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Modification of MDMX by Ubiquitination and Sumoylation

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Modification of MDMX by Ubiquitination and Sumoylation

by

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Dedication

To my family: my grandparents, my parents and my parents in law,
for their love and support.

To my husband, Linan, for everything.

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LIST OF ABREVIATIONS

ARF	alternative reading frame
CBP	CREB-binding protein
CDK	cyclin-dependent kinase
DUB	de-ubiquitinating protein
DSB	double strand break
FBS	fetal bovine serum
GFP	green florescent protein
HDAC	histone deacetylase
HECT	homologous to the E6-AP COOH terminus
HFF	human foreskin fibroblast
MDM2	mouse double minute 2
MEF	mouse embryo fibroblast
µl	micro liter
NLS	nuclear localization signal
NES	nuclear export signal
N_OLS	nucleolus localization signal
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIAS	protein inhibitors of activated STATs
PML	promyelocytic leukemia protein
Rb	retinoblastoma protein
RT	room temperature
SENP	sentrin-specific protease
SUMO	small ubiquitin-like molecule

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ABSTRACT

MDM2 and MDMX are two major negative regulators of tumor suppressor p53. Both MDM2 and MDMX can inactivate p53 and play important roles in mouse embryonic development in a p53 dependent manner. MDM2 possesses ubiquitin E3 ligase activity and mediates self-ubiquitination as well as ubiquitination and degradation of p53 by proteasome. We identify MDMX as another ubiquitin E3 ligase substrate of MDM2. MDM2 promotes the ubiquitination and degradation of MDMX through proteasome pathway. The RING domains of both MDMX and MDM2 are required and sufficient for MDM2-mediated MDMX ubiquitination. ARF overexpression, DNA damage or MDM2 overexpression can all stimulate MDMX ubiquitination and degradation. We present evidence that MDMX is also sumoylated. The sumoylation sites on MDMX are identified. ARF N-terminus is required for stimulating both MDMX ubiquitination and sumoylation. We also demonstrate that MDMX binds to ARF in an MDM2-dependent fashion.

Introduction

Tumorigenesis

The growth and proliferation of normal untransformed cells are the result of a balanced relationship between oncogenes and tumor suppressors compared to those of the tumor cells. Tumorigenesis is a progressive disease caused by the accumulative lesions in the genome, producing either gain of function oncogenes or loss of function of tumor suppressors, or both. The cancer cells are defective in their regulatory pathways, which lead to the abnormal cell growth and proliferation. A vast majority of cancer cells display a single or a combination of six traits: evasion from apoptosis, self-reliance in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, unlimited replication potential and incessant angiogenesis (Friend, Bernards et al. 1986; Hanahan and Weinberg 2000). While the uncurbed expression of oncogenes positively regulates cell proliferation, tumor suppressors play important roles as genomic guardians by restraining cell growth and proliferation, executing genetically impaired cells and maintaining genomic stability. Rb was the first tumor suppressor isolated in human and was found to be dysregulated in a majority of cancers (Friend, Bernards et al. 1986). Another intensively studies tumor suppressor is p53, which plays pivotal roles in maintaining homeostasis of the cells.

p53

p53 history

In 1979, p53 was originally recognized as a weak oncogene, which bound to the simian virus (SV40) large T antigen and accumulated in the cancer cells (DeLeo, Jay et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). Later, researchers found that the oncogenic activity was caused by the gain-of-function mutant p53. By forming tetramers with the wild type p53, mutant p53 suppresses wild type p53 function in a dominant-negative fashion (Sigal and Rotter 2000). In 1990, it was found that the E6 oncoprotein encoded by human papillomavirus promotes the degradation of p53 (Scheffner, Werness et al. 1990). The fact that viral system developed specific mechanisms using oncoproteins to target p53 for degradation underlines the importance of p53 inactivation for virus survival and proliferation.

P53 belongs to a family of transcription factors including p53, p63 and p73. While p63 and p73 take important responsibilities in maintaining normal development, only p53 evolved to prevent tumor formation (Yang and McKeon 2000). Now it is widely accepted that p53 is a classic tumor suppressor. P53 lost of function is found in about 50% of all human tumors (Hollstein, Sidransky et al. 1991; Levine, Momand et al. 1991). Functional analysis revealed p53 as a potent stress responder regulating cell-cycle arrest, DNA repair, cell senescence and apoptosis.

P53 function and transcription down stream targets

The structure of p53 represents a classic transcription factor (Ko and Prives 1996). It has transactivation domain in the N-terminus and the sequence-specific DNA binding domain in the central region (fig 1). The DNA-binding domain contains hot spots for mutation in tumor cells, indicating the tumor suppressor function of p53 relies heavily on its transcriptional activity. P53 induces the transcription and over-expression of a myriad of proteins through directly binding to their promoters, which leads to various biological functions (fig 1). Although the number of genes found regulated by p53 is still increasing, generally, these gene targets can be categorized into five groups according to their functions: (1) Cell cycle arrest genes, (2) Apoptosis genes, (3) DNA repair genes, (4) genes inhibiting angiogenesis and metastasis and (5) cellular senescence genes.

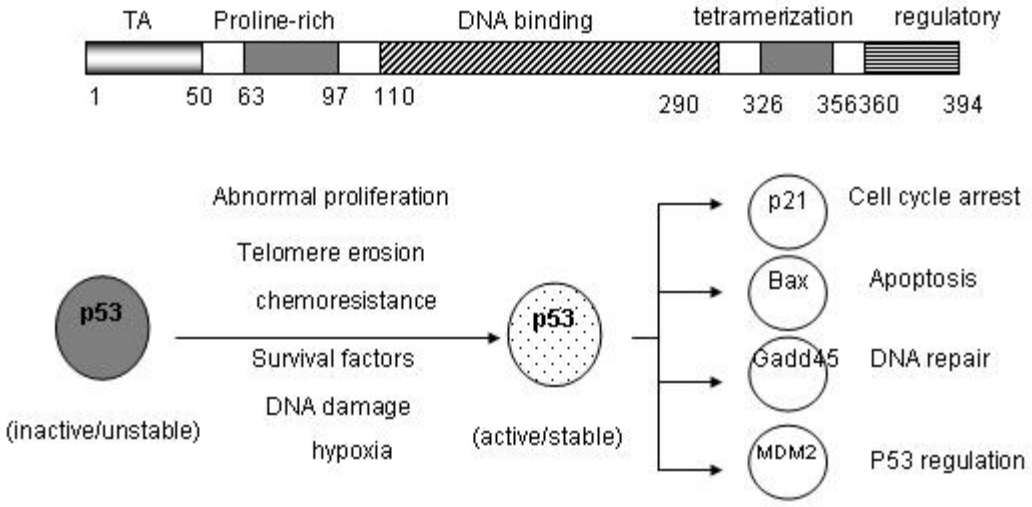


Figure 1 Structure and functions of p53 tumor suppressor

(1) Cell cycle arrest genes: CDKs or cyclin-dependent kinases are a group of enzymes evolved specifically to promote the cell cycle progression upon binding to correspondent cyclin proteins, thus supporting the mitosis and division of the cells. CDK inhibitors, for example, p16, p21 and p27, weigh the decision of going through S phase for DNA synthesis. P53 directly promotes the transcription of p21, which inhibits the transition from G1 to S and from G2 to M (el-Deiry, Tokino et al. 1993). 14-3-3 δ is another p53 transcriptional target that blocks the G2-M transition (Chan, Hermeking et al. 1999; Laronga, Yang et al. 2000).

