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Multiple modes of MDMX regulation affect p53 activation

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Multiple Modes of Mdmx Regulation Affect p53 Activation

by

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

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This work is dedicated to my mom, Marie, grandmother, Madeline, grandfather, Clement, and cousin, Cindy who all lost their brave battle with cancer. You are gone but not forgotten.

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ActD	Actinomycin D
ARF	alternative reading frame
ATM	ataxia-telangiectasia mutated
BER	base excision repair
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
Chk1	checkpoint kinase 1
Chk2	checkpoint kinase 2
DISC	death-receptor-inducing-signaling complex
DKC1	Dyskeratosis congenita
DUB	de-ubiquitinating protein
DSB	double strand break
FBS	fetal bovine serum
GFP	green florescent protein
HAUSP	herpesvirus-associated ubiquitin-specific protease
HDAC	histone deacetylase
HECT	homologous to the E6-AP COOH terminus
HIF-1	Hypoxia Inducible transcription factor
HFF	human foreskin fibroblast
HPV	Human Papilloma Virus
LOH	loss of heterozygosity
MDM2	mouse double minute 2
MDR1	multi-drug resistance gene
MEF	mouse embryo fibroblast
μl	micro liter
NLS	nuclear localization signal
NES	nuclear export signal
NoLS	nucleolus localization signal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pol I	RNA Polymerase I
Rb	retinoblastoma protein
SL1	promoter-selectivity factor
SV40	simian virus
TAD	transactivation domain
TBP	TATA-binding protein

TIF-IA   Transcription intermediary factor IA
UBF   upstream binding factor
UV   ultraviolet light

Multiple Modes of Mdmx Regulation Affect p53 Activation

Daniele M. Gilkes

ABSTRACT

MDMX has emerged as a negative regulator of p53 transcriptional activity following DNA damage, loss of ribosomal integrity, and aberrant mitogenic signaling. Disruption of rRNA biogenesis by ribosomal stress activates p53 by releasing ribosomal proteins from nucleoli which bind MDM2 and inhibit p53 degradation. We found that p53 activation by ribosomal stress requires degradation of MDMX by MDM2. This occurs by L11 binding to the acidic domain of MDM2 which promotes its E3 ligase function preferentially towards MDMX. Further, unlike DNA damage which regulates MDMX stability through ATM-dependent phosphorylation events, ribosomal stress does not require MDMX phosphorylation suggesting p53 may be more sensitive to suppression by MDMX under these conditions. Indeed, we find that tumor cells overexpressing MDMX are less sensitive to ribosomal stress-induced growth arrest by the addition of actinomycin D due to formation of inactive p53–MDMX complexes that fail to transcriptionally activate downstream targets such as p21. Knockdown of MDMX increases sensitivity to actinomycin D, whereas MDMX overexpression abrogates p53 activation. Furthermore, MDMX expression promotes resistance to the chemotherapeutic agent 5-fluorouracil (5-FU), which at low concentrations activates p53 by inducing ribosomal stress without significant DNA damage signaling. Knockdown of MDMX

abrogates HCT116 tumor xenograft formation in nude mice. MDMX overexpression does not accelerate tumor growth but increases resistance to 5-FU treatment in vivo.

In addition to MDMX regulation at the protein level, we found that regulation of cellular MDMX levels, like MDM2, can occur at the transcriptional level by inducing the Ras/Raf/MEK/ERK pathway. We found MDMX levels in tumor cell lines closely correlate with promoter activity and mRNA level. Activated K-Ras and growth factor IGF-1 induce MDMX expression at the transcriptional level through mechanisms that involve the MAPK kinase and c-Ets-1 transcription factors. Pharmacological inhibition of MEK results in down-regulation of MDMX in tumor cell lines. MDMX overexpression is detected in ~50% of human colon tumors and showed strong correlation with increased Erk phosphorylation. Taken together, the data show that MDMX has multiple modes of regulation, which ultimately determine the overall extent of p53 activation.

Chapter One
Introduction

Cancer

Although ancient Egyptians and their successors had knowledge of cancer as early as 400 BC, its prevalence could not be appreciated until more recently when average life expectancies have reached as high as 78 years old. Major causes of death in the past included common childhood and infectious diseases which have been eradicated by improved public healthcare and awareness ensuring the majority of the population will live beyond the age of 55. This is important because 77% of all cancers are currently diagnosed in people over the age of 55 (American Cancer Society 2007). The incidence of cancer rises dramatically with age, most likely due to risk accumulation over a life span accompanied by the tendency for cellular repair mechanisms to be less effective as a person grows older. Why some cancers occur in young children is still partially a mystery. Although it is now clear that some cancers occur in young children because of inherited predisposition.

Cancer is a leading cause of death worldwide. From a total of 58 million deaths recorded worldwide in 2005, cancer accounts for 7.6 million (or 13%) of all deaths (American Cancer Society 2007). The main types of cancer leading to overall cancer mortality are: lung (1.3 million deaths/year); Stomach (almost 1 million deaths/year); Liver (662,000 deaths/year); Colon (655,000 deaths/year) and Breast (502,000

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deaths/year). For Americans living in the United States this means men have a one in two lifetime risk of developing cancer; for women, the risk is one in three. These striking statistics exemplify the importance of researching the prevention and cure for cancer.

Tumorigenesis

Cancer is thought to arise from one single cell. The transformation from a normal cell into a tumor cell is now well accepted as a multistage process typically progressing from a pre-cancerous lesion to a malignant tumor. Tumor progression occurs via a sequence of sometimes-arbitrary events, which include both genetic and epigenetic DNA alterations. Based upon the observation that all cancers contain genetic alterations, it has been suggested that cancer cells are genetically unstable (Cahill, Kinzler et al. 1999). This instability may represent an early stage in cancer formation, suggesting that genetic instability results in a cascade of mutations to genes involved in cell growth, death, and differentiation. Successive accumulation of genetic abnormalities in a cell may be the overall driving force for tumor progression (Bishop 1987).

Genetic instability refers to abnormally increased tendencies for DNA to undergo mutations. When DNA damage rates supersede the rate of DNA repair, permanent mutations can occur. DNA mutations at the single nucleotide level occur in the form of base substitutions or deletions or insertions of a few nucleotides. Alternatively it can occur at the chromosomal level (chromosomal instability) resulting in losses and gains of whole chromosomes or large portions of a chromosome by translocation or amplifications (Lengauer, Kinzler et al. 1998). Epigenetic alterations can occur through DNA methylation, histone modifications or gene imprinting (Feinberg and Tycko 2004). Ultimately, DNA modifying events affecting genes responsible for cell growth, death,

and repair, such as the activation of oncogenes or inactivation of tumor suppressor genes lead to cancer progression. Furthermore, genetic changes have been linked to environmental factors such as:

physical carcinogens - ultraviolet (UV) and ionizing radiation

chemical carcinogens - asbestos and tobacco smoke

biological carcinogens - viral infections (Hepatitis B or Human Papilloma Virus (HPV)), bacteria (Helicobater pylori and gastric cancer) , contamination of food by mycotoxins such as aflatoxins

The culmination of deregulated genes controlling cellular homeostasis can lead to self-sufficiency in growth signals, insensitivity to antigrowth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and metastasis – the hallmarks of cancer (Vogelstein and Kinzler 1993; Hanahan and Weinberg 2000). Characterization of genes that promote or prevent cell proliferation is paramount to discovering treatments for cancer.

Tumor Suppressor Genes and Knudson's Two-Hit Hypothesis

Tumor suppressor genes reduce the probability that a normal cell will become a tumor cell. Therefore, it follows that mutations or deletions of tumor suppressor genes increase the probability of tumor formation. Indeed, experiments involving somatic cell fusion and chromosome segregation pointed to the existence of genes that could suppress tumorigenicity (Harris, Miller et al. 1969; Stanbridge 1976). Unlike oncogenes, tumor suppressor genes generally follow the 'Knudson's two-hit hypothesis,' which implies that both alleles that code for a particular gene must be affected before an effect is manifested (Knudson 1971). If one allele for the gene is damaged, the second can still produce the

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correct protein. In other words, tumor suppressors are usually not haploinsufficient (Comings 1973).

From these initial observations three properties were derived which are used to characterize 'classic tumor suppressors'. First, they are recessive and undergo biallellic inactivation in tumors. Second, inheritance of a single mutant allele accelerates tumor susceptibility, and only one additional mutation is required for complete loss of gene function (termed loss of heterozygosity (LOH)). Thus a germline mutation can be the underlying cause of a familial cancer syndrome that exhibits an autosomally dominant pattern of inheritance. Third, the same gene is frequently inactivated in sporadic cancers. The classic features of tumor suppression were first exemplified in studies of retinoblastoma and Wilm's tumor (Knudson 1971). Shortly thereafter, one of the most famous tumor suppressors studied to date, p53 was beginning to be characterized.

The Tumor Suppressor p53

P53 is a tumor suppressor gene located on the short arm of human chromosome 17 (17p13.1). P53 performs a variety of tumor surveillance functions in order to prevent the formation of tumors. Consequently, p53 mutations are shared by a wide variety of cancer types. When the integrity of genomic DNA is threatened by DNA damage, oncogene activation, nucleotide deprivation, or hypoxia, indolent p53 becomes stable and active. Upon activation, p53 triggers a variety of responses depending upon the type, extent, and duration of the imposed stress. The responses include but are not limited to cell cycle arrest, initiation of apoptosis, differentiation, senescence, and DNA repair. Cell cycle arrest and apoptosis are the most well-characterized effects of p53 activation (Vousden and Lu 2002).

Knocking out p53 in mice has highlighted the role of p53 in cellular homeostasis and tumor protection. Although most p53 knockout mice develop normally, they exhibit a high incidence of spontaneous lymphomas and sarcomas at an early age (Donehower, Harvey et al. 1992). Moreover, thymocytes (lymphocytes that derive from the thymus and are the precursor of a T cells) from p53 null mice are profoundly resistant to DNA-damage induced apoptosis. These observations indicate that a normal p53 gene is dispensable for embryonic development, but its absence predisposes the animal to neoplastic disease.

p53 History

Two independent groups identified p53 in 1979 as a cellular protein that bound to the simian virus (SV40) large T antigen and accumulated in the nuclei of cancer cells (Lane and Crawford 1979; Linzer and Levine 1979). Oren and Levine (1983) cloned the gene encoding p53 (TP53) from neoplastic rodent and human cells, and characterized it as having weak oncogenic activity (Lane and Crawford 1979; Oren and Levine 1983). This was further supported by the fact that p53 could cooperate with the activated Ha-Ras oncogene to transform normal embryonic cells (Eliyahu, Raz et al. 1984; Parada, Land et al. 1984). Other investigators found that the p53 gene was rearranged and inactivated in mouse erythroleukemia cells by insertion of the Friend murine leukemia virus into the gene locus (Mowat, Cheng et al. 1985). These changes were observed *in vivo* during the natural course of virus-induced disease, although the precise nature of the selective advantage conferred by p53 disruption remained unclear. Later, to further complicate earlier results, a murine p53 cDNA derived from F9 embryonal carcinoma cells failed to

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form foci in the presence of activated Ras unless it was mutated (Hinds, Finlay et al. 1987).

It took several years for researchers to determine that the first form of p53 discovered was actually a missense mutant of p53 and not the wild-type gene. Moreover, the missense mutations found in the original TP53 cDNA clones proved to be the key to understanding the pathobiological activity of p53. Mutant p53 can behave in a dominant-negative fashion. For example, the allele-producing mutant p53 suppresses the activity of wild-type p53 by binding and forming inactive tetramers. In 1989, a landmark study showed that p53's native function in a cell is actually as a suppressor of transformation (Finlay, Hinds et al. 1989).This was further exemplified in studies showing p53 was deleted in human colorectal cancers (Baker, Markowitz et al. 1990). Mutations of p53 were soon documented in many other forms of sporadic cancer and were revealed to be a causative genetic factor in patients with the familial Li-Fraumeni cancer susceptibility syndrome (Malkin, Li et al. 1990). Oncogenic human DNA viruses have also evolved a mechanism to inactivate p53 functions. Several viral oncoproteins including human papilloma virus (HPV) E6 and the adenovirus E1B 55K protein can bind to p53 and enhance its ubiquitin-dependent proteolysis (Zantema, Fransen et al. 1985; Werness, Levine et al. 1990). By the 1990s, p53 was widely recognized as a tumor suppressor gene, mutated or lost in ~50% of all human cancer cases worldwide making it a molecule worthy of intensive biomedical research studies in years to come.

Structure and Function of p53

Human p53 contains 393 amino acids with four functional domains including: amino-terminal transactivation domain (TAD), core DNA-binding domain (DBD),

carboxy-terminal oligomerization domain (OD) and a regulatory domain (RD). The first 42 amino acids of p53 encodes its transactivation function. This region is relatively acidic and has been shown to interact with components of the transcriptional machinery such as TATA-binding protein (TBP) (Lu and Levine 1995). The MDM2 oncoprotein has been shown to bind p53 in the N-terminal region where it negatively regulates p53's transactivation function (Lin, Chen et al. 1994). The proline-rich domain of p53 between residues 60 and 90 contains five copies of the sequence PXXP and is thought to play a role in p53-mediated suppression of cell growth and apoptosis (Sakamuro, Sabbatini et al. 1997; Venot, Maratrat et al. 1998).

The core DNA-binding domain allows p53 to bind to DNA in a sequence-specific manner. The consensus DNA binding sequence consists of two repeats of the 10 bp motif 5'-PuPuPuC(A/T)(A/T)GPyPyPy-3' separated by 0-13 bp (el-Deiry, Kern et al. 1992). The C-terminal region of p53(26 amino acids) is relatively basic in charge and regulates the ability of p53 to bind to specific DNA sequences at its core domain (Wang and Prives 1995; Wang, Vermeulen et al. 1996). Upon stabilization of p53, a discrete region within the C-terminal domain regulates oligomerization of p53. Deletion, phosphorylation, or binding of antibody to the C-terminal domain activates site-specific DNA binding by the central domain. The nuclear localization signals (NLS) are located within the c-terminal domain of p53 (Dang and Lee 1989).

Functions of p53

P53 functions by both transcriptionally-dependent and independent mechanisms. It plays a role in a wide variety of cell signaling mechanisms, which lead to cell-cycle arrest, DNA repair, cellular senescence, differentiation and apoptosis (Figure 1).

Ultimately, p53 can facilitate the repair and survival of damaged cells or eliminate severely damaged cells as a protection mechanism. When transactivated, p53 binds to DNA as a tetramer and stimulates expression of downstream genes that negatively regulate growth and invasion or mediate apoptosis (Vogelstein, Lane et al. 2000). Alternately, p53 acts as a transcriptional repressor. Transrepression by p53 relies on its ability to interact with basal transcriptional machinery, co-repressors or other DNA binding proteins.
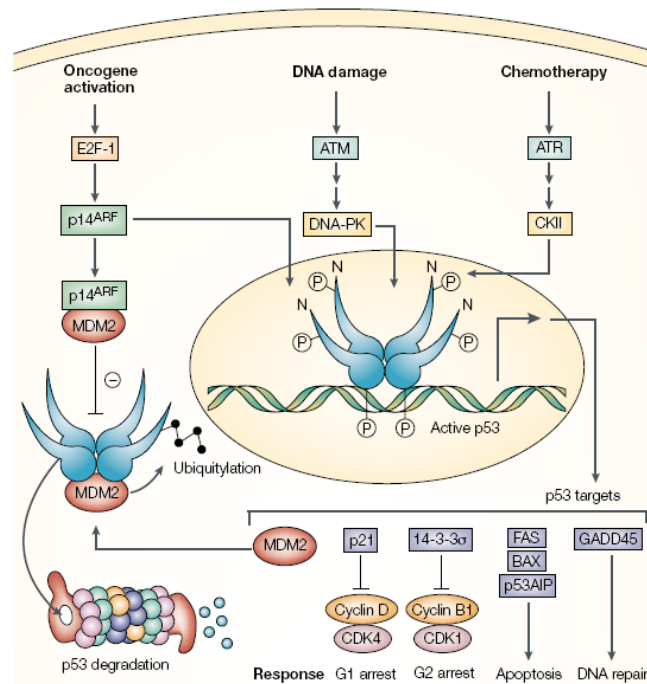
Figure 1. P53 Signaling. P53 plays a role in a wide variety of cell signaling mechanisms *(Bullock and Fersht 2001)*.

Cell Cycle Arrest

The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. Cyclins, cyclin dependent kinases (CDK), and CDK inhibitors

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are the major proteins, which control cell cycle progression. There are several cell cycle checkpoints which are key to preventing the proliferation of cells with flawed DNA(Kopnin 2000). P53 can transactivate proteins, which are responsible for monitoring both the G1/S and G2/M phases of the cell cycle. Perhaps the most well studied gene transduced by p53 is the cell cycle inhibitor p21^{WAF1}. Basal levels of p21^{WAF1} are required for cyclin/cdk complexes to assemble and be active; however, high levels block cdk activity thereby inhibiting cell cycle progression (Kastan, Onyekwere et al. 1991; Agarwal, Agarwal et al. 1995). The inhibitory effects of p21^{WAF1} are dominant, since induction or over-expression of p21^{WAF1} inhibits the activity of cdks, especially cyclin E/cdk2 complexes (Xiong, Hannon et al. 1993). Induction of p21^{WAF1} by p53 requires the transcription factor Sp1 and an intact p53 binding site localized far (> 1.9 kb) upstream of the coding sequence (el-Deiry, Harper et al. 1994; Macleod, Sherry et al. 1995; Koutsodontis, Tentes et al. 2001).

In addition to p21, 14-3-3σ (sigma) is a p53-response gene, which regulates cellular activity by binding and sequestering phosphorylated proteins. Upon induction, 14-3-3σ inactivates Cdc25 and Cdc2 by sequestering them in the cytoplasm causing a pre-mitotic G2/M cell cycle arrest upon DNA damage (Chan, Hermeking et al. 1999; Lopez-Girona, Furnari et al. 1999). Further, 14-3-3σ has been shown to promote the translocation of Bax out of the cytoplasm, delaying apoptotic signaling resulting in a G2 arrest (Samuel, Weber et al. 2001).

Apoptosis

Initial observations on the role of p53-induced apoptosis came from studies of the temperature sensitive mutant of p53 which acquires wild-type p53 conformation at the

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permissive temperature of 32°C. After shifting cells to 32°C, rapid cell death was observed (Yonish-Rouach, Resnitzky et al. 1991). The role of p53-dependent apoptosis was further eludicated using mouse models. Mice expressing SV40 Large T-antigen developed slow growing tumors due to suppression of p53-dependent growth arrest function. Mice cross-bred on a p53 null background developed more aggressive tumors (Symonds, Krall et al. 1994) suggesting p53 is needed to prevent tumor formation *in vivo*. In a study utilizing myc-driven lymphogenesis, blocking p53-mediated apoptosis prevented the selection of p53 mutations (Schmitt, Fridman et al. 2002). This suggests that p53 mutations are acquired as a means to overcome apoptosis. There are at least two broad pathways that lead to apoptosis, an "extrinsic" and an "intrinsic" pathway. P53 plays a role in both of these pathways.

The Intrinsic Pathway

The intrinsic apoptosis pathway begins when an injury occurs within the cell. It is governed by both pro- and anti-apoptotic members of the Bcl-2 family of proteins. The pro-survival (anti-apoptotic) family members are Bcl-2 and Bcl-XL. The pro-apoptotic family members include Bax, Bad, and Bid(Green and Evan 2002). There are also BH3 domain only apoptotic family members such as Puma and Bim. These proteins are involved in mitochondrial membrane potential and maintenance and the release of cytochrome C. Several proteins in the Bcl-2 family are transactivated by p53. Bax was the first Bcl-2 family member recognized as a target of p53 activation following cellular stress (Miyashita and Reed 1995). Upon induction Bax undergoes a conformational change forming homodimers which translocate to the mitochondria and promote the

release of Cytochrome C (Adams and Cory 2001). Mice deficient for Bax have increased tumor growth and a decrease in apoptosis (Yin, Knudson et al. 1997).

In addition to Bax, Puma is also upregulated following a p53 response to cellular stress (Nakano and Vousden 2001). Puma functions by promoting the oligomerization of Bax. On the other hand, Bax deficient cells are resistant to PUMA-mediated apoptosis (Yu, Wang et al. 2003). Other p53-inducible genes involved in the intrinisic apoptotic include Noxa, Bid, and APAF-1 (Oda, Ohki et al. 2000; Moroni, Hickman et al. 2001; Walensky, Pitter et al. 2006). Bid links the extrinsic and intrinsic pathways. Caspase-8 (involved in death receptor signaling) causes the cleavage of Bid. The truncated form of (tBid) induces Bax activation. Suprisingly, Bcl-2, an anti-apoptotic protein is also a transcriptional target of p53 but is repressed following p53 activation (Shen and Shenk 1994).

The Extrinsic Pathway

The extrinsic pathway begins outside a cell, when conditions in the extracellular environment signal the cell to undergo programmed cell death. Binding of Fas ligand (FasL or CD95L) to the Fas receptor (CD95) results in clustering of receptors and initiates the extrinsic pathway. Fas is a p53 response gene which is upregulated following chemically induced DNA-damage (Muller, Wilder et al. 1998). Fas clustering recruits FADD and pro-caspase 8 to form a complex. Formation of the death-receptor-inducing-signaling complex (DISC) results in activation of effector caspases. In addition, the death-domain-containing receptor DR5/KILLER of the TRAIL family is also a target of p53 activation (Wu, Kim et al. 2000).

Cellular Senescence

Following oxidative stress or telomere shortening, cells stop dividing and can undergo cellular senescence. Both p53 and Rb tumor suppressors are activated during senescence. Inactivation of p53 in mouse embryo fibroblast cells is sufficient to circumvent cellular senescence (Dirac and Bernards 2003). The induction of p21 by p53 is important for the activation of the RB pathway and for triggering senescence following DNA damage and telomere uncapping (Herbig, Jobling et al. 2004). Telomere shortening can be prevented by the overexpression of the human telomerase catalytic subunit, hTERT. Interestingly, hTERT has been shown to be downregulated by p53 whereas overexpression of hTERT can overcome p53-induced apoptosis (Xu, Wang et al. 2000).

DNA Repair

P53 has been linked to both the base excision repair (BER) and nucleotide excision repair mechanisms (NER). P53 induces GADD45 (usually induced following gamma irradiation in p53 wild-type cells) which can enhance NER (Smith, Ford et al. 2000). On the other hand, p53's interaction with DNA polymerase β and AP endonuclease (APE) substantiates its regulation of BER (Zhou, Ahn et al. 2001). Furthermore, following gamma irradiation, p53 induction is followed by an increase in 3-methyladenine (3-MeAde), an enzyme necessary for BER (Zurer, Hofseth et al. 2004).

Inhibition of Angiogenesis and Metastasis

Angiogenesis is the formation of new blood vessels which are required to sustain a tumor. It can be triggered by hypoxic conditions which activate the Hypoxia Inducible transcription factor (HIF-1). HIF-1 can induce the expression of VEGF, a potent endothelial mitogen necessary for vessel formation (Dachs and Tozer 2000). P53 can

inhibit this process in several ways. P53 can mediate the MDM2-dependent proteosomal degradation of the alpha subunit of HIF-1 (HIF-1α) (Ravi, Mookerjee et al. 2000). Moreover, p53 can down regulate the expression of VEGF and up regulate anti-agiogenic proteins such as Tsp-1 which has been shown to be suppressed in a variety of tumors (Bouvet, Ellis et al. 1998). P53 has also been shown to enhance the expression of the metastasis suppressor Nm23-H1. Further, the matrix metalloproteinases, MMP-1 and MMP-13, which promote tissue invasion by causing extra cellular matrix degradation are repressed by activated p53 (Sun, Sun et al. 1999; Sun, Cheung et al. 2000).

P53 Mediated Transcriptional Repression

Although gene transactivation has been the most intensively studied tumor suppressive function of p53 to date, p53 also plays an important role in gene repression. For instance, p53 mediated cell cycle arrest occurs not only by the upregulation of p21 and 14-3-3σ but by the transrepression of cyclin B and cdc2. Additionally, the putative caspase inhibitor Survivin is also downregulated by p53 (Hoffman, Biade et al. 2002). An elevated level of Survivin has been identified in numerous tumor types and is correlated with poor survival outcomes. The overexpression of Survivin has been shown to inhibit p53-inducible apoptosis. Other genes which are negatively regulated by p53 include the following: MDR1, Map4, stathmin, VEGF, PTGFβ, WT-1, and hTERT.

P53 has also been shown to repress transcription by competing for binding at target promoters. For example, repression of the alpha-fetoprotein gene (AFP) is the result of overlapping DNA binding sites for p53 and HNF-3 within the AFP promoter. Displacement of HNF-3 by activated p53 leads to a reduction in AFP transcription (Lee, Crowe et al. 1999). Repression by p53 can also occur in the absence of a specific p53

consensus sequence. For example, downregulation of hTERT occurs by p53 interfering with the coactivator, sp1 binding at the promoter (Xu, Wang et al. 2000).

P53 can also cause direct interference by interacting with basal transcriptional machinery. For example, cyclin B promoter deletions or mutations do not affect its repression by p53 suggesting that p53 is acting through the basal transcriptional machinery to inhibit transcription. Further, p53 has been shown to interact with TBP and certain TAFs which results in disruption of the pre-initiation complex assembly (Seto, Usheva et al. 1992).

P53 has also been shown to alter the chromatin structure by recruiting histone deactylases (HDAC) and the corepressor mSin3a to p53 target promoters. Moreover, in response to hypoxia the interaction between mSin3a and p53 is promoted resulting in downregulation of a subset of p53-repressed genes. HDAC inhibitors such as TSA has been shown to abrogate p53-mediated repression of MAP4. Further, p53 expression has been shown to decrease histone acetylation at the Survivin promoter (Murphy, Ahn et al. 1999).

Both the N and C terminus of p53 have been shown to play a role in its ability to repress gene transcription. Mutation of p53 serine 25 prevents its ability to repress MAP4 (Murphy, Ahn et al. 1999). On the other hand, mutating serine 386 C-terminal phosphorylation site of p53 attenuates p53-mediated repression of SV40 early promoters but not p53 activation (Hall, Campbell et al. 1996). Deletion of the PRD domain also impairs p53 transrepression function and is required for p53 and mSin3a interaction (Zilfou, Hoffman et al. 2001). This suggests that different regions are required to provide the specificity for p53-mediated transrepression under varying conditions.

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P53 Regulation

Following cellular insults, p53 must become rapidly activated in order to initiate

cell cycle arrest or apoptosis. However, in normal undamaged cells p53 is maintained at

low levels to prevent unwanted cell death and maintain cellular homeostasis. The ability

of p53 to act rapidly based on its microenvironment involves a variety of

posttranslational modifications including p53 ubiquitination, phosphorylation,

acetylation, sumoylation and neddylation (Figure 2). Although increases in the rate of

transcription or translation of p53 affect its cellular level, posttranslational modifications

have been shown to be the most efficient way to elevate both the activity and stability of

p53.
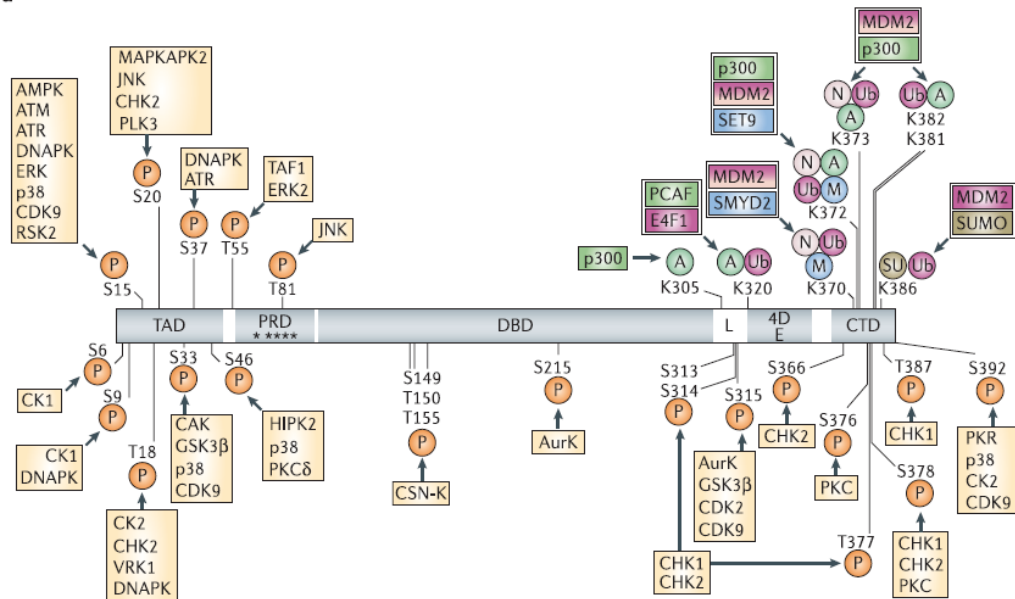
Figure 2. Post-translational Modifications of Human p53 *(Toledo and Wahl 2006).*

P53 Ubiquitination

The most well studied mechanism for the rapid turnover of p53 involves its negative regulator, MDM2. MDM2, a p53 target protein, directs p53's degradation via ubiquitin-dependent proteolysis. Thus, p53 directly activates expression of its own negative regulator, producing a potent negative feedback regulatory loop. Protein ubiquitination, including both mono- and polyubiquitination, is involved in many cellular processes. Polyubiquitination can target proteins for degradation by providing a recognition signal for the 26S proteasome. MDM2 acts as an E3 ligase, the final component of the enzyme cascade, conjugating ubiquitin to p53 to mark it for degradation via the proteosome (Haupt, Maya et al. 1997). Furthermore, ubiquitination by MDM2 has been shown to differentially catalyze monoubiquitination and polyubiquitination of p53 in a dosage-dependent manner (Li, Brooks et al. 2003). More specifically, low levels of MDM2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53. It is possible that these distinct mechanisms are exploited under different physiological settings or simply that poly-ubiquitination follows mono-ubiquitination.

Although, MDM2 is a major regulator of p53 protein stability, recent data suggests that MDM2-mediated ubiquitination, is not the only important factor for p53 regulation. In vitro, human p53 mutants in which all six highly conserved C-terminal lysine residues were mutated to arginine to prevent post-translational modifications including ubiquitylation and acetylation proved to be stable and more active than wild-type p53 (Nakamura, Roth et al. 2000). Likewise, knock-in experiments *in vivo* show that a p53 mutant protein, lacking the major ubiquitination sites for MDM2, has a normal half-life

and is stabilized and activated in response to stress (Feng, Lin et al. 2005; Krummel, Lee et al. 2005).

In addition to MDM2, other E3 ligases have been shown to promote p53 degradation. Pirh2, a RING-H2 domain-containing protein, interacts with p53 and promotes MDM2-independent p53 ubiquitination and degradation. Similar to MDM2, Pirh2 is a p53 responsive gene and participates in a similar autoregulatory negative feedback loop (Leng, Lin et al. 2003). COP1 is a direct ubiquitin ligase for p53 and is a p53-inducible target gene. Further, COP1 depletion by siRNA enhances p53-mediated G1 arrest and can sensitize cells to ionizing radiation (Dornan, Wertz et al. 2004). ARF-BP1 was recently identified as a HECT domain-containing E3 ligase that can ubiquitinate and degrade p53. ARF-BP1 was purified as a major ARF binding protein from p53-null cells. Interestingly, inactivation of ARF-BP1, in a manner reminiscent of ARF overexpression, induces tumor suppression in both p53 null and wild-type cells. This suggests that ARF-BP1 is involved in both p53-dependent and p53-independent functions of ARF (Chen, Kon et al. 2005). Together, MDM2, Pirh2, COP1 and ARF-BP1 represent an array of E3 ligases that the cell utilizes to regulate p53 stability. Thus, p53 directly activates expression of its own negative regulator, producing a potent negative feedback regulatory loop.

Recently, the discovery of deubiquitination enzymes (DUBs) added another layer of complexity to the ubiquitin-proteasome process. The herpesvirus-associated ubiquitin-specific protease (HAUSP) was found to bind to and stabilize p53 adding an additional layer of p53 regulation through the ubiquitination pathway. In the presence of HAUSP, p53 levels were sufficiently stabilized to induce growth arrest and apoptosis (Li, Chen et

al. 2002). On the other hand, siRNA-mediated reduction or knockout of HAUSP in HCT116 cells resulted in p53 stability. This can be explained by the observation that HAUSP can interact with MDM2. HAUSP exhibits strong deubiquitinase activity and stabilization of MDM2. These data suggest that HAUSP-mediated deubiquitination of MDM2 is required to maintain a sufficient level of the protein to act as an E3 ligase for p53 (Li, Brooks et al. 2004).

P53 Phosphorylation

There have been 23 different phosphorylation and dephosphorylation sites identified for p53 (Figure 2). Most residues are phosphorylated by different kinases following cellular stress. Upon DNA damage, one of the best characterized mechanisms of p53 stabilization and activation is its phosphorylation by activated checkpoint protein kinases. Upon activation by DNA damage, ATM, Chk1, and Chk2 phosphorylate p53 on several key serine residues. ATM phosphorylates p53 on serine 15 (Canman and Lim 1998) which induces a cascade of p53 phosphorylation. Chk2 phosphorylates p53 on serine 20 (Hirao, Kong et al. 2000). Following DNA damage signaling, MDM2 dissociates from p53 suggesting this is mediated through phosphorylation events. Indeed, tranfection studies indicate that phosphorylation of p53 by Chk2 is thought to disrupt MDM2-p53 binding (Unger, Juven-Gershon et al. 1999), but it has also been shown to be dispensable for efficient p53 induced G1 arrest (Jack, Woo et al. 2002). Further, *in vitro* studies with peptides from this region indicate that threonine 18 phosphorylation can significantly destabilize the MDM2-p53 association (Schon, Friedler et al. 2002). By contrast, other studies have shown that p53 mutants, in which all serine residues in the entire protein were changed to Alanine, displayed wild-type stability and transactivation

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(Wahl 2006). It is important to point out that mouse models with mutations of serine-15-to-Alanine (human serine 18) or serine-23-to-Alanine (human serine 20) exhibited modest, tissue-specific deficiencies, but did not substantially destabilize or inactivate p53 as would have been predicted if they were critical residues for reducing MDM2 binding (Wu, Earle et al. 2002; MacPherson, Kim et al. 2004; Sluss, Armata et al. 2004). This suggests that post-translational modifications to MDM2 following DNA damage may also be required to attenuate MDM2-p53 interactions. For example, c-Abl kinase phosphorylation of MDM2 on Tyrosine 394 has been shown to destabilize the MDM2-p53 interaction resulting in p53 stabilization and enhanced cell death (Goldberg, Vogt Sionov et al. 2002).

DNA-PK has also been shown to induce phosphorylation on N-terminal residues of p53 including serine 37 which is necessary but not sufficient for p53-DNA binding and transcriptional activity (Woo, McLure et al. 1998). C-terminal phosphorylation of p53 by CDKs, PKC, and CKII on serines 315, 378, 392 have been shown to mediate p53 sequence specific binding in vitro (Bischoff, Friedman et al. 1990; Delphin and Baudier 1994; Hall, Campbell et al. 1996). Phosphorylation of p53 on serine 46 by the autophosphorylating kinase, DRK2 following severe DNA damage results in apoptosis by p53-mediated transactivation of the pro-apoptotic gene p53AIP (Oda, Arakawa et al. 2000). HIPK2 also phosphorylates p53 on serine 46 dissociating the MDM2-p53 complex and inducing p53-mediated apoptosis (Di Stefano, Blandino et al. 2004). Conversely, the dephosphorylation of serine 215 by Aurora Kinase A reportedly inhibits the binding of p53 to DNA, overriding a DNA damage induced stress response (Liu, Kaneko et al. 2004). Likewise, the dephoshorylation of some residues of p53 have been

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correlated with p53 activation. For example, serine 376 is phosphorylated in unstressed cells, but following DNA damage, it becomes dephosphorylated enhancing its interaction with 14-3-3 (Stavridi, Chehab et al. 2001). Dephosphorylation of p53 can also lead to its inhibition. For example the intracellular domain of NOTCH-1 can bind to p53 inhibiting its phosphorylation on serine 15, 37, and 46 resulting in reduced p53 activation (Kim, Chae et al. 2007). The intricate control of p53 by a wide variety of kinases suggests a potential redundancy which would ensure that p53 is effectively activated under a multitude of stress conditions.

P53 Acetylation

Histone acetyltransferases such as p300/CBP and PCAF mediate acetylation of the C-terminal lysines of p53. DNA damage induced phosphorylation of the N-terminal of p53 increases its association with p300/CBP enhancing p53 acetylation and resulting in its activation (Barlev, Liu et al. 2001). MDM2 has been shown to inhibit the interaction of p53 and p300 in the absence of stress (Ito, Kawaguchi et al. 2002). The DNA damage inducible gene p33ING2, a potential tumor suppressor has been shown to increase Lysine 382 acetylation enhancing a G1/S specific checkpoint arrest (Garkavtsev, Grigorian et al. 1998). Additionally, PML, a protein induced by various stimuli, localizes to nuclear bodies together with p53 and CBP where it triggers N-terminal phosphorylation and C-terminal acetylation of p53 to facilitate its transcriptional activation (Guo, Salomoni et al. 2000; Pearson, Carbone et al. 2000).

P53 Neddylation and Sumoylation

P53 C-terminal lysine residues can also be altered by neddylation or sumoylation. Sumoylation is similar to ubiquitylation 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. The target for sumoylation in p53 is Lysine 386 and sumoylation was reported to modulate p53 transcriptional activity (Gostissa, Hengstermann et al. 1999). Sumoylation of p53 seems to be regulated by MDM2- and ARF mediated nuclear targeting (Chen and Chen 2003). It has been shown to induce senescence in normal human fibroblasts and apoptosis is Rb-deficient cell lines (Bischof, Schwamborn et al. 2006). Whether p53 can be de-sumoylated is not yet known.

Neddylation inhibits p53 transcriptional activation in a process promoted by MDM2 (Xirodimas, Saville et al. 2004). In this modification, the C-terminal glycine residue of the ubiquitin-like protein NEDD8 can be covalently linked to Lysines 370, 372, or 373 of p53. NEDD8 is conjugated to MDM2, which apparently promotes conjugation of NEDD8 to p53. Three of the neddylated lysine residues overlap with lysine residues that are ubiquitinated. Whether p53-specific de-neddylation pathways exist, or whether neddylation competes with acetylation or augments ubiquitylation is not yet clear. Other modifications that regulate p53 activity include methylation mediated by methyltransferases such as Set9 on Lysine 372 which causes stabilization and activation of p53 when overexpressed (Chuikov, Kurash et al. 2004).

P53 Cellular Localization

Nuclear import and export of p53 is a tightly regulated process. Nuclear localization is required for p53-mediated transcriptional regulation. P53 contains three nuclear localization signals (NLS) that upon stimulation enable its nuclear import whereas nuclear export of p53 to the cytoplasm is mediated by two nuclear export signals (NES) (O'Brate and Giannakakou 2003). However, efficient nuclear export of p53 to the

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cytoplasm requires the ubiquitin ligase function of MDM2 (Boyd, Tsai et al. 2000; Geyer, Yu et al. 2000). Mutations of the lysine residues in the C-terminus, where MDM2-mediated ubiquitination occurs, abrogates MDM2-directed nuclear export (Nakamura, Roth et al. 2000; Rodriguez, Desterro et al. 2000). This is thought to be due to the exposure or activation of a nuclear export sequence of p53 by MDM2. Nuclear export of p53 has been shown to be necessary for efficient p53 degradation.

In some tumor types, such as neuroblastomas, expression of wildtype p53 is coupled with its failure to accumulate in the nucleus. The observed nuclear exclusion may be an effect of hyperactive MDM2 or the activity of glucocorticoid receptors (GR) (Lu, Pochampally et al. 2000; Sengupta, Vonesch et al. 2000). The latter involves complex formation between p53 and GR, resulting in cytoplasmic sequestration of both p53 and GR. Dissociation of this complex by GR antagonists, results in accumulation of p53 in the nucleus, activation of p53-responsive genes, growth arrest and apoptosis. Other proteins that directly or indirectly effect p53 nuclear import/export are importin-α, PI3/Akt, p14[ARF], Pacr, actin, vimentin and mot2 (O'Brate and Giannakakou 2003).

P53 mutations in Human Cancer

P53 mutations have been identified in a broad range of tumors to date including cancers of the ovary (48%), colon (43%), esophagus (43%), lung (38%), stomach (32%) and breast (25%) (Figure 3) (Lim, Lim et al. 2007). Increasing numbers of sequences obtained from human cancers add to a database of over 10,000 somatic tumorigenic p53 mutations (Hainaut and Hollstein 2000). Point mutations have been identified in more than 250 codons of p53. About 95% of these lie in the core DNA-binding domain, revealing the key role that p53 has in transcriptional activation. Furthermore, 75% occur

as single missense mutations in one allele of p53 rather than deletions, insertions or frameshifts. So, the oncogenic form of p53 is predominantly a full-length protein with single amino-acid mutations. The result of these mutations is usually high expression of stable mutant p53. These mutations assert a dominant-negative effect over the remaining wild-type allele by generating a heterooligomer of wild-type p53 with mutant p53. The second wild-type allele of p53 is generally also lost by a process called loss-of-heterozygosis (LOH) resulting in genetic instability. Many of these mutations affect the structural integrity of p53 or its ability to interact with DNA at target promoters, leading to the partial or complete loss of protein function. Other evidence suggests that some mutants of p53 possess oncogenic activity by a gain-of function mechanism (Dittmer, Pati et al. 1993; Harvey, Vogel et al. 1995). Mutant p53 is capable of activating an alternate subset of promoters such as c-myc as well as MDR1 (multi-drug resistance) genes which facilitate cell proliferation even under unfavorable growth conditions (Frazier, He et al. 1998; Sampath, Sun et al. 2001). Mutant forms of p53 are still able to interact with cofactors such as p300 and CBP causing deregulated gene expression. Thus, human tumors favor selection for accumulation of p53 mutations to promote tumor progression.

Most of the p53 mutations (30%) are found at six specific codon 'hotspots': R175, R245, R248, R249, R273, R282 in the core domain. Overall the most prevalent mutation occurring in human cancer affects R248. Although the resulting protein maintains a wild-type conformation, its DNA-binding capability is severely compromised (Ory, Legros et al. 1994). Mutations to codon R175 result in an altered conformation in p53 with a more severe phenotype *in vitro* (Soussi and Beroud 2001).
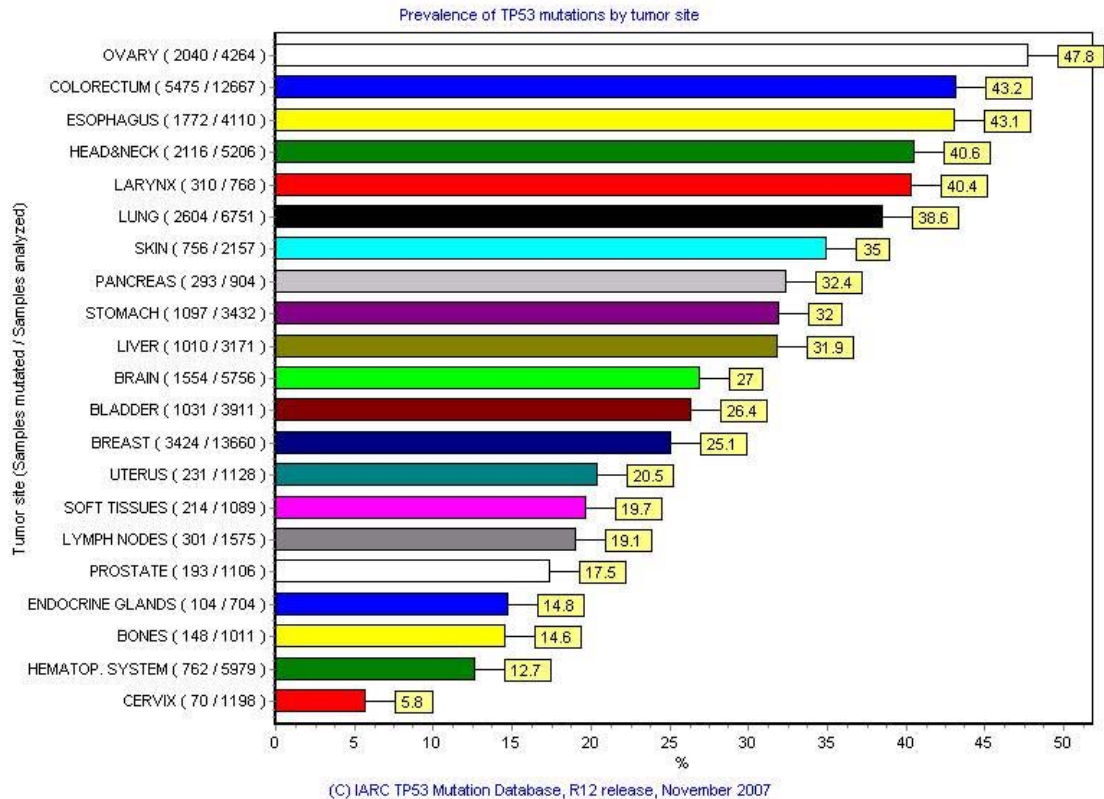
Figure 3. Prevalence of p53 mutation by site. *from IARC TP53 Mutation Database November 2007*

Clinical Consequences of p53 Mutation

There are severe clinical consequences for individuals when p53 mutations occur. According to several studies, specific p53 mutations can be associated with a poor prognosis or weak response to treatment. For example, colon cancer patients with p53 mutations in codon 175 have a lower probability of survival compared to patients with other mutations (Goh, Yao et al. 1995). Likewise breast cancer patients harboring p53 DNA-contact mutations also have poor prognosis compared to patients with p53 mutations which affect structural integrity (Takahashi, Tonoki et al. 2000).

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The specific amino acid substitution may also have prognostic significance. For example, polymorphisms in codon 72 can result in substitution of either an Arginine or Proline resulting in the expression of two different p53 proteins. The Arginine 72 form of p53 has been found to be more efficient at inducing apoptosis than the Proline 72 form. In contrast, the Proline 72 form appears to induce a higher level of G1 arrest. These results demonstrate significant differences in how the codon 72 polymorphism affects the biological activity of p53 (Pim and Banks 2004). Furthermore, the arginine form of p53 was found to be significantly more susceptible than the proline form to E6 mediated degradation. Moreover, allelic analysis of patients with HPV-associated tumors revealed a striking overrepresentation of homozygous arginine-72 p53 compared with the normal population, indicating individuals homozygous for arginine 72 are about seven times more susceptible to HPV-associated tumorigenesis than heterozygotes (Storey, Thomas et al. 1998).

Non-Mutated p53 in Human Cancer

While half of human tumors acquire a mutation in the p53 gene, the remaining 50% of cancers suppress p53 by disrupting its activation. For example, overexpression of the negative regulator MDM2 by gene amplification occurs in many tumor types (Momand, Jung et al. 1998; Dworakowska, Jassem et al. 2004; Ragazzini, Gamberi et al. 2004; Muthusamy, Hobbs et al. 2006). Increased expression of MDM2 leads to continuous degradation of p53 and therefore suppresses its activity. MDMX, a MDM2 homologue, has been found to be overexpressed in several tumor types and this leads to a reduction in p53 transcriptional activity (Danovi, Meulmeester et al. 2004).

Alternatively, upstream modulators of p53 are often inactivated in some tumor types. For example, mutations to ATM in the human disease ataxia-telangiectasia (AT) renders p53 unphosphorylated at serines 15 and 20 which can attenuate DNA-damage induced p53 stabilization (Maya, Balass et al. 2001). The AT-patients show multiple abnormalities including increased risk for lymphomas. Likewise a Chk2 germline mutation has been indentified in Li-Fraumeni-like syndrome patients that lack mutations in p53 (Bell, Varley et al. 1999). Additionally, the tumor suppressor ARF binds directly to MDM2, preventing MDM2-mediated degradation of p53 following mitogenic stress or oncogene activation (Sherr 2001). Loss of the INK4a/ARF/INK4b locus on chromosome 9p21 is among the most frequent cytogenetic events in human cancer (Kim and Sharpless 2006). Tumors which express wild-type p53 but lack ARF are unable to signal to p53 following oncogene activation (Ruas and Peters 1998).

Cancer Therapies involving p53

Current cancer treatments usually consist of heavy doses of chemo-or-radio therapies. These therapies primarily act to kill rapidly dividing cells but do not target specific aberrant pathways unique to tumor cells. As mentioned previously, to evade these therapies, about 50% of human tumors have mutated p53. Theoretically, it is possible to restore functional activity to p53 mutants by using second site suppressor mutations. These second site suppressor mutations can restore the stability and result in additional DNA contacts, and therefore restore the normal function to p53 mutants. One example is that the suppressor mutant N239Y can restore the 'hotspot' mutation G245S, and result in an improvement in DNA binding (Nikolova, Wong et al. 2000).

