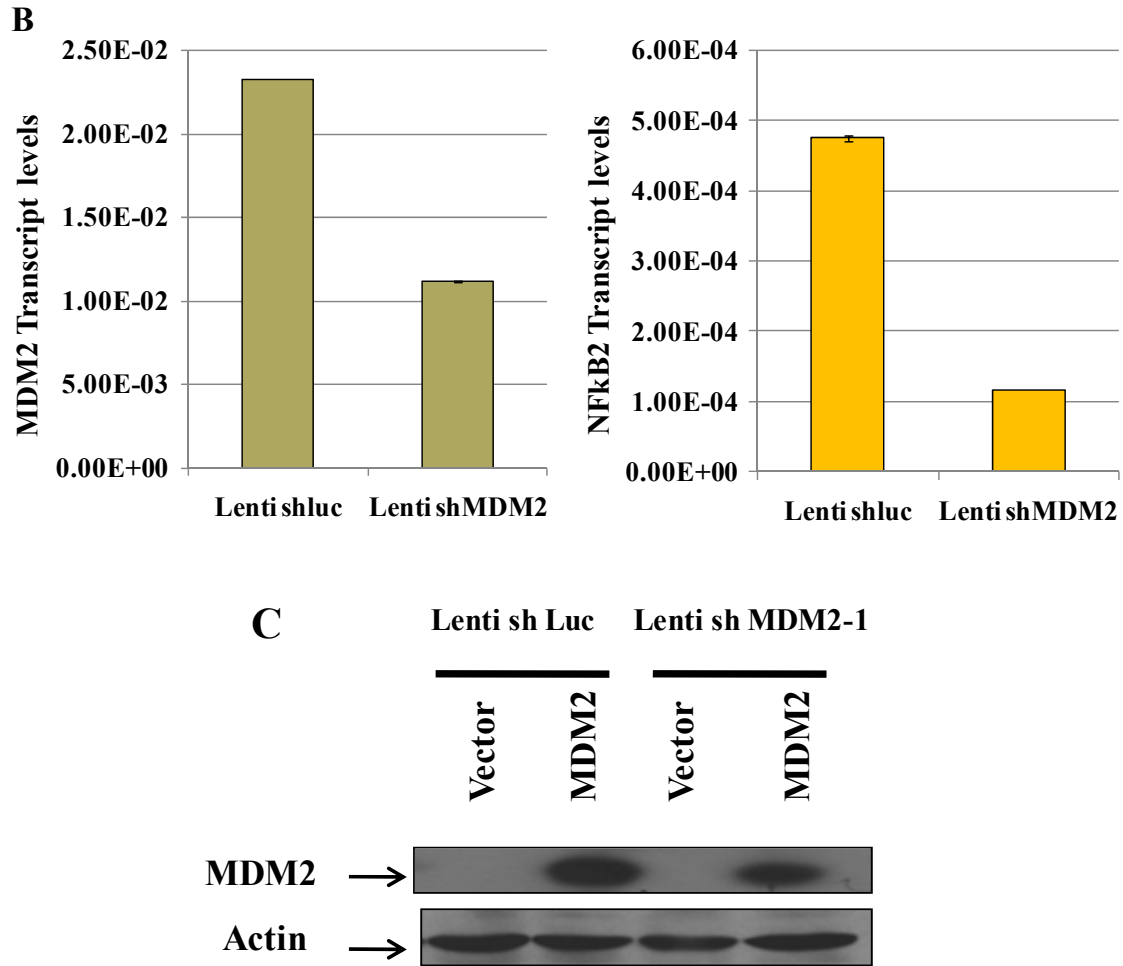
Figure 15: (B) Downregulation of MDM2 expression using lentivirus encoding shMDM2 downregulates NFκB2 expression in H460 cells. (C) Western blot shows silencing of MDM2 by the lentivirus shMDM2 at the protein level.

Figures 15 A and B indicate that shRNA against MDM2 and lentivirus encoding

shMDM2 downregulate MDM2 and NFκB2 expression proportionally at transcript level.

The QPCR analysis was done in triplicates and also the results were reproduced by three

independent experiments. The error bars shown correspond to the standard deviation

within the triplicates. These data suggest that endogenously expressed MDM2

upregulates NFκB2 expression. To check if the lentivirus silences MDM2 at the protein

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level, H460 cells were infected with lentivirus encoding the shMDM2 or non endogenous luciferase (control). After 48 hours, lentivirus was removed and replaced with new, complete media. Since the endogenous levels of MDM2 in H460 cells cannot be detected at the protein level, after 24 hours, both the control and the shMDM2 infected cells were transfected with empty vector plasmid or the MDM2 plasmid to observe a relative effect of MDM2 silencing in the cells. 16 hours after nucleofection, cells were harvested in 1X reporter lysis buffer and were run on a SDS PAGE gel. Figure 15 (C) shows the western blot showing silencing of MDM2 in the H460 cells on infection with shMDM2 lentivirus.

Silencing MDM2 in normal lung fibroblasts downregulates NFκB2 levels: We observed that silencing MDM2 in lung cancer cell line H460 lowered the levels of NFκB2. To determine if this is a phenomenon that occurs even in normal lung fibroblasts, WI38 cells were infected with lentivirus containing shMDM2 or lentivirus against the non endogenous luciferase gene as a control. The virus was removed and replaced with new media after 48 hours. The infected cells were then harvested for RNA extraction after 48 hours. cDNA prepared from lentiviral infected WI38 cells were analyzed for their MDM2 and NFκB2 transcript levels.
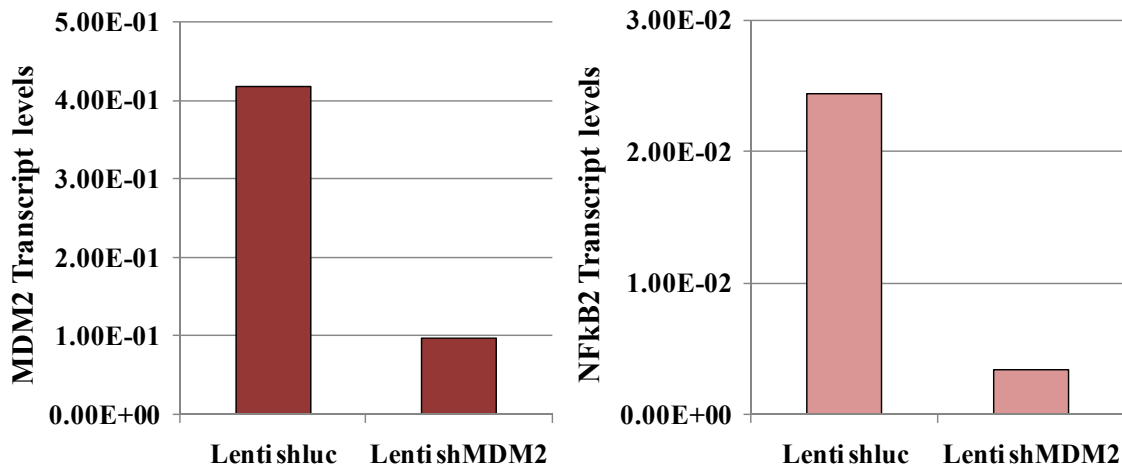
Figure 16: Downregulation of MDM2 expression downregulates NFκB2 expression in WI38 cells.

The figure above shows that silencing endogenous MDM2 in normal WI38 cells also downregulates the NFκB2 transcript levels. The QPCR analysis was done in triplicates and also the results were reproduced by three independent experiments. The error bars shown correspond to the standard deviation within the triplicates. These data suggest that MDM2-mediated NF kB2 upregulation is a normal cellular effect. In cancer cells that overexpress MDM2 this effect is drastic and may be a hindrance in chemotherapy.

MDM2 upregulates NFκB2 promoter activity: MDM2 harbors several structural properties of a transcription regulator. It has an acidic activation domain, Zn finger domain and a basic region. Although not a general regulator of transcription, MDM2 has been shown to regulate transcription when recruited to a promoter [98]. MDM2 represses telomerase RNA gene promoter and upregulates NFκBp65 expression [80, 99]. MDM2 also interacts and monoubiquinates histone H2B suggesting its ability to inhibit promoters [100]. NFκB is a ubiquitously expressed and a highly regulated transcription factor that

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regulates genes involved in immunity, stress and apoptosis. Transcriptional regulation of NFKB2, NFKB1 and IκB may be an important mechanism in regulating NFκB activity. This led us to determine if MDM2 induces NFκB2 levels by activating its promoter activity. To address this, H460 cells were transiently transfected with full length MDM2 plasmid or empty vector and the firefly luciferase gene under the control of the NFκB2 promoter [101]. The cells were also cotransfected with the Beta galactosidase (βgal) plasmid to verify transfection efficiency. Invitrogen Lipofectamine 2000 reagent was used for transfection and manufacturer's instructions were followed. The samples were harvested 30 hours after transfection in 1X Promega reporter assay lysis buffer. Protein concentrations were quantified in all the samples by using the Biorad protein assay reagent. Equal concentrations of cell extracts from samples were used for the reporter assay. The luciferase intensity was determined using the reporter luciferase kit. The samples were run on a SDS PAGE gel to confirm the overexpression of MDM2 in the samples.
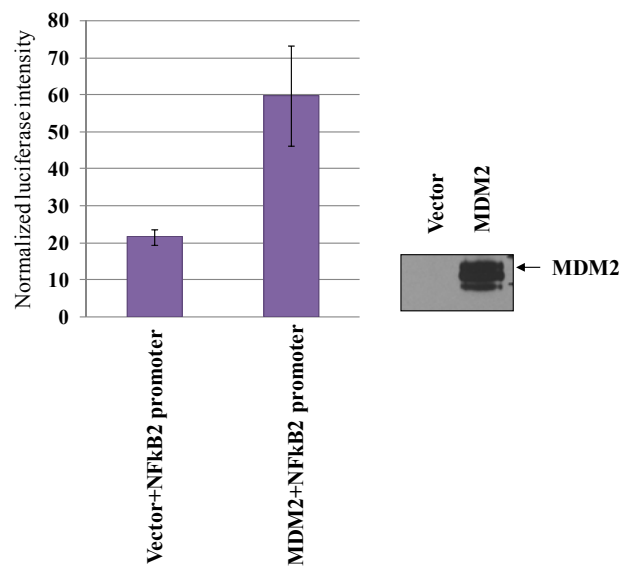
Figure 17: MDM2 overexpression transcriptionally upregulates the NFκB2 promoter.
Cells overexpressing MDM2 showed a 2.76 fold increase in the NFκB2 promoter activity when compared to the cells with the empty vector (Figure17). Samples were normalized with the beta galactosidase assay. The luciferase analysis and the transfection were done in triplicates. The experiment was repeated five times with comparable results that showed similar fold increase. Figure 17 is a representative of one such experiment. The error bars shown correspond to the standard deviation within the triplicates. This indicates that MDM2 upregulates NFκB2 at the promoter level.

MDM2 domains required for MDM2 mediated NFκB2 promoter activity: Data from the previous experiment suggests that full length MDM2 transcriptionally upregulates NFκB2. Our next step was to define the domain on the human MDM2 protein that was responsible for this activity. The MDM2 protein is a 491 amino acid protein and consists of different domains that attribute specific functional roles to MDM2. It consists of an N-terminal p53 binding domain that is responsible for inhibition of p53 transactivation. It has a central acidic domain that has certain residues whose phosphorylation regulates MDM2 function. It also contains a C- terminal RING finger domain which is essentially required for the ubiquitin ligase activity of MDM2. MDM2 interacts with several growth suppressors like p53, Retinoblastoma (Rb) and other proteins like TATA Binding Protein (TBP), p19, TFIIE. (Figure: 1). For example, the acidic domain of MDM2 binds to the C-terminal fragment of pRB is required for the growth suppression functions of pRB. The G1/S transition is also affected as unphosphorylated pRB binds to E2F1/DP1 heterodimers (involved in cell cycle progression) and suppresses their transactivating function [102].

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For similar MDM2 domain analysis, N- terminal and C- terminal deletion mutants of MDM2 generated by our laboratory for interaction studies were used to perform the experiments discussed below [103]. H460 cells were lipofected with the various MDM2 deletion mutants or the vector (control) with the NFκB2 promoter luciferase construct. The beta galactosidase plasmid was co-transfected to verify the transfection efficiency of the cells. The deletion mutants incorporated in the experiment have been listed in Figure 18.
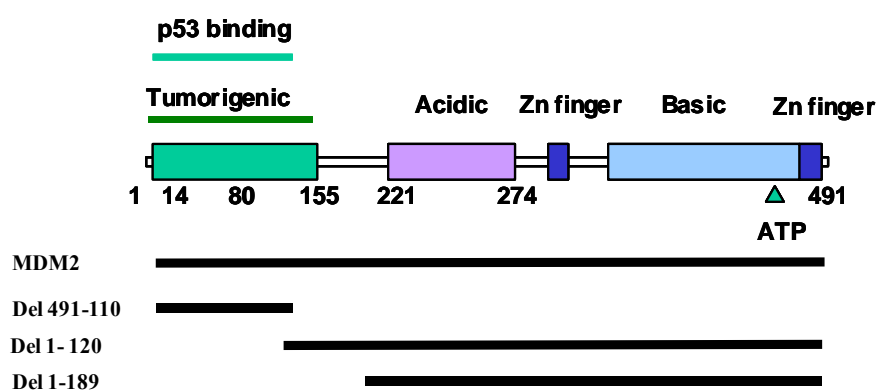
Figure 18: Schematic representation of the MDM2 deletion mutants used in NFκB2 promoter analysis.