(2) Apoptosis genes: When DNA damage is beyond repair, apoptosis is the last resolution to avoid the risk of having cells carrying damaged genetic signal and proliferating. The apoptotic process can proceed through two main pathways: the intrinsic and extrinsic pathways. The intrinsic pathway involves an apoptotic complex called apoptosome that is comprised of mitochondria-released cytochrome c, Apaf-1 and procaspase 9 (Kroemer and Reed 2000). The release of cytochrome c is a result of mitochondria depolarization, a process under control by two groups of Bcl-2 family proteins (Tsujimoto 2003). The proapoptotic group of proteins includes Bax and Bak residing on mitochondrial membrane and the BH3 domain only proteins such as PUMA, Noxa. The other group mainly contains anti-apoptotic Bcl-2 and Bcl-XL. The change in ratio between the two groups determines the life or death of the cell.

One major tumor suppressive function of p53 is to induce apoptosis, thereby preventing tumor cells from proliferating. P53 can transcriptionally induce proapoptotic genes that participate in both intrinsic and extrinsic apoptosis pathways. In the intrinsic

pathway, p53 induces proapoptotic genes such as Bax, PUMA and Noxa, which localize to the mitochondria and promote mitochondria depolarization (Miyashita, Krajewski et al. 1994; Oda, Ohki et al. 2000; Nakano and Vousden 2001). As a result, cytochrome c is released and the subsequent apoptosome formation and caspase activation eventually lead to cell death. Another group of p53-induced genes belong to the death-receptor mediated apoptosis pathway or extrinsic pathway, including DR5, Fas, PIDD.(Owen-Schaub, Zhang et al. 1995; Wu, Burns et al. 1997; Muller, Wilder et al. 1998; Lin, Ma et al. 2000). Initiation of the extrinsic pathway results from ligand binding to the death receptors, leading to the clustering of death receptors at the cell membrane and the consequent induction of caspase activity. By transcriptionally activating proapoptotic genes in both intrinsic and extrinsic pathways, p53 contribute significantly to apoptosis and the disruption of p53 activity can lead to tumorigenesis.

P53 promotes apoptosis not only by transcriptional activation, but also through transcriptional repression of the target genes. For instance, p53 represses the transcription of survivin, which is an inhibitor of apoptosis (Ambrosini, Adida et al. 1997). Although p53 transcriptional repression is not as well understood as its transactivation function, it is suggested that under certain circumstances, p53 transcription repression contributes more to apoptosis than does its gene transactivation function (Koumenis, Alarcon et al. 2001).

Additionally, p53 can also induce apoptosis via transcriptional-independent pathways (Caelles, Helmberg et al. 1994; Wagner, Kokontis et al. 1994; Haupt, Rowan et al. 1995; Kokontis, Wagner et al. 2001; Dumont, Leu et al. 2003). Evidence showed that the DNA-binding deficient p53 mutants could induce apoptosis almost as efficient as the

wild type. Furthermore, p53 regulates the intrinsic apoptotic pathway by directly translocating to the mitochondria (Mihara, Erster et al. 2003; Mihara and Moll 2003). The interaction between p53 and anti-apoptotic proteins such as Bcl-2 and Bcl-XL changes the membrane integrity, leading to cytochrome c release and apoptosis. Additionally, it has been reported that p53 promotes the transportation of Fas from cytoplasm to cell membrane, augmenting the extrinsic pathway (Bennett, Macdonald et al. 1998).

(3) DNA repair genes: The defect in DNA repair genes will eventually lead to genomic instability due to the accumulated errors in DNA left uncorrected. The mechanism by which p53 mediates DNA repair remains elusive. However, experimental data showed that P53 contributes to genomic stability by regulating the transcription of DNA repair genes such as p48 and GADD45. (Kastan, Zhan et al. 1992; Smith, Chen et al. 1994; Hwang, Ford et al. 1999).

(4) Genes inhibiting angiogenesis and metastasis: Angiogenesis is a hallmark for tumorigenesis in solid tumors. In order for the tumor to progress and grow to a certain size, ample blood supply is needed that can only be satisfied by the generation of new blood vessels, or angiogenesis. Tumor cells shed into the blood vessels where they can be carried to distant locations to metastasize. P53 promotes expression of genes to antagonize tumor angiogenesis and metastasis processes. Some of the anti-angiogenesis or anti-metastasis genes activated by p53 include MASPIN (Song, Lee et al. 2002), TSP1 (el-Deiry, Tokino et al. 1993; Nagayama, Shigematsu et al. 2000) and BAI-1 (Nishimori, Shiratsuchi et al. 1997). An example of anti-metastasis gene activated by p53 is KAI (Schindl, Birner et al. 2001).

(5) Cellular senescence: Normal human cells have a finite lifespan, which allows only certain numbers of DNA replications. This has been defined as the cellular replicative senescence and it is mainly caused by telomere shortening (Linskens, Harley et al. 1995). For example, human skin cell chromosomes lose 50-100 nucleotides at the telomere ends following each DNA replication cycle, due to the intrinsic mechanism built in the replication procedure of the DNA lagging strand. After certain generations, the continuous shortening of the DNA leads to intolerable level of DNA loss and causes genomic instability. As a result, the cells which inherited the unstable chromosomes quit the cell cycle and enter into a phase called replicative senescence (Levy, Allsopp et al. 1992). In this way, telomere, the repetitive DNA composing the chromosome ends provides a counting mechanism to prevent the infinite DNA replication and cell proliferation. It has been suggested that besides apoptosis, cellular senescence is another mechanism to prevent the cells bearing defective DNA from tumorigenic transformation (Sager 1991; Yeager, DeVries et al. 1998). Additionally, cellular senescence is also suggested to be involved in aging.