Other strategies have been developed to eliminate cells bearing mutant p53. For example, targeting malignant cells with oncolytic viruses (ONYX-015) genetically engineered to proliferate in cells containing mutant p53 genes have been identified as therapeutic approaches in previous animal studies (McCormick 2000). Initial clinical trials have confirmed functional activity and expression of the transgene product in tumors injected with a replication-deficient adenoviral vector containing wild-type p53 (Heise, Sampson-Johannes et al. 1997; Ries and Korn 2002; Crompton and Kirn 2007). Further, screening of a low-molecular-weight compound library yielded the identification of PRIMA-1, a compounds that can restore wild-type function to mutant p53. It is capable of inducing apoptosis in human tumor cells by restoring sequence-specific DNA binding and the active conformation to mutant p53 proteins *in vitro* and in living cells (Bykov, Issaeva et al. 2002). Several other investigators have identified small synthetic molecules or peptides that allow mutant p53 to maintain an active conformation (Foster, Coffey et al. 1999; Friedler, Hansson et al. 2002). With further work aimed at improving potency and deliverability, this class of compounds may be developed into anticancer drugs with broad utility.

As mentioned above, in tumors lacking p53 mutations, the maintenance of wild type p53 accompanies deficiencies in alternate components of the p53 pathway, such as amplification of MDM2. The crystal structure of MDM2 in complex with an N-terminal peptide of p53 shows that the p53 peptide forms an amphipathic α-helix that interacts with a hydrophobic pocket on MDM2 (Kussie, Gorina et al. 1996), suggesting small molecules could compete with MDM2 binding and activate p53. High throughput screening resulted in the recent development of Nutlin-1 and RITA, which restore the

apoptosis-inducing function of p53 by disrupting p53-MDM2 complex formation in tumor cells and xenograft models (Issaeva, Bozko et al. 2004; Vassilev, Vu et al. 2004). To date, a compound which can effectively inhibit MDMX-p53 binding has not been identified.

P53 Monitors Ribosomal Integrity

In addition to the nucleolar protein ARF, other components of the nucleolus interact with the p53 pathway resulting in p53 activation. The nucleolus serves as the processing center for rRNA synthesis and ribosomal assembly. Protein synthesis requires an available pool of rRNA causing cell growth and proliferation to be dependent on changes in ribosome production. rRNA synthesis requires three basal transcription factors: promoter-selectivity factor (SL1), upstream binding factor (UBF) and RNA Polymerase I (Pol I). A large family of small nucleolar RNAs extensively modifies and processes the pre-rRNA producing several rRNA intermediates, and finally, mature 18S, 5.8S and 28S rRNAs. The mature rRNA species associates with more than 70 ribosomal proteins to form the small (S, 40S) and the large (L, 60S) ribosomal subunits. After their assembly, the large and small subunits are transported to the cytoplasm to initiate protein synthesis (Nazar 2004). During G1, an increase in rRNA synthesis and ribosome assembly is necessary for protein synthesis during S phase. rRNA is maximal in S and G2 phases, repressed in mitosis and increased in G1. This link between cell-cycle progression and protein synthesis exists to ensure accurate cell growth and proliferation under appropriate conditions. Disrupting the pool of rRNA could subject the cell to deregulated growth conditions. The ability of the cell to recognize ribosomal stress and induce cell cycle arrest may be a determinant of cell survival in suboptimal conditions.

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This suggests that failure to recognize ribosomal stress could result in tumor initiation or oncogenic progression (Ruggero and Pandolfi 2003). In fact, nucleolar morphology has long been used a clinical marker for cell transformation. However, it remains to be determined whether this is a cause or a consequence of the transformation process.

Several lines of evidence suggest that the ribosome can signal the cell to regulate proliferation. A screen for cell lines generated from zebra fish which have a high propensity towards cancer incidence showed that ribosomal proteins can function as haploid-insufficient tumor suppressors (Amsterdam, Sadler et al. 2004). In addition, Germline mutation of DKC1, the gene altered in Dyskeratosis congenita, has a direct affect on ribosome assembly, and in humans has been associated with an increased risk of cancer (Ruggero, Grisendi et al. 2003; Ruggero and Pandolfi 2003). DKC1 mediates the posttranscriptional conversion of uridine to psuedouridine, which is required for proper rRNA folding and eventually ribosome biogenesis. In mouse models of Dyskeratosis congenita, over 50 percent of mutant mice develop tumors (Ruggero, Grisendi et al. 2003; Ruggero and Pandolfi 2003). Additionally, the small ribosomal subunit protein S19 has been shown to be mutated in Diamond-Blackfan anemia, a condition associated with an increased susceptibility to haematopoetic malignancies (Da Costa, Tchernia et al. 2003; Choesmel, Bacqueville et al. 2006). The direct mechanism of S19-induced tumorigenesis is not known, but it provides evidence that ribosomal defects can promote cancer susceptibility (Draptchinskaia, Gustavsson et al. 1999).

Ribosomal Stress and p53

The nucleolus has previously been regarded as a static entity, directing over 50% of cellular transcription, rRNA transcription. So, how is ribosome biogenesis coupled to cell

cycle progression? It has been demonstrated that serum-starvation of cycling cells results in both growth arrest and the inhibition of rDNA transcription. In addition, growth inhibitory stimuli represses RNA polymerase I transcription through pRB-UBF interactions (Hannan, Hannan et al. 2000; Ciarmatori, Scott et al. 2001). The Syrian hamster temperature cell line BHK21 is unable to produce a mature 28S RNA and 60S ribosome subunits and undergoes growth arrest at permissive temperatures (Toniolo and Basilico 1976; Mora, Darzynkiewicz et al. 1980). Inhibiting the nucleolar protein p120, a protein necessary for 60S ribosomal subunit formation, by siRNA induces G1 arrest of human lymphocytes (Fonagy, Swiderski et al. 1992). A conditional deletion of the S6 ribosomal protein in mice leads to defective ribosome biogenesis and reduced cellular proliferation (Volarevic, Stewart et al. 2000). The results of these studies imply that aberrant ribosome biogenesis may induce a checkpoint that prevents cell cycle progression. Since p53 is a cell sensor with capabalitities to quickly respond to both intrinsic and extrinsic cellular assaults, it follows that p53 could also play a role in monitoring ribosomal integrity.

Genetic Models of Ribosomal Stress and p53.

Several recent genetic models show that p53 is responsible for responding to rRNA perturbations. Transcription intermediary factor (TIF) IA is an RNA polymerase-I-specific transcription factor that is required for recruitment of polymerase I to the rRNA promoter. A dominant negative mutant of TIF-IA can suppress cell-cycle progression in proliferating HEK293T tumor cells, presumably by restricting ribosome production and thereby halting growth (Zhao, Yuan et al. 2003). The genetic inactivation of TIF-IA is embryonic lethal in mice. Cre-mediated depletion of TIF-IA in MEFs leads to disruption

of nucleoli, cell cycle arrest, upregulation of p53, and induction of apoptosis. RNAi-induced loss of p53 overcomes proliferation arrest and apoptosis in response to TIF-IA abrogation (Yuan, Zhou et al. 2005). Additionally, nucleolar stress induced by inactivating BOP1, using a BOP1 dominant negative mutant (BOP1D) first identified in a cDNA screen designed to isolate growth suppressors (Pestov, Grzeszkiewicz et al. 1998) led to a p53-dependent cell cycle arrest (Pestov, Strezoska et al. 2001). BOP1 is cell cycle regulated with peak levels at mid G1 phase (Strezoska, Pestov et al. 2000), concomitant with increased nucleolar function. Expression of BOP1D leads to cell growth arrest in the G(1) phase and results in specific inhibition of the synthesis of the 28S and 5.8S rRNAs without affecting 18S rRNA formation. Importantly, inhibition of p53 function results in the attenuation of cell cycle arrest induced by BOP1D (Strezoska, Pestov et al. 2002). The correlation between perturbation of rRNA biogenesis with elevated levels of p53 and induction of cell death supports the notion that the nucleolus can signal to p53 and direct cellular fate.

Actinomycin D induces Ribosomal Stress

In order to study the effects of ribosomal stress on p53 activation many laboratories utilize actinomycin D (ActD) to inhibit ribosome biogenesis (Iapalucci-Espinoza and Franze-Fernandez 1979). ActD is an agent widely used in combination chemotherapy for the treatment of choriocarcinoma (Kendall, Gillmore et al. 2003; Newlands 2003), testicular cancer (Miyazaki, Kawai et al. 2003), and soft tissue sarcomas (Bernstein, Kovar et al. 2006). ActD can induce DNA damage and inhibits general transcription at high concentrations (430 nM), but at low concentrations (5 nM) selectively inhibits RNA polymerase I and induces ribosomal stress. Low concentrations of ActD cause a

breakdown of nucleolar structure allowing release of L proteins (L5, L11, L23) from the nucleolus to the nucleoplasm where they become localized with and have increased binding affinity for MDM2. The MDM2-L protein interaction results in stabilization and activation of p53 (see MDM2 and Ribosomal Stress).

Nucleolin and Nucleophosmin/B23 signal to p53

In addition to L proteins, other components of ribosomal RNA processing such as nucleolin (Saxena, Rorie et al. 2006) and nucleophosmin/B23 (Itahana, Bhat et al. 2003; Korgaonkar, Hagen et al. 2005) have been implicated in signaling to the p53-MDM2 network. Nucleolin protein levels in unstressed cells correlate with levels of p53. Nucleolin directly binds to MDM2 and inhibits both p53 ubiquitination and MDM2 auto-ubiquitination. Increases in nucleolin levels in unstressed cells led to higher expression of p21, a reduced rate of cellular proliferation, and increased apoptosis (Saxena, Rorie et al. 2006). NPM/B23 (nucleophosmin), an abundant protein associated with ribosomal protein assembly, can activate p53 when overexpressed in many primary cells (Colombo, Marine et al. 2002). Conversely, knocking down B23 inhibits the processing of pre-ribosomal RNA and induces cell death (Itahana, Bhat et al. 2003). NPM affects p53 stability by interacting with ARF. NPM targets ARF to nucleoli and blocks ARF-mediated p53 activation and growth suppression in a dose-dependent manner. When NPM expression levels are reduced, ARF is released from its nucleolar constraints allowing it to bind to and suppress MDM2 resulting in p53 activation and growth-inhibition (Korgaonkar, Hagen et al. 2005). It is unclear why so many different proteins involved with ribosome biogenesis would interact with MDM2. Perhaps each protein

represents a signaling molecule, which is responsible for recognizing specific types of ribosomal stress.

MDM2-independent Ribosomal Stress Signaling

Although the mechanisms described thus far involve ribosomal signaling through the direct inhibition of MDM2, several MDM2-independent p53 signaling mechanisms exist. For example, ribosomal protein L26 can bind to the 5′ untranslated region of p53 mRNA enhancing p53 translation following DNA damage, increasing cell-cycle arrest and irradiation-induced apoptosis (Takagi, Absalon et al. 2005). Alternatively, the ribosomal protein S27L (S27-like protein) was identified as a p53 inducible gene in a genome-wide chip-profiling study. S27L harbors a concensus p53-binding site in the first intron. Further investigation revealed a p53-dependent induction of RPS27L in multiple cancer cell lines. In addition, expression of RPS27L promotes etoposide-induced apoptosis (He and Sun 2006). Lastly, a mitochondrial ribosomal protein L41 can directly bind to p53 and enhance translocation of p53 to the mitochondria, thus contributing to p53-induced apoptosis (Yoo, Kim et al. 2005). Overexpression of the mitochondrial ribosomal protein S36 also increases p53 expression and induces cell cycle arrest (Chen, Chang et al. 2006).

MDM2

The murine double minute (MDM2) oncogene, was originally cloned from the transformed mouse cell line 3T3-DM (Cahilly-Snyder, Yang-Feng et al. 1987; Fakharzadeh, Trusko et al. 1991). Three MDM genes were located on small, acentromeric extrachromosomal nuclear bodies, called double minutes, which were retained in cells only if they provided a growth advantage. MDM2 protein overexpression proved to be

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responsible for transformation of the 3T3-DM cell line. Additionally, the overexpression of MDM2 in mouse models showed a high risk of tumor formation, suggesting it may play a role in oncogenesis (Jones, Hancock et al. 1998). Later, MDM2 was co-purified with p53 and found to negatively regulate p53 stability and transcriptional activity (214). MDM2 overexpression, in cooperation with oncogenic Ras, promotes transformation of primary rodent fibroblasts, and leads to tumor formation in nude mice (Fakharzadeh, Trusko et al. 1991). Further supporting the role of MDM2 as an oncogene, several human tumor types have been shown to have increased levels of MDM2, including soft tissue sarcomas and osteosarcomas as well as breast tumors (Momand, Jung et al. 1998).

Structure and Function of MDM2

The full-length transcript of the MDM2 gene encodes a protein of 491 amino acids with a predicted molecular weight of 56 kDa. MDM2 is a member of the RING finger domain family of E3 ubiquitin ligases. It contains at least four functionally independent domains, including an N-terminal domain (a.a. 19-102) that recognizes the N-terminal Box-I domain of p53, a central acidic domain (a.a. 223-274), a putative zinc finger (a.a. 305-322), and a RING finger domain (a.a. 438-478) critical for its E3 ubiquitin ligase activity (Figure 4).

The interaction between the N-terminal domains of MDM2 and p53 has been extensively studied and several compounds have been reported to inhibit this interaction. Binding between MDM2 and p53 has been shown inhibit p53's transactivation function (Momand, Zambetti et al. 1992). However, recently studies using GST pull-down experiments have shown that MDM2 constructs without the N-terminal p53 binding domain still retain the ability to bind to p53 (Ma, Martin et al. 2006).

The nuclear export and import signals that are essential for proper nuclear-cytoplasmic trafficking of MDM2 are located between the N-terminal domain and the acidic domain (Hay and Meek 2000). The central acidic domain of MDM2 is required for its binding to a number of proteins, including p14^ARF, p300, and YY1 (Bothner, Lewis et al. 2001; Sui, Affar el et al. 2004). The phosphorylation of residues within this domain appears to be important for regulation of MDM2 function. Another conserved domain within the MDM2 protein is a zinc finger domain, the MDM2 central zinc finger plays a critical role in mediating MDM2's interaction with ribosomal proteins and its ability to degrade p53 under ribosomal stress conditions (Lindstrom, Jin et al. 2007).
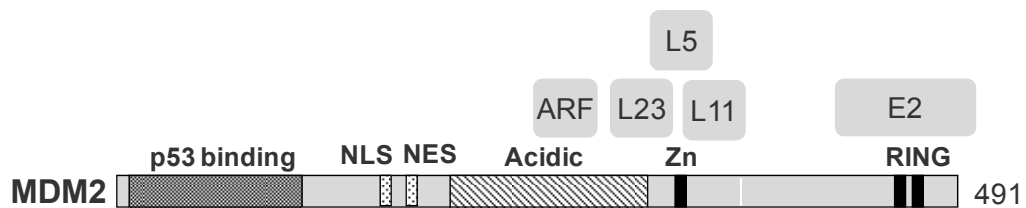
Figure 4. Structure of MDM2.

MDM2 also contains a C-terminal RING domain (amino acid residues 430-480) which is important for many functions of MDM2. First, it contains a Cis3-His2-Cis3 consensus that coordinates zinc binding which is essential for proper folding of the RING domain (Boddy, Freemont et al. 1994). Second, the RING domain of MDM2 is necessary and sufficient for its E3 ligase activity towards p53 as well as itself (Fang, Jensen et al. 2000). Third, the RING domain also binds specifically to 5S RNA, although the function of this is poorly understood (Elenbaas, Dobbelstein et al. 1996). Fourth, this region of MDM2 contains a cryptic nucleolar localization signal revealed upon protein interactions

with p14^{ARF} (Lohrum, Ashcroft et al. 2000). Last, an intact RING domain of MDM2 is required to interact with MDMX. Furthermore, the C-terminal 130 amino acids of MDM2 containing the RING domain are sufficient to ubiquitinate MDMX whereas deletion of the MDMX C-terminal RING domain (Δ394-490) can prevent polyubiquitination by MDM2 (Pan and Chen 2003).

The MDM2 RING domain also binds nucleotides with a strong preference for ATP and although such binding does not contribute to its E3 ubiquitin ligase activity, it is important for sub-nuclear translocation of MDM2 from the nucleoplasm to the nucleolus (Poyurovsky, Jacq et al. 2003). Lastly, the lysine residues within the RING domain of MDM2 have been shown to be substrates for CBP/p300-mediated acetylation leading to inhibition of the ubiquitin ligase activity (Wang, Taplick et al. 2004).

MDM2 Interacts with p53

MDM2 controls p53 through two distinct mechanisms, by directly binding and masking the N-terminal transactivation domain of p53 (Momand, Zambetti et al. 1992) and by promoting proteasomal degradation of p53 (Haupt, Maya et al. 1997). The direct interaction between the two proteins has been localized to a relatively small (aa 25–109) hydrophobic pocket domain at the N-terminus of MDM2 and a 15 amino acid amphipathic peptide at the N-terminus of p53 (Chen, Marechal et al. 1993; Kussie, Gorina et al. 1996). The minimal MDM2-binding site on the p53 protein was mapped within residues 18–26 (Chen, Marechal et al. 1993; Bottger, Bottger et al. 1997; Haupt, Maya et al. 1997). Site-directed mutagenesis has shown the importance of p53 residues Leu14, Phe19, Leu22, Trp23, and Leu26, of which Phe19, Trp23, and Leu26 are the most critical. Accordingly, the MDM2-binding site p53 mutants are resistant to degradation by

MDM2 (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997). Similarly, mutations of

MDM2 at residues Gly58, Glu68, Val75, or Cys77 result in a lack of p53 binding

(Freedman, Epstein et al. 1997). The interacting domains show a tight key-lock

configuration of the p53-MDM2 interface. The hydrophobic side of the amphipathic p53

α-helix, which is formed by amino acids 19–26 (with Phe19, Trp23, and Leu26 making

contact), fits deeply into the hydrophobic cleft of MDM2. The MDM2 cleft is formed by

amino acids 26–108 and consists of two structurally similar portions that fold up into a

deep groove lined by 14 hydrophobic and aromatic residues (Kussie, Gorina et al. 1996).

The interactions between p53 and MDM2 are tightly regulated and have been shown to

be disrupted by post-translational modifications to either protein.

MDM2 promotes p53 degradation

MDM2 functions as an E3 ubiquitin ligase toward p53 promoting its degradation

through a complex series of steps that involve E1, E2, and E3 proteins. The E1 enzyme

binds ubiquitin, a 76-amino acid protein, activating it for further processing. The E2

conjugating enzyme accepts the activated ubiquitin from E1 and transfers it to the E3

enzyme, a ligase that covalently bonds the ubiquitin to the substrate. However, mutants of

MDM2 lacking the E3 ubiquitin ligase activity can efficiently bind wild-type p53 and

inhibit p53-mediated transcriptional activation in transfection experiments (Leng, Brown

et al. 1995). MDM2 can also promotes its own degradation by autoubiquitination (Fang,

Jensen et al. 2000; Honda and Yasuda 2000). This is a second mechanism of promoting

p53 stabilization.

Although MDM2 was believed to polyubiquitinate p53 for protein degradation,

other evidence suggests that MDM2 mediates monomeric p53 ubiquitination on multiple

lysine residues rather than a polymeric ubiquitin chain (Lai, Ferry et al. 2001). This

suggests other proteins must aid in polyubiquitination of p53 (Thrower, Hoffman et al.

2000). Further research indicates that MDM2 requires p300 to catalyze p53

polyubiquitination, whereas alone MDM2 can only catalyze p53 monoubiquitination

(Grossman, Deato et al. 2003). MDM2 mutants lacking part of the acidic domain that

overlaps the p300/CBP-binding domain failed to degrade p53 but accumulated

monoubiquitinated p53 (Zhu, Yao et al. 2001). Interestingly, MDM2 can also bind to the

p53-related proteins P63 and P73, yet it does not mediate their degradation (Zeng, Chen

et al. 1999). As mentioned earlier, the E3 activity of MDM2 is dependent on its RING

finger domain and is abolished by mutations which delete the domain or substitute any of

the amino acids required for the coordination of zinc (Honda and Yasuda 2000).

Besides acting as an E3 ligase for p53, MDM2 also stimulates the ubiquitination of

additional proteins including MDMX (which will be discussed in more detail), b-arrestin,

PCAF and insulin-like growth factor 1 receptor (IGF1R) (74, 93, 94, 136, 275).

Furthermore, MDM2 promotes other forms of p53 posttranslational modifications such as

sumoylation, acetylation, and neddylation. Sumo-1 is a 110 amino acid protein belonging

to the ubiquitin-like family. MDM2 mediates p53 sumoylation which moderately

enhances p53 transcriptional activity (204). MDM2 suppresses p53 acetylation by

binding to and inhibiting the function of CBP/P300, rendering p53 more susceptible to

degradation (147). Furthermore, MDM2 promotes the conjugation of another ubiquitin-

like molecule, nedd8 to p53, leading to the transcriptional inhibition of p53 activity (79).

MDM2-p53 Negative Feedback Loop

MDM2 is transcriptionally activated by p53 by binding to and transcriptional activating the MDM2 P2 promoter, a response element situated downstream of the first exon of the MDM2 (Figure 1) (Barak, Juven et al. 1993; Perry, Piette et al. 1993). Ionizing irradiation, UV-irradiation as well as other DNA damaging agents induce MDM2 expression in a p53-dependent manner (Perry, Piette et al. 1993; Price and Park 1994; Bae, Smith et al. 1995). Because MDM2 inhibits p53 activity, this forms a negative feedback loop that tightly regulates p53 function. Likewise, decreasing p53 activity results in decreased MDM2 protein levels. In addition to transcriptional activation by p53, oncogenic Ras induces MDM2 through the Raf/MEK/MAP kinase pathway in a p53-independent manner (Ries, Biederer et al. 2000).

MDM2 Regulation by DNA Damage

Upon DNA damage, p53 is posttranslationally modified to inhibit its interactions with MDM2. Several kinases also phosphorylate MDM2 and modulate its interactions with p53 (Moll and Petrenko 2003). For example, ATM phosphorylates MDM2 at serine 395, disrupting the nuclear export signal that is needed for efficient p53 export into the cytoplasm (201). MDM2 can be phosphorylated by c-Abl on Tyr394 following DNA damage which contributes to apoptosis by blocking the ability of MDM2 to down-regulate p53 function (96). Other protein kinases that have been implicated in regulating MDM2 phosphorylation and function include AKT, p38 mitogen-activated kinase (MAPK), DNA-dependent protein kinase (DNA-PK), cyclin A-dependent kinases 1 and 2 (CDK1 and CDK2), and protein kinase CK2 (Meek and Knippschild 2003).

Growing evidence suggests that dephosphorylation of MDM2 is also likely to be a critical event following stress and there are now two striking examples of mechanisms where MDM2 dephosphorylation plays a key role in the p53 response. The first of these involves the cyclin G1 protein, the product of one of the first p53 responsive genes to be identified (Okamoto and Beach 1994). The cyclin G1-PP2A complex dephosphorylates MDM2 residue Thr216 resulting in p53 induction by attenuating MDM2 regulation, leading to restoration of p53 levels and re-establishment of the MDM2-p53 feedback loop (Okamoto, Li et al. 2002). Dephosphorylation of the acidic domain of MDM2 is also thought to play a role in the network of events mediating p53 induction. Following ionizing radiation, key phospho-serine residues in the acidic domain of MDM2 (serine 240, 242, 260, and 262) become rapidly dephosphorylated preceding p53 accumulation. Mutants of MDM2 with serine to Alanine substitutions at these phospho-serine residues alleviate degradation of p53 suggesting dephosphorylation of these residues results in a positive regulation of p53 (Blattner, Hay et al. 2002).

MDM2 regulation by Oncogenic Stress

Deregulated oncogenes, such as oncogenic Ras mutants, c-myc, or viral E1A, use yet another way of interfering with MDM2 regulation to stabilize and activate p53. Oncogenic stress stimulates an increase in the p14^{ARF} protein (p19^{ARF} in the mouse), the alternate product of the INK4A tumor suppressor locus. ARF binds to the RING finger domain of MDM2 and directly inhibits its E3 Ligase activity (Honda and Yasuda 1999). A model has been proposed in which ARF binds MDM2 and sequesters it into the nucleolus while p53 remains in the nucleoplasm resulting in enhanced p53 transcriptional activity (Tao and Levine 1999; Weber, Taylor et al. 1999). However, there is some

disagreement as to whether sequestration of MDM2 by ARF takes place in the nucleolus or in the nucleoplasm (Llanos, Clark et al. 2001). Whatever the actual mechanism of MDM2 inactivation by ARF, the major consequence is the stabilization of nuclear p53 levels. The ARF-MDM2-p53 relationship appears to be an integrated part of several cellular networks involving complex mitogenic signaling pathways, such as Wnt (via ß-catenin), Myc, and pRb-E2F (Sharpless and DePinho 1999; Sherr 2001).

MDM2 regulation by Ribosomal Stress

Ribosomal proteins such as L5 (Marechal, Elenbaas et al. 1997; Dai and Lu 2004), L11 (Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003; Bhat, Itahana et al. 2004; Dai, Shi et al. 2006), L23 (Dai, Zeng et al. 2004; Jin, Itahana et al. 2004) have also been implicated in p53 signaling (Figure 5). Ribosomal stress induced by inhibiting rRNA synthesis causes the release of L proteins from the nucleolus to the nucleoplasm. In the nucleoplasm, L proteins bind to the central acidic domain/zinc finger of MDM2 and inhibit its E3 ubiquitin ligase activity towards p53. Each of the L proteins when overexpressed independently can inhibit MDM2's repressive function toward p53 causing an in increase in p53 target genes and cell cycle arrest (Bhat, Itahana et al. 2004; Dai and Lu 2004). Likewise, knockdown of any of the L proteins by siRNA can cause a decrease in p53 activation. On the other hand, all three ribosomal proteins can bind in a quaternary complex to MDM2 simultaneously without the need for p53 suggesting that each of these proteins is essential for p53 activation (Jin, Itahana et al. 2004). Although previous studies report minor variations regarding the L protein-MDM2 binding region, an MDM2 zinc finger mutant (C305F) abrogates the interaction of MDM2 with L5 and L11 but not L23 (Lindstrom, Jin et al. 2006). The MDM2 mutant has decreased nuclear

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export capabilities, retains the functional ability to promote p53 ubiquitination but delayed degradation, and escapes inhibition by L11. Although some studies suggest that the MDM2-L protein interaction causes a steric hindrance preventing the transfer the ubiquitin moiety from E2 to p53 (Zhang, Wolf et al. 2003), the exact mechanism of p53 protection has yet to be determined.
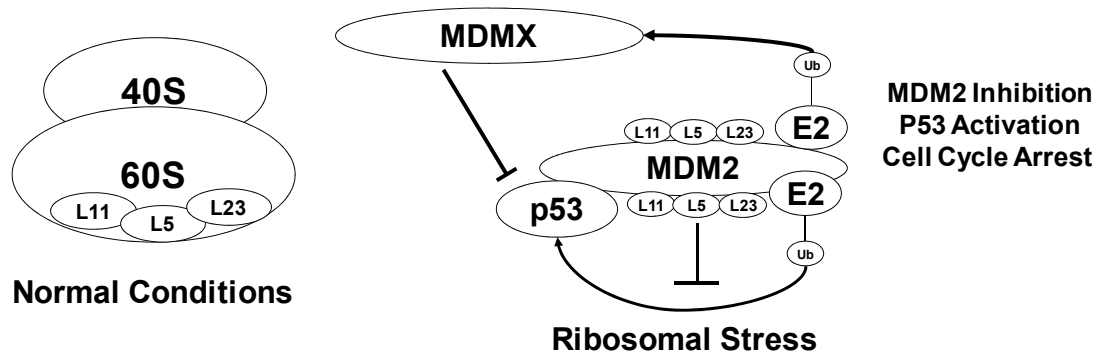
Figure 5. MDM2 Regulation by Ribosomal Stress. Under normal conditions the L proteins are associated with the large ribosomal subunit. Following ribosomal stress, the L proteins associate with MDM2 and attenuate its ability to degrade p53.

MDM2 Mouse Models

The importance of the MDM2/p53 interaction has been convincingly demonstrated in *in vivo* experiments. Mice lacking MDM2 are early embryonic lethal and die before implantation at 3.5 days post-coitum. This phenotype is completely rescued by concomitant deletion of p53, suggesting that the embryo lethality was due to overactive p53 (Jones, Roe et al. 1995; Leveillard, Gorry et al. 1998). Mice with a hypomorphic allele that expresses approximately 30% of the total MDM2 levels have a decreased body weight, defects in hematopoiesis, and are more radiosensitive than normal mice (Mendrysa, McElwee et al. 2003). MDM2^{+/-} heterozygous mice are more resistant to the development of lymphoid tumors induced by expression of the Eu-Myc transgene (Alt,

Greiner et al. 2003). These phenotypes are p53 dependent, emphasizing the importance of

regulating MDM2 levels in many cell types.

MDM2 Interacting Proteins

Besides MDMX, ARF, and the ribosomal proteins described above, several other

MDM2 interacting proteins have been identified in various systems. Hypoxia-inducible

factor 1a (HIF-1a) interacts with MDM2 and enhances p53 function by preventing the

nuclear export of p53 (Chen, Li et al. 2003). MDM2 was also identified as an RB

binding protein. MDM2 inhibits RB suppression of E2F1 function, causing cell cycle

arrest (Xiao, Chen et al. 1995; Hsieh, Chan et al. 1999). MDM2 also interacts with the

transcriptional activator Sp1 *in vivo* (Johnson-Pais, Degnin et al. 2001). MDM2/Sp1

binding prevents Sp1-DNA interactions thereby blocking transcription. Rb has been

shown to compete with Sp1 for binding to MDM2. However, there is no evidence that

MDM2 plays a role in the degradation of either RB or Sp1. Furthermore, MDM2 can

interact with the E2F1/DP1 complex to stimulate transcription (Martin, Trouche et al.

1995). Additional reports indicate that MDM2 blocks the apoptotic activity of E2F1

(Loughran and La Thangue 2000). Numb, a protein important for specifying cell fate

during development, has also been identified as an MDM2 interacting protein that is

degraded by MDM2 (Yogosawa, Miyauchi et al. 2003). The ability for MDM2 to

promote cell proliferation by regulating components of the cell cycle underscores its

importance.

<center>*MDMX*</center>

MDMX is emerging as a potent suppressor of p53 transcriptional activity following

stresses imposed by DNA damage, loss of ribosomal integrity and aberrant mitogenic

signaling. MDMX was first identified as a p53 (Shvarts, Steegenga et al. 1996) and later as an MDM2 (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999) binding protein. MDMX is structurally similar to MDM2 (Shvarts, Steegenga et al. 1996), but it does not have intrinsic E3 ligase activity nor does it promote p53 degradation (Stad, Little et al. 2001). MDMX forms heterodimers with MDM2 through C-terminal RING domain interactions (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999), and stimulates the ability of MDM2 to ubiquitinate and degrade p53 (Gu, Kawai et al. 2002; Linares, Hengstermann et al. 2003). Due to self-ubiquitination, MDM2 has a short half life; whereas MDMX is relatively stable in the absence of stress. Similar to its actions against p53, MDM2 can ubiquitinate and degrade MDMX (de Graaf, Little et al. 2003; Kawai, Wiederschain et al. 2003; Pan and Chen 2003) ultimately generating a steady state level of MDM2, MDMX, and p53 proteins.

MDMX overexpression is found in a number of tumors or tumor cell lines with wild-type p53 (Ramos, Stad et al. 2001; Danovi, Meulmeester et al. 2004). A study of a large series of gliomas revealed that MDMX is amplified/overexpressed in 5/208 tumor samples (Riemenschneider, Buschges et al. 1999) and more recently it has been found to be severely amplified or overexpressed in retinoblastomas (65%) (Laurie, Donovan et al. 2006). In approximately 30% of tumor cell lines tested, MDMX is either overexpressed or alternatively transcribed, and in general this correlates with the presence of wild-type p53 (Ramos, Stad et al. 2001). A recent analysis of a large series of tumors also revealed overexpression of MDMX in 19% of breast, colon , and lung cancers studied (Danovi, Meulmeester et al. 2004). In all cases, amplification of MDMX correlated with wild-type p53 status and lack of MDM2 amplification. In addition, MDMX overexpression can

prevent oncogenic Ras-induced premature senescence, and MDMX can cooperate with

Ras^{V12} to transform cells which are capable of forming tumors in nude mice (Danovi,

Meulmeester et al. 2004). Taken together this evidence suggests that MDMX can

suppress p53 and alleviate the need for its inactivation by mutation in order to promote

tumor progression.

Structure and Function of MDMX

      MDMX and MDM2 are structurally related proteins of 490 and 491 amino acids,

respectively (Figure 6). The greatest similarity between the two proteins is at the N-

terminus, a region encompassing the p53-binding domain (53.6% homology). The

residues required for interaction with p53 are conserved in MDM2 and MDMX (Shvarts,

Steegenga et al. 1996), and the same residues in p53 are required for both MDMX-p53

and MDM2-p53 interactions (Bottger, Bottger et al. 1999). Another well-conserved

region common to MDMX and MDM2 is a RING-finger domain, located at the C-

terminus of each protein. The RING-finger domain is essential for MDMX-MDM2

heterodimerization (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999). Like

MDM2, MDMX contains a zinc-finger domain which recent results suggest is necessary

for interaction between MDMX and casein kinase 1 alpha (CK1α) (Chen, Li et al. 2005).

The central regions of MDM2 and MDMX show no significant similarity, but both

regions are rich in acidic residues.
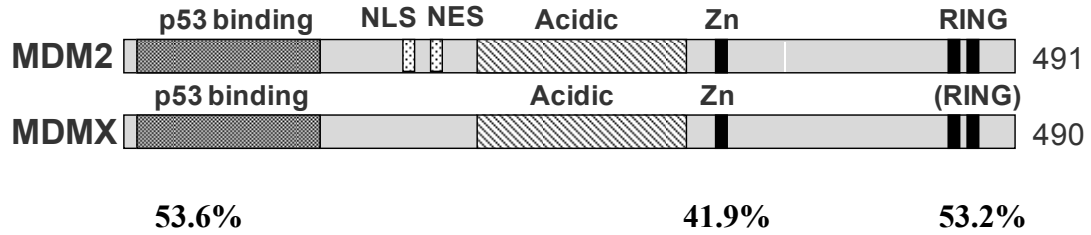
Figure 6. Comparison of the Structure of MDM2 versus MDMX. Both proteins contain a p53 binding domain, acidic region, zinc finger, and form heterodimers through their ring domains.

At the genomic level, exons 4-12 are well conserved between MDMX and MDM2. However, the 5' ends of the genes are quite distinct. Importantly, in contrast to MDM2, the MDMX promoter does not contain a p53-responsive element (Shvarts, Steegenga et al. 1996). One non-coding exon was found in the MDMX locus instead of two for MDM2. Also, the intron between exon 1 and 2 in MDMX is about 6 kb, while in MDM2 the first three exons are within 1 kb. The MDM2 gene has two promoters, the second of which (P2) is responsive to p53. Consistently, MDM2, but not MDMX is induced following p53 activation. This highlights the need to understand what makes these proteins so distinct.

MDMX Post Translational Modifications

The post-translational modifications of MDMX that have been characterized to date include phosphorylation, ubiquitination and sumoylation. Ubiquitination of MDMX by MDM2 was the first described post translational modification of MDMX. The RING domain of MDM2 is required both to interact with MDMX and to provide E3 ligase function (de Graaf, Little et al. 2003; Kawai, Wiederschain et al. 2003; Pan and Chen 2003). This effect is stimulated by ARF and DNA damage and correlates with the ability of ARF to bind MDM2. Interestingly, ARF inhibits MDM2 E3 ligase activity toward

p53 and MDM2, leading to stabilization of both proteins (139, 245). On the otherhand, ARF has been shown to promote MDM2-dependent degradation of MDMX (Pan and Chen 2003). This suggests that p53 activation by ARF occurs by both enhanced MDMX degradation as well as reduced p53 ubiquitination.

Phosphorylation of MDMX has functional implications for p53 activation and MDMX degradation. Efficient degradation of MDMX following DNA damage requires ATM-dependent phosphorylation on S342 and S367 by Chk2 and S403 by ATM (Chen, Gilkes et al. 2005; Okamoto, Kashima et al. 2005; Pereg, Shkedy et al. 2005; LeBron, Chen et al. 2006). Furthermore, Chk2-mediated phosphorylation of MDMX on S367 is important for stimulating 14-3-3 binding, MDMX nuclear import, and degradation by MDM2 (LeBron, Chen et al. 2006). Other studies suggest that ultra violet radiation results in Chk1-mediated phosphorylation of S367 (Jin, Dai et al. 2006). Phosphorylation of MDMX reduces its affinity for the deubiquitylating enzyme (DUB) HAUSP/USP7 which has been shown to be essential in for maintenance of both MDM2, MDMX, and p53 protein levels (Cummins, Rago et al. 2004; Meulmeester, Pereg et al. 2005). Basal phosphorylation of MDMX can also occur on serines 96 and 289 by kinases CDK2 and CK1α, respectively. Phosphorylation of serine 96 is proposed to regulate MDM2 localization, whereas CK1α mediated phosphorylation stimulates the MDMX-p53 interaction (Chen, Li et al. 2005; Elias, Laine et al. 2005).

Sumoylation of MDMX has been cited but its functional importance has yet to be described. MDMX is conjugated with SUMO-1 on K254 and K379, but conversion of K254 and K379 to arginine has no effect on MDMX function (Pan and Chen 2005). Further studies indicate that endogenous MDMX is modified by SUMO-2 on K254 and

K379. The role of post translational modifications of MDMX are continuing to be characterized. Mouse models involving these modifications may help to determine the physiological role of these modifications.

Mouse Models of MDMX

The physiological importance of MDMX's functional affect on p53 was characterized by the embryonic lethality of MDMX null mice, which can be rescued by the concomitant knockout of p53 (Parant, Reinke et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Lazzerini Denchi et al. 2002). Moreover, conditional alleles have recently been developed to offer further insight on MDMX regulation of p53. MDM2 and MDMX were conditionally inactivated in neuronal progenitors. Mice lacking MDM2 expression in the central nervous system suffered from apoptosis, whereas MDMX deletion enhanced cell cycle arrest and apoptosis at a later stage of embryonic development. The deletion of both genes contributed to an even earlier and more severe CNS phenotype (Xiong, Van Pelt et al. 2006). Similar studies in which p53 was conditionally expressed in neuronal progenitor cells or in post-mitotic cells of mice lacking MDMX or MDM2 showed that MDM2 prevents p53 accumulation while MDMX contributes to the overall inhibition of p53 activity, independent of MDM2 (Francoz, Froment et al. 2006). Interestingly, the phenotypes disappear in the absence of p53. This suggests that both MDM2 and MDMX are required to inhibit p53 activity in the same cell type, and MDM2 does not compensate for loss of MDMX *in vivo.* However, a recent paper suggests that overexpression of an MDM2 transgene rescues the embryonic lethality associated with MDMX-deficiency (Steinman, Hoover et al. 2005). MDMX has also been conditionally inactivated in cardiomyocytes and smooth muscle cells of the GI tract (Boesten, Zadelaar

et al. 2006; Grier, Xiong et al. 2006). In contrast to loss of MDM2, loss of MDMX leads to only minor defects in histogenesis and tissue homeostasis. Overall these studies suggest that the absence of MDMX enhances p53 transcriptional activity.

The analysis of mice encoding a mutant p53 lacking the proline-rich domain (p53Δ P) also enabled evaluation of MDMX function (Toledo, Krummel et al. 2006). This hypomorphic p53 mutant is able to fully rescue MDMX deficiency. In the absence of MDMX, the transcription of MDM2 is stimulated to some extent, leading to slightly increased MDM2 levels. While MDMX loss did not alter MDM2 stability, it significantly increased p53ΔP partially restoring cell cycle control. In contrast, decreasing MDM2 levels increased p53ΔP levels without altering p53ΔP transactivation. This suggests MDMX regulates p53 activity, while MDM2 controls p53 stability.

The difference between the MDM2-null and MDMX-null phenotypes may be a result of the fact that loss of MDM2 leads to dramatic accumulation of the p53 protein, whereas loss of MDMX does not significantly increase p53 levels *in vivo.*

MDMX –MDM2-p53 Pathway

The first reported activity of MDMX is the inhibition of p53-induced transcription following ectopic expression on both luciferase reporter genes and endogenous p53 targets (Shvarts, Steegenga et al. 1996). This effect is dependent on the p53-binding domain of MDMX. The same amino acids in p53 are required for both MDMX/p53 and MDM2/p53 interactions, and these amino acids are located in the transcriptional activation domain of p53 (Bottger, Bottger et al. 1999). This suggests that MDMX may inhibit p53 transcriptional activity by interfering with the ability of p53 to interact with the basal transcription machinery or to recruit essential coactivator (s) or it could inhibit

p53 binding at target promoters. MDMX abrogates p300/CBP-mediated acetylation of p53 even in MDM2-null cells and the same result is also observed with a mutant of MDMX defective for MDM2 binding (Sabbatini and McCormick 2002; Danovi, Meulmeester et al. 2004) resulting in stimulation of p53 activation.

MDMX binds to MDM2 through the MDM2 RING domain which could result in several different outcomes for p53 and MDM2 stability. One study suggests MDMX stabilizes MDM2 by interfering with its auto-ubiquitination (Stad, Little et al. 2001). Another study shows that knocking down MDMX expression with siRNA results in decreased MDM2 levels and an increase in p53 (Gu, Kawai et al. 2002). Alternatively, knocking down MDMX in U2OS and MCF-7 cells by siRNA increased both MDM2 and p53 levels (Linares, Hengstermann et al. 2003). Further studies demonstrate, that there is no significant effect on MDM2 or p53 levels after knocking down MDMX in MCF-7 cells (Danovi, Meulmeester et al. 2004). While still other studies have suggested that elevated levels of MDMX could stabilize p53 by inhibiting its degradation by MDM2, without interfering significantly with MDM2-dependent p53 ubiquitination (Jackson and Berberich 2000; Stad, Little et al. 2001; Migliorini, Lazzerini Denchi et al. 2002). This effect was proposed to be a consequence of reduced MDM2 induced p53 nuclear export, an event thought to be required for efficient p53 degradation (Boyd, Tsai et al. 2000; Geyer, Yu et al. 2000).

The discrepancies in these findings suggest that the ratio of MDMX to MDM2 may be necessary to determine the overall affect on p53. If the MDMX:MDM2 ratio is about 1:1, p53 undergoes MDM2-dependent proteasomal degradation. When MDMX is expressed at levels greater than MDM2, MDMX inhibits MDM2-mediated p53

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degradation. Furthermore, in the presence of high MDMX levels, MDM2 and MDMX

compete for p53 binding and MDM2 is likely to be displaced from p53. In these

conditions, MDMX inhibits p53 transcriptional activity independent of MDM2 (Marine

and Jochemsen 2005).

Our lab has studied the effects of p53 activation in cells which either have an

overexpression or knock down levels of MDMX following DNA damage or ribosomal

stress (Chen, Gilkes et al. 2005; Gilkes, Chen et al. 2006). These studies show that while

endogenous p53 levels show little change in response to altered MDMX levels, following

cellular stress the level of p53 activation is inversely correlated to the amount of MDMX

in these cells due to formation of inactive p53-MDMX complexes. In the presence of a

high level of MDMX these complexes fail to bind the DNA of target promoters. In

contrast, knockdown of MDMX abrogates HCT116 tumor xenograft formation in nude

mice. MDMX overexpression does not accelerate tumor growth but increases resistance

to 5-FU treatment *in vivo*. Our studies show that MDMX plays a negative role in p53

transcriptional activation.

MDMX Localization

Exogenous MDMX is mainly localized in the cytoplasm as determined by cell

fractionation and indirect immunofluorescence studies (Rallapalli, Strachan et al. 1999;

Migliorini, Danovi et al. 2002). Co-expression of MDM2 stimulates the recruitment of

MDMX into the nucleus (Gu, Kawai et al. 2002; Migliorini, Danovi et al. 2002). This

effect is independent of p53 but requires intact RING finger domains on both MDMX

and MDM2 proteins, and the NLS of MDM2. Other studies report that p53 can target

MDMX to the nucleus independent of MDM2 (Li, Chen et al. 2002). However, it is

important to note that MDMX nuclear entry is also observed following DNA damage in p53/MDM2 double-null MEFs, suggesting a mechanism of MDMX nuclear localization independent of both MDM2 and p53 (Li, Chen et al. 2002). Importantly, our lab recently showed that Chk2-mediated phosphorylation of MDMX on S367 and binding of 14-3-3 was important for MDMX nuclear import by exposing a cryptic nuclear import signal. Mutation of MDMX S367 to Arginine prevents MDMX nuclear import (LeBron, Chen et al. 2006). These results suggest that phosphorylation of MDMX is important for its localization in response to DNA damage.

MDMX Regulation by DNA Damage

Following DNA damage, both p53 and MDM2 are phosphorylated by several kinases; most notably, ATM which functions as the primary signal transducer of DNA double-strand breaks (Shiloh 2003). Until recently, little was known about the affects of DNA damage on MDMX. Although, it was recognized that DNA damage induces MDMX degradation in p53-deficient cells, without inducing MDM2 (Kawai, Wiederschain et al. 2003). Recently our laboratory and others showed that MDMX is phosphorylated at several key C terminal serine residues in an ATM-dependent manner following DNA damage. ATM modifies S403 (Pereg, Shkedy et al. 2005) and Chk2 modifies S342 and S367 (Chen, Gilkes et al. 2005) on MDMX. Chk1 has also been shown to modify S367 under certain conditions (Jin, Dai et al. 2006). Phosphorylation of MDMX led to increased binding to MDM2 followed by ubiquitination and degradation of MDMX. When HCT116-Chk2-/- cells were compared to wild-type HCT116 cells after gamma irradiation (5 Gy), MDMX phosphorylation and degradation were impaired showing that DNA damage-induced phosphorylation of S342 and S367 strictly requires

Chk2. The addition of Chk2 to Chk2-null cells increased MDMX phosphorylation and ubiquitination. Functionally, the degradation of MDMX was necessary for p53 activitation following DNA damage since cells overexpressing MDMX were unable to undergo a cell cycle arrest.
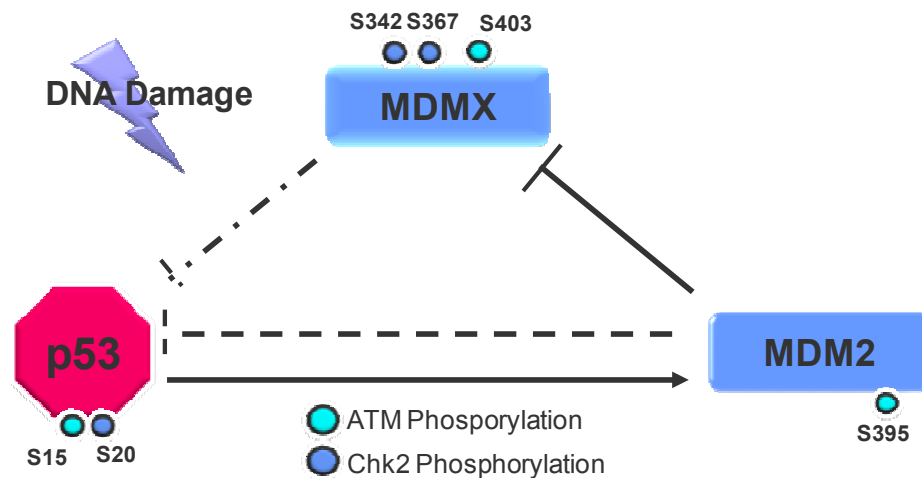
Figure 7. MDMX-MDM2-p53 pathway following DNA damage.

To further elucidate this mechanism, we showed that DNA-damage induced phosphorylation of S367 increased the affinity of MDMX/14-3-3 binding (LeBron, Chen et al. 2006). Mutating the MDMX S367 binding site abrogated the MDMX/14-3-3 interaction increasing MDMX stability. Furthermore, phosphorylation of S367 was required for MDMX nuclear import after DNA damage. The results suggested that 14-3-3 proteins regulated MDMX localization and abundance in response to DNA damage, and contribute to the efficient activation of p53.

Additional means by which MDM2 and MDMX become destabilized following DNA damage have also been proposed (Meulmeester, Pereg et al. 2005). The deubiquitinating enzyme HAUSP can directly interact with both MDMX and MDM2.

HAUSP deubiquitinates MDMX counteracting MDM2-mediated degradation. However, DNA Damage can inhibit the interactions between HAUSP and both MDMX and MDM2. Notably, ectopic expression of HAUSP was not able to rescue DNA damage-mediated degradation of MDMX.

MDMX Regulation by induction of ARF

Oncogenic insults can activate p53 by promoting the binding of ARF and MDM2. The interaction of ARF with MDM2 inhibits MDM2's E3 activity towards p53 (Honda and Yasuda 1999) and relocalizes MDM2 to the nucleolus (Weber, Taylor et al. 1999; Lohrum, Ashcroft et al. 2000; Rizos, Darmanian et al. 2000; Weber, Kuo et al. 2000). However, relocalization of MDM2 is not essential for the inhibition of MDM2 function by ARF in all cells (Llanos, Clark et al. 2001; Korgaonkar, Zhao et al. 2002). Our lab has shown that ARF binding to MDM2 selectively blocks p53-ubiquitination but promotes ubiquitination of MDMX (Pan and Chen 2003). Our data shows MDMX overexpressing cells have reduced induction of p21 and cell cycle arrest following E2F activation of ARF whereas MDMX siRNA can sensitize cells to ARF-induced cell cycle arrest (unpublished observations). A recent paper by Laurie et al. shows that inactivation of the Rb pathway in the developing mouse or human retina leads to activation of the ARF–MDM2–p53 tumor surveillance pathway. Genetic changes resulting in MDMX gene amplification can occur in the preneoplastic retinoblastoma cells causing the p53 pathway to be inactivated. Cells with amplified MDMX will have a growth advantage over those with an intact ARF–MDM2/MDMX–p53 pathway resulting in retinoblastoma development (Laurie, Donovan et al. 2006). This highlights the need to determine how MDMX is amplified as well as to identify specific inhibitors of the MDMX-p53 interaction.