In the experiment with full length MDM2 and the deletion mutants, we observed an increase in NFκB2 promoter activity with the full length MDM2 as expected, as well as with the C- terminal deletion mutant Del 491-110 that consists of only the p53 binding domain indicating that a p53 dependent NFκB2 promoter activity is one of the mechanisms. The N terminal deletion mutant, Del 1-120 that does not contain the p53 binding domain shows a promoter activity of 2.33 fold. This suggests that p53 mediated regulation is not the only mechanism by which NFκB2 upregulation occurs. However, another N- terminal deletion mutant Del 1-189 lacking an additional 69 amino acids showed no increase in the NFκB2 promoter activity (1.28 fold). This indicates that amino

acid residues 121-189 are necessary for the p53 independent upregulation of the NFκB2 promoter. The luciferase analysis and the transfection were done in triplicates. The experiment was repeated five times with comparable results that showed similar fold increase. Figure 19A is a representative of one such experiment. The error bars shown correspond to the standard deviation within the triplicates.
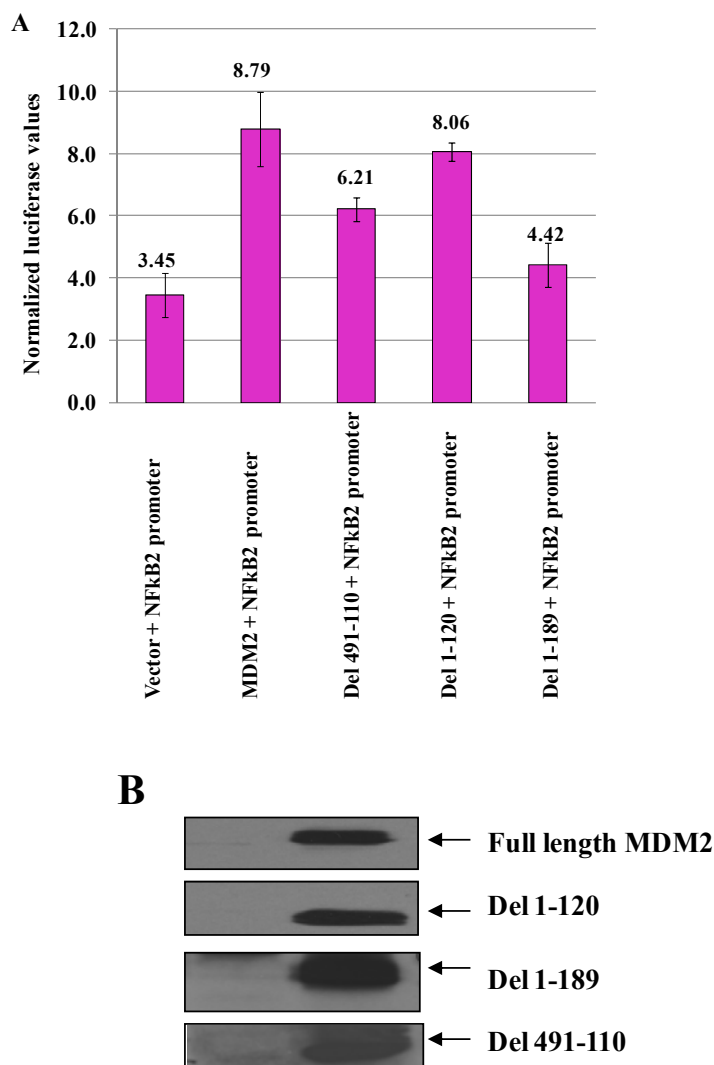
Figure 19: MDM2 domain analysis to identify the region responsible for increased NFκB2 promoter activity. (A) NFκB2 promoter luciferase activity by MDM2 deletion mutants. (B) Western blot showing MDM2 expression.

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As mentioned before, MDM2 interacts with other proteins, one of them being the TATA Binding Protein (TBP). The TBP binding domain of MDM2 is located within amino acids 120-276 (Figure 20) that coincides with the residues required for the p53 independent promoter activity (121-189) mentioned above. This region resides in the acidic domain of MDM2 raising the possibility that MDM2 may act as a transcriptional activator by interacting with TBP [104].

Specific NFκB2 promoter transcripts are upregulated by MDM2: The NFκB2 gene shows alternative transcription of exons 1a and 1b. It has been shown to have two different promoters P1 and P2 (Figure 21), both of which can be active in a cell [101].
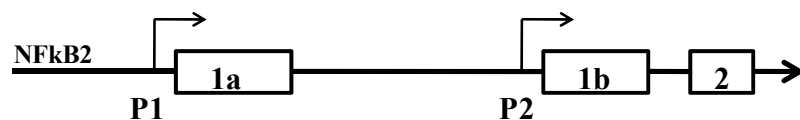
Figure 20: Schematic representation of the NFκB2 promoter.

To determine if MDM2 preferentially activates either of the promoters, QPCR analysis was performed using promoter-specific primers for both P1 and P2 with conditions described by Lombardi et al. [101]. For this experiment, H460 cells were infected with the lentivirus containing short hairpin against MDM2 (shMDM2) or lentivirus against the non endogenous luciferase gene (sh luciferase) as a control. The infected cells were then harvested for RNA. cDNA prepared from lentiviral infected H460 cells were analyzed for their transcript levels.

The QPCR analysis was done in triplicates. The experiment was repeated three times with comparable results that showed similar fold difference. Figure 21 is a

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representative of one such experiment. The error bars shown correspond to the standard deviation within the triplicates. Data shown in Figure 21 demonstrate that silencing MDM2 in the H460 cells decreases the levels of the NFκB2 P2 transcript proportionally, however the P1 levels are unaffected in comparison to lenti-luciferase infected (control) H460 cells suggesting that MDM2 influences the transcript levels of NFκB2 P2 promoter.
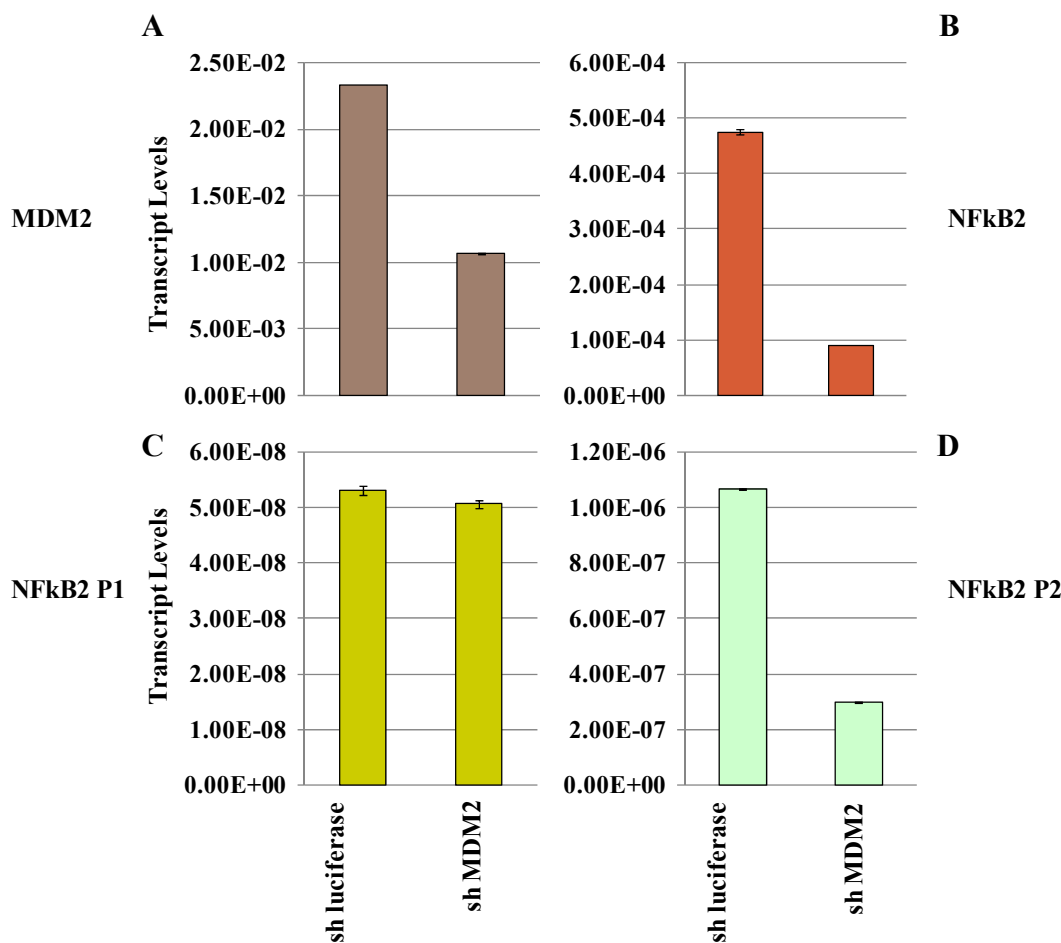
Figure 21: MDM2 regulates NFκB2 P2 transcripts.

MDM2 requires both P1 and P2 promoters to induce NFκB2 promoter activity. As mentioned previously, both NFκB2 P1 and P2 can be functional in the cell. To determine

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the promoter that MDM2 activates, transcriptional promoter analysis was done with the entire promoter consisting of both P1+ P2 and promoters and; P1 and P2 independently. Figure 22 is a schematic representation of the promoter sequences used.
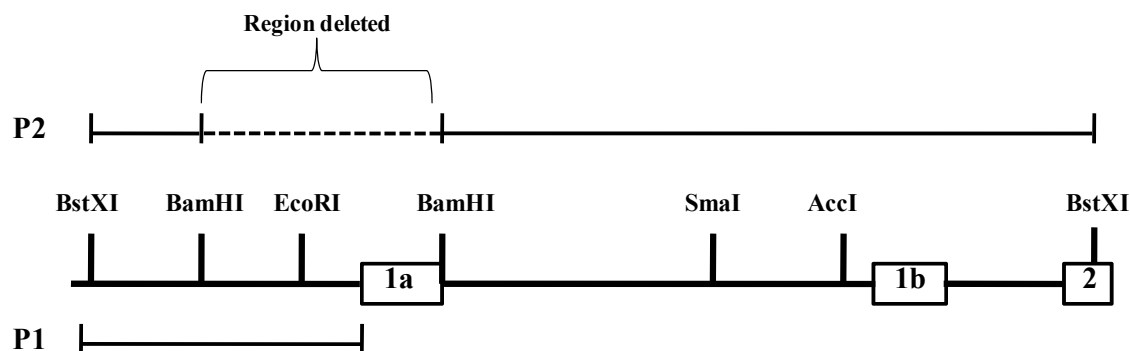
Figure 22: Structure of the 5' flanking region of the NFκB2 gene with restriction sites, untranslated exons and P1, P2 constructs.

H460 cells were transfected with full length MDM2 plasmid or the vector control along with the NFκB2 luciferase promoter construct (P1+P2, P1 or P2). Beta galactosidase plasmid was cotransfected to verify transfection efficiency in these cells. Figure 23 shows the fold increase in the NFκB2 luciferase activity with the indicated promoters. The P1 (0.84 fold) and the P2 (0.70) promoter independently were not activated by MDM2 overexpression. This suggests that although MDM2 upregulates the P2 promoter transcript of NFκB2, the upstream sequences near the P1 promoter may be required for optimal activity due to the consensus binding sequences for transcription factors NFκB, Sp1 and E2F (Figure 24). MDM2 is known to interact with the transcription factors Sp1 and E2F that mediate progression through S-phase. The luciferase analysis and the transfection were done in triplicates. The experiment was repeated three times with comparable results. Figure 19A is a representative of one such experiment. The error bars shown correspond to

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the standard deviation within the triplicates. Also, we observe a higher basal activity of the P2 promoter. This could possibly be due to the presence of inhibitory sequences present in the P1 promoter sequence that could affect the activity. However, this does not indicate an effect of MDM2, due to the upregulation in the basal level itself.
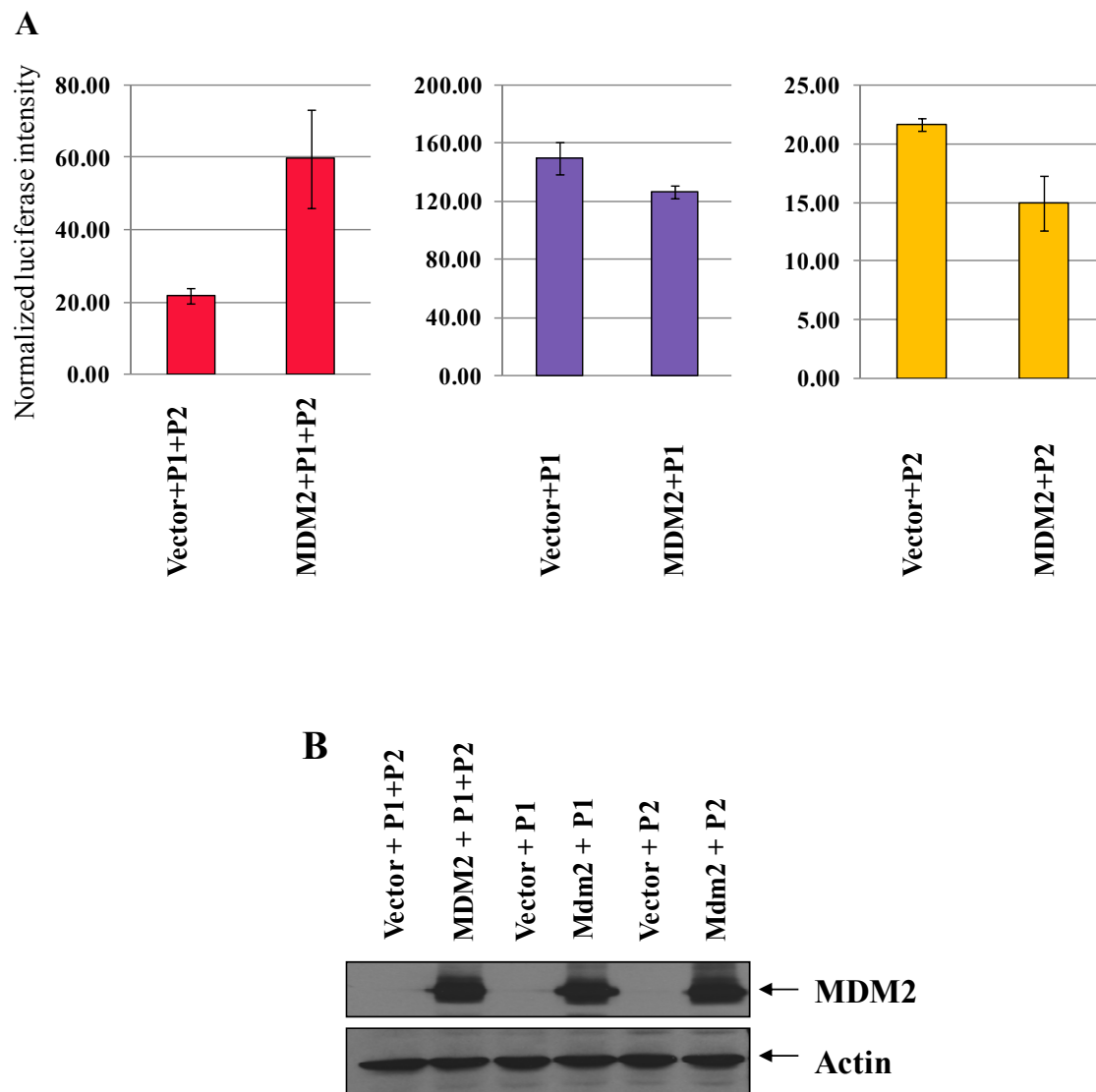
Figure 23: (A) MDM2 requires P1 and P2 promoter sequences for NFκB2 promoter activity (B) Western blot confirming MDM2 expression.
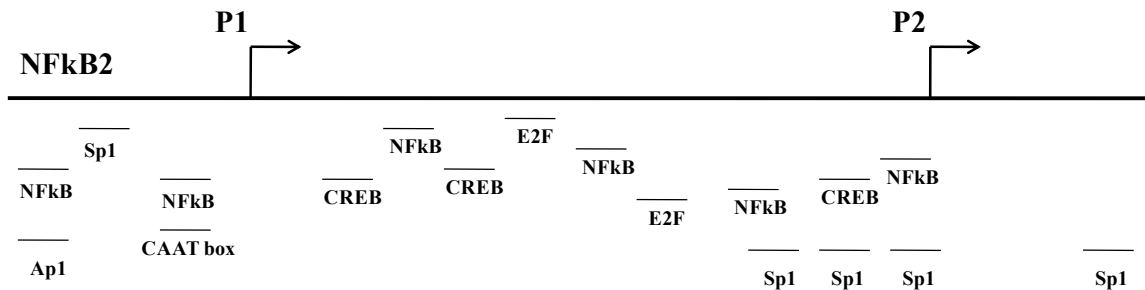
Figure 24: Transcription factor binding sites on the NFκB2 promoter.