Senescence can also be induced through telomere-independent pathways. A variety of stimuli including oncogenic or mitogenic signals, chromatin remodeling, DNA damage or tumor suppressor activity have all been reported to elicit cellular senescence. Some tumor suppressors such as p16 (the cyclin-dependent kinase inhibitor) and p14 ARF (the inhibitor of oncogene MDM2) respond to these senescence-inducing stimuli (McConnell, Starborg et al. 1998; Dimri, Itahana et al. 2000). Although the mechanism of p53 regulation of senescence remains elusive, there are indications that p53 responds to senescence-inducing stimuli through ATM and sensitizes the cells in a way similar to

DNA damage induced p53 activation (Vaziri 1997; Vaziri, West et al. 1997; Lansdorp 2000). It is very likely that p53 induces p21 expression, which in concert with p16 initiates and sustains the senescence growth arrest of the cells (Atadja, Wong et al. 1995; Vaziri, West et al. 1997; Stein, Drullinger et al. 1999).

Activation of p53

P53 is usually maintained at low levels in the cells by virtue of its short half-life. One of the most important functions of p53 in suppressing tumorigenesis is to respond to cellular stress, achieved by promoting the cell cycle arrest or apoptosis of the DNA-damaged cells by transcriptionally regulating target genes bearing the aforementioned functions. In this way, given the time by p53 induced cell cycle arrest, the cells containing damaged DNA are repaired or removed by apoptosis to keep the homeostasis of the cells. Thus, this DNA-damage response of p53 is crucial for avoiding neoplasia generated from DNA lesions.

One of the most potent DNA damages inflicted on the cells is DNA double strand break. This can be elicited from ionizing radiation, certain chemical agents or genotoxic stress. Ataxia telangiectasia is an autosomal recessive genetic disorder featured by neurodegeneration, immunodeficiency, radiation sensitivity and cancer predisposition (Crawford 1998; Becker-Catania and Gatti 2001). On genetic level, ataxia telangiectasia is caused by the loss or inactivation of ATM protein, which belongs to a conserved family of proteins, PI3 kinase like protein kinases or PIKK. Most of the PIKK family proteins possess serine/threonine kinase activity. ATM kinase responds to DNA double

strand breaks by phosphorylating proteins crucial to various DNA response pathways (Banin, Moyal et al. 1998; Canman, Lim et al. 1998).

One of the main targets of ATM kinase is p53, the activation of which mediates the G1-S cell cycle arrest by inducing the expression of CDK inhibitor p21. Upon DNA damage, ATM phosphorylates p53 on Serine 15 (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Khanna, Keating et al. 1998). Moreover, ATM also phosphorylates and activates CHK2, a checkpoint kinase that phosphorylates p53 on Serine 20. (Bartek, Falck et al. 2001; McGowan 2002). Another ATM phosphorylation target is MDM2 at serine 395, which disrupted the nuclear export signal that is needed for efficient p53 export into the cytoplasm (Maya, Balass et al. 2001). MDM2 affects p53 in both its function and its stability. In order to keep p53 inactive and at low protein level, MDM2 binds to the N-terminal transactivation domain and inhibits the transcription factor function of p53. On the other hand, MDM2 is the ubiquitin E3 ligase, mediating the ubiquitination and degradation of p53 by proteasome. Furthermore, the NES on MDM2 has been reported to enhance MDM2 mediated ubiquitination and degradation of p53. These aforementioned phosphorylations dissociate p53 from the binding to its major negative regulator MDM2, thus significantly contributes to the stabilization and activation of p53 as a transcription factor. Other PIKK family proteins such as ATR and ATX carry out similar functions in phosphorylating p53 (Hammond, Denko et al. 2002; Heffernan, Simpson et al. 2002). Even though they can collaborate with ATM in the double strand break response, ATR and ATX are also activated in response to different genotoxic stresses, such as UV-irradiation and hypoxia.

P53 regulation by MDM2 and MDMX

As a tumor suppressor, p53 functions to inhibit growth and proliferation of abnormal cells, which impose a potential threat for tumorigenesis. In mouse models, p53 null mice showed high incidence of tumor formation, supporting p53 as a tumor suppressor (Donehower, Harvey et al. 1992; Livingstone, White et al. 1992; Yin, Tainsky et al. 1992; Fukasawa, Wiener et al. 1997). The stability and activity of p53 are mostly regulated by MDM2. MDM2 is one of the down stream targets of p53 and the expression of MDM2 is often elevated subsequent to p53 activation (Barak, Juven et al. 1993; Zauberman, Flusberg et al. 1995). As mentioned above, MDM2 possesses E3 ubiquitin ligase activity towards p53 and acts as a major negative regulator to keep p53 stability and activity at low level (Oliner, Pietenpol et al. 1993; Zauberman, Barak et al. 1993; Haupt, Maya et al. 1997; Honda and Yasuda 1999). Thus, the relationship between p53 and MDM2 composes the classic negative feedback loop, whereas the elevation of MDM2 expression by p53 leads to the down regulation of p53 itself (Wu, Bayle et al. 1993). The importance of MDM2 as the major negative regulator of p53 is also revealed in mouse models. Nullifying MDM2 leads to embryonic lethality at day 3.5 due to the massive apoptosis induced by p53, supporting MDM2 as a major p53 negative regulator (Jones, Roe et al. 1995). Furthermore, the embryonic lethality of MDM2 null mice can be rescued by crossing into p53 null background, indicating the major role of MDM2 is to negate p53 functionality. This indicates the intrinsic mechanism in cells to keep p53 level under tight control.

The negative effect of MDM2 inflicted on p53 is not without control. As the inhibition of MDM2 favors p53 function, MDM2 inhibitor might possess tumor suppressor characteristics. ARF is such a tumor suppressor. ARF expression is elevated upon the induction by certain genetic stress signals such as E1A, E2F, myc, ras and v-abl. Consequently, as a major negative regulator of MDM2, ARF inhibits MDM2 mediated p53 ubiquitination and degradation while negating MDM2's inhibition of p53 transcriptional activity (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998).