MDMX Regulation by induction of Ribosomal Stress

Recent studies revealed a connection between ribosomal stress and p53-dependent cell cycle arrest, suggesting that aberrant rRNA and ribosome biogenesis are sensed by p53 (Marechal, Elenbaas et al. 1994; Pestov, Strezoska et al. 2001; Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003). Ribosomal stress induced by actinomycin D, serum starvation, or contact inhibition cause p53 stabilization and activation (Bhat, Itahana et al. 2004). These studies suggest a mechanism involving the translocation of ribosomal proteins, L5, L11, and L23 from the nucleolus to the nucleoplasm where they bind to MDM2 and prevent p53 degradation (Bhat, Itahana et al. 2004; Dai, Zeng et al. 2004; Jin, Itahana et al. 2004). Each of these L proteins when overexpressed can inhibit MDM2 degradation of p53. Results described in this dissertation suggest that activation of p53 by ribosomal stress requires down-regulation of MDMX. This process can be blocked by MDMX overexpression. As a result, tumor cells expressing high-level endogenous MDMX have less efficient p53 activation and growth arrest during ribosomal stress. Furthermore, we found that the widely used chemotherapy agent 5-FU activates p53 in part through inducing ribosomal stress. As such, MDMX overexpression can cause significant resistance to 5-FU in cell culture and tumor xenograft models. These observations suggest that MDMX plays a unique and important role in regulating p53 response to perturbations in ribosome biogenesis.

MDM2/MDMX Inhibitors

MDM2 has been an attractive target for the development of novel anti-tumor agents (Bond, Hu et al. 2005). Recently, high throughput screening was utilized to identify, Nutlins, a class of cis-imidazoline analogues, which can bind to MDM2 and inhibit the

p53-MDM2 interaction (Vassilev, Vu et al. 2004; Vassilev 2005). MDMX and MDM2

proteins share the highest degree of sequence homology at the N-terminal region within

the p53-binding domain. Since previous studies using peptide inhibitors suggested that

the p53-binding site on MDMX is similar to MDM2, it has been speculated that MDM2

inhibitors may perform a dual function also blocking the MDMX-p53 interaction

(Freedman, Epstein et al. 1997). However, three separate laboratories have shown that the

MDM2 inhibitor Nutlin-3 is ineffective at targeting MDMX-p53 binding (Hu, Gilkes et

al. 2006; Patton, Mayo et al. 2006; Wade, Wong et al. 2006). Additionally, elevated

MDM2 levels following Nutlin treatment are not able to degrade MDMX in several

tumor cell lines. More importantly, overexpression of MDMX prevents p53 activation by

Nutlin-3. However, using a phage display library, we recently identified a peptide

sequence, which blocks both MDM2 as well as MDMX binding. The peptide can activate

p53 and induce growth arrest more effectively than blocking MDM2 alone (Hu, Gilkes et

al. 2007). Since MDMX has been identified as an important negative regulator of p53

function, it will be necessary to design specific MDMX/p53 binding inhibitors. A dual

inhibitor of MDM2/MDMX-p53 binding would be even more effective.

Ras/MAPK Signaling Pathway

The MAP Kinase pathway participates in many diverse processes including cell

proliferation, differentiation, transformation, and apoptosis. At the center of this signaling

cascade is the small guanine nucleotide–binding protein, Ras. It is localized at the plasma

membrane and can exist in two conformations: a guanosine triphosphate (GTP)–bound

active state and the guanosine diphosphate (GDP)–bound inactive state. Receptors

regulate Ras through nucleotide exchange factors, such as the murine Son of Sevenless

(SOS) protein, that can load Ras with GTP, and through GTPase-activating proteins (GAPs) that facilitate the hydrolysis of GTP to GDP to inactivate Ras. The GTP-bound form of Ras signals by its preferential binding to several effector molecules, most notably c-Raf-1, a serine-threonine kinase. Raf-1 activation initiates a kinase cascade through MEK (mitogen-activated protein kinase or ERK kinase), a dual-specificity protein kinase, which in turn phosphorylates ERK (extracellular signal–regulated kinase), another serine-threonine kinase (Downward 1998). ERK can phosphorylate other kinases, such as Rsk2, and transcription factors, such as c-Fos and Elk1 . Thus, the original signal is not only amplified in signal strength through a succession of kinases but is also diversified by the number of kinase substrates. This leads to the multiple effects seen by extracellular stimuli and growth factor stimulation (Garrington and Johnson 1999).

The Ras-p53 connection

Expression of constitutively active forms of Ras in primary mouse or human fibroblasts leads to elevated levels of p53 which in turn induce the expression of target genes that can cause growth arrest. Although the precise mechanism by which Ras induces p53 is not fully elucidated, activated Ras and Raf have been shown to promote the expression of ARF. As mentioned previously, ARF binds to MDM2, allowing p53 to become stabilized and accumulate, leading to induction of p53 target genes that promote cell cycle arrest (Groth, Weber et al. 2000). In accordance with this model, ARF-null MEFs are also susceptible to Ras transformation since they do not undergo p53-induced senescence (Kamijo, Zindy et al. 1997). The mechanism by which Ras elicits ARF expression is unclear, but it is possible that the c-Myc, E2F, or DMP1 transcription factors may provide an important link.

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It has been shown that MEK activity is important for expression of p53 at the transcriptional level and also for p53 activation by genotoxic agents (Persons, Yazlovitskaya et al. 2000; Agarwal, Ramana et al. 2001). For example, overexpression of ERK2 in AP14 cells (low levels of MAPK phosphorylation and p53 compared to parental cells) restored both MAP kinase activity and p53 expression. Furhermore, The levels of p53 mRNA increased significantly when activated Ras was introduced into wild-type cells. The levels of the p53 and p21 proteins decreased substantially in wild-type cells treated with the MEK inhibitor U0216 (Agarwal, Ramana et al. 2001). Inhibition of ERK1/2 activation with the mitogen-activated protein kinase (MEK1) inhibitor PD98059 resulted in decreased p53 protein half-life and diminished accumulation of p53 protein during exposure to cisplatin. P53 protein can also be co-immunoprecipitated with ERK1/2 protein and phosphorylated by activated recombinant murine ERK2 in vitro (Persons, Yazlovitskaya et al. 2000).

The Ras-MDM2/MDMX connection

Proper regulation of MDM2 and MDMX expression levels is critical for the tumor suppressive function of p53. MDM2 expression is often increased following mitogenic activation. For example, cells exposed to IGF-I have enhanced levels of MDM2 (Leri, Liu et al. 1999). Likewise, cells treated with basic FGF show increased levels of MDM2 protein. Further, cells constitutively exposed to a basic FGF autocrine loop do not respond to cisplatin, which to a large extent occurs through p53-mediated apoptosis (Shaulian, Resnitzky et al. 1997). Interestingly, a screen for transcripts that accumulate in cells harboring a chimeric M-CSF/PDGF revealed MDM2 (Fambrough, McClure et al. 1999). This data eventually led to the finding that the MDM2 gene is also regulated by

the Ras/Raf/MEK/MAP kinase pathway in a p53-independent mannerRas-activated Raf–MEK–ERK pathway targets *cis*-acting AP-1 and Ets DNA elements in the first intron of the MDM2 gene. MDM2 induced by activated Raf degrades p53 and may account for the observation that cells transformed by oncogenic Ras are more resistant to p53-dependent apoptosis following exposure to DNA damage (Ries, Biederer et al. 2000). In normal MEFs, the ERK/MAP kinase pathway induces the expression of both MDM2 and ARF with no net consequence on the level of p53 expression. The biological significance of MDM2 regulation at the transcriptional level is exemplified by the effect of a single nucleotide polymorphism in the MDM2 promoter, which is associated with increased susceptibility to tumor development (Bond, Hu et al. 2004).

In contrast, MDMX expression is not induced by p53 and the regulation of its promoter is still largely unknown. Furthermore, MDMX gene amplification only accounts for a subset of the cases of protein overexpression in cell lines and tumors, indicating that regulation of promoter activity is also a critical means of overexpression. The results section of this dissertation shows that MDMX expression level is closely correlated with MDMX mRNA levels and MDMX promoter activity in different tumor cell lines. However, unlike the MDM2 promoter, a survey of a large cell line panel did not reveal a sequence polymorphism in the MDMX promoter region. We also found that the Ras oncogene and IGF1 growth factor induces MDMX expression through activation of mRNA transcription. Further, evaluation of the MDMX promoter showed that increased ERK phosphorylation led to increased levels of MDMX whereas inhibiting phosphorylation using a MEK inhibitor led to a decrease MDMX mRNA and protein.

Elk-1/c-Ets-1

The Ets family consists of a large number of evolutionarily conserved transcription factors, many of which have been implicated in tumor progression. Ets proteins have a conserved DNA-binding domain (GGAA/T) and regulate transcriptional initiation from a variety of cellular and viral gene promoter and enhancer elements. Interestingly, Ets family members can act as both upstream and downstream effectors of signaling pathways. As downstream effectors their activities are directly controlled by specific phosphorylations, resulting in their ability to activate or repress specific target genes. As upstream effectors they are responsible for the spacial and temporal expression of numerous growth factor receptors. Some members of the Ets family, Ets-1 and Ets-2, cooperate in transcription with the AP-1 transcription factor, the product of the proto-oncogene families, *fos* and *jun*, while others, Elk-1 and SAP-1, form ternary complexes with the serum response factor (SRF) (Macleod, Leprince et al. 1992).

Ets-1 is involved in both normal and pathological functions. It is expressed in a variety of cells, including endothelial cells, vascular smooth muscle cells and epithelial cells. Ets-1 regulates the expression of several angiogenic and extracellular matrix remodeling factors promoting an invasive phenotype. In fact, in many tumors such as breast cancer, expression of c-Ets-1 indicates a poor prognosis (Lincoln and Bove 2005).

Many Ets family members including c-Ets-1 and Elk-1 have been identified as substrates for MAPK phosphorylation. In vitro, MAP kinase phosphorylates the Elk-1 C-terminal region at multiple sites, which are also phosphorylated following growth factor stimulation *in vivo* (Marais, Wynne et al. 1993). Ets-1 has a single MAPK phosphorylation site located near the Pointed domain (Brunner, Ducker et al. 1994).

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Phosphorylation generally enhances their ability to activate transcription. Phosphorylation of Elk-1 by ERK both enhances its recruitment to DNA (Gille, Kortenjann et al. 1995; Sharrocks 1995) and potentiates its transcriptional activation activity (Hill, Marais et al. 1993; Marais, Wynne et al. 1993; Gille, Kortenjann et al. 1995). In order to enhance DNA-binding of Elk-1, phosphorylation by ERK can induce a conformational change (Yang, Shore et al. 1999). Likewise, enhanced ERK1/2 phosphorylation of Ets-1 has been shown to increase Ets-1 protein levels and induce target promoter activation (Liu, Liang et al. 2005).

In the results section of this dissertation, analysis of the human MDMX proximal promoter revealed a cluster of potential transcription factor binding sites which included both Ets-1 and Elk-1. These sites appeared to be critical for elevated MDMX expression in tumor cell lines. Chip assays revealed enhanced promoter binding of both Ets-1 and Elk-1 under conditions of increased erk-phosphorylation by IGF-1 stimulation. Taken together , our results suggest that both Ets-1 an Elk-1 may play a role in both endogenous and growth factor stimulated activation of MDMX.

Chapter Two
Materials & Methods

Cell Lines

Tumor cell lines H1299 (lung, p53-null), A549 (lung), U2OS (bone), SJSA (bone, MDM2 amplification), MCF-7 (breast), JEG-3 (placenta, MDM2 overexpression) were maintained in DMEM medium with 10% fetal bovine serum. HCT116-p53+/+ and HCT116-p53-/- cells were kindly provided by Dr Bert Vogelstein. Normal human skin fibroblasts (HFF) were provided by Dr Jack Pledger. MDMX/p53 double null (41.4), MDM2/p53 double null (174.1) and p53-null (35.8) MEFs were provided by Dr. Gigi Lozano. P53- null (35.8) and p53/ARF double null (DKO) cells expressing activated K-Ras were generated by infection with retrovirus pBabe-HA-K-Ras (12V). Infected cells were selected with 1 µg/ml puromycin and drug resistant colonies were pooled.

To generate cells with expression of lentiviral MDMX vector a Lentivirus vector expressing MDMX was generated using the ViraPower^{TM} T-REx^{TM} system following instructions from the manufacturer (Invitrogen). Overexpression of MDMX was achieved by infecting with the MDMX lentivirus and selection with Zeocin to obtain a pool of resistant colonies. Tetracycline inducible expression of MDMX in U2OS cells was achieved by first infecting with the T-REX regulator lentivirus and selection with Blasticidin, followed by infection with the MDMX lentivirus and selection with Zeocin. MDMX expression was subsequently induced with 0.1-1 µg/ml tetracycline.

To inhibit MDMX in human cell lines by RNAi, double-stranded oligonucleotide (5'GATCCC<u>GTGATGATACCGATGTAGA</u>TTCAAGAGA<u>TCTACATCGGTATCATC AC</u>TTTTTTGGAAA, MDMX sequence underlined) was cloned into the pSuperiorRetroPuro vector (OligoEngine) and the pSilencer. Cells expressing the pSuperiorRetroPuro were infected with the MDMX shRNA retrovirus and selected with 0.5-1 µg/ml puromycin. Cells expressing pSilencer siMDMX were selected with G418. Drug-resistant colonies were pooled for analysis. A virus expressing a scrambled shRNA (5'GATCCC<u>GCCGTCGTCGATAAGCAATAT</u>TTGATATCCG<u>ATATTGCTTATCGA CGACGGC</u>TTTTTTA) was used as control.

To transiently inhibit MDMX expression, U2OS or MCF-7 cells were transfected with 200 nM control siRNA (AATTCTCCGAACGTGTCACGT) or MDMX siRNA (AGATTCAGCTGGTTATTAA) using Oligofectamine (Invitrogen) as described below. L11 siRNA pool was purchased from Dharmacon.

<center>*Transfections*</center>

Calcium Phosphate

Calcium phosphate transfection was usually performed in H1299 cells because of their high transfection efficiency. In transient transfection assays, $> 2 \times 10^6$ cells were seeded into 10 cm tissue culture dishes for 24 hrs. For each transfection, a total amount of 40 µg of plasmid DNA was mixed with 450 µl of H2O and 125 mM calcium chloride. A mixture of 500 µl of HEPES (0.28 M NaCl, 0.05 M HEPES, 1.5mM CaCl2) was bubbled with air and the water/DNA mixture was added dropwise. Immediately after bubbling, the mixture was added to the cells and incubated for 16 hours. After incubation, transfected cells were washed 2 times with PBS, refed with complete medium and

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incubated for another 24 hours before harvest. To generate a stable cell line, 48 hours

after transfection, the cells were drug selected by complete medium containing 750 µg/ml

G418 or 0.5-1.0 µg/ml puromycin for two weeks. Drug-resistant colonies were either

pooled or cloned.

Lipofectamine Transfection

For Lipofectamine™ transfection experiments, 2×10^5 cells were seeded into 6 cm

tissue culture dishes for 24 hrs, washed with 3 ml of serum free media and refed with 2

ml of serum free medium. For each transfection, a total amount of 4 µg of plasmid DNA

was mixed with 250 µl of serum-free medium and 10 µl of lipofectamine plus reagent

and incubated for 15 min at room temperature. A pre-mix 10 µl of lipofectamine reagent

was mixed with 250 µl serum-free medium and then with the previously described DNA

solution. The mixture was incubated for another 15 min before being added to the cells.

The reaction mixtures were scaled according to the number of cells plated (i.e. 24-well,

6-well, 60-mm, or 100-mm plates) After 4 hrs incubation, the cells were refed with

complete medium and harvested 24-72 hours later.

Oligofectamine Transfection

For transfection of RNAi oligonucleotides Oligofectamine™ transfection reagent

was utilized. Approximately, 2×10^5 cells were seeded into a 6-cm tissue culture dishes

for 24 hrs, washed with 3 ml of serum free media and refed with 2 ml of serum free Opti-

MEM reduced serum medium. For each transfection, 10 µl of a 20 µM stock

oligonucleotide was mixed with 175 µl medium to give a 200 nM final oligonucleotide

concentration. In a separate tube, 4 µl of Oligofectamine™ Reagent was mixed into

medium without serum for a final volume of 15 µl and incubated for 10 min at room

temperature. The diluted Oligofectamine™ reagent was mixed with the diluted

oligonucleotide and incubated at room temperature for 15 before being added to the

cells. After 4 hrs incubation, the cells were refed with complete medium and harvested 72

hours later.

Protein Analysis

Western Blot

To detect proteins by Western blot, cells were lysed in lysis buffer (50mM Tris–

HCl (pH 8.0), 5mM EDTA, 150mM NaCl, 0.5% NP40, 1mM PMSF, and protease

inhibitors), centrifuged for 10 min at 10,000 g at 4ºC and the insoluble debris were

discarded. Cell lysate (10–50 ug protein) was fractionated by SDS–PAGE and transferred

to Immobilon P PVDF filters (Millipore). The filter was blocked for 1 hr with washing

buffer containing phosphate buffered saline (PBS), 5% non-fat dry milk and 0.1 %

Tween-20. The filter was then incubated for two hours to overnight with primary

antibodies diluted in blocking buffer. The filter was washed three times (10 min each)

with PBS containing 0.1 % Tween-20. Next, bound primary antibodies were conjugated

with secondary antibody HRP IgG goat-anti-mouse or HRP IgG goat-anti-rabbit by

incubating the filter with the secondary antibody diluted in blocking buffer for two hours.

After the filter was washed three times (10 min each), they were developed using either

ECL-plus reagent (Amersham) or Supersignal (Pierce). The following antibodies were

utilized for experiments:

- Human MDMX was detected using monoclonal 8C6 with a 1:40 dilution

- Mouse MDMX was detected using monoclonal 7A8 or 10C2 with a 1:40 dilution

- Human MDM2 was detected using monoclonal 3G9 with a 1:30 dilution

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

- ARF was detected by 14PO2 (Neomarkers) with a 1:500 dilution

- p21 was detected using anti-WAF1 at 1:1000 dilution

- Flag tagged proteins were detected with an α-Flag monoclonal antibody with a 1:5000 dilution

- Ets-1 was detected using c-Ets-1 (N-276) (Santa Cruz Biotechnology) at a 1:2000 dilution

- Ets-1 was detected using Elk-1 (H-160) (Santa Cruz Biotechnology) at a 1:5000 dilution

- Total ERK was detected using ERK-2 (Santa Cruz Biotechnology) at a 1:5000 dilution

- Phosphorylated ERK was detected using $ERK^{p42/p44}$ (Cell Signaling Technology) at a 1:1000 dilution

- Total ERK was detected using ERK2 (Santa Cruz Biotechnology) at a 1:5000 dilution

- HA-tagged K-Ras was detected using HA.11 (Covance Research Products) at a 1:2000 dilution

- L11 was detected using a rabbit polyclonal antibody provided by Dr. Yanping Zhang.

Affinity purification of MDMX and MDM2

Purification of MDMX complex was performed using HeLa cells stably transfected with FLAG-tagged MDMX ($\sim$2 $\times 10^8$ cells). Cells were lysed in 10 ml lysis buffer (50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 200 nM Okadaic acid). The lysate was precleared with 100 µl bed volume of protein A sepharose beads for 30 min, and then incubated with 50 µl bed volume of M2-agarose bead (Sigma) for 4 h at 4°C. The beads were washed and MDMX was eluted with 70 µl of 20 mM Tris pH 8.0, 2% SDS, 200 µg/ml FLAG epitope peptide for 15 min. The eluted proteins were fractionated on SDS–PAGE and stained with Coomassie Blue.

To purify MDM2 complexes, human MDM2 cDNA expression plasmid was transiently transfected into 293T cells. Two days after transfection, cells ($\sim$2x10^8) were treated with 30 µM MG132 for 4 hours, lysed in a total of 10 ml lysis buffer and centrifuged for 5 minutes at 10,000 g. The lysate was precleared with protein A Sepharose beads for 30 minutes, and then incubated with 40 µl protein A Sepharose beads and 0.5 ml 2A9 hybridoma supernatant for 4 hours at 4˚C. The beads were washed with lysis buffer and boiled in SDS sample buffer. The eluted proteins were fractionated on SDS-PAGE and stained with Coomassie Blue. Proteins co-purified with MDMX and MDM2 were cut out from the SDS-Page gel and identified by mass spectrometry.

Immunoprecipitation Assay

For immunoprecipitation assays, cells were lysed in lysis buffer (50mM Tris–HCl (pH 8.0), 5mM EDTA, 150mM NaCl, 0.5% NP40, 1mM PMSF, and protease inhibitors), centrifuged for 10 min at 10,000 g at 4ºC and the insoluble debris were discarded. Cell lysate (500–1000 ug protein) was immunoprecipitated using 100 µl Pab1801, 100 µl

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3G9, or 100 μl 8C6 hydridoma antibody and 40 μL protein A Sepharose bead slurry at

4ºC overnight with rotation. The beads were washed 5 times with lysis buffer, boiled in

SDS sample buffer, fractionated by SDS–PAGE, and analyzed by Western blot.

In vivo Ubiquitination

H1299 and U2OS cells in 10-cm plates were transfected with combinations of 1 μg

GFP expression plasmid, 5 μg His6-ubiquitin expression plasmid, 1-5 μg human MDMX,

5 μg MDM2 and 5 μg ARF or L11, L5 or L23 expression plasmids using calcium

phosphate precipitation method (see above). Thirty-two hours after transfection, cells

from each plate were collected into two aliquots. One aliquot (10%) was used for

conventional western blot to confirm expression and degradation of transfected proteins.

The remaining cells (90%) were used for purification of His6-tagged proteins by Ni_2^+-

NTA beads. The cell pellet was lysed in buffer A (6 M guanidinium-HCl, 0.1 M

Na_2HPO_4/NaH_2PO_4, 0.01 M Tris-Cl pH8.0, 5 mM imidazole, 10 mM β-mercaptoethanol)

and incubated with Ni_2^+-NTA beads (Qiagen) overnight at room temperature. The beads

were washed one time each with buffer A, B (8 M urea, 0.1 M Na_2PO_4/NaH_2PO_4, 0.01 M

Tris-Cl pH8.0, 10 mM β-mercaptoethanol), C (8 M urea, 0.1 M Na_2PO_4/NaH_2PO_4, 0.01

M Tris-Cl pH6.3, 10 mM β-mercaptoethanol), and C + 10% triton-X and bound proteins

were eluted with buffer D (200 mM imidazole, 0.15 M Tris-Cl pH6.7, 30% glycerol,

0.72M β-mercaptoethanol, 5% SDS). The eluted proteins were analyzed by western blot

for the presence of conjugated MDMX by 8C6 antibody, MDM2 by 3G9 antibody, or

p53 by DO-1 antibody .

Acid Extraction of DNA bound proteins

To detect chromatin bound proteins such as gamma-H2A.X acid extraction of proteins is necessary. After treated cells were washed and collected by scraping, they were resuspended in 300-500 µL of lysis buffer (50 mM Tris-Cl pH7.4, 10% Glycerol, 10mM KCl, 0.2% NP-40, 1mM EDTA, protease inhibitors). After spinning for 5 minutes at 14,000 rpm and removing the cytoplasmic fraction, the nuclear fraction was resuspended in 100 µL of nuclear lysis buffer (50 mM Tris-Cl pH7.4, 20% Glycerol, 10mM KCl, 0.4M NaCl, 0.2% NP-40, 1mM EDTA, protease inhibitors) and kept on ice for 30 minutes. The samples were spun at 14,000 rpm for 5 min at 4°C. The nuclear extract was removed and the insoluble chromatin bound proteins was then acid extracted in 20-50 µL of acid extraction buffer (0.25 M HCl, 10% Glycerol, 100mM β-mercaptoethanol). The supernatant was spun down and neutralized to pH = 7.0 before loading onto an SDS page gel.

Cell Viability and Growth Assays

Cell Cycle Analysis by Flow Cytometry

After treatment, cells were harvested by trypsinizing and washed once in PBS. The cells were resuspended in 1 ml of PBS and fixed by adding 4 mL of ethanol while slowly vortexing. Fixed cells were placed at -20°C overnight, but may be stored for several months in fixative. Cells were washed once in PBS and then resuspended in 1 mL of staining solution (50 µg/ml RNase A treatment and 50 µg/ml propidium iodide in PBS) and incubated for at least one hour at 4°C. Flow cytometry was performed on an argon laser-equipped Becton Dickinson (Sunnyvale, CA) FACScan instrument to determine the number of cells in sub G_o, G1/M, S, or G2 phase of the cell cycle.

BRDU Assay

In order to compare the number of cells which are capable of DNA synthesis, we utilized the 5-Bromo-2´-deoxy-uridine Labeling and Detection Kit III by Roche. Briefly, after experimental treatment, cells were incubated with 10 mmoles of BrdU for 2 to 4 hours. Then the samples were fixed with ethanol/HCl. Following fixation of cells cellular DNA is partially digested by nuclease treatment. Next a peroxidase labeled antibody to BrdU (anti-BrdU POD, Fab fragments) was added and binds to the BrdU label. In the final step, the peroxidase substrate is added. The peroxidase enzyme catalyses the cleavage of the substrate yielding a colored reaction product. The absorbance of the sample was determined using a microplate reader and is directly correlated to the level of BrdU incorporated into cellular DNA.

MTS Assays

U2OS, MCF-7 or HCT-116+/+ cells were plated in 24-well plates with approximately 10,000-30,000 cells plated per well. Fresh media (250 µL) containing 10 µL of MTT reagent was added to each well. The plates were kept in the 37ºC incubator for 15-30 min. The reactions were stopped by putting the plates on ice. From each well, 200 µL was transferred into a 96 well plate. The plate was measure using an OD of 490. The absorbance reading correlates with the number of live cells per well.

Colony Formation Assays

U2OS or HCT116 cells were plated with 100 cells per well in a 6-well plate. After treatment (24 hrs), cells were refed with complete media and allowed to grow for ~ 1 week. The media was removed and cells were washed in PBS. Each well was incubated with crystal violet (0.5% crystal in 50% Ethanol). After 15 minutes, each well was

carefully washed with distilled water and allowed to air dry. Visible colonies were counted for comparison.

<div align="center">*RNA Analysis*</div>

RNA Isolation

Total RNA was extracted from 10 cm plates of cells using the RNeasy Mini Kit by Qiagen following the manufacturer's protocol. Briefly, samples were lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate–containing buffer. Ethanol was added and then the sample was applied to an RNeasy Mini spin 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.

Real-time PCR

Reverse transcription of total RNA was performed using the SuperScript III kit (Invitrogen). The following PCR primers were used for qPCR analysis: p21F (5' CAGACCAGCATGACAGATTTC) and p21R (5' TTAGGGCTTCCTCTTGGAGA); MDM2FW (5' CCCTTAATGCCATTGAACCT) and MDM2REV (5' CATACTGGGCAGGGCTTATT); p53FW (5' GGCAGCTGGTTAGGTAGAGG) and p53REV (5' AGGTCGACCAAGAGGTTGTC); 18SFW (5'GATTAAGTCCCTGCCCTTTGTACA) and 18SREV (5' GATCCGAGGGCCTCACTAAAC). Samples were analyzed in triplicate.

<div align="center">*Promoter Analysis Assays*</div>

Genomic DNA isolation

Adherent cells were removed from plates by trypsinization followed by washing with PBS. Cells were resuspended in 1 volume of digestion buffer (25 mM EDTA, 10

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mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.5% SDS and 100 µg/ml proteinase-K) and incubated overnight at 50ºC. Each sample was extracted with an equal volume of phenol/chloroform/isoamyl alcohol and centrifuged at 1700 x g for 10 minutes. The aqueous top layer was transferred to a new tube. NaCl was added to a 0.1 M final concentration and two volumes of ethanol were added. DNA was recovered by centrifuging at 1700 x g for two minutes. The pellet was rinsed in 70% ethanol and air dried before being reconstituted in TE.

Construction of the MDMX promoter reporter plasmids.

To isolate the 5' upstream region of MDMX, PCR was performed using an antisense primer in exon 1 of the MDMX gene (5'AAGAGCCACACCTTACGGCA) and a sense primer in a 5' genomic sequence (5'CTATCTCGGCTCACTGCAAC) with genomic DNA isolated from MCF-7 cells as a template. The resulting 1100-bp fragment was cloned into pDrive vector (Qiagen) and then transferred to the luciferase reporter plasmid pGL2-Basic (pGL2-FL MDMX) and was confirmed by sequencing. The mutant promoter constructs were generated using the QuickChange Site-Directed Mutagenesis kit (Stratagene) in the context of the pGL2-FL MDMX according to the manufacturer's instructions.

Reporter Transfections

Cell lines were cultures in 24-well plates and transfected with a mixture containing 50 ng luciferase reporter plasmid (BP-100 or MDMX promoter fragments), 10 ng CMV– lacZ plasmid, 200ng of ssDNA and 5ng of GFP. Transfection was achieved using Lipofectamine PLUS reagents (Invitrogen) as described above. Forty eight hours after transfection, cells were analyzed for luciferase and Beta-galactosidase expression.

Chromatin immunoprecipitation

Two confluent 15 cm plates per cell line (approximately 2 x 10^7 - 2 x 10^8 cells)

were used per sample. 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 1X PBS plus protease inhibitors and PMSF, scraped and centrifuged to collect. The

pellet was resuspended in 7 mL of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCL,

0.5% NP40 plus the protease inhibitors PMSF (10 ul per ml), aprotinin (1 ul per ml)

leupeptin (1 ul per ml)), incubated on ice for 10 minutes, and centrifuged at 4,000 rpm

for 7 minutes at 4°C to pellet the nuclei. The nuclear pellet was resuspended in 500-900

μL of nuclei lysis buffer (50 mM Tris-Cl pH 8.1, 10 mM EDTA, 1% SDS, protease

inhibitors) and incubated on ice for 10 minutes. Next, samples were sonicated to an

average chromatin length of about 500-1000 bp and then centrifuged at 14,000 rpm for

10 minutes at 4°C. The supernatant was transfered to a new tube, precleared by adding

40 μL of protein A/DNA slurry (Upstate Biotechnology) and incubated on a rotating

platform at 4°C for 30 minutes. Samples were centrifuged at 14,000 rpm for 5 minutes

and divided into 100-200 μL aliquots for immunoprecipitation.

The following antibodies have been added and utilized for chromatin

immunoprecipitation:

MDM2: 100μL of 2A9, 5B10, and 4B11
MDMX: 100μL of 8C6 and 10C2
P53: 100μL of 1801 and 10 μL DO-1 (BD Pharmigen)
c-Ets: 5 μg of c-Ets-1 (N-276) (Santa Cruz Biotechnology)

Elk-1: 5 µg of Elk-1 (H-160) (Santa Cruz Biotechnology)

YY-1: 5 µg of YY-1 (c-20) (Santa Cruz Biotechnology)

The final volume of each sample was adjusted to 800 µL using IP dilution buffer (0.01% SDS, 1.1% Trition X 100, 1.2 mM EDTA, 16.7 mM Tris-Cl pH 8.1, 167 mM NaCl). Control samples that were used for experiments include a "no antibody" sample as well as "mock" samples which contain 1X dialysis buffer instead of chromatin. Samples were incubated on a rotating platform at 4°C overnight.

The following day 60 µL of protein A beads/DNA slurry (Upstate Biotechnology) was added and incubated on a rotating platform at 4°C for 2 hours. Samples were centrifuged and supernatant from the "no antibody" sample was saved as "total input chromatin". Beads were washed one time in low salt buffer, one time in high salt buffer, two times in LiCl buffer (100 mM Tris-Cl pH 8.0, 500 mM LiCl, 1% NP40, 1% deoxycholic acid) and 2 times in TE. For each wash, samples were rotated for 3 minutes then centrifuged at 14,000 rpm for 3 minutes at room temp. Chromatin was eluted two times by adding 250 µL of IP elution buffer (50 mM NaHCO$_3$, 1% SDS) while shaking for at least 15 minutes. The eluates were combined and then centrifuged at 14,000 rpm for 5 minutes to remove any traces of Protein A beads and transferred to a clean tube. RNase A (2µL of 10 mg/ ml) and 5M NaCl was added to a final concentration of 0.3 M. Samples were incubated at 67ºC for 4 hours to overnight to reverse formaldehyde crosslinks. Next, 20µL of 1.0M Tris, pH 6.5, 10 µL 0.5M EDTA, pH 8.0 and 5 µL of 10 mg/ml proteinase K were added per 500 µL sample and incubated at 45ºC for 2 hours. The DNA was purified using the Qiagen PCR purification kit or by phenol/chloroform extraction and reconstituted in 50 µL of water. For each PCR reaction, 1-3 µL was

utilized. For p53 promoter binding, samples were subjected to SYBR Green real-time PCR analysis using forward and reverse primers for the p53 binding sites in the MDM2 promoter (5'-CGGGAGTTCAGGGTAAAGGT and 5'-CCTTTTACTGCAGTTTCG) and p21 promoter (5'-TGGCTCTGATTGGCTTTCTG and 5'-TCCAGAGTAACAGGCTAAGG). For MDMX promoter studies, co-precipitated chromatin was analyzed by standard PCR (30-32 cycles) using primers (5' ACTCTCTCCCCGGACTAGGA and 5' CGAGTAATGAAGCCGCAACT) to amplify the human basal MDMX promoter containing the c-Ets-1 and Elk-1 binding sites. Primers located 3 kb upstream of the basal promoter (5' TAAACGATCCTCCCACCTTG and 5' CCTGGAGCCTTGGAATATGA) were used as negative PCR controls.

Immunohistochemistry Staining

Tissue microarrays were de-parrafinized in three changes of xylene, rehydrated using a decreasing gradient of ethanol, followed by incubation in sodium citrate buffer (10mM sodium citrate, 0.05% Tween-20, pH 6.0) at 95ºC for 20 min. Slides were cooled at room temperature for 20 minutes, washed in two changes of PBS and incubated in 1% H_2O_2 for 10 min to quench endogenous peroxidase activity. The ABC Staining system (Santa Cruz Biotechnology) was used for staining. Briefly, slides were blocked in 1.5% serum in PBS. A polyclonal MDMX antibody was affinity purified by the procedure described below and incubated at a 1:500 dilution overnight at 4ºC. After washing, slides were incubated with biotinylated secondary, then with AB enzyme reagent followed by incubation with DAB chromogen.

The polyclonal MDMX antibody was validated for its specificity using cell lines expressing different levels of endogenous and transfected MDMX. The tumor array was scored according to MDMX staining intensity (1 as low intensity, 2 intermediate, and 3 as intense staining). A phospho-ERK$^{p42/p44}$ antibody (1:100; Cell Signaling Technology) was used for the same array and scored as percent-positive tumor cells (0 as negative, 1 as 1–30%, 2 as 30–70% and 3 as 70–100% positive).

Affinity Purification of MDMX antibody

This protocol was used to purify MDMX polyclonal antibody in order to stain the colon tumor array. Recombinant MDMX protein (5 ug) diluted in 5mL of western blot transfer buffer was spotted onto a nitrocellulose filter. The filter was blocked in 3% BSA in 10mM TrisHCl (pH= 8.0), 150 mM NaCl, 0.2% Tween-20 (TBS-T) for 30 min at room temperature. Next, the nitrocellulose filter was incubated with 1 ml of MDMX rabbit anti-MDMX serum in 10 ml total volume of TBS-T. The filter was washed 4 times for 5 minutes with TBS-T. Antibody was eluted from the filter using 10 mM glycine-HCl (pH = 2.7), and neutralized by adding 1.5 M Tris.HCl (pH 8.8). The filter was washed two times and the procedure was repeated. The elutes were combined and dialyzed in PBS overnight, then concentrated to a 500 µL volume. For storage purposes, 10% normal goat serum was added to the concentrated antibody and stored at -20ºC.

Xenograft Studies

Athymic-NCr-nu female mice between 7 and 8 weeks were inoculated s.c. on both flanks with 5×10^6 of HCT116-p53+/+ control, MDMX, or MDMX siRNA cells. For 5-FU treatment response, control and Lenti-MDMX expressing tumors were grown for 10 days to ~0.1 cm^3 on both flanks. Mice were treated with 5-FU at 50 mg/kg/day for 4 days

by tail vain injection. Tumor size was measured every other day using a digital caliper, and tumor volume was calculated with the formula: (Average (Rmax, Rmin)^3)*0.5236, where Rmax and Rmin are the maximum and minimum tumor radii, respectively. Data were analyzed using the student paired t-test to assess differences in tumor growth rates.

Chapter Three
Mdmx Regulation of P53 Response to Ribosomal Stress

Abstract

Ribosomal stress such as disruption of rRNA biogenesis activates p53 by release of ribosomal proteins from the nucleoli, which bind to MDM2 and inhibit p53 degradation. We found that p53 activation by ribosomal stress requires degradation of MDMX in an MDM2-dependent fashion. Tumor cells overexpressing MDMX are less sensitive to actinomycin D-induced growth arrest due to formation of inactive p53-MDMX complexes. Knockdown of MDMX increases sensitivity to actinomycin D, whereas MDMX overexpression abrogates p53 activation and prevents growth arrest. Furthermore, MDMX expression promotes resistance to the chemotherapeutic agent 5-FU, which at low concentrations activates p53 by inducing ribosomal stress without significant DNA damage signaling. Knockdown of MDMX abrogates HCT116 tumor xenograft formation in nude mice. MDMX overexpression does not accelerate tumor growth but increases resistance to 5-FU treatment *in vivo*. Therefore, MDMX is an important regulator of p53 response to ribosomal stress and RNA-targeting chemotherapy agents.

Ribosomal proteins selectively bind MDM2 but not MDMX

In experiments aimed at identifying MDM2 and MDMX binding proteins, we performed affinity purification of MDM2 and MDMX from stable or transiently transfected cells followed my mass spectrometry to indentify the coprecipitating bands. As reported by others, MDM2 co-purified with several ribosomal proteins, the most prominent being L5, L11 and L23. This binding pattern was observed with transfected MDM2 (Figure 8a), or endogenous MDM2 from SJSA cells (not shown). In contrast, flag tagged MDMX co-purified with casein kinase 1 alpha and 14-3-3 under the same washing conditions (Figure 8b) (Chen, Li et al. 2005). Reproducibly absent from the MDMX immunoprecipitation (IP) was the binding of ribosomal proteins. These results indicated that ribosomal proteins directly target MDM2 but not MDMX.
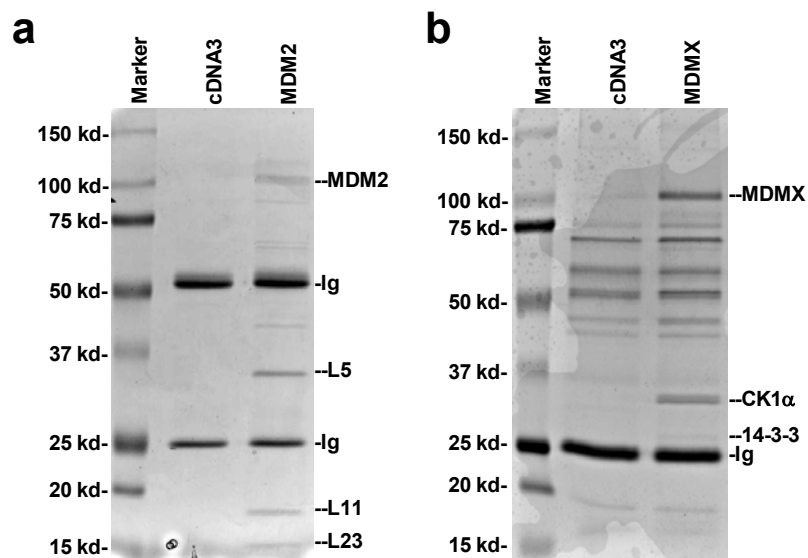

Figure 8. Differential binding of ribosomal proteins to MDM2 and MDMX. (a) MDM2 expression plasmid was transfected into 293T cells for 2 days. MDM2 complex was immunoprecipitated using 2A9 antibody and stained with Coomassie Blue. (b) FLAG-tagged MDMX stably expressed in Hela cells was purified using M2-agarose beads and eluted with FLAG epitope peptide.

To further confirm the results from the mass spectometry analysis, U2OS cells

stably expressing tetracycline-regulated MDMX and MDM2 were immunoprecipitated

using MDMX and MDM2 antibodies, followed by western blot for L11. MDMX and

MDM2 expression were induced to ~10-fold above endogenous levels using tetracycline.

Coprecipitation between MDM2 and L11 was detected when MDM2 was induced,

whereas MDMX-L11 interaction was not detectable (Figure 9). This result suggested that

MDMX-L11 interaction was negligible even in overexpression conditions. The dramatic

difference in ribosomal protein binding suggested that MDMX is regulated differently by
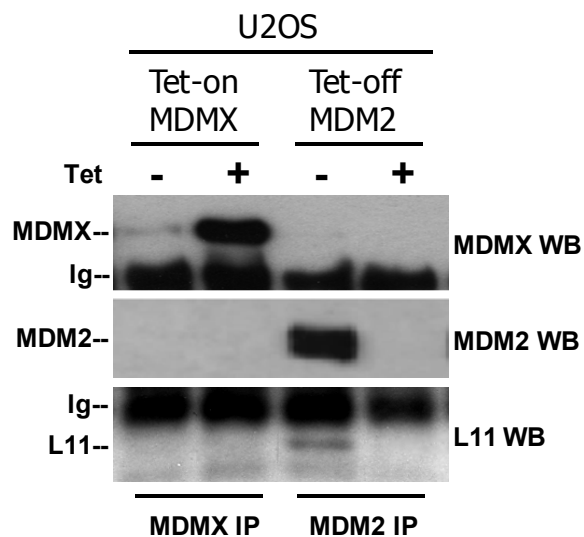
ribosomal stress compared to MDM2.

Figure 9. L11 binds to MDM2 not MDMX. U2OS cell lines expressing Tet-on MDMX
or Tet-off MDM2 were treated with tetracycline for 16 hours to modulate
expression levels, followed by MDMX or MDM2 IP and L11 western blot.

Ribosomal stress induces MDMX degradation

To determine the effect of ribosomal stress on MDMX, we used actinomycin D

(ActD) to inhibit ribosome biogenesis. ActD is a chemotherapeutic agent that can induce

DNA damage and inhibit general transcription at high concentrations (>30 nM), but at

low concentrations (5 nM) it selectively inhibits RNA polymerase I and induces

ribosomal stress (Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003).

When HCT116 and U2OS cells were treated with 5 nM ActD for 8-20 hours,

significant activation of p53 was observed, resulting in the induction of p21 and MDM2.

In contrast, MDMX level decreased significantly after ActD treatment (Figure 10).

MDMX was also down-regulated to the same degree in HCT116-p53-/- cells despite

much weaker induction of MDM2 (Figure 10), suggesting that additional mechanisms
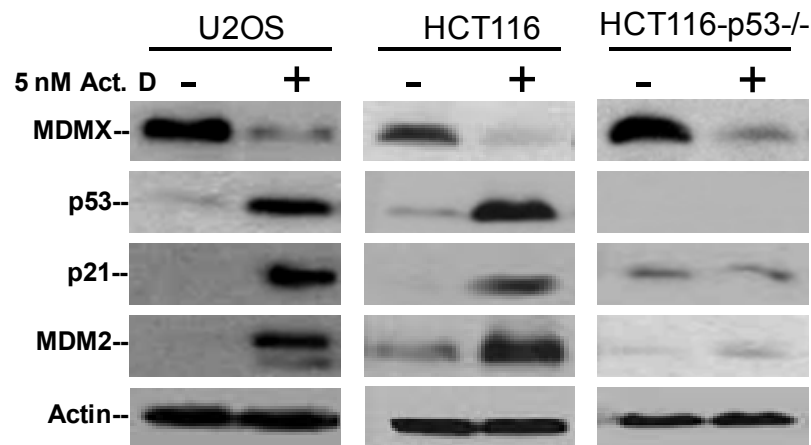
contributed to reduction in MDMX level.

Figure 10. Down regulation of MDMX by ribosomal stress. Cells were treated with 5
nM ActD for 16 hours and analyzed by western blot.

HCT116-p53-/- cells were treated with ActD and MG132 to block proteosomal

degradation, MDMX down regulation was partially inhibited (Figure 11). Gamma

irradiation has previously been shown to induce protein degradation of MDMX and was

utilized as a control to show MDMX degradation can be prevented with the addition of

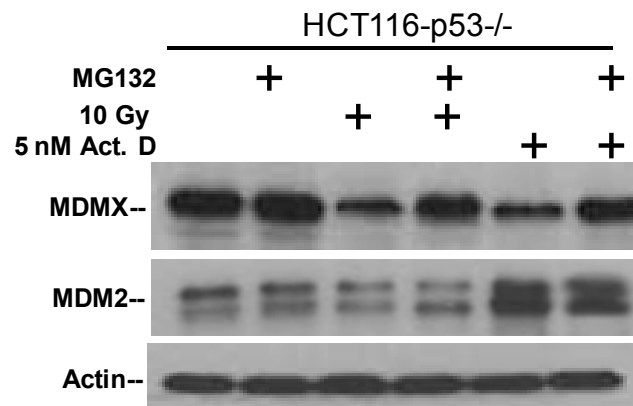MG132. These results suggested that ActD promotes degradation of MDMX.

Figure 11. MDMX is degraded by ribosomal stress. HCT116-p53-/- cells were treated
with 5 nM ActD for 8 hours, with or without 30 μM MG132 for the last 4
hours and analyzed by western blot.

Recent studies showed that phosphorylation of MDMX C terminus by ATM and

Chk2 promote MDMX degradation by MDM2 (Chen, Gilkes et al. 2005; Pereg, Shkedy

et al. 2005). We found that ActD (5 nM) and 5-FU (50 μM) did not induce significant

phosphorylation of histone gamma H2A.X which is a well accepted marker for DNA

damage (Figure 12). Cells treated with the DNA damaging agents CPT, and Gamma

Irradiation (10 Gy) were used as a positive control for phosphorylation of histone gamma
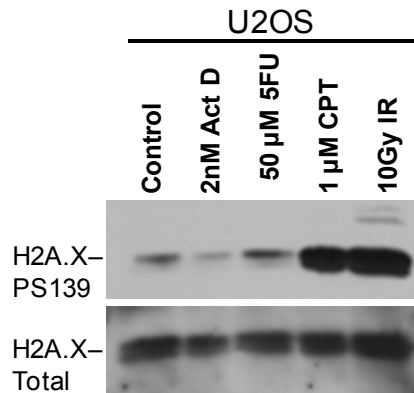
H2A.X.

Figure 12. Actinomycin D and 5-FU do not induce DNA Damage. U2OS cells treated with indicated agents for 18 hours or irradiated for 4 hours were analyzed by western blot using antibody against phosphorylated H2A.X, followed by reprobing for total H2A.X.

DNA damage induces ATM-dependent phosphorylation and degradation of MDMX. Phosphorylation of MDMX by CHK2 on S367 has been confirmed by phosphopeptide-specific antibodies and is enhanced followed by DNA damage ultimately leading to MDMX ubiquitination and degradation by MDM2 (Chen, Gilkes et al. 2005). To determine whether MDMX degradation following ribosomal stress enhances MDMX (S367) phosphorylation we compared phosphorylation levels following treatment with ActD, 5-FU, CPT, or Gamma Irradiation (Figure 13). Cells treated with ActD or 5-FU did not have enhanced MDMX phosphorylation suggesting an alternate mechanism for MDMX degradation under these conditions.
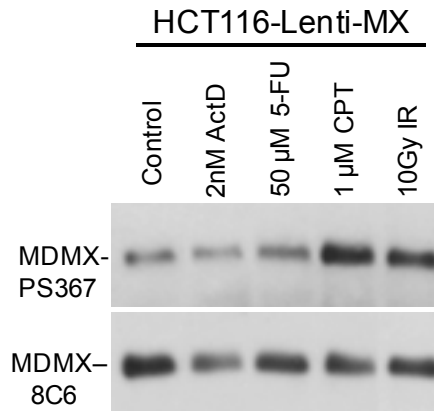
Figure 13. Ribosomal Stress does not Induce MDMX S367 phosphorylation.
HCT116 overexpressing MDMX was treated with indicated drugs and
analyzed by MDMX IP and western blot for phosphorylated S367, a target
site for Chk2 kinase. The membrane was reprobed for total MDMX level.

