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Brittany Lynne Cross
University of South Florida, brittany.cross@moffitt.org

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Inhibition of p53 DNA Binding Function by the MDM2 Acidic Domain

by

Brittany Lynne Cross

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cancer Biology
College of Arts and Sciences
University of South Florida

Major Professor: Jiandong Chen, Ph.D.
Kenneth L. Wright, Ph.D.
Alvaro Monteiro, Ph.D.
Jia Fang, Ph.D.

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DEDICATION

  I would like to dedicate this dissertation to all of my family and friends who have supported me through graduate school. I am especially grateful to my husband for his patience and support throughout this process, along with his constant ability to keep me grounded and extremely happy. Also, to my parents and sisters who have been a source of encouragement and amusement. Lastly, my Grandma and Grandpa Woodruff who lost there brave battle with cancer and are the reason I am where I am today.

ACKNOWLEDGMENTS

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LIST OF ABBREVIATIONS

Ab	antibody
ARF	alternative reading frame
ATM	atazia-telangiectasia mutated
CDK	cyclin-dependent kinase
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
DNA	deoxyribose nucleic acid
FBS	fetal bovine serum
GFP	green florescent protein
HAUSP	herpesvirus-associated ubiquitin-specific protease
HDAC	histone deacetylase
HIF-1	hypoxia inducible transcription factor
HPV	human papilloma virus
hTERT	human telomerase catalytic subunit
IP	immunoprecipitation
IR	ionizing radiation
LOH	loss of heterozygosity
MDM2	mouse double minutes 2
MDMX	mouse double minutes x
MEF	mouse embryo fibroblast
μl	microliter
μM	micromolar
ml	milliliter
mM	millimolar
NLS	nuclear localization signal
NES	nuclear export signal
NoLS	nucleolus localization signal
PBS	phosphate buffer saline
PCR	polymerase chain reaction
Rb	retinoblastoma protein
RNA	ribonucleic acid
SV40	simian virus
Ub	ubiquitin
UV	ultraviolet light
WCE	whole cell extract

ABSTRACT

MDM2 regulates p53 predominantly by promoting p53 ubiquitination. However, ubiquitination-independent mechanisms of MDM2 have also been implicated. Here we show that MDM2 inhibits p53 DNA binding activity *in vitro* and *in vivo*. MDM2 binding promotes p53 to adopt a mutant-like conformation, losing reactivity to antibody Pab1620, while exposing the Pab240 epitope. The acidic domain of MDM2 is required to induce p53 conformational change and inhibit p53 DNA binding. ARF binding to the MDM2 acidic domain restores p53 wild type conformation and rescues DNA binding activity. Furthermore, histone methyl transferase SUV39H1 binding to the MDM2 acidic domain also restores p53 wild type conformation and allows p53-MDM2-SUV39H1 complex to bind DNA. These results provide further evidence for an ubiquitination-independent mechanism of p53 regulation by MDM2, and reveal how MDM2-interacting repressors gain access to p53 target promoters and repress transcription. Furthermore, we show that the MDM2 inhibitor Nutlin cooperates with the proteasome inhibitor Bortezomib by stimulating p53 DNA binding and transcriptional activity, providing a rationale for combination therapy using proteasome and MDM2 inhibitors.

INTRODUCTION

Carcinogenesis

Carcinogenesis is a multi-step process by which normal cells are transformed into cancer cells, ultimately reprogramming a cell to undergo uncontrolled cell division. Under normal cellular conditions there is a balance between proliferation and programmed cell death that is tightly regulated to ensure cellular integrity. Both genetic and epigenetic DNA alterations that lead to cancer disrupt this balance, resulting in not only uncontrolled cellular division, but also genetic instability (Cahill et al., 1999). More than one mutation is necessary for carcinogenesis, and this genetic instability allows for successive accumulation of mutations in certain classes of genes. This series of mutations is the requirement for a normal cell to transform into a cancer cell (Fearon and Vogelstein, 1990).

Genetic instability is defined as a set of events that are capable of causing unscheduled DNA alterations within the genome. These genetic changes can occur at multiple levels, anywhere from gaining or losing an entire chromosome to mutating a single oligonucleotide in a particular gene. DNA mutations at a single nucleotide level include point mutations, deletions or insertions. These small changes have the potential to affect the expression of the gene, or alter the function or stability of the protein product. Large-scale mutations will occur at the chromosomal level, which results in the loss or gain of portions (or entire) chromosomes. Chromosomal instability can involve genomic amplification, causing a cell to gain multiple copies of one or more genes, or translocations, which occur when two or more separate chromosome regions become fused at uncharacteristic locations (Lengauer et al., 1998).

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Epigenetic alterations involve modifications in gene expression from mechanisms other than changes in the DNA sequence itself (Bird, 2007). These modifications occur through DNA methylation, histone modifications and gene imprinting (Feinberg and Tycko, 2004). Epigenetic changes occur naturally in eukaryotic biology during the process of cellular differentiation, creating multiple different cell types that will have activated some genes, yet inhibited others (Reik, 2007). In some instances the DNA modifying events may lead to the activation of oncogenes or inactivation of tumor suppressor genes, leading to cancer. A variety of compounds or environmental factors are also associated with epigenetic alterations and result in increased incidence of tumors. These include environmental factors such as UV and ionizing radiation, tobacco smoke or even viral infections.

Following tumor initiation via the culmination of mutations that deregulate genes necessary to control homeostasis and the selective clonal expansion of these unstable, abnormal cells there is tumor progression. This process results in a group of malignant cells that display the following biological properties: unchecked cellular growth (self-sufficiency in growth signals and loss of sensitivity to anti-grown signals), evasion from cell death, loss of senescence leading to limitless replicative potential, and the ability to invade and build metastases in other tissues (Hanahan and Weinberg, 2000). Many of the genes that lead to these malignant phenotypes are involved in cell cycle control, DNA damage responses and DNA repair, making discovery and characterization of these genes critical for development of cancer therapies. One such example is the "Guardian of the genome", or the tumor suppressor gene, TP53 (Lane, 1992) and oncogenes MDM2 and MDMX.

Tumor Suppressor and Oncogenes

Tumor suppressor genes code for proteins that suppress cell growth, protecting a normal cell from the path to cancer. Tumor suppressors have several functions, including repression of cell cycle progressing genes, coupling the cell cycle to DNA damage and initiating apoptosis if the cell is unable to repair the damage (Croce, 2008). When a tumor suppressor gene is mutated in

a way that causes it to lose or reduce its function, the cell has an increased probability of tumor formation, combined with other genetic changes. Tumor suppressor genes generally follow the Knudson two-hit hypothesis, first proposed by A.G. Knudson for cases of retinoblastoma. It implies that both alleles that code for a particular gene must be affected before an effect is manifested (Knudson, 1971). Therefore, mutated tumor suppressors are recessive, in that if only one allele is damaged, the second can still produce the correct protein. These observations propose that the development of cancer via mutations in tumor suppressors involves at least two mutational events. There are exceptions to this "two-hit" rule for tumor suppressors. These involve tumor suppressors with mutations that exhibit haploinsufficiency, where the mutation of a single allele can cause an increase in carcinogen susceptibility (Fero et al., 1998). Another exception would be genes that have a "dominate negative" function, meaning that the mutated protein can prevent the function of the normal protein from the un-mutated allele (Baker et al., 1990). Certain individuals with mutations in the tumor suppressor p53 gene product function with this dominate negative effect.

Unlike tumor suppressor genes, oncogenes encode for proteins that promote cell growth through a variety of ways and they also have the potential to cause cancer. Proto-oncogene functions include proteins that help regulate cell growth and differentiation and are involved in signal transduction and execution of mitogenic signals. Following activating mutations, the proto-oncogene then becomes an oncogene (Todd and Wong, 1999). There are three main activation mutation types. First, a mutation that causes a change in the protein structure, leading to an increase in enzyme activity or loss of regulation. Second, an increase in protein concentration caused by an increase in expression, mRNA stability or gene duplication. Third, a chromosomal translocation that can cause an increase in gene expression in the wrong cell or at the wrong time or expression of a constitutively active hybrid protein (Croce, 2008). Normally these genes are critical for growth, repair and homeostasis, it is only when they become mutated that growth signals become excessive. Under this condition

oncogenes can also inactivate specific tumor suppressor genes, as is the case with MDM2 and MDMX regulating p53 activity.

Tumor Suppressor p53

P53 is a tumor suppressor protein that, in humans, is encoded by the *TP53* gene located on the short arm of chromosome 17 (17p13.1) (Isobe et al., 1986; Kern et al., 1991; Matlashewski et al., 1984; McBride et al., 1986). The p53 protein belongs to a family of transcription factors that include p53, p63 and p73. All three are required for normal development, yet only p53 seems to prevent tumor formation (Yang and McKeon, 2000). It does this by inducing cell-cycle arrest, DNA repair or apoptosis in response to various types of damage (Levine, 1997; Levine et al., 2004; Vogelstein et al., 2000). Consequently, mutations or deletions in the p53 gene occur in over 50% of all human cancers (Soussi et al., 2006).

P53 History

P53 was identified in 1979 by two groups as a 55-kDa protein that co-precipitated with the large-T antigen of the simian virus (SV40) and accumulated in the nuclei of cancer cells (Lane and Crawford, 1979; Linzer and Levine, 1979). It was initially presumed to be an oncogene due to these observations and the use of cDNA containing missense mutated forms of p53, not the wild type, that acquired gain of function activities. These mutated forms can stabilize p53 due to its increased interaction with HSP90, which inhibits ubquitination by MDM2, and can suppress the activity of wild-type p53 by binding and forming inactive tetramers (Peng et al., 2001; Sigal and Rotter, 2000). This idea was further supported by the fact that p53 was shown to cooperate with the activated Ha-Ras oncogene by immortalizing normal embryonic cells, leading to cells sensitive to ras transformation (Eliyahu et al., 1984; Parada et al., 1984). In 1989, the Arnold Levine lab was the first to characterized p53 as a protein that suppresses cellular transformation (Finlay et al., 1989). This was further established in studies showing p53 was deleted in human colorectal cancers and that DNA viruses, such as HPV and the adenovirus E1B 55K, could inactivate p53 (Baker et al., 1990; Scheffner et al., 1990; Zantema et al., 1985). In 1992 a consensus

sequence to which human p53 could bind was discovered, defining p53 as a transcription factor (el-Deiry et al., 1992). The transcription factor, p53, is now widely recognized as a tumor suppressor that responds to a wide variety of stress signals and is thought to be the rate-limiting factor in specific steps of tumor development.

P53 Structure and Localization

P53 is a polypeptide chain of 393 amino acids, consisting of five major domains: a transactivation domain, a proline rich region, a sequence specific DNA binding domain, a tetramerization domain and a regulatory domain (**Figure 1**). The N-terminus transactivation domain (TAD, residues 1- 45) can facilitate transcription activity and regulate p53 function by interacting with transcription coactivators such as p300/CBP and p21 (Joerger and Fersht, 2008). This region is also involved in negative regulation of p53 by interacting with one of its negative regulators, MDM2 (Lin et al., 1994). The proline rich domain (PxxP, residues 61-94) is reportedly necessary for p53 induced cell cycle arrest and apoptosis (Sakamuro et al., 1997). The DNA binding domain (Core domain, residues 102-292) is required for sequence specific DNA binding and is basic in charge (Wang et al., 1996; Wang et al., 1995). The most common consensus DNA binding sequence consists of two tandem repeats separated by a 0-13 bp spacer (el-Deiry et al., 1992). The p53 core domain has poor thermo stability at physiological temperatures and can undergo spontaneous denaturation (Bullock et al., 1997; Canadillas et al., 2006; Hansen et al., 1996). Changes in p53 conformation can inhibit the ability of p53 to induce expression of its downstream transcriptional targets (Olivier et al., 2002; Soussi et al., 2000). The tetramerization domain (TD, residues 324-355) resides near the C-terminus and is required for the efficient oligomerization of p53 tetramers through hydrophobic interactions. The p53 regulatory domain (Carboxyl terminus, residues 311-393) is located at the extreme C-terminus and is rich in lysines and arginines, subjecting this domain to multiple post-translational modifications that are important for p53 regulation (Appella and Anderson, 2000; Brooks and Gu, 2003).
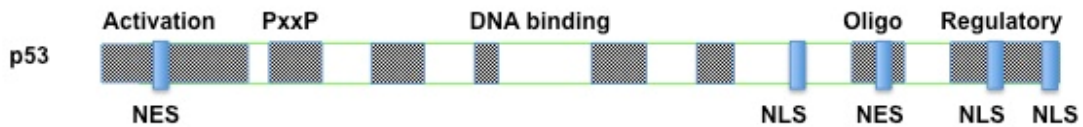
Figure 1: p53 Structure. P53 is a polypeptide chain consisting of five major domains: a transactivation domain, a proline rich region, a sequence specific DNA binding domain, a tetramerization domain and a regulatory domain. P53 also contains multiple nuclear export signals and nuclear localization signals.

Also located within the carboxyl terminus are nuclear localization signals (NLS), and there is a nuclear export sequence located in the oligomerization domain and activation domain (Cho et al., 1994; Dang and Lee, 1989; Hainaut et al., 1997). Working mainly as a transcription factor, p53 nuclear import is essential for normal function as a tumor suppressor. Therefore, there are multiple cellular mechanisms that exist to regulate the nuclear import and export of p53 (Knippschild et al., 1996; Moll et al., 1996; Ryan et al., 1994). Regulation of nuclear localization occurs either by direct modifications of the NLS or NES sites or interaction with other proteins that interfere with NLS or NES accessibility. For example, phosphorylation by insulin-like growth factor I, IGFI, can induce elevated levels of phosphorylated p53 resulting in high cytoplasmic levels in human breast cancer cells (Takahashi et al., 1993). MDM2 can regulate p53 localization both by protein-protein interaction and posttranslational modifications. MDM2 protein interaction itself mediates nuclear export and degradation of p53 by shuttling it to the cytoplasm, while ubiquitination of p53 in the nucleus unmasks the p53 NES (Inoue et al., 2005; Roth et al., 1998; Tao and Levine, 1999). It is important to note that there are conflicting studies reporting that nuclear export of p53 is a MDM2 independent event, yet knockdown of MDM2 results in nuclear import and retention, and mutations of the lysine residues ubiquitinated by MDM2 abrogate nuclear export (Nakamura et al., 2000; Stommel et al., 1999). It has been suggested that tumorigenesis could result from a defect in the regulation of p53 nuclear import and some studies have

shown that wild-type p53 is abnormally sequestered in the cytoplasm in a subset of human tumor cells (Bosari et al., 1995; Liang and Clarke, 2001; Moll et al., 1995; Moll et al., 1992).

P53 Function

P53 plays a role in a variety of cell signaling mechanisms by both transcription-dependent and independent mechanisms, which lead to cell cycle arrest, DNA repair, apoptosis-mediated cell death, inhibition of angiogenesis, differentiation and cellular senescence (**Figure 2**). The p53 protein is normally maintained at low levels in unstressed cells with a short half-life (Shu et al., 2007). Once cells encounter stress, post-translational modifications occur, leading to an increase in p53 levels due to stabilization (Lakin and Jackson, 1999; Saito et al., 2003). Once transcriptionally activated, the p53 protein binds to DNA as a tetramer and stimulates the expression of downstream genes, which facilitates the repair and survival of damaged cells or eliminates severely damaged cells through apoptosis (Ko and Prives, 1996; Levine, 1997; Vogelstein et al., 2000). Occasionally, p53 can act as a transcriptional repressor through interactions with basal transcription machinery, co-repressors or other DNA binding proteins (Chen et al., 2010; Seto et al., 1992).
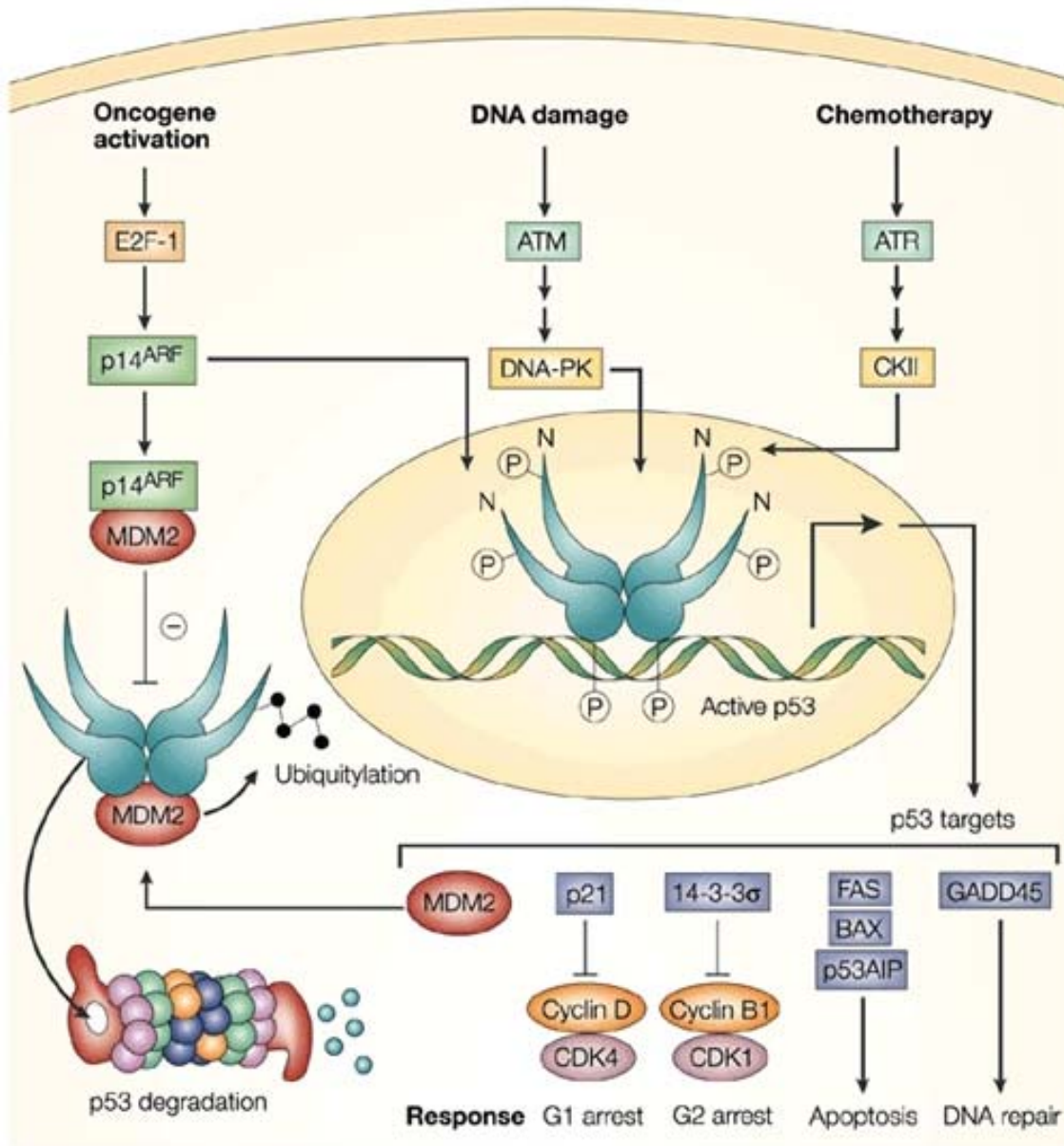
Figure 2: p53 Function. P53 plays a critical role in a variety of cell signaling mechanisms (Bullock and Fersht, 2001).

Cell Cycle Arrest

The cell cycle is a series of events that lead a cell to divide and duplicate. Regulating the cell cycle is a critical step to ensure that genetic material is correctly passed onto daughter cells, as well as for the detection and repair of

genetic damage and prevention of uncontrolled cellular division. Disregulation of any number of the cell cycle components can lead to tumor formation. There are four distinct phases to the cell cycle: G1-phase, S-phase, G2-phase and M-phase. Each phase has checkpoints that monitor and regulate the progress of the cell cycle and prevent cell cycle progression until all necessary phase processes and repair of DNA damage occur (Elledge, 1996). Two classes of regulatory molecules, cyclins and cyclin-dependent kinases (CDKs), along with CDK inhibitors, control cell cycle progression (Nigg, 1995). In order for the cell to move from G1 to S phase, DNA must be intact. Upon DNA damage recognition, the G1 checkpoint will stop the progression into S phase, giving the cell time to repair the damage. Following DNA damage activated p53 binds DNA and activates expression of several cell cycle inhibiting genes, including p21^{WAF1}. P21 binds to the G1-S/CDK and S/CDK complexes, inhibiting their activity, and preventing the cell from continuing on to the next stage of cell division (el-Deiry et al., 1993). When p53 is unable to bind to the DNA properly, the p21 protein will not be available at high enough levels to act as a stop signal for the cell cycle, leading to uncontrollable division and tumor formation.

Another transcriptional target of p53 that is involved in cell cycle arrest is the 14-3-3σ gene (Hermeking et al., 1997). The 14-3-3σ protein functions at both the G1/S and G2/M transitions and regulates cellular activity by binding and sequestering phosphorylated proteins. Following p53 induction, 14-3-3σ binds and sequesters CDC2 and Cdc25 into the cytoplasm, which are essential for transition into mitosis, causing cell cycle arrest (Chan et al., 1999; Lopez-Girona et al., 1999). The 14-3-3σ protein can also delay apoptotic signaling, resulting in G2 arrest, by promoting the translocation of Bax out of the cytoplasm (Samuel et al., 2001).

Apoptosis-Intrinsic and Extrinsic

Apoptosis is the process of programmed cell death involving biochemical events that lead to changes in cell morphology and eventually death. Apoptosis can be initiated by two distinct pathways: the intrinsic and extrinsic pathways. P53 plays a distinct role in both of these pathways. The intrinsic pathway is

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initiated from within the cell in response to cellular signals resulting from DNA damage and involves the release of pro-apoptotic proteins that activate caspase enzymes from the mitochondria (Coultas and Strasser, 2003; Fulda and Debatin, 2006; Thornberry and Lazebnik, 1998). This pathway involves a balance between the pro-apoptotic (Bax, Bad, Bid) and anti-apoptotic (Bcl-2, Bcl-XL) members of the Bcl-2 family of proteins, which ultimately regulate the permeability of the mitochondrial membrane and release of cytochrome C (Coultas and Strasser, 2003; Green and Evan, 2002). There are also BH3 domain-only proteins, such as PUMA and Bim. Several of the Bcl-2 proteins are transactivated by p53, the first one being Bax (Miyashita and Reed, 1995). Upon initiation of apoptotic signaling, p53 transcribed Bax undergoes a conformation shift, homodimerizes and inserts into the outer mitochondrial membrane, creating pores and promoting the release of cytochrome C (Adams and Cory, 2001; Wolter et al., 1997). P53 can also induce PUMA, Noxa and Bid (Moroni et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000a; Walensky et al., 2006). PUMA and Noxa also promote mitochondrial depolarization via homo- or heterodimerization, leading to cytochrome C release. BID interacts with Bax, leading to the insertion of Bax into the mitochondrial membrane. Recently it was shown that following extracellular stress signals, p53 can also translocate to the mitochondria, bind to Bcl-2 and MDMX, creating mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome C (Mancini et al., 2009).

The extrinsic pathway begins outside of the cell through activation of specific pro-apoptotic receptors on the cell surface, which are activated by molecules known as pro-apoptotic ligands (Fulda and Debatin, 2006). This leads to the induction of caspase 8 and 3 activities. An example of this would be the binding of the Fas ligand to the Fas receptor. P53 can induce the transcription of Fas following DNA damage, resulting in the formation of death-receptor-inducing-signaling-complexes (DISCs). This leads to the activation of effector caspases 8 and 3 (Bouvard et al., 2000; Muller et al., 1998). P53 can also induce the transcription of the death-domain-containing receptor DR5 in response to DNA damage (Wu et al., 1997).

DNA Repair

DNA repair refers to the multiple processes by which a cell identifies and corrects damage to the DNA that encodes its genome. P53 has been linked to both the base excision repair (BER), nucleotide excision repair (NER) and correction of double stranded breaks (Gatz and Wiesmuller, 2006). P53 induces transcription of GADD45, a protein that promotes NER mechanisms following gamma irradiation (Smith et al., 2000). Following DNA damage, p53 induction can also transcribe p53R2, a ribonucleotide reductase that is important for repair, and lead to an increase in 3-methyladenine (3-MeAde), an enzyme required for BER (Guittet et al., 2001; Zurer et al., 2004). When these normal repair processes fail to occur and cellar apoptosis also does not occur, irreparable DNA damage accumulates which can lead to tumor formation.

Senescence

Cellular senescence is the phenomenon by which normal diploid cells lose the ability to divide. Normally this occurs after a set number of cellular divisions in response to DNA damage (this includes telomere shortening) and the cells either age or self-destruct via apoptosis due to damage that cannot be repaired. This is called the "Hayflick phenomenon" or "Hayflick limit" (Hayflick, 1965). Early on, senescence was found to be mediated by two major tumor suppressor pathways, p53 and Rb (Gil and Peters, 2006; Kim and Sharpless, 2006) In fact, inactivating these proteins in mouse embryonic fibroblasts is sufficient to evade cellular senescence (Dirac and Bernards, 2003). P53 induces transcription of the p21 protein, leading to the activation of the Rb pathway and triggering senescence following DNA damage and telomere uncapping (Herbig et al., 2004). INK4a/ARF, a critical positive regulator of p53 stability, has been shown to have increasing mRNA and protein levels as tissues age, leading to increased p53 transcriptional activity (Herbig et al., 2004). Telomere shortening can be prevented by overexpression of the hTERT protein and has been shown to be downregulated when p53 is activated. Overexpression of hTERT can actually overcome p53-induced apoptotsis (Xu et al., 2000).

Metabolism

Metabolism is a set of chemical reactions that happen in living organisms necessary to maintain life and allow said organism to grow, reproduce, maintain their structure and respond to their environment. Metabolic alterations are common features of cancer cells and have recently been shown to have an important role in the maintenance of malignancies (Kroemer and Pouyssegur, 2008). Like any other stress signal, the metabolic responses to limited nutrients, energy or oxygen availability stimulate p53 activity. For example, reduced nutrients result in the failure to stimulate the MDM2 activator, AKT, leading to increased p53 stability by removing the negative regulation normally imposed by MDM2 (Mayo et al., 2002; Mayo and Donner, 2002). Under low oxidative stress conditions, p53 can transcribe TIGAR (TP-53 induced glycolysis and apoptosis regulator), which results in an inhibition of glycolysis and a decrease in overall reactive oxygen species (ROS) levels. TIGAR can then protect cells from ROS induced apoptosis and allow for repair and survival of the cells. Knockdown of TIGAR expression has been shown to sensitize cells to p53-induced death (Bensaad et al., 2006). Under acute oxidative stress, p53 induces pro-apoptotic genes, including Bax and PUMA, leading to the removal of the damaged cells (Liu et al., 2005). P53 can also activate AMPK, a protein that drives energy producing responses under conditions of metabolic stress, and negatively regulates mTOR, a protein that promotes protein synthesis and suppresses induction of autophagy (Feng et al., 2005; Hardie, 2007). A disruption in balance of these processes is associated with mutations in p53 and oncogenic transformation (Vousden and Ryan, 2009).

Angiogenesis and Metastasis

Angiogenesis is the physiological process involving the growth of new blood vessels from pre-existing ones and is the fundamental step in the transition of tumors to a malignant state (Weidner et al., 1991). It can by triggered by activation of Hypoxia-inducible factors (HIFS), which are transcription factors that respond to changes in available oxygen in the cellular environment (Smith et al., 2008). HIF-1 can induce the expression of VEGF (vascular endothelial growth

factor), which is necessary for the formation of new blood vessels (Dachs and Tozer, 2000). P53 can mediate the MDM2-dependent proteosomal degradation of the alpha subunit of HIF-1, therefore downregulating VEGF expression (Ravi et al., 2000). P53 can also upregulate a number of anti-angiogenic and metastasis suppressor proteins, such as Tsp-1 and Nm23-H1 (Bouvet et al., 1998). Moreover, a number of matrix metalloproteinases, such as MMP-1 and MMP-13, that promote tissue invasion by causing extra cellular matrix degradation are repressed by activated p53. (Sun et al., 2000; Sun et al., 1999). Therefore, loss of p53 activity could lead to uncontrolled blood vessel growth and eventually metastasis of tumor cells.

Transcriptional Repression

In addition to serving as a DNA binding transcriptional activator, p53 has been reported to play a role in repression at a variety of promoters. As previously mentioned, p53 can negatively regulate the hTERT gene by interfering with the coactivator sp-1 binding at its promoter, contributing to the induction of cellular senescence (Xu et al., 2000). P53 can also mediate cell cycle arrest by not only upregulating p21 and 14-3-3σ, but also by repressing cyclin B and cdc2 (Krause et al., 2000). P53 transcriptional repression can also influence apoptotic activity by downregulating Survivin, a putative caspase inhibitor (Hoffman et al., 2002). P53 can actually compete with other transcription factors for binding to certain target promoters, such as AFP (alpha-fetoprotein gene), which results in the overlapping of DNA binding for p53 and HNF-3. This leads to displacement of HNF-3 and a reduction of AFP transcription (Lee et al., 1999).

Transcriptional repression also seems to be partially due to the association of p53 with basal transcription machinery. For example, p53 has been shown to interact with TATA-box binding protein (TBP) and TBP-associated factors, which leads to p53 repressed basal transcription via disruption of the pre-initiation complex assembly (Seto et al., 1992). However, recent reports have suggested that the p53-TBO interaction is not sufficient for transcriptional repression by p53 and that it involves the interaction of other factors called TAFs (TATA box binding *protein* associated factor) (Farmer et al., 1996).

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P53 can also alter the chromatin structure by recruiting histone deactylases and methyltransferases. P53 has been shown to physically interact with HDACS in vivo and even decrease histone acetylation at certain promoters, such as Survivin (Murphy et al., 1999). Histone methyltransferases SUV39H1 and EHMT1 bind specifically to MDM2 and, following the formation of a SUV39H1/EHMT1-MDM2-p53 complex, increase H3 K9 methylation at p53 target promoters and cause a decrease in p53 mediated transcription (Chen et al., 2010). MDM2 also interacts with several other transcription repressors, including YY1 and KAP1, and it is presumed that under certain conditions these interactions can actively repress basal activity of p53 target genes by recruiting these corepressors to p53 target promoters (Sui et al., 2004; Wang et al., 2005).

Regulation of p53

The p53 protein is normally maintained at low levels in unstressed cells with a short half-life to prevent unnecessary cell death (Shu et al., 2007). Once cells encounter stress, post-translational modifications occur, leading to an increase in p53 levels due to stabilization (Lakin and Jackson, 1999; Saito et al., 2003). This accumulation, followed by p53 activation, results in the transcription of genes involved in multiple cellular processes necessary to prevent the accumulation of errors in the genome and the outgrowth of cells with malignant potential (Ko and Prives, 1996; Levine, 1997). Increases in rates of transcription and translation may affect the cellular level of p53, but post-translational modifications, such as ubiquitination, phosphorylation acetylation, methylation, sumolyation and neddylation are the most efficient ways to increase p53 stability and activity (Appella and Anderson, 2000). The fact that p53 is highly regulated at the post-translational level greatly underscores its importance in maintaining cellular homeostasis.
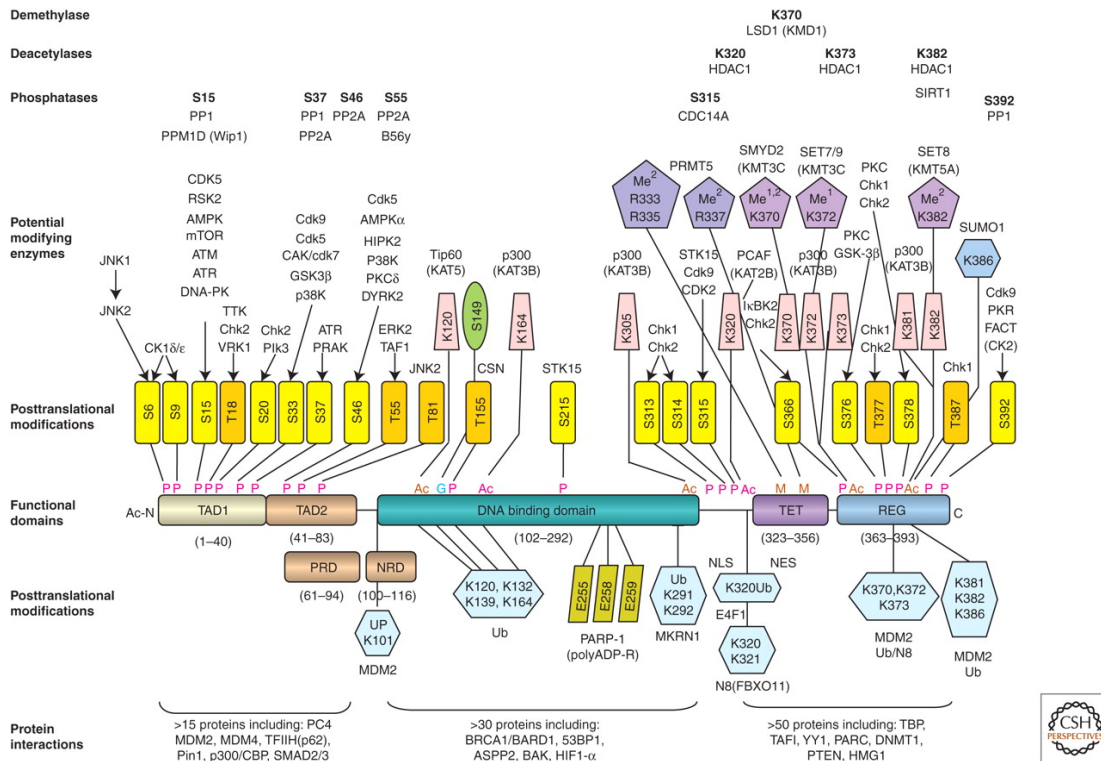
Figure 3: p53 Posttranslational Modifications. (Meek and Anderson, 2009).