c. Chapter Summary:

The focus of this chapter was to decipher the mechanism of MDM2 mediated upregulation of NFκB2 in human lung cancer. Results from the tumor samples distinctly indicated a significant correlation between the levels of MDM2 and NFκB2 in samples harboring wildtype p53. To study this further in an in vitro tissue culture system, experiments were performed in a non small cell lung cancer cell line, H460 that harbors wildtype p53. Overexpression of MDM2 in H460 cells elevated the expression levels of NFκB2 confirming the correlation between the two proteins. To determine if MDM2 was the factor responsible for the increase in NFκB2, a MDM2 silencing experiment was designed to measure the levels of NFκB2 after MDM2 silencing that suggested that endogenous MDM2 regulates the levels of NFκB2 in the cell.

Since H460 was a cancer cell line, it is a definite possibility that the other genetic defects in the cell play a role in this NFκB2 upregulation. To understand if this is an event that occurs in a normal cell, MDM2 was silenced in normal lung fibroblasts, WI38 to quantify the corresponding levels of NFκB2. In this experiment downregulation of MDM2 lowered the levels of NFκB2 suggesting that this is a mechanism that occurs in a normal

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cell; however the effect may be drastic in cancer cells conferring additional oncogenic properties like chemoresistance and cell proliferation to the cell.

Having established a correlation between MDM2 and NFκB2, a NFκB2 promoter luciferase reporter assay was done with H460 cells overexpressing MDM2 and control cells that showed an increase in the NFκB2 promoter activity in the presence of MDM2 suggesting that MDM2 transcriptionally upregulates NFκB2.

In order to identify the MDM2 domain responsible for upregulation in promoter activity, different N- and C- terminal deletion mutants of MDM2 were used in the NFκB2 promoter luciferase reporter assay. The p53 binding domain resides in the N- terminal region of MDM2. The C- terminal deletion mutant that consists of the p53 binding domain upregulates the promoter suggesting that the increase in the promoter activity may be p53 mediated. Two different N- terminal deletion mutants were used that consisted of amino acid residues 121-491 and 190-491 respectively. If MDM2 regulates the NFκB2 promoter due to its interaction with p53, the N- terminal deletion mutants of MDM2 would not show any increase in the promoter activity as both the mutants lack the p53 binding domain. On the contrary, the N-terminal deletion mutant Del 1-120 showed an elevated promoter activity, whereas the Del 1-189 MDM2 mutant that lacks an additional 70 amino acid residues does not show any upregulation. This information tells us that MDM2 mediated NFκB2 promoter upregulation may also have a p53 independent mechanism and that residues from 121 to 189 seem to be critical for this p53 independent activity.

As mentioned before, MDM2 interacts with various other proteins and transcription factors, one of them being the TATA Binding Protein (TBP) that interacts with MDM2

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between residues 120-275. Since this domain overlaps with the domain that is required for the p53-independent increase in NFκB2 promoter activity, it is possible that MDM2-TBP interaction is essential.

The NFκB2 promoter consists of two alternative promoters P1 and P2 both of which are functional in the cell. Based on this, we proceeded to analyze if MDM2 preferentially regulates one of these promoters. An experiment designed to study this involved PCR analysis of the H460 cells with promoter specific primers (for P1 and P2). Downregulation of endogenous MDM2 in the H460 cells, by MDM2 silencing proportionally decreased the levels of the P2 transcript but did not alter the levels of P1 transcript. This indicates that MDM2 specifically influences the P2 promoter transcripts. Presence of sp1 sites in the P2 promoter sequence could explain the effect of MDM2, as MDM2 is known to interact with sp1 and regulate transcription of genes. However a promoter luciferase reporter assay with both P1 and P2 and the two promoters independently suggested that MDM2 requires sequences of both P1 and P2 to upregulate the NFκB2 promoter raising the possibility that the consensus binding sequences of transcription factors present in the sequences upstream may be required for the MDM2 mediated upregulation of the NFκB2 promoter.

L. Chapter 5

Role of MDM2 in Oncogenesis and Effect on NFκB2 Downstream Target Genes

Partial work from this chapter is currently in a manuscript preparation.

a. Introduction:

Activation of the NFκB pathway has been linked with human cancers with respect to altered drug sensitivity [62]. Studies in the laboratory indicate that MDM2 induces Akt phosphorylation. Activated Akt is capable of phosphorylating several factors involved in transcriptional control, apoptosis and metabolic regulation. Also, activated Akt regulates processing of NFκB2 to form active p52 [105]. This suggests that it is possible that MDM2 not only upregulates NFκB2 expression but also leads to upregulation of downstream genes.

b. Experimental Results:

Silencing NFκB2 downregulates the rate of cell proliferation: Enhanced expression of NFκB2 has been related to oncogenesis [81]. Results from the previous chapter indicate that MDM2 overexpression correlates with elevated levels of NFκB2 in lung cancer patients (Figure 7), and MDM2 upregulates NFκB2 expression (Figure 13). To determine if NFκB2 promotes cancer by its effect on the rate of cell proliferation, H460 cells expressing MDM2 were nucleofected with siRNA against NFκB2 or scrambled siRNA. 48 hours after transfection equal numbers of cells were plated and harvested every 24 hours spanning over a period of five days. Cells were counted using a Beckman coulter counter. The rate of growth was determined by plotting a growth assay curve. The results of this experiment show that introduction of NFκB2 siRNA down regulates rate of cell

proliferation (Figure 25 A). Cells were plated in triplicates for every time point. The error

bars depict the standard deviation among the triplicates and Figure 25 is a representative of

two independent experiments. Western blot analysis of the cell extracts shows that the

siRNA against NFκB2 downregulates NFκB2 levels (Figure 25 B). This result suggests

that endogenous levels of NFκB2 determine the rate of cell multiplication implying that

MDM2 overexpression may alter cell proliferation by upregulating NFκB2 in human lung

cancer.

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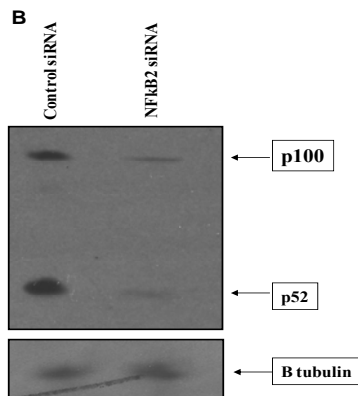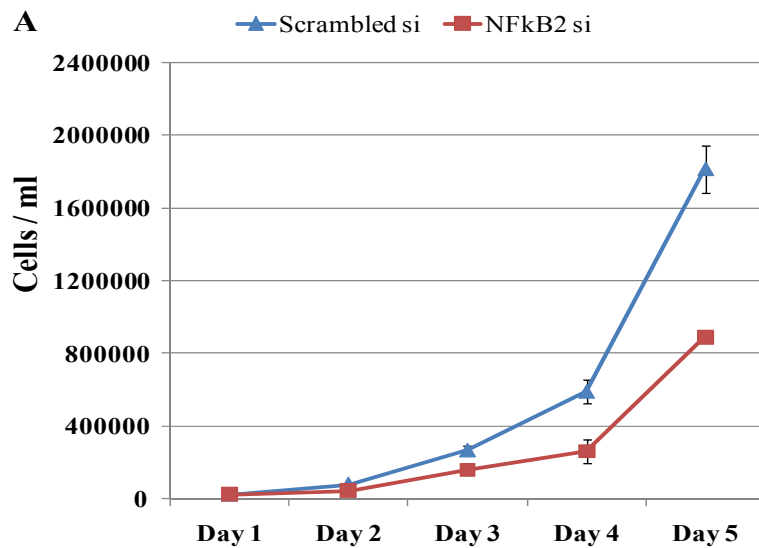
Figure 25: Silencing NFκB2 downregulates the rate of cell proliferation in lung cancer cell line.

Silencing MDM2 downregulates the rate of cell proliferation: The previous experiment indicates that MDM2 may regulate cell proliferation by upregulating the levels of NFκB2 in lung cancer. To determine if MDM2 plays a role in cell proliferation, a cell growth assay was performed. H460 cells were infected with lentivirus containing shRNA against MDM2 (shMDM2) or lentivirus with shRNA against the non endogenous luciferase gene as a control. Lentivirus was removed and replaced with new complete media after 48 hrs. After 24 hours cells were counted and equal numbers of control and MDM2 silenced cells were plated. Cells were harvested every 24 hours for a period of five consecutive days and cell numbers were counted using a coulter counter. A growth curve plotted with these cell numbers is shown in Figure 26. The curve clearly indicates that silencing MDM2 downregulates the rate of cell proliferation in the lung cancer cell line. Cells were plated in triplicates for every time point. The error bars depict the standard deviation among the triplicates and Figure 26 is a representative of two independent experiments.
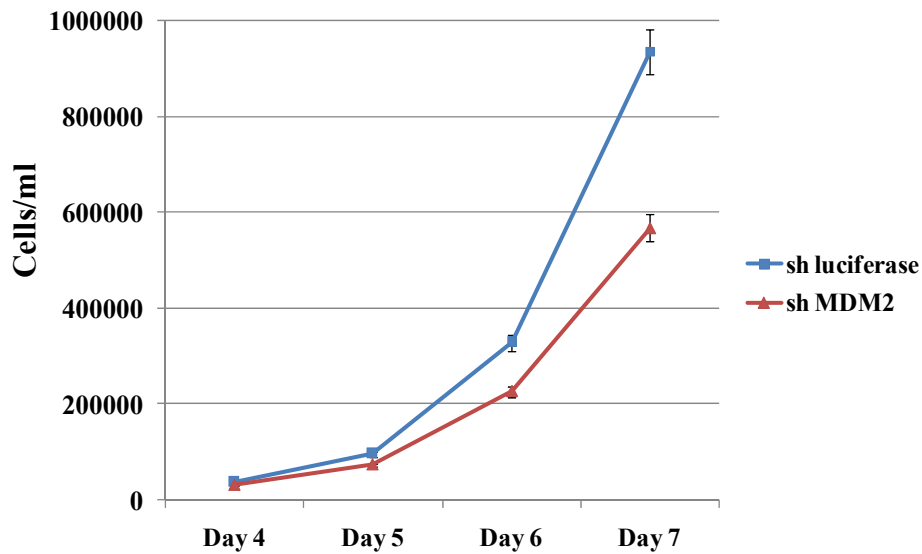
48

Figure 26: Silencing MDM2 downregulates the rate of cell proliferation in human lung cancer cell line.

Effect of MDM2 silencing on chemosensitivity in H460 cells: One of the major
obstacles in the success of lung cancer treatment is resistance of the tumor cells to classical
chemotherapeutic agents. It is possible that a subset of tumors with specific gene
abnormalities could be targeted more effectively with chemotherapeutic agents that would
counteract the abnormal function. In our study we analyzed if presence of MDM2 confers
chemoresistance to cells by colony formation assay. The drug assay was performed with
Etoposide, a topoisomerase II inhibitor [106] and Paclitaxel, a mitotic inhibitor [107], both
usually used for the treatment of non small cell lung cancer. In order to study this, H460
cells were infected with lentivirus containing shRNA against MDM2 (shMDM2) or
lentivirus with shRNA against the non endogenous luciferase gene as a control. Lentivirus
was removed and cells were harvested and counted after 48 hrs. 10^4 or 10^3 cells were
plated for drug or the vehicle (DMSO) treated plates respectively. We plate 10^3 cells for

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the DMSO plates as they grow at a faster rate than the plates treated with the drug. Cells were plated in triplicates. Either the drug or DMSO was added to the plates the next day. The colonies were counted and relative survival of cells was calculated after normalizing with the colonies in the DMSO plates that served as the plating control.