As an additional method to show that MDMX degradation following ribosomal

stress is independent of CHK2 we tested HCT116-Chk2 deficient cells for their ability to

prevent MDMX degradation following ribosomal stress. We found that while gamma

irradiation of MDMX requires CHK2, it had no effect on ActD or 5-FU induced MDMX

degradation (Figure 14). These results suggested that ribosomal stress induces MDMX

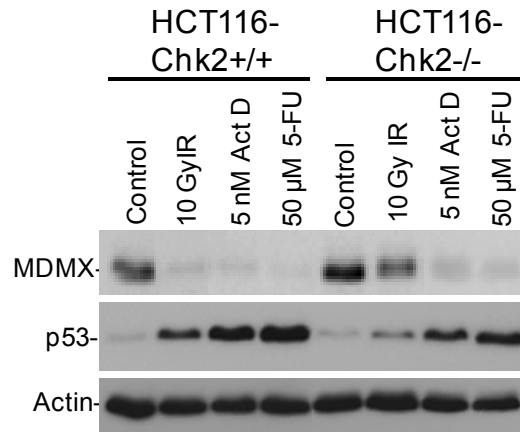degradation without causing DNA damage.

Figure 14. Ribosomal Stress induced MDMX degradation does not require CHK2.
HCT116 cells wild type or null for Chk2 were treated with indicated agents
for 16 hours and analyzed for MDMX degradation.

L11 promotes MDMX degradation by binding MDM2

Release of L11 from the nucleolus during ribosomal stress and binding to MDM2

was implicated in p53 activation. Therefore, we tested whether L11 stimulates MDMX

ubiquitination by MDM2. The results showed that in HCT116-p53-/- cells, exogenous

L11 stimulated MDMX poly-ubiquitination by MDM2 (Figure 15). L11 expression did

not increase MDM2 level, suggesting that the E3 ligase function of MDM2 was

stimulated by L11. These results suggested that L11-MDM2 interaction is unique in its

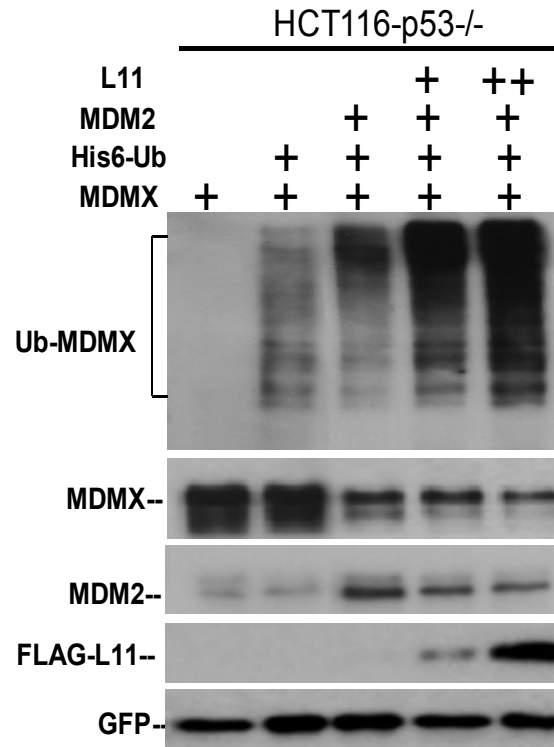ability to promote MDMX degradation during ribosomal stress.

Figure 15. L11 promotes MDMX ubiquitination. HCT116-p53-/- cells were transiently transfected with His6-ubiquitin, MDMX, MDM2 and L11 plasmids. MDMX ubiquitination was detected by Ni-NTA purification followed by MDMX western blot.

Next, the role of MDM2 was tested using MDM2-null mouse embryonic fibroblasts (174.1 cells) (McMasters, Montes de Oca Luna et al. 1996). ActD induced significant proteasome-dependent degradation of MDMX in MDM2+/+ MEFs compared to MDM2-/- control, suggesting that degradation of MDMX required MDM2 (Figure 16). The MDMX-/- cells were utilized as a control to show the specificity of the MDMX antibody. Further, knockdown of MDM2 in HCT116-p53-/- using a siRNA retrovirus against MDM2 also blocked MDMX degradation after ActD and 5-FU treatment (Figure 17). Taken together these experiments exemplify the role of MDM2 in MDMX degradation following ribosomal stress.
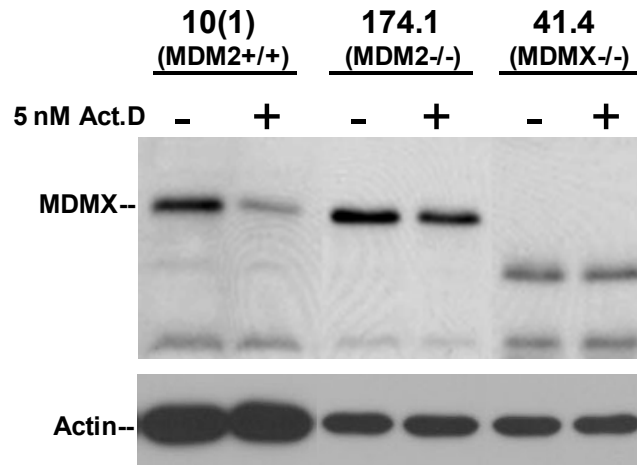
Figure 16. MDMX degradation by ribosomal stress requires MDM2. Mouse embryo fibroblasts with indicated genotypes were treated with ActD for 9 hours with MG132 for the last 6 hours and analyzed by western blot with the 7A8 antibody.

Figure 17. MDMX degradation by ribosomal stress requires MDM2. Knockdown of MDM2 prevents MDMX down regulation by ribosomal stress. HCT116-p53-/- cells stably transduced with retrovirus expressing MDM2 shRNA were treated with 2 nM ActD or 50 μM 5-FU for 16 hours, followed by analysis of MDM2 and MDMX levels.

To assess the specificity of L11 in degrading MDMX, MDMX was cotransfected with either L11 or L23. First, MDMX degradation was induced by L11 in MDM2+/+ cells (Figure 18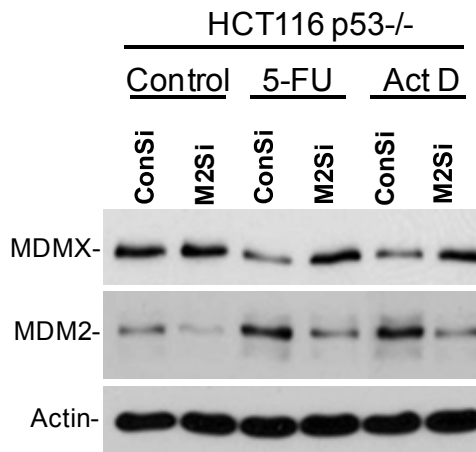b), but not in MDM2-/- cells unless MDM2 was restored by transfection (Figure 18a). Further, the addition of L23 or L5 (not shown) did not promote MDMX degradation suggesting that L11 is specific in its ability to degrade MDMX.
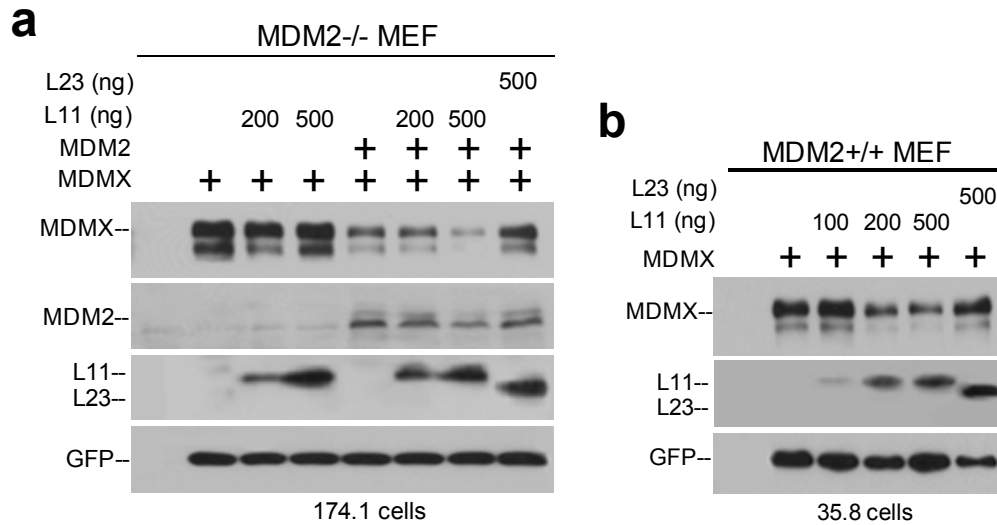
Figure 18. MDM2 and L11 mediate MDMX down regulation by ribosomal stress. (a, b) MEFs with and without MDM2 were transfected with 0.5 μg MDMX, 0.1 μg MDM2 and indicated amounts of L11 plasmids and analyzed by western blot.

To test the role of L11 in human cell lines, L11 was partially knocked down using a transient siRNA oligonucleotide in HCT116 cells. Cells were then treated with ActD overnight to assess the role of L11 in MDMX downregulation (Figure 19). MDMX was degraded in cells which maintained L11 expression.
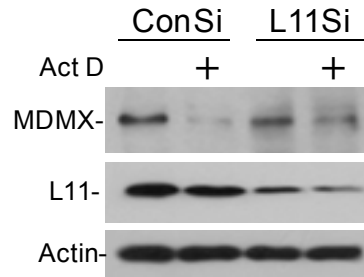
Figure 19. L11 is required for MDMX degradation in the presence of ActD. HCT116 were transfected with 100 nM L11 siRNA for 48 hours and treated with 5 nM ActD for 18 hours, followed by western blot analysis.

To further test the specificity of L11 regulation of MDM2 and MDMX, we generated the MDM2-C305S mutant with a mutated zinc finger in the L11 binding region. A similar mutation on MDMX (C306S) completely abrogated binding to casein kinase 1, revealing the structural importance of the zinc finger (Chen, Li et al. 2005). As expected, in transient transfection assays MDM2-C305S did not bind L11 but retained binding to L5, L23, and ARF (Figure 20). The ability of MDM2-C305S to ubiquitinate and degrade MDMX was no longer stimulated by L11, but remained responsive to ARF as expected (Figure 21). This result indicated that L11 stimulates MDMX degradation by binding to MDM2 and activating its ability to ubiquitinate MDMX.
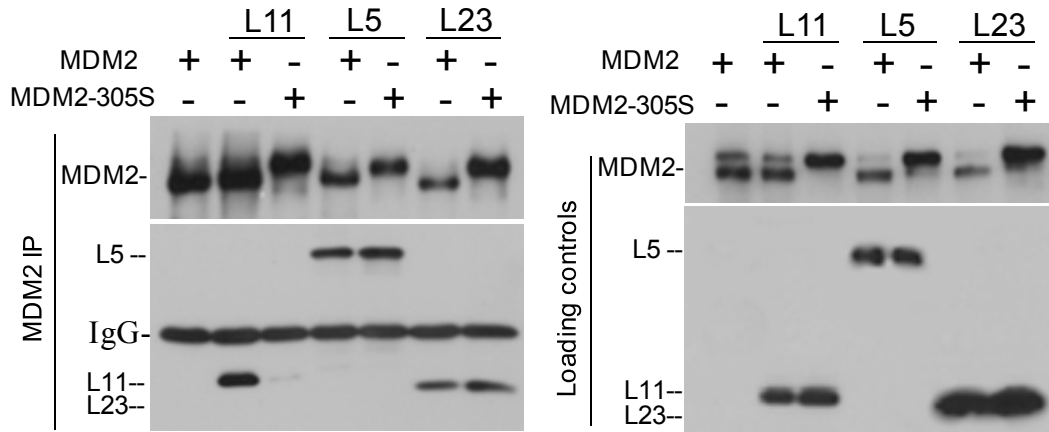
Figure 20. MDM2-305S mutant does not bind to L11. H1299 cells co-transfected with MDM2-C305S and FLAG-tagged L11, L5, and L23 were analyzed by MDM2 IP followed by FLAG western blot for coprecipitation of L proteins. Expression was verified by MDM2 and FLAG western blot of whole cell extract.
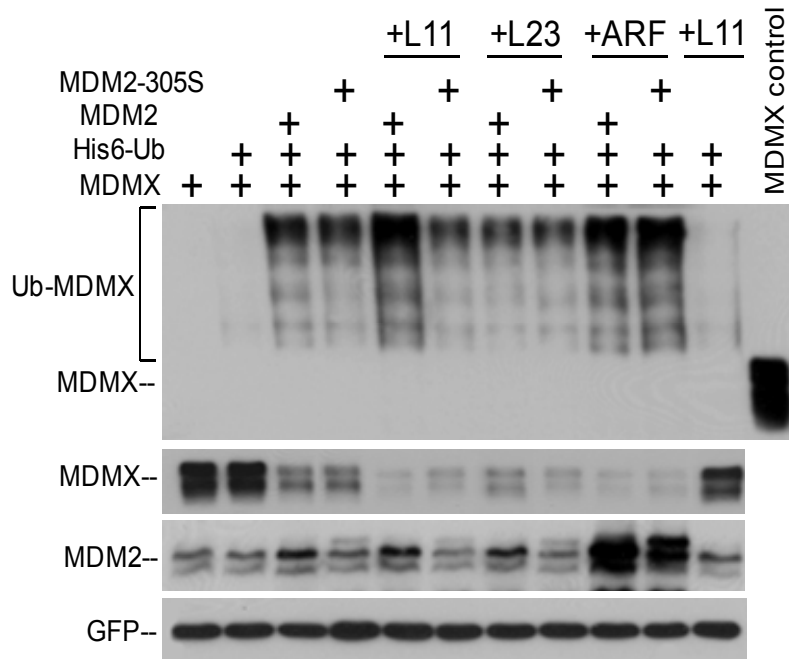
Figure 21. L11 does not enhance the ability of the MDM2-305S mutant to ubiquitinate MDMX. HCT116-p53-/- cells transfected with indicated plasmids were analyzed for MDMX ubiquitination, showing the loss of MDM2-C305S regulation by L11.

Since the lack of MDM2 did not completely prevent MDMX down regulation by

ActD (Figure 16), additional mechanisms for MDMX regulation were investigated. For

example, quantitative RT-PCR analysis of MDMX showed that ActD causes a 20%

reduction in MDMX mRNA level (Figure 22). The activity of a 1 kb human MDMX

promoter-luciferase construct was also inhibited 30% by ribosomal stress but not by

DNA damage (Figure 23). Therefore, although the mRNA level of MDMX seems to be

slightly reduced in the presence of ribosomal stress, MDM2-mediated degradation played

the major role in the rapid down-regulation of MDMX. mRNA levels for p53 target

genes, p21 and MDM2 are also included in Figure 22. Following ribosomal stress, p53

target genes are induced to similar levels as seen in DNA damage treated cells.
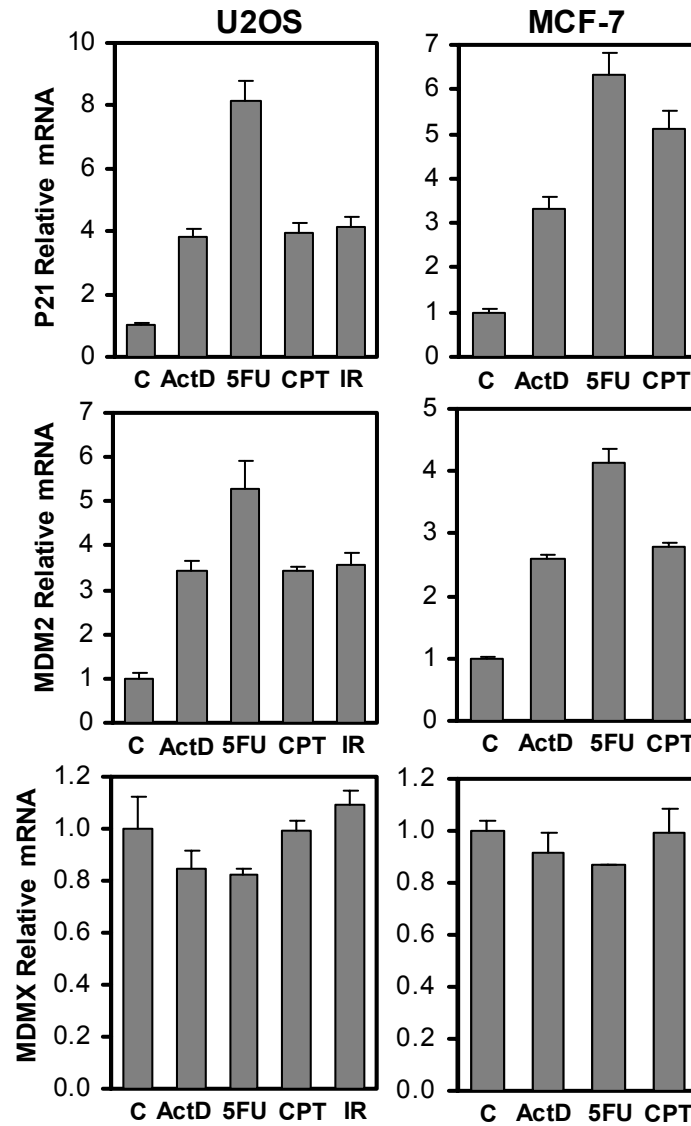
Figure 22. MDMX mRNA transcripts are reduced following ribosomal stress.
U2OS and MCF-7 cells were treated with 5 nM actinomycin D, 50 μM 5-FU, 0.5 μM CPT for 16 hours or irradiated with 10 Gy for 4 hours. The mRNA levels of indicated genes were analyzed by SYBR Green quantitative RT-PCR.
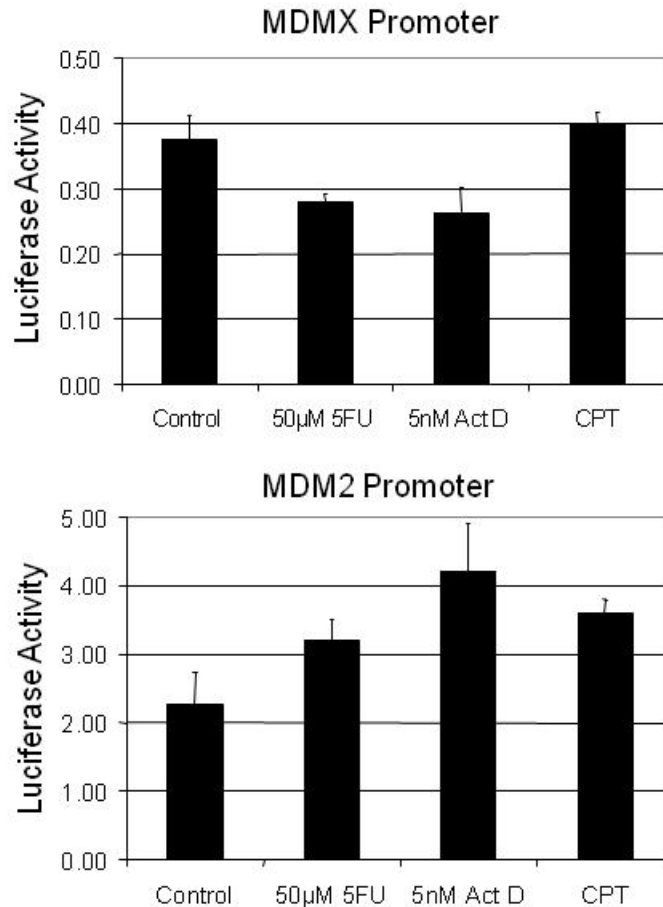
Figure 23. MDMX promoter activity is reduced following ribosomal stress. U2OS cells were transiently transfected with luciferase reporters driven by a 1 kb MDMX promoter or the p53-responsive MDM2 P2 promoter for 24 hours. Cells were treated with drugs for 16 hours and analyzed for the expression of luciferase and cotransfected CMV-lacZ. Luciferase levels are shown after normalization to beta galactosidase activity.

MDMX overexpression reduces p53 response to ribosomal stress

Since MDM2 and MDMX showed different expression and binding to ribosomal proteins, they likely have distinct effects on p53 response to ribosomal stress. To test this hypothesis, we compared tumor cell lines with different levels of MDMX and MDM2. In this panel, MDMX level can be ranked from highest to lowest in the order of JEG-3, MCF-7, U2OS, HCT116, A549, H1299, and SJSA. JEG-3 and SJSA have the highest

MDM2 levels due to gene amplification or increased translation (Leach, Tokino et al. 1993; Landers, Cassel et al. 1997). H1299 is p53-null and served as a control. After treatment with ActD, all cell lines showed p53 stabilization irrespective of MDM2 level. However, induction of p21 correlated inversely with the level of MDMX, but not MDM2 (Figure 24), suggesting that high MDMX levels kept the stabilized p53 in an inactive state.
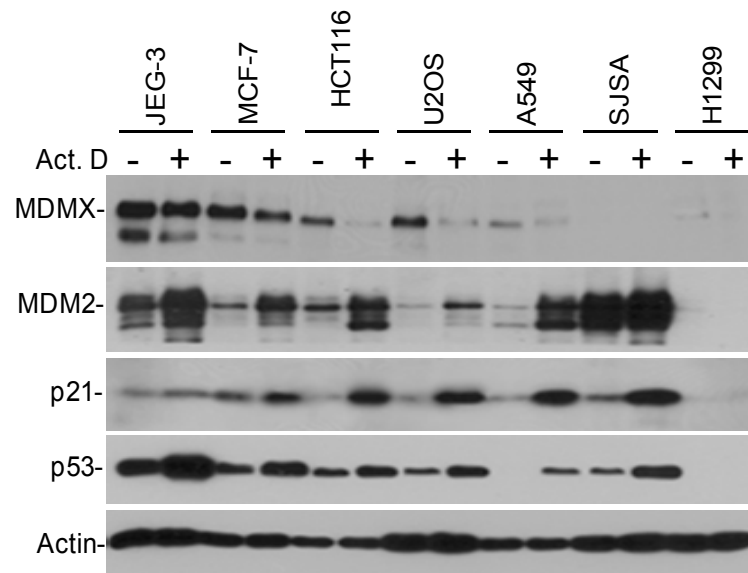
Figure 24. MDMX overexpression correlates with actinomycin D resistance. Cell lines were treated with 5 nM ActD for 18 hours and analyzed by western blot.

The same cell lines were also analyzed for cell cycle arrest after ActD treatment. Cell lines with high levels of MDMX (JEG-3, MCF-7) were unable to undergo cell cycle arrest. On the otherhand, cell lines with lower levels of MDMX (SJSA, A549, U2OS, HCT116) showed a more significant reduction in the number of cells in S-phase (MCF-7, JEG-3) (Figure 25-26). Interestingly, SJSA cells showed a strong response to ActD despite expressing the highest level of MDM2. As expected, p53-null H1299 did not

respond to ActD. Therefore, cell cycle sensitivity to ActD also correlated with MDMX

level, but not MDM2 level. These results suggested that MDMX overexpression has a

significant impact on p53 activation by ribosomal stress.
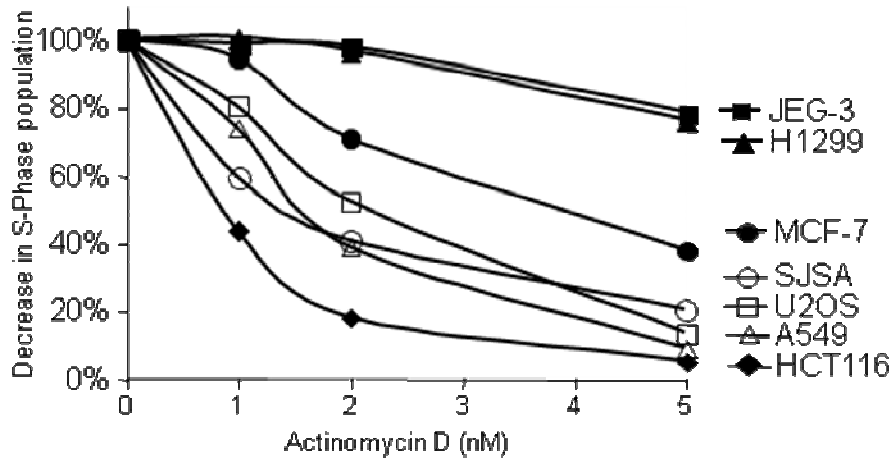
**Figure 25. MDMX overexpression correlates with cell cycle arrest after
Actinomycin D treatment.** Cells were treated with ActD for 18 hours and
analyzed for cell cycle distribution by FACS. The degree of growth arrest was
shown as the decrease of S phase population compared to untreated controls.
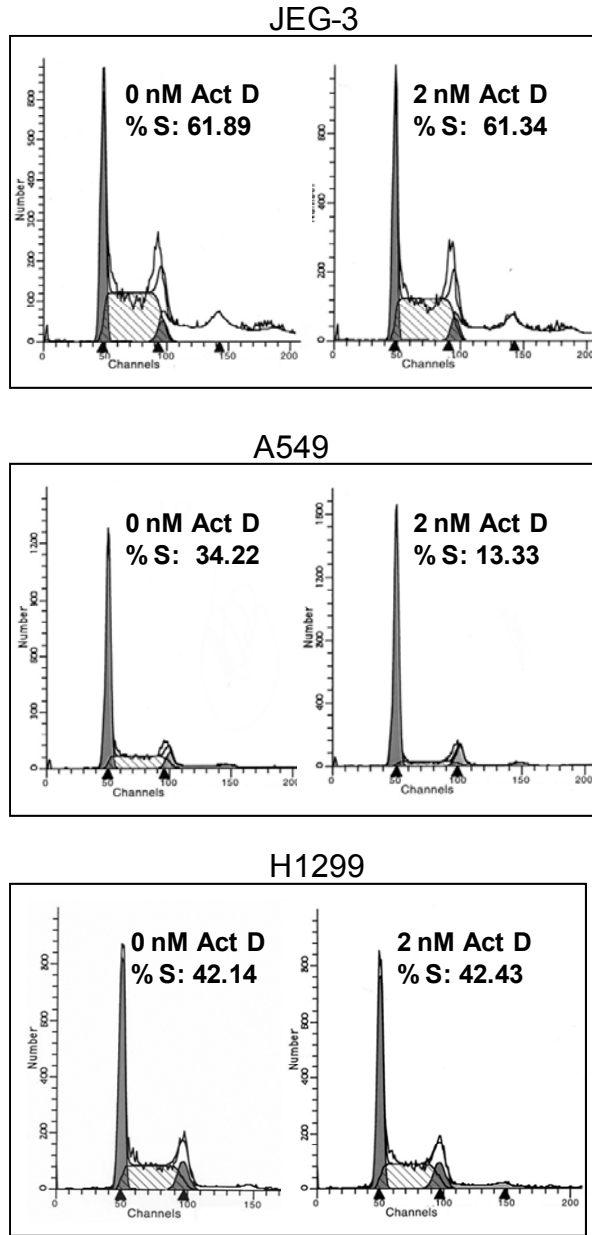
JEG-3

A549

H1299

Figure 26. Representative FACS histograms of cell cycle profile following ActD treatment. FACS profile of cell lines expressing high (JEG-3), and low (A549) levels of MDMX. H1299 cells do not have p53 and are used as a negative control.

Modulation of MDMX expression affects p53 activation by ribosomal stress

To further confirm that MDMX overexpression at a physiological level inhibits p53

activation and cell cycle arrest after ribosomal stress, HCT116 cells were infected with

MDMX cDNA lentivirus and siRNA retrovirus. Polyclonal cell lines expressing MDMX,

scrambled siRNA, or MDMX siRNA were analyzed. MDMX lentivirus provided ~5-fold

increase in MDMX levels in the HCT116 cell line. To show that this is a physiological

level of MDMX expression we compared it to the levels of MDMX in MCF-7 cells

(Figure 27).
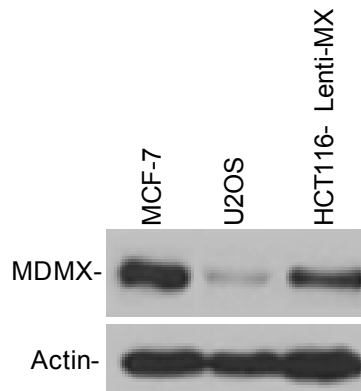
Figure 27. MDMX is expressed to physiological levels in HCT116-LentiMX cells.
Expression level of MDMX by lentivirus-mediated stable transduction
compared to endogenous levels in MCF-7 and U2OS. Identical amounts of
total protein were loaded in each lane.

Next we tested p53 activation status in each cell line following treatment with

ActD. MDMX overexpression reduced the sensitivity, whereas MDMX knockdown

sensitized cells to ActD induction of p21 (Figure 28a). Furthermore, ActD did not induce

p21 in HCT116-p53-/- cells, and MDMX overexpression or knockdown had no effect on

p21 expression (Figure 28b). Manipulation of MDMX level did not affect p53

stabilization by ActD. These results showed that MDMX overexpression blocked p53

activation, whereas MDMX knockdown increased sensitivity to ribosomal stress.

Next, the effect of MDMX on cell cycle arrest was analyzed. Treatment with 1-2

nM ActD for 18 hours caused significant reduction of S phase population in FACS

analysis. HCT116 cells with MDMX overexpression were efficiently protected from cell

cycle arrest by ActD, and knockdown of MDMX caused more efficient arrest (Figure

29).
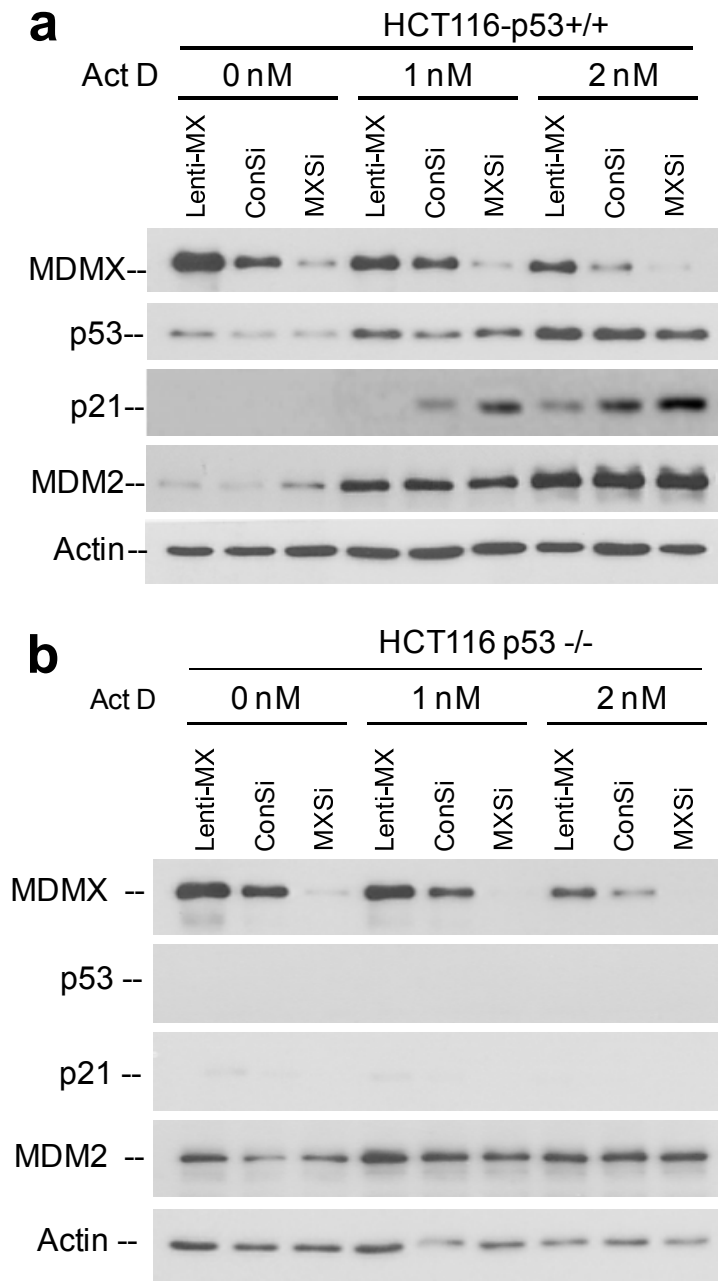
Figure 28. MDMX overexpression correlates with actinomycin D resistance in HCT p53 wild-type cells. (a) HCT116 p53-wildtype or (b) p53-null cells were infected with MDMX lentivirus, scrambled siRNA, and MDMX siRNA retrovirus. Pooled colonies were treated with either 1 or 2 nM of ActD for 18 hours and analyzed by western blot.
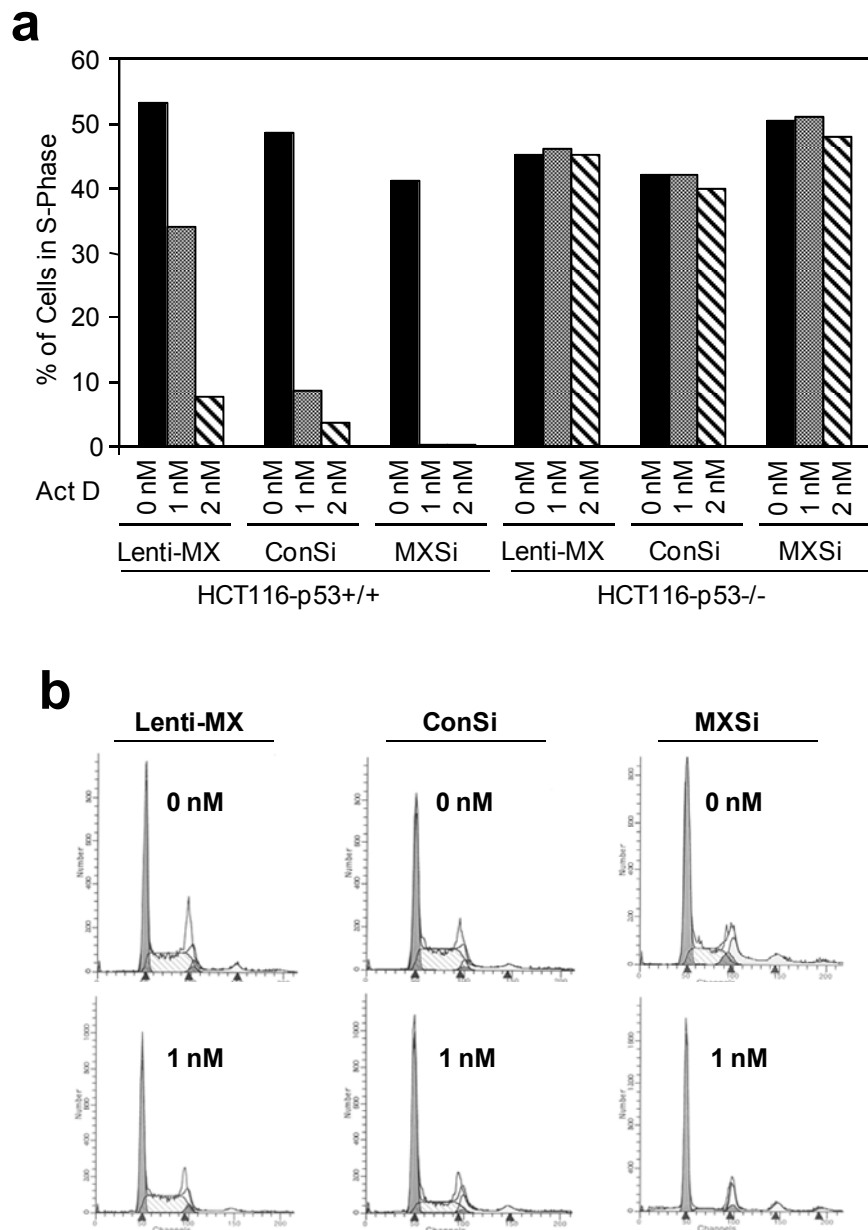
Figure 29. MDMX prevents cell cycle arrest following ActD treatment. (a) HCT116 cell lines expressing different levels of MDMX were treated with ActD for 18 hours and analyzed for cell cycle distribution by FACS. The percent of cells in S phase population is shown. (b) FACS histograms from data summarized in (a) showing cell cycle profiles from HCT116-p53+/+ cells before and after actinomycin D treatment.

We also used U2OS cells to knockdown or overexpress MDMX and examine p53 activation. Similar to HCT116 cells, MDMX overexpression reduced the sensitivity to ActD, whereas MDMX knockdown sensitized cells to ActD induction as measured by p53 target genes p21 and MDM2 (Figure 30). These results demonstrated that MDMX expression level has significant impact on p53 response to ribosomal stress. To confirm these results, we generate a stable pool of MCF-7 cells with MDMX knockdown. After treating with ActD, MCF-7 cells with a reduced dosage of MDMX were able to more readily undergo cell cycle arrest (Figure 31).
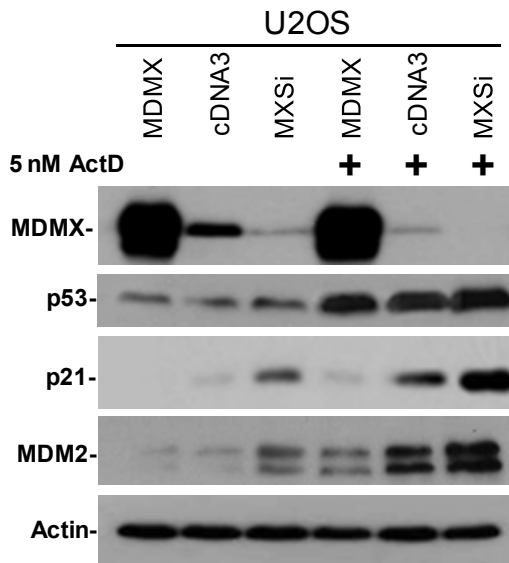
Figure 30. MDMX overexpression in U2OS cells prevents p53 activation. U2OS cells stably transfected with MDMX cDNA or siRNA plasmids were treated with 5 nM ActD for 16 hours and analyzed by western blot.
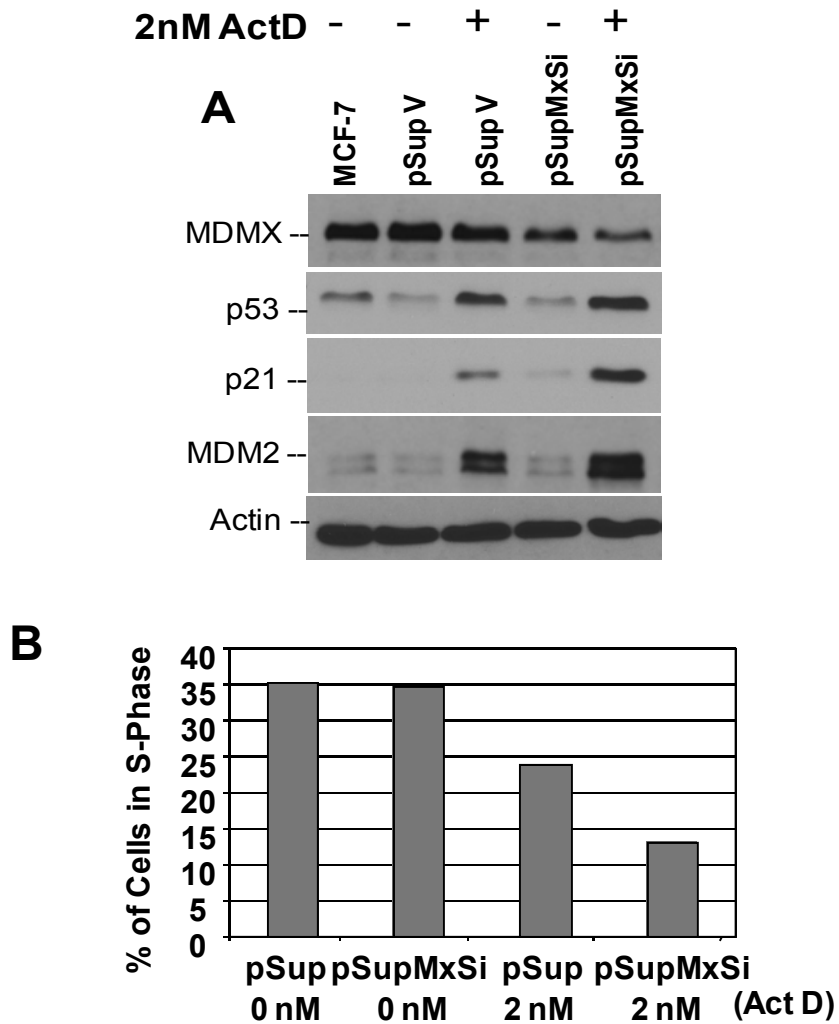
Figure 31. MDMX knockdown in MCF-7 cells enhances p53 activation. MCF-7 cells
stably infected with an MDMX siRNA virus were treated with 2 nM ActD for
16 hours and analyzed by (a) Western Blot and (b) FACS analysis

MDMX overexpression sustains cell proliferation after ribosomal stress

Cells contain a stockpile of ribosomes that can sustain normal protein synthesis for

at least 24 hours after inhibition of rRNA processing (Pestov, Strezoska et al. 2001).

Therefore, overcoming p53-mediated arrest should permit cell proliferation until

depletion of the ribosomes. To determine the maximum potential of MDMX in maintaining cell proliferation during ribosomal stress, we generated a U2OS cell line expressing MDMX at ~30-fold above endogenous level (Figure 30). BrdU labeling after 18 hours of ActD treatment showed that cells overexpressing MDMX continued to synthesize DNA (Figure 32). Conversely, MDMX knockdown caused more efficient shutdown of DNA synthesis even when treated with the lowest concentration (1 nM) of ActD.
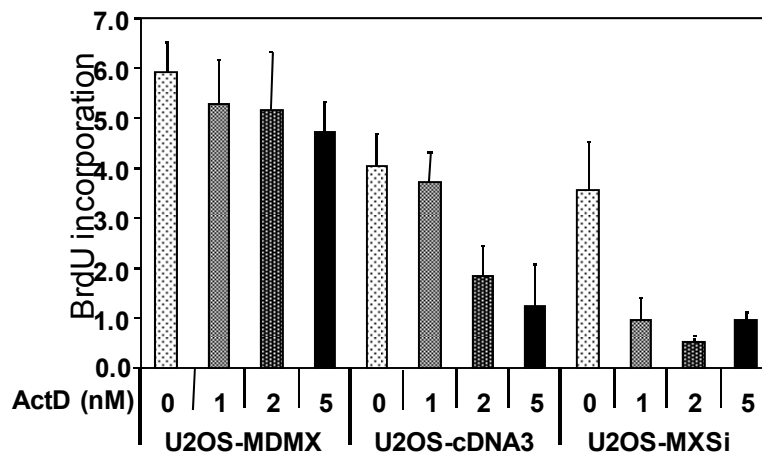
Figure 32. MDMX overexpression prevents cell cycle arrest. U2OS expressing different levels of MDMX were treated with ActD for 18 hours and analyzed for DNA synthesis by BrdU incorporation.

Next, we tested the effect of MDMX overexpression on cell proliferation during ribosomal stress. Starting at ~10% confluence, cells cultured in the continuous presence of ActD were analyzed by MTT assay over 4 days. After 1 day of treatment with ActD, U2OS cells expressing MDMX siRNA stopped proliferating, indicating activation of cell cycle checkpoints. Conversely, U2OS cells overexpressing MDMX continued to

proliferate at a significant rate, ultimately reaching confluency in the presence of ActD (Figure 33).
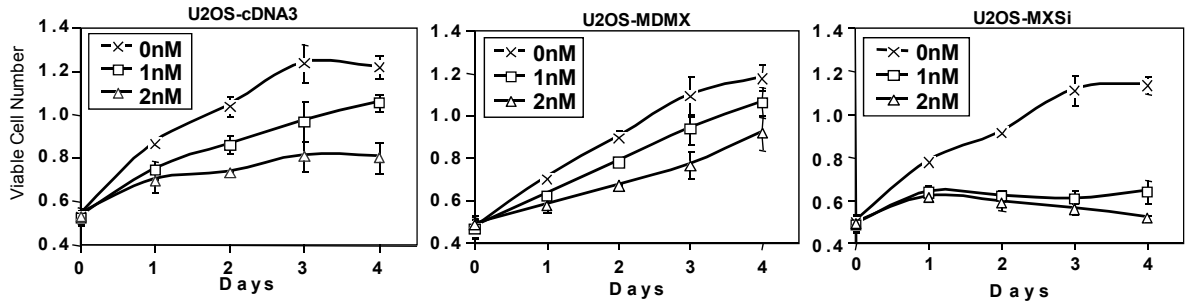
Figure 33. MDMX overexpression prevents growth arrest. Growth curve of U2OS cell lines in the presence of actinomycin D. Cells were plated at 10% confluency and cultured in the continuous presence of 1-2 nM ActD for 4 days. Cell proliferation was measured by MTT assay.

As expected, cell proliferation sustained by MDMX overexpression would eventually reach a limit as ribosomes were depleted. When cells were given unlimited space to proliferate by plating at a low density, MDMX-overexpressing cells were only able to give rise to micro colonies before cell proliferation stopped completely in 2 nM ActD (Figure 34). However, the growth arrest was reversible, as removal of ActD after 7 days of treatment allowed MDMX overexpressing cells to form large colonies (Figure 35). MDMX siRNA significantly reduced long-term viability after ActD treatment (data not shown). These results suggested that MDMX overexpression abrogated p53-mediated growth arrest and allowed cells to proliferate through multiple cycles after inhibition of ribosome biogenesis.

U2OS-MDMX   **U2OS-cDNA3**   **U2OS-MXSi**
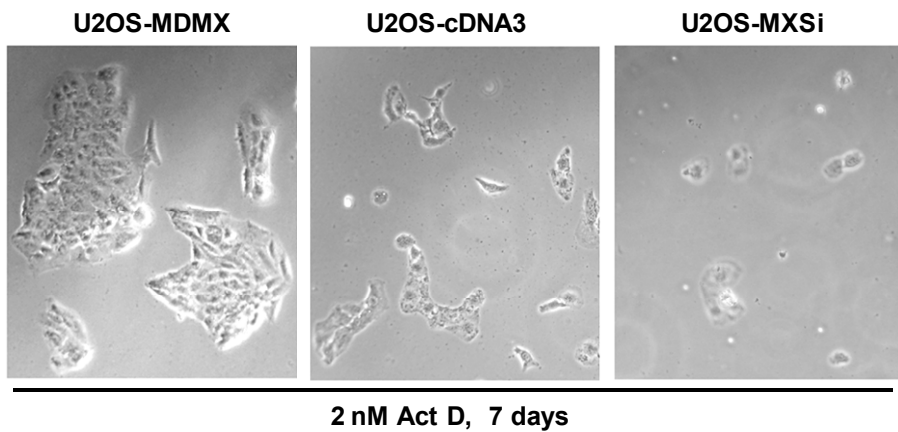
2 nM Act D, 7 days

Figure 34. MDMX overexpression allows cells to form microcolonies in the presence of ActD. Colony size of U2OS cell lines after continuous 2 nM actinomycin D treatment for 7 days.

U2OS

<u>+MDMX</u>   <u>Control</u>   <u>MXsiRNA</u>
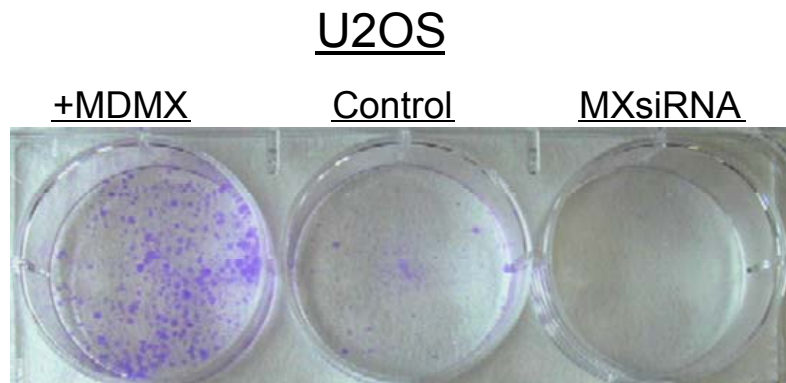
Figure 35. Cells overexpressing MDMX can recover after removal of ActD. U2OS cell lines after continuous 2 nM actinomycin D treatment for 7 days and then refed without drugs for 4 more days.

To test the growth advantage from having moderate MDMX overexpression,

HCT116 and HCT116-Lenti-MDMX cells were mixed at 20:1 ratio. Cells were treated

with 3 nM ActD for 4 days followed by normal medium for 4 days. After the treatment

cycle was repeated for a total of 30 days, the ActD-resistant colonies were pooled and

MDMX expression was determined. The results showed that the surviving cells were

predominantly HCT116-Lenti-MDMX cells (Figure 36). This suggests that cells

overexpressing MDMX have a clear survival advantage under conditions of ribosomal

stress.
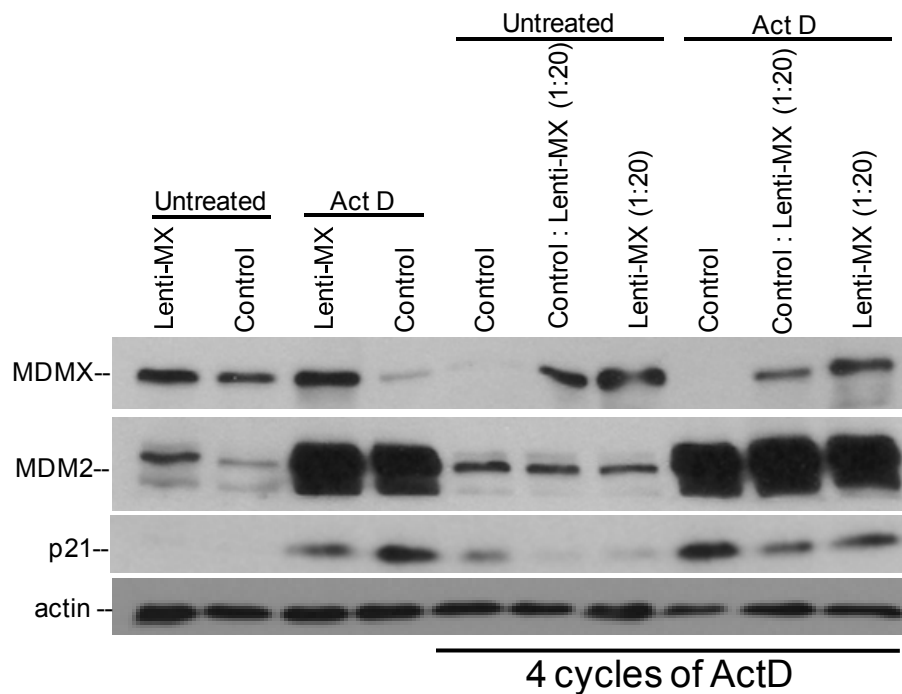
Figure 36. HCT 116+/+ Lenti-MX have a growth advantage when cycled with treatments of of ActD. 10,000 HCT116 positive cells and 200 HCT116-Lenti-MDMX cells were mixed and maintained on and off in ActD for 30 days then analyzed for their ability to respond to ActD.