P53 Ubiquitination

P53 was first shown to be ubiquitinated by cellular factors associated with viral E6 protein in papilloma virus infected cells (Scheffner et al., 1993). Later it was determined that p53 is ubiquitinated and degraded mainly through the proteosomal pathway (Chowdary et al., 1994). Ubiquitin begins with the formation of the thioester bond between a cysteine residue of an E1 ubiquitin-activating enzyme and the terminal glycine residue of ubiquitin. Next, the ubiquitin is transferred to a ubiquitin-conjugating enzyme E2, which links the ubiquitin molecular to the ε-amino group of a lysine residue on the target protein. Once activated E2 is able to ubiquitinate a substrate but needs the targeting function provided by E3 ligases. These E3 ligases are divided into two classes, depending on whether they contain a HECT domain or a RING domain (Pickart and Eddins, 2004). Once the polyubiquitin chains are attached to the target

protein, the protein is then subjected to 26S proteosomal degradation (Glickman and Ciechanover, 2002)

In normal cells the MDM2 E3 ligase has been shown to bind p53 and direct degradation via ubiquitin-dependent proteolysis (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). In the absence of stress signals the p53 protein is kept at low levels in order to allow normal cell proliferation and maintain cell viability. Therefore, p53 assists in its own gene regulation by driving the gene expression of MDM2, creating a negative feedback loop (Wu et al., 1993). (Haupt et al., 1997)Ubiquitination by MDM2 has actually been shown to catalyze either monoubquitination or polyubiquitination of p53 in a dose-dependent manner (Li et al., 2003). Low levels of MDM2 induce monoubquitination and nuclear export of p53, in contrast to high levels of oligomerized MDM2, which promote polyubiquitination and nuclear degradation of p53 by providing a recognition signal for the 26S proteosome (Cheng et al., 2009). It is likely that these distinct mechanisms are exploited under different physiological settings.

In addition to MDM2, other E3 ligases can mediate the ubiquitination and degradation of p53, including Pirh2 and COP1. Both these genes are also p53 inducible, thus, similar to MDM2, create a negative feedback loop (Barak et al., 1993; Dornan et al., 2004; Leng et al., 2003). Pirh2, a RING domain-H2 containing, interacts with p53 and promotes MDM2-independent p53 ubiquitination and degradation (Leng et al., 2003). COP1, also an E3 ligase, promotes MDM2-independent p53 ubiquitination and degradation, and knockdown of COP1 enhances p53 mediated G1 arrest and sensitizes cells to ionizing radiation (Dornan et al., 2004). ARF-BP1, a recently identified E3 ligase that contains a HECT domain that was purified as an ARF-BP1 binding protein, can also ubiquitinate and degrade p53. ARF-BP1 inactivation has been shown to induce tumor suppression in p53 wild-type cells. However, it is important to note that it has also been shown to induce tumor suppression in p53 null cells, suggesting that ARF-BP1 has both p53-independent and p53-dependent functions (Chen et al., 2005a). E4F1 is an atypical ubiquitin ligase, as it facilitates p53 ubiquitination, but not for degradation. Rather, ubiquitination at the

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Lysine 320 residue seems to affect only p53 dependent cell cycle arrest, not apoptosis (Le Cam et al., 2006). Together, these proteins represent an array of E3 ligases that the cell requires to regulate p53 stability, suggesting that the cell utilizes both MDM2-independent and MDM2-dependent mechanisms to maintain tight regulation of p53.

Deubiquitinates are specific protease enzymes that catalyze the removal of ubiquitin from substrates. The enzymes do this by cleaving the isopeptide bond between ubiquitin and the substrate. The Herpesvirus-Associated-Ubiquitin-Specific-Protease (HAUSP) was first identified as a cellular factor that bound to Herpes simplex virus type 1 regulatory protein ICP0 (Everett et al., 1997). HAUSP can bind to and stabilize p53 by directly removing the ubiquitin in order to induce growth arrest and apoptosis (Li et al., 2002a). It has also been demonstrated that the p53-HAUSP interaction is enhanced under DNA damage (Li et al., 2002a; Lim et al., 2004). However, siRNA knockout of HAUSP in wild-type p53 containing cells resulted in p53 stability. This is because HAUSP can also interact with MDM2 and deubiquitinate it under certain conditions, suggesting that HAUSP is required to maintain sufficient levels of MDM2 in order to keep p53 levels regulated (Li et al., 2004).

P53 Phosphorylation

There are multiple residues in both the amino terminus and the carboxyl terminus of p53 that are phosphorylated or dephosphorylated in response to genotoxic stress, for a total of 23 sites (Appella and Anderson, 2001). Upon DNA damage, ATM, Chk1 and Chk2 phosphorylate p53 on several serine residues. ATM phosphorylates Ser-15 and Chk2 phosphorylates Ser-20. These modifications eliminate the p53-MDM2 interaction following double strand DNA breaks (Canman and Lim, 1998; Hirao et al., 2000; Unger et al., 1999). Threonine 18 phosphorylation by Chk2 is also important for destabilizing the MDM2-p53 interaction (Schon et al., 2002). Phosphorylation at these three residues helps recruit other regulatory factors, such as p300, CBP and pCAF to p53 in order to facilitate p53 acetylation (Li et al., 2002b). However, there have been reports that when all serine residues were changed to alanine, p53

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continued to display wild-type stability and transactivation (Wahl, 2006). Mouse models with similar mutations at Ser-15 and Ser-23 (equivilant to Ser-18 and Ser-20) had modest, tissue-specific deficiencies and did not destabilize p53, as one would have predicted if they were critical residues for blocking MDM2-p53 binding following DNA damage (MacPherson et al., 2004; Sluss et al., 2004; Wu et al., 2002). The difficulty in showing strong phenotypic changes when these sites are mutated suggests a more complex role for these events. In fact, modifications to MDM2 may be required to efficiently block the MDM2-p53 interaction following stress. For example, MDM2 can also be phosphorylated on Ser-395 by the ATM pathway and can be phosphorylated on Tyrosine 394 by c-Abl, both shown to destabilize MDM2-p53, resulting in p53 stabilization (Goldberg et al., 2002; Mayo et al., 1997).

Phosphorylation of p53 on Ser-33, Thr-81 and Ser-315 promotes Pin1-p53 interaction, a regulator of p53 following genotoxic stress, and results in a p53 conformational change and activation of p53 (Zheng et al., 2002). DNA-PK has also been shown to induce phosphorylation on N-terminal residues of p53, which is necessary but not sufficient alone to initiate p53-DNA binding (Woo et al., 1998). Oncogenic stress signaling by Ras can stimulate Ser-33 and Ser-46 phosphorylation on p53 by stress kinase p38 and has been reported to result in senescence and tumor suppression (Bulavin et al., 2002; Ferbeyre et al., 2002). Phosphorylation has been shown to contribute to p53 gene target specificity, as is the case with CDKs, PKC and CKII phosphorylation on Ser-315, 378, and 392 (Bischoff et al., 1990; Delphin and Baudier, 1994; Hall et al., 1996). On Ser-46, p53 can be phosphorylated by DRK2 following severe DNA damage resulting in p53-mediated apoptosis. HIPK2, which leads to dissociation of MDM2-p53 also induces p53-mediated apoptosis (Di Stefano et al., 2004; Oda et al., 2000b). Inhibitory phosphorylation also exists. For example, phosphorylation of Ser-215 by aurora kinase A overrides p53 DNA damage induced stress response (Liu et al., 2004)

Conversely, there are also sites that are constitutively phosphorylated under normal conditions that undergo dephosphorylation during stress, resulting

in p53 activation. For example, Ser-376, when dephosphorylated following DNA damage, enhances p53-14-3-3ζ interaction and increases p53 DNA binding affinity (Stavridi et al., 2001). Dephosphorylation can also have inhibitory effects on p53 activity. The NOTCH-1 protein can bind p53 and inhibit phosphorylation on Ser-15, 37 and 46, leading to a decrease in p53 activation (Kim et al., 2007).

P53 Acetylation

Acetylation is the covalent linkage of an acetyl group to lysine residues located on histone tails and other proteins, such as transcription factors. It is involved with transcriptional regulation and correlates with an increase in transcriptional activity (Kouzarides, 2000). CBP/p300, a histone acetyl-transferase (HAT), acts as a coactivator of p53 and an increase in p53-CBP/p300 association is observed following DNA damage. This interaction enhances p53 acetylation and results in its activation (Barlev et al., 2001). PCAF also has been shown to acetylate the c-terminus of p53, resulting in its activation (Appella and Anderson, 2001). MDM2 has been shown to inhibt the interaction of p53 and p300 in normal cellular conditions (Ito et al., 2002). Phosphorylation at the N-terminus has been implicated in facilitating an increase in acetylation and in CBP/p300 and PCAF binding (Avantaggiati et al., 1997; Dumaz and Meek, 1999; Gu et al., 1997; Lill et al., 1997). Acetylation may have an even more direct role in p53 stabilization, as it was shown that c-terminal p53 acetylation can inhibit MDM2 mediated ubiquitination, further emphasizing how critically important this process is for the activation of p53 (Chuikov et al., 2004).

Histone deacetylase complexes (HDACs) are often associated with corepressor complexes and repress both histone and non-histone proteins by removing acetyl groups. Much less is known about HDAC activity on p53 function. However, it was recently shown that Sir2α (SIRT1), a NAD-dependent histone deacetylatse can deacetyate p53 and attenuate its transcriptional activity (Vaziri et al., 2001). It is not known if deacetylation can also serve as an important step in MDM2-mediated p53 degradation.

P53 Methylation

Histone and protein methylation is now recognized as an important modification linked to both transcriptional repression and activation (Margueron et al., 2005). It is believed that methylation of lysine residues on target proteins can influence the signaling potential of the modified protein, leading to diverse physiologic consequences (Shi et al., 2007). Recent reports indicate that there are multiple residues on p53 that can be methylated, affecting its transcriptional activity. Set7/9 monomethylation of p53 and Lys-372 activates p53 via stabilization of chromatin-associated p53 (Chuikov et al., 2004). Set 8 monomethylates p53 at Lys-382, which suppresses p53-mediated transcriptional activity. In fact, Set8 expression is downregulated upon DNA damage (Shi et al., 2007). Smyd2 monomethylated p53 at Lys-370, repressing p53 activity which can be impeded by Set7/9 methylation at Lys-372 (Huang et al., 2006). P53 can also be demethylated by the lysine demethylase, LSD1, removing both di-and monomethylation at Lys-370. This demethylase activity represses p53-mediated transcription and inhibits p53-dependent apoptosis (Huang et al., 2007). It is important to note that while we know monometylation occurs at Lys-370 via Smyd2 activity, it is not yet known what methyltransferase creates dimethylation at this particular site on p53.

P53 Neddylation and Sumoylation

The Nedd8 protein is in the ubiquitin-like family of proteins and uses E1 activating and E2 transferring enzymes, similar to that of ubiquitin. Neddylation inhibits p53 transcriptional activation via MDM2 (Xirodimas et al., 2004). MDM2 acts as a specific E3 Nedd8 ligase for p53 and also itself. C-terminal glycine residue of Nedd8 can be covalently linked to Lys-372, 372 and 373 of p53. P53 proteins with mutations at these residues are no longer transcriptionally inhibited. These neddylation residues overlap with lysine residues that can be ubiquitinated and acetylated, however, it is not known whether there are de-neddylation pathways or if neddylation is in competition with ubiquitin and acetylation, with one process favoring the other in certain conditions.

Sumolyation is similar to ubiquitination in that an isopeptide bond is formed between the C-terminal carboxy group of the small ubiquitin-like protein SUMO1 and the ε-amino group of a lysine residue in the target protein. It has been shown that p53 sumolyation on Lys-386 can enhance transcriptional activity and, similar neddylation, seems to be regulated by MDM2 and also ARF mediated nuclear targeting (Chen and Chen, 2003; Gostissa et al., 1999).

P53 Mutations in Cancer

P53 mutations occur in more than 50% of human cancers. A distribution of organ specific tumors and percentages of p53 gene mutations are depicted in **Figure 4**. The most common mechanism in which p53 is inactivated are point mutations (93.6%), which have been identified in more than 250 codons of p53. About 95% of these lie in the core DNA binding domain of p53, while only about 5% of the mutations are found in the regulatory domains (**Figure 5)**. These DNA binding domain mutations incapacitate the ability of p53 to transactivate the target genes that are necessary for mediation of p53 tumor suppressor functions. Of these point mutations, 75% occur as a single missense mutation in one allele of p53, rather than deletions, insertions or frameshifts. The second wild-type allele is generally also lost (loss of heterozygosis, LOH), resulting in genetic instability. In fact, a germ-line mutation in just one of the two p53 alleles abrogates p53 function and predisposes humans to cancer (Li-Fraumeni syndrome). This creates an extremely stable mutant p53 protein that is seen at high expression levels in cancer cells. There can also be a dominante negative effect from the mutant p53, forming inactive hetero-oligomers with the wild-type p53, rendering it inactive (de Vries et al., 2002). A majority of these point mutations will either affect the structural integrity of p53 or its ability to contact DNA directly.
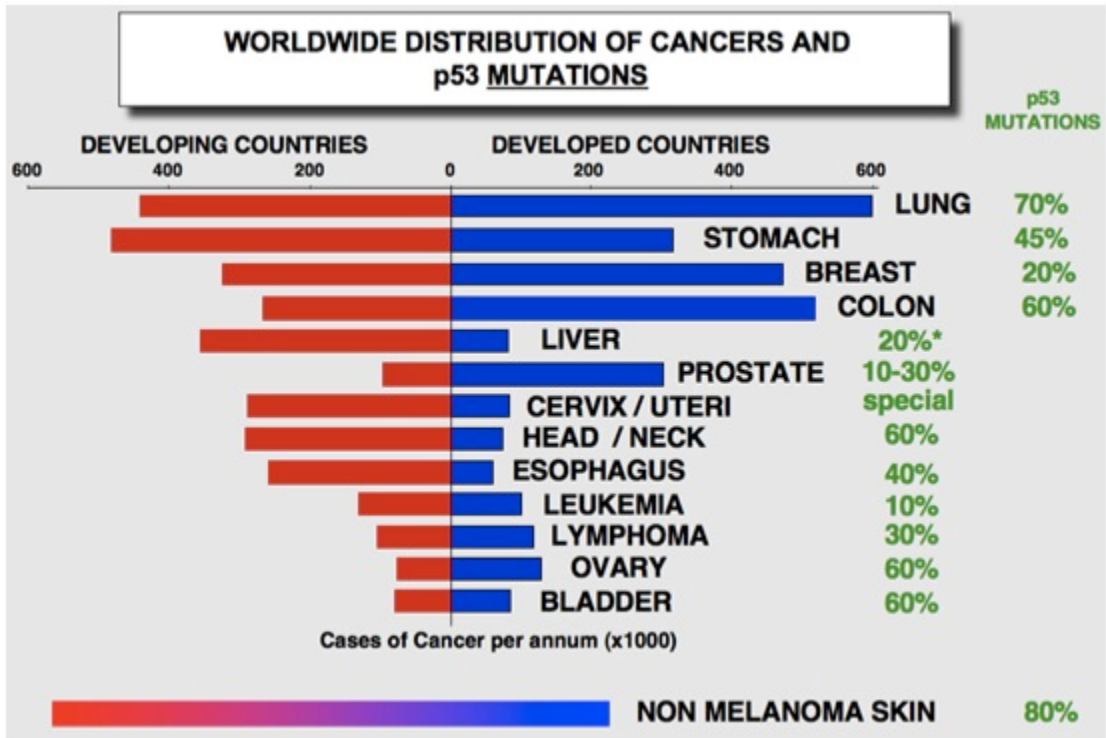
Figure 4: Distrubution of Cancers and p53 Mutations. Soussi, Thierry. Online Posting. *The TP53 Website*. n.d. Web. 14 Oct. 2010.
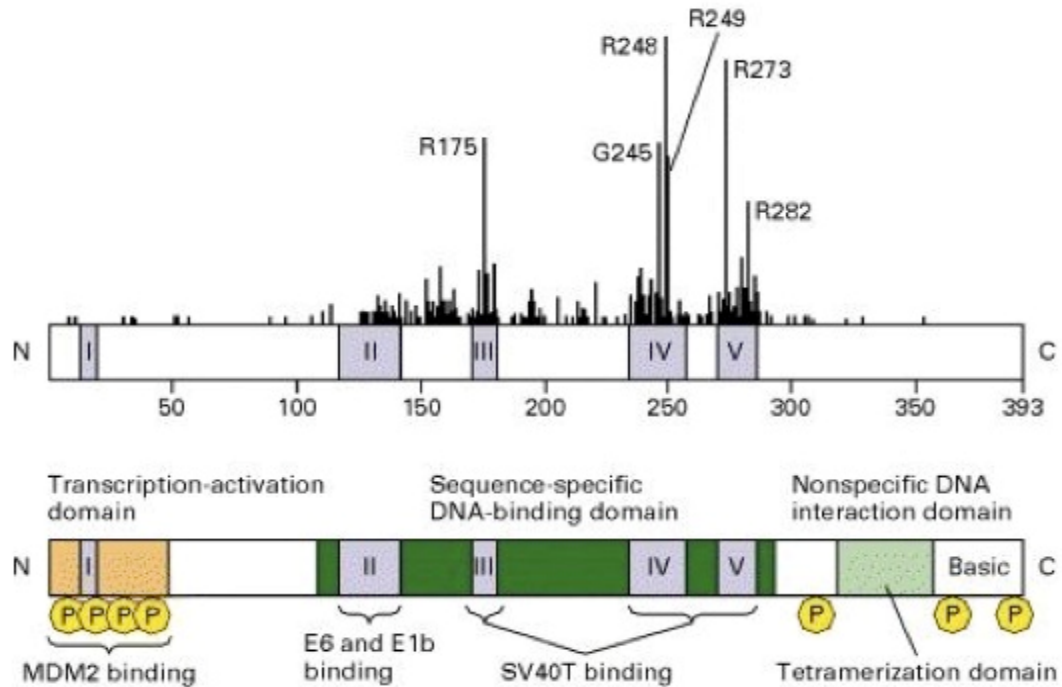
Figure 5: Distribution of p53 point mutations. Lodish, et. al. <u>Molecular Cell Biology 4th Addition</u>. New York: W. H. Freeman, 2000.

There are six hotspot mutations that are clustered in the DNA binding surface; two affect p53 DNA contact – Arg248 and Arg273 – and four affect the structure of the p53 DNA binding surface – Arg175, Gly245, Arg249 and Arg282. The contact mutations are capable of maintaining a wild-type conformation, however, the p53 DNA-binding capability is severely compromised. In fact, the most prevalent point mutation that occurs in human cancer is Arg 248 (Ory et al., 1994). Structural mutations result in an altered conformation in p53, also decreasing its ability to bind DNA (Legros et al., 1994). There are also less common point mutations that affect p53 thermostability, oligomerization and interactions with other proteins. Some mutants of p53 have even been suggested to have oncogenic activity by gain-of-function mechanisms (Dittmer et al., 1993; Harvey et al., 1995). For example, mice with the Val135 mutation in one allele exhibited accelerated tumor development and an altered tumor spectrum, compared to the wild-type only counterparts (Harvey et al., 1995).

Non-Mutated p53 in Cancer

The remaining 50% of cancers that do not contain mutant p53 actually suppress p53 by disrupting its activity. One of the mechanisms is through inhibition by viral oncoprotiens. In adenovirus transformed cells, the EIB oncoprotein can bind to p53 and inhibit its transcriptional transactivation by blocking PCAF acetylation (Liu, Colosimo et al. 2000; Zhao and Liao 2003). The E6 protein of human papillomavirus types 16 and 18 facilitates the rapid degradation of p53 via ubiquitin dependent proteolytic degradation. The E6 protein binds to the cellular E6-AP protein, and this complex interacts with p53 to induce ubiquitination (Scheffner et al., 1993).

Likewise, cellular proteins that regulate the activity of p53 cause many wild-type p53-inactivating lesions. For example, MDM2 overexpression through gene duplication enhances transcription or translation and is frequently observed in tumors that retain wild-type p53 (Landers, Haines et al. 1994; Momand, Jung et al. 1998; Phelps, Darley et al. 2003). Increased expression of MDM2 leads to the continuous degradation of p53 and therefore suppresses its activity, even under conditions of cellular stress. Hypomorphic MDM2 mice that express reduced levels of MDM2 show enhanced p53 activity, but without an increase in p53 protein levels. This suggests that MDM2 mediated degradation is not the only way it inactivates p53 (Mendrysa, McElwee et al. 2003). MDMX, a MDM2 homologue, is also overexpressed in several tumor types, particularly retinoblastomas. MDMX overexpression does not lead to a reduction in p53 levels, but rather a reduction in transcriptional activity (Danovi, Meulmeester et al. 2004).

Proteins that are further upstream in the p53 pathway are inactivated in some tumors. For example, the loss of the INK4A locus through deletions, mutations or DNA methylation is frequently seen in cancers (Sherr, 2001). This gene codes for the ARF protein that binds directly to MDM2, preventing MDM2-mediated degradation of p53 following mitogenic stress. Without ARF, tumors with oncogene activation signaling cannot reach p53 (Ruas and Peters, 1998). Mutations in upstream proteins critical in the DNA damage pathway are also

frequently inactivated in cancers with wild-type p53. For example, AT (human disease ataxia-telangiectasia) patients have mutations in the ATM protein, leaving p53 unphosphorylated following DNA damage and unable to transcribe proteins needed for its tumor suppressor activity (Maya, Balass et al. 2001). Li-Fraumeni-like syndrome patients that have wild-type p53 have Chk2 germline mutations that behave in a similar manner to those with ATM mutations (Bell, Varley et al. 1999). Thus, human tumors frequently select for not only p53 mutations itself, but mutations in its regulatory proteins to promote tumor progression.

P53 as a therapeutic Target

The p53 pathway provides an important target for drug therapy because a damaged p53 pathway provides a key difference between tumor cells and normal cells (Bell and Ryan, 2007). In fact, loss of p53 function has been linked with unfavorable prognosis and is indicated by more aggressive tumors, early metastasis and decreased survival rate (Gallagher and Brown, 1999). Multiple studies have indicated that loss of p53 function confers increased resistance to anti-tumor agents (Lowe, Bodis et al. 1994; Ruley 1996). There are two main strategies employed by researchers to reactivate p53 signaling: restore wild-type function to mutant p53 and rescue the functionality of wild-type p53 that has been compromised by loss or over-expression of its regulators.

There are three types of cancer therapies that target mutations within p53 itself. One cancer p53-oriented therapy targets the p53 dysfunction itself using pharmacological agents. PRIMA-1 and CP-31398 are compounds that have been found to restore transcriptional transactivation function and reduce tumor size in animal models with both DNA binding and conformational mutants (Foster, Coffey et al. 1999; Bykov, Issaeva et al. 2002). Another method would be to restore functional activity of p53 mutants using a second site suppressor mutation. In theory, these second site mutations will restore the stability of mutant p53, resulting in the restoration of normal p53-DNA binding function. One example is the second site mutant N239Y that can restore the hotspot mutation G245S, resulting in increased p53-DNA binding (Nikolova, Wong et al. 2000).

Unfortunately, the practicality in creating these second site suppressors in a clinical setting is not optimal.

A third approach to treating mutant p53 containing tumors is the use of gene replacement therapy with wild-type p53 or proteins that selectively target mutant p53 cells. Using genetic engineering, researchers are looking at both viral and non-viral ways to introduce wild-type p53 in hopes to restore its tumor suppressor function and to increase sensitivity to conventional anti-tumor agents, such as chemotherapy or radiation. A candidate for this type of therapy are retroviruses, which integrate stably into the genome of infected cells, require cell division for transduction and are used in the majority of approved gene transfer clinical protocols (Roth and Cristiano, 1997). It has been demonstrated that retrovirus gene transfer or wild-type p53 into human lung tumor cells and xenograft models could lead to inhibition of tumor cell growth, which was limited to tumor cells null or mutated for p53 (Cai, Mukhopadhyay et al. 1993; Fujiwara, Grimm et al. 1993; Fujiwara, Cai et al. 1994). Although promising, this type of treatment is in its infancy and requires further improvements before becoming part of the standard care. Another gene therapy that specifically targets mutant p53 cancers cells involves an adenovirus called ONYX-015, which encodes the E1b protein that specifically targets p53. In this case the virus is engineered with a specific defect that causes it to selectively target cells with or without mutant p53. Preliminary research on animal models were promising, however, preclinical testing has been less effective, although better results have been obtained when combined with conventional chemotherapies (Heise, Sampson-Johannes et al. 1997; Yang, You et al. 2001).

Rescuing the functionality of wild-type p53 that has been compromised by loss or overexpression of its regulators is another popular approach for the development of p53 based cancer therapies. MDM2 is an essential regulator of p53 and is a key target in discovering therapeutic ways to activate p53, particularly in tumors that retain wild-type p53 but have a functionally inactivated pathway through MDM2 overexpression or amplifications. Reactivating p53 could serve as a means to reduce tumor size or eliminate tumors all together.

The discovery of a small molecular compund, called Nutlin-3 (a cis-imidazoline analog), has been shown to inhibit the p53-MDM2 binding with a low IC50 (Bottger et al., 1996). Nutlin-3 treatment results in p53 stabilzation and transcriptional activation, leading to cells that undergo cell cycle arrest and inhibition of tumor growth in vivo (Vassilev 2004; Vassilev, Vu et al. 2004; Vassilev 2005). It is important to note that this compound would be unable to target tumor cells overexpressing the MDM2 homolog, MDMX. Although they have similar N-terminal binding regions for p53, Nutlin does not disrupt the MDMX-p53 interaction, therefore more work needs to be done to identify small molecular inhibitors specific for disruption of MDMX-p53 binding (Hu et al., 2006). For instance, using a phage display library, our lab identified a peptide sequence that blocks both MDM2 and MDMX-p53 binding. P53 activation and growth arrest are greater with the addition of the peptide than with blocking MDM2-p53 binding alone (Hu et al., 2007).

The E3 ligase activity of MDM2 has also been used as a therapeutic target and identification of small molecules that interfere with this activity have resulted in suppression of ubiquitination (Lai, Yang et al. 2002; Yang, Ludwig et al. 2005). One would conjecture that a problem would be lack of specificity, however, preliminary studies revealed that one of the compounds was selective for MDM2 with no off target effects and the compounds differentiated between MDM2 ubiquitination of p53 and self-ubiquitination.

Oncogene MDM2

History of MDM2

The MDM2 (murine double minute 2) protein was first identified as the product of gene amplification on murine double-minute chromosomes in a 3T3DM spontaneously transformed mouse cell line (Cahilly-Snyder et al., 1987). Overexpression of MDM2 resulted in tumor formation in nude mice and leads to transformation of primary rodent fibroblasts in cooperation with Ras, suggesting an oncogenic role for MDM2 (Fakharzadeh, Trusko et al. 1991; Finlay 1993). The human homologue of MDM2 (also referred to as HDM2) has been mapped to chromosome 12q14.3-15. In a subsequent study MDM2 was co-purified with

p53 and found to inhibit p53 transcriptional activity and negatively regulate its stability, providing a mechanism for its oncogenic properties (Momand, Zambetti et al. 1992; Oliner, Pietenpol et al. 1993). Concurrently, several tumor types have been shown to have amplified MDM2, including many osteosarcomas and soft tissue sarcomas that retained wild-type p53 (Momand et al., 1998).

MDM2 Structure

The MDM2 gene is conserved across mammalian species and found in other multicellular organisims (Momand et al., 2000). Expression of MDM2 is found ubiquitously and at low levels in normal human tissues. The human MDM2 gene is composed of 12 exons and 491 amino acids in length with a predicted molecular weight of 56 kDa. The MDM2 gene can be transcribed from two different promoters: an upstream constitutive promoter (P1) and a second (P2) promoter within the first intron that is responsive to p53 (Barak et al., 1994). MDM2 is a member of the RING finger domain family of E3 ubiquitin ligases that contains four domains. These include: an N-terminal domain (aa 19-102), a central acidic domain (aa 223-274), a zinc finger (aa 305-322) and a C-terminal RING domain (aa 438-478) (**Figure 6**).

Figure 6: MDM2 Structure. The MDM2 protein contains an N-terminal/p53-binding domain, a central acidic domain, a zinc finger domain and C-terminal RING finger domain. MDM2 also has a nuclear localization and nuclear export signal.

The N-terminus of MDM2 contains a p53-binding domain that interacts with the transactivation domain of p53. The binding between MDM2 and p53 has been shown to prevent the association of transcriptional co-activators, inhibiting p53 transactivation function (Momand, Zambetti et al. 1992). The binding of MDM2 to p53 also mediates the translocation of p53 from the nucleus to the

cytoplasm, which is dependent on the ability of MDM2 to bind to and ubiquitinate p53 (Geyer et al., 2000). However, there are recent studies that have shown MDM2 constructs without the N-terminus p53-binding domain retain the ability to bind to and inhibit p53 activity (Ma, Martin et al. 2006). The p53 amino acids essential for p53 binding to MDM2 include phenylalanine 19, tryptophan 23 and lysine 26 (Lin, Chen et al. 1994).

MDM2 contains both a nuclear localization and a nuclear export signal between the N-terminal domain and the central acidic domain, however, the protein is primarily localized in the nucleus (Hay and Meek 2000). There is also a nucleolar localization signal found in the C-terminus of MDM2, which is required for MDM2 to localize to the nucleolus in the presence of the ARF tumor suppressor. This leads to stabilization of p53 in response to oncogene activation (Lohrum, Ashcroft et al. 2000; Weber, Kuo et al. 2000).

The MDM2 central acidic domain has been shown to play an important role in p53 ubiquitination and degradation (Argentini, Barboule et al. 2001; Kawai, Wiederschain et al. 2003; Meulmeester, Frenk et al. 2003). The acidic domain is also required for MDM2 binding to a number of proteins that can either attenuate or enhance its activity, including p14^{ARF}, p300, YY1, ribosomal subunits, KAP1 and SUV39H1 (Pomerantz, Schreiber-Agus et al. 1998; Grossman, Deato et al. 2003; Zhang, Wolf et al. 2003; Jin, Itahana et al. 2004; Sui, Affar el et al. 2004; Wang, Ivanov et al. 2005; Chen, Li et al. 2010). The central domain also contains a cluster of serine and threonine residues that are sites for phosphorylation and important for regulation of MDM2 function. Serines 240, 242 and 246 are CK1δ phosphorylation sites, Ser-256 is a site targeted by GSK3β, and Ser-269 is targeted by CHK2 following DNA damage, impairing the MDM2-Rb interaction and subsequent proteosomal degradation of pRb (Winter, Milne et al. 2004; Allende-Vega, Dias et al. 2005; Kulikov, Boehme et al. 2005). In addition, it has been revealed that the central acidic domain contains a second p53 interaction site that binds to the core of p53 and is sufficient to target p53 for ubiquitination in vitro (Burch, Midgley et al. 2000; Shimizu, Burch et al. 2002; Ma, Martin et al. 2006).

MDM2 harbors a central zinc finger domain between the acidic domain and the C-terminal RING domain. In the case of MDM2, the zinc finger domain is critical for mediating the MDM2-ribosomal protein interaction and its ability to degrade p53 under ribosomal conditions (Lindstrom, Jin et al. 2007).

MDM2 also contains a C-terminal RING domain with two zinc ions coordinated by a Cis3-His2-Cis3 consensus that is essential for proper folding of the domain (Boddy et al., 1994). The RING domain is typically involved in protein-protein interactions (Borden, 2000). CBP/p300 interaction and subsequent acetylation occurs at particular lysine residues in the RING domain leading to inhibition of the ubiquitin ligase activity (Wang et al., 2004). The RING can also bind specifically to 5S RNA, although this interaction and its function is poorly understood (Elenbaas et al., 1996). Likewise, the domain is critical for binding nucleotides, strongly preferring ATP, which is important for translocation of MDM2 from the nucleoplasm to the nucleolus (Poyurovsky, Jacq et al. 2003).