The other important p53 regulator is MDMX, an MDM2 homolog. Recently, MDMX was found as another P53 binding protein besides MDM2 (Shvarts, Steegenga et al. 1996). MDMX and MDM2 share significant homology in several functional domains, such as P53 binding domain, Zn finger and the RING domain. MDMX was also found to be able to inhibit P53 transactivation. However, no feedback loop is formed between p53 and MDMX because MDMX does not contain p53-responsive element on the promoter and is not induced by p53 activation. On the other hand, MDMX has not been found to possess ubiquitin E3 ligase activity so it does not directly affect p53 stability (Jackson and Berberich 2000; Stad, Ramos et al. 2000). Nonetheless, MDMX has been established as another important negative regulator of p53. The MDMX knockout mice died at embryonic day 7 due to severe proliferation deficiency in contrast of massive apoptosis as in the case of MDM2 knockout mice. Similar to MDM2 null mice, this embryonic lethality can be rescued by nullifying P53 simultaneously (Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002). This

demonstrated the non-overlapping functionality of MDMX with MDM2 in embryonic development as well as its major function in P53 regulation.

P53 posttranslational modification

P53 responds to DNA damage signals through an increase in transcriptional activity and protein stability. The accumulation of active p53 largely relies on p53 posttranslational modifications (Appella and Anderson 2001). Compared to the change in transcriptional or translational rate, posttranslational modification is a time-efficient way to elevate the activity of p53 as a transcription factor in stress response. So far, reported posttranslational modifications of p53 include phosphorylation, ubiquitination, acetylation, neddylation and sumoylatoin, each of them has profound effect on p53 stability and/or activity.

P53 phosphorylation is pivotal for its activation after DNA damage. The N-terminus of p53 is phorsphorylated by at least three different protein kinases, ATM, ATR and DNA-PK (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Tibbetts, Brumbaugh et al. 1999; Nagayama, Shigematsu et al. 2000). The central event in this process is the phosphorylation of p53 Serine 15 and Serine 37, leading to the dissociation of MDM2 from p53 (Avantaggiati, Ogryzko et al. 1997; Shieh, Ikeda et al. 1997; Lambert, Kashanchi et al. 1998; Dumaz and Meek 1999; Zhang and Xiong 2001). Alternatively, MDM2 as the major negative regulator of p53 can also be phorsphorylated, favoring its dissociation from p53 (Mayo, Turchi et al. 1997). In addition, the C-terminal serine residue 378 undergoes dephosphorylation upon DNA damage, leading to the recruitment

of 14-3-3 adaptor protein followed by the enhanced DNA binding affinity of p53 (Waterman, Stavridi et al. 1998).

P53 acetylation also regulates the protein-protein interaction and activity of p53. It has been suggested that the phosphorylation in p53 N-terminus potentiates the acetylation by transcriptional co-activators CBP/P300 and PCAF, thus enhancing p53 transcriptional activity and stability (Avantaggiati, Ogryzko et al. 1997; Gu, Shi et al. 1997; Lill, Grossman et al. 1997; Sakaguchi, Herrera et al. 1998; Dumaz and Meek 1999). P53 activity is also under the control of HDACs, which often associate with transcription co-repressors and remove acetyl groups from substrates (Murphy, Ahn et al. 1999; Juan, Shia et al. 2000). The deacetylation of p53 may represent a mechanism to help restore cell biological functions when the damage is repaired and hyperactive p53 is no longer needed. As the major negative regulator of p53, MDM2 blocks p53 acetylation both in vivo and in vitro, suppressing the tumor suppressor function of p53 (Kobet, Zeng et al. 2000; Ito, Lai et al. 2001).

P53 stability is most directly regulated by its ubiquitination. Aforementioned MDM2-mediated ubiquitination sends p53 to 26S proteasome degradation and explains the particularly short half-life of p53. Besides MDM2, Pirh2 and COP1 are both p53-inducible genes and could mediate the ubiquitination and degradation of p53, forming negative feedback loops (Leng, Lin et al. 2003; Dornan, Wertz et al. 2004). The fact that multiple proteins converge on the negative regulation of p53 emphasizes the importance of keeping p53 level under strict control. Alternatively, the mechanism de-ubiquitinating p53 should hypothetically restore p53 stability. HAUSP is one such antagonist to MDM2

ubiquitination activity of p53, removing ubiquitin molecules from p53 and increases the protein half-life (Li, Chen et al. 2002).

Besides ubiquitin, ubiquitin-like small molecules such as Nedd8 or sumo have also emerged as p53 modifiers that regulate p53 activity (Melchior and Hengst 2002; Harper 2004; Xirodimas, Saville et al. 2004). Both neddylation and sumoylation utilize parallel mechanisms to ubiquitination in that the ubiquitin-like molecules need to be activated and transferred to substrates by E1 and E2 enzymes, respectively. MDM2 promotes conjugation of both sumo and nedd8 to p53. However, p53 sumoylation and neddylation have opposite effects on its transcription activity whereas sumoylation of p53 moderately enhances its transactivation while MDM2-mediated neddylation of p53 inhibits its function (Melchior and Hengst 2002; Harper 2004; Xirodimas, Saville et al. 2004).

P53 as a cancer therapy target

Cancer is among the top 3 causes of death in United States. To date, the treatment of cancer still heavily relies on chemotherapies and radiotherapies, which primarily converge to the functionality and activity of p53. In order to evade from p53 tumor suppressive functions, about 50% of human tumors have p53 mutated (Hollstein, Sidransky et al. 1991; Levine, Momand et al. 1991). In the rest of the cases, the maintenance of wild type p53 accompanies deficiencies of other components in the p53 regulatory pathway, such as the amplification of MDM2 or the loss of function of ARF.

Realizing the pivotal role of p53 in cancer control, scientists have endeavored to develop p53-targeting therapeutic strategies. They can be categorized into two classes.

The first tactic targets cancer cells containing mutant p53. This is based on the hypothesis that the function of mutant p53 can be restored to that of the wild type. Theoretically, cells bearing mutant p53 not only risk loss of function of the tumor suppressor, but also obtain the gain of function activity of oncogene as the p53-mutant allele exhibits dominant negative activity to silence the proper function of the wild type p53. Strategies were developed to either eliminate cells bearing mutant p53, exemplified by Onyx-015, or correction of mutant p53 functions, represented by PRIMA-1 (Heise, Sampson-Johannes et al. 1997; Ries, Brandts et al. 2000; Rogulski, Freytag et al. 2000; Bykov, Issaeva et al. 2002; Lebedeva, Su et al. 2003; Haupt and Haupt 2004).

The second class aims to boost the activity of wild type p53. In a significant percentage of tumors, p53 status remains wild type, but accompanied with overexpression or amplification of MDM2. MDM2 is one major negative regulator for both p53 stability and activity. To this end, peptide and small molecule inhibitors have been developed to achieve the abrogation of MDM2-P53 binding, alleviating the suppression of p53 (Haupt and Haupt 2004).