MDMX sequesters p53 into inactive complexes

Since MDMX does not significantly affect p53 stability, we investigated the mechanism by which p53 is inactivated by MDMX overexpression. The fractions of free p53 and p53-MDMX complex were analyzed by MDMX immuno-depletion followed by p53 IP. The results showed that overexpression of MDMX in U2OS sequestered the majority of p53 into MDMX-p53 complexes. After treatment with ActD or 5-FU, the majority of p53 remained bound to MDMX. In contrast, DNA damage by CPT released ~50% of p53 into a free form (Figure 37). This assay also revealed that >50% of p53 in MCF-7 can be co-precipitated with endogenous MDMX after ActD treatment (Figure 38), confirming that physiological MDMX overexpression is sufficient to quantitatively sequester p53.
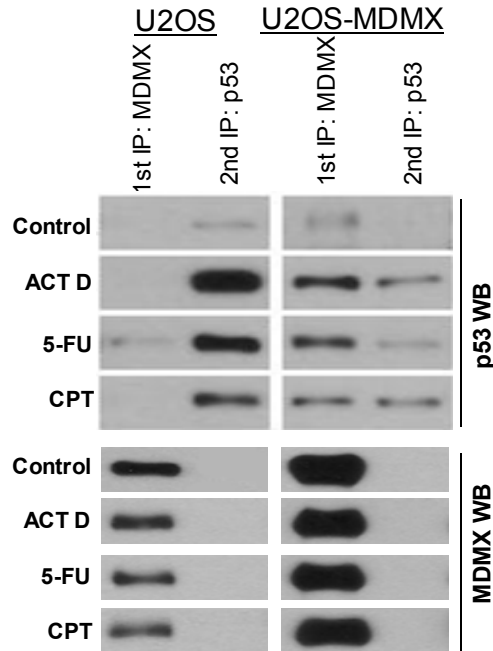
Figure 37. MDMX sequesters p53 into MDMX-p53 complexes. Lysate of U2OS-MDMX, U2OS or U2OS-MxSi cells treated with the indicated drugs for 16 hours were immuno-depleted with MDMX antibody to detect MDMX-p53 complex, followed by IP with p53 antibody to detect free p53. The precipitates were analyzed by p53 western blot.
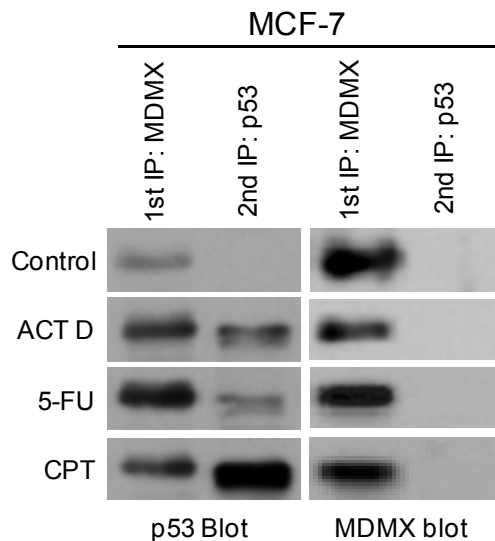
Figure 38. Endogenous MDMX sequesters p53 into MDMX-p53 complexes. MCF-7 cells treated with the indicated drugs for 16 hours followed by immuno-depletion with MDMX antibody to detect the MDMX-p53 complex, followed by IP with p53 antibody to detect free p53.

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To further test whether MDMX interferes with p53 binding to DNA, U2OS cells expressing different levels of MDMX were analyzed by ChIP assay using p53 antibodies and PCR primers for MDM2 and p21 promoters. The results of p53 ChIP showed that MDMX overexpression reduced p53 DNA binding to both MDM2 and p21 target promoters after ActD treatment compared to control cells, whereas MDMX knockdown increased p53 DNA binding in both untreated and ActD-treated cells (Figure 39). These results suggested that MDMX inhibits the DNA binding activity of p53. However, the difference in p53 binding to the p21 promoter appeared insufficient to account for the large difference in p21 expression level (Figure 30). This suggests that MDMX may also function by blocking p53 interaction with basal transcription factors at the promoter. We currently cannot confirm or rule out the presence of MDMX-p53 complex on DNA because ChIP assay using MDMX antibodies was inconclusive.
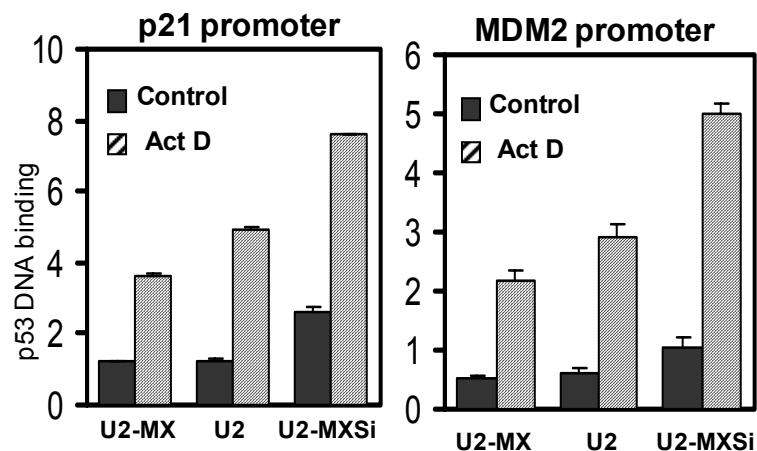
Figure 39. MDMX prevents p53 binding to target promoters. U2OS cells expressing different levels of MDMX were treated with 5 nM ActD for 16 hours and analyzed by ChIP to detect p53 binding to the MDM2 and p21 promoters.

MDMX prevents p53 activation by serum starvation and contact inhibition

To test the role of MDMX in p53 response to other types of ribosomal stress, we expressed MDMX in primary human foreskin fibroblasts (HFF) using lentivirus vector. Infection of HFF with MDMX lentivirus increased expression to a level similar to that of U2OS (data not shown). Therefore, this represents a physiologically achievable level of MDMX up-regulation. Normal human fibroblasts undergo p53 activation and G1 arrest during serum starvation or contact inhibition. A recent study showed that inhibition of rRNA expression and release of L11 was responsible for p53 activation during serum starvation (Bhat, Itahana et al. 2004). Other studies have shown that contact inhibition of normal fibroblasts causes a decrease in rRNA synthesis by inhibiting the recruitment of UBF to the rDNA promoter (Hannan, Hannan et al. 2000; Hannan, Kennedy et al. 2000).

HFF and HFF-Lenti-MDMX were compared for p53 activation after culturing in 0.5% serum for 18 hours (serum starvation), maintained at 100% density for 3 days (contact inhibition), or treated with 2 nM ActD for 18 hours. Western blot showed that all three treatments resulted in an increase in p53 and p21 levels in control HFFs. However, p21 induction was significantly weaker in HFF-lenti-MDMX cells (Figure 40), indicating ineffective p53 activation. Cell cycle analysis by FACS shows that HFF-Lenti-MDMX cells were desensitized to all three growth inhibitory conditions resulting in inefficient cell cycle arrest (Figure 41).
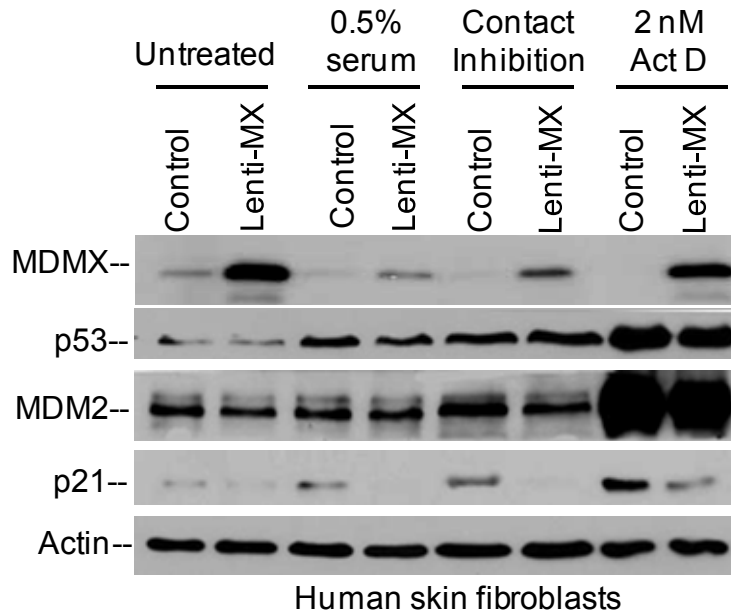
Figure 40. Effects of MDMX overexpression on p53 activation in normal human fibroblasts. Control HFF or Lenti-MDMX infected HFF were cultured in 0.5% serum for 24 hours, contact inhibited for 3 days, or treated with 2 nM of actinomycin D for 18 hours and analyzed by western blot.
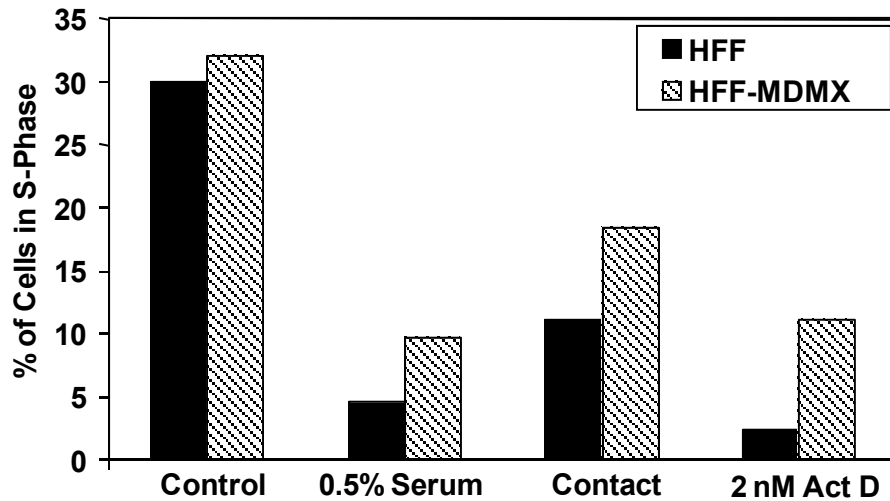
Figure 41. Effects of MDMX overexpression on cell cycle arrest in normal human fibroblasts. The fraction of cells in S phase measured by FACS analysis of serum starved, contact inhibited and actinomycin D treated HFF and HFF-lenti-MDMX cells.

Furthermore, an MTT assay was utilized to quantify the cell growth of HFF or HFF-Lenti-MX cells serum starved over a three-day period. This experiment revealed that MDMX promotes cell proliferation in normal cells under growth inhibitory conditions. Interestingly, under non-stress conditions MDMX overexpression provides minimal growth advantage. These results demonstrated that a tumor-equivalent level of MDMX overexpression in normal cells was sufficient to interfere with p53 response to abnormal ribosomal biogenesis.
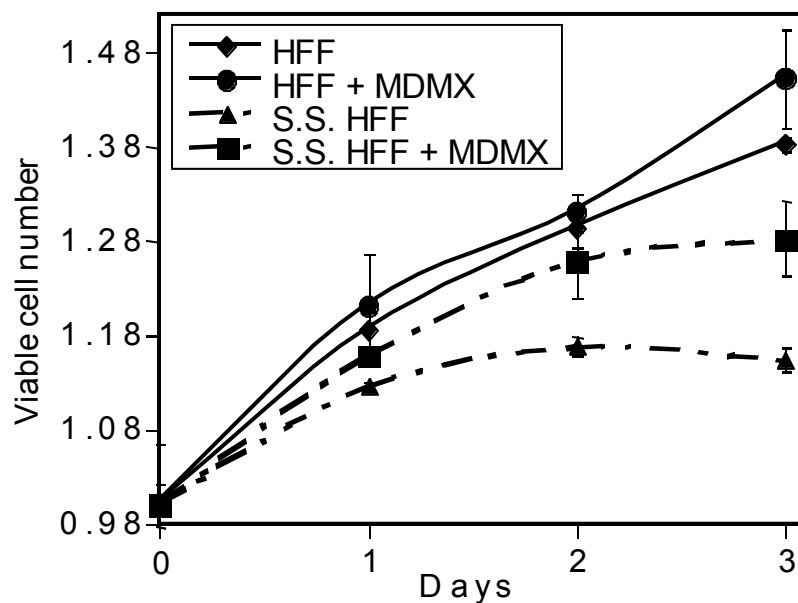
Figure 42. MDMX overexpression promotes cell proliferation during serum starvation. Growth of HFF and HFF-lenti-MDMX in 0.5% serum for 3 days. Cell number was quantified by MTT assay.

MDMX overexpression confers resistance to 5-fluorouracil

　　To investigate the relevance of MDMX overexpression in cancer chemotherapy, we tested its effect on sensitivity to 5-fluorouracil (5-FU). Inhibition of thymidylate synthase and DNA metabolism was thought to be responsible for the cytotoxicity of 5-FU (Parker and Cheng 1990). However, recent studies suggested that inhibition of RNA metabolism is responsible for its pro-apoptotic activity (Ghoshal and Jacob 1994; Longley, Boyer et al. 2002). Cell death by 5-FU can be prevented by uridine but not thymidine (Pritchard, Watson et al. 1997). Numerous reports showed that 5-FU at 100-500 μM induce p53 phosphorylation at serine 15, possibly through DNA damage and ATM activation. However, it has also been suggested that lower concentrations of 5-FU (10-100 μM) activates p53 through mechanisms independent of DNA damage or ATM activation

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(Longley, Boyer et al. 2002; Kurz and Lees-Miller 2004). We hypothesized that 5-FU may activate p53 by inhibiting rRNA synthesis and inducing ribosomal stress.

Tests using unmodified tumor cell lines showed that high endogenous MDMX levels were associated with reduced p21 induction after p53 activation following 5-FU treatment (Figure 43). This pattern was similar to ActD, and different from the DNA-damaging drug camptothecin which was sufficiently able to induce p21 in a variety of cell lines. 5-FU also induced proteasome-dependent degradation of MDMX which is partially rescued by treating with the proteosome inhibitor MG132 (data not shown).
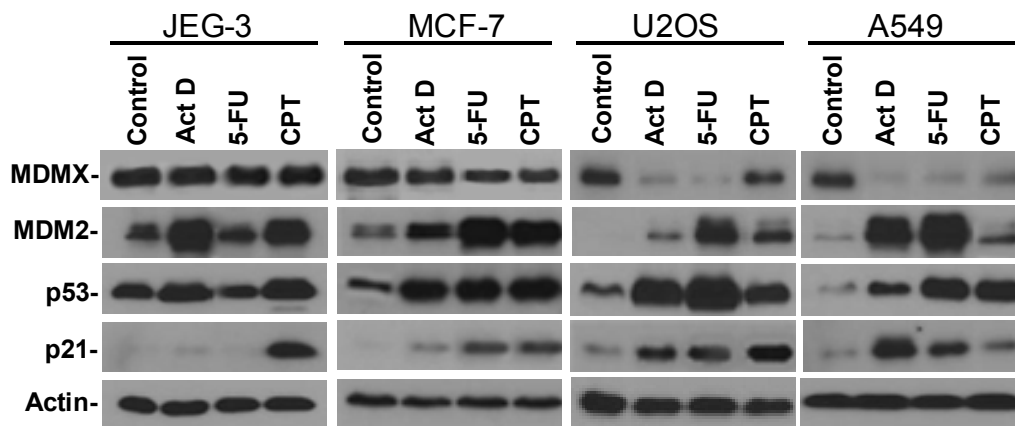
Figure 43. MDMX expression in tumor cell lines correlates with response to 5-FU not CPT Tumor cell lines were treated with 5 nM ActD, 50 µM 5-FU, and 0.5 µM CPT for 18 hours and analyzed by western blot.

Using a U2OS cell line expressing tetracycline-inducible Lenti-MDMX, we found that expression of MDMX 5-fold above endogenous levels resulted in significant inhibition of p21 induction by 5-FU and ActD, but had less of an effect on response to several DNA damaging agents (Figure 44). MDMX overexpression also sustained DNA replication in the presence of 5-FU, while MDMX knockdown increased sensitivity (data

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not shown). Compared to DNA damaging agents, 50 µM 5-FU induced very little p53

serine 15 phosphorylation, gamma H2A.X phosphorylation, and MDMX S367

phosphorylation (Figure 44, Figures 12-13), confirming the absence of significant DNA

damage.
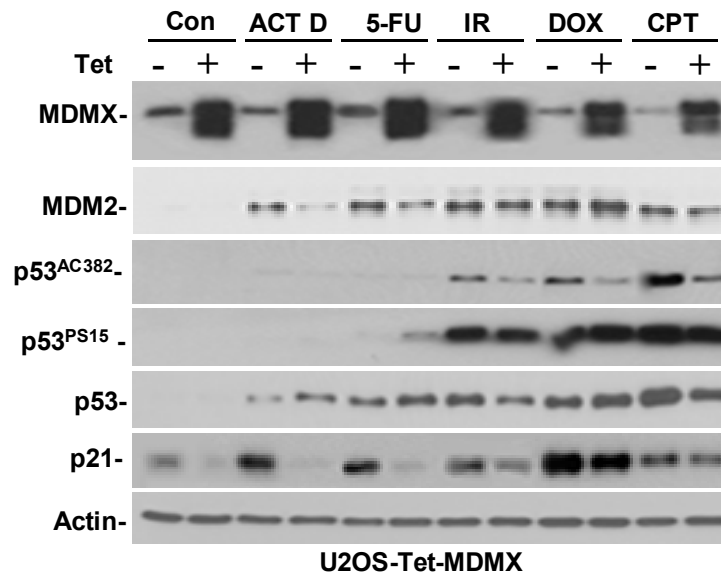
Figure 44. MDMX overexpression affects p53 response to ribosomal stress more than DNA damage. U2OS expressing tetracycline-inducible MDMX was treated with 1.0 µg/ml tetracycline and 5 nM ActD, 50 µM 5-FU, 1 µM doxorubicin or 0.5 µM CPT for 18 hours, or 10 Gy IR for 4 hours and analyzed by western blot.

The effects of MDMX overexpression on 5-FU and ActD responses suggested that

low concentrations of 5-FU mainly act by inducing ribosomal stress. To confirm that 5-

FU activates p53 by inhibiting RNA metabolism, HCT116 cells were treated with 5-FU

in the presence of uridine, which bypassed inhibition of uridine synthesis by 5-FU

(Longley, Harkin et al. 2003). Addition of uridine but not thymidine prevented p53

stabilization and p21 induction by 5-FU in a dose-dependent fashion (Figure 45),

suggesting that inhibition of RNA metabolism and ribosomal biogenesis was responsible

for p53 activation. Treatment with 5-FU also increased the amount of endogenous

binding between MDM2 and L11 (Figure 46), and induced release of nucleolin from the

nucleolus similar to ActD (Figure 47), consistent with nucleolar stress. These results

suggested that low concentrations of 5-FU activate p53 by inducing ribosomal stress.
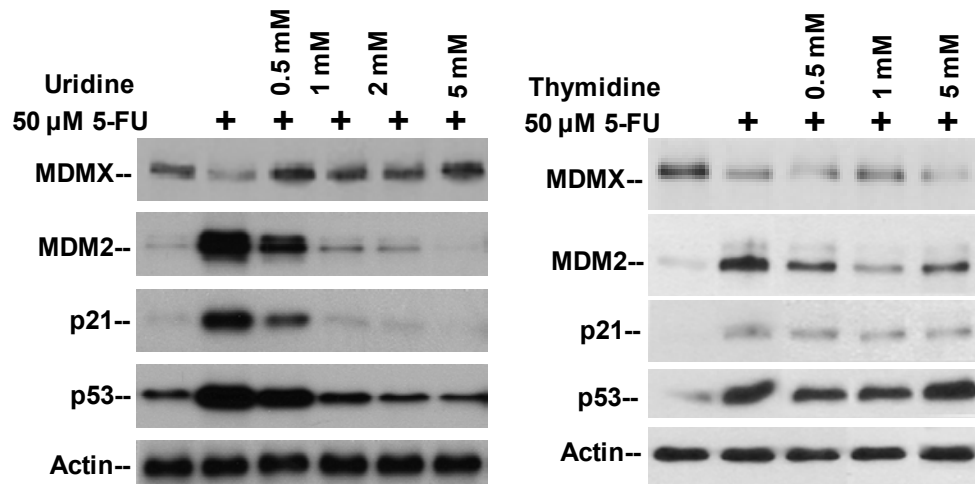
Figure 45. Uridine, but not Thymidine can reverse the actions of 5-FU. U2OS cells
were treated with 5-FU and uridine or thymidine for 8 hours and analyzed for
activation of p53.
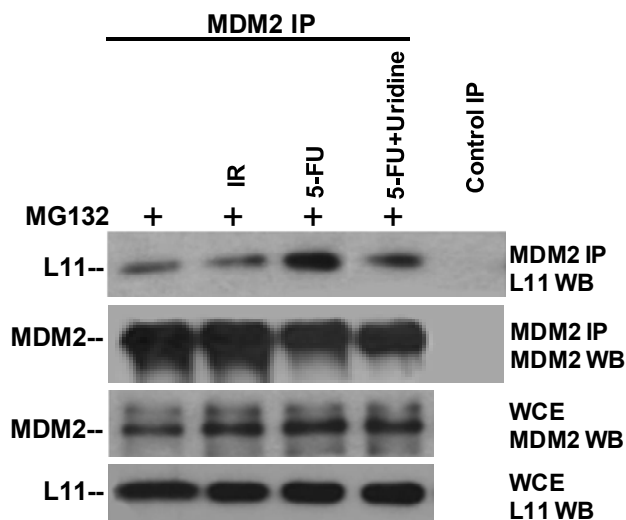
Figure 46. 5-FU enhances the binding between MDM2 and L11. U2OS cells were treated with 5-FU and uridine for 8 hours or irradiated with 10 Gy for 4 hours and analyzed for MDM2-L11 binding by MDM2 IP and L11 western blot. MG132 was added for 4 hours to obtain similar levels of MDM2.
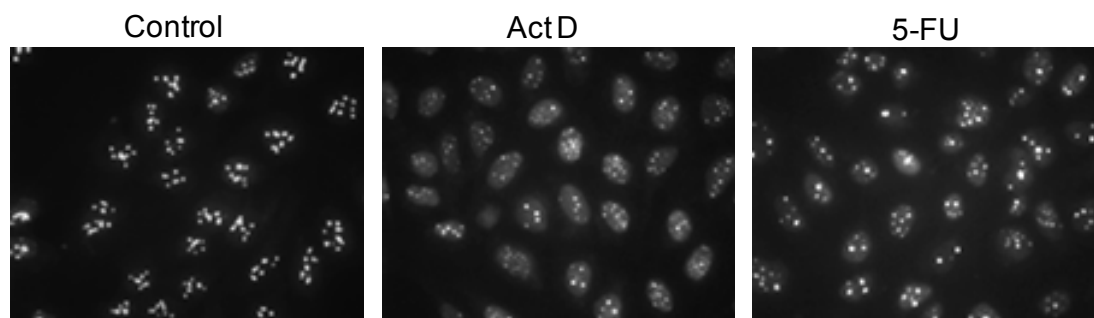
Figure 47. Release of nucleolin into nucleoplasm after 5-FU treatment. U2OS cells were treated with 2 nM actinomycin D and 100 µM 5-FU for 18 hours and stained using an antibody against the nucleolar protein nucleolin

5-FU is a major chemotherapy agent for colorectal cancer. When HCT116 cells

with overexpression and knockdown of MDMX were treated with 50 µM 5-FU, MDMX

was degraded and p21 expression was induced in a p53-dependent fashion. Similar to

ActD response, MDMX expression level showed an inverse correlation with p21

induction (Figure 48). As expected, HCT116 cells null for p53 still had reduced MDMX

levels following treatment but were unable to respond to treatment through activation of

p21 and MDM2.
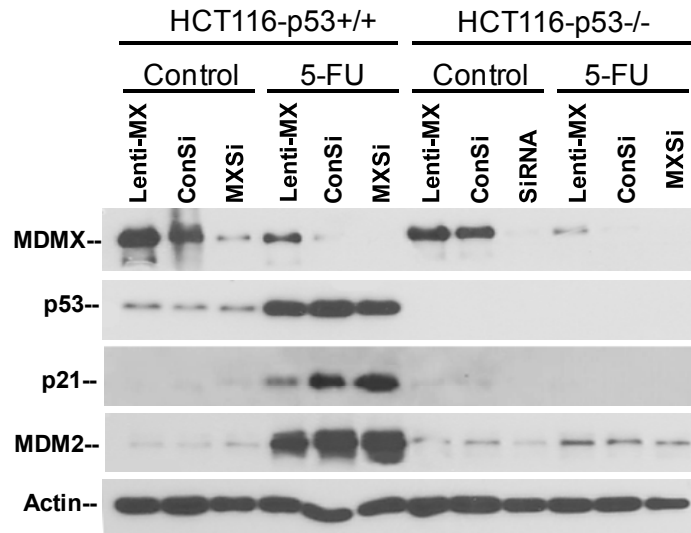
Figure 48. MDMX expression levels showed an inverse correlation with p21 induction following 5-FU treatment. HCT116 cell lines expressing different levels of MDMX were treated with 50 µM 5-FU for 18 hours and analyzed by western blot.

HCT116 cells undergo apoptosis after 5-FU treatment. Knockdown of MDMX

resulted in enhanced cell death, whereas MDMX overexpression blocked apoptosis in the

presence of 5-FU (Figure 49a). MDMX overexpression also increased resistance against

the DNA-damaging drug doxorubicin in short-term MTT assay (Figure 49b). However,

the impact of MDMX on 5-FU sensitivity was more significant, particularly at low drug

concentrations (compare Figure 49a and 49b). In colony formation assays, MDMX

overexpression improved long-term survival after treatment with 5-FU, but not

doxorubicin (Figure 50). These results suggested that MDMX is an important

determinant of sensitivity to 5-FU.
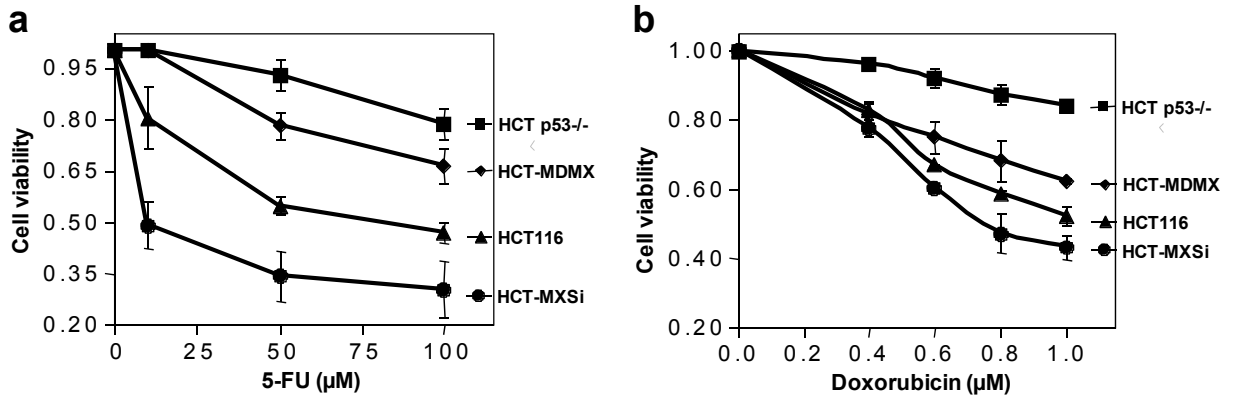
Figure 49. MDMX prevents apoptosis in HCT116 following 5-FU or Doxorubicin treatment. HCT116 cell lines were treated with (a) 5-FU or (b) doxorubicin for 48 hours and analyzed for cell viability by MTT assay.
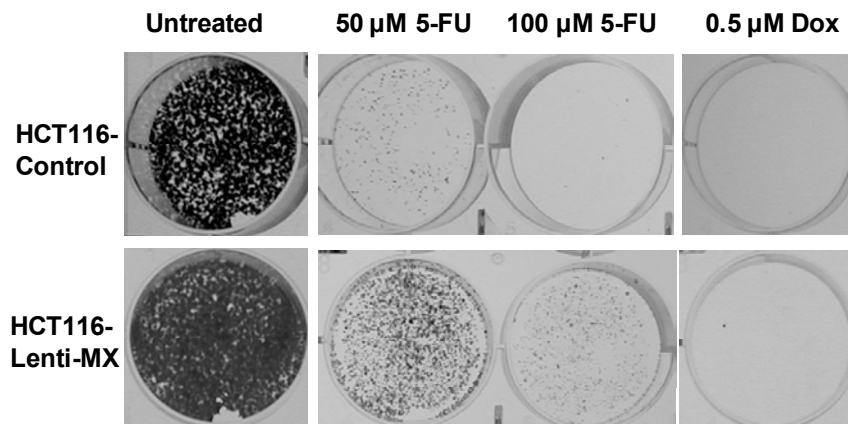
Figure 50. MDMX overexpression allows colony formation in the presence of 5-FU. Control and MDMX overexpressing HCT116 were plated at 5,000/well for 24 hours, treated with drugs for 24 hours, incubated in drug-free medium for 7 days, and stained for colony formation efficiency.

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MDMX regulates tumor formation and drug resistance in vivo

To test the role of MDMX in tumor formation *in vivo*, HCT116 cells expressing scrambled or MDMX siRNA (Figure 48) were inoculated subcutaneously on the dorsal flanks of athymic nude mice. Each animal received both control and test cell lines. The scrambled siRNA had no effect on tumor formation compared to the unmodified HCT116 cells (data not shown). In contrast, MDMX siRNA expressing cells showed significantly reduced tumorigenic potential (n=13, p=0.0005, Figure 51a, 51b). A second repeat of the experiment also generated similar results (not shown). Very few MDMX siRNA tumors were capable of reaching a dissectable size. However, dissectible tumors which form did form from the MDMX siRNA expressing cells were analyzed by Western blot. Interestingly, they showed an MDMX expression level similar to control HCT116 tumors (Figure 52). Since the MDMX siRNA cell line was a polyclonal pool of retrovirus infected colonies, it is likely that some of the cells regained normal MDMX expression and tumorigenic potential. These results demonstrated that partial knockdown of MDMX effectively blocked tumor formation *in vivo.* The results also suggested that the tumor environment caused unknown physiological stress that required suppression of p53 by MDMX.
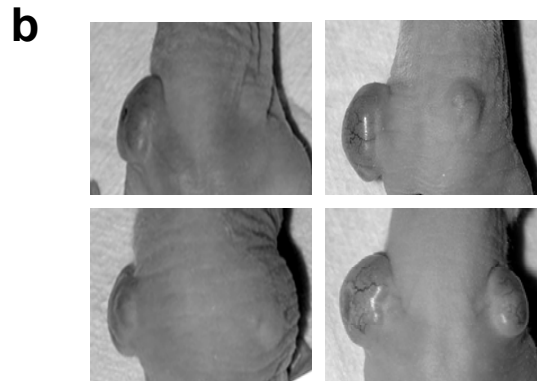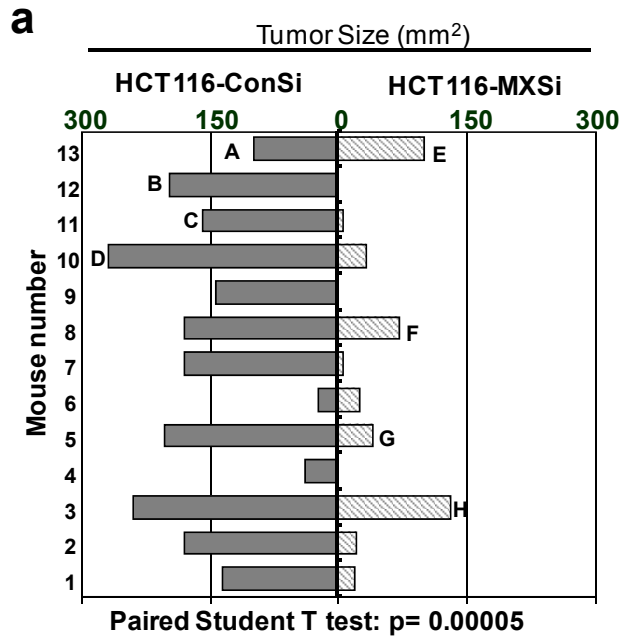
Figure 51. MDMX expression is required for tumor formation. (a) HCT116 cells expressing control and MDMX siRNA were inoculated into athymic nude mice (5×10^6/site). Tumor growth was measured after 14 days. Tumors marked with A-to-H were analyzed for MDMX expression. (b) Representative pictures of tumor bearing animals. Left side: HCT116-control siRNA. Right side: HCT116-MDMX siRNA.
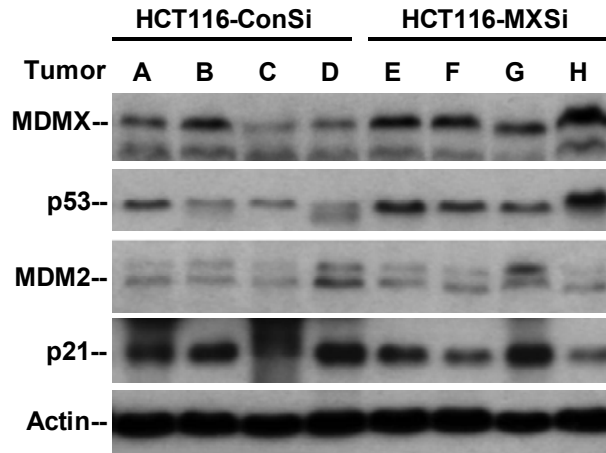
Figure 52. Tumors dissected 14 days after inoculation. Tumor samples recovered 14 days after inoculation were analyzed by western blot for MDMX and indicated markers.

To further test the effects of MDMX overexpression on tumor growth and treatment response in vivo, mice were inoculated with HCT116-vector and HCT116-Lenti-MDMX cells. The mice were treated with 5-FU by i.v. injection for four consecutive days when all tumors had reached an average of ~0.1 cm^3 in size. In untreated animals, HCT116-Lenti-MDMX cells did not show increased tumor growth compared to HCT116-vector control (Figure 53), suggesting that the level of endogenous MDMX was sufficient for growth in vivo. However, MDMX overexpression resulted in statistically significant tumor resistance (p-value = 0.01) to 5-FU treatment (Figure 53). These results further demonstrated that MDMX inhibits tumor response to RNA-targeting chemotherapy drugs in vivo.
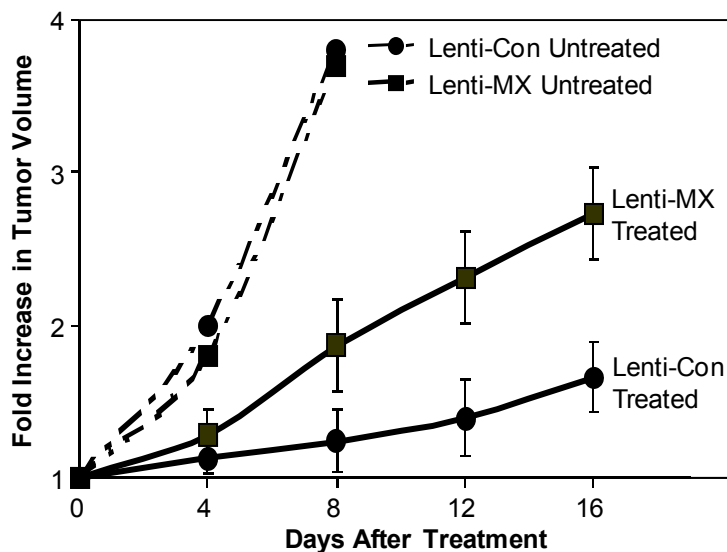
Figure 53. MDMX overexpression promotes tumor growth in the presence of 5-FU.
HCT116 cells stably infected with lentivirus vector or lenti-MDMX were inoculated into nude mice. Mice with ~0.1 cm^3 size tumors were treated with 5-FU at 50 mg/kg/day for 4 days and tumor growth were measured during the indicated time frame.

Discussion

Results described above show that MDMX is an important regulator of p53 activation by ribosomal stress. MDMX overexpression at physiologically relevant levels significantly desensitizes cells to ribosomal stress-inducing agents. In contrast, physiological level of MDM2 overexpression (from gene amplification) does not confer resistance to ActD. Our results also demonstrated that endogenous MDMX expression in HCT116 cells is necessary for tumor formation, suggesting that MDMX is a useful drug target.

Differences in structure and function of MDM2 and MDMX may be responsible for their distinct effects on ribosomal stress response. MDM2 is an ubiquitin ligase that functions mainly by promoting p53 degradation. This mechanism may be highly sensitive to inhibition by ribosomal proteins. Therefore, physiological levels of MDM2

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overexpression are effectively neutralized during ribosomal stress, resulting in p53 stabilization. In contrast, MDMX is a stable protein that regulates p53 mainly by sequestering p53 into complexes (Francoz, Froment et al. 2006; Toledo, Krummel et al. 2006). Because ribosomal stress does not induce p53 phosphorylation or block p53-MDMX binding, MDMX overexpression will trap p53 in inactive complexes and prevent p21 induction, sustaining cell proliferation. We should note that our results do not rule out p53-independent effects of MDMX on p21 expression, such as by targeting it for degradation.

The biological significance of ribosomal stress in regulating cell proliferation *in vivo* is still not clearly defined. The ability of MDMX to attenuate p53 activation and cell cycle arrest during growth factor deprivation and other ribosomal stress conditions may provide an advantage in a tumor environment. It is possible that different regions of a tumor undergo cycles of proliferation, growth arrest, and cell death due to imbalance in the supply of growth factors and nutrients. MDMX overexpression would suppress p53 activation by ribosomal stress, allowing additional rounds of cell division. The cumulative effect of such limited growth would be significant after repeated cycles of stress selection, as suggested by our mixing experiment.

MDMX overexpression may also interfere with p53 activation by other growth regulators. It has been shown that the retinoblastoma protein pRb inhibits RNA polymerase I-mediated transcription by binding to the UBF factor, thus inhibiting rRNA expression (Voit, Schafer et al. 1997). This function should lead to ribosomal stress and contribute to growth arrest by pRb during contact inhibition (Hannan, Kennedy et al. 2000). In addition, p53 itself has been shown to inhibit rRNA transcription (Budde and

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Grummt 1999), which would have a positive feedback effect through release of ribosomal proteins. Abnormal expression of MDMX may block p53 activation and weaken the effects of multiple tumor suppressor pathways.

The ability of MDMX to abrogate p53 activation by 5-FU may have significant clinical relevance. This drug is a mainstay compound in the chemotherapy of colon cancer. 5-FU cytotoxicity depends on conversion to 5-fluoroUTP, 5-fluoro-dUMP, and 5-fluoro-dUTP. Binding of 5-fluoro-dUMP to the enzyme thymidylate synthase inhibits the synthesis of thymidine nucleotides, giving rise to DNA strand breaks (Parker and Cheng 1990), this was believed to be the major mechanism of cytotoxicity. However, 5-FU also inhibits rRNA processing (Ghoshal and Jacob 1994). *In vitro* studies have shown that 5-FU incorporation into RNA but not DNA was associated with cell death (Geoffroy, Allegra et al. 1994). Incorporation into RNA is responsible for the gastrointestinal toxicity of 5-FU in mice (Houghton, Houghton et al. 1979). A study using p53-null mice showed that intestinal epithelial apoptosis induced by 5-FU is p53-dependent, and involves interference of RNA metabolism (Pritchard, Watson et al. 1997). Experiments using HCT116 cells also suggested a p53-dependent cytotoxicity of 5-FU through inhibition of RNA metabolism (Bunz, Hwang et al. 1999). Here we show that 5-FU activation of p53 is abrogated by uridine but not thymidine, and is highly sensitive to MDMX overexpression. These results suggest that induction of ribosomal stress and p53 activation is an important mechanism of 5-FU cytotoxicity, although DNA damage may also be a contributing factor at high drug doses.

In light of the findings described above, it will be important to investigate whether there is a correlation between MDMX expression level and tumor response to 5-FU or

other RNA-directed drugs in the clinic. MDMX overexpression has been observed in both tumor cell lines and primary tumor biopsies (Ramos, Stad et al. 2001; Danovi, Meulmeester et al. 2004). MDMX gene amplification does not appear to be the major mechanism of overexpression (~5% in breast tumors) (Danovi, Meulmeester et al. 2004). Analyses of MDMX promoter suggested that MDMX expression level in tumor cell lines correlates with promoter activity (unpublished observations). The drug sensitization and anti-tumor effects of MDMX siRNA suggest that targeting MDMX-p53 interaction with small molecules may have therapeutic value. To this end, it is noteworthy that the MDM2 inhibitor Nutlin 3 does not target MDMX-p53 binding (Vassilev 2004; Patton, Mayo et al. 2006), suggesting a need to develop novel MDMX inhibitors.

Chapter Four
Regulation of Mdmx Expression by Mitogenic Signaling

Abstract

MDMX is an important regulator of p53 transcriptional activity and stress response. MDMX overexpression and gene amplification are implicated in p53 inactivation and tumor development. Unlike MDM2, MDMX is not inducible by p53 and little is known about its regulation at the transcriptional level. We found that MDMX levels in tumor cell lines closely correlate with promoter activity and mRNA level. Activated K-Ras and growth factor IGF-1 induce MDMX expression at the transcriptional level through mechanisms that involve the MAPK kinase and c-Ets-1 transcription factors. Pharmacological inhibition of MEK results in down-regulation of MDMX in tumor cell lines. MDMX overexpression is detected in ~50% of human colon tumors and showed strong correlation with increased ERK phosphorylation. Therefore, MDMX expression is regulated by mitogenic signaling pathways. This mechanism may protect normal proliferating cells from p53 but also hamper p53 response during tumor development.

Results

MDMX level in tumor cell lines correlates with promoter activity

Recent studies demonstrated that MDMX expression is needed for the proliferation of tumor cell lines with wild type p53 in culture (Danovi, Meulmeester et al. 2004), and formation of tumor xenografts in nude mice (Gilkes, Chen et al. 2006). MDMX protein overexpression has been found in 40% of tumor cell lines (Ramos, Stad et al. 2001). MDMX mRNA overexpression has also been observed in 18.5% of breast, colon, and lung tumor samples as determined by in situ hybridization (Danovi, Meulmeester et al. 2004). However, MDMX gene amplification only occurs in 5% of breast tumors (Danovi, Meulmeester et al. 2004), suggesting that in most cases activated transcription is responsible for its overexpression. Therefore, we decided to investigate the pathways that regulate MDMX transcription.

A survey of a panel of cell lines that express high (JEG-3, MCF-7), moderate (U2OS, HCT116), and low (A549, SJSA, H1299) levels of MDMX revealed that MDMX protein level correlated with its mRNA levels but showed no correlation with MDM2 (Figure 54). The mRNA level was measured using Real-Time PCR with SYBR green chemistry and normalized to the expression of 18S ribosomal protein mRNA.
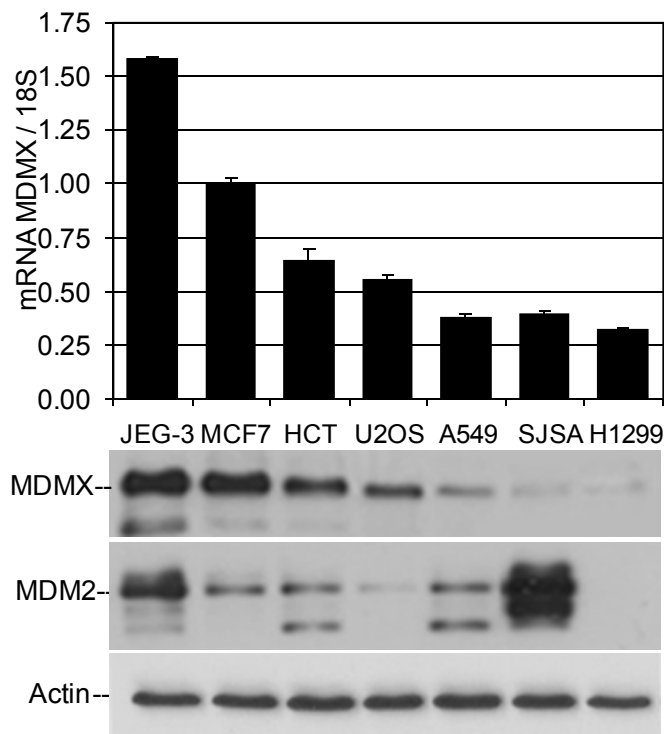
Figure 54. MDMX overexpression occurs at the transcriptional level. Total RNA and protein from tumor cell lines were analyzed by quantitative PCR and western blot. MDMX mRNA level was normalized to 18S rRNA (n=3).

To determine the stability of existing MDMX protein, we prevented cells from generating new MDMX protein by treating them with the protein synthesis inhibitor cyclohexamide. MDMX protein stability appeared much greater than MDM2 after treatment with cycloheximide (half life 4-8 hours), and is unrelated to cellular MDM2 levels (Figure 55). Furthermore, blocking protein degradation with the proteasome inhibitor MG132 for 4 hours dramatically increased the level of MDM2 but not MDMX (Figure 56), indicating that MDM2 but not MDMX undergoes rapid turn over in unstressed cells. Therefore, the rate of MDMX turn over is inherently slow in unstressed cells and its overexpression in a subset of cell lines is not due to higher stability. These

results suggest that mRNA expression is an important determinant of MDMX level in

unstressed cells.
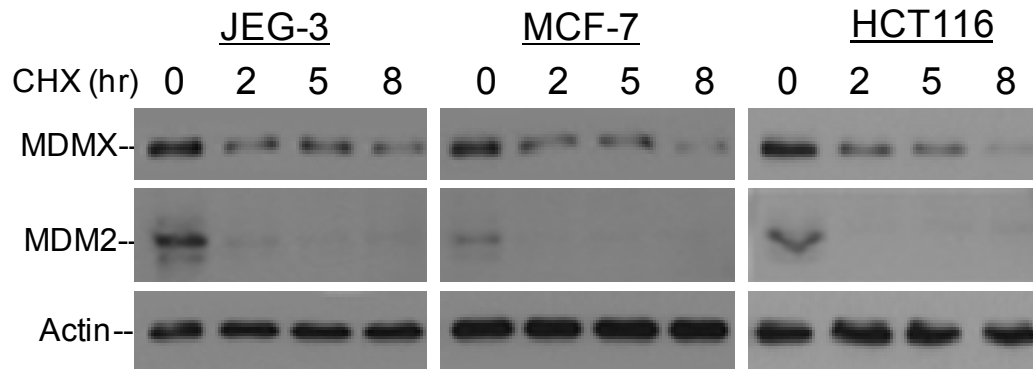
Figure 55. MDMX turnover is slower than MDM2. JEG-3, MCF-7, and HCT116 cells
were treated with cycloheximide (CHX; 50 µg/ml) for 0, 2, 5 or 8 hours and
analyzed by western blot.
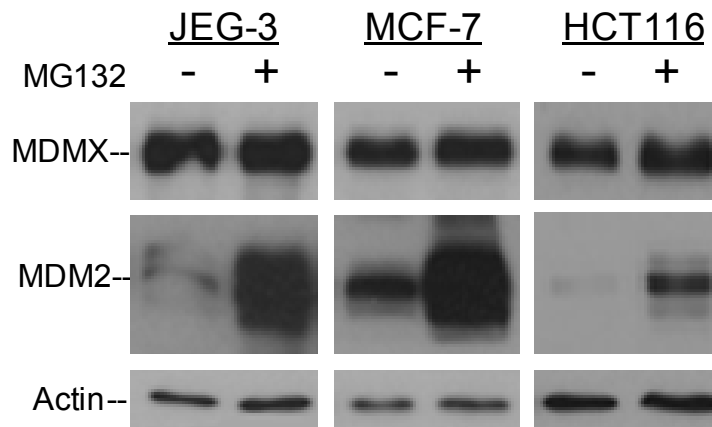
Figure 56. MDMX stability is greater than MDM2. JEG-3, MCF-7, and HCT116 cells
were treated with MG132 (25 µM) for 4 hours and analyzed by western blot.