MDM2 is also capable of self-oligomerization through its RING domain and heterodimerizing with MDMX. The RING domain is also necessary for MDM2 ubiquitin E3 ligase activity towards p53 and MDMX, as well as itself (Tanimura, Ohtsuka et al. 1999; Borden 2000; Fang, Jensen et al. 2000; Honda and Yasuda 2000; Poyurovsky, Priest et al. 2007). A knock-in mouse model of MDM2 with a point mutation inactivating the RING domain functions is embryonic lethal and is rescued in a p53 null background, indicating it is essential for MDM2-mediated suppression of p53 (Itahana, Mao et al. 2007). This area also contains a cryptic nucleolar localization signal that is only revealed upon interaction with p14[ARF] (Lohrum, Ashcroft et al. 2000).

MDM2-p53 Interaction

MDM2 is a vital regulator of p53, thus this interaction is controlled through several mechanisms. The direct interaction between p53 and MDM2 is mediated by a hydrophobic pocket domain located in the N-terminus of MDM2 and residues 18-26 in the N-terminus of p53 (Chen, Marechal et al. 1993; Kussie, Gorina et al. 1996). p53 mutants that can not bind MDM2 are resistant to degradation by MDM2, and mutations of critical N-terminal residues in MDM2

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lack p53-binding (Freedman, Epstein et al. 1997; Kubbutat, Jones et al. 1997). There are three critical p53 hydrophobic residues that fit into the hydrophobic cleft of MDM2 (Phe-19, Trp-23 and Leu-26) and are the target of the previously described MDM2-p53 small molecule inhibitor Nutlin.

The MDM2-p53 interaction allows MDM2 to control p53 through two distinct mechanisms: directly binding and masking the N-terminal transactivation domain and promoting ubiquitin-dependent proteosomal 26S degradation (Momand, Zambetti et al. 1992; Haupt, Maya et al. 1997). By binding to the p53 transactivation domain, MDM2 is able to transcriptionally inactivate p53 by blocking the recruitment of coactivators and transcriptional machinery. The most prominent target of MDM2 E3 ubiquitin ligase activity is p53, which is constantly being ubiquitinated by MDM2. However, mutants of MDM2 that lack E3 ligase activity can still bind wild-type p53 and, although significantly hindered, inhibit p53-mediated transcriptional activation (Leng et al., 1995). The extent of ubiquitination can also determine the fate of the p53 protein. Polyubiquitination is believed to target p53 for proteosomal degradation and monoubiquitination assists in nuclear export, both regulating p53 localization and its function as a transcription factor (Freedman and Levine 1998; Roth, Dobbelstein et al. 1998; Yang and Yu 2003). Recent studies have shown that MDM2 requires p300 to catalyze p53 polyubiquitination, but MDM2 alone can only catalyze monoubiquitination (Zhu, Yao et al. 2001). MDM2 can also promote its own degradation by autoubiquitination and ubiquitination of MDMX, promoting p53 stabilization and transcriptional activation (Fang, Jensen et al. 2000).

MDM2 can influence other forms of p53 posttranslational modifications, such as acetylation, sumolyation and neddylation. MDM2 suppresses CBP/p300 mediated p53 acetylation, increasing its ability to be ubiquitinated and degraded (Grossman, Perez et al. 1998). MDM2 mediated p53 sumoylation enhances p53 activity, while promotion of neddylation leads to the inhibition of p53 transcriptional activity (Chen and Chen 2003; Xirodimas, Saville et al. 2004).

MDM2 is also a transcriptional target of p53, creating an autoregulatory feedback loop (Momand, Zambetti et al. 1992; Barak, Juven et al. 1993; Wu,

Bayle et al. 1993). The MDM2 gene contains two promoter regions: P1 that is thought to be constitutively on, and P2 that contains two p53 responsive elements (Montes de Oca Luna et al., 1995). The ability of the cell to maintain p53 at low levels is critical for cellular survival and to allow for cell cycle progression. Similarly, decreasing p53 activity will lead to a decrease in MDM2 protein levels. Therefore, the ratio of MDM2 to p53 protein is critical for the correct response to multiple cellular conditions. In support of this relationship was the observation that the lethality of MDM2 null mice is rescued by simultaneous knockout of p53 (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995).

Several reports suggest that MDM2 has additional non-degradation mechanisms for regulating p53 activity. A previous study showed that a temperature-sensitive p53 mutant does not bind to DNA after forming a complex with MDM2 (Zauberman et al., 1993). EMSA experiments showed that full-length MDM2 does not interact with p53-DNA complex, suggesting that p53 binding to DNA and MDM2 are mutually exclusive (Burch et al., 2000). However, a GST-MDM2-1-188 fragment was able to super-shift p53-DNA complex (Bottger et al., 1997). More recent work shows that MDM2-hsp90 complex inhibits DNA binding by p53 in vitro and induces p53 unfolding (Burch et al., 2004). However, conflicting results suggest that MDM2 acts as a chaperone to promote p53 folding and stimulates p53 DNA binding in vitro (Wawrzynow et al., 2007). A recent study monitored p53 conformation under conditions in which MDM2 mediated degradation was inhibited and showed that MDM2 binding promotes conformational change, which preceded p53 ubiquitination and degradation (Sasaki et al., 2007). MDM2-mediated conformational change may expose lysine residues on p53 for ubiquitination, which can be opposed by overexpression of hsp90 (Nie et al., 2007; Sasaki et al., 2007).

MDM2 Regulation

DNA Damage

MDM2 is also controlled at the post-translational level. Upon DNA damage, several kinases can phosphorylate MDM2 on several of its serine or

threonine residues which modulate the p53-MDM2 interaction (Moll and Petrenko, 2003) (**Figure 7**). The DNA-activated protein kinase (DNA-PK) has been shown to phosphorylate MDM2 at serine 17, located in the p53-binding site leading to decreased binding affinity to p53 (Meek and Knippschild, 2003). ATM, an important DNA damage responsive kinase, phosphorylates MDM2 at Ser-395 in the RING domain, disrupting the nuclear export signal necessary for efficient p53 export to the cytoplasm (Maya, Balass et al. 2001). The tyrosine kinase c-Abl, whose activity is stimulated by ATM, phosphorylates MDM2 on Tyr-394, suppressing MDM2 mediated degradation of p53 and contributing to DNA damage induced apoptosis (Goldberg, Vogt Sionov et al. 2002). In response to growth factors, the PI-3 kinase pathway activated a signaling cascade that promotes cell growth and survival. This included activation of AKT, a serine/threonine kinase, that phosphorylates MDM2 on multiple residues leading to MDM2 stabilization and increased p53 suppression (Ogawara, Kishishita et al. 2002). The mitogenic p38 kinase (MAPK) also phosphorylated MDM2 at the same sites of AKT, resulting in a similar outcome (Weber et al., 2005). Modifications of MDM2 by cyclin-CDK complexes (cyclin A-CDK2 and cyclin A-CDK1) stimulated the interaction between MDM2 and ARF and decreased the interaction between MDM2 and p53 (Meek and Knippschild, 2003). The CK2 kinase has also been shown to phosphorylate MDM2, although the significance of this modification is currently unknown (Meek and Knippschild, 2003).
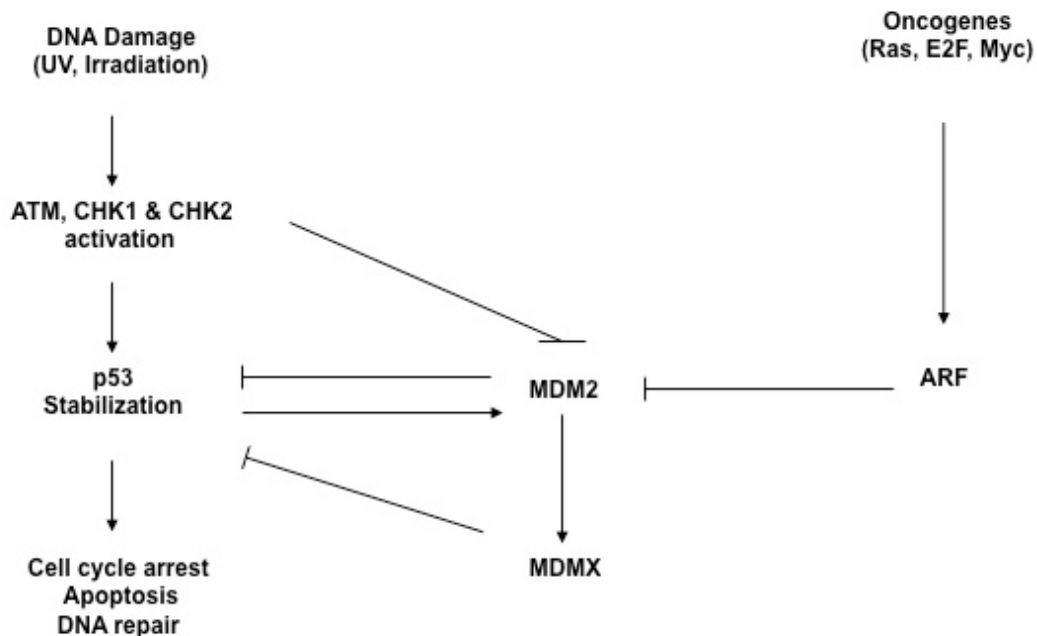
Figure 7: MDM2 Regulation. Regulation of the MDM2 and p53 interaction following DNA damage and mitogenic stress.

Dephosphorylation is also a potential mechanism for MDM2 regulation. The association of MDM2 with cyclin G-protein phosphatase 2A (G1-PP2A), a serine/threonine protein phosphatase and one of the first p53 responsive genes identified, results in dephosphorylation of Ser-166 and Thr-216 (Okamoto and Beach, 1994). This leads to the generation of a positive feedback loop where stress activated p53 increases G1-PP2A expression, which then associates with MDM2, dephosphorylating it and destabilizing MDM2. This process leads to the liberation of p53 where it can further transcribe target genes (Okamoto, Li et al. 2002). Wip1, a nuclear serine/threonine phosphatase, is also a downstream target of p53 whose expression is stimulated by stress. Wip1 dephosphorylates MDM2 on Ser-395, the same site that is phosphorylated by ATM following DNA damage, resulting in the stabilization and suppression of p53 and returning it to basal levels (Lu, Ma et al. 2007). The acidic domain is phosphorylated under

unstressed conditions and dephosphorylation of the acidic domain is also thought to play a role in mediating p53 induction. Ck2 phosphorylates Ser-262 and 269 reducing Rb binding, GSK3β phosphorylates Ser-256 enhancing MDM2 activity toward p53 degradation, and Ser-240, 242 and 246 are Ck1δ/ε targets (Winter, Milne et al. 2004; Gotz, Kartarius et al. 2005; Kulikov, Boehme et al. 2005). Following ionizing radiation, Ser-240, 242, 260 and 263 are rapidly dephosphorylated before p53 starts to accumulate, and alanine mutations at these phospho-sites alleviate degradation of p53 suggesting that these modifications result in positive regulation of p53 (Blattner, Hay et al. 2002). These findings seem to add another layer of complexity to the MDM2-p53 negative regulatory feedback loop.

Oncogenic Stress/ARF Induction

Deregulation of oncogenes, such as Ras mutants, pRb-E2F, c-myc or viral E1A, is another way of interfering with the MDM2-p53 interaction, allowing for the stabilization and activation of p53. Following oncogenic stress there is an increase in the p14[ARF] protein, which binds to the acidic domain of MDM2 and inhibits its E3 ligase activity (Honda and Yasuda 1999; Sherr 2006). It is thought that ARF binding sequesters MDM2 in the nucleolus while p53 remains in the nucleoplasm resulting in stabilization of nuclear p53 and increased p53 transcriptional activity (Tao and Levine 1999; Weber, Taylor et al. 1999).

Ribosomal Stress

Ribosomal stress induced by inhibiting rRNA synthesis causes the release of ribosomal proteins from the nucleolus to the nucleoplasm. Once in the nucleoplasm, ribosomal large subunit proteins such as L5, L11 and L23, bind to the central acidic domain/zinc finger of MDM2 (Marechal, Elenbaas et al. 1997; Lohrum, Ludwig et al. 2003; Dai, Zeng et al. 2004). This leads to the inhibition of MDM2 E3 ligase activity towards p53, increasing the expression of p53 target genes and cell cycle arrest (Bhat, Itahana et al. 2004; Dai, Zeng et al. 2004). It is believed that MDM2-L protein interactions cause a steric hindrance that prevents the transfer of ubiquitin from E2 to p53 (Zhang, Wolf et al. 2003). Knockdown of these L proteins by siRNA can lead to decreased p53 activation following

ribosomal stress. Also, point mutations in the MDM2 zinc finger domain (C305F) can abrogate L5 and L11, not L23 binding. This creates an MDM2 protein with decreased export capabilities and delayed ubiquitination and degradation of p53.
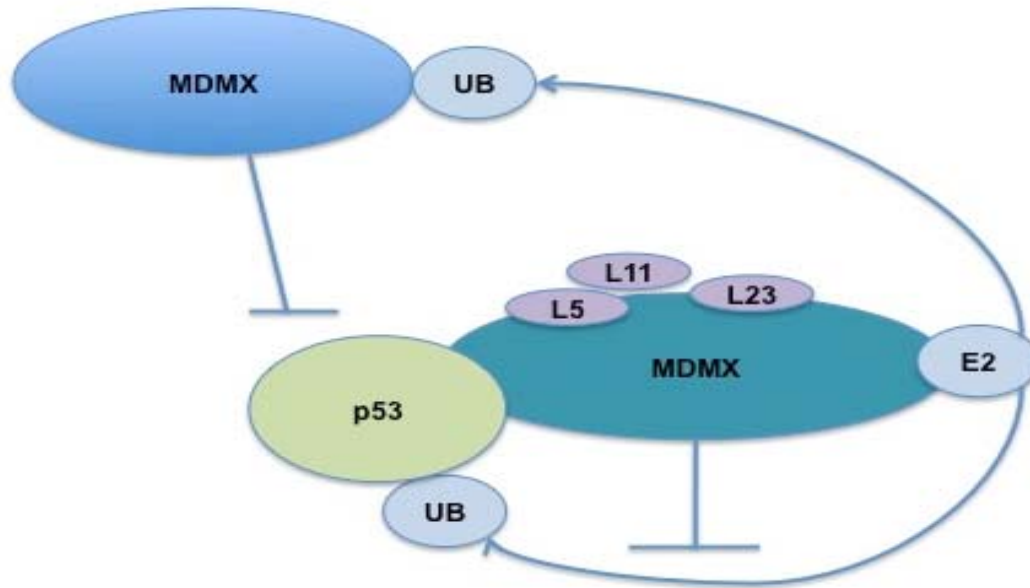
Figure 8: MDM2 Regulation by Ribosomal Stress. Following ribosomal stress the L proteins move from the large ribosomal subunit and bind to the MDM2 acidic domain. This attenuates MDM2's ability to degrade p53. It also increases MDM2 ubiquitination of MDMX.

MDM2 Mouse Models

The importance of MDM2 in regulation of p53 is best exemplified in mouse models. Homozygous knockouts of MDM2 result in embryonic lethality at day 3.5, mainly due to uncontrolled apoptosis induced by p53. This lethality is rescued in a p53 null background, indicating that the primary role of MDM2 is to control p53 activity (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). Other conditional knockout mouse models have shown some tissue specificity with regard to MDM2 function. Conditional deletion of MDM2 in osteoblasts results in severe skeletal defects during development and embryonic lethality, suggesting MDM2 is required to control p53 activity in these cells (Lengner, Steinman et al. 2006). MDM2 knockout in cardiomyoctyes, red blood

36

cells, smooth muscle cells and in the central nervous system all lead to p53-dependent lethality at birth, suggesting MDM2 plays a critical role in p53 regulation in multiple cell types (Boesten, Zadelaar et al. 2006; Grier, Xiong et al. 2006; Xiong, Van Pelt et al. 2006; Maetens, Doumont et al. 2007).

Mice with a hypomorphic allele that expressed 30% of the total MDM2 levels have decreased body weight, defects in hematopoeisis and increased radiosensitivity due to decreased p53 inhibition (Mendrysa, McElwee et al. 2003). Also in a p53-dependent manner, MDM2 heterozygous mice are more resistant to lymphoid tumor development when crossed to an Eµ-Myc mouse model (Alt, Greiner et al. 2003). Mice overexpressing MDM2 in the mammary epithelium were prone to tumor formation and increased hypertrophy, as well as reducing the anti-tumor suppressor functions of p53 (Lundgren, Montes de Oca Luna et al. 1997; Jones, Hancock et al. 1998).

MDM2 Interacting Proteins

ARF

The ARF tumor suppressor is a protein that is transcribed from an alternate reading frame of the INK4a/ARF locus (CDKN2A) (**Figure 9)**. The gene is located on the short arm of chromosome 9 and translated into a 14kDa, 132 amino acid protein. ARF is a highly basic and hydrophobic protein with more than 20% of its amino acids being arginine, making it a largely unstructured protein unless bound to other targets (Sherr, 2001). It therefore makes sense that ARF interacts with the acidic domain of MDM2. This interaction directly inhibits MDM2 E3 ligase activity (Honda and Yasuda 1999). ARF binding also causes MDM2 to be sequestered into the nucleolus, while p53 remains in the nucleoplasm, therefore blocking MDM2 mediated exportation of p53 from the cell nucleus to the cytoplasm for degradation (Tao and Levine 1999; Weber, Taylor et al. 1999).
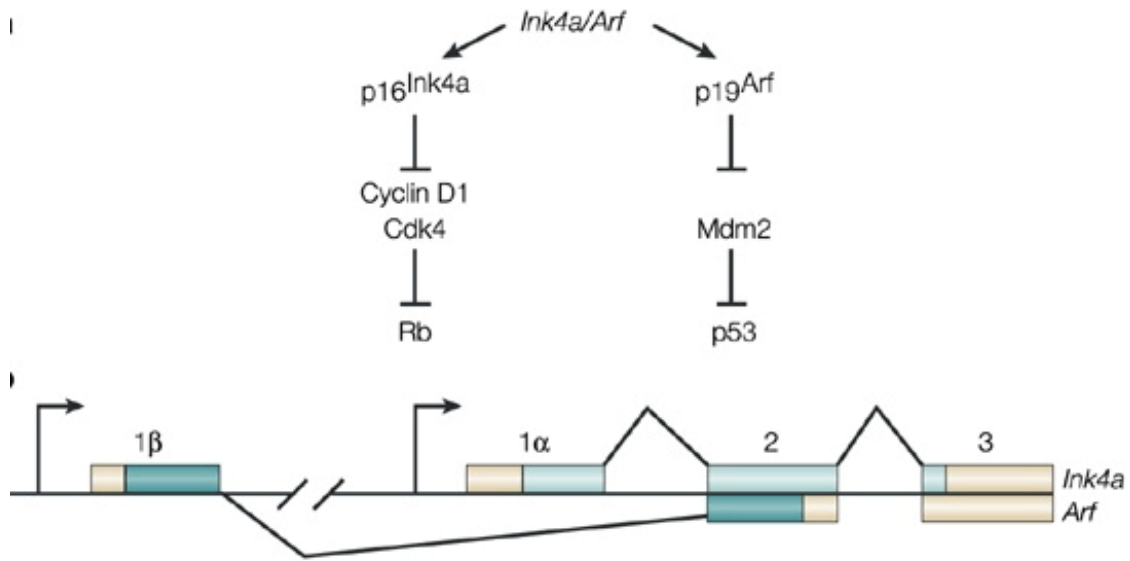
Figure 9: Ink4a/ARF locus. The ARF tumor suppressor is transcribed from an alternate reading frame of the INK4a/ARF locus. Following mitogenic stress ARF expression is increased. ARF then binds and antagonizes MDM2, permitting the transcriptional activation of p53 (Sherr, 2001).

ARF expression is increased following aberrant mitogenic stimulation, such as overexpression of MYC or Ras or enforced E2F expression. By antagonizing MDM2, ARF permits the transcriptional activity of p53, which leads to cell cycle arrest and apoptosis. Therefore, loss of function in ARF is commonly associated with cancer (Lowe and Sherr, 2003). In fact, the INK4a/ARF locus is deleted or silenced in many kinds of tumors. For example, 41% of breast carcinomas have p14ARF defects, while 32% of colorectal adenomas have p14ARF inactivation due to hypermethylation of the promoter (Yi, Shepard et al. 2004). Mouse models that lack p19^{ARF} are also more prone to tumor development. This is because without ARF, MDM2 inappropriately inhibits p53, leading to increased cell survival. Therefore, ARF has become a popular target in the search for novel cancer treatments. Some examples would include small molecular compounds that would act as ARF and bind to MDM2 in cells with INK4a/ARF deletions, or treatments that could reverse hypermethylation in cancers with silenced INK4a/ARF loci.

SUV39H1

Histone-lysine N-methyltransferase SUV39H1 is an enzyme that, in humans, is encoded by the SUV39H1 gene (Aagaard, Laible et al. 1999). The SUV39H1 gene is the human ortholog to the *Drosphila* Su(var)3-9 histone methyltransferase. Mice and humans also express a second isoform, SUV39H2 (O'Carroll, Scherthan et al. 2000). The gene encodes a protein with a chromodomain and a C-terminal SET domain. The chromodomain is about 40-50 amino acid residues in length and found in proteins associated with remodeling and manipulation of chromatin. These domain-containing proteins can recognize and bind methylated histones (Nielsen, Nietlispach et al. 2002). The function of the SET-domain (130 aa long) is to transfer a methyl group from *S*-adenosyl-L-methionine (AdoMet) to the amino group of a lysine residue on a histone or another protein. This serves as a post-translational epigenetic modification that can then control the expression of genes or the activity of proteins. It can also lead to recruitment of certain protein complexes that direct the organization of chromatin, or can influence protein function (Dillon et al., 2005).

Mouse knockout experiments have shown that the SUV39H1/2 genes are required for viability and proper H3 K9 tri-methylation at heterochromatin regions (Peters, O'Carroll et al. 2001). Overall, SUV39H1 plays a vital role in heterochromatin organization, chromosome segregation and mitotic progression. SUV39H1 null mice are viable but tumor prone (Peters, O'Carroll et al. 2001). Recently, our lab has shown that MDM2 recruits SUV39H1 through binding of the methyltransferase to the MDM2 acidic domain, leading to the formation of a trimeric complex between MDM2, SUV39H1 and p53 (Chen, Li et al. 2010). This interaction promotes histone H3 K9 methylation at p53 target promoters, leading to transcriptional repression of p53 target genes (**Figure 10**). Knockdown of SUV39H1 following DNA damage can further enhance p53 transcriptional output. Likewise, overexpression of the tumor suppressor ARF inhibits the MDM2-SUV39H1 interaction, leading to functionally activated p53. So far, SUV39H1

has shown increased expression in 25% of primary colorectal cancers indicating it has proto-oncogene characteristics (Kang, Lee et al. 2007).
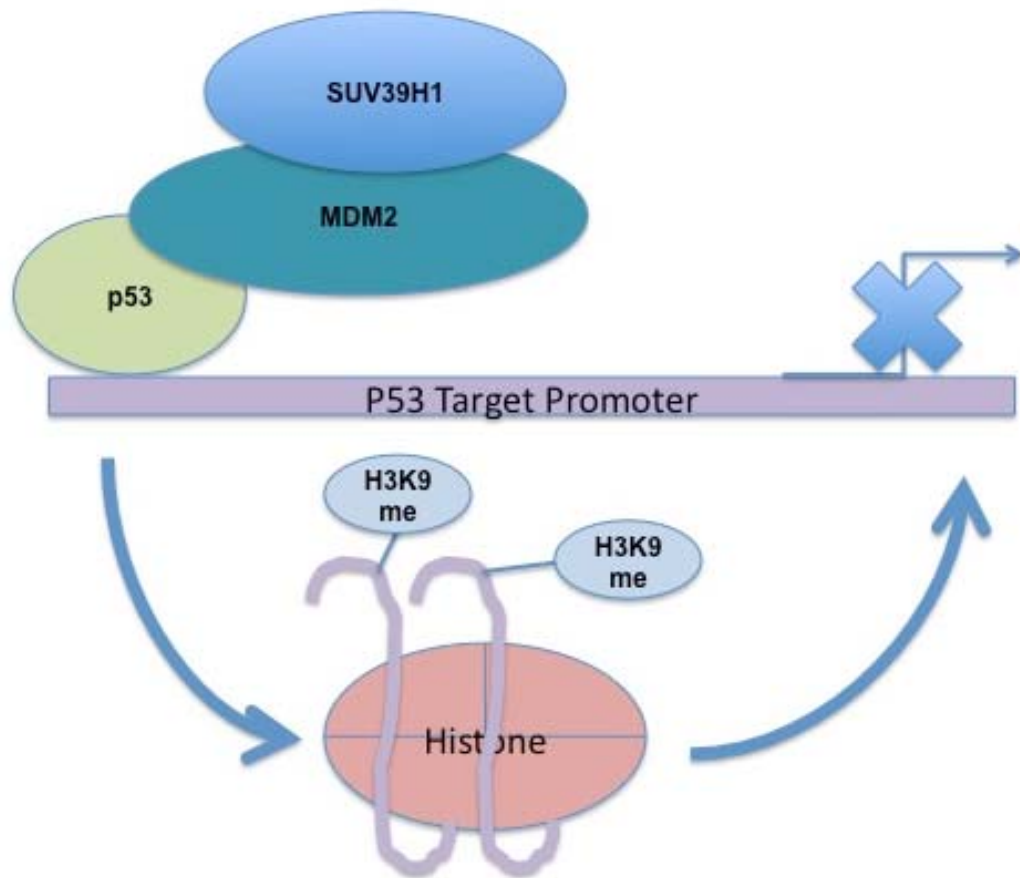
Figure 10: SUV39H1-MDM2 Interaction. SUV39H1 binds to the MDM2 acidic domain, creating a trimeric protein complex (SUV39H1-MDM2-p53). This interaction promotes histone H3K9 methylation at p53 target promoters, leading to transcriptional repression of p53 target genes.

Oncogene MDMX

History of MDMX

In 1996, through a mouse cDNA library screen, a p53 binding protein that shared 60% homology at the DNA and protein level with MDM2 was discovered. The protein was named MDMX (murine double minute X), also known as MDM4, (Shvarts, Bazuine et al. 1997). Although MDMX is structurally similar to MDM2, it

does not have intrinsic E3 ligase activity, nor does it promote p53 degradation (Stad, Little et al. 2001). However, MDMX has emerged as another critical regulator of p53 and overexpression of MDMX leads to suppression of p53 transcriptional activity following stress imposed by DNA damage, aberrant mitogenic signaling and ribosomal stress. Early studies also found that MDMX is constitutively expressed during cell proliferation, differentiation and stress (Jackson and Berberich 1999). In addition, overexpression of MDMX resulted in cellular transformation when the H-Ras oncogene was also co-expressed (Danovi, Meulmeester et al. 2004).

Several studies have implicated MDMX functions as oncogenic and implicated it in tumor formation. HDMX is located on chromosome 1q32, which is an area frequently aberrant in cervical and ovarian carcinomas (Danovi, Meulmeester et al. 2004). A subset of gliomas that were wild-type for p53 were found to have amplifications of MDMX, not MDM2 (Riemenschneider et al., 1999). Increased MDMX levels were also found in pre-B acute lymphoblastic leukemias, tumor cell lines and primary tumors that retained wild-type p53 (Ramos, Stad et al. 2001; Han, Garcia-Manero et al. 2007). Notably, elevated MDMX levels were found in 65% of retinoblastomas containing wild-type p53 (Laurie, Donovan et al. 2006). These data indicate that there is a clear link between MDMX overexpression and p53 suppression, making mutations in p53 unnecessary for tumor formation.

MDMX Structure and Function

The MDMX gene has 11 exons that encodes a protein of 490 amino acids in length. MDMX and MDM2 share significant homology in several functional domains (**Figure 11**). The highest homology is located in the N-terminal p53-binding domain (54.6%, aa 42-94). The residues required for interaction with p53 are conserved between the two proteins (Shvarts, Steegenga et al. 1996). Binding of MDMX to p53 occurs at the N-terminus of both proteins and inhibits p53 transactivation function (Jackson and Berberich 2000; Stad, Little et al. 2001).
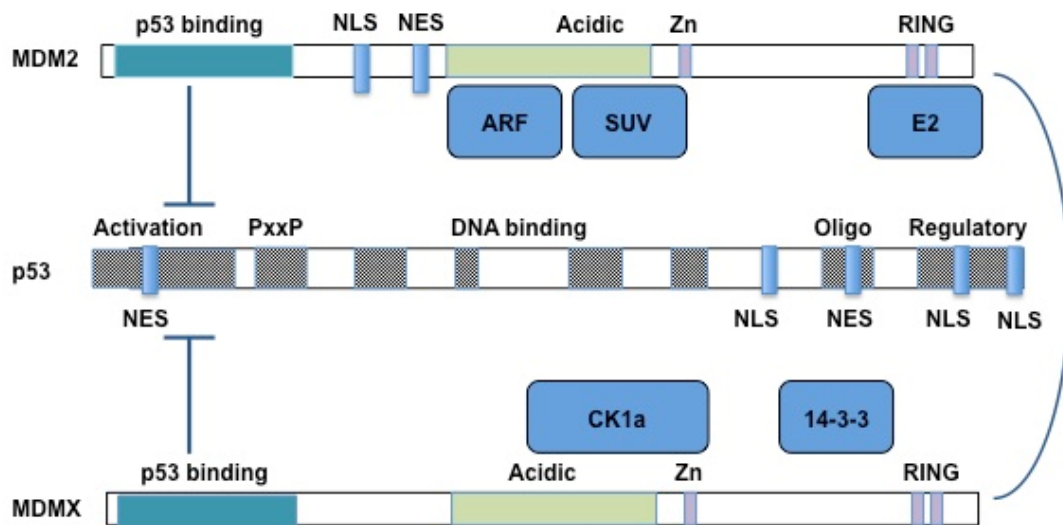
Figure 11. Structure Homology of MDM2 and MDMX. Both proteins contain N-terminal p53-binding domains, central acidic domains, zinc finger domains and form heterodimers through their RING finger domains. Unlike MDM2, MDMX does not have distinct nuclear localization and nuclear export signals.

The central domain of MDMX shares little sequence homology with MDM2, yet it does contain several acidic residues (Parant, Reinke et al. 2001). The MDMX acidic domain, to date, has a relatively unknown function. A MDM2 protein containing the MDMX acidic domain is incapable of rescuing the loss of degradation that occurs with the deletion of the MDM2 acidic domain (Kawai et al., 2003). The binding of casein kinase 1 alpha (CK1α) to MDMX, not MDM2, occurs at the zinc finger domain and phosphorylates Ser-289 in the acidic domain, leading to an enhanced MDMX:p53 interaction (Chen et al., 2005c). ARF interaction with the MDMX acidic domain is somewhat controversial. Some studies suggest that the binding of ARF to the MDMX acidic domain can translocate MDMX to the nucleolus, while another study suggests that there is no direct binding between MDMX and ARF (Jackson, Lindstrom et al. 2001; Wang, Arooz et al. 2001). Therefore, there is still work to be done in determining the exact function of the MDMX acidic domain.

The zinc finger domain (aa 301-329) shares 41.9% homology to MDM2 (Marine et al., 2007). The function of this domain is largely unclear, however, a p53-induced caspase cleavage site lies between the zinc-finger and RING finger domains that regulates MDMX stability (Gentiletti, Mancini et al. 2002).

The RING domain (aa 444-483) shares 53.2% homology with MDMX (Marine et al., 2007). MDMX forms heterodimers with MDM2 through the C-terminal RING:RING interactions (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999). It was long thought the MDMX itself does not contain a functional nuclear localization or nuclear export signal, and depends on binding to MDM2 through the RING domain to localize to the nucleus (Migliorini, Lazzerini Denchi et al. 2002). However, a recent study by our lab has shown that, in response to DNA damage, 14-3-3 binds MDMX revealing a cryptic nuclear localization signal and leading to MDMX translocation to the nucleus (LeBron, Chen et al. 2006). A major difference between the MDM2 and MDMX RING domain is that MDMX lacks E3 ligase activity. Yet although MDMX does not have intrinsic E3 ligase activity, it can enhance the ability of MDM2 to ubiquitinate substrates, including p53 (Badciong and Haas 2002). Recent studies have found that the key difference between the MDM2 and MDMX RING lies in the extreme C-terminal residues, and the mutation of these key amino acids abrogates MDM2 E3 ligase activity, which interestingly is rescued by oligomerization with MDMX (Singh, Iyappan et al. 2007; Uldrijan, Pannekoek et al. 2007).

At the genomic level, the 5' ends of MDM2 and MDMX are quite distinct. Unlike MDM2, the expression of MDMX is not dependent on p53 activity as it does not contain a p53 responsive element (Parant, Reinke et al. 2001). However there have been recent reports indicating that MDMX is a weak p53 responsive gene (Li et al., 2010). Unlike MDM2 the mRNA levels of MDMX do not correlate with a stress response, suggesting the MDMX has a different regulatory mechanism (Jackson and Berberich 1999). In fact, following stimulation the mitogen-activated protein kinase pathway upregulates MDMX

mRNA levels via activation of c-Ets-1 transcription factors (Gilkes, Pan et al. 2008).