MDM2

MDM2 History

MDM2 or Murine Double Minute Clone 2 was originally cloned from the abnormal chromosomes called double minute (Cahilly-Snyder, Yang-Feng et al. 1987; Fakharzadeh, Trusko et al. 1991). These chromosomes frequently harbor amplified genes, leading to uncontrolled cell proliferation and tumorigenesis. The amplification of MDM2 in mice resulted in high risk of tumor formation, giving scientists the first sign that MDM2 is an oncogene. Later, MDM2 was co-purified with p53 and found to negatively regulate p53 stability and transcriptional activity (Momand, Zambetti et al. 1992). Since p53 has been defined as a tumor suppressor gene, the inactivation of which by MDM2 established MDM2 as an oncogene.

MDM2 mouse model and clinical relevance

One of the crucial experiments established MDM2 as a major regulator of p53 tumor suppressor was done in mouse models. P53 -/- mice were viable but developed tumors after birth, supporting the role of p53 as a tumor suppressor. MDM2-/- mice were embryonic lethal, died at embryonic day 3.5. If the lethality of MDM2 null mice is caused by the loss of MDM2 negative regulation of p53, then crossing p53 to the MDM2 null background should be able to rescue MDM2 null mice from lethality. This was proven true (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). Beside, concerning tumor formation, MDM2 knockout and MDM2/p53 double knockout mice

are similar. Thus, the mouse model supports the hypothesis that MDM2 is an oncogene and its essential role is to negatively regulate p53.

If MDM2 is the major inhibitor of the tumor suppressor p53, then p53 loss of function alone is sufficient for tumorigenesis, precluding the need for amplification of MDM2 function. Indeed, the mutation of p53 and gene amplification of MDM2 do not often coincide. MDM2 gene amplification was first detected in a significant number of osteosarcomas and soft tissue tumors and the research up to date shows a 7% overall amplification frequency of MDM2 (Momand, Jung et al. 1998). Furthermore, in some of the tumors that maintain both functional p53 and normal level of MDM2, MDM2 can still be hyperactive due to the loss of function of another tumor suppressor ARF (Pomerantz, Schreiber-Agus et al. 1998; Zhang, Xiong et al. 1998). As a major negative regulator of MDM2, ARF is mutated at high frequency in human cancers (Momand, Jung et al. 1998).

MDM2 inhibits p53 transcriptional activity

Although p53 can induce apoptosis independent of its transcription activity, P53 tumor suppressor function is largely dependent on its role as a transcription factor. Upon genotoxic stresses, p53 induces apoptosis, cell cycle arrest, DNA repair and inhibition of angiogenesis. Some of the genes containing p53 responsive element and induced by p53 include p21, GADD45, 14-3-3 σ, Bax, PUMA, and MDM2 (Kastan, Zhan et al. 1992; Barak, Juven et al. 1993; Perry and Levine 1993; Perry, Piette et al. 1993; Miyashita, Krajewski et al. 1994; Chan, Hermeking et al. 1999; Nakano and Vousden 2001). Distinct from other p53 inducible genes, MDM2 binds to the transcriptional activation

domain on the N-terminus of p53, inhibiting p53 transcription of its downstream targets, thus established a negative feedback loop between p53 and MDM2 (Barak, Juven et al. 1993; Juven, Barak et al. 1993; Wu, Bayle et al. 1993; Barak, Gottlieb et al. 1994). This is necessary for the proper growth and proliferation of the cells under normal condition because p53 overexpression will lead to cell death or growth arrest. However, upon DNA damage, rather high level of p53 is needed to harness the growth of damaged cells and send them either to DNA repair or apoptosis pathway. At this point, the physical association between MDM2 and p53 is broken by the phosphorylation of p53 at serines critical for p53/MDM2 binding. Without the inhibition by MDM2, p53 is transcriptionally active and elicits cell cycle arrest or apoptosis to the damaged cells. In this sense, the override of p53 by MDM2 allows DNA damaged cells to proliferate, leading to tumorigenesis.

MDM2 is an ubiquitin E3 ligase for multiple proteins

In the ubiquitination pathway, E3 ubiquitin ligases are the group of enzymes catalyzing the conjugation of ubiquitin moiety to the protein substrates. To date, there are two major groups of ubiquitin E3 ligases. One is the HECT domain containing E3, catalyzing ubiquitin conjugation by forming thio-ester intermediates between E2 and the substrates. The other class is the RING finger domain ubiquitin E3 ligases, featured by the consensus sequence $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$. MDM2 is a RING domain type of E3.

So far, proteins diverse in their functions have been found to be the ubiquitin ligase substrates of MDM2. These proteins include p53, MDM2 itself, MDMX, b-

arrestin, PCAF and insulin-like growth factor 1 receptor (IGF1R) (Momand, Zambetti et al. 1992; Fang, Jensen et al. 2000; Shenoy, McDonald et al. 2001; Girnita, Girnita et al. 2003; Pan and Chen 2003; Jin, Zeng et al. 2004). The ubiquitination of these substrates by MDM2 leads to the rapid turnover of the proteins through proteasome degradation.

MDM2 promotes other posttranslational modifications of p53

Besides being an E3 ligase for p53 and other substrate proteins, MDM2 also stimulates other forms of posttranslational modifications. Sumo-1 is a 110 amino acid protein belonging to an ubiquitin-like family. The conjugation of sumo to a variety of proteins changes protein localization or activity. It was reported that MDM2 mediates p53 sumoylation, which moderately enhances p53 transcriptional activity (Melchior and Hengst 2002). Upon DNA damage, p53 is also acetylated by acetyl transferase CBP/P300. The acetylation of p53 contributes to its stabilization. MDM2 suppresses p53 acetylation by binding to and inhibiting the function of CBP/P300, rendering p53 more susceptible to degradation (Kobet, Zeng et al. 2000; Ito, Lai et al. 2001). Furthermore, MDM2 promotes the conjugation of another ubiquitin-like molecule, nedd8 to p53, leading to the transcriptional inhibition of p53 activity (Harper 2004; Xirodimas, Saville et al. 2004).

MDM2 nuclear import and export

MDM2 contains both nuclear localization signal (NLS) and nuclear export signal (NES), making it possible for transportation in and out of the nucleus independently (Roth, Dobbelstein et al. 1998; Tao and Levine 1999). In recent years, MDM2 has been

reported to mediate the nuclear export of p53 into cytoplasm, rendering p53 more susceptible for proteasome degradation in the cytoplasm (Roth, Dobbelstein et al. 1998; Tao and Levine 1999). Experiments using Leptomycin B blocked the nuclear export of proteins, as a result, p53 was stabilized and its transcriptional activity enhanced (Freedman and Levine 1998). However, MDM2-independent p53 nuclear export might also exist (Zhang and Xiong 2001).