The relative survival of cells normalized to DMSO plates on staining after three weeks did not indicate a significant increase in sensitivity to 6 µM Etoposide or 25 nM Paclitaxel on silencing MDM2 in H460 cells. This suggests that MDM2 does not influence chemosensitivity in the lung cancer cell line H460; however, this observation may vary based on cell line, cell type and also the type of cancer.
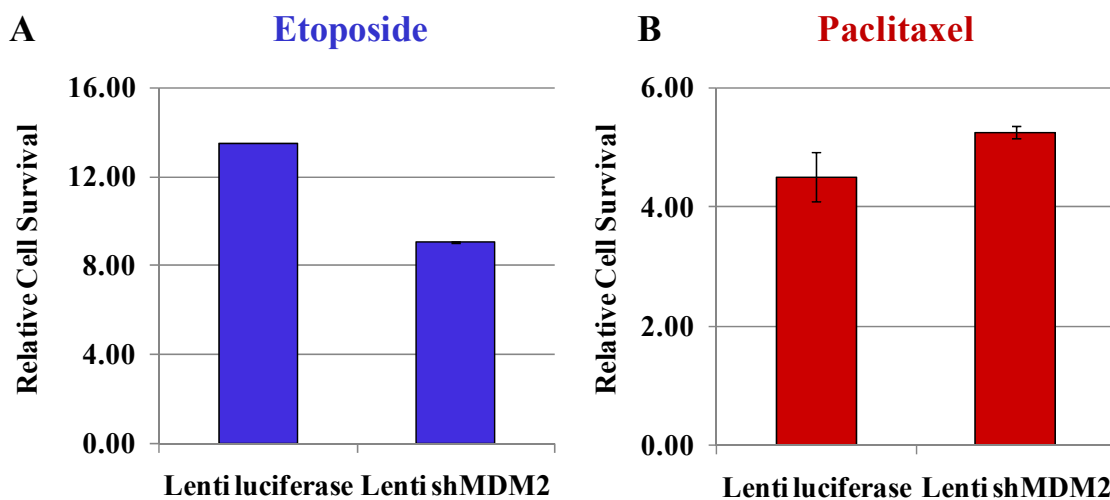
Figure 27: MDM2 silencing does not influence the chemosensitivity of H460 cells.

MDM2 induces nuclear translocation of NFκB2 in H460 cells: NFκB2 is present in the cell as a precursor form p100 that is further processed to form the active molecule p52, which is translocated to the nucleus and is involved in transcription of genes. Processing of p100 to p52 occurs by two mechanisms: constitutive and induced processing. Constitutive processing is known to be regulated by active nuclear shuttling function,

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however induced processing may be nuclear localization signal (NLS) dependent or independent [108]. Our next question was to determine if MDM2 overexpression induces nuclear translocation of p100/p52. This was tested in H460 cells nucleofected with either the full length MDM2 plasmid or the vector (control). 16 hours post nucleofection cells were lysed and fractionated into cytoplasmic and nuclear fractions using a previously published protocol [109]. Levels of NFκB2 p100 and p52 in cytosolic and nuclear fractions were analyzed using polyacrylamide gel electrophoresis. The levels of p100 and p52 ascertained by Western blot analysis with a p100/p52 specific antibody are shown by a representative blot in Figure 28.
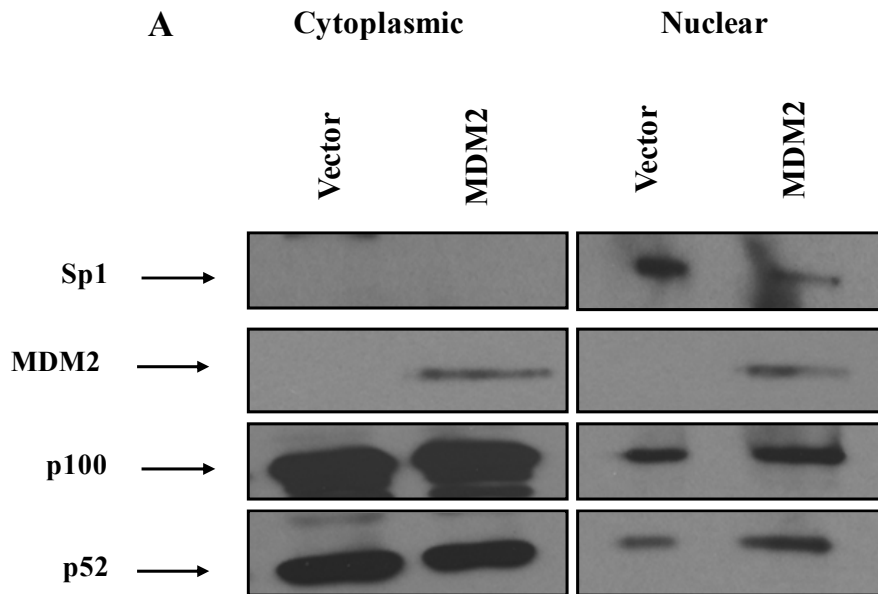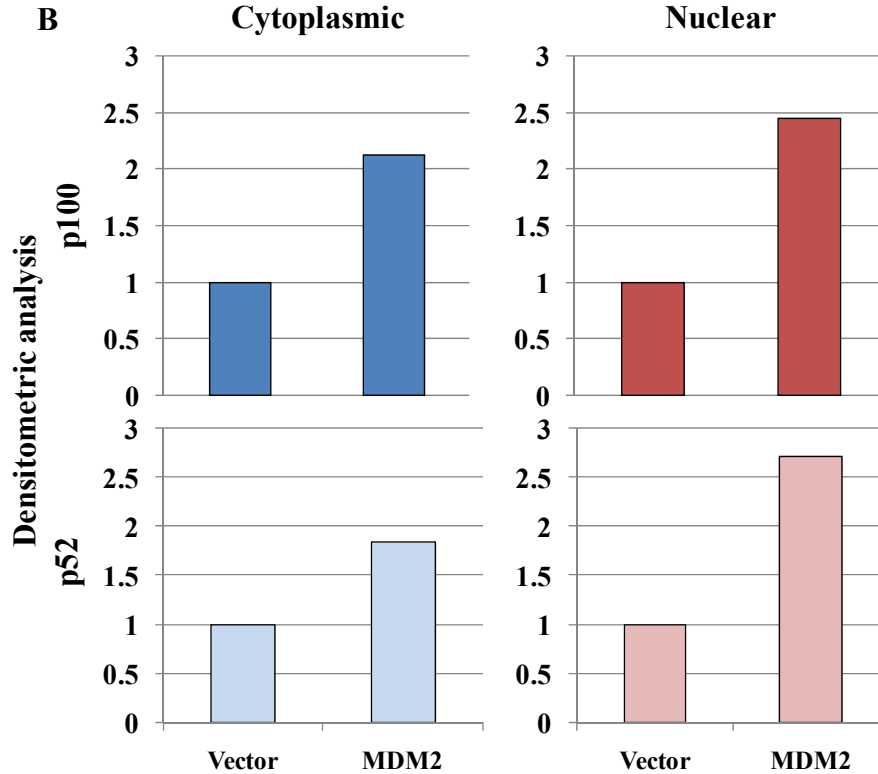
Figure 28: MDM2 enhances nuclear localization of NFκB2 p100/p52.

The densitometric analysis of the western blot suggests that overexpression of MDM2 increases the nuclear expression of p100 by 2.45 fold compared to 2.12 fold in the cytoplasm. We also see a 2.71 fold increase in the protein expression of nuclear p52 compared to 1.85 fold in the cytoplasmic fraction on MDM2 overexpression indicating that MDM2 enhances nuclear translocation of NFκB2 p100/p52. This suggests that MDM2 may induce transcription of downstream target genes of the NFκB2 pathway, following enhanced nuclear translocation of NFκB2 p100/p52.

Overexpression of MDM2 increases Bcl2 levels: In vitro experiments performed with the H460 cells in the previous chapter, show that MDM2 overexpression induces

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NFκB2 at the transcript levels (Figure 13) and the protein levels (Figure 14). We know that NFκB2 p100/p52 induces Bcl2 [67]. Data from the human lung tumor samples clearly indicate the correlation between overexpression of MDM2 and elevated levels of the anti-apoptotic gene, Bcl2 (Figure 3). To determine if MDM2 overexpression can elevate Bcl2 levels, H460 cells were nucleofected with the full length MDM2 expression plasmid (or vector plasmid). The cells were harvested and cDNA synthesized was subjected to QPCR for quantifying the levels of Bcl2.
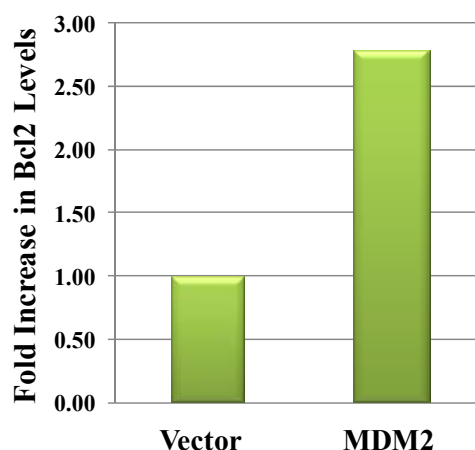
Figure 29: MDM2 overexpression elevates Bcl2 transcript levels.

QPCR results (Figures 29) showed that Bcl2 transcript levels normalized to GAPDH were significantly higher (2.76 fold) in the H460 cells transfected with MDM2 expression plasmid over control cells nucleofected with the vector plasmid. The QPCR analysis was done in triplicates and also the results were reproduced by three independent experiments. The error bars shown correspond to the standard deviation within the triplicates. This suggests that MDM2 overexpression induces Bcl2 levels.

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MDM2 silencing downregulates Bcl2 levels: In order to determine if silencing MDM2 decreases the levels of Bcl2 in H460 cells, these cells were infected with the lentivirus containing shMDM2 or lentivirus against the non endogenous luciferase gene as a control for 48 hours. The lentivirus was then removed and replaced with new complete media. The infected cells were then harvested for RNA after 48 hours. cDNA prepared from lentiviral infected H460 cells were analyzed for their MDM2 and Bcl2 transcript levels.
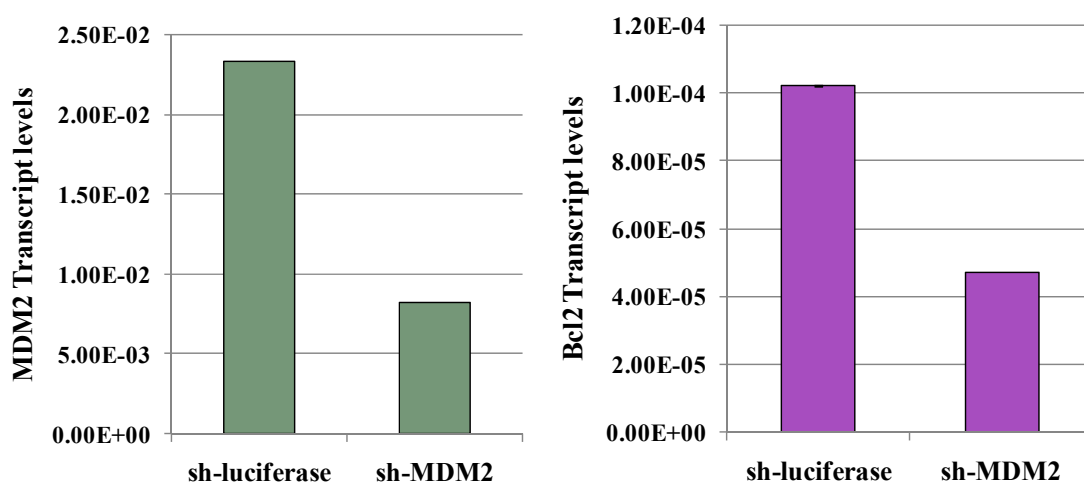
Figure 30: Downregulation of MDM2 expression downregulates Bcl2 expression in H460 cells.

Figure 30 indicates that the shRNA against MDM2 downregulates MDM2 and Bcl2 expression proportionally. The QPCR analysis was done in triplicates and also the results were reproduced by three independent experiments. The error bars shown correspond to the standard deviation within the triplicates of a representative experiment. This data suggests that endogenously expressed MDM2 upregulates Bcl2 expression.

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MDM2 silencing downregulates Bcl2 levels in WI38 cells: MDM2 induces

Bcl2 levels in H460 cells, which is a cancer cell line. In order to understand if this

phenomenon occurs in normal cells, an MDM2 silencing experiment was done in normal

lung fibroblasts, WI38. As done with H460 cells, WI38 cells were infected with

lentivirus containing shMDM2 or lentivirus against the non endogenous luciferase gene

as a control. The virus was removed and replaced with new media after 48 hours. The

infected cells were then harvested for RNA extraction after 48 hours. cDNA prepared

from lentiviral infected WI38 cells were analyzed for their MDM2 and Bcl2 transcript
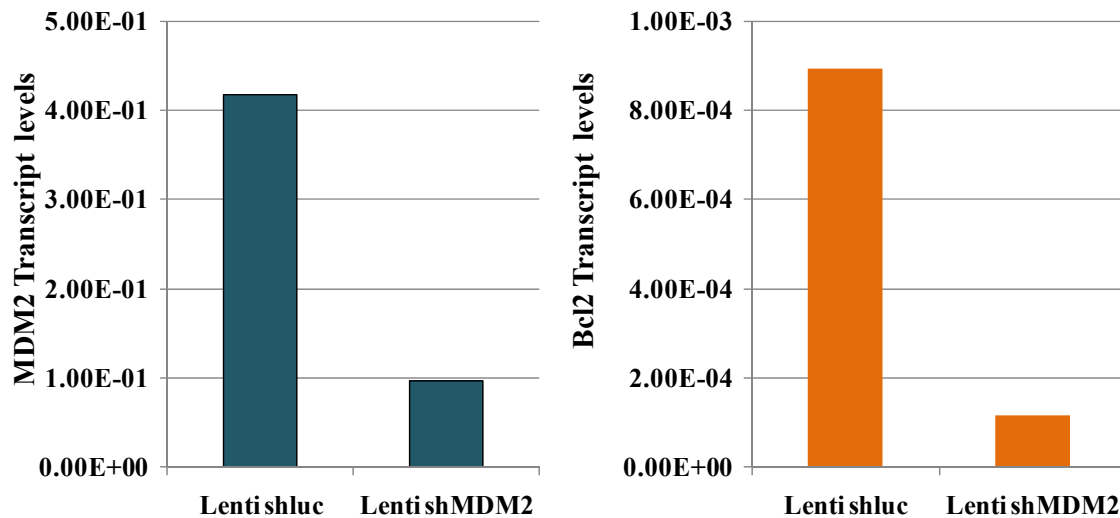
levels.