MDMX-p53-MDM2 Interaction

The importance of MDMX was established when MDMX-null mice were found to be embryonic lethal, and that this phenotype can be rescued by crossing to the p53-null background. This process suggests a unique role in the regulation of p53 (Parant, Reinke et al. 2001; Migliorini, Lazzerini Denchi et al. 2002). MDMX was first reported to inhibit p53-induced transcription following ectopic expression on p53 target genes (Shvarts, Steegenga et al. 1996). MDMX does this by binding to the same amino acids in the transactivation domain that are required for MDM2 binding and are located at the N-terminus of p53 (Bottger et al., 1999). This binding suggests that, similar to MDM2, MDMX can inhibit p53 transcriptional activity by interfering with the ability of p53 to interact with basal transcription machinery, recruitment of coactivators and binding to target promoters. Indeed, in MDM2 null cells or cells with MDMX unable to bind MDM2, MDMX abrogates CBP/p300 acetylation of p53, resulting in the stimulation of p53 activation (Sabbatini and McCormick 2002; Danovi, Meulmeester et al. 2004).

MDM2 binds MDMX through RING:RING domain interactions, resulting in changes to the MDM2-p53 autoregulatory feedback loop. It is well known that p53 transcriptionally activates MDM2, which negatively regulates p53 by inhibiting its transactivation and affecting its stabilization through ubiquitin-dependent proteosomal degradation. Although MDMX does not contain intrinsic E3 ligase activity, it has been shown in some studies to stabilize MDM2 by blocking auto-ubiquitination. This relationship leads to an increase in MDM2 ubiquitination and degradation of p53 (Stad, Little et al. 2001; Badciong and Haas 2002; Gu, Kawai et al. 2002). However, other studies have shown that knockdown of MDMX via siRNA in certain cancer cell lines either increased or had no significant effect on MDM2 or p53 levels and ubiquitination (Jackson and Berberich 2000; Stad, Little et al. 2001; Danovi, Meulmeester et al. 2004).

Notably, MDMX itself is also a target of MDM2 and is polyubiquitinated by MDM2, followed by proteosomal degradation (Pan and Chen 2003).

It seems that MDMX works along side MDM2 to maintain correct p53 stability and transcriptional activity. The discrepancies in the above findings suggest that the key factor is the ratio of MDMX to MDM2 when determining the overall effect of the proteins on p53. When the MDMX to MDM2 ratio is lesser or equal to 1, p53 preferentially undergoes MDM2-dependent proteosomal degradation. When MDMX expression is greater than MDM2, MDMX inhibits this process. Because MDM2 and MDMX share a binding site on p53, when MDMX is overexpressed it competes with MDM2 for p53 binding, rendering p53 more stable (Jackson and Berberich 2000). This scenario leads to MDMX inhibiting p53 transcriptional activity, independent of MDM2 (Marine and Jochemsen, 2005). The importance of these ratios and their ability to work together to correctly regulate p53 is also evident in a number of mouse model studies. These studies show neither protein can compensate for the other in regulating p53 function. Likewise, studies of the CNS with knockouts to both MDM2 and MDMX showed a more dramatic phenotype than single knockouts (Xiong, Van Pelt et al. 2006). However, in conditional knockout systems it seems that MDMX is only required for the inhibition of p53 in a subset of cell types and only under certain physiological conditions. In fact, one study found that the overexpression of the MDM2 transgene could compensate for the embryonic lethality that occurs in the MDMX knockout mice, however, the opposite remains embryonically lethal (Steinman, Hoover et al. 2005).

In the absence of cellular stress it seems that the primary function of MDM2 is to maintain p53 at low levels, whereas the function of MDMX is to inhibit p53 transcriptional activity. When DNA damage or other stresses occur, p53 is post-translationally modified, which leads to the inhibition of the MDM2-p53 interaction and p53 stability. Although MDM2 is a direct target of p53 transcription, there is a lag between DNA damage p53 activation and an increase in MDM2 protein levels, possibly because of the p53-MDMX interaction. Our lab has shown that, following cellular stress, the level of p53 activation is inversely

correlated with the amount of MDMX in these cells due to the formation of inactive p53-MDMX complexes (Chen, Gilkes et al. 2005; Gilkes, Chen et al. 2006). Conversely, MDMX become very unstable following DNA damage. This is due to phosphorylation, enhanced degradation via MDM2 ubiquitination and not being transcriptionally induced by p53.

The MDM2-MDMX-p53 interaction also influences MDMX localization. Exogenous MDMX is mainly localized in the cytoplasm (Rallapalli, Strachan et al. 1999). Co-expression of MDM2 recruits MDMX into the nucleus, independent of p53, but does require the RING finger domain of both proteins and the NLS of MDM2 (Migliorini, Danovi et al. 2002). Another study reports that MDMX is targeted to the nucleus in a p53-dependent, MDM2-independent manner (Li, Chen et al. 2002). However, this same study showed that MDMX nuclear entry was also observed following DNA damage in p53/MDM2 null MEFs, suggesting that MDMX nuclear localization can also be independent of both p53 and MDM2.

MDMX Regulation

DNA Damage

The primary mechanism of MDMX regulation occurs at the post-translational level. The destabilization of both MDMX and MDM2 are required for proper p53 response to DNA damage. Although MDMX expression is constant under non-stressed conditions, there are dramatic changes to the MDMX protein levels in response to stress. Not only is MDMX ubiquitinated and targeted for degradation via MDM2 following DNA damage, but phosphorylation also plays a key role in efficient degradation (**Figure 12**). Activation of ATM in response to DNA damage leads to phosphorylation on Ser-403 and activation of downstream kinases, such as Chk2, phosphorylates Ser-342 and 367 (Chen et al., 2005b; Pereg et al., 2005). DNA damage by ultraviolet radiation results in Chk1 phosphorylation at Ser-367 (Jin et al., 2006). Phosphorylation led to increased MDMX-MDM2 binding, followed by ubiquitination and degradation of MDMX. The increase in MDM2-MDMX binding was necessary for p53 activation following DNA damage since cells overexpressing MDMX were unable to undergo cell cycle arrest. (Chen et al., 2005b). Interestingly, phosphorylation at Ser-367 led

to 14-3-3 binding, yet did not lead to direct MDMX degradation. The phosphorylation does, however, stimulate nuclear import of MDMX by exposing a cryptic NLS region where it is preferentially degraded (LeBron et al., 2006). Another suggestion is that following ionizing radiation, the 14-3-3 binding prevents the association of HAUSP with MDMX, rendering it less stable (Meulmeester et al., 2005a; Meulmeester et al., 2005b). However, in response to UV radiation, where Chk1 leads to 14-3-3 binding, MDMX is sequestered mainly in the cytoplasm (Jin et al., 2006). Although there are multiple pathways, these mechanisms explain the ability of DNA damage to preferentially inactivate MDMX and stimulate p53 transcription.
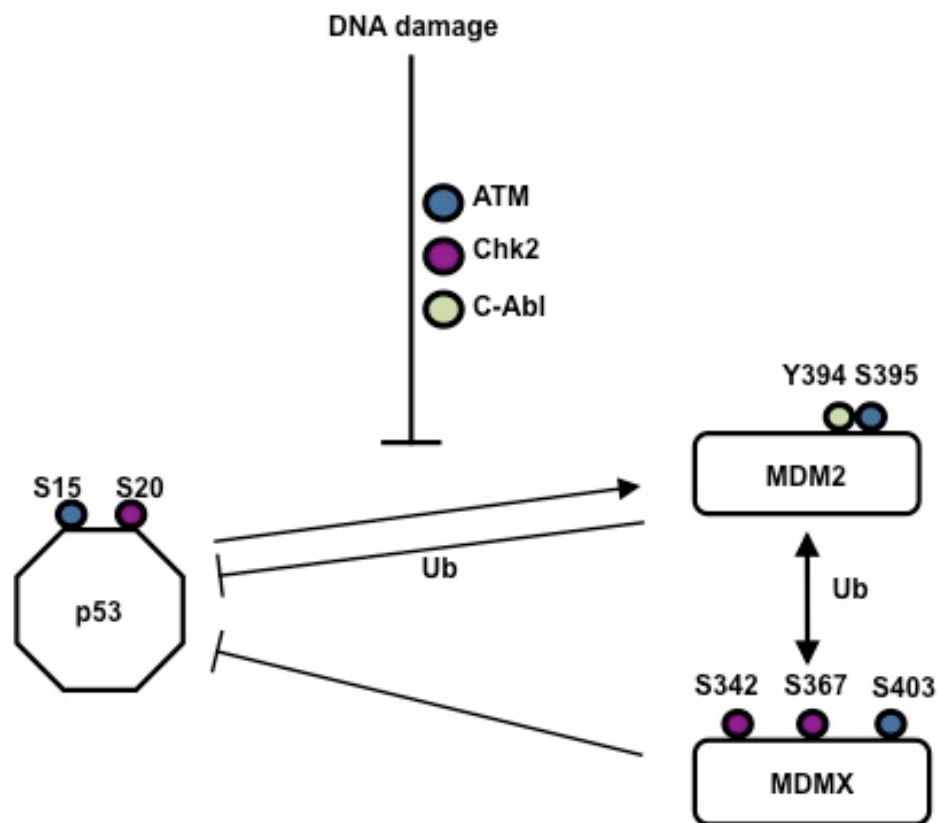
Figure 12: **Regulation of MDMX.** Following DNA damage protein kinases phosphorylate p53, MDM2 and MDMX on the indicated residues. MDM2-MDMX binding increases leading to MDMX ubiquitination and degradation. MDM2 also undergoes auto ubiquitination. This leads to enhanced p53 stability and transcriptional activity.

There have been reports of other kinases phosphorylaing MDMX. Ck1α phosphorylates MDMX on Ser-289 and stimulates MDMX-p53 binding (Chen et al., 2005c). CDK2 phosphorylates Ser-96 and results in nuclear export of MDMX and consequently the export of MDM2 (Elias et al., 2005). Growth factor stimulation of the PI-3 kinase/AKT pathway leads to phosphorylation at Ser-367, 14-3-3 binding and stabilization of MDMX and MDM2 (Lopez-Pajares et al., 2008).

Oncogenic Stress/ARF Induction

Mitogenic stimulation can also regulate MDMX protein levels. Our lab has shown that, following oncogenic stress, ARF binds to MDM2 selectively and blocks p53-ubiquitination, yet promotes ubiquitination of MDMX (Pan and Chen, 2003) (**Figure 7**). In cells overexpressing MDMX there is a reduction of p21 and cell cycle arrest following E2F activation of ARF. MDMX knockdown has the opposite effect, sensitizing the cells to ARF-induced cell cycle arrest. Another study showed that MDMX gene amplification occurs in preneoplastic retinoblastoma cells, causing the p53-MDM2-ARF pathway that is usually activated following Rb inactivation to become inactive. Cells with the amplified MDMX had a growth advantage over those with an intact ARF-MDM2-MDMX-p53 pathway resulting in retinoblastoma development (Laurie et al., 2006). These studies highlight the importance in finding inhibitors specifically for the MDMX-p53 interaction.

Ribosomal Stress

Recent studies have shown that the p53 response to ribosomal stress is another important factor in tumor suppression due to aberrant rRNA and biogenesis sensed by p53 (Lohrum et al., 2003; Marechal et al., 1994; Zhang et al., 2003). Following the addition of Actinomycin D, a drug that induces ribosomal stress, there is an activation of p53 leading to induction of p21 and MDM2 target proteins. However, the MDMX levels decrease due to increased L11 stimulating MDM2 to ubiquitinate MDMX (**Figure 8**). This process can be blocked by overexpression of MDMX in both cell culture and xenograft models

(Gilkes et al., 2006). These observations suggest that MDMX plays an important role in regulating the p53 response to ribosomal stress.

MDMX Mouse Models

The physiological importance of MDMX negative regulation of p53 was characterized by the embryonic lethality of MDMX null mice at day 8.5, and this lethality can be rescued by a concomitant p53 deletion (Parant et al., 2001). This lethality resulted from a severe proliferation deficiency, rather than the uncontrolled apoptosis seen in MDM2 null mice. These results also suggest that MDM2 and MDMX have non-redundant roles, as they do not compensate for the loss of each other. In these models MDMX contributes to p53 dependent regulation of both proliferating and quiescent cells (Francoz et al., 2006).

Studies have also examined the role of MDM2 and MDMX using tissue-specific knockout systems, some of which were described earlier. While MDM2 appears to be important in a majority of different cell types, the only severe phenotype observed when MDMX was knocked out occurred in neuronal cells and in the CNS (Boesten et al., 2006; Migliorini et al., 2002; Xiong et al., 2006). Knockout of MDMX in cardiomyocytes and smooth muscle cells of the GI tract only caused minor defects (Boesten et al., 2006; Migliorini et al., 2002; Xiong et al., 2006).

Despite some increased apoptosis observed in neuronal cells that lack MDMX, it is believed that the primary role of MDMX is to regulate cell cycle arrest specific p53 transcriptional activity. In support of this, concomitant deletion of the CDK inhibitor, p21, partially rescued the lethality of MDMX knockout mice in these cells (Steinman et al., 2004). It is thought that these mice would retain wild-type p53 transcription capabilities and stimulate MDM2 expression, thus leading to higher MDM2 levels that can compensate for the loss of MDMX. In fact, this has been observed in transgenic MDM2 mice that rescue the loss of MDMX (Steinman et al., 2005). This phenotype and the difference between the MDM2 and MDMX null phenotypes seems to result from the fact that loss of MDM2 leads to the accumulation of the p53 protein, whereas loss of MDMX does not significantly increase p53 levels in vivo.

MDMX interacting Proteins

CK1α

Casein kinase I (CKI) was one of the first serine/threonine protein kinases to be isolated and characterized, and functions as a regulator of multiple signal transduction pathways. CK1 is a protein that is highly conserved from *Saccharomyces cerevisiae* to the human and has been genetically linked to the regulation of DNA repair, cell cycle progression and cytokines in yeast (Gross and Anderson, 1998). There are seven mammalian isoforms that have been cloned and characterized, including: α, β, γ1, γ2, γ3, δ and ε (Rowles et al., 1991). CK1α is expressed equally in all tissues and detected in all cellular compartments (Zhang et al., 1996). In addition to containing a catalytic domain the CK1α protein posseses a small carboxyl terminus area. Following DNA damage p53 can be phosphorylated by CK1α in vitro, provided that p53 is modified at Ser-15 by ATM (Knippschild et al., 1997). Other isoforms of the CK1 family of proteins have been linked to phosphorylation of p53 and MDM2 upon cellular stress, resulting in a weakening of their interaction (Knippschild et al., 2005). Recent studies by our lab have shown that CK1α is a novel MDMX-binding protein. Specifically, it binds to the zinc finger domain of MDMX and stimulates MDMX inhibition of p53 by increasing the formation of the MDMX-p53 complex. The binding of CK1α also leads to phosphorylation on Ser-289, although the significance of this is yet to be determined (Chen et al., 2005c). These observations suggest that the CK1 family kinases are potential drug targets for sensitizing p53 to DNA damaging agents.

MATERIALS AND METHODS

Cell Lines and Plasmids

MDM2, MDMX, p53, ARF and SUV39H1 constructs used in this study were of human origin. MDM2-MDMX hybrid constructs were described previously (Kawai et al., 2003). Human pCIN4-HA-FLAG-p53 was kindly provided by Dr. Wei Gu (Brooks et al., 2007). NARF6 (U2OS expressing IPTG inducible ARF) was provided by Dr. Dawn Quelle. MDM2 and MDMX deletion mutants were generated by PCR amplification and subcloning. H1299 (non-small cell lung carcinoma, p53-null), U2OS (osteosarcoma, wild-type p53), NARF6, SJSA, (osteosarcoma, wild-type p53, amplified MDM2), DLD1 (colon carcinoma, mutant p53), MDA-231 (human breast adinocarcinoma, mutant p53), HCT116 +/+ (human colon carcinoma, wild-type p53), and HCT116 -/- (human colon carcinoma, p53 null) and H1299-V138 (H1299 stably transfected with temperature-sensitive mutant p53-V138) were maintained in DMEM with 10% fetal bovine serum.

Drug Treatments

MDM2 inhibitor Nutlin (Cayman) was used at 5–10 µM and the cells were incubated for 5h. Proteosomal inhibitor Velcade (Selleck) was used at 50 nM (unless otherwise indicated) and the cells were incubated for 5 h. Proteosomal inhibitor MG132 (BIOMOL Research) was used at 30 uM for 5h.

Transfections

Calcium Phosphate

Calcium phosphate transfections were performed in H1299 cells due to their high transfection efficiency and the fact that they are p53 null with undetectable levels of MDM2 and MDMX. In transient transfection assays > 2 x 10^6 cells were seeded into 10 cm tissue culture dishes for 24 h. For each transfection 40 ug of plasmid DNA was mixed with 450 ul of H_2O and 125 mM

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calcium chloride. A mixture of 500 ul of HEPES (0.28 M NaCl, 0.05 M HEPES, 1.5 mM CaCl$_2$) was bubbled with air and the water/DNA mixture was added dropwise to the HEPES. After a 10 minute incubation the mixture was added to the cells and incubated for 16 h. After incubation the transfected cells were washed 3 times with PBS and refed with complete medium. The cell cultures were then incubated for another 10 h before harvest.

Lipofectamine

For the Lipofectamine^{TM} 2000 (Invitrogen) transfection experiments 5 x 10^4 U2OS cells were seeded into 24 well tissue culture plates for 24 h. The following day the cells were washed once with serum free media and refed with 0.4 ml of serum free media. For each transfection a total amount of 0.6 μg of plasmid DNA was mixed with 37.5 μl of Opti-MEM I Reduces Serum Medium. Lipofectomine 2000 was diluted (1.4 μl) in 37.5 μl of the Opti-MEM I Reduces Serum Medium and incubated for 5 min. After incubation the lipofectomine mix was combined with the diluted DNA (total volume = 75 μl) and incubated for 20 min at room temperature. The mixture was added to each well and mixed gently by rocking the plate back and forth at room temperature. The cell culture plates were then incubated at 37°c for 20 h prior to testing for transgene expression.

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Protein Analysis

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Western Blot

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. Cell lysate (10 to 50 μg of protein) was fractionated by sodium dodecyl sulfate (SDS)-polyacrylammide gel electrophoresis and transferred to Immobilion P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.1% Tween 20. The filter was then incubated overnight with primary antibodies diluted in blocking buffer.   The filter was washed three times (5 min each) with PBS containing 0.1% Tween-20. Next, bound primary antibodies were conjugated with secondary antibody horseradish peroxidase IgG goat-anti-mouse or goat-anti-rabbit by incubating the filter with

the secondary antibody diluted in blocking buffer for two hours. The filter was then washed three times (5 min each) with PBS containing 0.1% Tween-20. The filters were developed using either Supersignal (Pierce) or ECL-plus reagent (Amersham Biosciences). The following antibodies were used:

- Human p53 was detected using DO-1 (mouse, Pharmigen) with 1:5,000 dilution or FL393 (rabbit, Santa-Cruz) with a 1:10,000 dilution

- Human MDM2 was detected using monoclonal mouse 5B10, 3G9 or 4B2 with a 1:40 dilution or a rabbit polyclonal antibody at a 1:20,000 dilution

- Human MDMX was detected using monoclonal mouse 10G11 or 8C6 with a 1:40 dilution or a rabbit polyclonal antibody at a 1:20,000 dilution

- ARF was detected by 14P02 (mouse, Neomarkers) with a 1:1,000 dilution

- FLAG-tagged proteins were detected with an α-FLAG monoclonal mouse antibody (Sigma) at a 1:5000 dilution and a rabbit polyclonal antibody with a 1:10,000 dilution

- Myc-tagged proteins were detected (Sigma) with mouse 9B11 antibody at a 1:40 dilution and a rabbit polyclonal antibody at a 1:10,000 dilution

- P21 was detected using anti-WAF1 (BD Bioscience) at a 1:1,000 dilution

- PUMA was detected using NT (ProScilnec) at a 1:1,000 dilution

- Actin was detected using a mouse monoclonal antibody (Sigma) at a 1:30,000 dilution

Immunoprecipitation

Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 150mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride), centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. Cell lysate (200-1000 µg of protein) was immunoprecipitated with 30 µl slurry of protein A agarose beads (Sigma) and MDM2 antibodies overnight at 4°C. For immunoprecipitations using conformation-specific p53 antibodies the lysate was divided into equal halves and each immunoprecipitated for 18 h at 4°C with wild-type conformation specific antibody (Pab1620) or mutant conformation specific antibody (Pab240). For immunoprecipitation of FLAG tagged proteins the lysate was incubated with 50 µl slurry of M2-agarose beads overnight at 4°C (Sigma).

The beads were washed three times with lysis buffer, boiled in SDS sample buffer, fractionated by SDS-PAGE and analyzed by Western blot.

DNA Binding Assays

Chromatin Immunoprecipitation

Two confluent 10 cm plates per cell line ($2 \times 10^7 – 2 \times 10^8$ cells) were used per sample, one for the chromatin immunoprecipitation assay and one for protein analysis via Western blot. Formaldehyde was added directly to tissue culture media to a final concentration of 1% and incubated on a shaking platform for 10 minutes at room temperature. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125 M and mixing for 5 minutes. The plates were rinsed twice with cold PBS plus protease inhibitors and PMSF, scraped and centrifuged to collect. The pellets were resuspended in 0.45 ml of RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium doexycholate) and incubated on ice for 10 min. Next, samples were sonicated to an average chromatin length of about 500-1000 base pairs and then centrifuged at 14,000 rpm for 1 minute at 4°c. The supernatant was transferred to a new tube, precleared by adding 60 μl of protein A agarose/salmon sperm DNA beads (Millipore) and incubated on a rotating platform at 4°c for 30 min. Samples were centrifuged at 4,000 rpm for 1 minute and divided into the appropriate aliquots for immunoprecipitation and 50 μl per sample was set aside for input control. The protein-DNA complexes from transiently transfected H1299 cells were immunoprecipitated with 2 μg DO-1 antibody for p53 or 9B11 for MYC tagged proteins. The final volume of each sample was adjusted to 1 ml using IP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.0, 167 mM NaCl). Control samples that were used for experiments included an untransfected plate of p53 null-H1299 cells, as well as samples that were immunoprecipited with 2 μg of IgG specific antibody. Samples were incubated on a rotating platform overnight at 4°c.

The following day a 40 μl slurry of protein A agarose/salmon sperm DNA beads (Millipore) was added and incubated on a rotating platform overnight at 4°c. Samples were centrifuged at 4,000 rpm for 1 min at room temperature.

Beads were washed one time in low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl), one time in high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl), one time in LiCl buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0) and two times in TE (1 mM EDTA, 10 mM Tris-HCl pH 8.0). For each wash samples were rotated for 3 minutes at 4°c and then centrifuged at 4,000 rpm for 1 min at room temperature. Chromatin was eluted two times by added 100 µl elution buffer (1% SDS, 0.1 M $NaHCO_3$) while shaking at 1000 rpm for 15 min. To the input control 150 µl of elution buffer was also added. The elutes were combined and then centrifuged at 4,000 rpm for 1 min at room temperature to remove any remaining beads and transferred to a new tube. The elutes received an addition of 0.3 M NaCl and were incubated at 65°c overnight, shaking at 650 rpm, to reverse the crosslinking. The following morning 1 ul of a 10 mg/ml RNAse A solution was added and the elutions were incubated for 30 min at 37°c, shaking at 640 rpm. Next, 16 µl of 1.0 M Tris-HCl pH 6.8, 2 µl 1 M EDTA and 1 µl of 10 mg/ml proteinase K were added per sample and incubated at 45°c for 2 h. The DNA was purified using the Qiagen PCR purification kit and reconstituted in 50 µl of water. The samples were then further diluted 1:4 in water and then 4 µl was utilized per PCR reaction. For p53, ARF and SUV39H1 promoter binding, samples were subjected to SYBR Green real-time PCR analysis using the following forward and reverse primers: (5'AGGAAGGGGATGGTAGGAGA and 5'ACACAAGCACACATGCATCA) to amplify the human p21 promoter and (5'CTGTGGCCTTGTGTCTGTGAGTAC and 5'CCTAGCCCAAGGCAAGGAGGAC) to amplify the human PUMA promoter, both containing the p53-binding site. The readouts were normalized using 10% input chromatin for each sample [ΔCt [normalized ChIP] = ($Ct_{[ChIP]}$-($Ct_{[input]}$ – Log_2(input dilution factor)))]. The % Occupancy was then calculated for each ChIP fraction [% Occupancy =$2^{(-\Delta Ct\ [Normalized\ ChIP])}$]. Samples were analyzed in triplicate and standard deviation was calculated [% Occupancy x (($2^{Ct\ Dev.}$)-1)]. Error bars represent mean ± standard deviation. Each sample was treated with

the indicated 'n' values and the difference between each condition was determined by calculating the p values using a two-tailed student's t-test.

DNA Affinity Immunoblotting

H1299 cells were transiently transfected with FLAG tagged MDM2, MDMX and p53, along with Myc-tagged ARF and SUV39H1 when indicated. Cells from a 10 cm plate were lysed in 1 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1 mM phenylmethylsulfonyl fluoride], centrifuged for 10 minutes at 14,000 x g and the insoluble debris was discarded. The lysate was incubated with 40 µl slurry of M2-agarose beads (Sigma) for 18 h at 4°C. The beads were washed with lysis buffer and the FLAG-tagged proteins with their binding partners were eluted with 150 µl of lysis buffer containing 50 µg/ml FLAG epitope peptide (Sigma) for 2 h at 4°C. An aliquot of the eluted proteins were analyzed for expression levels by western blot. Lysates containing equal levels of p53 were added to a 200 µl DNA binding reaction mixture and incubated at 4°C for 30 min. The DNA binding reaction mixture contains 25 nM double stranded biotinylated oligonucleotide DNA representing the p53 binding site at the p21 promoter (Biotin-5'-TCGAGAGGCATGTCTAGGCATGTCTC annealed with 5'-GAGACATGCCTAGACATGCCTCTCGA), 2 µg poly(dI•dC), 5 mM DTT, 150 mM NaCl, 20 mM Tris-HCl (pH 7.2), 1 mM EDTA, 0.1% Triton X-100, and 4% glycerol. Mutant control oligonucleotide contains Biotin-5'-TCGAGAGGTCGCTCTAGGTCGCTCTC annealed with 5'-GAGAGCGACCTAGAGCGACCTCTCGA. The DNA/protein complexes were captured with 0.1 mg of magnetic Streptavidin beads (Promega) at 4°C for 30 min. The beads were collected using a magnet and washed 3 times with DNA binding buffer. The bound proteins were eluted by boiling in sample buffer (4% SDS, 20% glycerol, 200 mM DTT, 120 mM Tris pH 6.8, 0.002% bromophenol blue). The protein complexes were resolved by SDS-PAGE, and p53 was detected by western blot using DO-1 antibody.

Luciferase Assay

U2OS cells (50,000/well) were cultured in 24-well plates and transfected with a DNA plasmid mixture containing 10 ng p53-responsive BP100-luciferase reporter (Freedman et al., 1997), 5 ng CMV1-lacZ, 10 ng MDM2, or 20 ng MDMX, 5 ng of GFP and up to 600 ng using ssDNA. Transfection was performed using Lipofectamine 2000 reagent (Invitrogen) as described above and cells were analyzed for luciferase and beta-galactosidase expression after 24 h. The ratio of luciferase/beta-galactosidase was used as an indicator of p53 transcriptional activity.

RNA Isolation and Analysis

To determine the levels of p21, PUMA and GAPDH expression, total RNA was extracted using the Qiagen Rnease Mini Kit per the manufacturer instructions. Briefly, samples were lysed and homogenized using the QIAshredder columns in a highly denaturing guanidine-thiocyanate containing buffer. Ethanol was added and the samples were applied to an RNeasy Mini sping column. The total RNA was bound to the membrane and contaminants were washed away. The RNA was eluted from the column using 50 μl of water. cDNAs were prepared by reverse transcription of total RNA using the SuperScript III Invitrogen kit. The primers used for Sybrgreen quantitative PCR of human p21, PUMA and GAPDH mRNA were as follows. Human p21 forward: 5'ATGAAATTCACCCCCTTTCC; reverse: 5'AGGTGAGGGGACTCCAAAGT. PUMA forward: 5'TTGGGTGAGACCCAGTAAGG; reverse: 5'TTGTTACTTCCTGCCCTGCT. GAPDH forward: 5'GAGTCAACGGATTTGGTCGT; reverse: 5'GACAAGCTTCCCGTTCTCAG. Samples were analyzed in triplicate. Samples were normalized to the GAPDH Ct [$\Delta Ct = Ct_{[Target]} - Ct_{[Gapdh]}$], the ΔCt was converted to the fold difference between samples [$\Delta\Delta Ct = \Delta Ct_{[untreated]} - \Delta Ct_{[sample\ being\ compared]}$; FOLD=$2^{(-\Delta\Delta Ct)}$] and samples were analyzed in triplicate [Standard Deviation Error Bar = -FOLD + FOLD x ($2^{Ct\ Dev.}$)].

MTT Cell Viability Assay

Cell viability was measured by a MTT colorimetric survival assay. SJSA, U2OS, DLD1, MDA-231, HCT +/+ and HCT-/- cells (10,000 per well) were seeded into 24 well plates and then treated the next day with different concentrations of Velcade (5-150 nM) and/or Nutlin (3-8 μM) for 48 h. After treatment, cells were washed once with 0.5 ml PBS and then incubated with 0.25 ml PBS containing 10 μl CellTiter 96 Aqueous One Solution Reagent (Promega) for 15 min. Reaction was stopped by placing the plates on ice and the OD490 was measured using Microplate Manager Version 5.2.1 Build 106 software (BioRad). The absorbance reading correlates with the number of live cells per well.

RESULTS

MDM2 inhibits p53 DNA binding

A previous study using the McKay assay suggested that MDM2-p53 complex did not bind DNA (Zauberman et al., 1993). However, several studies reported detection of MDM2 binding to p53 target gene promoters, indicating that the MDM2-p53 complex has DNA binding activity *in vivo* (Arva et al., 2005; Jin et al., 2003; Tang et al., 2008; Wang et al., 2004; White et al., 2006). To further investigate the effect of MDM2 on p53 DNA binding, we purified MDM2-p53 complex from H1299 cells cotransfected with p53 and FLAG-MDM2 using M2 beads and eluted with FLAG peptide. The MDM2-p53 complex was incubated with biotinylated oligonucleotide DNA containing p53-binding sequence from the p21 promoter. P53-DNA complex was captured by streptavidin beads and analyzed by western blot. The analysis showed that p53 purified as FLAG-MDM2-p53 complex had very low DNA binding activity, whereas FLAG-p53 directly purified without MDM2 bound DNA efficiently (**Figure 13**). In the same assay, purified FLAG-MDMX-p53 complex retained significant DNA binding, suggesting that only MDM2 has strong ability to inhibit p53 DNA binding. As a specificity control, FLAG-p53 did not bind to oligonucleotide with mutated p53 consensus sequence.
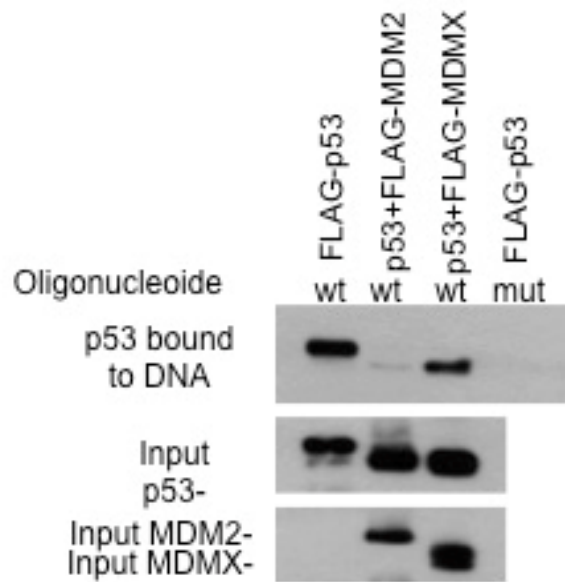
Figure 13: Inhibition of p53-oligonucleotide binding by MDM2. H1299 cells were transfected with FLAG-p53, or un-tagged p53 with FLAG-MDM2 or FLAG-MDMX plasmids. The protein complexes were purified using M2 Beads and eluted using a FLAG peptide. Elutes containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site (wt) or a mutated sequence (mut). An aliquot of eluate was analyzed to confirm similar levels of p53 expression.