MDMX

MDMX Protein structure

In 1996, a novel p53-binding protein was identified and named MDMX for its structural similarity with the well-established p53-binding protein, MDM2 (Shvarts, Steegenga et al. 1996). MDMX shares significant homology with MDM2 in its protein structure. Compared with MDM2, which is 491 amino acids, MDMX is a 490 amino acid protein that shares several functional domains with MDM2 (Shvarts, Bazuine et al. 1997). Over the years, it has been recognized that MDMX and MDM2 share highly conserved domains in three main regions (fig 2). The first region spans amino acid 42-94, comprising the p53-binding domain. This domain is strictly conserved between MDM2 and MDMX, sharing 53.6% identity (Bottger, Bottger et al. 1999). This conserved region enables both MDMX and MDM2 to bind p53 on its N-terminal transactivation domain and inhibit p53 transcription activity. The second conserved domain or, the Zinc binding domain locates in the central region of both proteins, which is comprised of residues 301-329. The function of the Zinc finger domain is still largely enigmatic. The third highly conserved region is in the C-terminus of both proteins, between amino acid 444-483. This RING finger domain is characterized by its $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$ consensus sequence (Sharp, Kratowicz et al. 1999; Tanimura, Ohtsuka et al. 1999). MDMX and MDM2 share 53.2% identity in this region, which enables the homo- and hetero-dimerization of the two. By using the yeast two-hybrid analysis system, *Tanimura et al* demonstrated that the MDMX-MDM2 heterodimer is more stable than the homodimers of each. The RING finger domain of

MDM2 is necessary and sufficient for its ubiquitin E3 ligase activity (Momand, Zambetti et al. 1992). However, in contrast to MDM2, no E3 ligase activity has been found for MDMX (Jackson, Lindstrom et al. 2001; Migliorini, Danovi et al. 2002; Linares, Hengstermann et al. 2003).

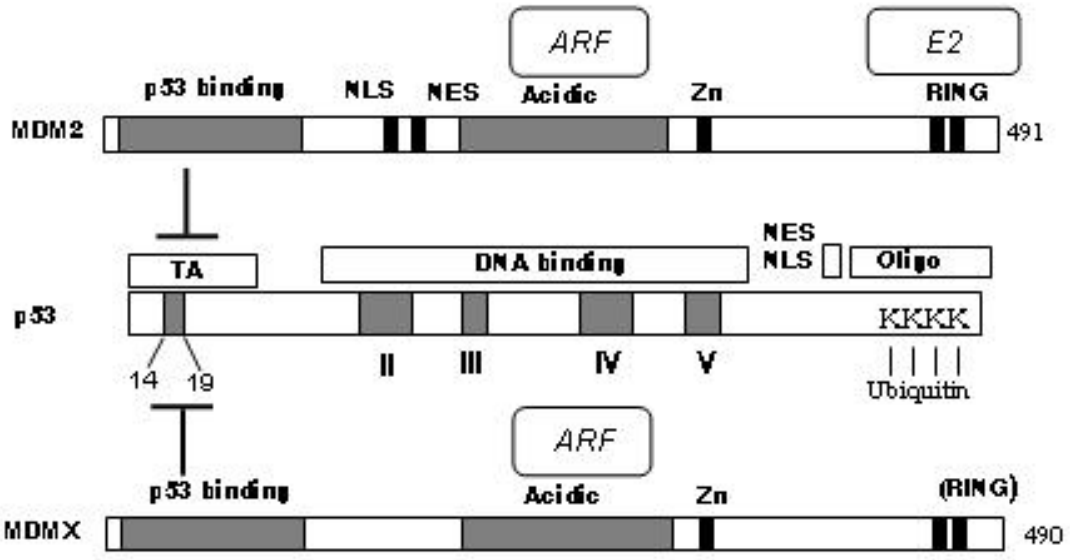


Figure2. MDM2 and MDMX share homology in their protein structures

Among the regions that are distinct between MDMX and MDM2 is the central acidic domain. The acidic domain of MDM2 provides a binding site for ARF. The interaction between MDM2 and ARF results in the inhibition of MDM2 E3 ligase activity and the stabilization of both MDM2 and p53 (Weber, Taylor et al. 1999; Zhang and Xiong 1999). ARF/MDM2 binding also relocates MDM2 from nucleus to nucleolus, physically separates MDM2 from p53 (Pomerantz, Schreiber-Agus et al. 1998).

However, the binding between ARF and MDMX remains controversial (Jackson, Lindstrom et al. 2001; Wang, Arooz et al. 2001). The research of *Kawai et al* and *Meulmeester et al* shed some light on the functional significance of the central acidic domain of MDM2. Both groups demonstrated that the central acidic region is indispensable for MDM2 E3 ligase activity, making it a possible explanation for MDMX's lack of ubiquitin E3 ligase function (Kawai, Wiederschain et al. 2003; Meulmeester, Frenk et al. 2003). Other structural discrepancies between MDM2 and MDMX include the nuclear localization signal (NLS) and nuclear export signal (NES) in MDM2, which are not conserved in MDMX (Jackson and Berberich 2000; Migliorini, Danovi et al. 2002; Migliorini, Denchi et al. 2002).

MDMX gene structure

The homology on protein level between MDMX and MDM2 can be explained by the conservation of the two genes at genomic level (Parant, Chavez-Reyes et al. 2001). The exons 4-12 between MDMX and MDM2 are well conserved. The last exon, exon 12 encodes RING finger domains of MDM2 and MDMX, resulting in the well-conserved RING finger domains on protein level. However, the 5' ends of the genes are quite distinct. In contrast to MDM2, no p53-responsive element was found on MDMX (Shvarts, Steegenga et al. 1996). Consistently, MDM2, but not MDMX is induced upon the activation of p53 after DNA damage.

MDMX as an oncogene

In order to evade the genomic surveillance from tumor suppressor p53, tumor cells selectively mutate or delete p53 gene, escaping from the checkpoint control to proliferate. Alternatively, members of p53 regulatory pathway are involved for the advantage of tumorigenesis. As the major negative regulator of p53 function and stability, MDM2 has been shown to have approximately 7% amplification rate in 28 tumor types (Momand, Jung et al. 1998). Moreover, MDM2 amplification and p53 mutation did not concur in these tumors, implicating that MDM2 overexpression is sufficient for p53 inactivation and tumor formation. In other cases, wild type p53 is accompanied by the loss of function of ARF, which is the main inhibitor for MDM2, leading also to hyperactive MDM2 (Momand, Jung et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Zhang, Xiong et al. 1998).