To test whether the activity of the MDMX promoter is responsible for its

expression level, a 1.1 kb genomic DNA fragment upstream of the MDMX mRNA

coding region was cloned by PCR and inserted into the pGL2 promoter-less luciferase

reporter. Transient transfection of the MDMX promoter construct into different tumor

cell lines showed activity levels (normalized to cotransfected CMV and RSV promoters) that generally correlated with their endogenous MDMX protein and mRNA levels (Figure 57). For example, the promoter is highly active in cells overexpressing MDMX. Therefore, MDMX promoter activity is responsible for or contributes to the variations in protein levels in the tumor cell lines. It is noteworthy that MCF-7 cells in which the MDMX promoter is highly active also have abnormal MDMX gene copy number (5 copies instead of 2) (Danovi, Meulmeester et al. 2004), which may contribute to overexpression. Cotransfection of the MDMX promoter with several genes commonly involved in transformation (E2F1, c-myc, Stat3, Src, Akt,) did not show activation (data not shown), ruling out a direct role for these factors in MDMX overexpression.
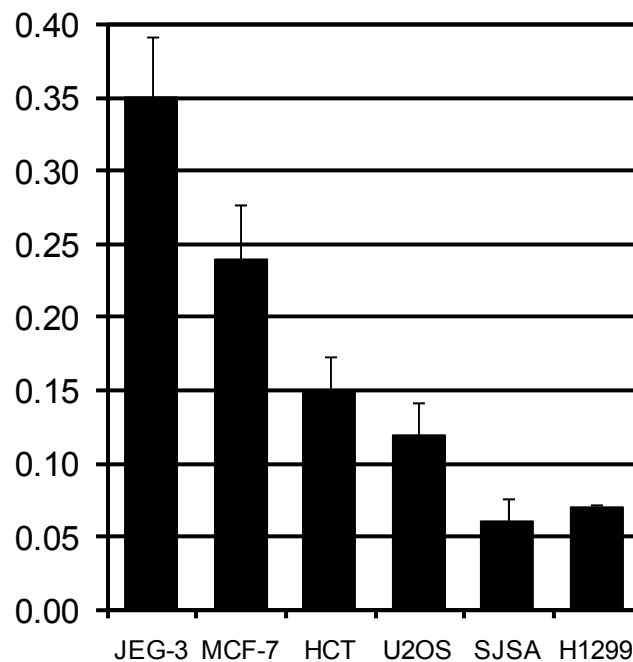
Figure 57. MDMX promoter analysis. Cell lines were transfected with the 1.1 kb MDMX promoter-luciferase construct and CMV-LacZ. The luciferase/lacZ activity ratio is shown (n=3).

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Identification of key transcription factor binding sites in the MDMX promoter

To identify MDMX promoter elements necessary for promoter activity in the cell lines expressing high levels of MDMX, a series of 5' deletion mutants were generated and transiently transfected into MCF-7 and H1299 cells (Figure 58). The full-length reporter fragment showed strong luciferase activity in MCF-7 cells, which have a high level of MDMX. Conversely, H1299 cells with low level MDMX expressed weak luciferase activity. Serial deletion from –990 bp to -120 bp (0 bp being the putative transcription start site based on promoter prediction analysis and the 5' end of two longest cDNA sequences in Genbank: NM_002393 and BC067299) showed little effect on promoter activity in MCF-7 and H1299 cells. However, deleting the region from -120 bp to 0 bp resulted in a greater than 90% loss of promoter activity in both cell lines. These results suggest that transcription factors binding between the -120 bp to 0 bp region are critical for regulating both basal and cell line-specific hyperactivation of the MDMX promoter.
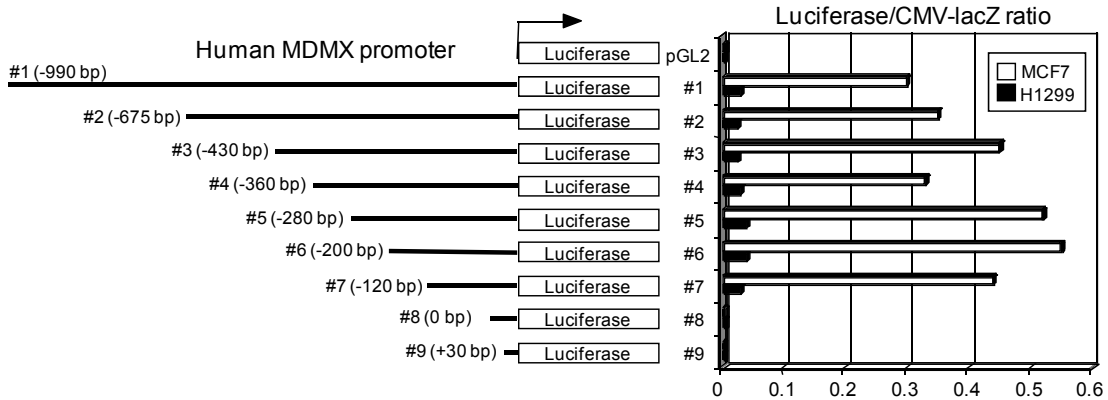
Figure 58. Luciferase expression of promoter deletion constructs in MCF-7 and H1299 cells. MCF-7 (high endogenous MDMX expression) and H1299 (low endogenous MDMX expression) cells were transfected with MDMX promoter deletion constructs and normalized by CMV-LacZ expression (n=3).

A database search for putative transcription factors which potentially bind to the -120 to 0 bp region revealed Aml-1, Cdxa, c-Ets-1, and Elk-1 consensus sequences. These sites are largely conserved in the putative mouse MDMX promoter (Figure 59), suggesting that they are important for regulation of MDMX expression. c-Ets-1 and Elk-1 are downstream targets of Ras, regulated via ERK-mediated phosphorylation (Gille, Kortenjann et al. 1995; Liu, Liang et al. 2005). To confirm the function of these sites, point mutations at each of the potential transcription factor binding sites were introduced into the 1.1 kb promoter construct as bolded in Figure 59.

```
                        -120
            Human  CCCCGGACTAGGATCTACTCTATGGTAGTG
            Mouse  CCCCGGACTAGTGTGTACTCAATGGTAGAT
                                            AML-1
                        -90
            Human  AGCTCAGCCTTTCTAGCTCTCTAGTCCTCC
            Mouse  TCT TCAGCCTTTCTAGCTCTCTAGTCCTCC
                                 CDXA
                        -60
            Human  CATTGCTCCATCTATGGTTTCCGGAGGCCT
            Mouse  _AG_GGTCCGTCTATGGTTTCCCCCGGCCT
                                      c-Ets-1 / Elk-1 (EE1)
                        -30
            Human  CACCGGAAGCCCTCGTGTGAGGCCGTGTGG
            Mouse  CCCCGGAAGCTCTTGCGA _ACGCTGTTTGA
                   c-Ets-1 / Elk-1 (EE2)
```

**Figure 59. Sequence (–120 to 0 bp) of the human and mouse MDMX basal
promoter.** The positions of putative transcription factor binding sites
underlined and mutated nucleotides are bolded.

The mutated promoter constructs were expressed in JEG-3, MCF-7, and H1299

cells along with the unmutated full length promoter and deletion mutants. Mutation of the

Cdxa or Aml-1 sites resulted in a 2-3-fold decrease in promoter activity, whereas c-Ets-

1/Elk-1 individual site mutants had 4-fold reduced activity in JEG-3 and MCF-7 cells

(Figure 60). Furthermore, 20 bp serial deletions of the –120 bp to 0 bp region caused a

step-wise decrease in promoter activity (data not shown), suggesting that multiple

transcription factor binding sites are necessary for MDMX promoter activity. Compound

mutations of the Aml-1 and c-Ets-1/Elk-1 sites resulted in greater than 90% reduction in

promoter activity, similar to the activity of the 0 bp construct or full-length reporter with

mutations in all four transcription factor binding sites (quad mutant) (Figure 60). The

activity of mutant luciferase reporter constructs also changed in a similar pattern in

H1299 cells but at a reduced magnitude (Figure 61), presumably because the same set of

factors are functioning at a reduced level.

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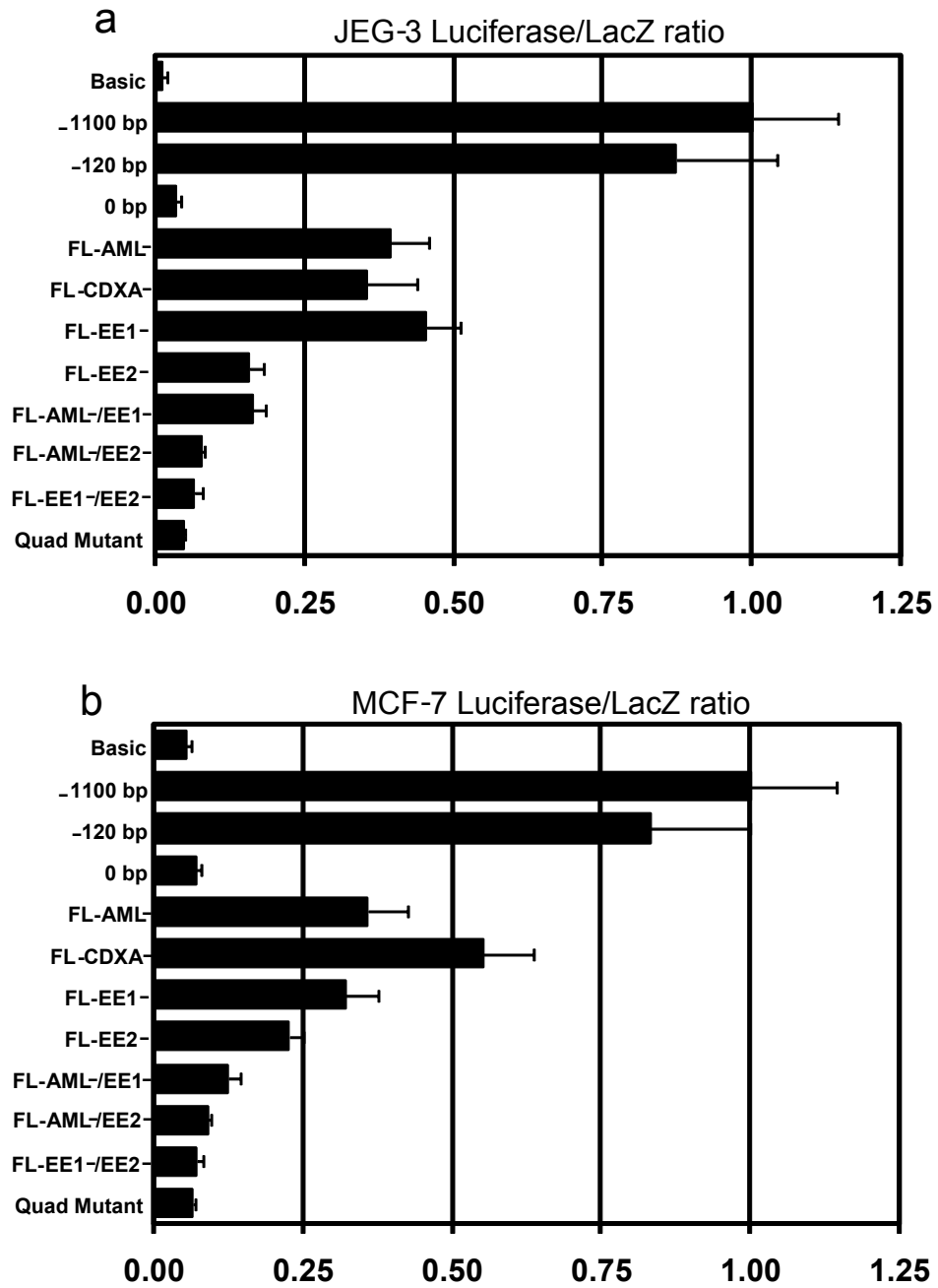
Figure 60. MDMX promoter mutation analysis. Full-length, deletion, as well as single, double, and quadruple point mutations in the full-length MDMX luciferase reporter construct were tested for activity in JEG-3 (a) and MCF-7 (b) cells (n=3).
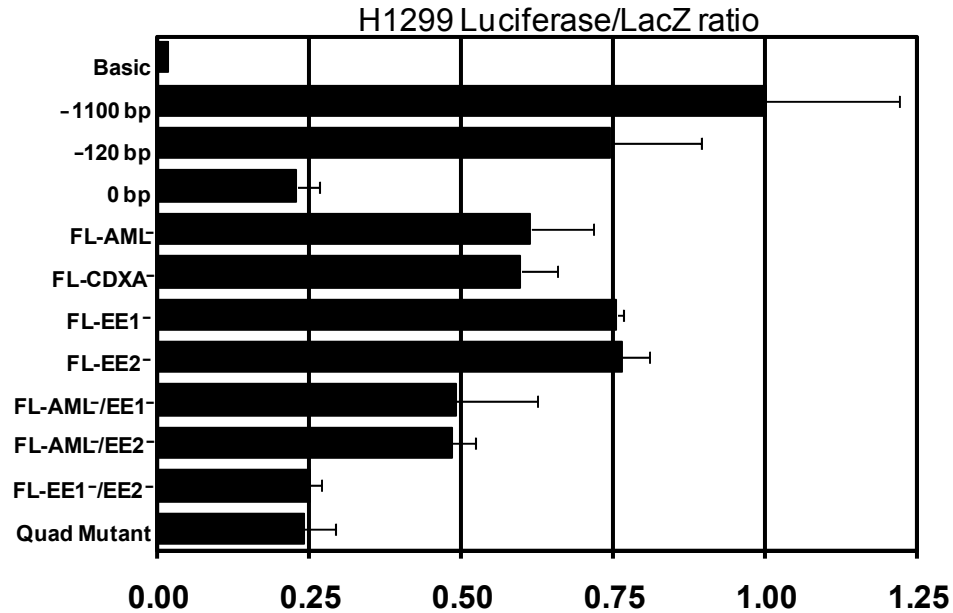
Figure 61. MDMX promoter mutation analysis in H1299 cells. Full-length, deletion, as well as single, double, and quadruple point mutations in the full-length MDMX luciferase reporter construct were tested for activity in H1299 cells (n=3).

Next we considered whether c-Ets-1 was sufficient to induce MDMX promoter activity in cells with endogenously low levels of MDMX. MDMX reporter constructs were cotransfected with a c-Ets-1 expression plasmid in H1299 cells (Figure 62). c-Ets-1 induced the 1.1 kb promoter activity but was not able to activate the c-Ets-1/Elk-1 mutant promoter construct. To test the roles of endogenous c-Ets-1 and Elk-1 in regulating MDMX expression and p53 activity, MCF7 cells were treated with siRNA. The results showed that transient knockdown of c-Ets-1 and Elk-1 expression reduced MDMX expression in MCF7 cells (Figure 63). This was associated with increased expression of p53 target genes p21 and MDM2 without changes in p53 level. Low concentration of actinomycin D induces MDMX degradation and p53 activation by causing ribosomal stress (Gilkes, Chen et al. 2006). Ets-1 and Elk-1 knockdown cooperated with

actinomycin D in further reducing MDMX level and increasing p53 activity similar to the

effect of MDMX knockdown (Figure 63). Therefore, c-Ets-1 and Elk-1 control MDMX

transcription and contribute to the suppression of p53 activity.
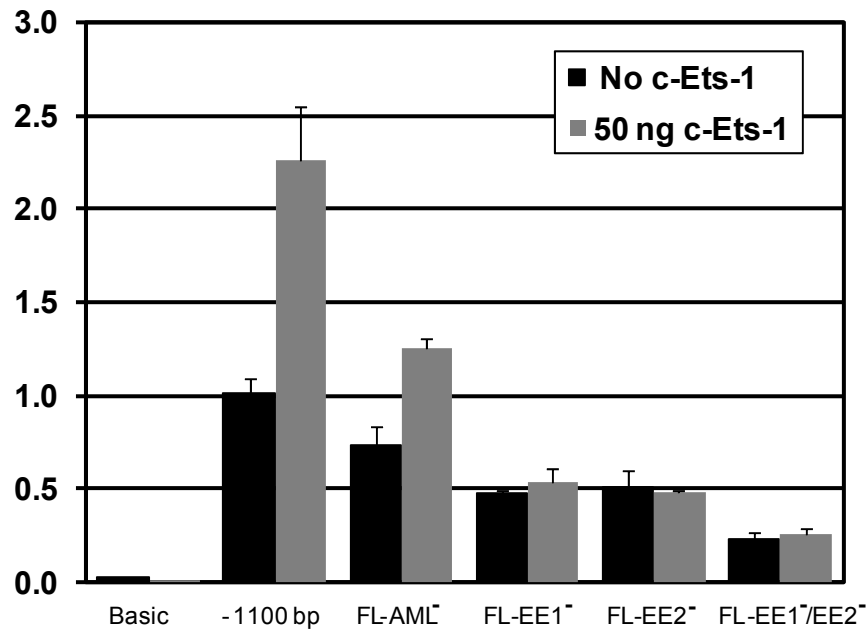
Figure 62. c-Ets-1 enhances MDMX basal promoter activity. H1299 cells were
transfected with the full-length MDMX promoter and 50 ng of c-Ets-1
plasmid to induce MDMX promoter activity. MDMX promoter mutants were
also transfected with 50 ng c-Ets-1 to determine the response of each binding
site mutant to c-Ets-1 expression.
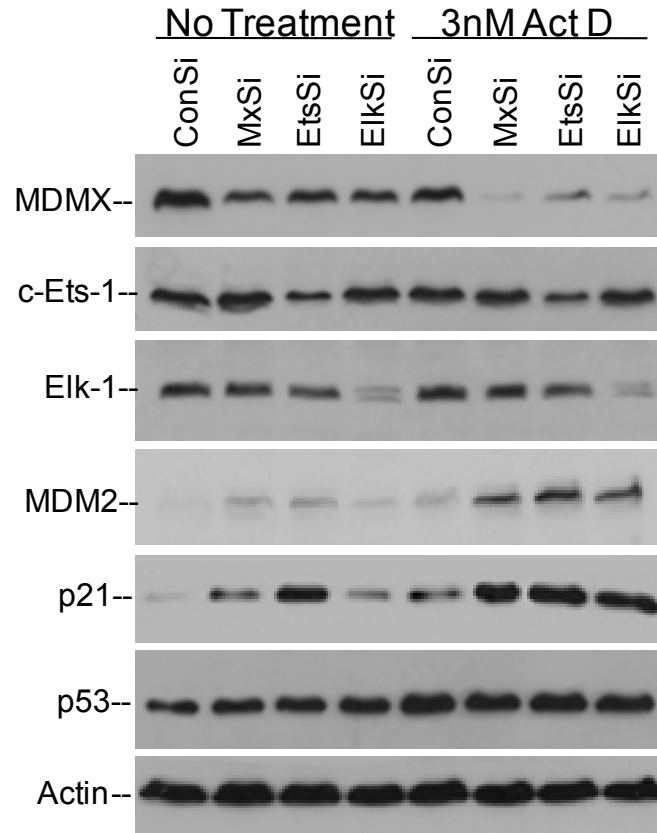
Figure 63. MDMX suppression of p53 requires c-Ets-1 and Elk-1. Synthetic RNAi oligonucleotides targeted to Elk-1, c-Ets-1, and MDMX mRNA were transfected into MCF7 cells using Oligofectamine reagent. After 48 hrs, cells were treated with actinomycin D for 20 hrs and analyzed for the expression level of indicated markers by Western blot.

Activation of MAP kinase pathway induces MDMX expression

MDM2 expression is induced by oncogenic H-Ras through activation of MAPK and c-Ets-1 (Ries, Biederer et al. 2000). Because c-Ets-1 also appeared to be critical for MDMX promoter activity, we tested the role of the Ras-MAPK pathway in MDMX induction. P53-null (35.8) and p53/ARF double-null (DKO) mouse embryo fibroblasts were stably infected with retrovirus expressing HA-tagged mutant K-Ras oncogene (12V), which is more frequently involved in human cancer than H-Ras (Sebolt-Leopold

and Herrera 2004). K-Ras expression resulted in significant induction of MDMX in both 35.8 and DKO cells. Additionally, phosphorylated ERK and downstream targets c-Ets-1 and Elk-1 levels were elevated in K-Ras expressing cells (Figure 64a). Half-life comparison by cycloheximide treatment did not indicate a change in MDMX stability after K-Ras expression (data not shown). RT-PCR analysis showed that MDMX mRNA was increased by over 2-fold in K-Ras overexpressing cells when compared to vector-infected control (Figure 64b), suggesting that the induction occurred at the transcriptional level.
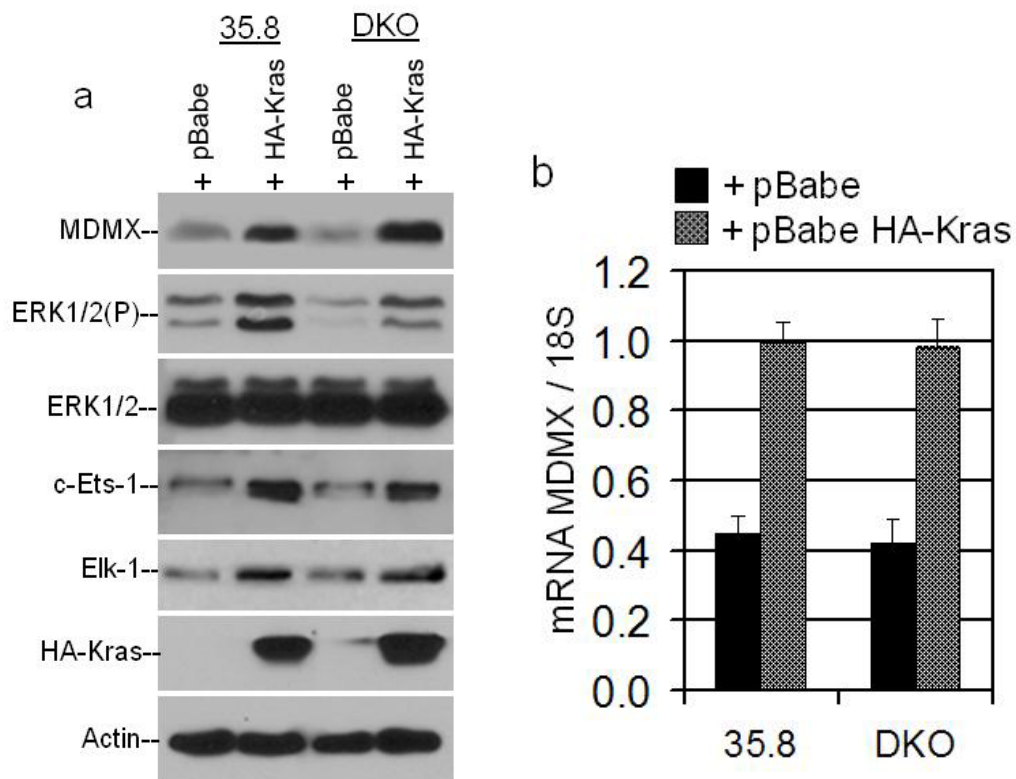
Figure 64. K-Ras induces MDMX protein and mRNA expression. (a) 35.8 (p53-null) and DKO (p53/ARF–double null) MEFs stably infected with pBabe-HA-K-Ras (12V) virus were analyzed by western blot for indicated markers. (b) Total RNA from 35.8 and DKO cells expressing activated K-Ras were analyzed for MDMX mRNA level by qPCR (n=6).

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To further test whether downstream factors of the Ras signaling pathway have the same effect as activated Ras, H1299 cells with low endogenous MDMX were transiently transfected with constitutively active mutants of B-Raf (V600E) and MEK1. The results showed that expression of active B-Raf and MEK1 led to ERK phosphorylation and significant induction of MDMX protein level as expected (Figure 65a). Furthermore, the MDMX promoter construct was stimulated by cotransfection with activated Ras, MEK1, and B-Raf, whereas a promoter with mutated Ets-1 binding sites was not responsive (Figure 65b). These results suggest that activation of the Ras-Raf-MAPK pathway is sufficient to activate the MDMX promoter.
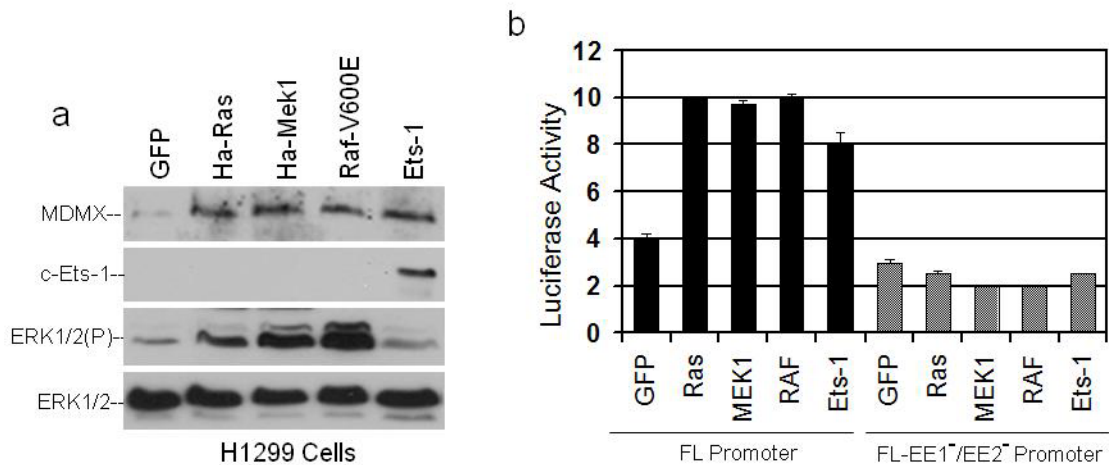
Figure 65. The Ras Pathway induces endogenous MDMX. (a) H1299 cells were transiently transfected with HA-K-Ras, HA-Mek1, B-RafV600E, or c-Ets-1. Endogenous expression of MDMX, phospho-ERK, ERK1/2, and actin were analyzed by Western blot. (b) H1299 cells were transfected with full-length or EE1/EE2 mutant MDMX reporter constructs and expression vectors for HA-K-Ras, HA-Mek1, B-RafV600E, or c-Ets-1. The luciferase reporter activity for each of the transfection conditions is shown (n=3).

Inhibitors of the MAP kinase pathway down regulate MDMX expression

To confirm that K-Ras induction of ERK phosphorylation mediated the increase in

MDMX level, 35.8-K-Ras cells were treated with the MEK inhibitor U0126 for 8 hours.

Inhibition of MEK/ERK pathway by U0126 has been shown to prevent the effects of

oncogenic H-Ras and K-Ras (Zhang and Lodish 2004). U0126 treatment caused a

reduction of MDMX in 35.8-K-Ras expressing cells to levels equivalent to 35.8 control

cells (Figure 66). Additionally, treatment of MCF-7 cells (high MDMX) with the MEK

inhibitor PD98059 led to a time-dependent decrease in MDMX protein expression

(Figure 67). Therefore, the results of the inhibitors were as predicted from the activation

experiments.
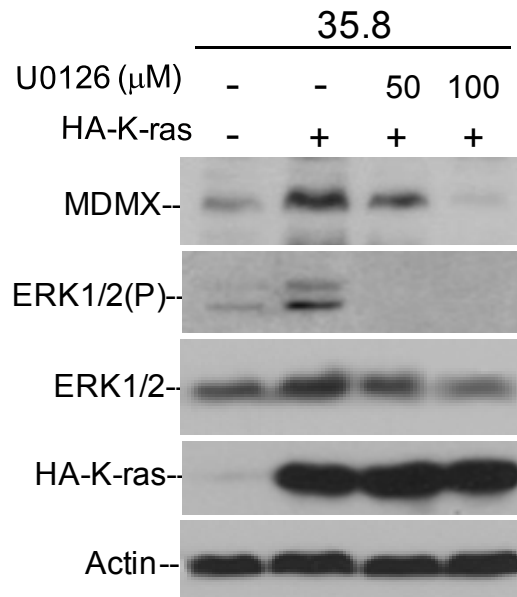
Figure 66. Oncogenic K-Ras induces MDMX expression in an ERK-dependent manner. 35.8 cells stably transfected with HA-K-Ras were treated with U0126 for 8 hours and analyzed for expression of MDMX and phospho-ERK.
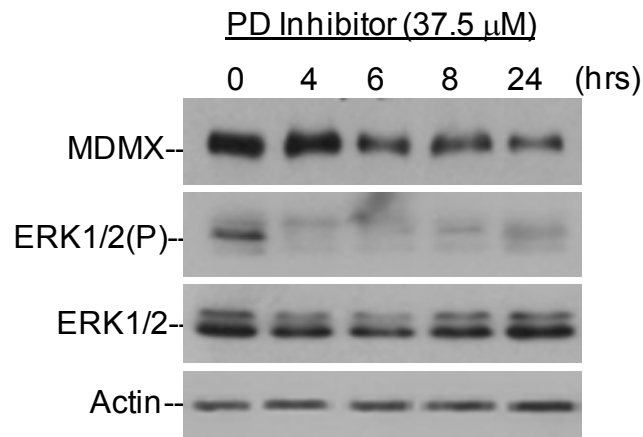
Figure 67. Mek inhibition results in downregulation of MDMX. MCF-7 cells were treated with 37.5 µM PD98059 for the indicated time points followed by western blot analysis.

To test whether there is a correlation between MAPK activation and MDMX overexpression, cell lines were compared for their p-ERK level, MDMX level, and response to UO126. The results from this small cell panel suggested a general association between high-level p-ERK and MDMX expression (Figure 68). Furthermore, UO126 inhibited MDMX expression in most cell lines. As expected, MDM2 levels were also decreased in the presence of U0126 (Ries, Biederer et al. 2000). Therefore, strong activation signaling from the Ras/Raf/MEK/MAP kinase pathway may be responsible for MDMX overexpression in a majority of tumor cell lines. However, JEG-3 is an exception with low p-ERK, high MDMX, and insensitivity to UO126 (Figure 68), suggesting additional mechanism of MDMX overexpression independent of hyperactive MAPK pathway. The activity of MDMX promoter in JEG-3 cells still requires the Ets-1 and Elk-

1 binding sites (Figure 60a), suggesting that these transcription factors are activated by

MAPK-independent mechanisms in JEG-3 cells.
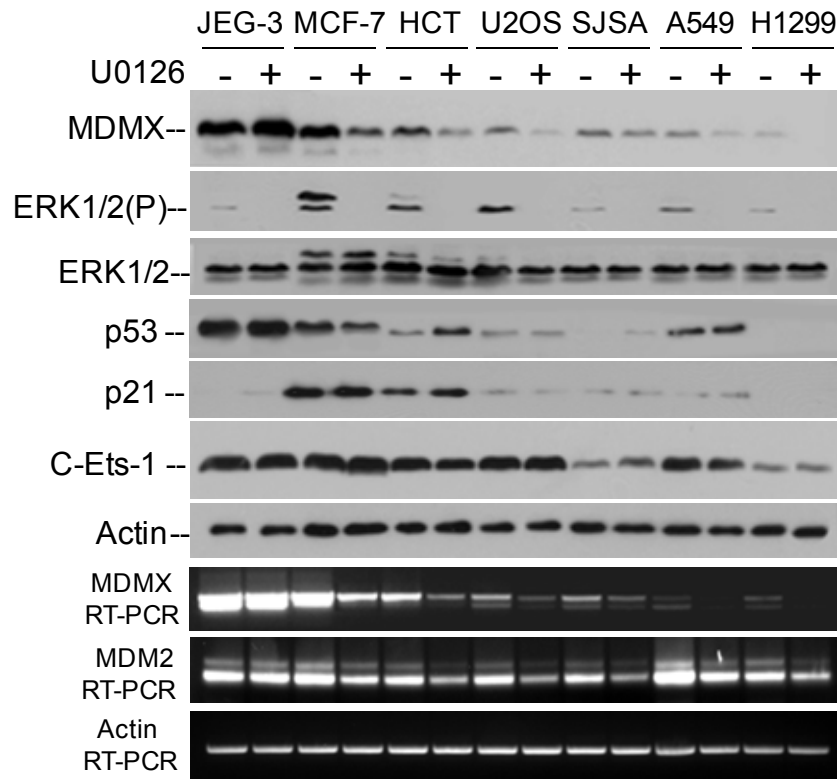
Figure 68. Mek inhibition downregulates MDMX in panel of cancer cell lines. A
panel of cell lines expressing different endogenous levels of MDMX were
treated with 30 μM U0126 for 18 hrs and compared for expression of the
indicated proteins and MDMX mRNA.

Although MAPK inhibitors suppressed MDMX expression, they did not lead to

reproducible increase in p53 activity and p21 expression (Figure 68). U0126 treatment

also failed to induce several other p53 target genes (PUMA, 14-3-3 sigma, cyclin G, PIG-

3) when tested by RT-PCR, and did not activate p53-response promoter in reporter gene

assay (data not shown). In contrast, direct knock down of MDMX by siRNA consistently

induced p21 expression in the same panel of cell lines (Figure 69). These results suggest

that although MAPK pathway regulates MDMX expression, targeting this pathway by

MAPK inhibitors does not provide a net activation of p53. This may be due to the

complex biological effects or lack of specificity by the kinase inhibitors.
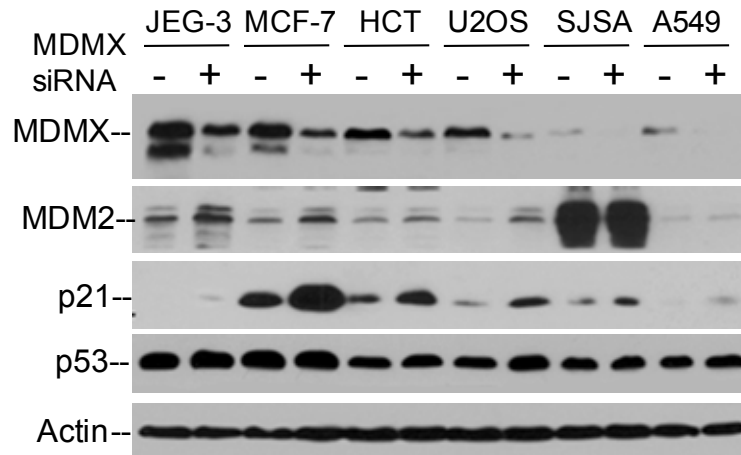
Figure 69. MDMX knockdown induces p21 and MDM2 expression. Cells were
transfected with MDMX siRNA for 72 hrs and analyzed for expression of
indicated markers by western blot.

MDMX promoter activation correlates with increased Ets-1 and Elk-1 binding

Knockdown of Ets-1 and Elk-1 by siRNA resulted in reduced MDMX expression

and p53 activation, suggesting that Ets-1 and Elk-1 are important factors in mediating

MDMX induction by mitogenic signals. To determine whether promoter occupancy of

Ets-1 and Elk-1 correlates with MDMX promoter activity and expression level, cell lines

with high and low levels of MDMX were analyzed by ChIP using Ets-1 and Elk-1

antibodies. The results confirmed that high-level MDMX expression was associated with

increased promoter binding by Ets-1 and Elk-1, whereas YY1 binding was similar

(Figure 70). The binding difference was specific for the basal promoter region and was

not observed using PCR primers 3 kb upstream from the basal promoter.
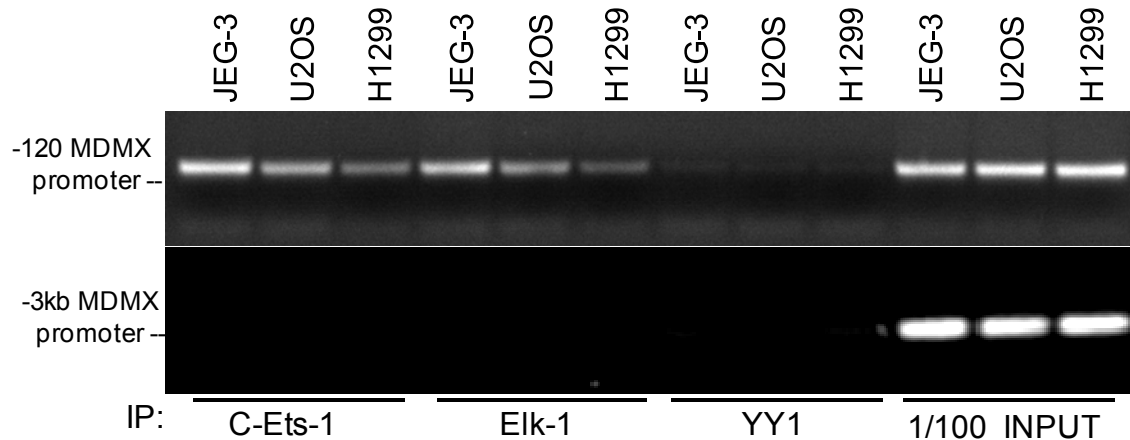
Figure 70. c-Ets-1 and Elk-1 bind to the MDMX promoter. JEG-3 (high MDMX expression), U2OS, and H1299 (low MDMX expression) cells were analyzed by ChIP to detect binding of endogenous c-Ets-1 and Elk-1 to the basal MDMX promoter. YY1 was used as a negative control. PCR of a promoter element 3 kb upstream of the basal MDMX promoter was performed as a specificity control.

When MCF-7 cells were treated with UO126 (MEK inhibitor) and SB203580 (p38

inhibitor), Ets-1 and Elk-1 binding to MDMX promoter was specifically reduced by

UO126 but not by SB203580 (Figure 71). These results were also consistent with the

effects of the inhibitors on MDMX expression level and promoter activity (Figure 72).

These results provide additional evidence that MAP kinase signaling stimulates Ets-1 and

Elk-1 binding to the MDMX basal promoter, inducing MDMX expression.
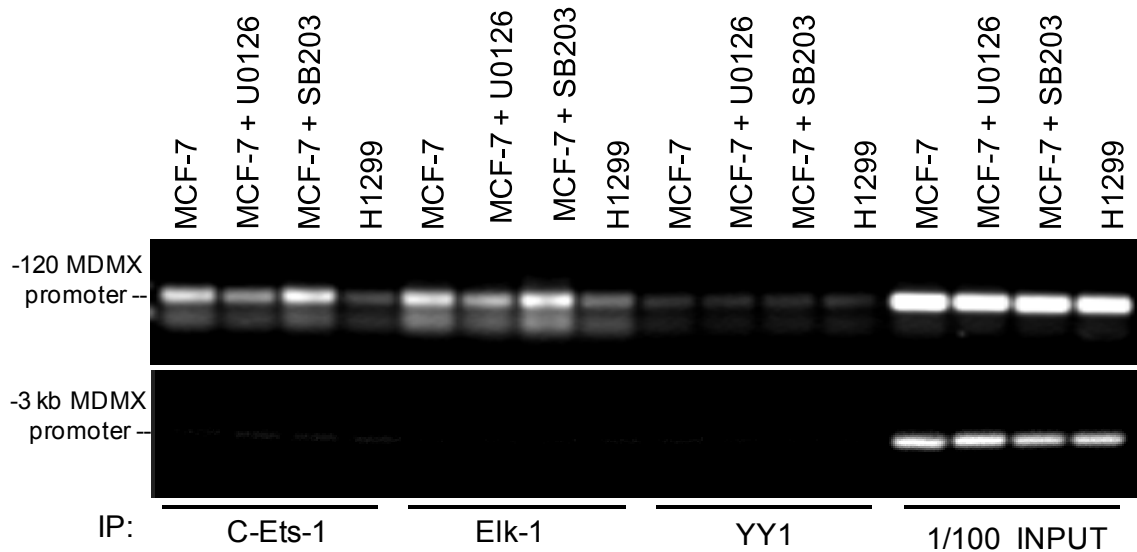
Figure 71. c-Ets-1 and Elk-1 binding is phosphor-erk dependent. MCF-7 cells treated with 30 µM of MAPK (U0126) or p38 stress kinase inhibitor (SB203580) were compared to H1299 cells by ChIP analysis for c-Ets-1 and Elk-1 binding to the MDMX promoter.

IGF-1 increases expression of MDMX in a MAPK-dependent manner

The involvement of Ras-MAPK pathway in stimulating MDMX expression

suggests that extracellular growth factors may also influence MDMX expression.

Mitogenic stimulation by FGF, IGF-1 or activated PDGF receptor has been shown to

inhibit the p53 pathway by inducing MDM2 transcription (Shaulian, Resnitzky et al.

1997; Fambrough, McClure et al. 1999). Additionally, IGF-1-induced cell division

correlates with nuclear exclusion of p53 and enhanced p53 degradation (Jackson, Patt et

al. 2006). MCF-7 cells have been shown to have up-regulated IGF receptor (IGFR-I)

mRNA and protein (Clarke, Howell et al. 1997). To address the role of MDMX in

mitogenic signaling to p53, serum starved MCF-7 cells were treated with IGF-1. This led

to a marked increase of MDMX expression after 8 hours (Figure 72a). Additionally, IGF-

1 stimulation of MCF-7 also led to an increase in activity of the transfected MDMX

promoter (Figure 72b). These results indicate that MDMX expression can be regulated by

extracellular growth factors.
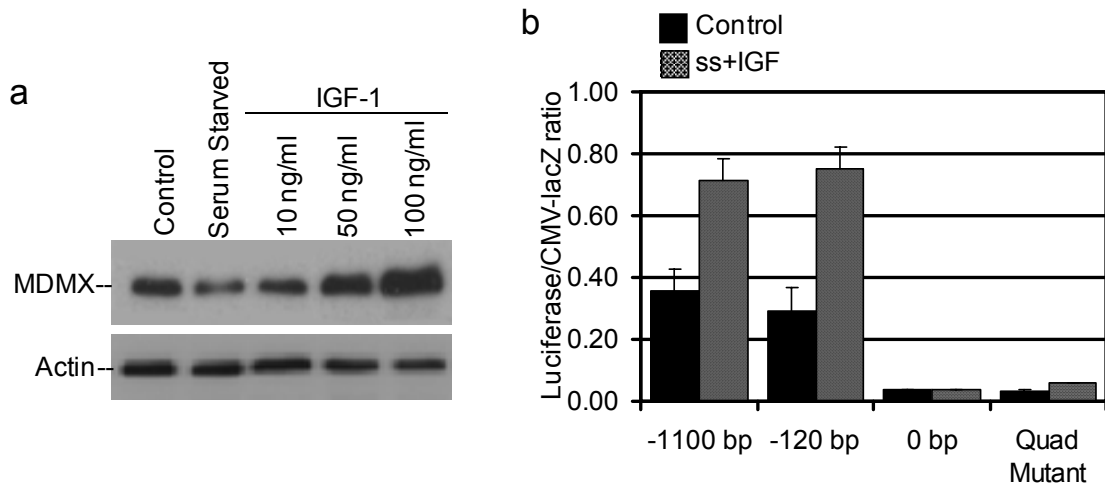
Figure 72. IGF-1 induces MDMX expression (a) MCF-7 cells were starved in DMEM
with 0% serum for 24 hrs. IGF-1 (10-100 ng/ml) was added and cells were
analyzed 8 hrs later by western blot. (b) MCF-7 cells were transfected with
MDMX promoter constructs for 24 hrs, serum starved for 24 hrs, and treated
with 100 ng/ml IGF-1 for 8 hrs. Promoter activity was compared to MCF-7
cells in 10% serum (n=3).

The biological effects of IGF-1 are mediated by the activation of the IGF-1

receptor, a transmembrane tyrosine kinase linked to the Akt and Ras-Raf-MAPK

cascades (Datta, Brunet et al. 1999). To evaluate which signaling pathway is involved in

IGF-1-mediated MDMX induction, serum starved MCF-7 cells were stimulated with

IGF-1 in the presence of PI3K inhibitor (LY294002), MEK inhibitor (PD98059), or p38

stress kinase inhibitor (SB203580). IGF-1 induction of MDMX was completely blocked

by the MEK inhibitor PD98059 but not by the PI3K or p38 inhibitors (Figure 73). The

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level of phosphorylated ERK, which decreased following serum starvation, was increased

upon the addition of IGF-1, confirming that IGF-1 was activating the MAPK pathway.

As expected, PD98059 completely abrogated ERK phosphorylation whereas LY294002

and SB203580 had no effect (Figure 73). Quantitative RT-PCR analysis showed that

IGF-1 induced MDMX mRNA expression by 3-fold over unstimulated MCF-7 cells

(Figure 74). Additionally, the MAPK inhibitor PD98059 completely abrogated the

induction of MDMX mRNA, while the p38 inhibitor had no effect. The PI3K inhibitor

LY294002 was able to partially suppress MDMX mRNA expression. This is likely due to

the cooperation between the PI3K and Ras activation pathways, which may act

synergistically to increase ERK phosphorylation.
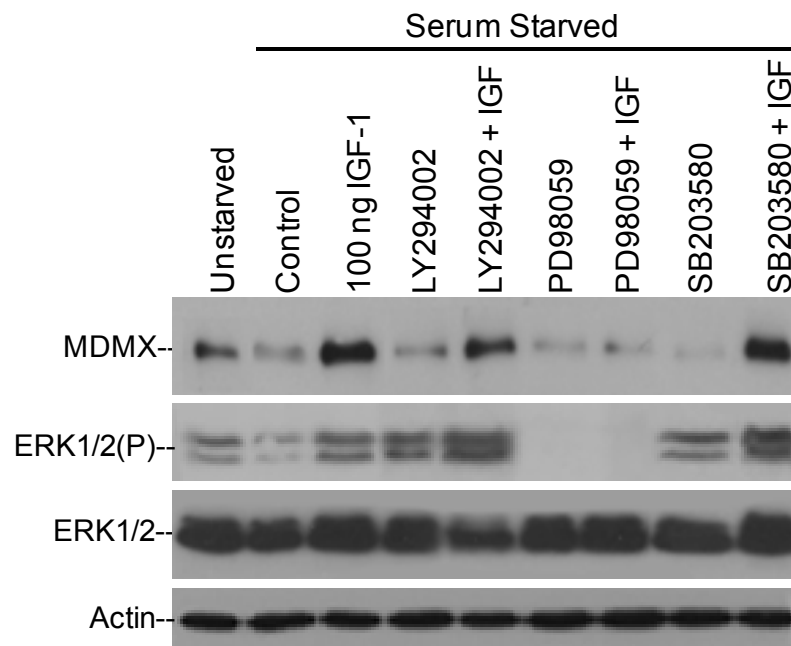
Figure 73. IGF-1 induces MDMX in serum starved cells. MCF-7 cells were serum
starved for 24 hrs, and treated with IGF-1 and inhibitors against PI3K (30 μM
LY294002), MAPK (37.5 μM PD98059), or p38 kinase (30 μM SB203580)
for 8 hrs. Cell lysate was analyzed by western blot.
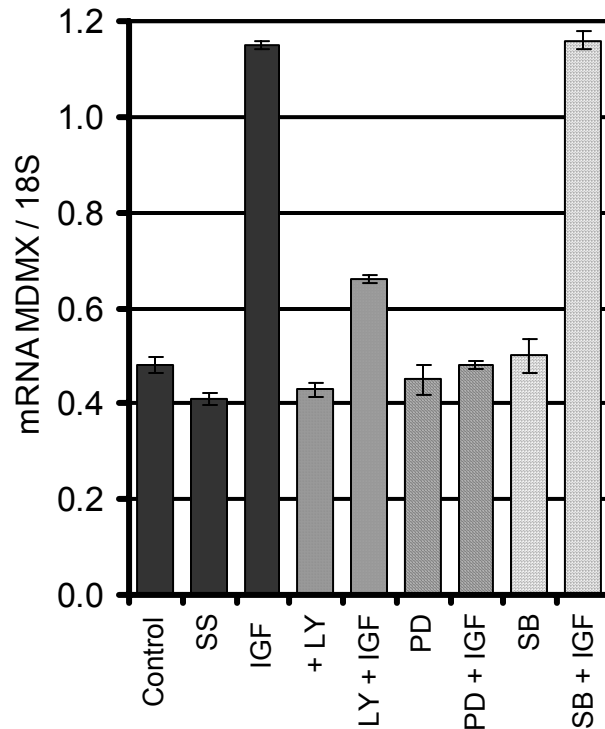
Figure 74. IGF-1 induces erk-dependent MDMX expression. Total RNA from serum starved MCF-7 treated with IGF-1, 30 µM LY294002, 37.5 µM PD98059, and 30 µM SB203580 were analyzed for MDMX mRNA levels by qPCR (n=3).

MDMX overexpression correlates with ERK phosphorylation in colorectal tumors

MDMX mRNA overexpression has been observed in 18.5% of breast, colon, and lung tumor samples as determined by in situ hybridization (Danovi, Meulmeester et al. 2004). MDMX protein expression was also observed in ~80% of adult pre-B acute lymphoblastic leukemia by immunohistochemical staining (Han, Garcia-Manero et al. 2007), suggesting that its expression is associated with common changes in signaling pathways in tumor cells.