To test whether MDM2 inhibits p53 DNA binding *in vivo*, H1299 cells were cotransfected with p53 and MDM2, treated with MG132 to block MDM2 mediated degradation and then followed by p53 ChIP and quantitative PCR (qPCR) analysis. Western blot of duplicate samples confirmed comparable expression of p53, MDM2 and MDMX levels (**Figure 14 c, lower panels**). The p53 ChIP showed that coexpression of MDM2 significantly inhibited p53 binding to the p21 (n = 3, p = 0.01) and PUMA (n = 3, p = 0.05) promoter (**Figure 14 a & b**), whereas MDMX had a less dramatic effect (n = 3, p = 0.07 and 0.08). P21 protein expression was also significantly repressed by MDM2, compared to the modest decrease by MDMX (**Figure 14 c**).
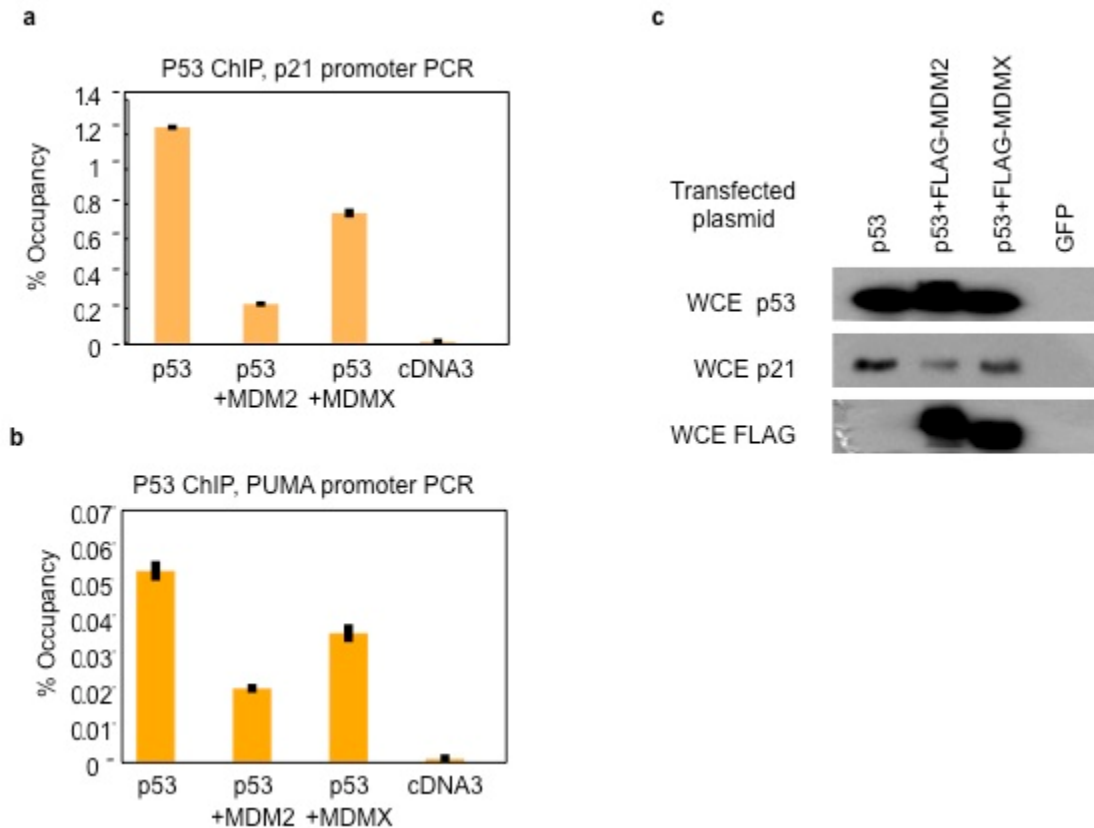
Figure 14: Inhibition of p53-DNA binding by MDM2. (a) & (b) H1299 cells were transfected with p53, MDM2 or MDMX and analyzed by ChIP, followed by quantitative PCR (qPCR) to detect p53 DNA binding to the p21 promoter. (c) WCE (whole cell extracts) of parallel samples were analyzed to confirm levels of protein expression.

Next, to test the effect of MDM2 or MDMX over expression on endogenous p53 transcriptional activity, U2OS cells were transfected with the BP100-luciferase reporter. Co-transfection with the MDM2 plasmid resulted in strong inhibition of luciferase expression, while MDMX only weakly inhibited p53 activity (n = 4, p = 0.002 compared to p = 0.07) (**Figure 15**). These results corroborated the *in vitro* and *in vivo* DNA binding assay and showed that MDM2, not MDMX, efficiently inhibits p53 DNA binding and transcriptional activity.

P53 Target Promoter MDM2 – Luciferase Assay
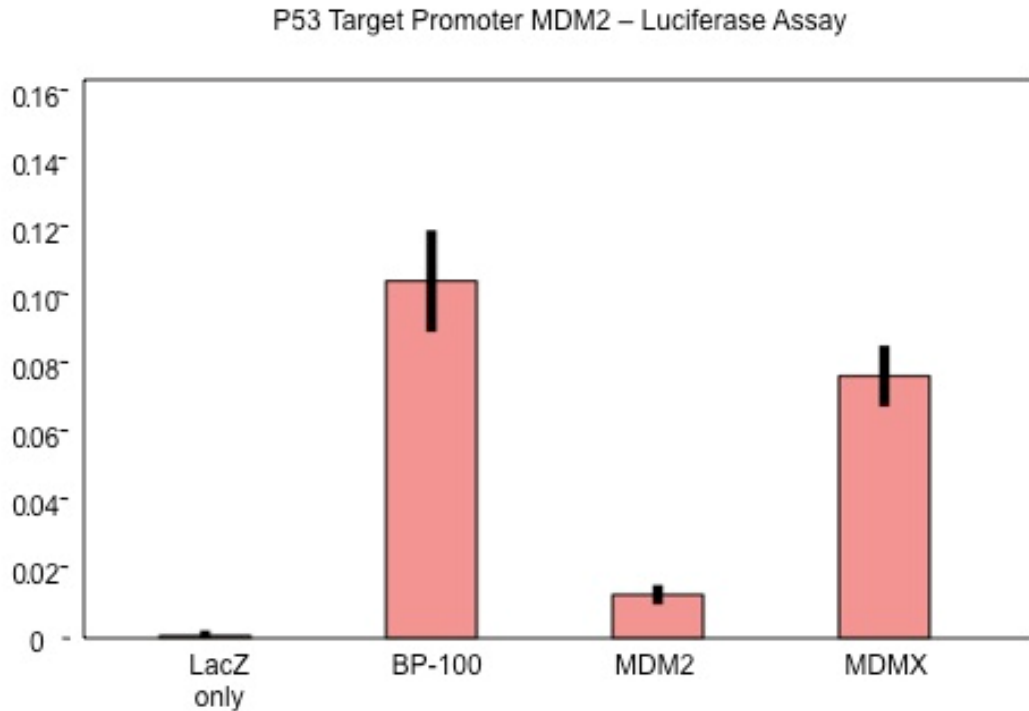
Figure 15: MDM2 overexpression inhibits endogenous p53 activity.
Endogenous p53 activity in U2OS cells was measured by transfection of BP100-luciferase reporter. Inhibition by MDM2 or MDMX was determined by co-transfection and luciferase assay.

MDM2 acidic domain is critical for inhibiting p53 DNA binding

To identify the domain on MDM2 necessary for blocking p53 DNA binding, different C terminal mutants of FLAG-MDM2 were tested. The results showed that the MDM2 RING domain mutant (457S) was as active as wt MDM2. MDM2-1-300 (without the RING domain and zinc finger) still retained strong activity in blocking p53 DNA binding despite being expressed at lower levels. However, further deletion of the acidic domain (MDM2-1-200) completely abrogated the ability to inhibit p53 DNA binding (**Figure 16 a**). In a control experiment, different C terminal truncation mutants of FLAG-MDMX were also tested. The results showed that full length or fragments of MDMX had no effect on p53 DNA binding despite forming complexes with p53 efficiently (**Figure 16 b**). It is important to note that in this assay sample input was equalized for the amount of co-

precipitated p53. Some samples may have excess free MDM2, but is not expected to influence the result.
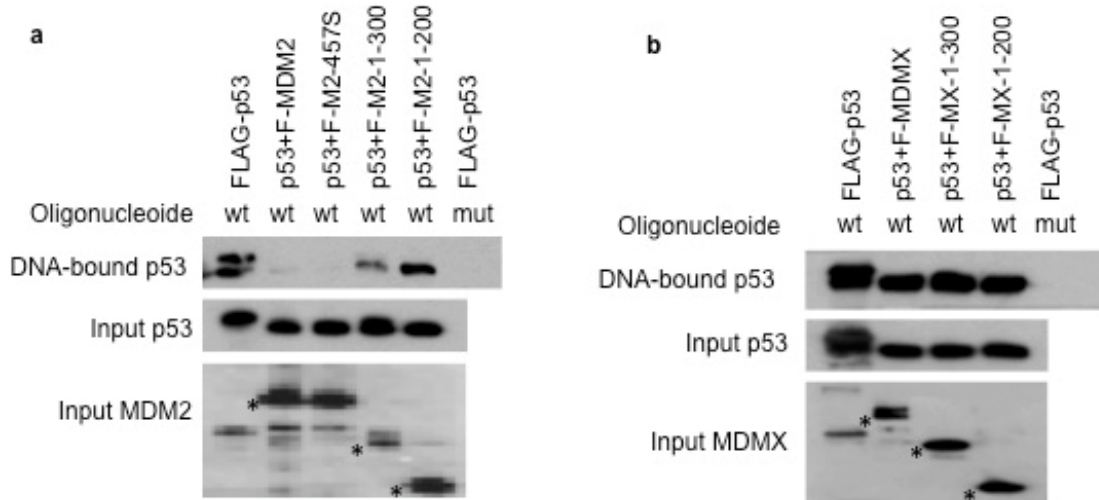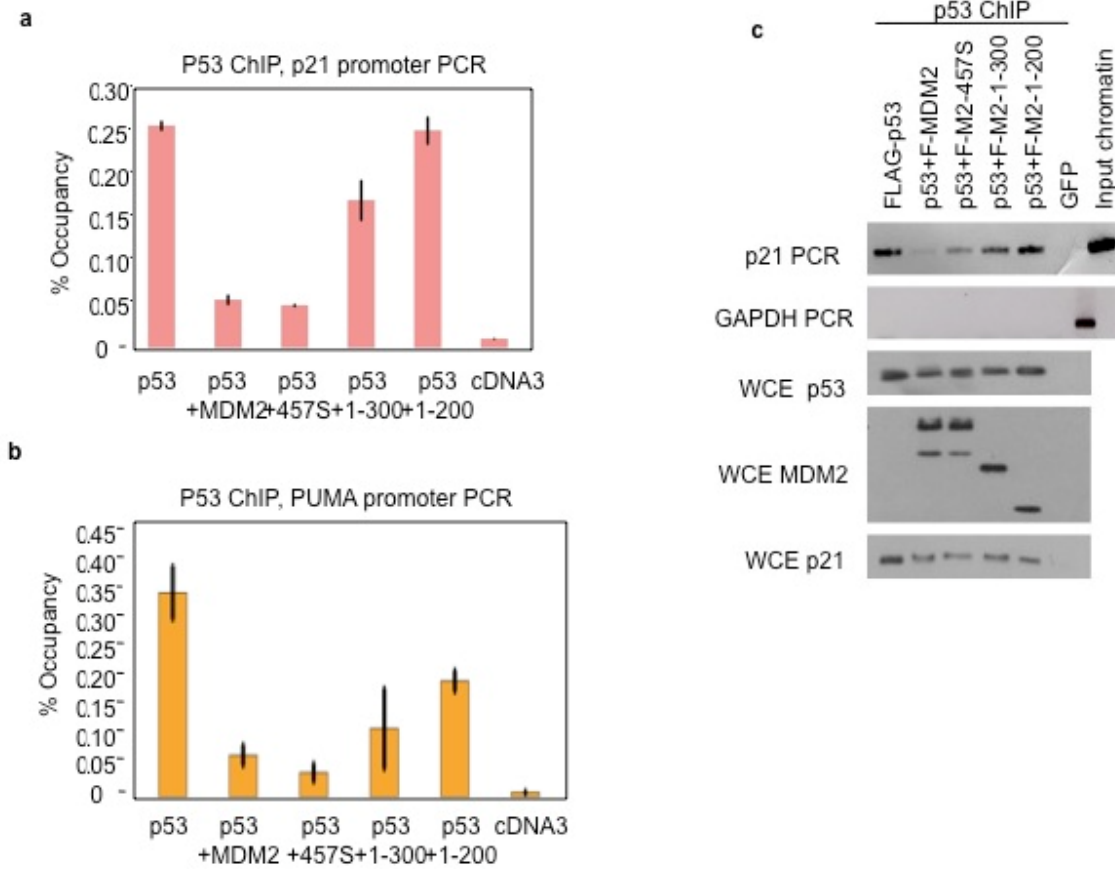

Figure 16: MDM2 acidic domain inhibits p53-oligonucleotide binding. (a) & (b) H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. An aliquot of eluate was analyzed to confirm similar levels of p53 expression.

When p53 DNA binding *in vivo* was analyzed by ChIP in transfected cells, the effects of MDM2 mutants were consistent with the *in vitro* DNA pull down assay. The full length and MDM2-457S E3 mutant were both efficient in blocking p53 DNA binding (n = 3, p = 0.03 and p = 0.08 for p21 and p = 0.06 and p = 0.09 for PUMA). However, deletion of both acidic domain and RING domains (MDM2-1-200) completely restored p53 DNA binding (n = 3, p = 0.55 for p21 and p = 0.2 for PUMA) (**Figure 17 a, b & c**). Treatment with MG132 for 5 hours before ChIP analysis produced similar results (n = 3, p = 0.02 and 0.05 versus p = 0.17 for p21 and p = 0.24 and 0.33 versus p = 0.53 for PUMA) (**Figure 18 a & b**), suggesting that MDM2 can inhibit p53 DNA binding *in vivo* without targeting it for degradation. As expected, MDMX wild type or mutants showed no difference in regulating p53 DNA binding in ChIP assay, consistent with the *in vitro* DNA binding result (n = 3, p = 0.31, 0.2 and 0.25 for p21 and p = 0.4, 0.21, 0.2 for

PUMA) (**Figure 19 a, b & c**).   These results showed that the MDM2 acidic domain has an important role in regulating p53 DNA binding.

Figure 17: MDM2 acidic domain inhibits DNA binding. (a) & (b) H1299 cells were transiently transfected with the indicated plasmids, treated with MG132 for 5 h and analyzed by ChIP qPCR to detect p53 DNA binding to the p21 and PUMA promoter. (c) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP to detect p53 DNA binding to the p21 promoter. GAPDH promoter primers were used for a specificity control. Input is 1% of the starting lysate material.   WCE of parallel samples were analyzed to confirm levels of protein expression.
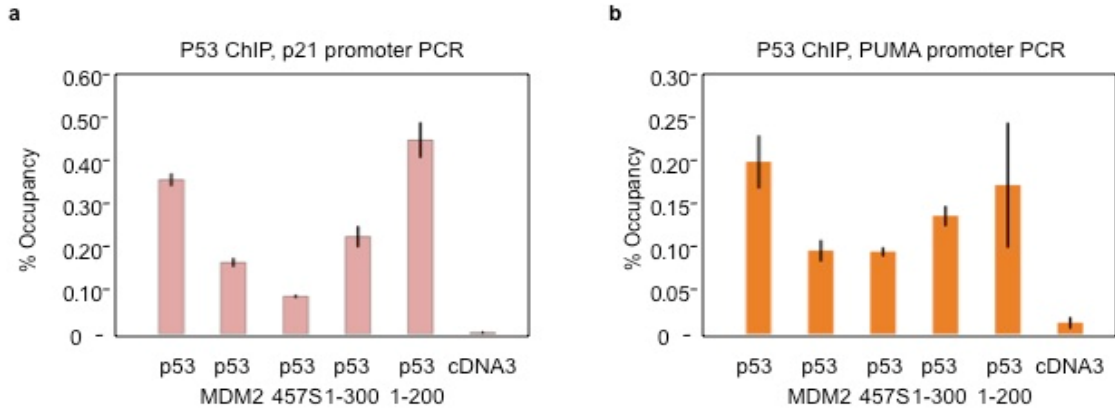
Figure 18: MDM2 inhibits DNA binding prior to proteosomal degradation. (a) & (b) H1299 cells were transiently transfected with the indicated plasmids, treated with 30 μM MG132 for 5 h and analyzed by ChIP qPCR to detect p53 DNA binding to the p21 and PUMA promoter.
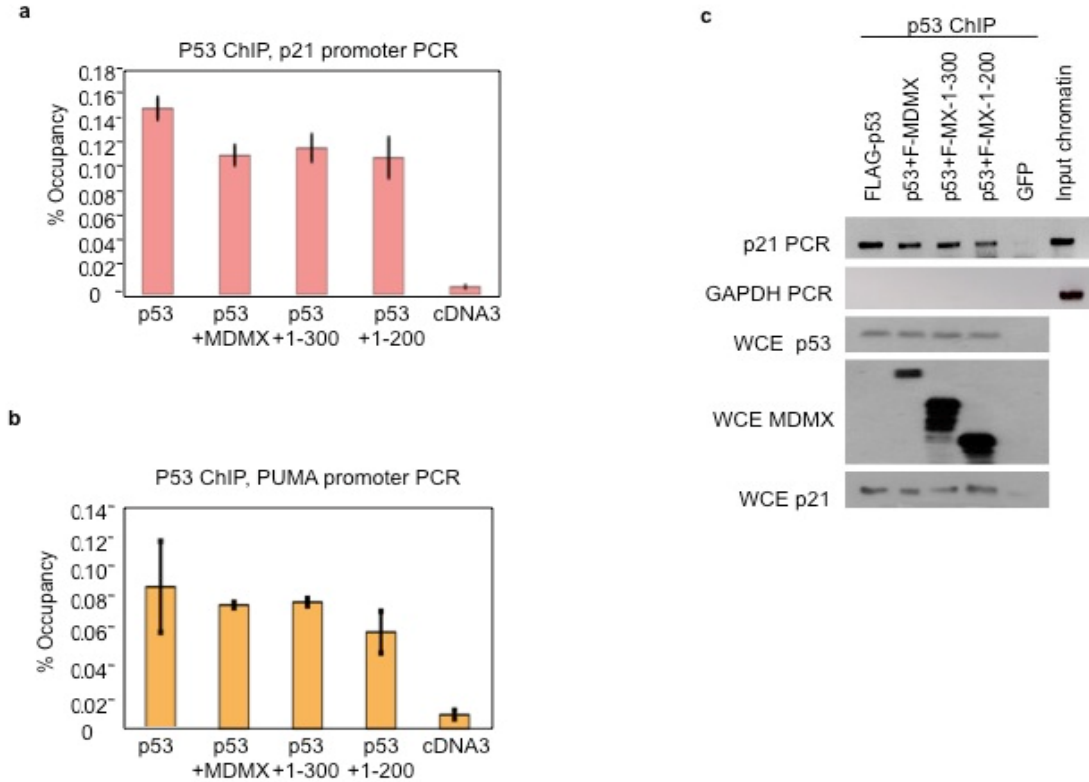
Figure 19: MDMX acidic domain does not inhibit p53-DNA binding. (a) & (b) H1299 cells were transiently transfected with the indicated plasmids, treated with MG132 for 5 h and analyzed by ChIP qPCR to detect p53 DNA binding to the p21 and PUMA promoter. (c) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP to detect p53 DNA binding to the p21 promoter. GAPDH promoter primers were used for a specificity control. Input is 1% of the starting lysate material. WCE of parallel samples were analyzed to confirm levels of protein expression.

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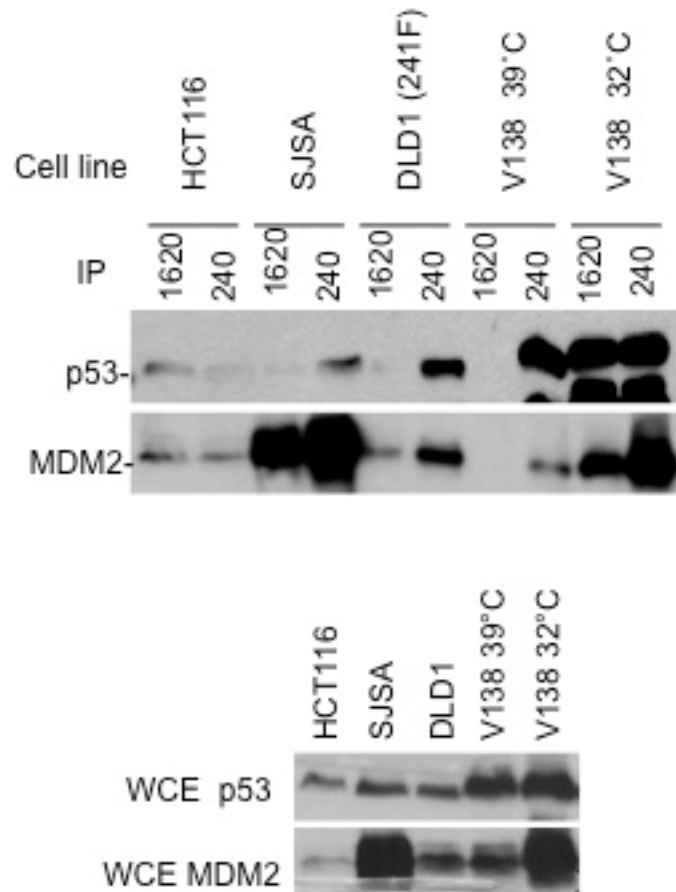## MDM2 but not MDMX induces p53 conformational change

DNA binding by p53 requires proper folding of a conformation-labile core domain. To determine how MDM2 inhibits p53 DNA binding, we tested the ability of MDM2 and MDMX in inducing p53 conformational change. A recent study showed that MDM2 induces p53 conformational change as measured by reactivity to wild type-specific Pab1620 and mutant-specific Pab240, which recognize epitopes located in the p53 core domain (Sasaki et al., 2007). Using this assay, p53 was cotransfected with MDM2 or MDMX into H1299 cells, incubated without or with MG132 to prevent p53 degradation, and analyzed by Pab1620/Pab240 IP. The ratios of p53 with wild type and mutant conformation were determined by western blot with pan-specific p53 antibody FL393.

The results showed that p53 was predominantly in a wild type conformation when expressed alone (**Figure 20 a**). Coexpression with MDM2 induced a significant switch from wild type to mutant conformation as reported previously (Sasaki et al., 2007). In addition, MDM2-457S RING mutant retained full effect, indicating that E3 ligase function was not required for inducing p53 conformational change. Furthermore, MDMX expression did not alter p53 conformation (**Figure 20 a**). In the absence of MG132, p53 with mutant conformation did not accumulate, presumably because of subsequent ubiquitination and degradation by the proteasome (**Figure 20 a**). In contrast, MDM2-457S-induced p53 conformational change was readily detectable in the absence of MG132 because it did not promote p53 ubiquitination and degradation (**Figure 20 a**). Direct loads for each transfection were run as well to determine p53, MDM2 and MDMX protein expression (**Figure 20 b**). These results showed that MDM2 inhibition of p53 DNA binding correlates with induction of conformational change before ubiquitination.
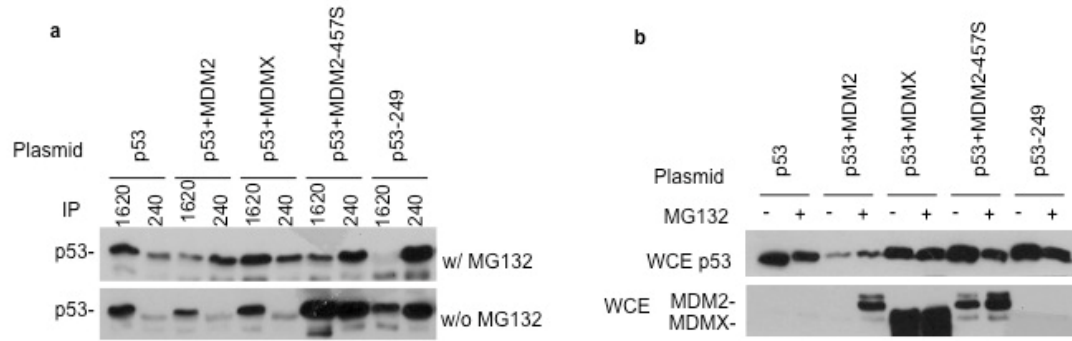
Figure 20: MDM2 but not MDMX induces p53 conformational change. (a) H1299 cells were transfected with the indicated plasmids and left untreated or treated with 30 μM MG132 for 5 h. Cell lysates were immunoprecipitated with wild-type conformation specific (Pab1620) or mutant conformation specific (Pab240) antibodies. The precipitated p53 was detected by western blot. (b) WCE were analyzed for protein expression levels. The amount of sample used for the IP was empirically adjusted to compensate for large changes in expression levels p53- 249S is a conformational mutant as a positive control.

To test whether MDM2-induced p53 conformational change occurs with endogenous proteins, SJSA cells (MDM2 amplification) was analyzed in comparison to HCT116 cells (no MDM2 amplification). The result showed that whereas p53 was present mostly in wild type conformation in HCT116, >50% of endogenous p53 in SJSA was present in the mutant conformation (**Figure 21**). In comparison, endogenous mutant p53 in DLD1 cells was nearly 100% in the mutant conformation. As another control, the temperature-sensitive p53-V138 mutant stably expressed in H1299 cells showed conformational switch between permissive and non-permissive temperatures as expected (**Figure 21**).

Figure 21: Amplified MDM2 induces p53 conformational change. Cell lines expression different levels of endogenous MDM2 and wt or mutant p53 were treated with 30 μM MG132 for 5 h and immunoprecipitated with Pab1620 or Pab240. Protein expression was confirmed by western blot of WCE.

Nutlin blocks MDM2 mediated p53 conformational change

To further test whether mutant conformation p53 in SJSA cells and transient transfections was due to interaction with overexpressed MDM2, cells were treated with Nutlin to disrupt p53-MDM2 binding. The result showed that Nutlin treatment not only increased total p53 level, but also increased the ratio of Pab1620-positive p53 over Pab240-positive p53 in both transient transfection assays and SJSA cells (**Figure 22 a & b**). As expected, MDM2 coprecipitation with p53 was reduced, but not eliminated after Nutlin treatment, presumably due to the fact that Nutlin induces very high level MDM2 expression (**Figure 22 b,**

right panels).   Taken together, the data suggests that a direct interaction between endogenous MDM2 and p53 is needed to change p53 conformation.
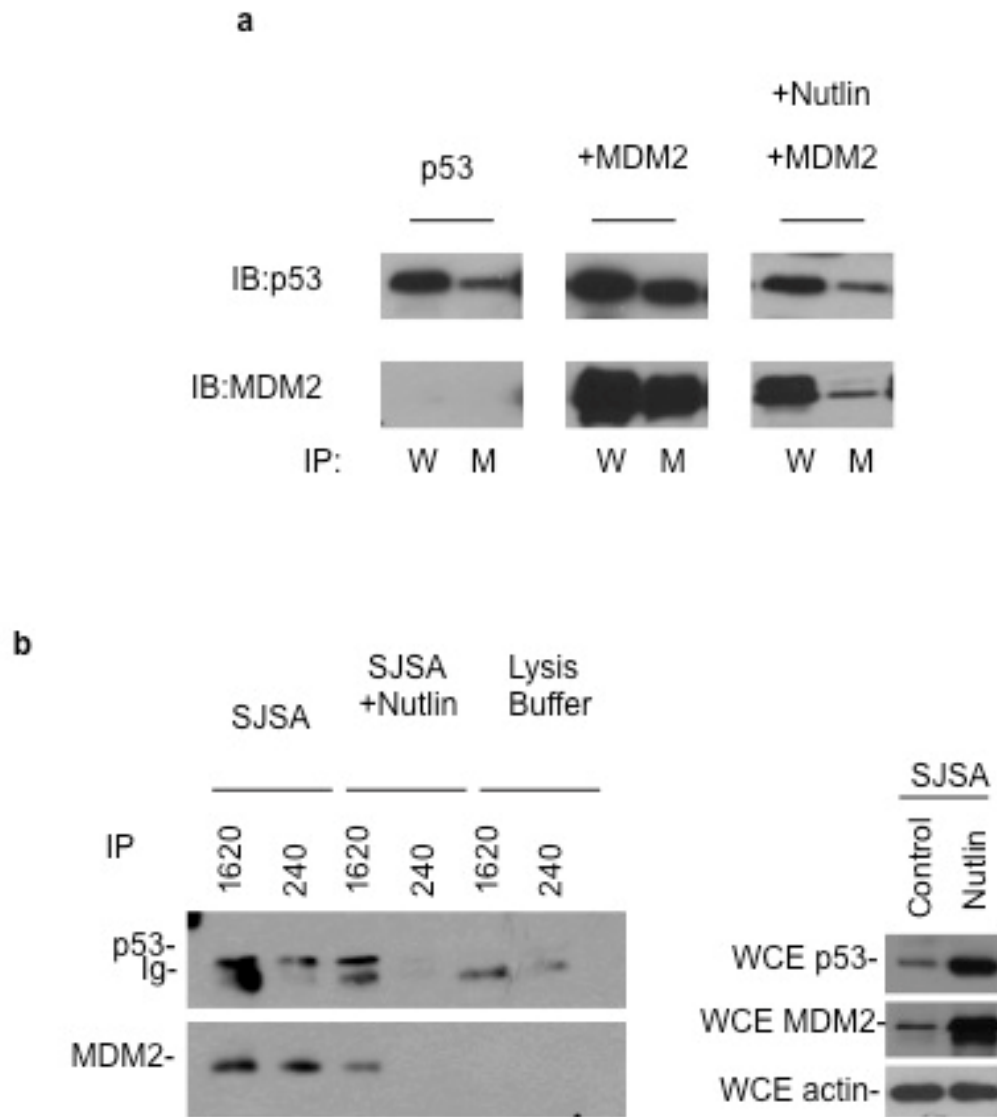
Figure 22: Nutlin blocks MDM2 induced p53 conformational change. (a) & (b) Transiently transfected H1299 cells and SJSA cells were treated with 30 µM MG132 for 5 h with or without 5 µM Nutlin and analyzed for p53 conformation by Pab1620 and Pab240 IP.   MDM2 coprecipitated with p53 was detected by western blot. Protein expression was confirmed by western blot of WCE.

MDM2 acidic domain is critical for inducing conformational change

To map the MDM2 domain that mediates p53 conformational change a panel of MDM2 deletion mutants were co-expressed with p53 in H1299 cells (**Figure 23 a**). Conformational analysis of p53 showed that deletion of the MDM2 acidic domain (1-230, 1-200, Δ220-325) abrogated the conformational effect (**Figure 23 b**). As expected, deleting the N terminal p53-binding domain (50-491, 230-491) also abrogated the activity, whereas mutations affecting the RING domain (1-440, 1-290, 457S) had no effect. The expression and binding between p53 and MDM2 mutants were confirmed by IP-western blot analysis (**Figure 23 c & d**). Therefore, the MDM2 acidic domain has a critical role in inducing the p53 switch to mutant conformation, but requires targeting by the high-affinity N terminal p53-binding domain.
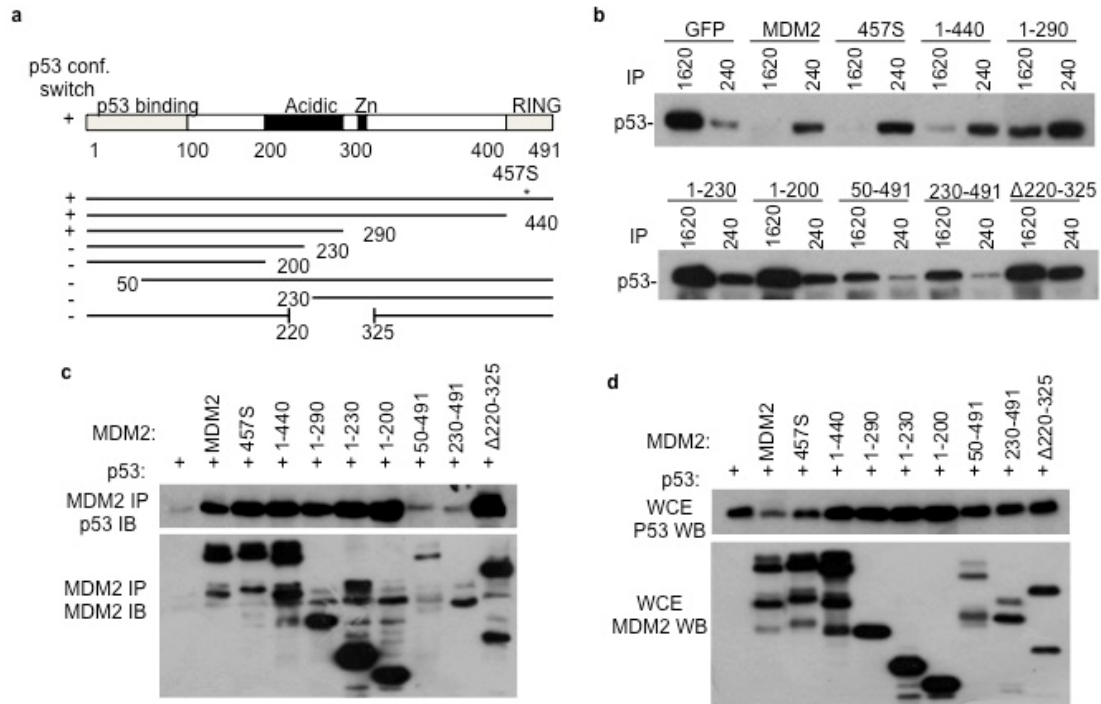
Figure 23: Mapping the MDM2 domain that mediates p53 conformational change. (a) Diagram of MDM2 deletion and point mutants. (b) H1299 cells were transfected with the indicated plasmids, treated with MG132 (30 μM, 5 h) and analyzed by Pab1620 and Pab240. (c) MDM2 mutants were co-transfected with p53 into H1299 cells. MDM2 was immunoprecipitated with a mixture of 4B2, 5B10 and 3G9 antibodies and co-precipitation of p53 was detected by western blot. Immunoprecipitation of MDM2 mutants were confirmed by western blot (bottom panel). (d) Expression of p53 and MDM2 mutations were confirmed by western blot of WCE.