The mouse models demonstrated that MDMX and MDM2 play non-overlapping roles in negating p53 function (Parant, Chavez-Reyes et al. 2001; Parant, Reinke et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002). MDMX gene amplification was found in a subset of malignant gliomas (Riemenschneider, Buschges et al. 1999). MDMX localizes on chromosome 1q32, which contains frequently amplified sequences including GAC1*, REN* and *RBBP5* in malignant gliomas. In analogy to MDM2 amplification, no p53 mutation concurred with MDMX amplification in the malignant gliomas investigated, suggesting that MDMX overexpression alone is sufficient for p53 inactivation. Furthermore, no MDM2 amplification was found in the presence of MDMX amplification, supporting the non-overlapping regulation of MDMX and MDM2 on p53.

Aberrations of chromosome 1q frequently occur in cervical and ovarian carcinoma (Danovi, Meulmeester et al. 2004). MDMX overexpression was found in a significant number of these cancer cell lines and seems to correlate with the presence of wild type p53 in those cells. In a comprehensive experiment performed by *Danovi et al*, over 500 different human primary tumors were investigated for their MDMX status. It was found that MDMX is overexpressed at the protein level and correlates with 19% of breast cancers, 19% of colon cancers and 18% of lung cancers. None of the MDMX overexpression cell lines contains p53 mutation and only few contain MDM2 gene amplification. Additionally, in the retrovirus-infected MEFcells, MDMX overexpression caused the immortalization of MEF without the selective pressure for the loss of p53 or ARF functions. Furthermore, the transformation of MEF by $HRas^{A12}$ requires p53 loss of function, which can be achieved by concomitant overexpression of $HRas^{A12}$ and MDMX. These findings argue for an important and non-overlapping role of MDMX with MDM2 in negatively regulating p53 tumor suppressive function and implicate MDMX as a putative oncogene.

MDMX mouse models

In order to elucidate the functional significance of MDMX in vivo, MDMX heterozygous mice were generated and crossed for the homozygous MDMX knockout offspring (Parant, Chavez-Reyes et al. 2001; Parant, Reinke et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002). Although mice bearing heterozygous MDMX seemed to develop normally, the MDMX -/- mice were not viable, died at E7.5-

8.5 dpc due to lack of cell proliferation and massive apoptosis in central nervous system. However, when MDMX null allele was crossed into the p53 null background, the generated MDMX/ p53 double null mice were rescued from the embryonic lethality but were prone to tumorigenesis. Moreover, the loss of MDMX cannot be compensated by the expression of MDM2. This evidence strongly argues for the function of MDMX as a major p53 negative regulator in vivo and that MDMX and MDM2 serve to regulate p53 by distinct mechanisms.

MDMX upon DNA damage

Both MDMX and MDM2 proteins contain a p53-binding domain and inhibit the growth-suppressive function of p53. However, unlike MDM2, MDMX gene does not possess p53-responsive element thus cannot be induced in a p53-dependent manner after DNA damage (Shvarts, Steegenga et al. 1996). Early studies have found that MDMX is constitutively expressed at both mRNA and protein levels during cell proliferation, differentiation or after DNA damage (Jackson and Berberich 1999). MDMX lacks the nuclear localization signal (NLS) and is predominantly cytoplasmic in unstressed cells whereas both p53 and MDM2 possess functional NLS and localize in nucleus (Migliorini, Danovi et al. 2002). DNA damage induced p53 or MDM2 facilitates the nucleus translocation of MDMX by forming heterodimeric or heterotrimeric complexes with MDMX (Migliorini, Danovi et al. 2002). However, MDMX nuclear entry can also be independent of either MDM2 or p53, demonstrated by the efficient MDMX nuclear translocation in p53-/-MDM2-/- mouse embryo fibroblasts after DNA damage (Li, Chen

et al. 2002). Overexpression of MDMX under cellular stress has been shown to enhance p53 stability and activity due to the competitive binding between MDMX and MDM2 to p53. Under stressed conditions, overexpressed MDMX dominates the binding to p53, allowing a p53 population free of potent MDM2 inhibition, leading to enhanced p53-dependent apoptosis (Gu, Kawai et al. 2002; Mancini, Gentiletti et al. 2004). Although this result reflects a potential positive regulation of p53 by MDMX, the MDMX overexpression under DNA damage is an artificial effort. Studies in our lab and others found that MDMX is polyubiquitinated and degraded by proteasome in an MDM2-dependent fashion after DNA damage (Kawai, Wiederschain et al. 2003; Pan and Chen 2003). Furthermore, this MDM2-dependent polyubiquitination of MDMX can be enhanced by ARF (Kawai, Wiederschain et al. 2003; Pan and Chen 2003). ARF was shown to inhibit MDM2 E3 ligase activity toward p53 and MDM2 polyubiquitination and degradation, leading to stabilization of both proteins (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998). Additionally, ARF stimulates p53 growth suppressive function and can inhibit MDM2 or MDMX mediated p53 suppression (Jackson, Lindstrom et al. 2001). By stimulating the polyubiquitination of MDMX, ARF presumably enhances p53 functions by targeting both of its negative regulators namely MDMX and MDM2.

MDMX-MDM2-p53

The negative feedback loop between p53 and MDM2 has been well established. Explicitly, p53 transcriptionally activates MDM2, which negatively regulates p53 by

inhibiting its transactivation and destabilizing p53 by mediating its proteasome degradation (Barak, Juven et al. 1993; Juven, Barak et al. 1993; Wu, Bayle et al. 1993; Barak, Gottlieb et al. 1994). The mouse models experiments support MDMX as another major negative regulator of p53 function in vivo besides MDM2 (Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002; Parant and Lozano 2003). Moreover, it is well recognized that MDMX can also bind to the N-terminus transactivation domain of p53, thus inhibiting p53 transcriptional function (Shvarts, Steegenga et al. 1996; Bottger, Bottger et al. 1999; Stad, Ramos et al. 2000). We now know that MDMX is another substrate of MDM2 E3 ligase activity (Kawai, Wiederschain et al. 2003; Pan and Chen 2003). MDMX is polyubiquitinated by MDM2 and degraded in a proteasome-dependent pathway. However, two main questions remain elusive. (1) How does MDMX regulate MDM2 function and stability? (2) How do MDMX and MDM2 cooperate to regulate p53 function and stability?