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To verify the observation of MDMX overexpression in human tumors and test the association with hyperactive MAPK signaling, we performed immunohistochemical staining of a panel of colon tumors and normal colon mucosa controls. The results showed that normal mucosa expressed low levels of MDMX, whereas ~49% (49/99) of colon tumors expressed high-level MDMX as a diffused stain in both nucleus and cytoplasm (Figure 75a). MDMX overexpression was more frequently observed in high-grade tumors (Figure 75b). The tumor array was also analyzed for p53 overexpression, which serves as an indicator of p53 mutation. The results revealed that p53 and MDMX overexpression were independently associated with high-grade tumors (data not shown). These results suggest that MDMX expression is elevated in aggressive tumors and occurs independent of p53 mutation status.
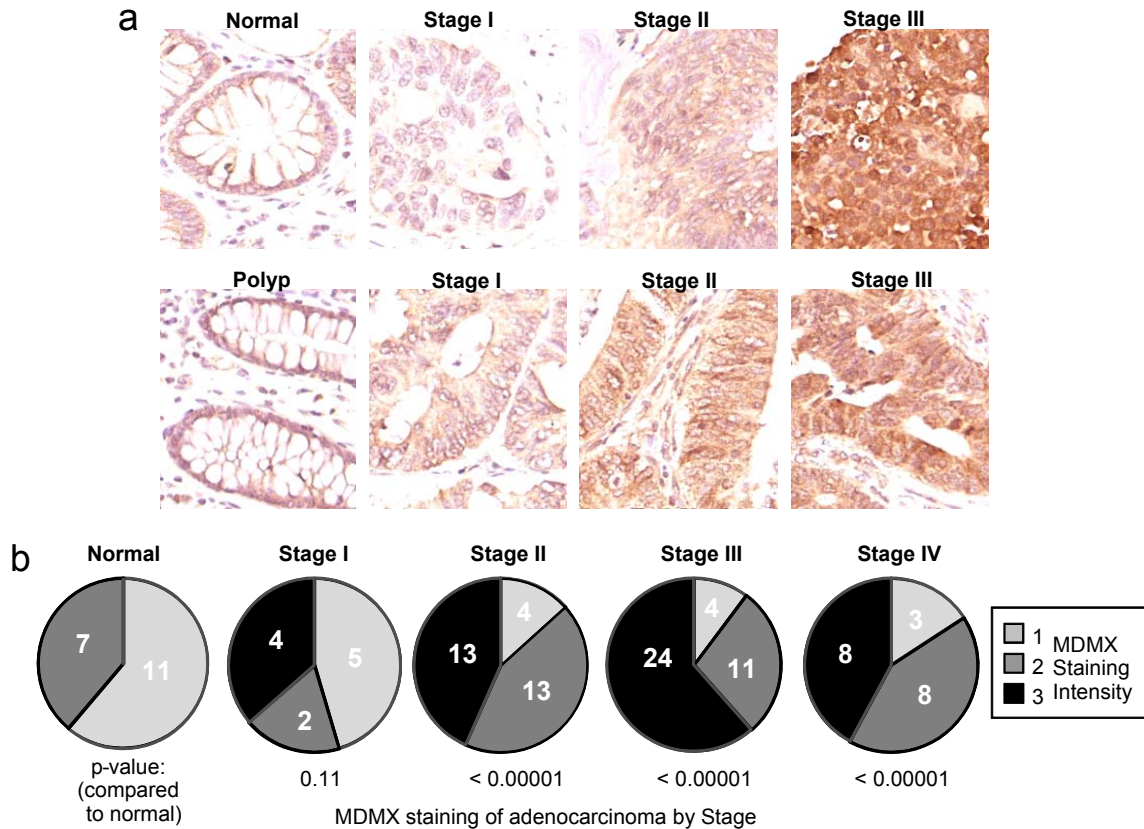
Figure 75. MDMX expression increases with tumor stage. (a) Representative MDMX immunohistochemical staining of normal colon mucosa and stage I-III tumors from a colon cancer tissue microarray (brown). An increased staining intensity as a function of tumor stage was observed. (b) Each tumor in the array was manually scored according to MDMX staining intensity from 1 to 3, and displayed according to the stage of colon cancer progression. The correlation between intensity of MDMX staining and the stage of colon cancer was calculated using Spearman's correlation analysis (n=117; $r2 =0.36$; $p < 0.0001$). Staining of MDMX intensity in each stage was compared to normal colon mucosa and the p-value is indicated.

Staining of the same tumor array using a phospho-ERK monoclonal antibody revealed a mosaic pattern of staining (20-40% cells positive) in a subset of tumors (Figure 76a). Tumors stained positive for phospho-ERK are 2-fold more likely to also have MDMX overexpression (Figure 76b). Unlike phospho-erk, the intensity of phospho-AKT staining could not be correlated with MDMX staining (data not shown). These

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results are consistent with cell culture analysis and suggest that hyperactive MAPK

signaling may stimulate MDMX overexpression and compromise the p53 pathway.
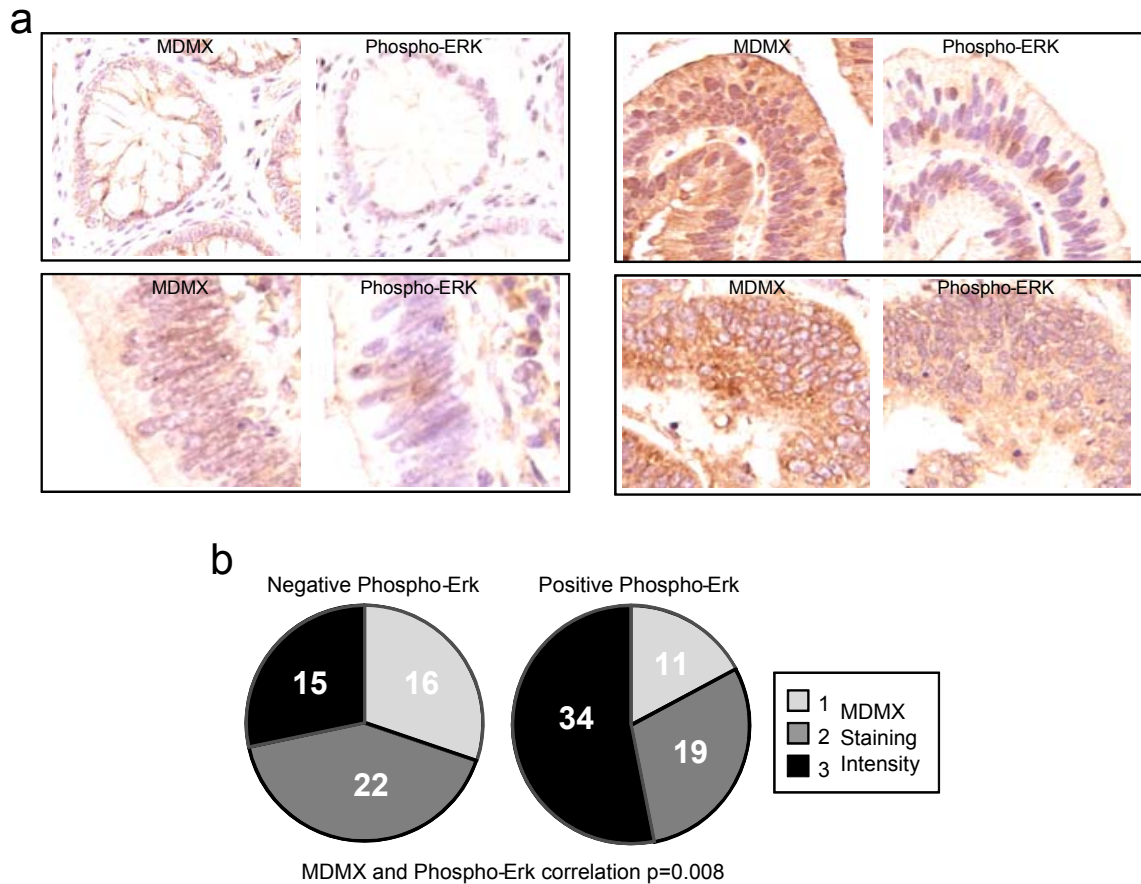
Figure 76. MDMX expression correlates with phospho-ERK level in colon cancer.
(a) Representative pictures of colon tumors stained for MDMX (left) or phospho-ERK (right). Each pair of pictures is from consecutive sections of the same tumor at the same position. (b) Intensity of MDMX staining in the phospho-ERK -positive and negative colon carcinomas. The correlation between intensity of MDMX staining and phospho-ERK was calculated using Spearman's correlation analysis (n=117; r^2 = 0.24; p=0.008).

Absence of sequence polymorphism in the MDMX basal promoter

The MDM2 P2 promoter (p53-responsive) contains a single nucleotide

polymorphism (SNP309) which is heterozygous in 40% and homozygous in 12% of the

sample population which results in increased binding by the Sp1 transcription factor and increased MDM2 expression (Bond, Hu et al. 2004). Importantly, the SNP309 allele is associated with higher risk for cancer, presumably due to attenuated p53 function. Therefore, we asked whether promoter sequence polymorphism contributes to different levels of MDMX expression in tumor cell lines. A 0.7 kb region of the MDMX promoter (0 to -700 bp) was amplified from the genomic DNA of 30 human cell lines (27 tumor cell lines, 3 skin fibroblasts) and analyzed by DNA sequencing. The analysis identified only one cell line (K562) with a SNP, which is located outside of the -120 bp to 0 bp region (data not shown). Sequencing results further upstream of the basal promoter were uninformative due to artifacts caused by multiple poly-T tracks. Therefore, the MDMX basal promoter does not contain significant sequence polymorphism.

Discussion

A significant difference between MDM2 and MDMX regulation is that MDMX transcription is not activated by p53. However, results described above identified important similarities in the induction of both MDM2 and MDMX by the Ras-MAPK and growth factor pathways (Leri, Liu et al. 1999; Ries, Biederer et al. 2000; Heron-Milhavet and LeRoith 2002). This finding provides an explanation for the frequent overexpression of MDMX in tumors, often in the absence of gene amplification. Induction of MDM2 and MDMX expression by the mitogenic pathways may serve to prevent unwanted p53 activation during normal cell proliferation in development and homeostasis. However, when inappropriately activated, this pathway also has oncogenic potential by blocking the tumor suppression functions of p53 during abnormal cell proliferation. Recent studies show that increased circulating IGF-1 levels put individuals

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at a higher risk for developing numerous types of cancers (Larsson, Girnita et al. 2005). Induction of MDM2 and MDMX may play a role in this process.

Following an initial oncogenic insult such as Ras mutation, MDMX and MDM2 induction by MAPK pathway may suppress p53 activity and facilitate initial tumor progression. MDMX induction may also attenuate ARF activation of p53 (Li, Chen et al. 2002). However, the lack of association between MDMX overexpression and p53 mutation in colon tumors suggests that MDMX is not sufficient to bypass the selection for p53 mutations. Previous study also showed that MDM2 gene amplification does not obviate the need for silencing ARF expression (Lu, Lin et al. 2002). It is possible that in advanced stage tumors, strong ARF induction by multiple activated oncogenes is dominant over the MAPK-MDM2/MDMX pathway, creating selection pressure for p53 mutation or ARF silencing. Consistent with this notion, ARF overexpression stimulates MDMX ubiquitination and degradation by MDM2 (unpublished results) (Pan and Chen 2003). Therefore, loss of ARF by epigenetic silencing or deletion is a key event that unleashes the oncogenic potential of the MAPK-MDM2/MDMX pathway, giving tumor cells with hyperactive MAPK an advantage in terms of resistance to p53.

MDM2 promoter polymorphism is prevalent among the human population, probably due to a certain level of evolutionary advantage it confers to the carriers at the expense of increased cancer risk. It is unclear whether MDMX expression level is affected by promoter polymorphism. Our sequence analysis of 30 human cell lines did not reveal significant variation in a 1.4 kb region including the proximal promoter and transcription factor binding sites necessary for basal and Ras-induced expression. Therefore, it is possible that sequence variations in this region that lead to increased or

decreased MDMX expression do not confer selection advantage and failed to accumulate in the population. However, these results do not rule out the presence of sequence polymorphism in other parts of the MDMX gene that may affect its transcription, splicing, and ability to regulate p53.

Recent studies have demonstrated the therapeutic potential of MDMX as a drug target in cancer. Knockout experiments suggest that elimination of MDMX leads to significant activation of p53. Reduction of MDMX gene dosage delays myc-induced lymphoma in mice (Terzian, Wang et al. 2007). Furthermore, shRNA knockdown of MDMX expression activates p53 in cell culture and abrogates tumor xenograft formation by HCT116 cells (Gilkes, Chen et al. 2006). Therefore, down regulation of MDMX expression is a useful therapeutic strategy. However, our results in this report suggest that although MAPK pathway regulates MDMX expression, targeting this pathway by kinase inhibitors may not provide a net activation of p53. This may be due to the complexity of the MAPK pathway, involvement of Ets-1 in regulating p21 expression (Zhang, Kavurma et al. 2003), and toxicity of the kinase inhibitors. It has been shown that MEK activity is required for expression of p53 at the transcriptional level and also for p53 activation by genotoxic agents (Persons, Yazlovitskaya et al. 2000; Agarwal, Ramana et al. 2001). Therefore, more specific approaches that directly target MDMX expression or activity are necessary for effective p53 activation.

Scientific Significance

P53 is a transcription factor that can be activated by a variety of stress signals. Upon activation, it induces a group of genes necessary to inhibit cell proliferation or induce cell death. The tumor suppressor p53 is mutated in a wide variety of human cancers at a frequency of about 50 percent. In tumors which retain wild-type p53, p53's functional activity can potentially be attenuated by upregulation, overexpression, or amplification of either of its two major negative regulators, MDM2 and/or MDMX. In support of this notion, we found that overexpressing MDMX in cells maintaining functionally active non-mutated p53 inhibits p53-induced cell cycle arrest following ribosomal stress. Likewise, reducing the gene dosage of MDMX by siRNA stimulates p53 activity following ribosomal stress. Additionally, staining MDMX in a colon tissue tumor array revealed a positive correlation between MDMX staining intensity and increased tumor grade. Taken together, our data shows that MDMX has the potential to suppress p53 in favor of tumor progression.

Under "normal" conditions, a well accepted hypothesis for p53 maintenance posits that MDM2 acts as an E3 ligase towards p53 directing its degradation by the proteosome. While MDMX does not degrade p53 on its own, it can bind to p53's transactivation domain blocking the induction of p53 target genes. It is not fully clear whether MDMX and MDM2 function independently or in a synergistic manner to prevent p53 activation.

Several labs have suggested that the relative abundance of MDMX versus MDM2 may be critical for the outcome of p53 protein levels. When they are expressed at equivalent ratios, p53 undergoes MDM2-dependent proteasomal degradation. In the presence of high MDMX levels, it is reasonable to suggest that MDM2 and MDMX may compete for p53 binding resulting in more MDMX-p53 interactions. Indeed, our experiments showed that overexpressing MDMX leads to an increase in MDMX-p53 complex formation. If MDMX outcompeted MDM2 for p53 binding, one may expect p53 protein levels to remain the same or even increase. However, we actually found the opposite to be true. Overexpressing MDMX does not appear to modulate p53 protein levels whereas knocking down MDMX causes a modest increase in p53 levels. Futhermore, in a small panel of cell lines tested for MDMX and MDM2 expression, the ratio of MDM2-to-MDMX does not correlate with p53 levels. This suggests that rather than competing for binding, MDMX and MDM2 may form complexes that bind to p53 and promote its degradation. Several studies show MDM2 forms hetero- and homodimers through ring domain interactions contributing to MDM2-mediated degradation of p53(Tanimura, Ohtsuka et al. 1999; Dang, Kuo et al. 2002; Linares, Hengstermann et al. 2003). Interestingly, two recent studies showed that heterodimers of a C-terminal point mutant of MDM2 (no E3 ligase activity) and MDMX are capable of targeting p53 for degradation, suggesting that the C-terminus of MDMX can substitute for MDM2 (Poyurovsky, Priest et al. 2007; Uldrijan, Pannekoek et al. 2007). Although we do not know the optimum ratio of MDMX-to-MDM2 which can prevent p53 induction under normal conditions but allow it to become be quickly activated following stress, we do know that these proteins do not play redundant roles in regulation of p53. This was well

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demonstrated in mouse models showing the abrogation of either MDM2 or MDMX leads to embryonic lethality, but after crossing into p53 null background mice are viable.

Following cellular stress, it is important for p53 to quickly become activated and induce cell cycle arrest or apoptosis. This should require inhibition of both MDM2 and MDMX. MDM2 can stimulate the polyubiquitination and degradation of MDMX through the proteasome pathway. It is well recognized that p53 binding to MDM2 is weakened after DNA damage due to phosphorylation of both p53 and MDM2. Recently, our lab showed that following DNA damage, ATM- dependent phosphorylation of MDMX enhances the degradation of MDMX by MDM2 an effect related to 14-3-3 binding and increased binding to the deubiquitinating enzyme HAUSP. On the other hand, the regulation of MDM2 and MDMX and activation of p53 following ribosomal stress has not been as intensely studied. Several studies have shown that ribosomal stress causes enhanced binding of MDM2 to several ribosomal proteins. The interaction of MDM2 with ribosomal proteins reduces its E3 ligase activity towards p53. Interestingly, we show that ribosomal stress-induced p53 induction is associated with rapid down-regulation of the MDMX protein. We found that the interaction of MDM2 with ribosomal protein L11 is enhanced following ribosomal stress and promotes the ubiquitination of MDMX. Further, inducing ribosomal stress with the addition of either actinomycin D or 5-FU does not lead to p53 or MDMX phosphorylation suggesting that DNA damage and ribosomal stress are completely unique in their ability to regulate MDM2, MDMX, and p53. Our data suggests that physiological levels of MDM2 overexpression can be effectively neutralized during ribosomal stress, resulting in p53 stabilization. In contrast,

MDMX is a stable protein that regulates p53 mainly by sequestering p53 into complexes which are not disrupted by ribosomal stress.

The level of MDM2 and MDMX in a cell is clearly paramount to p53 activation. The level of MDM2 in cells is determined by the following main mechanisms: (1) P53-dependent transactivation of the MDM2 gene (2) mitogen-dependent activation of factors such as Erk that also transactivate MDM2 (3) mitogen-dependent post-translational modifications that modulate MDM2 stability (4) self-ubiquitination and (5) interaction with HAUSP. The factors that influence MDMX abundance have not been widely studied. As mentioned previously, MDMX is targeted for degradation by MDM2. This is further stimulated following DNA damage and ribosomal stress. Furthermore, HAUSP, first identified as a P53-associated protein, appears to contribute to stabilization of both MDM2 and MDMX. Data from our current study show that like MDM2, MDMX can also be transactivated by components of the mitogen-activated protein kinase pathway such as Ras, Raf, and Erk and involves the transcription factors Ets and Elk. In addition, unlike MDM2, MDMX is a very stable protein with a long half-life (>2hrs). This can be partially explained by the fact that it does not have self-ubiquitination activity. Taken together, the data suggests that while MDM2 has dynamic control of p53, MDMX may have a more stable and long term affect on p53 activation.

One of the interesting discoveries which came out of our current research was the ability of MDMX to be degraded by MDM2 without its stabilization or upregulation by p53 induction. For example, MDMX is degraded to similar levels in both HCT p53 wild-type and p53-null cells following actinomycin D treatment even though MDM2 levels remain low in HCT p53-null cells. Studies addressing MDMX degradation following

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DNA damage induced by gamma irradiation have shown similar results. Interestingly, the degradation of MDMX is strongly attenuated in MEF cells lacking both p53 and MDM2. Furthermore, MDMX ubiquitination is increased following ribosomal stress due to an enhanced interaction of MDM2 with L11. This shows that the cellular level of MDM2 may not be directly coupled to its potential E3 ligase activity for MDMX. In support of this notion, we find after treatment with the MDM2-p53 inhibitor Nutlin-3, MDM2 levels are increased upon p53 activation but this does not lead to MDMX degradation in tumor cells. Interestingly, MDMX degradation appears to occur in non-tumor derived cell lines following Nutlin-3 treatment. The complexity of MDM2's E3 Ligase activity is not surprising. For example, studies have shown that the ring domain of MDM2 is necessary but not sufficient for p53 degradation while it is sufficient for degradation of MDMX. Therefore, further investigation of MDM2 E3 ligase activity is not only warranted but necessary to discern how it is potentiated under different stress conditions as well as its specificity for MDMX versus p53.

Another important finding from our studies was the importance of MDMX on tumor xenograft formation. To test the role of MDMX in tumor formation *in vivo*, HCT116 cells expressing scrambled or MDMX siRNA were inoculated subcutaneously on the dorsal flanks of athymic nude mice. While tumors derived from the scrambled siRNA cell lines formed readily, MDMX siRNA expressing cells showed significantly reduced tumorigenic potential. Likewise, a study by an independent group utilized MEF cells expressing a homozygous deleted PRD (proline rich domain) of p53 to introduce E1A and RAS and assess MDMX expression in a tumor xenograft model (Toledo, Krummel et al. 2006). E1A and Ras transformed p53$^{\Delta P/\Delta P}$ cells showed no suppression of

oncogene-induced tumors when compared to E1A and Ras transformed p53$^{+/+}$ cells. However, the number and size of tumors generated from E1A and Ras transformed p53$^{\Delta P/\Delta P}$ MDMX$^{-/-}$ MEFs were similar to those formed from E1A and Ras p53$^{+/+}$ MEFs. These studies highlight the importance of MDMX suppression of p53 during tumor progression. Interestingly, in cell culture, HCT116 cells expressing an MDMX siRNA grow at rates comparable to scrambled siRNA cells. This suggests that the tumor environment causes a physiological stress that required suppression of p53 by MDMX. It is possible that tumors are constantly under ribosomal stress. A tumor cell has an increased demand for protein synthesis as they continue to undergo cycles of proliferation. This may lead to an increase in ribosome and therefore ribosomal protein synthesis. In this scenario, decreasing MDMX levels will be an important mechanism for stimulating p53 activation. This suggests that agents which decrease the MDMX-p53 interaction may be important for future cancer therapies. While inhibitors such as Nutlin-3 have been designed to target the MDM2-p53 interaction, it is important to note that they do not effectively inhibit MDMX-p53 binding (Vassilev 2004; Patton, Mayo et al. 2006). This highlights the need for the development of novel dual inhibitors which block both MDMX and MDM2 interactions with p53.

List of References

Adams, J. M. and S. Cory (2001). "Life-or-death decisions by the Bcl-2 protein family." *Trends Biochem Sci* **26**(1): 61-6.

Agarwal, M. L., A. Agarwal, W. R. Taylor and G. R. Stark (1995). "p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts." *Proc Natl Acad Sci U S A* **92**(18): 8493-7.

Agarwal, M. L., C. V. Ramana, M. Hamilton, W. R. Taylor, S. E. DePrimo, L. J. Bean, A. Agarwal, M. K. Agarwal, A. Wolfman and G. R. Stark (2001). "Regulation of p53 expression by the RAS-MAP kinase pathway." *Oncogene* **20**(20): 2527-36.

Alt, J. R., T. C. Greiner, J. L. Cleveland and C. M. Eischen (2003). "Mdm2 haplo-insufficiency profoundly inhibits Myc-induced lymphomagenesis." *Embo J* **22**(6): 1442-50.

Amsterdam, A., K. C. Sadler, K. Lai, S. Farrington, R. T. Bronson, J. A. Lees and N. Hopkins (2004). "Many ribosomal protein genes are cancer genes in zebrafish." *PLoS Biol* **2**(5): E139.

Bae, I., M. L. Smith and A. J. Fornace, Jr. (1995). "Induction of p53-, MDM2-, and WAF1/CIP1-like molecules in insect cells by DNA-damaging agents." *Exp Cell Res* **217**(2): 541-5.

Baker, S. J., S. Markowitz, E. R. Fearon, J. K. Willson and B. Vogelstein (1990). "Suppression of human colorectal carcinoma cell growth by wild-type p53." *Science* **249**(4971): 912-5.

Barak, Y., T. Juven, R. Haffner and M. Oren (1993). "mdm2 expression is induced by wild type p53 activity." *Embo J* **12**(2): 461-8.

Barlev, N. A., L. Liu, N. H. Chehab, K. Mansfield, K. G. Harris, T. D. Halazonetis and S. L. Berger (2001). "Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases." *Mol Cell* **8**(6): 1243-54.

Bell, D. W., J. M. Varley, T. E. Szydlo, D. H. Kang, D. C. Wahrer, K. E. Shannon, M. Lubratovich, S. J. Verselis, K. J. Isselbacher, J. F. Fraumeni, J. M. Birch, F. P. Li, J. E. Garber and D. A. Haber (1999). "Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome." *Science* **286**(5449): 2528-31.

Bernstein, M., H. Kovar, M. Paulussen, R. L. Randall, A. Schuck, L. A. Teot and H. Juergens (2006). "Ewing's sarcoma family of tumors: current management." *Oncologist* **11**(5): 503-19.

Bhat, K. P., K. Itahana, A. Jin and Y. Zhang (2004). "Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation." *Embo J* **23**(12): 2402-12.

Bischof, O., K. Schwamborn, N. Martin, A. Werner, C. Sustmann, R. Grosschedl and A. Dejean (2006). "The E3 SUMO ligase PIASy is a regulator of cellular senescence and apoptosis." *Mol Cell* **22**(6): 783-94.

Bischoff, J. R., P. N. Friedman, D. R. Marshak, C. Prives and D. Beach (1990). "Human p53 is phosphorylated by p60-cdc2 and cyclin B-cdc2." *Proc Natl Acad Sci U S A* **87**(12): 4766-70.

Bishop, J. M. (1987). "The molecular genetics of cancer." *Science* **235**(4786): 305-11.

Blattner, C., T. Hay, D. W. Meek and D. P. Lane (2002). "Hypophosphorylation of Mdm2 Augments p53 Stability." *Mol. Cell. Biol.* **22**(17): 6170-6182.

Boddy, M. N., P. S. Freemont and K. L. Borden (1994). "The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger." *Trends Biochem Sci* **19**(5): 198-9.

Boesten, L. S., S. M. Zadelaar, S. De Clercq, S. Francoz, A. van Nieuwkoop, E. A. Biessen, F. Hofmann, S. Feil, R. Feil, A. G. Jochemsen, C. Zurcher, L. M. Havekes, B. J. van Vlijmen and J. C. Marine (2006). "Mdm2, but not Mdm4, protects terminally differentiated smooth muscle cells from p53-mediated caspase-3-independent cell death." *Cell Death Differ* **13**(12): 2089-98.

Bond, G. L., W. Hu, E. E. Bond, H. Robins, S. G. Lutzker, N. C. Arva, J. Bargonetti, F. Bartel, H. Taubert, P. Wuerl, K. Onel, L. Yip, S. J. Hwang, L. C. Strong, G. Lozano and A. J. Levine (2004). "A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans." *Cell* **119**(5): 591-602.

Bond, G. L., W. Hu and A. J. Levine (2005). "MDM2 is a central node in the p53 pathway: 12 years and counting." *Curr Cancer Drug Targets* **5**(1): 3-8.

Bothner, B., W. S. Lewis, E. L. DiGiammarino, J. D. Weber, S. J. Bothner and R. W. Kriwacki (2001). "Defining the molecular basis of Arf and Hdm2 interactions." *J Mol Biol* **314**(2): 263-77.

Bottger, A., V. Bottger, C. Garcia-Echeverria, P. Chene, H. K. Hochkeppel, W. Sampson, K. Ang, S. F. Howard, S. M. Picksley and D. P. Lane (1997). "Molecular characterization of the hdm2-p53 interaction." *J Mol Biol* **269**(5): 744-56.

Bottger, V., A. Bottger, C. Garcia-Echeverria, Y. F. Ramos, A. J. van der Eb, A. G. Jochemsen and D. P. Lane (1999). "Comparative study of the p53-mdm2 and p53-MDMX interfaces." *Oncogene* **18**(1): 189-99.

Bouvet, M., L. M. Ellis, M. Nishizaki, T. Fujiwara, W. Liu, C. D. Bucana, B. Fang, J. J. Lee and J. A. Roth (1998). "Adenovirus-mediated wild-type p53 gene transfer down-regulates vascular endothelial growth factor expression and inhibits angiogenesis in human colon cancer." *Cancer Res* **58**(11): 2288-92.

Boyd, S. D., K. Y. Tsai and T. Jacks (2000). "An intact HDM2 RING-finger domain is required for nuclear exclusion of p53." *Nat Cell Biol* **2**(9): 563-8.

Brunner, D., K. Ducker, N. Oellers, E. Hafen, H. Scholz and C. Klambt (1994). "The ETS domain protein pointed-P2 is a target of MAP kinase in the sevenless signal transduction pathway." *Nature* **370**(6488): 386-9.

Budde, A. and I. Grummt (1999). "p53 represses ribosomal gene transcription." *Oncogene* **18**(4): 1119-24.

Bullock, A. N. and A. R. Fersht (2001). "Rescuing the function of mutant p53." *Nat Rev Cancer* **1**(1): 68-76.

Bunz, F., P. M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. Williams, C. Lengauer, K. W. Kinzler and B. Vogelstein (1999). "Disruption of p53 in human cancer cells alters the responses to therapeutic agents." *J Clin Invest* **104**(3): 263-9.

Bykov, V. J., N. Issaeva, A. Shilov, M. Hultcrantz, E. Pugacheva, P. Chumakov, J. Bergman, K. G. Wiman and G. Selivanova (2002). "Restoration of the tumor suppressor function to mutant p53 by a low-molecular-weight compound." *Nat Med* **8**(3): 282-8.

Cahill, D. P., K. W. Kinzler, B. Vogelstein and C. Lengauer (1999). "Genetic instability and darwinian selection in tumours." *Trends Cell Biol* **9**(12): M57-60.

Cahilly-Snyder, L., T. Yang-Feng, U. Francke and D. L. George (1987). "Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line." *Somat Cell Mol Genet* **13**(3): 235-44.

Canman, C. E. and D. S. Lim (1998). "The role of ATM in DNA damage responses and cancer." *Oncogene* **17**(25): 3301-8.

Chan, T. A., H. Hermeking, C. Lengauer, K. W. Kinzler and B. Vogelstein (1999). "14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage." *Nature* **401**(6753): 616-20.

Chen, D., N. Kon, M. Li, W. Zhang, J. Qin and W. Gu (2005). "ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor." *Cell* **121**(7): 1071-83.

Chen, D., M. Li, J. Luo and W. Gu (2003). "Direct interactions between HIF-1 alpha and Mdm2 modulate p53 function." *J Biol Chem* **278**(16): 13595-8.

Chen, J., V. Marechal and A. J. Levine (1993). "Mapping of the p53 and mdm-2 interaction domains." *Mol. Cell. Biol.* **13**(7): 4107-4114.

Chen, L. and J. Chen (2003). "MDM2-ARF complex regulates p53 sumoylation." *Oncogene* **22**(34): 5348-57.

Chen, L., D. M. Gilkes, Y. Pan, W. S. Lane and J. Chen (2005). "ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage." *Embo J* **24**(19): 3411-22.

Chen, L., C. Li, Y. Pan and J. Chen (2005). "Regulation of p53-MDMX interaction by casein kinase 1 alpha." *Mol Cell Biol* **25**(15): 6509-20.

Chen, Y. C., M. Y. Chang, A. L. Shiau, Y. T. Yo and C. L. Wu (2006). "Mitochondrial ribosomal protein S36 delays cell cycle progression in association with p53 modification and p21(WAF1/CIP1) expression." *J Cell Biochem*.

Choesmel, V., D. Bacqueville, J. Rouquette, J. Noaillac-Depeyre, S. Fribourg, A. Cretien, T. Leblanc, G. Tchernia, L. Dacosta and P. E. Gleizes (2006). "Impaired ribosome biogenesis in Diamond-Blackfan anemia." *Blood*.

Chuikov, S., J. K. Kurash, J. R. Wilson, B. Xiao, N. Justin, G. S. Ivanov, K. McKinney, P. Tempst, C. Prives, S. J. Gamblin, N. A. Barlev and D. Reinberg (2004). "Regulation of p53 activity through lysine methylation." *Nature* **432**(7015): 353-60.

Ciarmatori, S., P. H. Scott, J. E. Sutcliffe, A. McLees, H. M. Alzuherri, J. H. Dannenberg, H. te Riele, I. Grummt, R. Voit and R. J. White (2001). "Overlapping functions of the pRb family in the regulation of rRNA synthesis." *Mol Cell Biol* **21**(17): 5806-14.

Clarke, R. B., A. Howell and E. Anderson (1997). "Type I insulin-like growth factor receptor gene expression in normal human breast tissue treated with oestrogen and progesterone." *Br J Cancer* **75**(2): 251-7.

Colombo, E., J. C. Marine, D. Danovi, B. Falini and P. G. Pelicci (2002). "Nucleophosmin regulates the stability and transcriptional activity of p53." *Nat Cell Biol* **4**(7): 529-33.

Comings, D. E. (1973). "A general theory of carcinogenesis." *Proc Natl Acad Sci U S A* **70**(12): 3324-8.

Crompton, A. M. and D. H. Kirn (2007). "From ONYX-015 to armed vaccinia viruses: the education and evolution of oncolytic virus development." *Curr Cancer Drug Targets* **7**(2): 133-9.

166

Cummins, J. M., C. Rago, M. Kohli, K. W. Kinzler, C. Lengauer and B. Vogelstein (2004). "Tumour suppression: disruption of HAUSP gene stabilizes p53." *Nature* **428**(6982): 1 p following 486.

Da Costa, L., G. Tchernia, P. Gascard, A. Lo, J. Meerpohl, C. Niemeyer, J. A. Chasis, J. Fixler and N. Mohandas (2003). "Nucleolar localization of RPS19 protein in normal cells and mislocalization due to mutations in the nucleolar localization signals in 2 Diamond-Blackfan anemia patients: potential insights into pathophysiology." *Blood* **101**(12): 5039-45.

Dachs, G. U. and G. M. Tozer (2000). "Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation." *Eur J Cancer* **36**(13 Spec No): 1649-60.

Dai, M. S. and H. Lu (2004). "Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5." *J Biol Chem* **279**(43): 44475-82.

Dai, M. S., D. Shi, Y. Jin, X. X. Sun, Y. Zhang, S. R. Grossman and H. Lu (2006). "Regulation of the MDM2-p53 pathway by ribosomal protein L11 involves a post-ubiquitination mechanism." *J Biol Chem* **281**(34): 24304-13.

Dai, M. S., S. X. Zeng, Y. Jin, X. X. Sun, L. David and H. Lu (2004). "Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition." *Mol Cell Biol* **24**(17): 7654-68.

Dang, C. V. and W. M. Lee (1989). "Nuclear and nucleolar targeting sequences of c-erb-A, c-myb, N-myc, p53, HSP70, and HIV tat proteins." *J Biol Chem* **264**(30): 18019-23.

Dang, J., M. L. Kuo, C. M. Eischen, L. Stepanova, C. J. Sherr and M. F. Roussel (2002). "The RING domain of Mdm2 can inhibit cell proliferation." *Cancer Res* **62**(4): 1222-30.

Danovi, D., E. Meulmeester, D. Pasini, D. Migliorini, M. Capra, R. Frenk, P. de Graaf, S. Francoz, P. Gasparini, A. Gobbi, K. Helin, P. G. Pelicci, A. G. Jochemsen and J. C. Marine (2004). "Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity." *Mol Cell Biol* **24**(13): 5835-43.

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Datta, S. R., A. Brunet and M. E. Greenberg (1999). "Cellular survival: a play in three Akts." *Genes Dev* **13**(22): 2905-27.

de Graaf, P., N. A. Little, Y. F. Ramos, E. Meulmeester, S. J. Letteboer and A. G. Jochemsen (2003). "Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2." *J Biol Chem* **278**(40): 38315-24.

Delphin, C. and J. Baudier (1994). "The protein kinase C activator, phorbol ester, cooperates with the wild-type p53 species of Ras-transformed embryo fibroblasts growth arrest." *J Biol Chem* **269**(47): 29579-87.

Di Stefano, V., G. Blandino, A. Sacchi, S. Soddu and G. D'Orazi (2004). "HIPK2 neutralizes MDM2 inhibition rescuing p53 transcriptional activity and apoptotic function." *Oncogene* **23**(30): 5185-92.

Dirac, A. M. and R. Bernards (2003). "Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53." *J Biol Chem* **278**(14): 11731-4.

Dittmer, D., S. Pati, G. Zambetti, S. Chu, A. K. Teresky, M. Moore, C. Finlay and A. J. Levine (1993). "Gain of function mutations in p53." *Nat Genet* **4**(1): 42-6.

Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel and A. Bradley (1992). "Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours." *Nature* **356**(6366): 215-21.

Dornan, D., I. Wertz, H. Shimizu, D. Arnott, G. D. Frantz, P. Dowd, K. O'Rourke, H. Koeppen and V. M. Dixit (2004). "The ubiquitin ligase COP1 is a critical negative regulator of p53." *Nature* **429**(6987): 86-92.

Downward, J. (1998). "Ras signalling and apoptosis." *Curr Opin Genet Dev* **8**(1): 49-54.

Draptchinskaia, N., P. Gustavsson, B. Andersson, M. Pettersson, T. N. Willig, I. Dianzani, S. Ball, G. Tchernia, J. Klar, H. Matsson, D. Tentler, N. Mohandas, B. Carlsson and N. Dahl (1999). "The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia." *Nat Genet* **21**(2): 169-75.

Dworakowska, D., E. Jassem, J. Jassem, B. Peters, R. Dziadziuszko, M. Zylicz, J. Jakobkiewicz-Banecka, G. Kobierska-Gulida, A. Szymanowska, J. Skokowski, A.

Roessner and R. Schneider-Stock (2004). "MDM2 gene amplification: a new independent factor of adverse prognosis in non-small cell lung cancer (NSCLC)." *Lung Cancer* **43**(3): 285-95.

el-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang and et al. (1994). "WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis." *Cancer Res* **54**(5): 1169-74.

el-Deiry, W. S., S. E. Kern, J. A. Pietenpol, K. W. Kinzler and B. Vogelstein (1992). "Definition of a consensus binding site for p53." *Nat Genet* **1**(1): 45-9.

Elenbaas, B., M. Dobbelstein, J. Roth, T. Shenk and A. J. Levine (1996). "The MDM2 oncoprotein binds specifically to RNA through its RING finger domain." *Mol Med* **2**(4): 439-51.

Elias, B., A. Laine and Z. Ronai (2005). "Phosphorylation of MdmX by CDK2/Cdc2(p34) is required for nuclear export of Mdm2." *Oncogene* **24**(15): 2574-9.

Eliyahu, D., A. Raz, P. Gruss, D. Givol and M. Oren (1984). "Participation of p53 cellular tumour antigen in transformation of normal embryonic cells." *Nature* **312**(5995): 646-9.

Fakharzadeh, S. S., S. P. Trusko and D. L. George (1991). "Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line." *Embo J* **10**(6): 1565-9.

Fambrough, D., K. McClure, A. Kazlauskas and E. S. Lander (1999). "Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes." *Cell* **97**(6): 727-41.

Fang, S., J. P. Jensen, R. L. Ludwig, K. H. Vousden and A. M. Weissman (2000). "Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53." *J Biol Chem* **275**(12): 8945-51.

Feinberg, A. P. and B. Tycko (2004). "The history of cancer epigenetics." *Nat Rev Cancer* **4**(2): 143-53.

Feng, L., T. Lin, H. Uranishi, W. Gu and Y. Xu (2005). "Functional analysis of the roles of posttranslational modifications at the p53 C terminus in regulating p53 stability and activity." *Mol Cell Biol* **25**(13): 5389-95.

Finch, R. A., D. B. Donoviel, D. Potter, M. Shi, A. Fan, D. D. Freed, C. Y. Wang, B. P. Zambrowicz, R. Ramirez-Solis, A. T. Sands and N. Zhang (2002). "mdmx is a negative regulator of p53 activity in vivo." *Cancer Res* **62**(11): 3221-5.

Finlay, C. A., P. W. Hinds and A. J. Levine (1989). "The p53 proto-oncogene can act as a suppressor of transformation." *Cell* **57**(7): 1083-93.

Fonagy, A., C. Swiderski, M. Dunn and J. W. Freeman (1992). "Antisense-mediated specific inhibition of P120 protein expression prevents G1- to S-phase transition." *Cancer Res* **52**(19): 5250-6.

Foster, B. A., H. A. Coffey, M. J. Morin and F. Rastinejad (1999). "Pharmacological rescue of mutant p53 conformation and function." *Science* **286**(5449): 2507-10.

Francoz, S., P. Froment, S. Bogaerts, S. De Clercq, M. Maetens, G. Doumont, E. Bellefroid and J. C. Marine (2006). "Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo." *Proc Natl Acad Sci U S A* **103**(9): 3232-7.

Frazier, M. W., X. He, J. Wang, Z. Gu, J. L. Cleveland and G. P. Zambetti (1998). "Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain." *Mol Cell Biol* **18**(7): 3735-43.

Freedman, D. A., C. B. Epstein, J. C. Roth and A. J. Levine (1997). "A genetic approach to mapping the p53 binding site in the MDM2 protein." *Mol Med* **3**(4): 248-59.

Friedler, A., L. O. Hansson, D. B. Veprintsev, S. M. Freund, T. M. Rippin, P. V. Nikolova, M. R. Proctor, S. Rudiger and A. R. Fersht (2002). "A peptide that binds and stabilizes p53 core domain: chaperone strategy for rescue of oncogenic mutants." *Proc Natl Acad Sci U S A* **99**(2): 937-42.

Garkavtsev, I., I. A. Grigorian, V. S. Ossovskaya, M. V. Chernov, P. M. Chumakov and A. V. Gudkov (1998). "The candidate tumour suppressor p33ING1 cooperates with p53 in cell growth control." *Nature* **391**(6664): 295-8.

Garrington, T. P. and G. L. Johnson (1999). "Organization and regulation of mitogen-activated protein kinase signaling pathways." *Curr Opin Cell Biol* **11**(2): 211-8.

Geoffroy, F. J., C. J. Allegra, B. Sinha and J. L. Grem (1994). "Enhanced cytotoxicity with interleukin-1 alpha and 5-fluorouracil in HCT116 colon cancer cells." *Oncol Res* **6**(12): 581-91.

Geyer, R. K., Z. K. Yu and C. G. Maki (2000). "The MDM2 RING-finger domain is required to promote p53 nuclear export." *Nat Cell Biol* **2**(9): 569-73.

Ghoshal, K. and S. T. Jacob (1994). "Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil." *Cancer Res* **54**(3): 632-6.

Gilkes, D. M., L. Chen and J. Chen (2006). "MDMX regulation of p53 response to ribosomal stress." *Embo J* **25**(23): 5614-25.

Gille, H., M. Kortenjann, O. Thomae, C. Moomaw, C. Slaughter, M. H. Cobb and P. E. Shaw (1995). "ERK phosphorylation potentiates Elk-1-mediated ternary complex formation and transactivation." *Embo J* **14**(5): 951-62.

Goh, H. S., J. Yao and D. R. Smith (1995). "p53 point mutation and survival in colorectal cancer patients." *Cancer Res* **55**(22): 5217-21.

Goldberg, Z., R. Vogt Sionov, M. Berger, Y. Zwang, R. Perets, R. A. Van Etten, M. Oren, Y. Taya and Y. Haupt (2002). "Tyrosine phosphorylation of Mdm2 by c-Abl: implications for p53 regulation." *Embo J* **21**(14): 3715-27.

Gostissa, M., A. Hengstermann, V. Fogal, P. Sandy, S. E. Schwarz, M. Scheffner and G. Del Sal (1999). "Activation of p53 by conjugation to the ubiquitin-like protein SUMO-1." *Embo J* **18**(22): 6462-71.

Green, D. R. and G. I. Evan (2002). "A matter of life and death." *Cancer Cell* **1**(1): 19-30.

Grier, J. D., S. Xiong, A. C. Elizondo-Fraire, J. M. Parant and G. Lozano (2006). "Tissue-specific differences of p53 inhibition by Mdm2 and Mdm4." *Mol Cell Biol* **26**(1): 192-8.

Grossman, S. R., M. E. Deato, C. Brignone, H. M. Chan, A. L. Kung, H. Tagami, Y. Nakatani and D. M. Livingston (2003). "Polyubiquitination of p53 by a ubiquitin ligase activity of p300." *Science* **300**(5617): 342-4.

Groth, A., J. D. Weber, B. M. Willumsen, C. J. Sherr and M. F. Roussel (2000). "Oncogenic Ras induces p19ARF and growth arrest in mouse embryo fibroblasts lacking p21Cip1 and p27Kip1 without activating cyclin D-dependent kinases." *J Biol Chem* **275**(35): 27473-80.

Gu, J., H. Kawai, L. Nie, H. Kitao, D. Wiederschain, A. G. Jochemsen, J. Parant, G. Lozano and Z. M. Yuan (2002). "Mutual dependence of MDM2 and MDMX in their functional inactivation of p53." *J Biol Chem* **277**(22): 19251-4.

Guo, A., P. Salomoni, J. Luo, A. Shih, S. Zhong, W. Gu and P. P. Pandolfi (2000). "The function of PML in p53-dependent apoptosis." *Nat Cell Biol* **2**(10): 730-6.

Hainaut, P. and M. Hollstein (2000). "p53 and human cancer: the first ten thousand mutations." *Adv Cancer Res* **77**: 81-137.

Hall, S. R., L. E. Campbell and D. W. Meek (1996). "Phosphorylation of p53 at the casein kinase II site selectively regulates p53-dependent transcriptional repression but not transactivation." *Nucleic Acids Res* **24**(6): 1119-26.

Han, X., G. Garcia-Manero, T. J. McDonnell, G. Lozano, L. J. Medeiros, L. Xiao, G. Rosner, M. Nguyen, M. Fernandez, Y. A. Valentin-Vega, J. Barboza, D. M. Jones, G. Z. Rassidakis, H. M. Kantarjian and C. E. Bueso-Ramos (2007). "HDM4 (HDMX) is widely expressed in adult pre-B acute lymphoblastic leukemia and is a potential therapeutic target." *Mod Pathol* **20**(1): 54-62.

Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." *Cell* **100**(1): 57-70.

Hannan, K. M., R. D. Hannan, S. D. Smith, L. S. Jefferson, M. Lun and L. I. Rothblum (2000). "Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1." *Oncogene* **19**(43): 4988-99.

Hannan, K. M., B. K. Kennedy, A. H. Cavanaugh, R. D. Hannan, I. Hirschler-Laszkiewicz, L. S. Jefferson and L. I. Rothblum (2000). "RNA polymerase I transcription in confluent cells: Rb downregulates rDNA transcription during confluence-induced cell cycle arrest." *Oncogene* **19**(31): 3487-97.

Harris, H., O. J. Miller, G. Klein, P. Worst and T. Tachibana (1969). "Suppression of malignancy by cell fusion." *Nature* **223**(5204): 363-8.

Harvey, M., H. Vogel, D. Morris, A. Bradley, A. Bernstein and L. A. Donehower (1995). "A mutant p53 transgene accelerates tumour development in heterozygous but not nullizygous p53-deficient mice." *Nat Genet* **9**(3): 305-11.

Haupt, Y., R. Maya, A. Kazaz and M. Oren (1997). "Mdm2 promotes the rapid degradation of p53." *Nature* **387**(6630): 296-9.

Haupt, Y., R. Maya, A. Kazaz and M. Oren (1997). "Mdm2 promotes the rapid degradation of p53." *Nature* **387**(6630): 296-299.

Hay, T. J. and D. W. Meek (2000). "Multiple sites of in vivo phosphorylation in the MDM2 oncoprotein cluster within two important functional domains." *FEBS Lett* **478**(1-2): 183-6.

He, H. and Y. Sun (2006). "Ribosomal protein S27L is a direct p53 target that regulates apoptosis." *Oncogene*.

Heise, C., A. Sampson-Johannes, A. Williams, F. McCormick, D. D. Von Hoff and D. H. Kirn (1997). "ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents." *Nat Med* **3**(6): 639-45.

Herbig, U., W. A. Jobling, B. P. Chen, D. J. Chen and J. M. Sedivy (2004). "Telomere shortening triggers senescence of human cells through a pathway involving ATM, p53, and p21(CIP1), but not p16(INK4a)." *Mol Cell* **14**(4): 501-13.

Heron-Milhavet, L. and D. LeRoith (2002). "Insulin-like growth factor I induces MDM2-dependent degradation of p53 via the p38 MAPK pathway in response to DNA damage." *J Biol Chem* **277**(18): 15600-6.

Hill, C. S., R. Marais, S. John, J. Wynne, S. Dalton and R. Treisman (1993). "Functional analysis of a growth factor-responsive transcription factor complex." *Cell* **73**(2): 395-406.

Hinds, P. W., C. A. Finlay, A. B. Frey and A. J. Levine (1987). "Immunological evidence for the association of p53 with a heat shock protein, hsc70, in p53-plus-ras-transformed cell lines." *Mol Cell Biol* **7**(8): 2863-9.

Hirao, A., Y. Y. Kong, S. Matsuoka, A. Wakeham, J. Ruland, H. Yoshida, D. Liu, S. J. Elledge and T. W. Mak (2000). "DNA damage-induced activation of p53 by the checkpoint kinase Chk2." *Science* **287**(5459): 1824-7.

Hoffman, W. H., S. Biade, J. T. Zilfou, J. Chen and M. Murphy (2002). "Transcriptional repression of the anti-apoptotic survivin gene by wild type p53." *J Biol Chem* **277**(5): 3247-57.

Honda, R. and H. Yasuda (1999). "Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53." *Embo J* **18**(1): 22-7.

Honda, R. and H. Yasuda (2000). "Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase." *Oncogene* **19**(11): 1473-6.

Houghton, J. A., P. J. Houghton and R. S. Wooten (1979). "Mechanism of induction of gastrointestinal toxicity in the mouse by 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2'-deoxyuridine." *Cancer Res* **39**(7 Pt 1): 2406-13.

Hsieh, J. K., F. S. Chan, D. J. O'Connor, S. Mittnacht, S. Zhong and X. Lu (1999). "RB regulates the stability and the apoptotic function of p53 via MDM2." *Mol Cell* **3**(2): 181-93.