We also attempted to determine whether sub regions of the MDM2 acidic domain are critical for p53 conformational change using a series of acidic domain small internal deletion mutants (**Figure 24 a**). The results showed that all small internal deletions had similar defects on the p53 conformational change as the complete acidic domain deletion (**Figure 24 b & c**). This suggests that different parts of the MDM2 acidic domain contribute to p53 conformational change in an additive fashion. Consistent with this notion, a study showed that the binding

affinity of MDM2 acidic domain to p53 core is proportional to the length of the acidic domain tested (Yu et al., 2006).
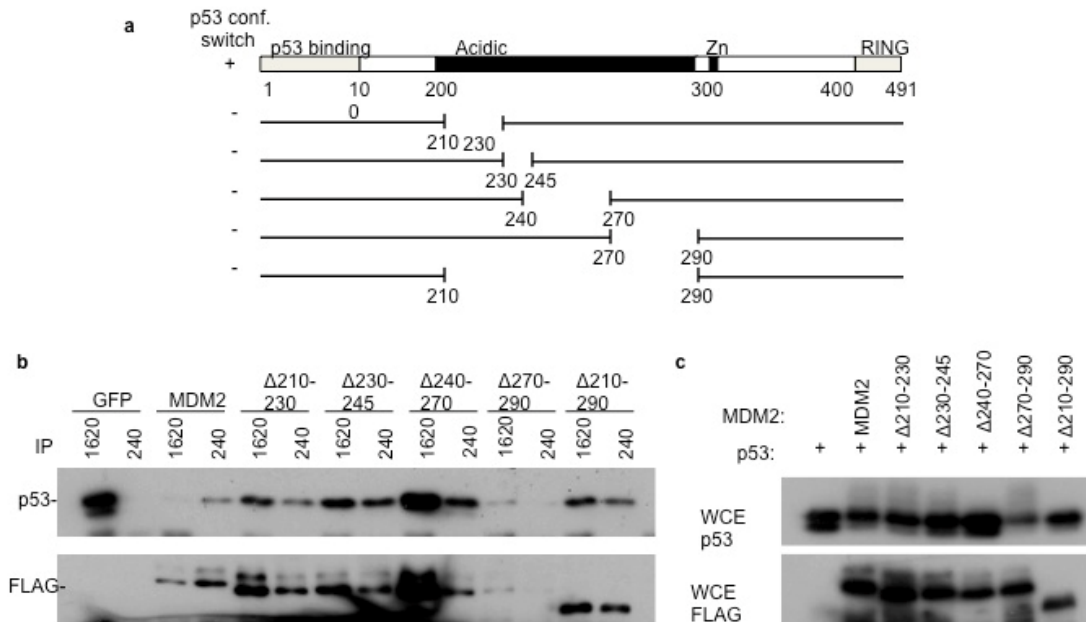
Figure 24: **Small deletions in the MDM2 acidic domain partially inhibit p53 conformational change.** (a) Diagram of MDM2 and internal deletions in the acidic domain. (b) H1299 cells were transiently transfected with the indicated expression plasmids and then treated with 30 µM MG132. The lysates were immunoprecipitated with Pab1620 or Pab240 antibodies, followed by immunoblotting for total p53 and FLAG-MDM2. (c) Western blot of whole cell extract showing expression levels of p53 and MDM2 mutants.

To further test the functional significance of the MDM2 acidic domain, we took advantage of the fact that MDMX has negligible activity in p53 conformational change. A panel of MDM2-MDMX hybrid constructs was analyzed for induction of p53 mutant conformation (**Figure 25 a & c**). The results showed that although MDMX did not alter p53 conformation, replacing its acidic domain and C terminal half with the MDM2 counterpart conferred partial to full activity (**Figure 25 a & c, #4, #6**). In contrast, transplanting the MDM2 RING domain alone to MDMX had no effect (**Figure 25 a & c, #8, #10**). Additionally, the MDMX acidic domain had no activity if transplanted to MDM2 (**Figure 25 a & c, #3, #5**). We should note that the phenotypes of #5 and #6 were somewhat

ambiguous in multiple experiments, possibly because placement of the junction was not optimal for protein function. Direct loads were run as well to show expression of all plasmids in the indicated transient transfections (**Figure 25 b**). Overall, these results corroborate the MDM2 deletion analysis and reveal a critical role of the MDM2 acidic domain in p53 conformational change. Furthermore, the p53-binding domain of MDM2 simply provided a targeting function, which can be replaced by the MDMX N terminus.
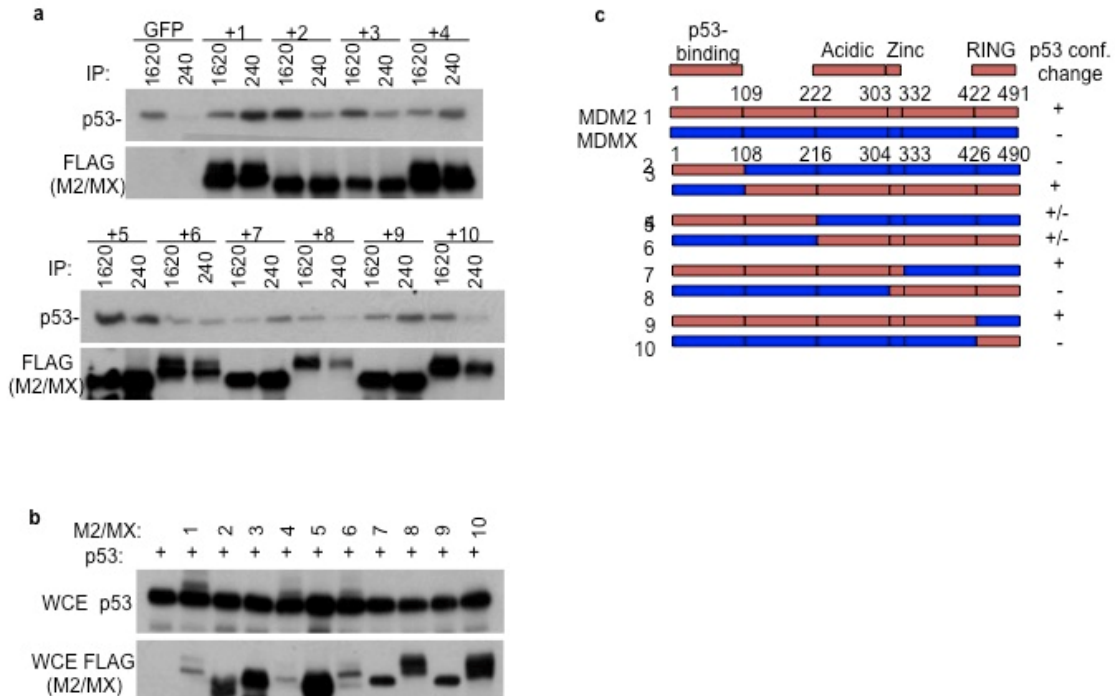
Figure 25: Induction of p53 conformational change by MDM2-MDMX hybrid constructs. (a) H1299 cells were tranfected with p53 and FLAG tagged MDM2-MDMX hybrid constructs. Cells were treated with MG132 (30 µM, 5 hr) and immunoprecipitated with Pab1620 or Pab240 followed by western blot for p53. Co-immunoprecipitation of the hybrid proteins was detected by western blot. (b) Expression of p53 and FLAG-tagged MDM2-MDMX hybrid proteins were confirmed by western blot of WCE. (c) Diagram of MDM2-MDMX hybrid constructs and summary of results in (a).

Previous studies suggested that phosphorylation of MDM2 acidic domain on S256 is needed for efficient degradation of p53 (Blattner et al., 2002). Our mass spectrometric analysis of MDM2 identified two additional phosphorylation sites S232 and S290 in the acidic domain (unpublished results). While S232A

and S290A mutants had no obvious effect on p53 degradation, S256A mutation caused a moderate defect in p53 degradation as reported (data not shown). To test whether phosphorylation of these sites play a role in p53 conformational change, alanine substitution mutants were analyzed. The result showed no significant difference compared to wild-type MDM2 (**Figure 26**), suggesting that acidic domain phosphorylation is not necessary for induction of p53 conformational change.
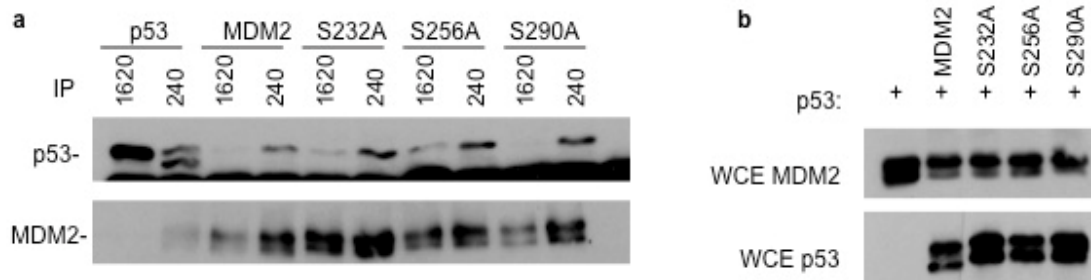
Figure 26: Analysis of MDM2 acidic domain phosphorylation mutants. (a) H1299 cells were tranfected with the indicated plasmids, treated with MG132 (30 µM, 5 hr) and analyzed by Pab1620 and Pab240 IP. (b) WCE of samples was analyzed to confirm similar levels of protein expression for the MDM2 mutants.

CK1α-MDMX binding increases MDMX ability to block p53-DNA binding

Next we wanted to determine if increasing MDMX-p53 binding would create an MDMX-dependent shift in the p53 conformation. Despite the N-terminal switching of the MDM2 and MDMX protein in **Figure 25 (#3 & #4)** having no effect on p53 conformational change, it is possible that the lack of shift in p53 to a mutant conformation, when co-transfected with MDMX, is due to inefficient overall binding efficiency. Therefore, we decided to look at a major MDMX binding protein that has been shown to increase MDMX-p53 DNA binding (Chen et al., 2005c). Affinity purification of MDMX revealed stable bind of the CK1α in HeLa cells stably overexpressing wild-type MDMX (**Figure 27 a**). CK1α binds to the zinc finger domain of MDMX (data not shown) and a point mutation of the conserved cysteine residue (C306S), located in the MDMX-CK1α binding

region, was deficient for CK1α binding in a coimmunoprecipitation assay (**Figure 27 b**).
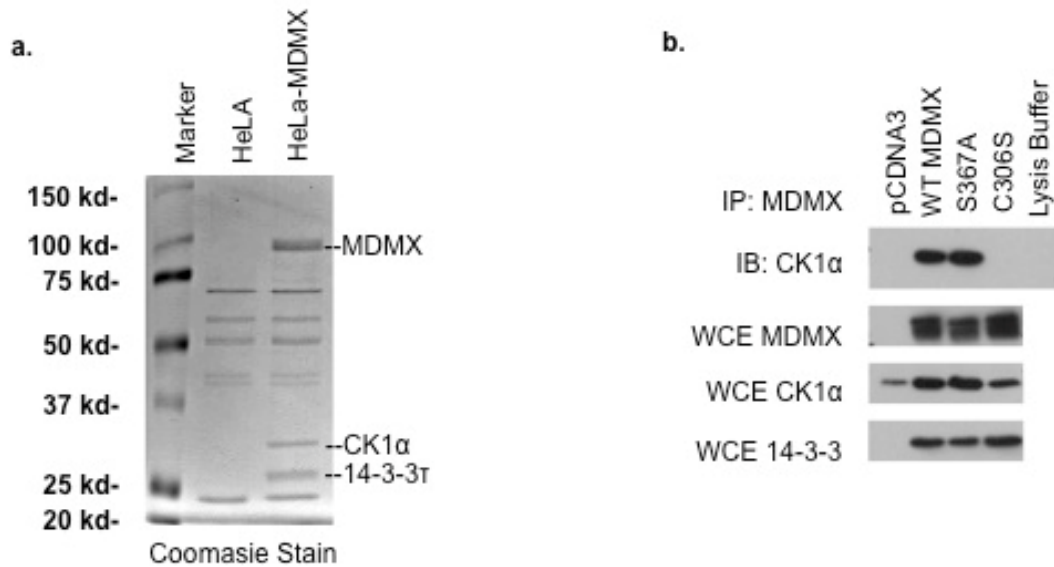
Figure 27: CK1α binds to MDMX and requires the 306 cysteine residue. (a) HeLa cells alone or stably transfected with human MDMX. Molecular mass marker (M) (in kilodaltons) is shown to the left of the gel. Coomassie blue staining of affinity-purified MDMX and associated proteins. The two marked bands were identified as CK1α and 14-3-3τ by mass spectrometric peptide sequencing. (b) The indicated plasmids were transiently transfected into H1299 cells, IPed for MDMX and analyzed for CK1α binding. WCE were analyzed to confirm protein expression.

To determine whether MDMX-p53 binding can be regulated by the CK1α-MDMX interaction, a panel of MDMX plasmids that retained the p53-binding domain was used to determine their p53-binding response when co-expressed with CK1α. Co-transfection of CK1α with wild-type MDMX and p53 showed a significant increase in p53-MDMX binding compared to the MDMX-p53 only transfection (**Figure 28 a**). The C306S mutation, which abrogates MDMX-CK1α binding, showed no difference in MDMX-p53 binding whether or not CK1α was also transfected (**Figure 28 a**). Previous reports indicated that, once bound to MDMX, CK1α can phosphorylate Ser-289 located in the MDMX acidic domain (Chen et al., 2005c). The significance of this phosphorylation has yet to be

established. Therefore, we also looked at how mutating the serine to an alanine residue might affect the ability of CK1α to increase MDMX-p53 binding. Compared to similar transfections with wild-type MDMX, there was a significant decrease in the ability of CK1α to increase MDMX-p53 binding when Ser-289 phosphorylation was blocked (**Figure 28 a**). Therefore, the MDMX-CK1α interaction and its subsequent Ser-289 phosphorylation are critical for increasing the MDMX-p53 binding interaction (**Figure 28 b**). This raises the question: does this increase in MDMX-p53 binding via the MDMX-CK1α interaction lead to an increase in MDMX-mediated inhibition of p53 activity?
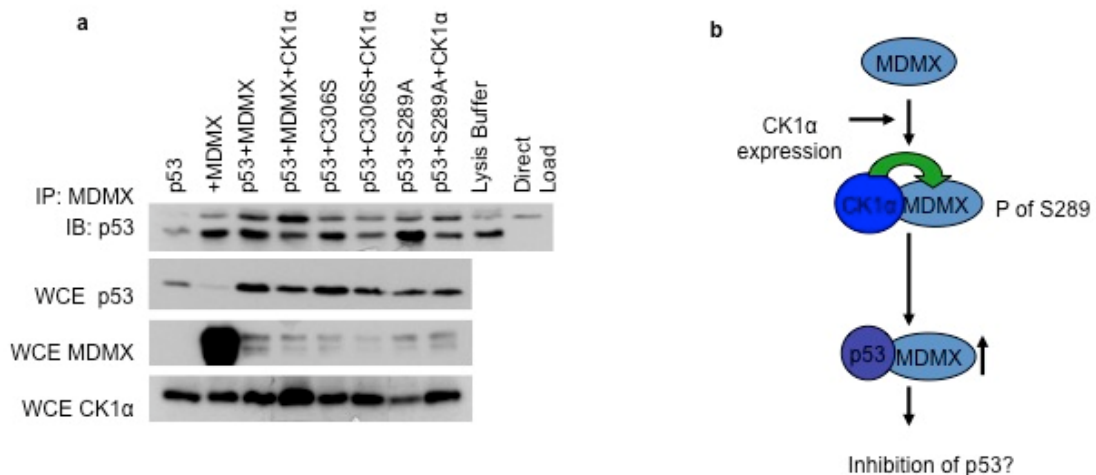
Figure 28: CK1α-MDMX binding increases MDMX-p53 binding. (a) Binding efficiency between MDMX mutants and p53 was determined by transient cotransfection of H1299 cells with MDMX, p53 and CK1α expression plasmids, followed by MDMX IP/p53 western blotting. WCE were analyzed to confirm protein expression. (b) Diagram of the MDMX-CK1α-p53 interaction.

Therefore, to further investigate the effect of MDMX-CK1α binding on p53 DNA binding activity, we purified MDMX-p53 complex from H1299 cells cotransfected with p53 and FLAG-MDMX, with or without CK1α, using M2 beads and eluted with FLAG peptide. The protein complex was incubated with biotinylated oligonucleotide DNA containing p53-binding sequence from the p21 promoter. P53-DNA complex was captured by streptavidin beads and analyzed by western blot. The analysis showed that p53 purified as FLAG-MDMX-p53

complex still had significant DNA binding activity compared to FLAG-p53 only, whereas FLAG-p53 copurified with MDMX and CK1α had a significant decrease in p53 bound to DNA (**Figure 29 a).** As a specificity control, FLAG-p53 did not bind to an oligonucleotide with a mutated p53 consensus sequence.   To test whether MDMX inhibits p53 DNA binding *in vivo*, H1299 cells were cotransfected with p53 and MDMX, with or without CK1α, and analyzed by p53 ChIP-PCR analysis.   Western blot of duplicate samples confirmed comparable expression of p53, MDMX and CK1α levels (**Figure 29 c, red bars**).   The p53 ChIP showed that coexpression of MDMX with CK1α significantly inhibited p53 binding to the p21 promoter (**Figure 29 b**), whereas the MDMX-only transfection had a less dramatic effect.   P21 protein expression was also significantly repressed by MDMX-CK1α, compared to the modest decrease by MDMX only (**Figure 29 c**).
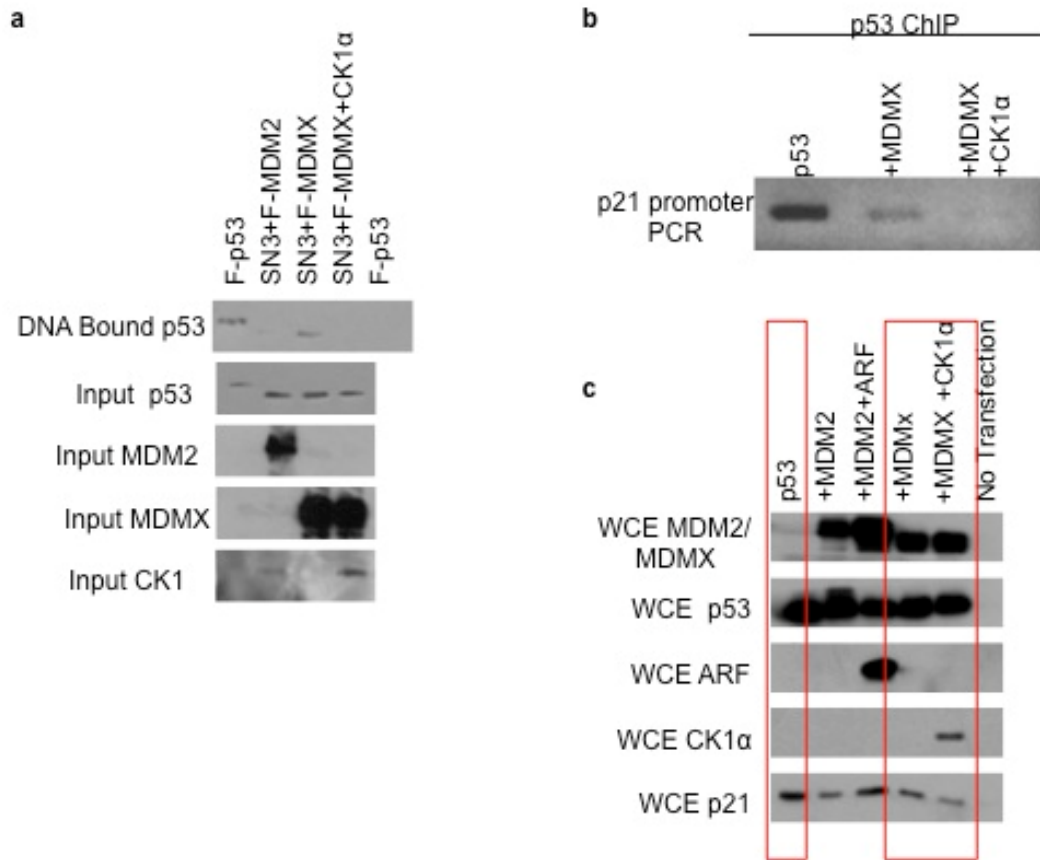
Figure 29: MDMX-CK1α binding decreases p53-DNA binding. (a) H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. An aliquot of eluate was analyzed to confirm similar levels of p53 expression. (b) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP to detect p53 DNA binding to the p21 promoter. (c) WCE of parallel samples were analyzed to confirm levels of protein expression.

In order to specifically determine the function of the CK1α-MDMX interaction, the effect of CK1α expression on transcriptional activity of p53 was examined by our lab using mouse embryo fibroblasts null for both MDMX and p53. Transfection with p53 alone with the Bp100-luciferase reporter containing the p53-binding site derived form the MDM2 P2 promoter resulted in strong activation of luciferase expression, with or without CK1α co-transfection (**Figure 30**). Cotransfection of MDMX expression plasmid resulted in only a weak

inhibition of p53 function, whereas cotransfection with CK1α resulted in more efficient inhibition of p53 activity (**Figure 30**). MDMX-C306S mutant was found to be nonresponsive to CK1α stimulation (**Figure 30**). Therefore the MDMX-CK1α interaction is critical for MDMX to efficiently block p53-DNA binding and transcriptional activity.
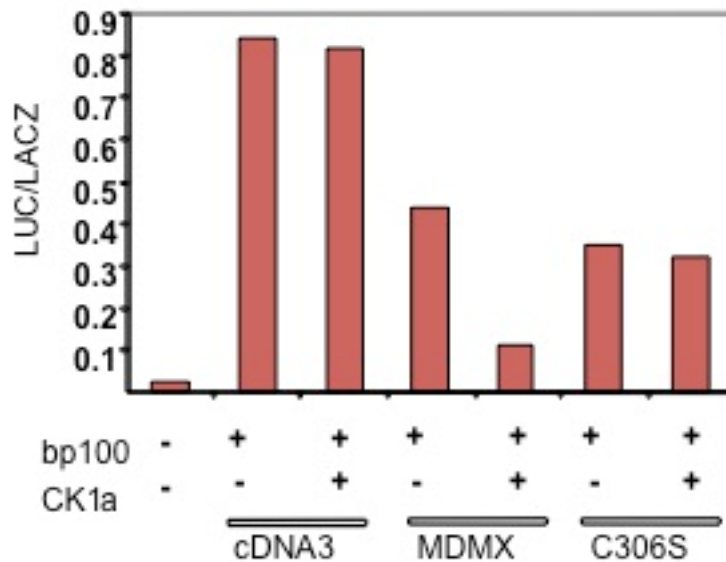
Figure 30: CK1α binding to MDMX is required for p53 regulation. P53 activation of BP100-luciferase reporter was detected after transient transfection into MDMX/p53 double null mouse embryonic fibroblast cells. The effects of MDMX mutants and CK1α were determined by cotransfection.

Increasing MDMX-p53 binding does not induce conformational change

Since CK1α can increase p53-MDMX binding, which leads to a decrease in p53-DNA binding and transcriptional activity, could it also cause MDMX to switch p53 to a mutant conformation? To investigate this H1299 cells were co-transfected with p53, MDMX and CK1α in the indicated combinations and surprisingly there was no significant shift in the ratio of wild-type to mutant p53 when all three proteins were co-transfected (**Figure 31 a & b**). Another transient transfection assay using increasing amounts of CK1α also showed no p53 shift towards a more mutant conformation (**Figure 31 c & d**). Therefore, MDMX deficiency in causing a p53 conformational shift has little to do with its overall

binding efficiency. This observation leads to a significant question: what is the difference between MDM2 and MDMX that allows for only a MDM2-mediated p53 conformational change?
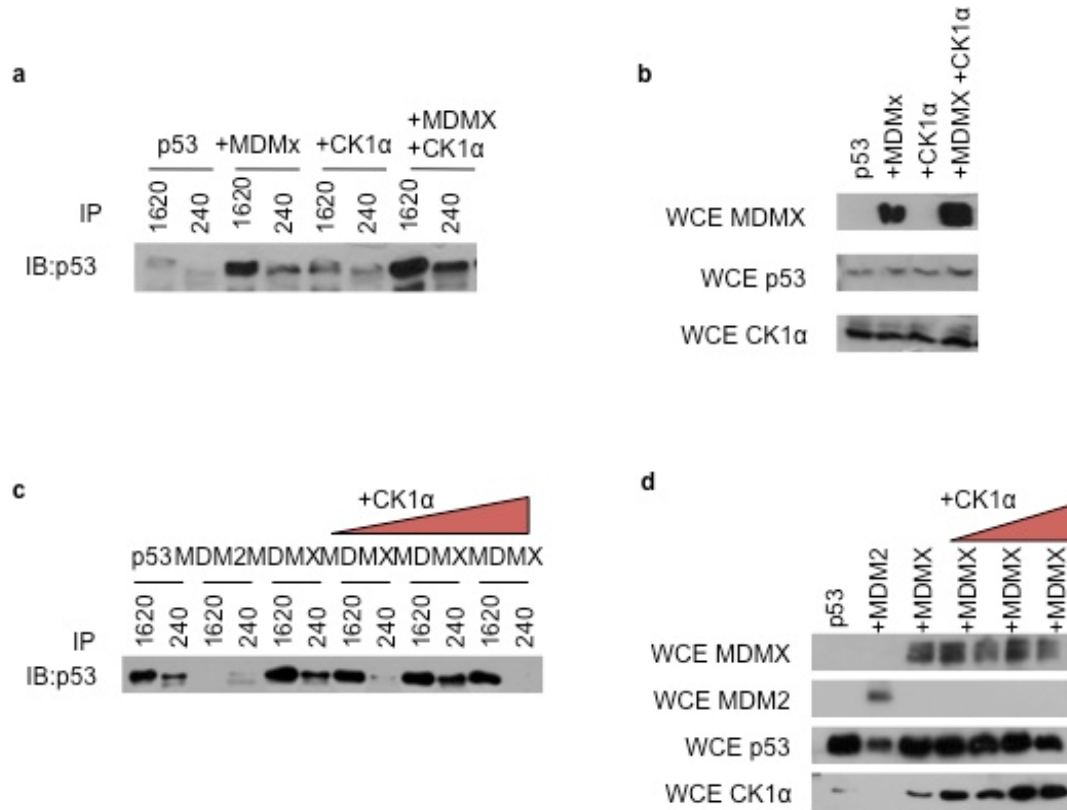
Figure 31: CK1α expression does not induce MDMX-mediated p53 conformational change. (a) & (c) H1299 cells were transiently transfected with the indicated expression plasmids and then treated with 30 μM MG132. The lysates were immunoprecipitated with Pab1620 or Pab240 antibodies, followed by immunoblotting for total p53 and FLAG-MDM2. (b) & (d) Western blot of whole cell extract showing expression levels of p53 and MDM2 mutants.

Acidic domain-p53 binding correlates with p53 conformational change

MDM2 acidic domain has been shown to interact weakly with p53 core domain (Ma et al., 2006; Wallace et al., 2006; Yu et al., 2006). Whether MDMX acidic domain has such activity has not been reported. To further test whether the ability to induce p53 conformational switch correlates with p53 core domain binding, MDM2-100-361 and MDMX-100-361 fragments were tested for p53 binding after cotransfection into H1299 cells. The result showed that MDM2-100-

80

361 coprecipitated with p53 at a clearly detectable level, although much weaker than full-length MDM2 as expected (**Figure 32**). In contrast, MDMX-100-361 showed no interaction with p53. This result adds further evidence that the MDM2 acidic domain is unique compared to MDMX in its ability to bind p53, which may be why MDM2, not MDMX, promotes p53 misfolding and inhibits p53 DNA binding.
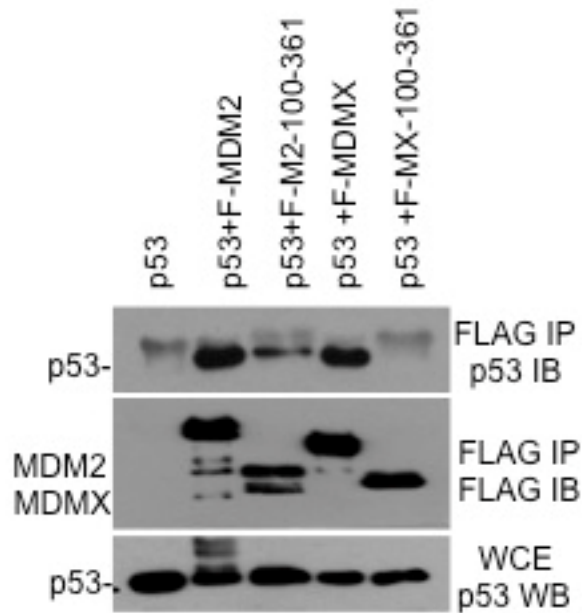
Figure 32: Acidic domain-p53 binding correlates with p53 conformational change. The indicated FLAG tagged proteins were transiently co-transfected with p53 into H1299 cells. FLAG-MDM2 or MDMX was immunoprecipitated using M2 Beads. Co-precipitated p53 was detected by western blot.

DNA damage abrogates the ability of MDM2 to shift p53 conformation

We also examined how this conformational response to high levels of MDM2 expression would be affected by phosphorylation of the RING domain of MDM2 following DNA damage. P53 was transfected with or without MDM2 and either left untreated or exposed to 10 Gy irradiation. These results showed that following DNA damage the 1620/240 ratio of p53, alone or with MDM2, began shifting towards a more wildtype conformation (**Figure 33 a & b**). Previous

reports have indicated that ATM phosphorylation of MDM2 is important for stabilization of p53 following DNA damage (Cheng, et al., 2009). Therefore, we also transfected p53 with the ATM resistant MDM2 6A mutant containing alanine substitution of six ATM phosphorylation sites near the RING domain. In unstressed cells this mutant is still able to shift the p53 conformation, however, following DNA damage the increase in 1620-reactive p53 that was observed when co-transfected with wild type MDM2 no longer occurs (**Figure 33 a & b**).
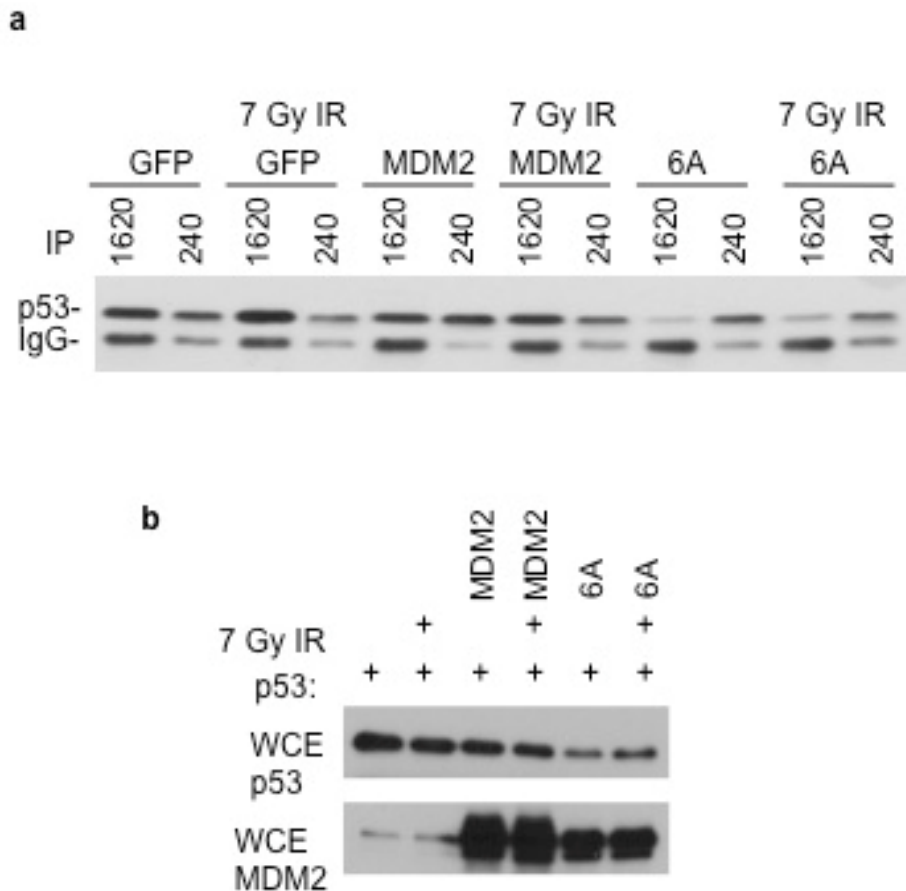
Figure 33: DNA damage and ATM phosphorylation of the MDM2 RING domain inhibit p53 conformational change. (a) H1299 cells were tranfected with the indicated plasmids, treated with MG132 (30 µM, 5 hr) and analyzed by Pab1620 and Pab240 IP. (b) WCE of samples was analyzed to confirm similar levels of protein expression for the MDM2 mutants.