Figure 31: Downregulation of MDM2 expression downregulates Bcl2 expression in WI38 cells.

The figure above shows that silencing of endogenous MDM2 in normal WI38 cells also

downregulates Bcl2 transcript levels, suggesting that induction of Bcl2 by MDM2 is a

normal occurrence in the cell. The QPCR analysis was done in triplicates and also the

results were reproduced by three independent experiments. The error bars shown correspond to the standard deviation within the triplicates of a representative experiment.

Correlation between MDM2 and MDMX: MDMX is a structurally related protein to MDM2. MDM2 interacts with MDMX through the RING finger domain and this complex stabilizes MDM2 resulting in steady, increased levels of MDM2. In contrast to MDM2, MDMX lacks ubiquitin E3 ligase activity and is unable to target p53 for ubiquitin-proteasome-dependent proteolysis, however it has the ability to inhibit p53 induced transcription on overexpression. MDMX overexpression has been observed in cancers in the presence of p53 [85]. These characteristics may attribute oncogenic properties to MDMX.

Results from the transcript levels and statistical analysis of the human lung tumor samples indicated a very significant correlation between MDM2 and MDMX. To study this in an in vitro system, H460 cells were infected with the lentivirus containing shMDM2 or lentivirus against the non endogenous luciferase gene as a control for 48 hours. The lentivirus was then removed and replaced with new complete media. The infected cells were then harvested for RNA after 48 and 72 hours. cDNA prepared from lentiviral infected H460 cells were analyzed for their MDM2 and MDMX transcript levels. The QPCR analysis was done in triplicates and also the results were reproduced by two independent experiments. The error bars shown correspond to the standard deviation within the triplicates of a representative experiment.

This experiment shows that silencing MDM2 also decreases MDMX levels, confirming the correlation observed in the lung tumor samples. This suggests a possibility

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that MDMX stabilizes MDM2 and may in turn influence NFκB2 levels or may directly regulate NFκB2 levels.
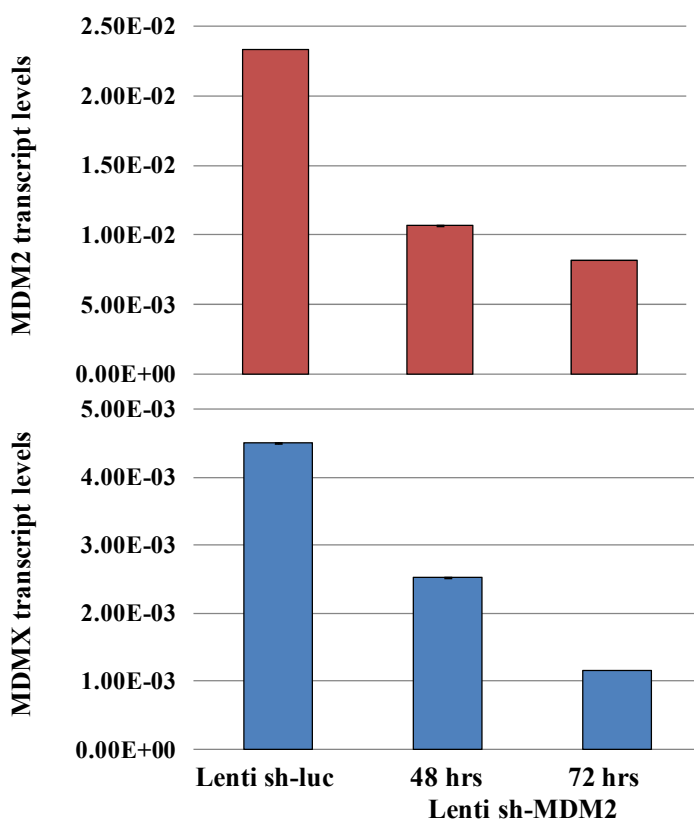
Figure 32: Downregulation of MDM2 decreases the levels of MDMX.

c. Chapter Summary:

MDM2 is a well known oncoprotein and results clearly suggest its role in the NFκB2 pathway. On basis of the observation that MDM2 elevates NFκB2 levels, both at the mRNA and the translational level and also upregulates the NFκB2 promoter, this chapter primarily focuses on the role of this MDM2 mediated NFκB2 upregulation on oncogenic properties like cell proliferation and drug resistance.

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To determine the role of NFκB2 on cell proliferation, a cell growth assay was done with lung cancer cell line, H460 after silencing NFκB2. This experiment showed that silencing NFκB2 levels downregulates the rate of cell proliferation. As MDM2 regulates the levels of NFκB2, the next step was to see if MDM2 has a similar effect on cell proliferation. For this, H460 cells were silenced for MDM2 and the growth assay was performed for five consecutive days. Results suggest that MDM2 silencing in lung cancer cells downregulates the rate of cell proliferation.

Another oncogenic property to be investigated was the role of MDM2 in chemoresistance. The experiment to address this property involved studying the effect of MDM2 silencing on cell response to drug treatment. A colony formation assay was done on treatment with a topoisomerase II inhibitor, Etoposide and a mitotic inhibitor, Paclitaxel. No significant increase in drug sensitivity was observed in MDM2 silenced cells on treatment with either drug. This raises the possibility that MDM2 may not influence chemoresistance in lung cancer cells as the property of chemoresistance could be cell type specific.

The NFκB pathway plays a role in apoptosis by regulating expression of genes that control apoptosis. Bcl2 is a downstream target of NFκB2 and is an important regulator of programmed cell death [110] . Leukemic patients show an association between the levels of NFκB2 p100 and Bcl2. NFκB2 p52 binds and transactivates the Bcl-2 promoter [82]. Results from the lung cancer samples suggested that there is a significant correlation between the levels of MDM2 and Bcl2 in human lung cancer as well. This correlation was confirmed in an in vitro system by the overexpression of MDM2 in H460 cells. Elevated

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levels of MDM2 proportionally increased the Bcl2 transcript levels and silencing MDM2 in the lung cancer cells reduced Bcl2 levels. This suggests that MDM2 mediated upregulation of NFκB2 also influences its downstream target Bcl2.

Constitutive processing of NFκB2 precursor p100 to form its active component p52, is a pathogenic process and involves nuclear shuttling. MDM2 overexpression induces nuclear translocation of NFκB2 p100 and p52 and possibly enhances transcription of target genes by active p52.

Though not related to the NFκB2 pathway, it is interesting to note that the levels of the structurally similar counterpart of MDM2- MDMX and p53 significantly correlate in the lung tumor samples. In H460 cells, MDM2 silencing correspondingly decreased MDMX levels. It is a possible that MDMX may play a role in MDM2 stability that may favor NFκB2 upregulation in a wildtype p53 background.

M. Chapter 6. Discussion.

Cancer is a disease that involves instability and deregulation of the normal functions and state of the cell. It usually encompasses multiple genetic abnormalities including mutations of tumor suppressor genes and transition of proto-oncogenes to form oncogenes. The human homologue of Mouse Double Minute-2 (MDM2) is a well known oncoprotein and is overexpressed in a wide range of carcinomas, soft tissue sarcomas, gliomas and other cancers.

It is a well accepted fact that tumor progression is usually a consequence of cumulative genetic mutations [111, 112]. Cancers with elevated levels of MDM2 also display other genetic abnormalities. There are reports that associate MDM2 overexpression with increase in VEGF levels and decreased Cadherin levels. This increase in VEGF levels may seem to be important for neo-angiogenesis and thus provide survival advantage to tumor cells. Low E-Cadherin levels render cancer cells more motile and invasive eventually leading to distant metastasis.

In an attempt to determine the levels of MDM2 overexpression and its frequency in human lung cancer, RNA was isolated from lung cancer samples and MDM2 transcripts (m-RNA) were quantified using RT-PCR using standard protocol. Results indicated that MDM2 is over expressed in almost a third of the tumor samples (30%) when compared to tissues from non tumorigenic regions (Figure 6). This result shows a strong correlation of MDM2 with oncogenesis in lung cancers.

Having established correlation with MDM2, our next effort was to identify genetic alterations and abnormalities that co-occur with MDM2 overexpression in human lung

cancer that would confer specific survival advantage to these cancer cells. Since p53 is one of the most commonly mutated tumor suppressor genes and its expression is tightly coupled to MDM2 by an auto-regulatory feedback loop, it was important to determine the p53 status of the human lung tumor samples obtained. Some of the hotspots for p53 mutations are codons 158, 175, 248, 273 in cancers such as lung cancer, gastric cancers, breast carcinoma and colorectal cancer. p53 sequencing of the samples identified 18 samples harboring WT p53 and 12 with point mutations in the DNA binding domain of p53. P53 mutations observed in our lung tumor samples showed various mutations and not a higher frequency of any specific mutation. Mutations of the p53 gene are usually known to be GC to TA transversions. Studies suggest a strong correlation between the frequency of these GC to TA transversions and lifetime cigarette smoking leading to formation of adducts at codon 157, 248 and 273 in the p53 gene [113, 114].

After demonstrating the significant overexpression of MDM2 and the p53 status, our next experiments were designed to elucidate the signaling pathway that may be involved. Since typical pathways implicated in cancers are MAPK and PI3K/Akt pathways, our initial effort was to identify the pathway affected. Members of the NFκB family of transcription factors play a role in cellular transformations [115]. Signaling via NFκB pathway can be canonical or non-canonical. There is evidence that MDM2 induces NFκB/p65 expression transcriptionally in a p53-independent role and leads to doxorubicin resistance in acute lymphoblastic leukemia [80]. However, less is known about the interaction of NFκB and members of the alternate NFκB pathway NFκB1 and NFκB2. High levels of NFkB2 expression were observed in mammary carcinoma cell lines and

primary tumors [67]. Another study suggests that knockout mice that lack the inhibitory C-terminal domain of NFκB2/p100 constitutively express p52 and have dramatic hyperplasia of the gastric epithelium [116]. Chromosomal rearrangements that affect the NFKB2 locus have been associated with a variety of B- and T-cell lymphomas, including chronic lymphocytic leukaemia (CLL), multiple myeloma, T-cell lymphoma and cutaneous B- and T-cell lymphomas [117]. However, there have been no reports so far suggesting a relation between NFκB2 and lung cancer.

Data from our laboratory suggest that MDM2 participates in the PI3K pathway by inducing Akt phosphorylation. Since NFκB pathway is further downstream in the PI3K/Akt pathway, we hypothesized that MDM2 may in fact play a role in NFκB pathway. We started by comparing the transcript levels of NFκB2 p100 and MDM2 in lung tumor samples with wildtype and mutant p53. Statistical analysis indicated a significant correlation between the levels of MDM2 and NFκB2 in lung cancer samples especially those harboring WT p53. This correlation further led us to investigate if the two proteins, MDM2 and NFκB2 are mutually regulated in lung cancer cells. In order to support the above evidence from lung tumor samples, we repeated the experiments in an in-vitro culture setting in lung cancer cell line, H460. We first overexpressed and then silenced MDM2 in these cell lines and studied the effects on NFκB2, both at the transcript and protein levels. Our results show a corresponding increase and decrease in the NFκB2 levels at the transcript level (Figure 14, 15) and an increase in protein levels of NFκB2 on MDM2 overexpression. We were unable to verify the effect of MDM2 silencing on NFκB2 at the protein level, as endogenous expression of MDM2 in H460 was undetectable by western

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blotting. These data confirm the hypothesis that MDM2 influences the member of the non-canonical NFκB pathway, NFκB2.

Since MDM2 mediates upregulation of Akt phosphorylation and subsequent over expression of NFκB2, we expected a corresponding upregulation of NFκB2 target genes including Bcl2 and c-myc [82, 105]. In addition, since both MDM2 and Bcl2 have been implicated in suppressing p53 mediated transactivation of target genes they may act in concert to render cells oncogenic. In order to confirm this relation, we analyzed the effects of MDM2 overexpression on Bcl2 and c-myc. Transcript analysis showed that both Bcl2 and c-myc were overexpressed in lung cancer samples. More significant was the observation that increased levels of Bcl2 (seen in 26% of cases) showed a significant correlation with MDM2 (Figure 8) while c-myc did not show any correlation. This suggests that MDM2 may not influence all the downstream target genes of NFκB pathway to the same degree. MDM2 interacts with various other proteins and transcription factors thereby regulating their expression. Transcriptional regulators may recognize a very similar set of DNA binding sites, however, minor differences caused by interaction with another proteins and cofactors can result in different levels of correlation with their respective target genes [118].

 MDM2 overexpression and silencing showed corresponding increase and decrease respectively in the transcript levels of Bcl2 in lung cancer cells (Figure 29, 30). These observations suggest that MDM2 is capable of upregulating Akt-NFκB2-Bcl2 pathway, which may cause growth proliferation and confer drug resistance to cancer cells. In spite of its oncogenic role, human MDM2 induces G1 arrest in normal human cells. Cell lines

bearing known genetic mutations are insensitive to MDM2-mediated growth arrest. This suggests that the cancer cells that overexpress these oncoproteins must have acquired genetic damages to evade the growth arrest function of the overexpressed oncoproteins. Consistent with the complexity of its normal function, MDM2 has been reported to interact with a number of factors [71]. Similar MDM2 downregulation experiments were performed in normal lung fibroblasts, WI38. Though not an expected result, normal cells also exhibited the MDM2 – NFkB2 correlation (Figure 16).