Hu, B., D. M. Gilkes and J. Chen (2007). "Efficient p53 activation and apoptosis by simultaneous disruption of binding to MDM2 and MDMX." *Cancer Res* **67**(18): 8810-7.

Hu, B., D. M. Gilkes, B. Farooqi, S. M. Sebti and J. Chen (2006). "MDMX overexpression prevents p53 activation by the MDM2 inhibitor Nutlin." *J Biol Chem* **281**(44): 33030-5.

Iapalucci-Espinoza, S. and M. T. Franze-Fernandez (1979). "Effect of protein synthesis inhibitors and low concentrations of actinomycin D on ribosomal RNA synthesis." *FEBS Lett* **107**(2): 281-4.

Issaeva, N., P. Bozko, M. Enge, M. Protopopova, L. G. Verhoef, M. Masucci, A. Pramanik and G. Selivanova (2004). "Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors." *Nat Med* **10**(12): 1321-8.

Itahana, K., K. P. Bhat, A. Jin, Y. Itahana, D. Hawke, R. Kobayashi and Y. Zhang (2003). "Tumor suppressor ARF degrades B23, a nucleolar protein involved in ribosome biogenesis and cell proliferation." *Mol Cell* **12**(5): 1151-64.

Ito, A., Y. Kawaguchi, C. H. Lai, J. J. Kovacs, Y. Higashimoto, E. Appella and T. P. Yao (2002). "MDM2-HDAC1-mediated deacetylation of p53 is required for its degradation." *Embo J* **21**(22): 6236-45.

Jack, M. T., R. A. Woo, A. Hirao, A. Cheung, T. W. Mak and P. W. Lee (2002). "Chk2 is dispensable for p53-mediated G1 arrest but is required for a latent p53-mediated apoptotic response." *Proc Natl Acad Sci U S A* **99**(15): 9825-9.

Jackson, M. W. and S. J. Berberich (2000). "MdmX protects p53 from Mdm2-mediated degradation." *Mol Cell Biol* **20**(3): 1001-7.

Jackson, M. W., L. E. Patt, G. A. LaRusch, D. B. Donner, G. R. Stark and L. D. Mayo (2006). "Hdm2 nuclear export, regulated by insulin-like growth factor-I/MAPK/p90Rsk signaling, mediates the transformation of human cells." *J Biol Chem* **281**(24): 16814-20.

Jin, A., K. Itahana, K. O'Keefe and Y. Zhang (2004). "Inhibition of HDM2 and activation of p53 by ribosomal protein L23." *Mol Cell Biol* **24**(17): 7669-80.

Jin, Y., M. S. Dai, S. Z. Lu, Y. Xu, Z. Luo, Y. Zhao and H. Lu (2006). "14-3-3gamma binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation." *Embo J* **25**(6): 1207-18.

Johnson-Pais, T., C. Degnin and M. J. Thayer (2001). "pRB induces Sp1 activity by relieving inhibition mediated by MDM2." *Proc Natl Acad Sci U S A* **98**(5): 2211-6.

Jones, S. N., A. R. Hancock, H. Vogel, L. A. Donehower and A. Bradley (1998). "Overexpression of Mdm2 in mice reveals a p53-independent role for Mdm2 in tumorigenesis." *Proc Natl Acad Sci U S A* **95**(26): 15608-12.

Jones, S. N., A. E. Roe, L. A. Donehower and A. Bradley (1995). "Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53." *Nature* **378**(6553): 206-8.

Kamijo, T., F. Zindy, M. F. Roussel, D. E. Quelle, J. R. Downing, R. A. Ashmun, G. Grosveld and C. J. Sherr (1997). "Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF." *Cell* **91**(5): 649-59.

Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein and R. W. Craig (1991). "Participation of p53 protein in the cellular response to DNA damage." *Cancer Res* **51**(23 Pt 1): 6304-11.

Kawai, H., D. Wiederschain, H. Kitao, J. Stuart, K. K. Tsai and Z. M. Yuan (2003). "DNA damage-induced MDMX degradation is mediated by MDM2." *J Biol Chem* **278**(46): 45946-53.

Kawai, H., D. Wiederschain and Z. M. Yuan (2003). "Critical contribution of the MDM2 acidic domain to p53 ubiquitination." *Mol Cell Biol* **23**(14): 4939-47.

Kendall, A., R. Gillmore and E. Newlands (2003). "Chemotherapy for trophoblastic disease: current standards." *Expert Rev Anticancer Ther* **3**(1): 48-54.

Kim, S. B., G. W. Chae, J. Lee, J. Park, H. Tak, J. H. Chung, T. G. Park, J. K. Ahn and C. O. Joe (2007). "Activated Notch1 interacts with p53 to inhibit its phosphorylation and transactivation." *Cell Death Differ* **14**(5): 982-91.

Kim, W. Y. and N. E. Sharpless (2006). "The regulation of INK4/ARF in cancer and aging." *Cell* **127**(2): 265-75.

Knudson, A. G., Jr. (1971). "Mutation and cancer: statistical study of retinoblastoma." *Proc Natl Acad Sci U S A* **68**(4): 820-3.

Kopnin, B. P. (2000). "Targets of oncogenes and tumor suppressors: key for understanding basic mechanisms of carcinogenesis." *Biochemistry (Mosc)* **65**(1): 2-27.

Korgaonkar, C., J. Hagen, V. Tompkins, A. A. Frazier, C. Allamargot, F. W. Quelle and D. E. Quelle (2005). "Nucleophosmin (B23) targets ARF to nucleoli and inhibits its function." *Mol Cell Biol* **25**(4): 1258-71.

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Korgaonkar, C., L. Zhao, M. Modestou and D. E. Quelle (2002). "ARF function does not require p53 stabilization or Mdm2 relocalization." *Mol Cell Biol* **22**(1): 196-206.

Koutsodontis, G., I. Tentes, P. Papakosta, A. Moustakas and D. Kardassis (2001). "Sp1 plays a critical role in the transcriptional activation of the human cyclin-dependent kinase inhibitor p21(WAF1/Cip1) gene by the p53 tumor suppressor protein." *J Biol Chem* **276**(31): 29116-25.

Krummel, K. A., C. J. Lee, F. Toledo and G. M. Wahl (2005). "The C-terminal lysines fine-tune P53 stress responses in a mouse model but are not required for stability control or transactivation." *Proc Natl Acad Sci U S A* **102**(29): 10188-93.

Kubbutat, M. H. G., S. N. Jones and K. H. Vousden (1997). "Regulation of p53 stability by Mdm2." *Nature* **387**(6630): 299-303.

Kurz, E. U. and S. P. Lees-Miller (2004). "DNA damage-induced activation of ATM and ATM-dependent signaling pathways." *DNA Repair (Amst)* **3**(8-9): 889-900.

Kussie, P. H., S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A. J. Levine and N. P. Pavletich (1996). "Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain." *Science* **274**(5289): 948-53.

Lai, Z., K. V. Ferry, M. A. Diamond, K. E. Wee, Y. B. Kim, J. Ma, T. Yang, P. A. Benfield, R. A. Copeland and K. R. Auger (2001). "Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization." *J Biol Chem* **276**(33): 31357-67.

Landers, J. E., S. L. Cassel and D. L. George (1997). "Translational enhancement of mdm2 oncogene expression in human tumor cells containing a stabilized wild-type p53 protein." *Cancer Res* **57**(16): 3562-8.

Lane, D. P. and L. V. Crawford (1979). "T antigen is bound to a host protein in SV40-transformed cells." *Nature* **278**(5701): 261-3.

Larsson, O., A. Girnita and L. Girnita (2005). "Role of insulin-like growth factor 1 receptor signalling in cancer." *Br J Cancer* **92**(12): 2097-101.

Laurie, N. A., S. L. Donovan, C. S. Shih, J. Zhang, N. Mills, C. Fuller, A. Teunisse, S. Lam, Y. Ramos, A. Mohan, D. Johnson, M. Wilson, C. Rodriguez-Galindo, M. Quarto, S. Francoz, S. M. Mendrysa, R. K. Guy, J. C. Marine, A. G. Jochemsen and M. A. Dyer (2006). "Inactivation of the p53 pathway in retinoblastoma." *Nature* **444**(7115): 61-6.

Leach, F. S., T. Tokino, P. Meltzer, M. Burrell, J. D. Oliner, S. Smith, D. E. Hill, D. Sidransky, K. W. Kinzler and B. Vogelstein (1993). "p53 Mutation and MDM2 amplification in human soft tissue sarcomas." *Cancer Res* **53**(10 Suppl): 2231-4.

LeBron, C., L. Chen, D. M. Gilkes and J. Chen (2006). "Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3." *Embo J* **25**(6): 1196-206.

Lee, K. C., A. J. Crowe and M. C. Barton (1999). "p53-mediated repression of alpha-fetoprotein gene expression by specific DNA binding." *Mol Cell Biol* **19**(2): 1279-88.

Leng, P., D. R. Brown, C. V. Shivakumar, S. Deb and S. P. Deb (1995). "N-terminal 130 amino acids of MDM2 are sufficient to inhibit p53-mediated transcriptional activation." *Oncogene* **10**(7): 1275-82.

Leng, R. P., Y. Lin, W. Ma, H. Wu, B. Lemmers, S. Chung, J. M. Parant, G. Lozano, R. Hakem and S. Benchimol (2003). "Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation." *Cell* **112**(6): 779-91.

Lengauer, C., K. W. Kinzler and B. Vogelstein (1998). "Genetic instabilities in human cancers." *Nature* **396**(6712): 643-9.

Leri, A., Y. Liu, P. P. Claudio, J. Kajstura, X. Wang, S. Wang, P. Kang, A. Malhotra and P. Anversa (1999). "Insulin-like growth factor-1 induces Mdm2 and down-regulates p53, attenuating the myocyte renin-angiotensin system and stretch-mediated apoptosis." *Am J Pathol* **154**(2): 567-80.

Leveillard, T., P. Gorry, K. Niederreither and B. Wasylyk (1998). "MDM2 expression during mouse embryogenesis and the requirement of p53." *Mech Dev* **74**(1-2): 189-93.

Li, C., L. Chen and J. Chen (2002). "DNA damage induces MDMX nuclear translocation by p53-dependent and -independent mechanisms." *Mol Cell Biol* **22**(21): 7562-71.

Li, M., C. L. Brooks, N. Kon and W. Gu (2004). "A dynamic role of HAUSP in the p53-Mdm2 pathway." *Mol Cell* **13**(6): 879-86.

Li, M., C. L. Brooks, F. Wu-Baer, D. Chen, R. Baer and W. Gu (2003). "Mono- versus polyubiquitination: differential control of p53 fate by Mdm2." *Science* **302**(5652): 1972-5.

Li, M., D. Chen, A. Shiloh, J. Luo, A. Y. Nikolaev, J. Qin and W. Gu (2002). "Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization." *Nature* **416**(6881): 648-53.

Lim, Y. P., T. T. Lim, Y. L. Chan, A. C. Song, B. H. Yeo, B. Vojtesek, D. Coomber, G. Rajagopal and D. Lane (2007). "The p53 knowledgebase: an integrated information resource for p53 research." *Oncogene* **26**(11): 1517-21.

Lin, J., J. Chen, B. Elenbaas and A. J. Levine (1994). "Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein." *Genes Dev* **8**(10): 1235-46.

Linares, L. K., A. Hengstermann, A. Ciechanover, S. Muller and M. Scheffner (2003). "HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53." *Proc Natl Acad Sci U S A* **100**(21): 12009-14.

Lincoln, D. W., 2nd and K. Bove (2005). "The transcription factor Ets-1 in breast cancer." *Front Biosci* **10**: 506-11.

Lindstrom, M. S., A. Jin, C. Deisenroth, G. White Wolf and Y. Zhang (2007). "Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation." *Mol Cell Biol* **27**(3): 1056-68.

Lindstrom, M. S., A. Jin, C. Deisenroth, G. W. Wolf and Y. Zhang (2006). "Cancer-Associated Mutations in MDM2 Zinc Finger Domain Disrupt Ribosomal Protein Interaction and Attenuate MDM2-Induced p53 Degradation." *Mol Cell Biol*.

Linzer, D. I. and A. J. Levine (1979). "Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells." *Cell* **17**(1): 43-52.

Liu, Q., S. Kaneko, L. Yang, R. I. Feldman, S. V. Nicosia, J. Chen and J. Q. Cheng (2004). "Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215." *J Biol Chem* **279**(50): 52175-82.

Liu, S., Y. Liang, H. Huang, L. Wang, Y. Li, J. Li, X. Li and H. Wang (2005). "ERK-dependent signaling pathway and transcriptional factor Ets-1 regulate matrix metalloproteinase-9 production in transforming growth factor-beta1 stimulated glomerular podocytes." *Cell Physiol Biochem* **16**(4-6): 207-16.

Llanos, S., P. A. Clark, J. Rowe and G. Peters (2001). "Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus." *Nat Cell Biol* **3**(5): 445-52.

Lohrum, M. A., M. Ashcroft, M. H. Kubbutat and K. H. Vousden (2000). "Contribution of two independent MDM2-binding domains in p14(ARF) to p53 stabilization." *Curr Biol* **10**(9): 539-42.

Lohrum, M. A., M. Ashcroft, M. H. Kubbutat and K. H. Vousden (2000). "Identification of a cryptic nucleolar-localization signal in MDM2." *Nat Cell Biol* **2**(3): 179-81.

Lohrum, M. A., R. L. Ludwig, M. H. Kubbutat, M. Hanlon and K. H. Vousden (2003). "Regulation of HDM2 activity by the ribosomal protein L11." *Cancer Cell* **3**(6): 577-87.

Longley, D. B., J. Boyer, W. L. Allen, T. Latif, P. R. Ferguson, P. J. Maxwell, U. McDermott, M. Lynch, D. P. Harkin and P. G. Johnston (2002). "The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates." *Cancer Res* **62**(9): 2644-9.

Longley, D. B., D. P. Harkin and P. G. Johnston (2003). "5-fluorouracil: mechanisms of action and clinical strategies." *Nat Rev Cancer* **3**(5): 330-8.

Lopez-Girona, A., B. Furnari, O. Mondesert and P. Russell (1999). "Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein." *Nature* **397**(6715): 172-5.

Loughran, O. and N. B. La Thangue (2000). "Apoptotic and growth-promoting activity of E2F modulated by MDM2." *Mol Cell Biol* **20**(6): 2186-97.

Lu, H. and A. J. Levine (1995). "Human TAFII31 protein is a transcriptional coactivator of the p53 protein." *Proc Natl Acad Sci U S A* **92**(11): 5154-8.

Lu, W., J. Lin and J. Chen (2002). "Expression of p14ARF overcomes tumor resistance to p53." *Cancer Res* **62**(5): 1305-10.

Lu, W., R. Pochampally, L. Chen, M. Traidej, Y. Wang and J. Chen (2000). "Nuclear exclusion of p53 in a subset of tumors requires MDM2 function." *Oncogene* **19**(2): 232-40.

Ma, J., J. D. Martin, H. Zhang, K. R. Auger, T. F. Ho, R. B. Kirkpatrick, M. H. Grooms, K. O. Johanson, P. J. Tummino, R. A. Copeland and Z. Lai (2006). "A second p53 binding site in the central domain of Mdm2 is essential for p53 ubiquitination." *Biochemistry* **45**(30): 9238-45.

Macleod, K., D. Leprince and D. Stehelin (1992). "The ets gene family." *Trends Biochem Sci* **17**(7): 251-6.

Macleod, K. F., N. Sherry, G. Hannon, D. Beach, T. Tokino, K. Kinzler, B. Vogelstein and T. Jacks (1995). "p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage." *Genes Dev* **9**(8): 935-44.

MacPherson, D., J. Kim, T. Kim, B. K. Rhee, C. T. Van Oostrom, R. A. DiTullio, M. Venere, T. D. Halazonetis, R. Bronson, A. De Vries, M. Fleming and T. Jacks (2004). "Defective apoptosis and B-cell lymphomas in mice with p53 point mutation at Ser 23." *Embo J* **23**(18): 3689-99.

Malkin, D., F. P. Li, L. C. Strong, J. F. Fraumeni, Jr., C. E. Nelson, D. H. Kim, J. Kassel, M. A. Gryka, F. Z. Bischoff, M. A. Tainsky and et al. (1990). "Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms." *Science* **250**(4985): 1233-8.

Marais, R., J. Wynne and R. Treisman (1993). "The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain." *Cell* **73**(2): 381-93.

Marechal, V., B. Elenbaas, J. Piette, J. C. Nicolas and A. J. Levine (1994). "The ribosomal L5 protein is associated with mdm-2 and mdm-2-p53 complexes." *Mol Cell Biol* **14**(11): 7414-20.

Marechal, V., B. Elenbaas, L. Taneyhill, J. Piette, M. Mechali, J. C. Nicolas, A. J. Levine and J. Moreau (1997). "Conservation of structural domains and biochemical activities of the MDM2 protein from Xenopus laevis." *Oncogene* **14**(12): 1427-33.

Marine, J. C. and A. G. Jochemsen (2005). "Mdmx as an essential regulator of p53 activity." *Biochem Biophys Res Commun* **331**(3): 750-60.

Martin, K., D. Trouche, C. Hagemeier, T. S. Sorensen, N. B. La Thangue and T. Kouzarides (1995). "Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein." *Nature* **375**(6533): 691-4.

Maya, R., M. Balass, S. T. Kim, D. Shkedy, J. F. Leal, O. Shifman, M. Moas, T. Buschmann, Z. Ronai, Y. Shiloh, M. B. Kastan, E. Katzir and M. Oren (2001). "ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage." *Genes Dev* **15**(9): 1067-77.

McCormick, F. (2000). "Interactions between adenovirus proteins and the p53 pathway: the development of ONYX-015." *Semin Cancer Biol* **10**(6): 453-9.

McMasters, K. M., R. Montes de Oca Luna, J. R. Pena and G. Lozano (1996). "mdm2 deletion does not alter growth characteristics of p53-deficient embryo fibroblasts." *Oncogene* **13**(8): 1731-6.

Meek, D. W. and U. Knippschild (2003). "Posttranslational modification of MDM2." *Mol Cancer Res* **1**(14): 1017-26.

Mendrysa, S. M., M. K. McElwee, J. Michalowski, K. A. O'Leary, K. M. Young and M. E. Perry (2003). "mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation." *Mol Cell Biol* **23**(2): 462-72.

Meulmeester, E., Y. Pereg, Y. Shiloh and A. G. Jochemsen (2005). "ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation." *Cell Cycle* **4**(9): 1166-70.

Migliorini, D., D. Danovi, E. Colombo, R. Carbone, P. G. Pelicci and J. C. Marine (2002). "Hdmx recruitment into the nucleus by Hdm2 is essential for its ability to regulate p53 stability and transactivation." *J Biol Chem* **277**(9): 7318-23.

Migliorini, D., E. Lazzerini Denchi, D. Danovi, A. Jochemsen, M. Capillo, A. Gobbi, K. Helin, P. G. Pelicci and J. C. Marine (2002). "Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development." *Mol Cell Biol* **22**(15): 5527-38.

Miyashita, T. and J. C. Reed (1995). "Tumor suppressor p53 is a direct transcriptional activator of the human bax gene." *Cell* **80**(2): 293-9.

Miyazaki, J., K. Kawai, H. Hayashi, M. Onozawa, S. Tsukamoto, N. Miyanaga, S. Hinotsu, T. Shimazui and H. Akaza (2003). "The limited efficacy of methotrexate, actinomycin D and cisplatin (MAP) for patients with advanced testicular cancer." *Jpn J Clin Oncol* **33**(8): 391-5.

Moll, U. M. and O. Petrenko (2003). "The MDM2-p53 interaction." *Mol Cancer Res* **1**(14): 1001-8.

Momand, J., D. Jung, S. Wilczynski and J. Niland (1998). "The MDM2 gene amplification database." *Nucleic Acids Res* **26**(15): 3453-9.

Momand, J., G. P. Zambetti, D. C. Olson, D. George and A. J. Levine (1992). "The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation." *Cell* **69**(7): 1237-45.

Mora, M., Z. Darzynkiewicz and R. Baserga (1980). "DNA synthesis and cell division in a mammalian cell mutant temperature sensitive for the processing of ribosomal RNA." *Exp Cell Res* **125**(1): 241-9.

Moroni, M. C., E. S. Hickman, E. Lazzerini Denchi, G. Caprara, E. Colli, F. Cecconi, H. Muller and K. Helin (2001). "Apaf-1 is a transcriptional target for E2F and p53." *Nat Cell Biol* **3**(6): 552-8.

Mowat, M., A. Cheng, N. Kimura, A. Bernstein and S. Benchimol (1985). "Rearrangements of the cellular p53 gene in erythroleukaemic cells transformed by Friend virus." *Nature* **314**(6012): 633-6.

Muller, M., S. Wilder, D. Bannasch, D. Israeli, K. Lehlbach, M. Li-Weber, S. L. Friedman, P. R. Galle, W. Stremmel, M. Oren and P. H. Krammer (1998). "p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs." *J Exp Med* **188**(11): 2033-45.

Murphy, M., J. Ahn, K. K. Walker, W. H. Hoffman, R. M. Evans, A. J. Levine and D. L. George (1999). "Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a." *Genes Dev* **13**(19): 2490-501.

Muthusamy, V., C. Hobbs, C. Nogueira, C. Cordon-Cardo, P. H. McKee, L. Chin and M. W. Bosenberg (2006). "Amplification of CDK4 and MDM2 in malignant melanoma." *Genes Chromosomes Cancer* **45**(5): 447-54.

Nakamura, S., J. A. Roth and T. Mukhopadhyay (2000). "Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination." *Mol Cell Biol* **20**(24): 9391-8.

Nakano, K. and K. H. Vousden (2001). "PUMA, a novel proapoptotic gene, is induced by p53." *Mol Cell* **7**(3): 683-94.

Nazar, R. N. (2004). "Ribosomal RNA processing and ribosome biogenesis in eukaryotes." *IUBMB Life* **56**(8): 457-65.

Newlands, E. S. (2003). "The management of recurrent and drug-resistant gestational trophoblastic neoplasia (GTN)." *Best Pract Res Clin Obstet Gynaecol* **17**(6): 905-23.

Nikolova, P. V., K. B. Wong, B. DeDecker, J. Henckel and A. R. Fersht (2000). "Mechanism of rescue of common p53 cancer mutations by second-site suppressor mutations." *Embo J* **19**(3): 370-8.

O'Brate, A. and P. Giannakakou (2003). "The importance of p53 location: nuclear or cytoplasmic zip code?" *Drug Resist Updat* **6**(6): 313-22.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi and N. Tanaka (2000). "Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis." *Science* **288**(5468): 1053-8.

Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura and Y. Taya (2000). "p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53." *Cell* **102**(6): 849-62.

Okamoto, K. and D. Beach (1994). "Cyclin G is a transcriptional target of the p53 tumor suppressor protein." *Embo J* **13**(20): 4816-22.

Okamoto, K., K. Kashima, Y. Pereg, M. Ishida, S. Yamazaki, A. Nota, A. Teunisse, D. Migliorini, I. Kitabayashi, J. C. Marine, C. Prives, Y. Shiloh, A. G. Jochemsen and Y. Taya (2005). "DNA damage-induced phosphorylation of MdmX at serine 367 activates p53 by targeting MdmX for Mdm2-dependent degradation." *Mol Cell Biol* **25**(21): 9608-20.

Okamoto, K., H. Li, M. R. Jensen, T. Zhang, Y. Taya, S. S. Thorgeirsson and C. Prives (2002). "Cyclin G recruits PP2A to dephosphorylate Mdm2." *Mol Cell* **9**(4): 761-71.

Oren, M. and A. J. Levine (1983). "Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen." *Proc Natl Acad Sci U S A* **80**(1): 56-9.

Ory, K., Y. Legros, C. Auguin and T. Soussi (1994). "Analysis of the most representative tumour-derived p53 mutants reveals that changes in protein conformation are not correlated with loss of transactivation or inhibition of cell proliferation." *Embo J* **13**(15): 3496-504.

Pan, Y. and J. Chen (2003). "MDM2 promotes ubiquitination and degradation of MDMX." *Mol Cell Biol* **23**(15): 5113-21.

Pan, Y. and J. Chen (2005). "Modification of MDMX by sumoylation." *Biochem Biophys Res Commun* **332**(3): 702-9.

Parada, L. F., H. Land, R. A. Weinberg, D. Wolf and V. Rotter (1984). "Cooperation between gene encoding p53 tumour antigen and ras in cellular transformation." *Nature* **312**(5995): 649-51.

Parant, J. M., V. Reinke, B. Mims and G. Lozano (2001). "Organization, expression, and localization of the murine mdmx gene and pseudogene." *Gene* **270**(1-2): 277-83.

Parker, W. B. and Y. C. Cheng (1990). "Metabolism and mechanism of action of 5-fluorouracil." *Pharmacol Ther* **48**(3): 381-95.

Patton, J. T., L. D. Mayo, A. D. Singhi, A. V. Gudkov, G. R. Stark and M. W. Jackson (2006). "Levels of HdmX expression dictate the sensitivity of normal and transformed cells to Nutlin-3." *Cancer Res* **66**(6): 3169-76.

Pearson, M., R. Carbone, C. Sebastiani, M. Cioce, M. Fagioli, S. Saito, Y. Higashimoto, E. Appella, S. Minucci, P. P. Pandolfi and P. G. Pelicci (2000). "PML regulates p53 acetylation and premature senescence induced by oncogenic Ras." *Nature* **406**(6792): 207-10.

Pereg, Y., D. Shkedy, P. de Graaf, E. Meulmeester, M. Edelson-Averbukh, M. Salek, S. Biton, A. F. Teunisse, W. D. Lehmann, A. G. Jochemsen and Y. Shiloh (2005). "Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage." *Proc Natl Acad Sci U S A* **102**(14): 5056-61.

Perry, M. E., J. Piette, J. A. Zawadzki, D. Harvey and A. J. Levine (1993). "The mdm-2 gene is induced in response to UV light in a p53-dependent manner." *Proc Natl Acad Sci U S A* **90**(24): 11623-7.

Persons, D. L., E. M. Yazlovitskaya and J. C. Pelling (2000). "Effect of extracellular signal-regulated kinase on p53 accumulation in response to cisplatin." *J Biol Chem* **275**(46): 35778-85.

Pestov, D. G., T. M. Grzeszkiewicz and L. F. Lau (1998). "Isolation of growth suppressors from a cDNA expression library." *Oncogene* **17**(24): 3187-97.

Pestov, D. G., Z. Strezoska and L. F. Lau (2001). "Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition." *Mol Cell Biol* **21**(13): 4246-55.

Pim, D. and L. Banks (2004). "p53 polymorphic variants at codon 72 exert different effects on cell cycle progression." *Int J Cancer* **108**(2): 196-9.

Poyurovsky, M. V., X. Jacq, C. Ma, O. Karni-Schmidt, P. J. Parker, M. Chalfie, J. L. Manley and C. Prives (2003). "Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization." *Mol Cell* **12**(4): 875-87.

Poyurovsky, M. V., C. Priest, A. Kentsis, K. L. Borden, Z. Q. Pan, N. Pavletich and C. Prives (2007). "The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin ligase activity." *Embo J* **26**(1): 90-101.

Price, B. D. and S. J. Park (1994). "DNA damage increases the levels of MDM2 messenger RNA in wtp53 human cells." *Cancer Res* **54**(4): 896-9.

Pritchard, D. M., A. J. Watson, C. S. Potten, A. L. Jackman and J. A. Hickman (1997). "Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation." *Proc Natl Acad Sci U S A* **94**(5): 1795-9.

Ragazzini, P., G. Gamberi, L. Pazzaglia, M. Serra, G. Magagnoli, F. Ponticelli, C. Ferrari, C. Ghinelli, M. Alberghini, F. Bertoni, P. Picci and M. S. Benassi (2004). "Amplification of CDK4, MDM2, SAS and GLI genes in leiomyosarcoma, alveolar and embryonal rhabdomyosarcoma." *Histol Histopathol* **19**(2): 401-11.

Rallapalli, R., G. Strachan, B. Cho, W. E. Mercer and D. J. Hall (1999). "A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity." *J Biol Chem* **274**(12): 8299-308.

Ramos, Y. F., R. Stad, J. Attema, L. T. Peltenburg, A. J. van der Eb and A. G. Jochemsen (2001). "Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53." *Cancer Res* **61**(5): 1839-42.

Ravi, R., B. Mookerjee, Z. M. Bhujwalla, C. H. Sutter, D. Artemov, Q. Zeng, L. E. Dillehay, A. Madan, G. L. Semenza and A. Bedi (2000). "Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha." *Genes Dev* **14**(1): 34-44.

Riemenschneider, M. J., R. Buschges, M. Wolter, J. Reifenberger, J. Bostrom, J. A. Kraus, U. Schlegel and G. Reifenberger (1999). "Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification." *Cancer Res* **59**(24): 6091-6.

Ries, S., C. Biederer, D. Woods, O. Shifman, S. Shirasawa, T. Sasazuki, M. McMahon, M. Oren and F. McCormick (2000). "Opposing effects of Ras on p53:

transcriptional activation of mdm2 and induction of p19ARF." *Cell* **103**(2): 321-30.

Ries, S. and W. M. Korn (2002). "ONYX-015: mechanisms of action and clinical potential of a replication-selective adenovirus." *Br J Cancer* **86**(1): 5-11.

Rizos, H., A. P. Darmanian, G. J. Mann and R. F. Kefford (2000). "Two arginine rich domains in the p14ARF tumour suppressor mediate nucleolar localization." *Oncogene* **19**(26): 2978-85.

Rodriguez, M. S., J. M. Desterro, S. Lain, D. P. Lane and R. T. Hay (2000). "Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation." *Mol Cell Biol* **20**(22): 8458-67.

Ruas, M. and G. Peters (1998). "The p16INK4a/CDKN2A tumor suppressor and its relatives." *Biochim Biophys Acta* **1378**(2): F115-77.

Ruggero, D., S. Grisendi, F. Piazza, E. Rego, F. Mari, P. H. Rao, C. Cordon-Cardo and P. P. Pandolfi (2003). "Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification." *Science* **299**(5604): 259-62.

Ruggero, D. and P. P. Pandolfi (2003). "Does the ribosome translate cancer?" *Nat Rev Cancer* **3**(3): 179-92.

Sabbatini, P. and F. McCormick (2002). "MDMX inhibits the p300/CBP-mediated acetylation of p53." *DNA Cell Biol* **21**(7): 519-25.

Sakamuro, D., P. Sabbatini, E. White and G. C. Prendergast (1997). "The polyproline region of p53 is required to activate apoptosis but not growth arrest." *Oncogene* **15**(8): 887-98.

Sampath, J., D. Sun, V. J. Kidd, J. Grenet, A. Gandhi, L. H. Shapiro, Q. Wang, G. P. Zambetti and J. D. Schuetz (2001). "Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1." *J Biol Chem* **276**(42): 39359-67.

Samuel, T., H. O. Weber, P. Rauch, B. Verdoodt, J. T. Eppel, A. McShea, H. Hermeking and J. O. Funk (2001). "The G2/M regulator 14-3-3sigma prevents apoptosis through sequestration of Bax." *J Biol Chem* **276**(48): 45201-6.

Saxena, A., C. J. Rorie, D. Dimitrova, Y. Daniely and J. A. Borowiec (2006). "Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization." *Oncogene* **25**(55): 7274-88.

Schmitt, C. A., J. S. Fridman, M. Yang, E. Baranov, R. M. Hoffman and S. W. Lowe (2002). "Dissecting p53 tumor suppressor functions in vivo." *Cancer Cell* **1**(3): 289-98.

Schon, O., A. Friedler, M. Bycroft, S. M. Freund and A. R. Fersht (2002). "Molecular mechanism of the interaction between MDM2 and p53." *J Mol Biol* **323**(3): 491-501.

Sebolt-Leopold, J. S. and R. Herrera (2004). "Targeting the mitogen-activated protein kinase cascade to treat cancer." *Nat Rev Cancer* **4**(12): 937-47.

Sengupta, S., J. L. Vonesch, C. Waltzinger, H. Zheng and B. Wasylyk (2000). "Negative cross-talk between p53 and the glucocorticoid receptor and its role in neuroblastoma cells." *Embo J* **19**(22): 6051-64.

Seto, E., A. Usheva, G. P. Zambetti, J. Momand, N. Horikoshi, R. Weinmann, A. J. Levine and T. Shenk (1992). "Wild-type p53 binds to the TATA-binding protein and represses transcription." *Proc Natl Acad Sci U S A* **89**(24): 12028-32.

Sharp, D. A., S. A. Kratowicz, M. J. Sank and D. L. George (1999). "Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein." *J Biol Chem* **274**(53): 38189-96.

Sharpless, N. E. and R. A. DePinho (1999). "The INK4A/ARF locus and its two gene products." *Curr Opin Genet Dev* **9**(1): 22-30.

Sharrocks, A. D. (1995). "ERK2/p42 MAP kinase stimulates both autonomous and SRF-dependent DNA binding by Elk-1." *FEBS Lett* **368**(1): 77-80.

Shaulian, E., D. Resnitzky, O. Shifman, G. Blandino, A. Amsterdam, A. Yayon and M. Oren (1997). "Induction of Mdm2 and enhancement of cell survival by bFGF." *Oncogene* **15**(22): 2717-25.

Shen, Y. and T. Shenk (1994). "Relief of p53-mediated transcriptional repression by the adenovirus E1B 19-kDa protein or the cellular Bcl-2 protein." *Proc Natl Acad Sci U S A* **91**(19): 8940-4.

Sherr, C. J. (2001). "The INK4a/ARF network in tumour suppression." *Nat Rev Mol Cell Biol* **2**(10): 731-7.

Shiloh, Y. (2003). "ATM: ready, set, go." *Cell Cycle* **2**(2): 116-7.

Shvarts, A., W. T. Steegenga, N. Riteco, T. van Laar, P. Dekker, M. Bazuine, R. C. van Ham, W. van der Houven van Oordt, G. Hateboer, A. J. van der Eb and A. G. Jochemsen (1996). "MDMX: a novel p53-binding protein with some functional properties of MDM2." *Embo J* **15**(19): 5349-57.

Sluss, H. K., H. Armata, J. Gallant and S. N. Jones (2004). "Phosphorylation of serine 18 regulates distinct p53 functions in mice." *Mol Cell Biol* **24**(3): 976-84.

Smith, M. L., J. M. Ford, M. C. Hollander, R. A. Bortnick, S. A. Amundson, Y. R. Seo, C. X. Deng, P. C. Hanawalt and A. J. Fornace, Jr. (2000). "p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes." *Mol Cell Biol* **20**(10): 3705-14.

Soussi, T. and C. Beroud (2001). "Assessing TP53 status in human tumours to evaluate clinical outcome." *Nat Rev Cancer* **1**(3): 233-40.

Stad, R., N. A. Little, D. P. Xirodimas, R. Frenk, A. J. van der Eb, D. P. Lane, M. K. Saville and A. G. Jochemsen (2001). "Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms." *EMBO Rep* **2**(11): 1029-34.

Stanbridge, E. J. (1976). "Suppression of malignancy in human cells." *Nature* **260**(5546): 17-20.

Stavridi, E. S., N. H. Chehab, A. Malikzay and T. D. Halazonetis (2001). "Substitutions that compromise the ionizing radiation-induced association of p53 with 14-3-3

proteins also compromise the ability of p53 to induce cell cycle arrest." *Cancer Res* **61**(19): 7030-3.

Steinman, H. A., K. M. Hoover, M. L. Keeler, A. T. Sands and S. N. Jones (2005). "Rescue of Mdm4-deficient mice by Mdm2 reveals functional overlap of Mdm2 and Mdm4 in development." *Oncogene* **24**(53): 7935-40.

Storey, A., M. Thomas, A. Kalita, C. Harwood, D. Gardiol, F. Mantovani, J. Breuer, I. M. Leigh, G. Matlashewski and L. Banks (1998). "Role of a p53 polymorphism in the development of human papillomavirus-associated cancer." *Nature* **393**(6682): 229-34.

Strezoska, Z., D. G. Pestov and L. F. Lau (2000). "Bop1 is a mouse WD40 repeat nucleolar protein involved in 28S and 5. 8S RRNA processing and 60S ribosome biogenesis." *Mol Cell Biol* **20**(15): 5516-28.

Strezoska, Z., D. G. Pestov and L. F. Lau (2002). "Functional inactivation of the mouse nucleolar protein Bop1 inhibits multiple steps in pre-rRNA processing and blocks cell cycle progression." *J Biol Chem* **277**(33): 29617-25.

Sui, G., B. Affar el, Y. Shi, C. Brignone, N. R. Wall, P. Yin, M. Donohoe, M. P. Luke, D. Calvo, S. R. Grossman and Y. Shi (2004). "Yin Yang 1 is a negative regulator of p53." *Cell* **117**(7): 859-72.

Sun, Y., J. M. Cheung, J. Martel-Pelletier, J. P. Pelletier, L. Wenger, R. D. Altman, D. S. Howell and H. S. Cheung (2000). "Wild type and mutant p53 differentially regulate the gene expression of human collagenase-3 (hMMP-13)." *J Biol Chem* **275**(15): 11327-32.

Sun, Y., Y. Sun, L. Wenger, J. L. Rutter, C. E. Brinckerhoff and H. S. Cheung (1999). "p53 down-regulates human matrix metalloproteinase-1 (Collagenase-1) gene expression." *J Biol Chem* **274**(17): 11535-40.

Symonds, H., L. Krall, L. Remington, M. Saenz Robles, T. Jacks and T. Van Dyke (1994). "p53-dependent apoptosis in vivo: impact of p53 inactivation on tumorigenesis." *Cold Spring Harb Symp Quant Biol* **59**: 247-57.

Takagi, M., M. J. Absalon, K. G. McLure and M. B. Kastan (2005). "Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin." *Cell* **123**(1): 49-63.

Takahashi, M., H. Tonoki, M. Tada, H. Kashiwazaki, K. Furuuchi, J. Hamada, Y. Fujioka, Y. Sato, H. Takahashi, S. Todo, N. Sakuragi and T. Moriuchi (2000). "Distinct prognostic values of p53 mutations and loss of estrogen receptor and their cumulative effect in primary breast cancers." *Int J Cancer* **89**(1): 92-9.

Tanimura, S., S. Ohtsuka, K. Mitsui, K. Shirouzu, A. Yoshimura and M. Ohtsubo (1999). "MDM2 interacts with MDMX through their RING finger domains." *FEBS Lett* **447**(1): 5-9.

Tao, W. and A. J. Levine (1999). "P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2." *Proc Natl Acad Sci U S A* **96**(12): 6937-41.

Terzian, T., Y. Wang, C. S. Van Pelt, N. F. Box, E. L. Travis and G. Lozano (2007). "Haploinsufficiency of Mdm2 and Mdm4 in tumorigenesis and development." *Mol Cell Biol* **27**(15): 5479-85.

Thrower, J. S., L. Hoffman, M. Rechsteiner and C. M. Pickart (2000). "Recognition of the polyubiquitin proteolytic signal." *Embo J* **19**(1): 94-102.

Toledo, F., K. A. Krummel, C. J. Lee, C. W. Liu, L. W. Rodewald, M. Tang and G. M. Wahl (2006). "A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network." *Cancer Cell* **9**(4): 273-85.

Toledo, F. and G. M. Wahl (2006). "Regulating the p53 pathway: in vitro hypotheses, in vivo veritas." *Nat Rev Cancer* **6**(12): 909-23.

Toniolo, D. and C. Basilico (1976). "Processing of ribosomal RNA in a temperature sensitive mutant of BHK cells." *Biochim Biophys Acta* **425**(4): 409-18.

Uldrijan, S., W. J. Pannekoek and K. H. Vousden (2007). "An essential function of the extreme C-terminus of MDM2 can be provided by MDMX." *Embo J* **26**(1): 102-12.

Unger, T., T. Juven-Gershon, E. Moallem, M. Berger, R. Vogt Sionov, G. Lozano, M. Oren and Y. Haupt (1999). "Critical role for Ser20 of human p53 in the negative regulation of p53 by Mdm2." *Embo J* **18**(7): 1805-14.

Vassilev, L. T. (2004). "Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics." *Cell Cycle* **3**(4): 419-21.

Vassilev, L. T. (2005). "p53 Activation by small molecules: application in oncology." *J Med Chem* **48**(14): 4491-9.

Vassilev, L. T., B. T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi and E. A. Liu (2004). "In vivo activation of the p53 pathway by small-molecule antagonists of MDM2." *Science* **303**(5659): 844-8.

Venot, C., M. Maratrat, C. Dureuil, E. Conseiller, L. Bracco and L. Debussche (1998). "The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression." *Embo J* **17**(16): 4668-79.

Vogelstein, B. and K. W. Kinzler (1993). "The multistep nature of cancer." *Trends Genet* **9**(4): 138-41.

Vogelstein, B., D. Lane and A. J. Levine (2000). "Surfing the p53 network." *Nature* **408**(6810): 307-10.

Voit, R., K. Schafer and I. Grummt (1997). "Mechanism of repression of RNA polymerase I transcription by the retinoblastoma protein." *Mol Cell Biol* **17**(8): 4230-7.

Volarevic, S., M. J. Stewart, B. Ledermann, F. Zilberman, L. Terracciano, E. Montini, M. Grompe, S. C. Kozma and G. Thomas (2000). "Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6." *Science* **288**(5473): 2045-7.

Vousden, K. H. and X. Lu (2002). "Live or let die: the cell's response to p53." *Nat Rev Cancer* **2**(8): 594-604.

Wade, M., E. T. Wong, M. Tang, J. M. Stommel and G. M. Wahl (2006). "Hdmx modulates the outcome of p53 activation in human tumor cells." *J Biol Chem* **281**(44): 33036-44.

Wahl, G. M. (2006). "Mouse bites dogma: how mouse models are changing our views of how P53 is regulated in vivo." *Cell Death Differ* **13**(6): 973-83.

Walensky, L. D., K. Pitter, J. Morash, K. J. Oh, S. Barbuto, J. Fisher, E. Smith, G. L. Verdine and S. J. Korsmeyer (2006). "A stapled BID BH3 helix directly binds and activates BAX." *Mol Cell* **24**(2): 199-210.

Wang, X., J. Taplick, N. Geva and M. Oren (2004). "Inhibition of p53 degradation by Mdm2 acetylation." *FEBS Lett* **561**(1-3): 195-201.

Wang, X. W., W. Vermeulen, J. D. Coursen, M. Gibson, S. E. Lupold, K. Forrester, G. Xu, L. Elmore, H. Yeh, J. H. Hoeijmakers and C. C. Harris (1996). "The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway." *Genes Dev* **10**(10): 1219-32.

Wang, Y. and C. Prives (1995). "Increased and altered DNA binding of human p53 by S and G2/M but not G1 cyclin-dependent kinases." *Nature* **376**(6535): 88-91.

Weber, J. D., M. L. Kuo, B. Bothner, E. L. DiGiammarino, R. W. Kriwacki, M. F. Roussel and C. J. Sherr (2000). "Cooperative signals governing ARF-mdm2 interaction and nucleolar localization of the complex." *Mol Cell Biol* **20**(7): 2517-28.

Weber, J. D., L. J. Taylor, M. F. Roussel, C. J. Sherr and D. Bar-Sagi (1999). "Nucleolar Arf sequesters Mdm2 and activates p53." *Nat Cell Biol* **1**(1): 20-6.

Werness, B. A., A. J. Levine and P. M. Howley (1990). "Association of human papillomavirus types 16 and 18 E6 proteins with p53." *Science* **248**(4951): 76-9.

Woo, R. A., K. G. McLure, S. P. Lees-Miller, D. E. Rancourt and P. W. Lee (1998). "DNA-dependent protein kinase acts upstream of p53 in response to DNA damage." *Nature* **394**(6694): 700-4.

Wu, G. S., K. Kim and W. S. el-Deiry (2000). "KILLER/DR5, a novel DNA-damage inducible death receptor gene, links the p53-tumor suppressor to caspase activation and apoptotic death." *Adv Exp Med Biol* **465**: 143-51.

Wu, Z., J. Earle, S. Saito, C. W. Anderson, E. Appella and Y. Xu (2002). "Mutation of mouse p53 Ser23 and the response to DNA damage." *Mol Cell Biol* **22**(8): 2441-9.

Xiao, Z. X., J. Chen, A. J. Levine, N. Modjtahedi, J. Xing, W. R. Sellers and D. M. Livingston (1995). "Interaction between the retinoblastoma protein and the oncoprotein MDM2." *Nature* **375**(6533): 694-8.

Xiong, S., C. S. Van Pelt, A. C. Elizondo-Fraire, G. Liu and G. Lozano (2006). "Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development." *Proc Natl Acad Sci U S A* **103**(9): 3226-31.

Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi and D. Beach (1993). "p21 is a universal inhibitor of cyclin kinases." *Nature* **366**(6456): 701-4.

Xirodimas, D. P., M. K. Saville, J. C. Bourdon, R. T. Hay and D. P. Lane (2004). "Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity." *Cell* **118**(1): 83-97.

Xu, D., Q. Wang, A. Gruber, M. Bjorkholm, Z. Chen, A. Zaid, G. Selivanova, C. Peterson, K. G. Wiman and P. Pisa (2000). "Downregulation of telomerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells." *Oncogene* **19**(45): 5123-33.

Yang, S. H., P. Shore, N. Willingham, J. H. Lakey and A. D. Sharrocks (1999). "The mechanism of phosphorylation-inducible activation of the ETS-domain transcription factor Elk-1." *Embo J* **18**(20): 5666-74.

Yin, C., C. M. Knudson, S. J. Korsmeyer and T. Van Dyke (1997). "Bax suppresses tumorigenesis and stimulates apoptosis in vivo." *Nature* **385**(6617): 637-40.

Yogosawa, S., Y. Miyauchi, R. Honda, H. Tanaka and H. Yasuda (2003). "Mammalian Numb is a target protein of Mdm2, ubiquitin ligase." *Biochem Biophys Res Commun* **302**(4): 869-72.

Yonish-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi and M. Oren (1991). "Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6." *Nature* **352**(6333): 345-7.

Yoo, Y. A., M. J. Kim, J. K. Park, Y. M. Chung, J. H. Lee, S. G. Chi, J. S. Kim and Y. D. Yoo (2005). "Mitochondrial ribosomal protein L41 suppresses cell growth in association with p53 and p27Kip1." *Mol Cell Biol* **25**(15): 6603-16.

Yu, J., Z. Wang, K. W. Kinzler, B. Vogelstein and L. Zhang (2003). "PUMA mediates the apoptotic response to p53 in colorectal cancer cells." *Proc Natl Acad Sci U S A* **100**(4): 1931-6.

Yuan, X., Y. Zhou, E. Casanova, M. Chai, E. Kiss, H. J. Grone, G. Schutz and I. Grummt (2005). "Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis." *Mol Cell* **19**(1): 77-87.

Zantema, A., J. A. Fransen, A. Davis-Olivier, F. C. Ramaekers, G. P. Vooijs, B. DeLeys and A. J. Van der Eb (1985). "Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies." *Virology* **142**(1): 44-58.

Zeng, X., L. Chen, C. A. Jost, R. Maya, D. Keller, X. Wang, W. G. Kaelin, Jr., M. Oren, J. Chen and H. Lu (1999). "MDM2 suppresses p73 function without promoting p73 degradation." *Mol Cell Biol* **19**(5): 3257-66.

Zhang, C., M. M. Kavurma, A. Lai and L. M. Khachigian (2003). "Ets-1 protects vascular smooth muscle cells from undergoing apoptosis by activating p21WAF1/Cip1: ETS-1 regulates basal and and inducible p21WAF1/Cip: ETS-1 regulates basal and inducible p21WAF1/Cip1 transcription via distinct cis-acting elements in the p21WAF/Cip1 promoter." *J Biol Chem* **278**(30): 27903-9.

Zhang, J. and H. F. Lodish (2004). "Constitutive activation of the MEK/ERK pathway mediates all effects of oncogenic H-ras expression in primary erythroid progenitors." *Blood* **104**(6): 1679-87.

Zhang, Y., G. W. Wolf, K. Bhat, A. Jin, T. Allio, W. A. Burkhart and Y. Xiong (2003). "Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway." *Mol Cell Biol* **23**(23): 8902-12.

Zhao, J., X. Yuan, M. Frodin and I. Grummt (2003). "ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth." *Mol Cell* **11**(2): 405-13.

Zhou, J., J. Ahn, S. H. Wilson and C. Prives (2001). "A role for p53 in base excision repair." *Embo J* **20**(4): 914-23.

Zhu, Q., J. Yao, G. Wani, M. A. Wani and A. A. Wani (2001). "Mdm2 mutant defective in binding p300 promotes ubiquitination but not degradation of p53: evidence for the role of p300 in integrating ubiquitination and proteolysis." *J Biol Chem* **276**(32): 29695-701.

Zilfou, J. T., W. H. Hoffman, M. Sank, D. L. George and M. Murphy (2001). "The corepressor mSin3a interacts with the proline-rich domain of p53 and protects p53 from proteasome-mediated degradation." *Mol Cell Biol* **21**(12): 3974-85.

Zurer, I., L. J. Hofseth, Y. Cohen, M. Xu-Welliver, S. P. Hussain, C. C. Harris and V. Rotter (2004). "The role of p53 in base excision repair following genotoxic stress." *Carcinogenesis* **25**(1): 11-9.

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