To test if this DNA damage induced shift takes place with endogenous proteins we treated SJSA cells with DNA damage and MG132. Compared to untreated cells, there is a significant shift back to the wild-type conformation following DNA damage, regardless of the high levels of MDM2 observed in the cell line (**Figure 34**). To determine if DNA damage leads to a decrease in the MDM2 acidic domain-p53 interaction, p53 was transfected with or without full length or a.a. 50-491 MDM2 and either left untreated or exposed to 10 Gy irradiation. The results showed that there was a significant decrease in the p53 co-immunoprecipitated with the a.a. 50-491 MDM2 protein following gamma irradiation (**Figure 35**)**.** This data suggests that phosphorylation of the MDM2 RING domain also plays a role in prevention of p53 conformational shift following DNA damage by decreasing the MDM2 acidic domain-p53 binding interaction. This observation helps explain one way that DNA damage results in a transcriptionally activated p53 protein that can bind DNA and stimulate expression of downstream genes, facilitating the repair and survival of damaged cells or eliminate severely damaged cells through apoptosis (Ko and Prives, 1996; Levine, 1997; Vogelstein et al., 2000).
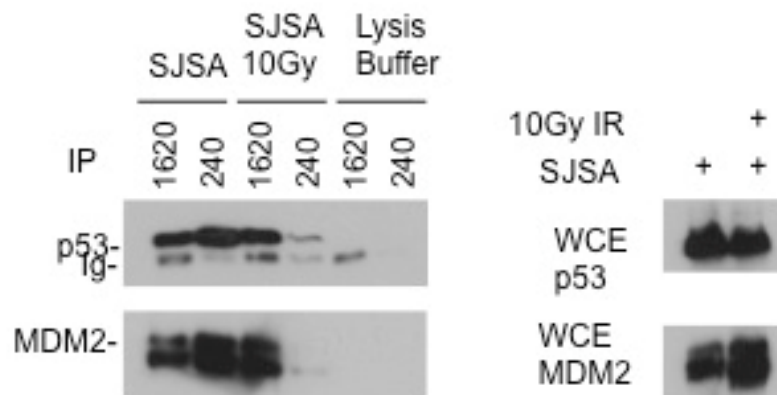
Figure 34: DNA damage inhibits MDM2 induced p53 conformational change. SJSA cells were treated with 30 uM of MG132, with or without 10 Gy irradiation, for 5 hours. Lysate was analyzed by Pab1620 and Pab240 IP. WB of WCE used to confirm protein expression.
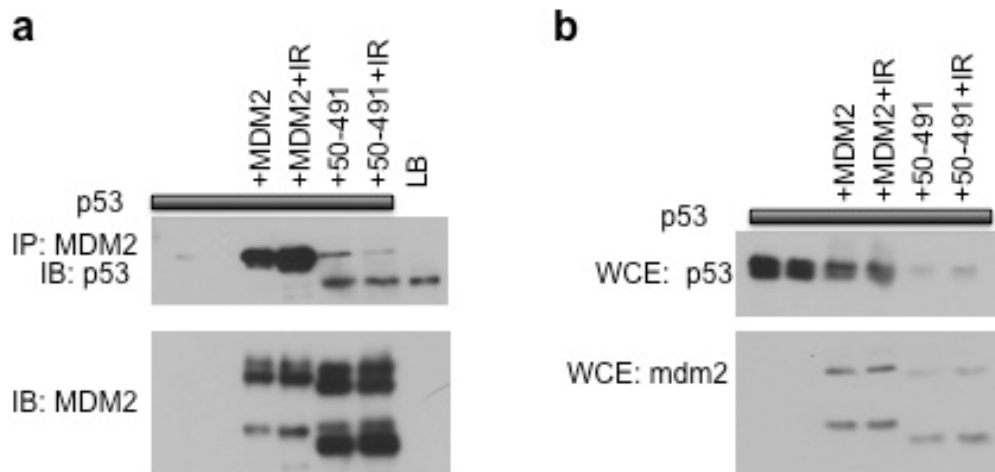
Figure 35: DNA damage inhibits the MDM2 acidic domain-p53 interaction.
(a) H1299 cells were tranfected with the indicated plasmids, treated with MG132
(30 μM, 5 hr) and analyzed by MDM2 IP. (b) WCE of samples was analyzed to
confirm similar levels of protein expression for the transfected plasmids.

ARF prevents p53 conformational change and restores DNA binding

The ARF protein is critical for p53 activation in response to hyper
proliferative stress through interacting with MDM2 acidic domain (Sherr, 2006).
Although the best-established effect of ARF is to cause p53 stabilization, we
asked whether ARF also stimulates p53 DNA binding by interfering with the
acidic domain of MDM2. When tested in the oligonucleotide DNA pull-down
assay, coexpression of ARF significantly rescued the DNA binding activity of
p53-MDM2 complex (**Figure 36a**). Furthermore, ARF expression also stimulated
p53 binding to the p21 and PUMA promoter *in vivo* (n = 3, p = 0.07 versus p =
0.24 for p21 and p = 0.06 versus p = 0.4 for PUMA) (**Figure 36 b, c & d**).
Western blots of duplicate samples were used to verify equal p53 expression and
confirm expression of ARF and MDM2 (**Figure 36 c, bottom panels**)
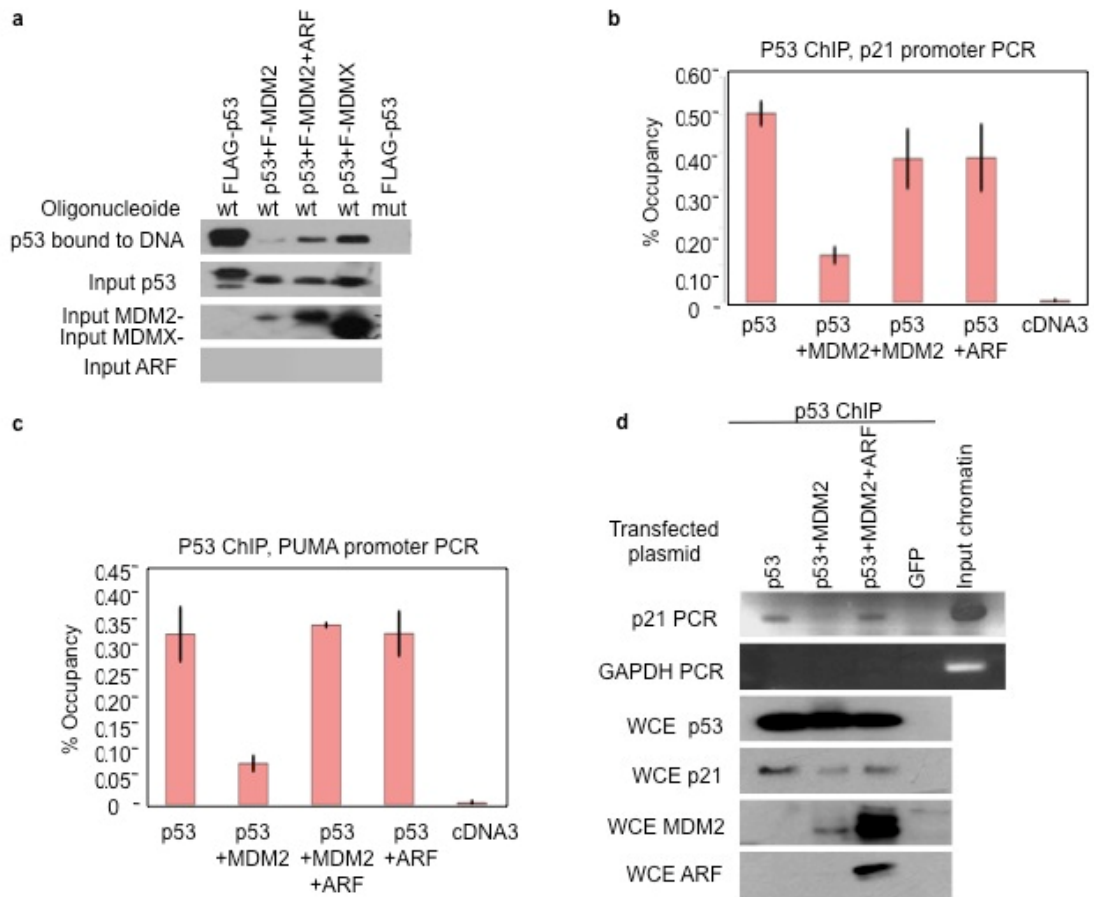
Figure 36: ARF expression stimulates p53-DNA binding. (a) H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site. (b) & (c) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP-qPCR to detect p53 DNA binding to the p21 and PUMA promoter. (c) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP to detect p53 DNA binding to the p21 promoter. Input is 1% of the starting lysate material. GAPDH promoter primers were used for a specificity control. WCE of parallel samples was analyzed to confirm levels of protein expression.

To test the effect of ARF expression on endogenous p53 transcriptional activity, U2OS cells were transfected with the BP100-luciferase reporter. Co-transfection with the MDM2 plasmid, again, resulted in strong inhibition of luciferase expression (n = 4, p = 3.75 x 10^{-6}), while the addition of ARF partially restored p53 transcriptional activity (n = 4, p = 1.4 x 10^{-4}) (**Figure 37**). These results corroborated the *in vitro* and *in vivo* DNA binding assay and showed that ARF efficiently increases p53 DNA binding and transcriptional activity, even in the presence of overexpressed MDM2.
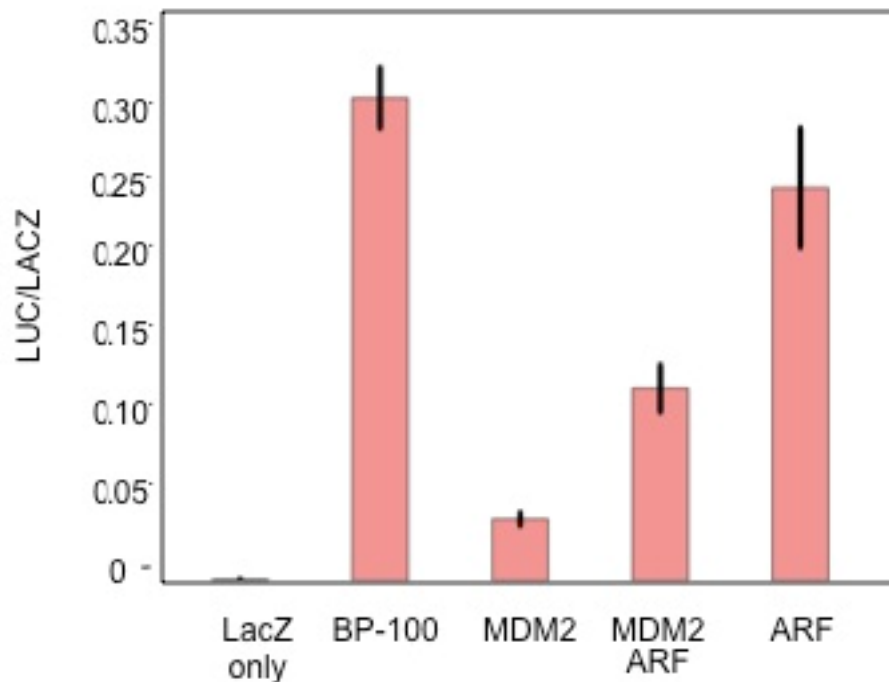
Figure 37: ARF increases p53 transcriptional activity. Endogenous p53 activity in U2OS cells was measured by transfection of BP100-luciferase reporter. Inhibition or stimulation by MDM2 or MDM2 + ARF was determined by co-transfection and luciferase assay.

Next, the effect of ARF on MDM2-mediated p53 conformational change was analyzed by co-transfection in H1299. The result showed that ARF blocked p53 conformational change in a dose-dependent fashion (**Figure 38 a**). This

occurred without affecting p53-MDM2 complex formation. In fact, ARF coprecipitated with p53 in the presence of MDM2, indicating formation of p53-MDM2-ARF trimeric complex (**Figure 38 a**). Furthermore, restoring ARF expression in SJSA cells (ARF-negative, MDM2 amplification) by infection with ARF adenovirus partially reverted endogenous p53 to wild type conformation (**Figure 38 b**). In additional experiments, expression of ARF in U2OS cells with IPTG-inducible ARF (NARF6 cells) also showed that endogenous p53 reverted back to wild-type conformation (**Figure 39**). To confirm the trimeric complex formation FLAG-p53 was transiently transfected with the indicated plasmid combinations and only when MDM2 was co-transfected with ARF did p53 co-precipitate the ARF protein (**Figure 40 a & b).** These results showed that ARF has a novel function in preventing p53 conformational change mediated by MDM2.
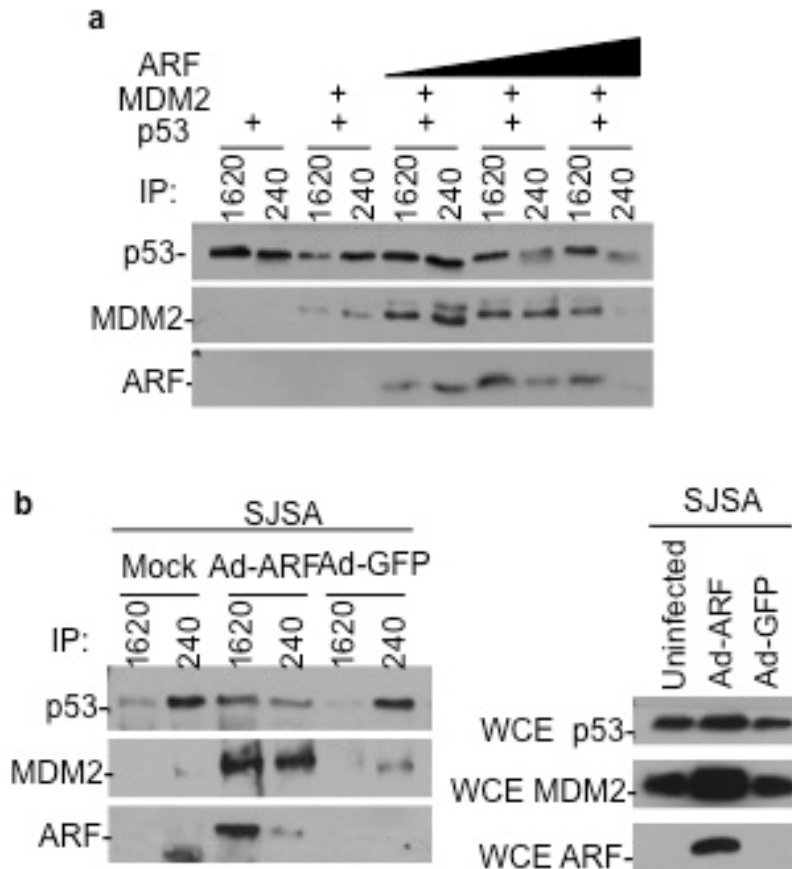
Figure 38: ARF inhibits MDM2-mediated p53 conformational change. (c) H1299 cells were transiently transfected with the indicated plasmids and increasing amounts of ARF. Cells were treated with MG132 (30 µM, 5 hr) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of ARF and MDM2 were detected by western blot. (d) SJSA cells were infected with adenovirus expressing ARF or GFP (MOI=50) for 24 hr. Cells were treated with MG132 (30 µM, 5 hr) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of ARF and MDM2 were detected by western blot. Expression levels were confirmed by western blot of WCE (right panels).
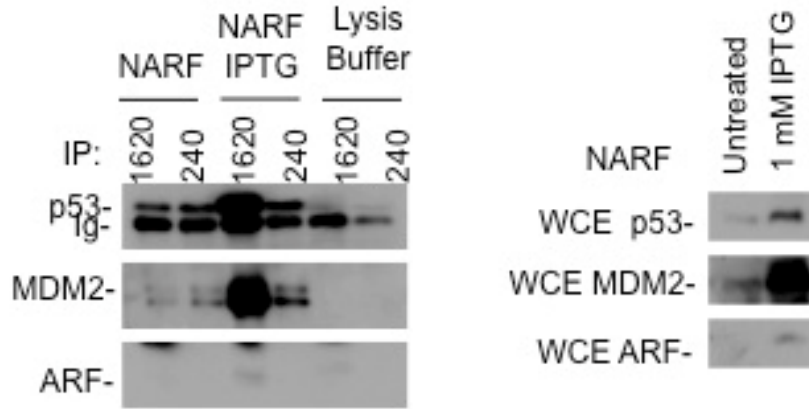
Figure 39: ARF blocks p53 conformational change. ARF restores p53 wild type conformation. NARF6 cells (U2OS with stable IPTG-inducible ARF) were treated with or without 1 mM IPTG for 24 hours to induce ARF expression, followed by 5 hrs of 30 µM MG132. Lysates were immunoprecipitated with Pab1620 and Pab240 antibodies, followed by immunoblotting for p53, MDM2 and ARF. Expression of each protein was confirmed by western blot of WCE.
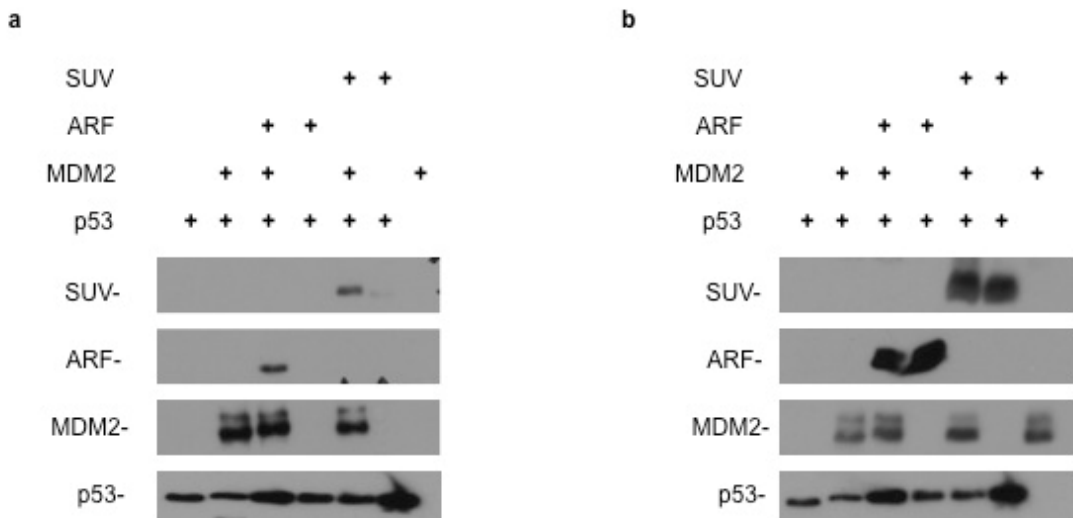
Figure 40: p53-MDM2-ARF form a trimeric complex. (a) H1299 cells were transiently transfected with the indicated plasmids followed by 5 h of 30 µM MG132. Lysates were immunoprecipitated with M2Beads, followed by immunoblotting for p53, MDM2, ARF and SUV39H1. (b) Expression of proteins were confirmed by western blot of WCE.

Since p53-MDM2-ARF trimeric complex was detectable after coexpression (**Figure 38, 39 & 40**), ARF may be recruited to DNA by binding to MDM2 and keeping p53 in a wild type conformation. As expected, ChIP analysis using Myc-tagged ARF showed that it was detected at the p21 and PUMA promoters in an MDM2 and p53-dependent manner (n = 3, p = 0.05 for p21 and p = 0.15 for PUMA) (**Figure 41 a & b**). Besides restoring p53 DNA binding, it remains to be determined whether chromatin recruitment of ARF has other functional consequence.
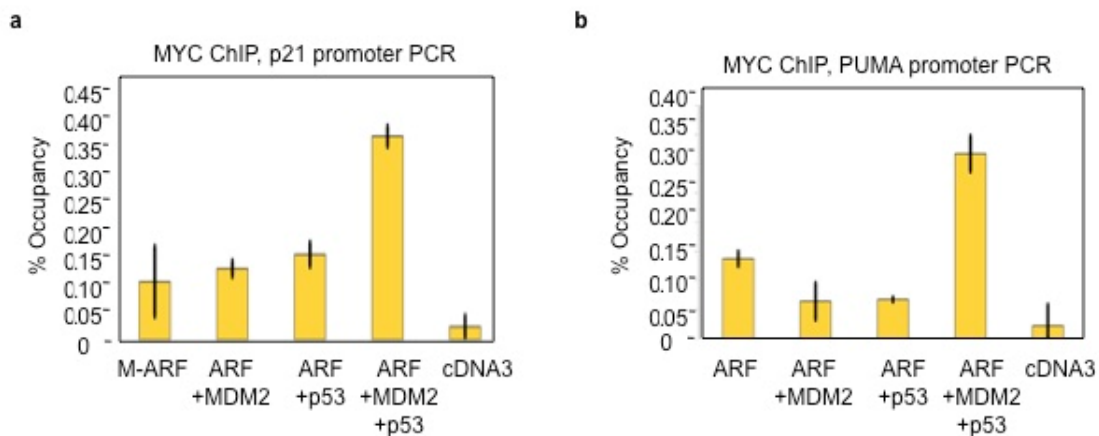
Figure 41: ARF binds at p53 target promoters via trimeric complex formation. (a) & (b) H1299 cells were transfected with Myc-ARF, MDM2 and p53. Cells were analyzed by ChIP using the anti-Myc antibody. ARF recruitment to p21 and PUMA promoters by p53 and MDM2 were detected by qPCR of ChIP samples.

SUV39H1 blocks p53 conformational change to access p53 target promoters

The MDM2 acidic domain interacts with several transcription repressors including YY1, KAP1 and SUV39H1. These interactions suggest that MDM2 may actively repress p53 targets by recruiting corepressors to promoters. We hypothesized that similar to ARF, co-repressors may also prevent MDM2 acidic domain from blocking p53 DNA binding, thus allowing recruitment of the p53-MDM2-repressor complex to promoters and repress transcription.

We tested this hypothesis using SUV39H1 as an example due to its efficient MDM2 binding (Chen et al., 2010). *In vitro* DNA binding assay showed that co-expression of SUV39H1 with MDM2 partially restored p53 DNA binding function (**Figure 42**). Co-expression of SUV39H1 with MDM2 and p53 also led to an increase in p53-DNA binding *in vivo* (n = 3, p = 0.06 versus p = 0.13 for p21 and p = 0.06 versus p = 0.1 for PUMA) (**Figure 43 a, b & c**).
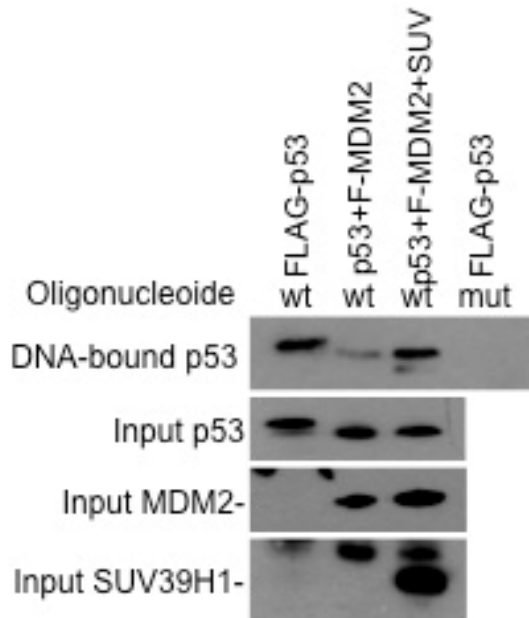
Figure 42: SUV restores p53-oligonucleotide binding. H1299 cells were transiently transfected and the FLAG-tagged protein complexes were immunopurified. Eluates containing equal amounts of p53 were captured with biotinylated oligonucleotide DNA containing p53 binding site.
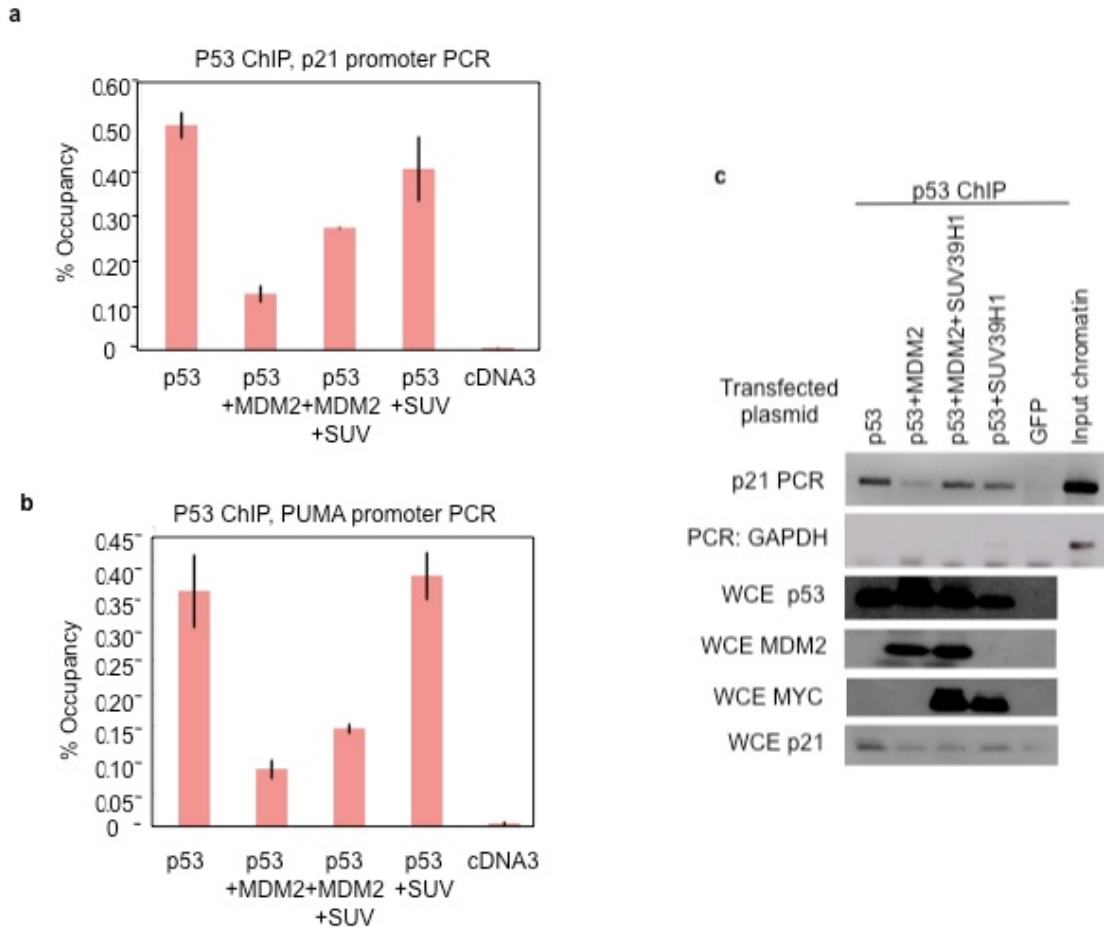
Figure 43: SUV39H1 restores p53-DNA binding. (a) & (b) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP-qPCR to detect p53 DNA binding to the p21 and PUMA promoter. (c) H1299 cells were transiently transfected with the indicated plasmids and analyzed by ChIP to detect p53 DNA binding to the p21 promoter. Input is 1% of the starting lysate material. WCE of parallel samples were analyzed to confirm levels of protein expression.

Next, to test the effect of SUV39H1 expression on endogenous p53 transcriptional activity, U2OS cells were transfected with the BP100-luciferase reporter. Co-transfection with the MDM2 plasmid, again, resulted in strong inhibition of luciferase expression and the addition of SUV39H1 was unable to restore p53 transcriptional activity (n = 4, p = 0.017 for MDM2 transfected alone compared to p = 0.015 for MDM2 and SUV39H1) (**Figure 44**). These results corroborated the *in vitro* and *in vivo* DNA binding assay and showed that

SUV39H1 efficiently increases p53 DNA binding, even in the presence of overexpressed MDM2. However, SUV39H1 still acts as a transcriptional repressor and blocks p53 transcriptional activity.
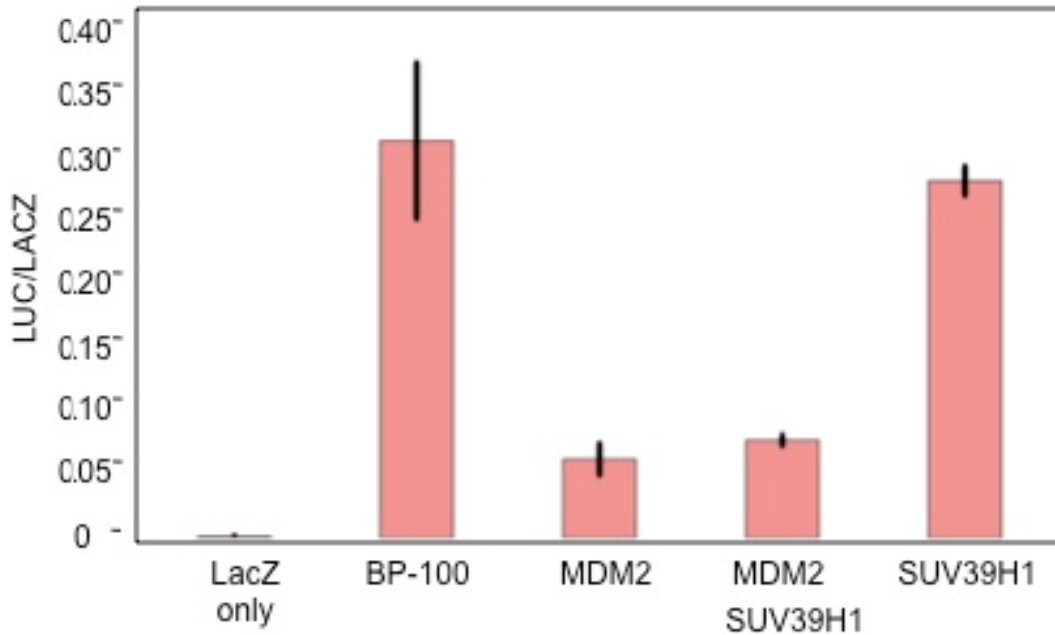
Figure 44: SUV39H1 increases p53 transcriptional activity. Endogenous p53 activity in U2OS cells was measured by transfection of BP100-luciferase reporter. Inhibition or stimulation by MDM2 or MDM2 + SUV39H1 was determined by co-transfection and luciferase assay.