NFκB2 is an important mediator in non-canonical pathway of NFκB pathway. It is present as a precursor molecule-p100, in the cytoplasm, that gets processed by ubiquination and proteosomal degradation to form the active molecule p52 that then translocates to the nucleus to induce gene transcription. Activation of p100 is a critical step in a tightly regulated signaling pathway. Loss of the Ankyrin Repeat Domain (ARD) located in the C-terminal region of p 100, leads to constitutive processing and active nuclear translocation resulting in over expression of target genes [108].

First, we demonstrated that MDM2 overexpression upregulates NFκB2 p100/p52 levels. Studies suggest that the nuclear localization signal (NLS) and translocation of NFkB2 to nucleus is essential for constitutive p100 processing. Nuclear translocation is also partially involved in inducible processing of wildtype p100 [108]. Epstein–Barr virus (EBV) latent infection membrane protein 1 (LMP1)-induced NFkB activation involved induced p100 processing in human lymphoblasts cells [119]. Similarly, we verified if MDM2 affects nuclear shuttling of NFκB2 and induces processing of p100. Our results indicate that overexpression of MDM2 may induce nuclear localization of NFκB2

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p100/p52 in human lung cancer cell line (Figure 29). This may favor transcription of genes by active p52.

It is well known that though MDM2 is not a general regulator of transcription, it regulates transcription when recruited to a promoter through interactions with other proteins and transcription factors as so far there is no evidence suggesting direct DNA binding characteristic of MDM2. In order to investigate the effect of MDM2 on the NFκB2 promoter, we designed promoter experiments tagged with luciferase as a reporter. These experiments showed that MDM2 indeed upregulated NFκB2 at the promoter level. MDM2 affects p53 as 1) it directly interacts with p53 to inhibit p53-mediated transactivation and 2) binds and degrades p53 by ubiquination.

Studies have been done using various deletion mutants of MDM2 to identify the domain involved in transcriptional activity of promoters. Experiments done by others have shown that different sequences of MDM2 are required for inhibition of the cyclin A and c-fos promoters. This difference in sequence is due to interaction of the acidic domain of MDM2 with TBP and C- terminus of MDM2 with TAFII250 [120].

Similar to these studies, we tested if p53-interaction domain and ubiquitin ligase domain of MDM2 is responsible for NFκB2 promoter upregulation. Our first deletion mutant was from 491-110, containing the p53 interaction domain (1-109): this was shown to retain function demonstrating p53 dependent mechanism of promoter activity. The proposed model suggests that p53 probably recruits co-repressors at the promoter that leads to promoter repression. In presence of MDM2, the MDM2-p53 interaction relieves

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the repression on the promoter. Activators now are recruited to the promoter and activate the NFκB2 promoter.

Deletion of p53 binding domain of MDM2 (Del 1-120) is capable of up-regulating the NFκB2 promoter, demonstrating p53 independent mechanism for activation of NFκB2 promoter. Further, deletion mutant Del 1-189 resulted in loss of increase in promoter activity. This suggests that residues 120 to 189 of MDM2 are critical in up-regulating NFκB2 promoter by a p53 independent mechanism. These observations imply that MDM2 upregulates NFκB2 promoter in a p53 dependent and independent mechanism.

A   Model for p53 dependent mechanism

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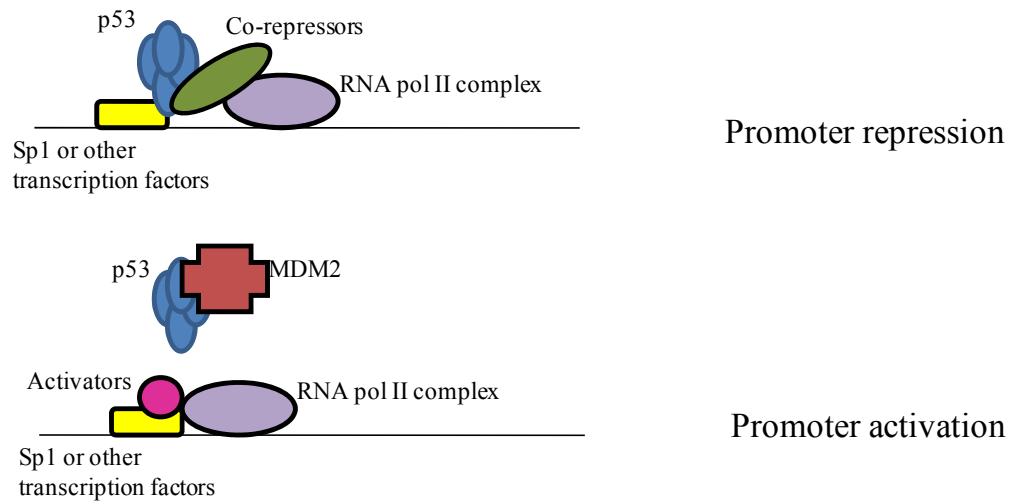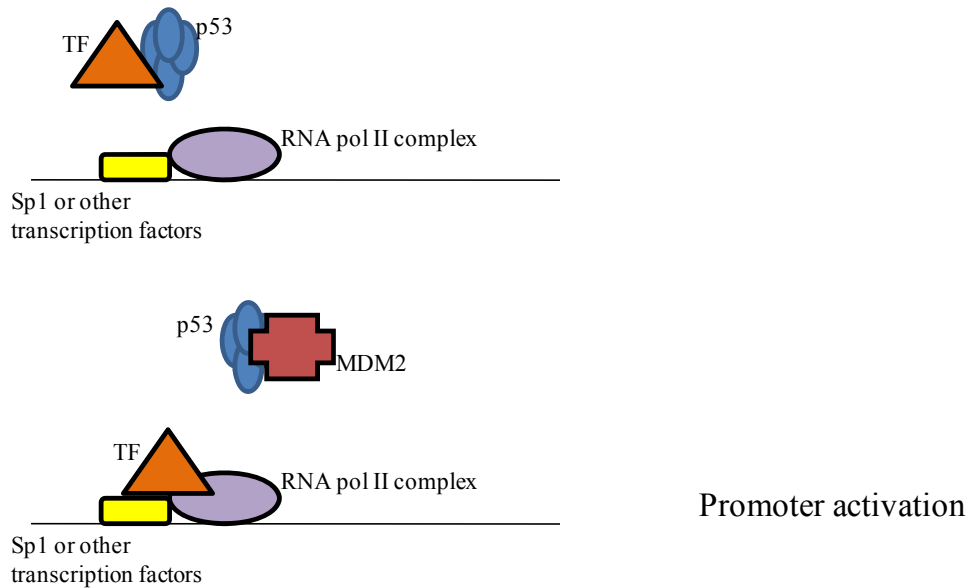
B   Model for p53 independent mechanism
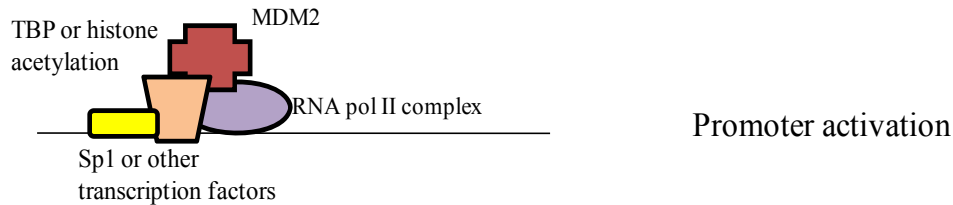
Figure 33: Proposed model for (A) p53 dependent and (B) p53 independent mechanism for MDM2 mediated upregulation of NFκB2 promoter.

TATA Binding Protein (TBP) is a transcription factor that is an integral member of Transcription Initiation Complex Assembly. It is known that TBP participates in transcription from TATA-containing and TATA-less promoters [121]. Since residues 120-276 of MDM2 have been shown to bind TBP and NFκB2 is a TATA-less promoter, we propose that MDM2 binds to TBP and recruits it to the promoter to induce NFκB2 promoter activity.
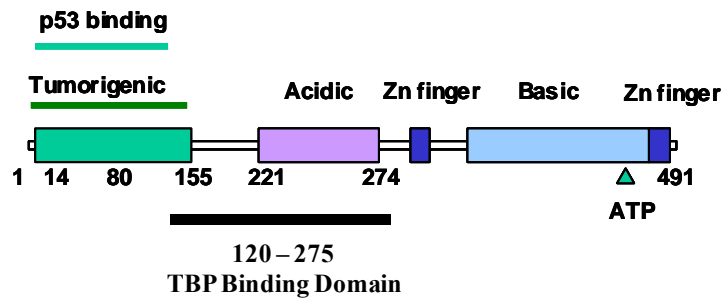
Figure 34: The TATA Binding Protein (TBP) interaction domain of MDM2 is essential for MDM2 mediated upregulation of the NFκB2 promoter.

MDM2 may also induce histone acetylation, activating transcription of the NFκB2 promoter. In addition, studies in our lab show that a deletion mutant of MDM2 (Del 491-155), containing only 154 amino acid residues at the N terminus, harboring the tumorigenic domain enhances cell proliferation.

Since NFκB2 promoter consists of two alternate promoters P1 and P2, our next efforts were to investigate if MDM2 preferentially upregulates one of these two promoters. Silencing MDM2 decreased the transcript levels of P2 without affecting the amounts of P1 suggesting that MDM2 upregulates NFκB2 by activating the P2 promoter (Figure 22). The ankyrin repeat region of NFκB2 prevents its nuclear translocation and is important in providing a tight control in the levels of functional p52 and alteration in this critical region leads to constitutive processing of NFκB2. Since MDM2 upregulates the P2 promoter, it is possible that transcription from the P2 promoter generates a transcript of NFκB2 with alteration in the ankyrin repeat region.

However, results from NFκB2 promoter assay show no activation in response to MDM2 if only P1 or P2 was used. If both P1 and P2 (entire promoter) are present, MDM2

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induces a significant upregulation, indicating that MDM2 requires both P1 and P2 promoter sequences for its affect on the NFkB2 activity. The difference in the effect of MDM2 on the NFkB2 transcript levels and the actual promoter activity is not clear.

The NFκB2 promoter contains binding sites for several known transcription factors, such as NFκB, Sp1 and E2F. Several of these transcription factors have been implicated in p53-mediated promoter repression [122]. The MDM2 interaction site of p53 interacts with several transcription factors such as TFIID and TBP [112, 115, 123]. It is likely that MDM2 may function by interfering with p53 to repress the function of these DNA-binding factors. Since the NFκB2 promoter does not harbor any WT p53 binding site, MDM2 may release a transcription factor from the MDM2-interaction domain of p53 making the factor available for the NFκB2 promoter.

Since expression of MDM2 is strongly correlated to upregulation of NFκB2 in lung cancer samples with WT p53 and in-vitro experiments suggest p53 requirement for MDM2-mediated upregulation of NFκB2, the hypothesis that MDM2 mediated NFκB regulation is p53 dependent is strongly favored. In this circumstance, one of the promoter regions (P1, P2) may be responsive to WT p53-mediated transcription repression. As shown in figure 34, it is very likely that MDM2 modulates nucleation of transcription factors in the promoter region responsive to WT p53 repression.

In order to determine the role of MDM2 mediated upregulation of NFκB2 in promoting oncogenesis, cell proliferation and chemoresistance assays were performed. Cell growth assay after silencing NFκB2 reduced the rate of cell proliferation in human lung cancer cell line (Figure 26). This proves that NFκB2 is required for cell proliferation.

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Similar but separate experiments silencing MDM2 showed decreased rate of cell proliferation (Figure 27). However, to determine if the decrease in cell proliferation in MDM2 silenced cells is NFκB2 mediated, the cell growth assay should be performed with cells overexpressing MDM2 plasmid in the presence of NF-κB2 siRNA. In this case, if proliferation is NFκB2 mediated, there should be no increase in cell growth.

Over-expression of MDM2 in breast and lung cancer cells harboring WT p53 reduces chemotherapeutic sensitivity, implicating MDM2-mediated inactivation and degradation of p53 in the oncogenic function [111, 124]. Since MDM2 has been shown (in separate studies in our laboratory) to induce Akt phosphorylation and NFκB2 upregulation, we tested the hypothesis if MDM2 silencing would confer chemoresistance to cancer cells with WT p53. Knockdown of MDM2 stabilizes Topoisomerase II and usually decreases resistance to TopoII-targeting drugs. In contrast to this, on treatment with etoposide and paclitaxel, lung cancer cells with silenced MDM2 did not show a significant increase in chemosensitivity.

Since MDM2 has multiple domains that can interact with other proteins (p19, MDMX, Rb, ribosomal protein L5), we investigated the possibility of such interactions in regulating their cellular levels and function. For example, MDM2 and MDMX interact with each other and this interaction prevents auto-ubiquination of MDM2 thereby stabilizing MDM2 levels. MDMX also interacts with p53 and inhibits its function however, it is neither a transcriptional target of p53 nor does it degrade p53. MDMX also blocks MDM2 mediated p53 degradation. These observations indicate that MDM2, MDMX and p53 are capable of forming a complex and alter the expression and activity of

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the proteins [56, 86]. In an effort to better understand MDM2-MDMX interaction, MDMX transcript levels were analyzed in the lung tumor samples. Statistical analysis indicated a strong correlation between the levels of MDM2 and MDMX (Figure 10). An in vitro experiment also confirmed that MDM2 silencing proportionally downregulates MDMX levels (Figure 31). However, detailed experiments studying the influence of MDM2-MDMX-p53 expression levels on the regulation of NFκB2 in human lung cancer would shed more light on the signaling pathways in oncogenesis and needs to be performed.

Tumor suppressor p53 has alternative splice forms, one of them being Delta N p53. Since interaction of this isoform with p53 abrogates its tumor suppressor activity, we analyzed transcript levels of Delta N p53 in lung cancer samples. Results indicated negative correlation between MDM2 and Delta Np53. Since p21 is a potent cell cycle inhibitor and is an inducible target for p53, we expected to see a correlation between p21 and MDM2 in the presence of WT or mutant p53. Statistical analyses comparing the transcript levels of MDM2 with p21 did not find any significant correlation irrespective of p53 status in contrast to existing literature. These results suggest that p21 levels can be p53-independent and need not be necessarily influenced by MDM2 levels. There is a possibility that p21 can also be regulated by other growth inhibitory transcription factors and tumor suppressors that exhibit p53 dependent and independent mechanisms [91].

In conclusion, MDM2 upregulates the expression levels and the promoter activity of NFκB2 in human lung cancer cells with WT p53 by at least two mechanisms: p53 dependent and p53 independent. Two distinct, non overlapping domains of MDM2 are responsible for this upregulation of NFκB2 promoter activity. Our proposed model

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suggests that the p53 binding domain leads to a p53 dependent increase where as, the TBP binding domain of MDM2 induces promoter activity in a p53 independent mechanism. MDM2 requires sequences of both the NFκB2 promoters P1 and P2 for its effect on the promoter. MDM2 mediated upregulation of NFκB2 regulates the cell proliferation of lung cancer cells and also influences the downstream target gene of the NFκB2 pathway, Bcl2. This study could be further extended to identify other downstream targets in the pathway. Experiments could also be performed to determine the effect of inhibitors on this pathway. Since Akt activation could lead to the activation of the NFkB2 pathway, inhibitors of Akt phosphorylation such as Perifosine could be used to prevent the activation of the pathway. Other taxols such as doxorubicin could also be used to inhibit the functions of Bcl2 in the pathway. Use of a proteasome inhibitor could also be analyzed to prevent the activation of the NFκB pathway that is dependent on proteasomal degradation of inhibitors of κB. Information from this study and further research can be used to customize treatment approaches involving inhibitors against specific members of the NFκB pathway based on individual genetic makeup of patients.

N. Figure Legends.

Figure 1: MDM2 domains and its various interaction proteins. N-terminal p53-binding domain and the tumorigenic domain are shown. The putative acidic, basic, Zn-finger and ATP-binding domains predicted by computer analysis have been depicted. Regions of MDM2 that interact with other tumor suppressors and proteins have been shown.

Figure 2: Structure and domains of the tumor suppressor p53. The three distinct regions of p53 comprising of the transactivation domain, the central DNA binding domain and the oligomerization domain have been shown. The nuclear localization signals are also indicated in the figure.

Figure 3: Interaction between MDM2 and p53: An auto-regulatory loop. MDM2 binds to p53 and degrades it by ubiquination. However, elevated p53 levels induce MDM2 levels. This autoregulatory loop between MDM2 and p53 has been illustrated in this figure.

Figure 4: Members of the NFκB pathway. The structure of the members of the canonical pathway – RelA, RelB, c-Rel and the non canonical pathway- NFκB1 p105, NFκB2 p100 are shown in the figure.

Figure 5: NFκB pathways: canonical and non–canonical. The overall view of the canonical and the non- canonical pathway with its different players- NFκB Inducing Kinase (NIK), Inhibitor of kappa B kinase (IKK), Inhibitor of kappa B (IκB) have been illustrated in this figure.

Figure 6: MDM2 overexpression in human lung tumor samples with wildtype and mutant p53. cDNA synthesized from 33 human lung tumor samples (VLU) were analyzed by Quantitative PCR (QPCR) to quantify the mRNA levels of MDM2. The transcript

values were normalized with Glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The bar graph represents the normalized MDM2 transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N2, N7, N13) and were considered as normal control samples.

Figure 7: (A) NFκB2 transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of NFκB2. The values were normalized with GAPDH. The bar graph represents the normalized NFκB2 transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N2, N7, N13) and were considered as normal control samples. **(B) Scatter plot of MDM2 and NFκB2 transcript levels in tumor samples with wildtype p53.** A scatter plot was generated with the normalized transcript levels of MDM2 and NFκB2 to depict the correlation between the two in human lung tumor samples with wildtype p53.

Figure 8: Scatter plot of MDM2 and Bcl2 transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of Bcl2. The values were normalized with GAPDH levels. The normalized MDM2 levels (obtained from the Figure 1) and the Bcl2 transcript levels were analyzed to obtain the ranked gene expression values. The scatter plot projects the correlation between the normalized levels of MDM2 and Bcl2 by plotting their ranked expression levels.

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Figure 9: (A) c-myc transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of c-myc. The values were normalized with GAPDH. The bar graph represents the normalized c-myc transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N2, N7, N13) and were considered as normal control samples. **(B) Scatter plot of MDM2 and c-myc transcript levels in human lung tumor samples.** The normalized MDM2 levels and the c-myc transcript levels were analyzed to obtain the ranked gene expression values. The scatter plot shows the ranked expression levels of MDM2 and c-myc indicating no significant correlation between the normalized levels of MDM2 and c-myc in the lung tumor samples.

Figure 10: (A) MDMX transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of MDMX. The values were normalized with GAPDH. The bar graph represents the normalized MDMX transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N2, N7, N13) and were considered as normal control samples. **(B) Scatter plot of MDM2 and MDMX in human lung tumor samples.** The normalized MDM2 levels and the MDMX transcript levels were analyzed to obtain the ranked gene expression values. The scatter plot projects the correlation between the normalized levels of MDM2 and Bcl2 by plotting their ranked expression levels suggesting a significant correlation between them

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Figure 11: (A) Delta Np53 transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of Delta Np53. The values were normalized with GAPDH. The bar graph represents the normalized Delta Np53 transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N30) and were considered as normal control samples. **(B) Scatter plot of MDM2 and Delta Np53 transcript levels in human lung tumor samples.** The normalized MDM2 levels and the Delta Np53 transcript levels were analyzed to obtain the ranked gene expression values. The scatter plot shows the ranked expression levels of MDM2 and Delta Np53 indicating a negative correlation between the normalized levels of MDM2 and Delta Np53 in the lung tumor samples.

Figure 12: (A) p21 transcript levels in human lung tumor samples with wildtype and mutant p53. Human lung tumor samples (VLU) were analyzed by QPCR to quantify the transcript levels of the CDK inhibitor p21. The values were normalized with GAPDH. The bar graph represents the normalized p21 transcript levels categorized into samples harboring wildtype and mutant p53. Lung tissue samples from the non tumorigenic region of the patients were analyzed similarly (N2, N7, N13) and were considered as normal control samples. **(B) Scatter plot of MDM2 and p21 transcript levels in human lung tumor samples.** The normalized MDM2 levels and the p21 transcript levels were analyzed to obtain the ranked gene expression values. The scatter plot shows the ranked expression levels of MDM2 and p21 indicating no correlation between the normalized levels of MDM2 and p21 in the lung tumor samples.

Figure 13: MDM2 overexpression in lung cancer H460 cell line elevates NF-κB2 gene expression: (A) Western blot analysis of H460 cell extracts for expression of NFκB2 p100, p52 and MDM2 after nucleofection with MDM2 expression plasmid or vector plasmid. Plasmids used are shown at the top. MDM2, p100 and actin were identified using respective antibodies. Migration of the control MDM2, p100 p52 and actin bands are shown by arrows. **(B)** Densitometric analyses of p100 and p52 expression are also shown. Band intensities were normalized for levels of actin.

Figure 14: MDM2 overexpression in lung cancer H460 cell line elevates NFκB2 p100 and transcripts: Transcript levels determined by QPCR are shown by bar graphs. An endogenous GAPDH control was used to ensure equal mRNA levels in each sample. The normalized transcript levels are shown. Plasmids used are shown at the top of each bar graph. The assays were performed in triplicates. The error bars are shown.

Figure 15: Silencing MDM2 downregulates NFκB2 expression in H460 cells. (A) Using short hairpin RNA (shRNA) against MDM2: cDNA from H460 cells transfected with a shRNA against MDM2 or a shRNA against the non endogenous luciferase gene (control) were quantified for MDM2 and NFκB2 transcript levels. The bar graph on the left shows the decrease in the transcript levels of MDM2 on silencing with shMDM2 and graph on the right shows the corresponding decrease in the NFκB2 transcript levels. **(B) Using lentivirus encoding the shRNA against MDM2:** cDNA from H460 cells infected with the lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene was measured for the MDM2 and NFκB2 transcript levels. In (A) and (B) the bar graphs show the transcript levels normalized by the endogenous GAPDH levels. The

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assays were performed in triplicates. The error bars are shown. **(C) Silencing of MDM2 by the lentivirus at the protein level:** Western blot to show MDM2 silencing after infecting H460 cells with the control or shMDM2 lentivirus followed by subsequent transfection with empty vector or MDM2 plasmid to indicate a relative difference in the levels of MDM2 after silencing.

Figure 16: Silencing MDM2 downregulates NFκB2 expression in normal lung fibroblast (WI38) cells: Normal human lung fibroblasts, WI38 cells were infected with lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene as the control. cDNA prepared from the infected cells were measured for MDM2 and NFκB2 transcript levels. The bar graph on the left shows the decrease in the transcript levels of MDM2 on silencing with shMDM2 and graph on the right shows the corresponding decrease in the NFκB2 transcript levels in WI38 cells. In (A) and (B) the bar graphs show the transcript levels normalized by the endogenous GAPDH levels. The assays were performed in triplicates. The error bars are shown.

Figure 17: MDM2 overexpression transcriptionally upregulates the NFκB2 promoter: H460 cells were transfected with a plasmid encoding luciferase reporter gene under the control of NF-κB2 promoter and MDM2 expression (or vector) plasmid. The cells were cotransfected with the beta galactosidase plasmid to check for transfection efficiency. The luciferase intensity values normalized with its corresponding beta gal values is shown in the bar graph. Plasmids used are shown at the top. The assays were performed in triplicates. The error bars are shown. The right panel shows the western blot analysis to ensure MDM2 expression.

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Figure 18: Schematic representation of the MDM2 deletion mutants used in the NFκB2 promoter analysis. The figure shows the following domains of the MDM2: N-terminal p53 binding domain, central acidic domain and the C- terminal basic domain with the Zn fingers. The C- terminal mutant Del 491-110 and the N- terminal mutants Del 1-120 and Del 1-189 have been depicted in the figure below the structure of MDM2 protein.

Figure 19: MDM2 domain analysis to identify the region responsible for increased NFκB2 promoter activity. (A) NFκB2 promoter luciferase activity by MDM2 deletion mutants: H460 cells were transfected with full length MDM2 or the N- and C- terminal deletion mutants along with the luciferase reporter plasmid under the control of the NFκB2 promoter. The cells were cotransfected with the beta galactosidase plasmid to check for transfection efficiency. The luciferase intensity values normalized with its corresponding beta gal values for each sample has been depicted in the bar graph. The deletion mutants of MDM2 and the vector control are shown at the bottom. The assays were performed in triplicates. The error bars are shown**. (B) Western blot showing MDM2 expression:** Western blot analysis of the transfected H460 cells to confirm expression of full length and the deletion mutants of MDM2 is shown in the figure.

Figure 20: Schematic representation of the NF-κB2 promoter: Boxes represent the exons 1a and 1b and the arrows indicate the two promoters, P1 and P2.

Figure 21: MDM2 regulates NFκB2 P2 transcripts: H460 cells were infected with lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene as the control. cDNA prepared from the infected cells were analyzed for transcript levels of (A) MDM2 (B) NFκB2 (C) Promoter P1 and (D) Promoter P2. The bar graphs show the

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transcript levels normalized by the endogenous GAPDH levels. The assays were performed in triplicates. The error bars are shown.

Figure 22: Structure of the 5' flanking region of the NFκB2 gene: The schematic shows a restriction map of the genomic NFκB2 clone including the untranslated exons 1a and 1b.

Figure 23: MDM2 requires P1 and P2 promoter sequences for NFκB2 promoter activity: H460 cells were transfected with full length MDM2 or the empty vector plasmid along with the luciferase reporter plasmid under the control of the NFκB2 promoter (P1+P2) or P1 and P2 independently. The cells were cotransfected with the beta galactosidase plasmid to check for transfection efficiency. The luciferase intensity values normalized with its corresponding beta gal values for each sample has been depicted in the bar graph. The graphs to the left, center and to the right correspond the normalized luciferase intensity due to promoter constructs (P1+P2), P1 and P2 respectively. MDM2 and the vector plasmid with the respective promoter constructs are shown at the bottom. The assays were performed in triplicates. The error bars are shown**. (B) Western blot confirming MDM2 expression:** Western blot analysis of the transfected H460 cells to confirm expression of MDM2 is shown in the figure. Actin acts as the loading control.

Figure 24: Transcription factor binding sites on the NFκB2 promoter: The NF-κB2 promoter sequence was searched using the web-based program TFSEARCH (www.cbrc.jp/research/db/TFSEARCH.html) for putative transcription factor binding sites. Sites with a score >90 were graphed.

Figure 25: Silencing NFκB2 downregulates the rate of cell proliferation in H460 cells.
Equal numbers of H460 cells nucleofected with siRNA against NFκB2 and scrambled
siRNA (control) were plated for the cell proliferation assay 48 hours after nucleofection
and were counted every 24 hours for a period of five days. The assay was done in
triplicates. **(A)** The figure on the left panel shows the cell growth curve. **(B)** The right
panel shows the western blot to confirm NFκB2 p100/p52 silencing (β-tubulin was the
loading control).

**Figure 26: Silencing MDM2 downregulates the rate of cell proliferation in human
lung cancer cell line.** Equal numbers of H460 cells infected with lentivirus encoding
shMDM2 or the non endogenous luciferase gene as the control were plated for the cell
proliferation assay 72 hours after infection and were counted every 24 hours for a period of
five days. The assay was done in triplicates. The figure shows the cell growth curve with
the cells per ml plotted on the Y axis.

Figure 27: MDM2 silencing does not influence the chemosensitivity of H460 cells.
H460 cells infected with lentivirus encoding shMDM2 or the non endogenous luciferase
gene (control) were plated for treatment with the drug and the vehicle (DMSO). 6μM
Etoposide and 25nM of paclitaxel was added to the respective plates. After 48 hours the
drug was removed and plates maintained for a period of 3 weeks to permit colony
formation. The assay was done in triplicates. The bar graphs show the relative colony
numbers after treatment with **(A)** Etoposide and **(B)** Paclitaxel after normalization with
colony numbers in the DMSO plate (plating control). Error bars are shown.