To determine if the increase in p53-DNA binding when SUV39H1 is overexpressed correlates with a block in MDM2-mediated p53 conformational change a p53 conformational analysis was conducted. Overexpression of SUV39H1 partially inhibited the ability of MDM2 to induce p53 mutant conformation (**Figure 45**). Co-preciptation of p53, MDM2 and SUV39H1 indicated that these proteins also formed a trimeric complex (**Figure 45 & 40**). SUV39H1 ChIP analysis showed that coexpression of p53 and MDM2 promoted SUV39H1 recruitment to p21 and PUMA promoters (n = 3, p = 0.06 for p21 and p = 0.1 for PUMA) (**Figure 46 a & b**). Additional attempts to detect recruitment of endogenous SUV39H1 to the p21 and PUMA promoters by p53 and MDM2 were

not informative, possibly limited by the SUV39H1 antibody.   These results showed that similar to ARF, SUV39HI binding to MDM2 acidic domain prevents p53 misfolding by MDM2, thus allowing the trimeric repressive complex to bind p53 target promoters.
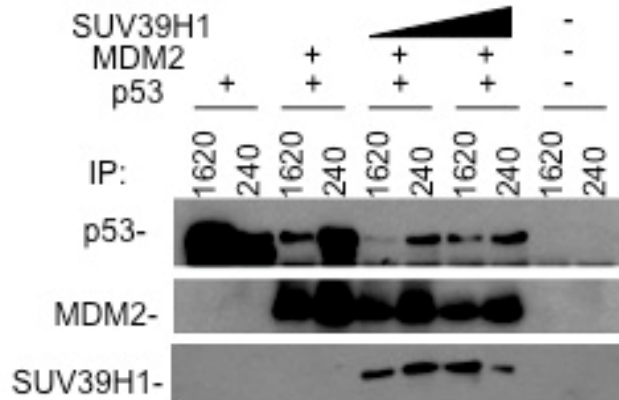
Figure 45: SUV39H1 restores p53 wild-type conformation. H1299 cells were transiently transfected with the indicated plasmids and increasing amounts of SUV39H1. Cells were treated with MG132 (30 µM, 5 hr) and analyzed by Pab1620 and Pab240 IP. Co-immunoprecipitation of SUV39H1 and MDM2 was detected by western blot.
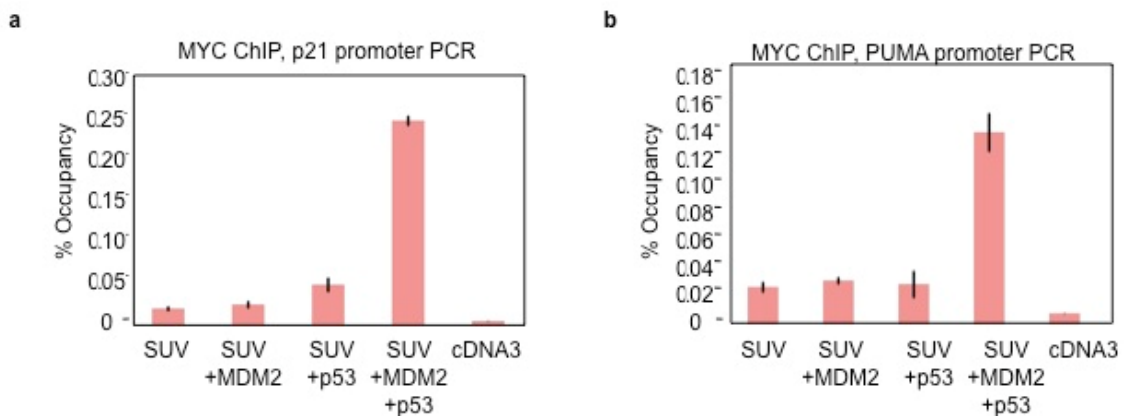
Figure 46: SUV39H1 binds at p53 target promoters via trimeric complex formation. (a) & (b) H1299 cells were transfected with Myc-SUV39H1, MDM2 and p53. Cells were analyzed by ChIP using the anti-Myc antibody. SUV39H1 recruitment to p21 and PUMA promoters by p53 and MDM2 was detected by qPCR of ChIP samples.
**Inhibition of MDM2 cooperates with Bortezomib to activate p53**

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Given the observation that p53 accumulated after proteasome inhibition is partially misfolded due to MDM2 binding, we asked whether this phenomenon has clinical relevance. The proteasome inhibitor Bortezomib (Velcade) induces cell death independent of p53 (Hideshima et al., 2003b). Our results suggest that proteasome inhibition stabilizes both p53 and MDM2, forming complexes that are partially deficient for DNA binding or transcription activation. Thus the p53-mediated anti-tumor activity is not fully exploited. If this is the case, Nutlin should cooperate with Bortezomib to induce p53 targets by inhibiting MDM2.

When SJSA and U2OS cells were treated with Bortezomib, there was negligible to weak increase in protein levels of PUMA and p21. Combination of Bortezomib and Nutlin resulted in strong induction of PUMA and p21 compared to either drug alone, without further increasing p53 level (**Figure 47**). RT-PCR analysis showed that in MDM2-overexpressing SJSA cells, Bortezomib caused p53 accumulation but did not induce PUMA and p21 mRNA. Bortezomib and Nutlin combination induced PUMA and p21 mRNA to levels similar to Nutlin alone (**Figure 48 a & b**). ChIP analysis showed that p53 DNA bind was moderately induced by Bortezomib in SJSA cells, and was further enhanced by Nutlin (n = 3, p = 0.001 increased to p = 0.004 for p21 and p = 0.0085 increased to p = 0.009 for PUMA) (**Figure 48 c & d**).
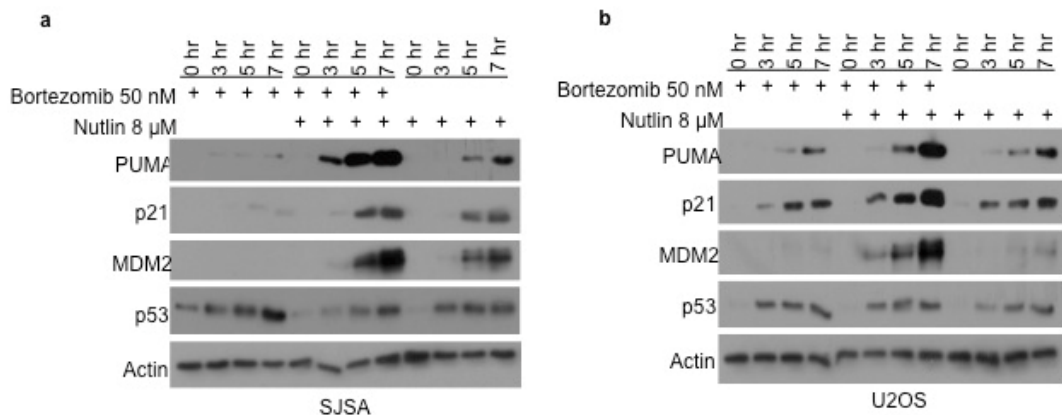
Figure 47: Nutlin activates p53 in Bortezomib-treated cells. (a) SJSA & (b) U2OS cells were treated with the indicated drugs and analyzed by western blot at indicated time points for expression of p53 targets.

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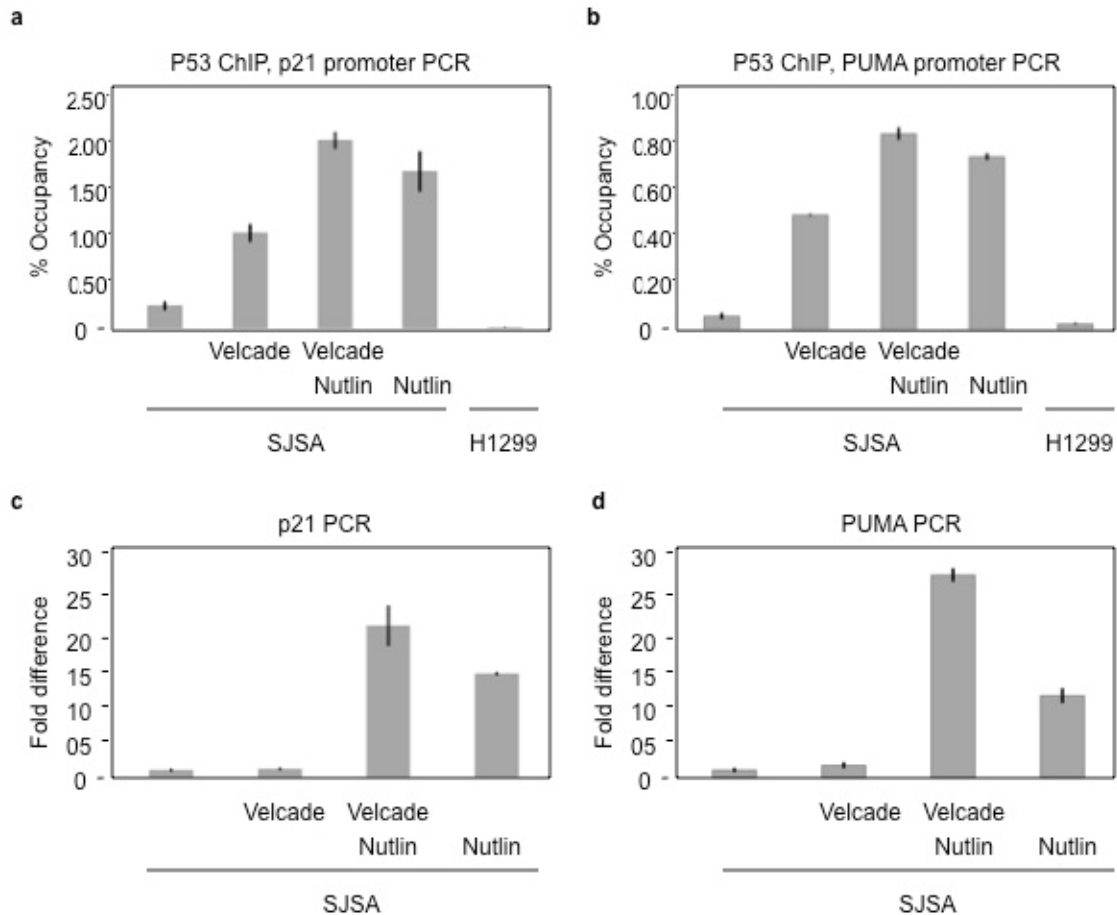
Figure 48: Nutlin increases p53-DNA binding and transcription of target genes in Bortezomib treated SJSA cells. (a) & (b) SJSA cells were treated with 8 μM Nutlin and 50 nM Bortezomib for 5 hours. Cells were analyzed by p53 ChIP and qPCR of PUMA and p21 promoters. (c) & (d) SJSA cells were treated with 8 μM Nutlin and 50 nM Bortezomib for 5 hours. Total RNA isolated from the cells was analyzed by quantitative RT-PCR. PUMA and p21 mRNA levels were normalized to GAPDH.

Similar results were observed in U2OS cells (n = 3, p = 0.001 increased to p = 0.007 for p21 and p = 0.006 increased to p = 0.05 for PUMA), although higher level of PUMA and p21 mRNA were induced by Bortezomib alone (**Figure 49 a - d**), as expected from the lower level of MDM2 in this cell line. These results showed that p53 stabilized by Bortezomib has poor DNA binding and poor transcriptional activity due to interaction with MDM2. Inhibition of MDM2 restores the DNA binding and activation functions of p53 in Bortezomib-treated cells.
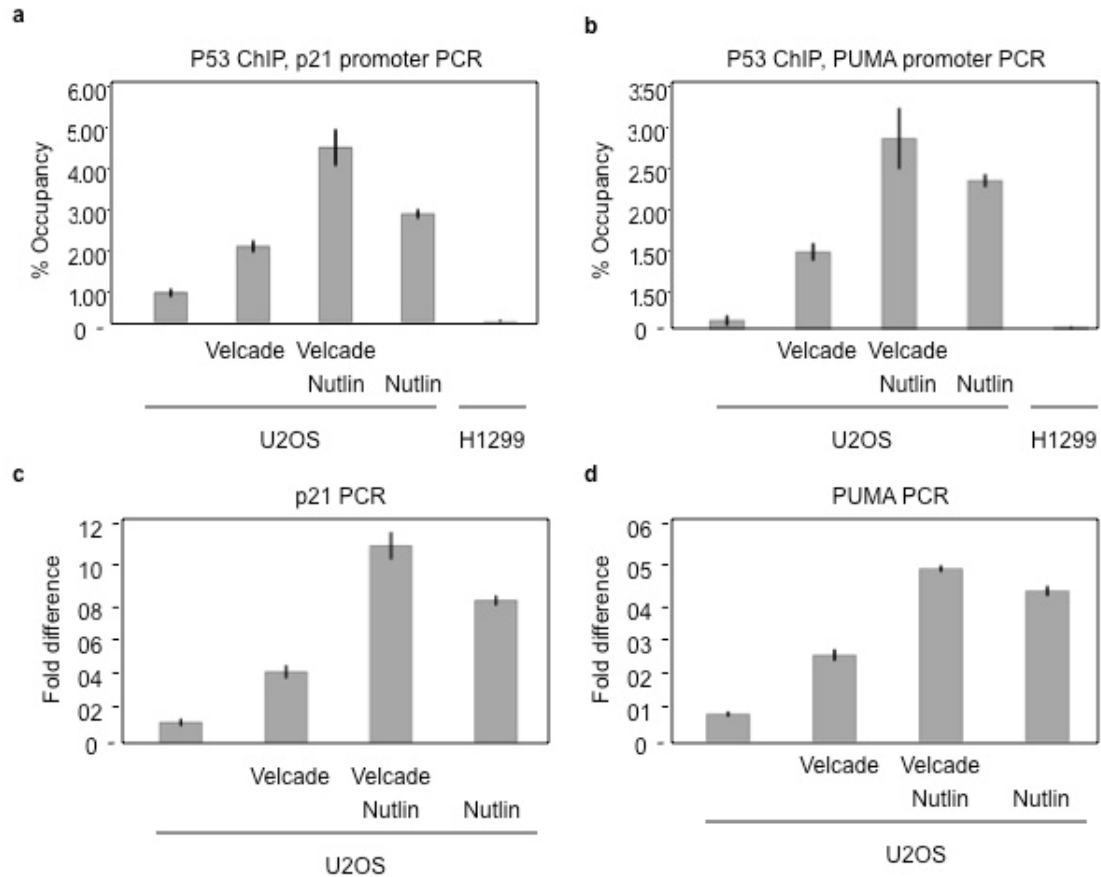
Figure 49: Nutlin increases p53-DNA binding and transcription of target genes in Bortezomib treated U2OS cells. (a) & (b) U2OS cells were treated with 8 μM Nutlin and 50 nM Bortezomib for 5 hours. Cells were analyzed by p53 ChIP and qPCR of PUMA and p21 promoters. (c) & (d) U2OS cells were treated with 8 μM Nutlin and 50 nM Bortezomib for 5 hours. Total RNA isolated from the cells was analyzed by quantitative RT-PCR. PUMA and p21 mRNA levels were normalized to GAPDH.

To establish if the inhibition of MDM2-p53 interaction by Nutlin might potentiate the effects of the proteosomal inhibitor Bortezomib in a p53-dependent manner we assessed the effect of combining the drugs on cell viability using the MTT assay. Following a 48 h exposure of Bortezomib or Nutlin alone, and in combination, on cells containing wild type p53 (SJSA, U2OS & HCT +/+) or mutant and null p53 (DLD1, MDA-231 & HCT -/-), we showed that the combination produced a strong synergistic cytotoxic effect on the wild-type containing cell lines, compared to the mutant and null p53 cell lines (**Figure 50 a,**

b & e compared to Figure 50 c, d & f). Nutlin alone was slightly cytotoxic to the wild-type p53 containing cell lines, however, the combination treatment increased the cytotoxic effects, as indicated by the drastic decline in cell viability from 0 to 5 μM Bortezomib, compared to Bortezomib only treatment from 0 to 5 μM. These results indicate that the inhibition of MDM2 from Nutlin treatment, which restores DNA binding and activation functions of p53 in Bortezomib-treated cells, ultimately leads to decreased cell viability in wild-type-p53 containing cancer cell lines.
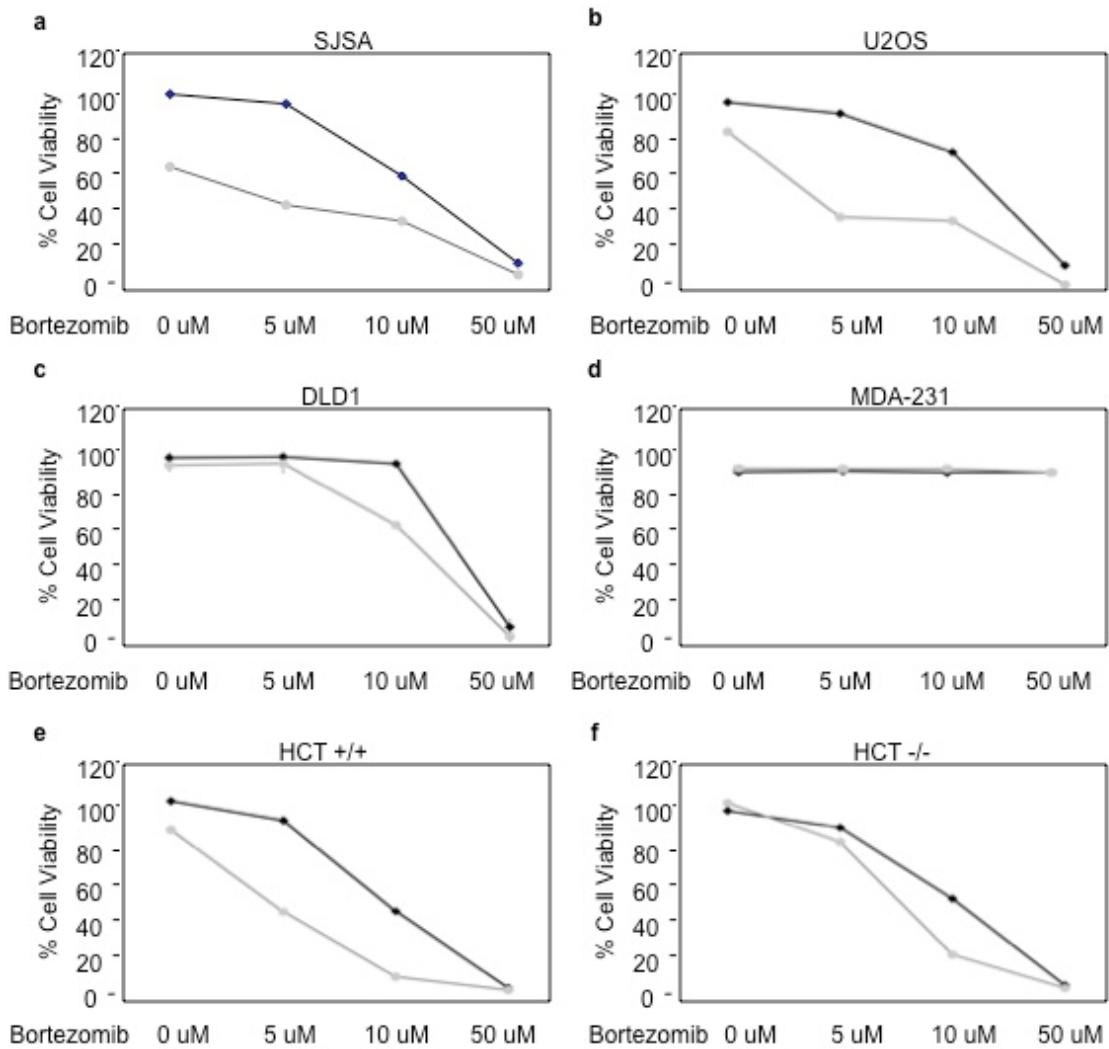
Figure 50: **Combination of Bortezomib and Nutlin treatment lead to increased cytotoxicity in p53 wild type containing cell lines.** (a), (b), (c), (d), (e) & (f) Cell viability of SJSA, U2OS, DLD1, MDA-231, HCT +/+ and HCT -/- cell lines after exposure to the indicated drug treatments for 48 h was assessed by MTT. The black line indicates Bortezomib only treatment, the grey line indicates combination 8 μM Nutlin and Bortezomib.

DISCUSSION

The experiments described above showed that MDM2 binding to p53 leads to its conformational change to a state similar to misfolded mutant p53. This activity does not require the ubiquitin E3 ligase activity of MDM2, consistent with recent reports from the Maki lab (Nie et al., 2007; Sasaki et al., 2007). Furthermore, our study produced several new observations: (1) MDM2 inhibits p53 binding to DNA through an E3-independent mechanism. (Gilmore et al., 2008) The acidic domain of MDM2 is critical for inducing p53 misfolding and inhibiting p53 DNA binding. (3) The MDMX acidic domain does not bind p53 or induce p53 misfolding. (4) DNA damage blocks MDM2-mediated p53 conformational change. (5) ARF prevents p53 misfolding by MDM2. (6) SUV39H1 binding to MDM2 acidic domain prevents p53 misfolding and allows its recruitment to p53 target promoters.

The detailed mechanism by which MDM2 binding induces p53 conformational change remains to be determined. The core domain of p53 is known to have poor thermo stability and spontaneously denatures at physiological temperature *in vitro* (Bullock et al., 1997). Presumably, p53 in cells also exist in a dynamic equilibrium as wild type and mis-folded forms. A simple scenario is that the MDM2 acidic domain preferentially interacts with p53 at a mutant-like conformation with exposed internal residues. The presence of MDM2 will alter the p53 conformation equilibrium and trap it in a mutant conformation. It is also possible that MDM2 binding to p53 N terminus causes allosteric changes in its core domain, which is then trapped by subsequent interaction with the MDM2 acidic domain. Molecular chaperones that normally promote protein folding, such as hsp90, have been shown to antagonize the effect of MDM2 (Sasaki et al., 2007).

It is noteworthy that the use of conformation-sensitive p53 antibodies resulted in the arbitrary definition of wild type (Pab1620-positive) and mutant (Pab240-positive) conformational states. In reality, p53 is likely to also exist in many intermediate states between Pab1620 and Pab240-reactive conformations. After binding to MDM2, p53 may rapidly lose DNA binding activity before fully adopting a Pab240-positive conformation. Consistent with this notion, MDM2 inhibits p53 DNA binding in ChIP assay in the absence of MG132, when most p53 complexed with MDM2 are still Pab1620-positive. The MDM2-457S E3 mutant also efficiently inhibits p53 DNA binding *in vivo* while only switching a fraction of p53 to Pab240-positive conformation. Therefore, MDM2-induced p53 misfolding may be more efficient than the Pab240 reactivity suggests. This distinct function may enable MDM2 to act rapidly without relying on ubiquitination and degradation of p53, or serve as backup mechanism as a counter balance to p53 deubiquitinating enzymes.

Our results underscore the importance of the MDM2 acidic domain in p53 regulation. The central region between residues 200-300 of MDM2 is a busy hub for binding multiple transcription corepressors and coactivators, and contains several phosphorylation sites (Blattner et al., 2002; Kulikov et al., 2005). Important p53 activators such as ARF and many ribosomal proteins also bind to the acidic domain. The region is predicted to be structurally disordered, which is a pre-requisite for interaction with multiple partners by adopting different conformations upon binding (Dunker et al., 2008). The acidic domain has also been shown to bind to p53 core domain, and our results add a new function to this interaction, which is blocking DNA binding by p53. Surprisingly, the MDMX central domain does not have this activity, probably because of low sequence homology to MDM2 acidic domain. In fact, most MDM2 acidic domain-binding partners do not bind MDMX (ARF, L5, L11, L23, SUV39H1, EHMT1). Although it is not clear which is the primitive member of the MDM2/X family, we speculate that MDM2 may be an evolved version of MDMX that gained a multitude of new regulatory capabilities to control p53. Alternatively, MDMX may have evolved in a different direction to perform distinct but essential functions in p53 regulation.

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The specific ability of MDM2 to switch p53 to a mutant conformation should enable more effective p53 inhibition than MDMX. This is consistent with findings in animal models that MDM2 is a more critical regulator of p53 than MDMX in adult organs (Grier et al., 2006; Maetens et al., 2007). By simultaneously promoting p53 ubiquitination and blocking DNA binding, MDM2 efficiently neutralizes p53 function without entirely relying on its degradation. This function is further enhanced by recruitment of corepressors to p53 target genes that directly repress transcription. It is possible that p53 misfolding by MDM2 also serves to increase ubiquitination efficiency by increasing access to lysine residues in the core or C terminal domains. Although the novel activities of MDM2 acidic domain are most evident in overexpression assays, they are likely to be physiologically relevant, as shown using endogenous proteins in SJSA cells.

Our results reveal novel mechanisms by which p53 can be regulated (**Figure 51**). It has been well document that the tumor suppressor, p53, has the ability to couple the cell cycle to DNA damage and initiate apoptosis if the cell is unable to repair the damage (Croce, 2008). In fact, following DNA damage p53 plays a critical role in prevention against malignant transformation by inducing not just apoptosis, but also cell cycle arrest and DNA repair depending on the level and type of cellular damage that has occurred (Levine, 1997; Levine et al., 2004; Vogelstein et al., 2000). Many studies have shown that once the cells encounter DNA damage, multiple post-translational modifications occur, leading to an increase in p53 levels due to stabilization and an increase in p53 transcriptional activity (Lakin and Jackson, 1999; Saito et al., 2003). There are still questions as to how DNA damage stabilizes and activates p53. Here we show that one possible mechanism would be through blocking MDM2-mediated conformational change. This would allow p53, now in a mainly wild-type conformation, to bind DNA and transcribe target genes. It is also a possible explanation for the observations by our lab and other groups that show DNA damage leads to p53 stabilization, yet MDM2-p53 binding is not significantly decreased. DNA damage might prevent the secondary MDM2 acidic domain-

p53 interaction, decreasing p53 ubiquitination and proteosomal degradation while also allowing for the protein complex to bind DNA.
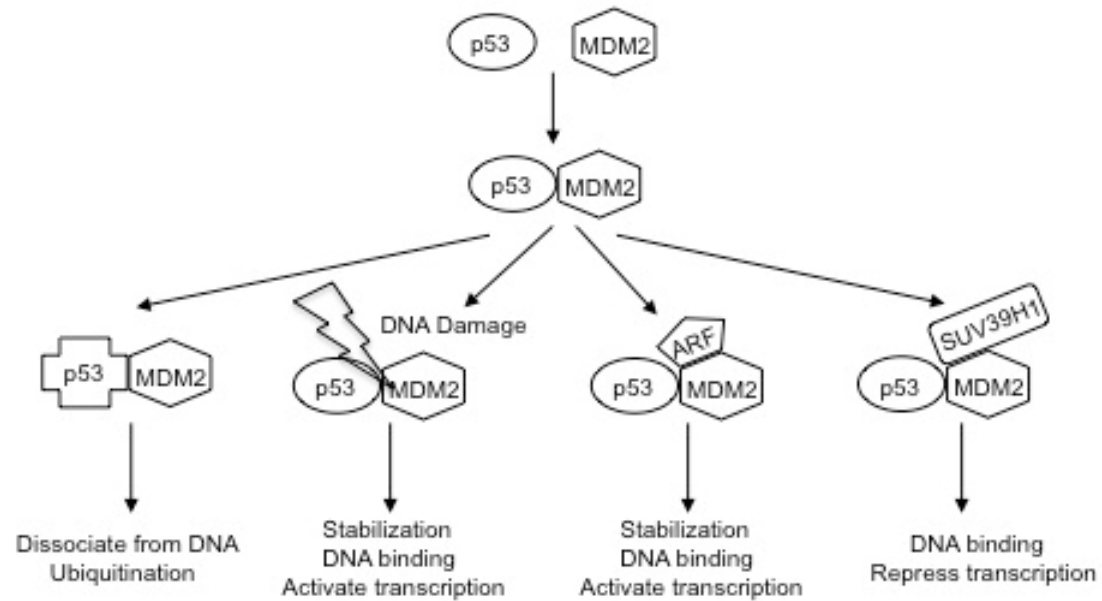
Figure 51: A model for ubiquitin-independent regulation of p53 by MDM2. MDM2 binding to p53 leads to subsequent conformational change of p53 core domain, resulting in loss of DNA binding. Following DNA damage MDM2 is not longer able to shift p53 to a mutant conformation, leading to DNA binding and transcriptional activity. ARF binding to MDM2 acidic domain inhibits ubiquitination of p53, but also prevents p53 conformational change and loss of DNA binding activity, resulting in stabilized p53 competent in transcription activation. SUV39H1 binding to MDM2 acidic domain prevents p53 conformational change, which allows p53-MDM2-SUV39H1 trimeric complex to bind DNA and repress p53 target promoters.

The ARF tumor suppressor is best known for its ability to block p53 ubiquitination by MDM2. Here we showed that ARF binding to the MDM2 acidic domain also prevents p53 misfolding, and stimulates p53 DNA binding. ARF binding to MDM2 has been shown to promote stable beta sheet formation (Bothner et al., 2001; Sivakolundu et al., 2008), which may trap MDM2 acidic domain in a conformation that cannot bind p53 core domain or induce p53 misfolding. It is still not clear how p53-MDM2-ARF complex can activate transcription after binding DNA. Possibly because p53 binds DNA as a tetramer,

a fraction of the p53 molecules have N terminal domain that are not concealed by MDM2. Furthermore, p53-DNA binding may be more stable than p53-MDM2 interaction, eventually leaving free p53 on the DNA.

If MDM2-p53 complex does not bind DNA, why is endogenous MDM2 detected on p53 target promoters in tumor cells devoid of ARF (Arva et al., 2005; Jin et al., 2003; Tang et al., 2008; Wang et al., 2004; White et al., 2006)? Our results suggest that binding of MDM2 acidic domain by SUV39H1 has effects similar to ARF, preventing p53 misfolding and allowing p53-MDM2 complex to bind DNA. It is possible that other proteins that interact with the acidic domain of MDM2 also protect p53 from misfolding. Therefore, when MDM2 is detected on p53 target promoters, it is likely to be in complexes with acidic domain binding partners. In the case of p53 activators such as ARF and ribosomal proteins, these interactions promote p53 DNA binding and transcription activation. In the case of corepressors such as KAP1 and SUV39H1, the interactions allow their recruitment to p53 target promoters to repress transcription. Furthermore, ARF has been shown to displace KAP1 and SUV39H1 from MDM2 (Chen et al., 2010; Wang et al., 2005), suggesting that ARF binding to p53-MDM2 complex on chromatin may block the recruitment of corepressors by MDM2. These mechanisms enable the MDM2 acidic domain to function as an important signal conduit for both activators and inhibitors of p53.

Our findings also have obvious translational implications. The proteasome inhibitor Bortezomib was initially shown to induce cell death independent of p53 status (Hideshima et al., 2003b). However, there is evidence that Bortezomib induces pro-apoptotic protein NOXA more efficiently in the presence of Wt p53 (Perez-Galan et al., 2006). Bortezomib acts as a reversible inhibitor of the chymotrypsin-like activity of the 26S proteasome in mammalian cells. The ubiquitin proteasome pathway plays an essential role in regulating the intracellular concentration of specific proteins, thereby maintaining homeostasis within the cells. Inhibition of the 26S proteasome can affect multiple signaling cascades within the cell, leading to disruption of normal homeostatic mechanisms and eventually cell death. Bortezomib has been reported to induce

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cytotoxicity in a variety of cancer cell types *in vitro* and delay tumor growth *in vivo* in pre-clinical tumor models, including multiple myeloma and mantel cell lymphoma cells (Perez-Galan et al., 2006; Wang et al., 2008). Although the drug has a variety of effects, bortezomib is capable of activating the p53 apoptotic pathway by stabilizing p53 through proteosomal blockade (Lowe et al., 1993).

Recent studies showed that Nutlin synergizes with Bortezomib to induce cell death in multiple myelomas and mantel cell lymphoma. The molecular mechanism of this cooperation was either not investigated (Ooi et al., 2009; Tabe et al., 2009), or was shown to be due to induction of p53 target genes (Saha et al., 2010). Our results suggest that proteasome inhibition stabilizes both p53 and MDM2, forming complexes that are partially deficient for DNA binding due to conformational switch by MDM2, or bind DNA but inactive for transcription due to MDM2 recruitment of repressors. As such, Nutlin cooperates with Bortezomib by relieving the repressive effects of MDM2, resulting in high-level expression of p53 target genes and increased p53-dependent cytotoxicity. This finding provides a molecular rationale for combination therapy using proteasome and MDM2 inhibitors against tumors that express wild type p53.

On the basis of a phase 3 clinical trial, Bortezomib is currently approved for the treatment of newly diagnosed and relapsed multiple myeloma patients (Lee et al., 2008; Richardson et al., 2005; San Miguel et al., 2008). Nutlin-like MDM2 disruptor is also being investigated in a phase 2 clinical trial but at this time there is no published data. Although bortezomib has been shown to be active against multiple myeloma, resistance to bortezomib, plus lack of response in solid tumors, have been an issue and an obstacle to overcome in order to achieve better clinical results. Our data indicates that the combination treatment of Nutlin-3 with bortezomib results in cooperative cytotoxicity in cells from solid tumor harboring wild-type p53 and high levels of MDM2. This combination treatment may be able to restore bortezomib sensitivity in advanced multiple myeloma patients and extend bortezomib activity against a larger spectrum of tumors. The results also explain why p53 does not seem to play a major role as a mediator of the effects of bortezomib in mono therapy. All though the total p53

mRNA and protein levels are increased, it is still functionally inactivated by MDM2 (Hideshima et al., 2003a). Therefore, the combination therapy includes the addition of MDM2 inhibition to proteasomal inhibition, not only stablizing p53 but also increasing its DNA binding activity. This ultimately makes p53 a more functional protein and is now able to induce apoptosis in previously functionally compromised cancer cells.

This information supports the need for a combination clinical trial in order to improve patient outcomes with not only multiple myeloma and lymphoma malignancies, but also in patients with solid tumors containing wt p53 and high levels of MDM2. Also of significance, the addition of MDM2 inhibitor treatment with bortezomib may allow a reduction in the therapeutic dose of this and other genotoxic drugs, which has the potential to reduce unwanted side effects. Similarly, because bortezomib has already been shown to induce an excellent response in some multiple myeloma and mantal cell lymphoma patients, the combinatorial effects from the addition of MDM2 inhibitor may allow for more flexibility in dosing patients on clinical trials and an increase in quality of life.

In summary, our basic research on the biochemical activity of MDM2 has led to unexpected insight directly relevant to cancer treatment. With the anticipated advance of MDM2 inhibitors currently in development, our results provide the rationale for implementing clinical trials of novel combination of approved and experimental drugs in the near future.

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