Figure 28: MDM2 enhances nuclear localization of NFκB2 p100/p52. (A) H460 cells nucleofected with MDM2 or the empty vector plasmid were harvested into cytoplasmic and nuclear fractions. The lysates were run on a SDS PAGE gel and developed with antibodies for MDM2, NFκB2 p100/p52 and Sp1 (nuclear marker). A representative blot of the experiment is shown in this figure. **(B)** The graph shows the densitometric analysis of the western blot for the cytoplasmic and nuclear NFκB2 p100/p52.

Figure 29: MDM2 overexpression elevates Bcl2 transcript levels. H460 cells nucleofected with MDM2 or the empty vector control were harvested for RNA. cDNA synthesized was used to determine the transcript levels by QPCR and are shown by bar graphs. Endogenous GAPDH control was used to ensure equal mRNA levels in each sample. The normalized transcript levels are shown. The assays were performed in triplicates. The error bars are shown.

Figure 30: Silencing MDM2 downregulates Bcl2 expression in H460 cells. cDNA from H460 cells infected with the lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene was measured for the MDM2 and Bcl2 transcript levels. The bar graphs show the transcript levels normalized by the endogenous GAPDH levels. The assays were performed in triplicates. The error bars are shown.

Figure 31: Silencing MDM2 downregulates Bcl2 expression in WI38 cells. Normal human lung fibroblasts, WI38 cells were infected with lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene as the control. cDNA prepared from the infected cells were measured for MDM2 and Bcl2 transcript levels. The bar graph on the left shows the decrease in the transcript levels of MDM2 on silencing with shMDM2

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and graph on the right shows the corresponding decrease in the Bcl2 transcript levels in WI38 cells. In (A) and (B) the bar graphs show the transcript levels normalized by the endogenous GAPDH levels. The assays were performed in triplicates. The error bars are shown.

Figure 32: Silencing MDM2 downregulates MDMX expression in H460 cells. cDNA from H460 cells infected with the lentivirus containing shRNA against MDM2 or the non endogenous luciferase gene was measured for the MDMX (above) and MDM2 (below) transcript levels with cells harvested at 48 and 72 hours. The line graphs show the transcript levels normalized by the endogenous GAPDH levels. The assays were performed in triplicates. The error bars are shown.

Figure 33: Proposed model for (A) p53 dependent and (B) p53 independent mechanism for MDM2 mediated upregulation of NFκB2 promoter. The models in Figure A show the p53 dependent mechanism. The figure suggests that p53 probably recruits co-repressors at the NFκB2 promoter, leading to promoter repression. In the presence of MDM2, p53 interacts with MDM2 and its transactivation function is inhibited. This leads to removal of p53 repression and activation of the promoter. The models shown in Figure B show the p53 independent mechanism. MDM2 interacts with many transcription factors including TBP. The model suggests that MDM2 may bind to TBP and recruiting it to the NFκB2 promoter or induce histone acetylation leading to enhanced transcriptional activity.

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Figure 34: The TATA Binding Protein (TBP) interaction domain of MDM2 is essential for MDM2 mediated upregulation of the NFκB2 promoter: The figure shows the TBP interaction domain of MDM2 that spans over amino acid residues 120-275.

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O. Experimental Design

Chapter 3: Methods.

Human lung cancer samples: The Tissue and Data Acquisition Core (TDAAC) laboratory at the Virginia Commonwealth University (VCU) acquires human residual lung tumor samples under a VCU IRB-approved protocol (IRB number 2471). Thirty human lung cancer specimens consisting of 21 adenocarcinomas and 9 squamous cell carcinomas were used in the present study. All tumors were classified according to standard histopathological criteria [125]. Histological evaluation of the frozen tumor tissues showed that on an average all specimens studied consisted of $68\pm15\%$ tumor cells. As control tissues for the expression studies adjacent non-neoplastic tissues were collected from three patients.

RNA extraction: RNA preparation from lung tumor samples was performed using a method described by Scian et al [81]. Total RNA was isolated from the tissues using TRIzol reagent (Life Technologies, Invitrogen) following a protocol supplied by the manufacturer, and was checked by 1.2% agarose Tris-borate-EDTA gel electrophoresis.

Generation of cDNA and QPCR: cDNA was synthesized using a Thermoscript reverse transcription- PCR system (Invitrogen) and amplification of the cDNA by PCR using sequence-specific primers. QPCR was conducted using a LightCycler system (Roche) as described previously [81]. Primers were designed using OLIGO 5 software (Molecular Biology Insights) and synthesized by Sigma Genosys. Reactions were performed in triplicate utilizing SYBR green dye, which exhibits a higher fluorescence upon binding of double-stranded DNA. The QPCR primers used were as follows: (a) NFκB2, 5'- GGG

GCA TCA AAC CTG AAG ATT TCT- 3' and 5'- TCC GGA ACA CAA TGG CAT ACT

GT -3'; (b) c-myc: 5'-GCCGCCGCCAAGCTCGTCTCAGAG-3' and 5'-

GCTGCTGGTGGTGGGCGGTGTCTC- 3'; (c) MDM2, 5'-

CCCAAGACAAAGAAGAGAGTGTGG- 3' and 5'-

CTGGGCAGGGCTTATTCCTTTTCT- 3'; (d) p21, 5'-

TTAGCAGCGGAACAAGGAGT -3' and 5'- AGCCGAGAGAAAACAGTCCA 3'; (e)

Bcl2, 5'- CAACATCGCCCTGTGGAT -3' and 5'- GCCAAACTGAGCAGAGTCTTC –

3'.

Determination of p53 status by DNA sequencing: We have sequenced the p53 gene in

tumor samples following the method described by Sjogren et al [126]. To identify p53

mutations, four sets of primers (Fragment 1: 5'- GACACGCTTCCCTGGATTGGC -3'

and 5'- GCAAAACATCTTGTTGAGGGCA -3', Fragment 2: 5'-

GTTTCCGTCTGGGCT TCTTGCA -3'and 5'- GGTACAGTCAGAGCCAACCTC -3',

Fragment 3: 5'- TGGCCCCTCCTCAGCATCTTA -3' and 5'-

CAAGGCCTCATTCAGCTCTC -3', Fragment 4: 5'-

CGGCGCACAGAGGAAGAGAATC 3' and 5'- CGCACACCTATTGCAAGCAAGGG -

3') were used to amplify four overlapping regions of p53 mRNA spanning the entire

reading frame. Reverse transcription and polymerase chain reaction (RT-PCR) was

performed using the method described above. The amplified fragments were analyzed by

agarose gel electrophoresis and sequenced.

Statistical analysis: Relationship between the MDM2 expression (independent variable)

and NFκB2 p100, Bcl2, c-myc, MDMX, Delta Np53 and p21 expression levels (dependent

variables) were determined using ranked spearman's correlation. The relationships were also examined by grouping the lung cancer samples into WT and mutant p53 harboring tumor samples.

Chapter 4: Methods.

Cells: H460 and WI38cells were purchased from American Type Culture Collection and were maintained in media suggested by the supplier. H460 cells were maintained in RPMI with 10% fetal bovine serum. W138 cells were maintained in Minimum Essential Media (with Earle's salts) with 10% fetal bovine serum.

Plasmids and MDM2 deletion mutants: Construction of plasmids expressing full-length MDM2 and the deletion mutants has previously been described in detail [103, 127]. Construction of NFκB2 promoter has been described earlier [81]. The sequences present in the independent P1and P2 promoter construct of NFκB2 has been shown by a schematic representation in Figure 23.

Transient transfections: For transfection of expression plasmids (vector control, MDM2), H460 cells were seeded 48 hours before transfection and 3×10^6 cells were used per transfection with the Nucleofector and kit reagents (Amaxa) following supplier's protocol. The NFκB2 siRNA was delivered by nucleofection using a nucleofector as described above. The short interfering (si) RNA directed against human NFκB2 (5'-gacaaggaagaggugcagctt- 3' and 5'- gcugcaccucuuccuuguctt -3') and control RNA (5'-caugucaugugucacaucuctt -3' and 5'- gagaugugacacaugacaugtt – 3') was designed using a program suggested by Qiagen Inc, and was purchased from Proligo.

Western Blot Analysis: Cells were washed with cold phosphate buffered saline (PBS) and harvested in a cell lysis buffer (Promega) containing 50 mM NaF, 0.1M phenylmethylsulfonyl fluoride, Na pyrophosphate, Na orthovanadate and protease inhibitors (Calbiochem protease inhibitor Cocktail I consisting of 500 mM AEBSF, Hydrochloride; 150 nM Aprotinin; 1mM E-64 Protease inhibitor; 0.5 mM EDTA, Disodium; 1 mM Leupeptin, Hemisulphate) and transferred to a microfuge tube. Cells were then spun at 900 rcf for 10 minutes. The lysates were analyzed for protein concentrations; laemmli loading dye was added to equal amounts of the lysate and then boiled for five minutes. Cells were separated in a 10% polyacrylamide gel and transferred to 0.45 pm nitrocellulose membrane. The membranes were treated with the antibodies of interest and were developed using ECL purchased from Amersham.

Antibodies: Antibody against MDM2 (Ab-1) (purchased from Calbiochem, San Diego, CA) was used in 1:200 dilution. NFκB2 antibody (purchased from Upstate) was used in a 1:5000 dilution and β-actin antibody (A-5441, purchased from Sigma, St. Louis, MO) was used in a 1:200 dilution. Mouse secondary antibody was used for all the antibodies in a 1:10000 dilution.

Transient transcription assays: MDM2-mediated upregulation of the NFκB2 promoter was determined by transient transcription assays. The sequences present in the promoter plasmids have been shown in Figure 23. H460 cells were cotransfected with 200 ng of the reporter plasmid containing the NFκB2 promoter upstream of the luciferase gene, 200 ng of the beta galactosidase plasmid, pCMV β gal (for normalizing transfection efficiency) and 2μg of MDM2 expression plasmid or the deletion mutants of MDM2 (or

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vector plasmid) using LipofectAMINE 2000 as per supplier's recommendations. H460 cells were plated 24 hours before transfection and were harvested 30 hours post lipofection. Cell lysates were prepared using reporter lysis buffer (Promega). Cell extracts containing equal amounts of protein were assayed for luciferase and beta galactosidase activity as described earlier [81]. Luciferase activities were normalized to the beta galactosidase activity.

Lentiviral transfection: Human Embryonic Kidney 293 cells are a cell line derived from human embryonic kidney cells grown in tissue culture. 293T is a variant of the cell line that contains in addition the SV40 large T-antigen. These cells are grown in RPMI and 10% hyclone FBS. 293T cells are split for 60 percent confluency. The next day plates are replaced with fresh media 6-7 hours prior transfection. A calcium chloride transfection is done with Hepes Buffered Saline (HBS) (Hepes 1g, NaCl 1.6 g, 0.25 M Na_2HPO_4 0.72 ml, 1M KCl and make up volume to 100 ml, pH 7.12) and 2M $CaCl_2$. The plasmid DNA used for the transfection are the plasmids of interest-shMDM2 DNA or shluciferase DNA (from Open biosystems), the envelope glycoprotein vesicular stomatitis virus (VSV-G) and the packaging plasmid Delta 8.7. A cocktail is made that consists of plasmid of interest (10ug), VSVG (6ug), Delta 8.7 (10ug), 2M $CaCl_2$ and sterile water. The cocktail is added to pre-warm HBS by vortexing. The mixture is added to the plates and incubated at 37°C for 48 hours. The supernatant containing the virus is collected after 48 hours.

Chapter 5: Methods.

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Cell Growth Assay: Cells were seeded at a density of 10^4 cells per 60-mm plate 48 hours after transfection of NFκB2 siRNA or control RNA. For the growth assay after MDM2 silencing, the cells were infected with lentivirus for 48 hours and then counted and plated for the assay. Triplicate sets of plates were harvested at 24-hour intervals for five consecutive days and counted using Coulter counter.

Drug sensitivity assays: H460 cells were infected with lentivirus expressing shMDM2 or short hairpin against non-endogenous luciferase as the control. 48 hours after infection cells were plated at equal densities and treated with final concentrations of 6 μM etoposide or 25 nM Paclitaxel (Sigma) or equal volumes of dimethyl sulfoxide (DMSO vehicle) for 48 hours. For the colony formation assay, cells were plated at a density of 10^4 cells for treatment with etoposide/paclitaxel and 10^3 cells for DMSO treatment per 10-cm dish. Drug/DMSO was removed from the plates after 48 hours and was replaced with new complete media. Media was changed every five days allowing colony formation for a period of two to three weeks. On observing detectable colonies the media was removed, plates were washed with 1X Phosphate buffered saline (PBS), fixed with ice cold methanol for 20 minutes followed by methylene blue staining. DMSO treated plates were assessed for plating efficiency and effects of DMSO on cell growth.

Cytoplasmic and nuclear fractionation: H460 cells were nucleofected with empty vector plasmid and the full length MDM2 plasmid with Amaxa nucleofector and harvested after 18 hours. Cells were washed once with PBS and lysed in hypotonic buffer (20 mM Hepes-KOH [pH 8.0], 5 mM KCl, 1.5 mM MgCl2, 5 mM Na butyrate, 0.1 mM dithiothreitol [DTT]). Nuclei were collected by centrifugation (10 min, 14,000g, 4°C) and resuspended

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in nuclear extraction buffer (15 mM Tris-HCl [pH 7.5], 1 mM EDTA, 0.4MNaCl, 10% sucrose, 1 mM DTT). After 30 min on ice, insoluble proteins were removed from the nuclear extract by high-speed centrifugation (40 min, 14,000g, 4°C). The cytoplasmic and the nuclear fractions were quantified and equal concentrations of the lysate were run on a SDS PAGE gel and treated with antibodies against MDM2, NFκB2 p100/p52 and Sp1 (nuclear marker).

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