One model proposed that MDMX and MDM2 regulate p53 in a mutually dependent manner. MDM2 is a very unstable protein due to its intrinsic E3 ligase activity. MDMX stabilizes MDM2 by inhibiting the ubiquitin E3 ligase function of MDM2 toward itself. On the other hand, MDM2 recruits MDMX to the nucleus where MDMX retains a nuclear pool of p53 through direct binding. Since MDM2-p53 interaction leads to re-localization of p53 to cytoplasm, where p53 is efficiently degraded, MDMX stabilizes p53 by retaining it in the nucleus. Meanwhile, MDMX and MDM2 synergistically inactivate p53 transcriptional activity in the nucleus through direct binding to p53's transactivation domain. Therefore, MDMX and MDM2 cooperatively inhibit p53 transcription activity but antagonize each other in regulating p53 stability. This

model is supported by a majority of the labs employing overexpression technique (Jackson and Berberich 2000; Stad, Ramos et al. 2000; Stad, Little et al. 2001). The other model agrees on the observation that MDMX stabilizes p53 but assumes a distinct mechanism for the stabilization. It is suggested that MDMX over-expression enhances p53 stability and even functionality through the competitive binding of MDMX to p53 with MDM2. MDMX overexpression has a squelching effect on p53-MDM2 binding, resulting in more p53 free from MDM2 association and inhibition (Gu, Kawai et al. 2002; Mancini, Gentiletti et al. 2004). The third model is supported by in vitro assays, showing that MDMX stimulates the E3 ligase activity of MDM2, explaining the destabilization of both p53 and MDM2 at the overexpression of MDMX. All except for one group excluded the possible E3 function of MDMX (Badciong and Haas 2002).

ARF

$P16^{INK}$ is a cell cycle checkpoint gene that inhibits cyclin-dependent kinase 4 (Sherr and Roberts 1995; Weinberg 1995). The gene locus of $p16^{INK}$ on chromosome 9 is mutated at high frequency in human tumors (Kamb, Gruis et al. 1994; Nobori, Miura et al. 1994). Later it was found that the necessity in tumor cells to mutate p16 locus lies in the fact that this locus encodes a second gene transcribed with an alternative reading frame, namely ARF (fig 3) (Serrano, Hannon et al. 1993; Quelle, Zindy et al. 1995). Although ARF is completely unrelated to p16, the mutation of ARF also caused high frequency of tumor genesis independent of p16, supporting ARF as a tumor suppressor (Foulkes, Flanders et al. 1997; Ruas and Peters 1998; Sharpless and DePinho 1999).

The mechanism of ARF induced cell cycle arrest is distinct from that of $p16^{INK}$. A myriad of genetic stress including E1A, E2F, myc, ras and v-abl induce the expression of ARF. ARF activates the function of tumor suppressor p53 by harnessing p53 inhibitor, MDM2 (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998). ARF plays a major role in inhibiting the negative regulation of MDM2 on p53 through at least three mechanisms. One relies on the nucleolus localization signal (NoLS) contained within 85-101 residues on the C-terminal domain of human ARF, enables ARF nucleolus-localization (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998). By binding to MDM2 central region, ARF recruits MDM2 into nucleolus, thus

physically separated MDM2 from p53. The second mechanism involves the abrogation of MDM2 E3 ligase activity by ARF (Weber, Taylor et al. 1999; Zhang and Xiong 1999). The third mechanism is supported by the experiments showing that ARF attenuates the suppression of p53 acetylation by MDM2, contributing to the stabilization of p53 (Ito, Lai et al. 2001). The end result of both mechanisms is that ARF stabilizes and activates p53, inducing the transcription of a myriad of p53-dependent genes that function in DNA repair, apoptosis, cell cycle arrest and anti-angiogenesis.

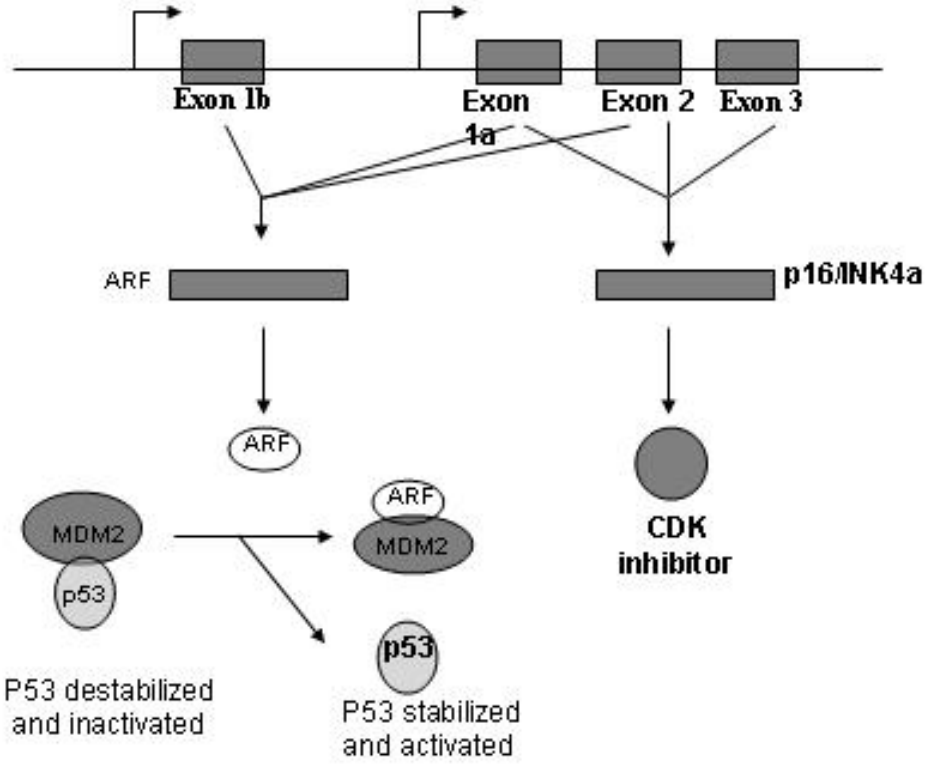


Figure3. p16 and ARF are two tumor suppressors encoded in the same gene locus but working by distinct mechanisms

ARF is not a stereotypical inhibitor of MDM2 and the interplay between the two proteins is still being explored. Nevertheless, previous studies showed that ARF works on all aspects of MDM2-mediated posttranslational modification. MDM2 ubiquitination of the substrates including self-ubiquitination is inhibited by ARF, resulting in the stabilization of the MDM2 E3 substrates (Kamijo, Weber et al. 1998). In addition, in response to stress signals, p53 is acetylated by CBP/P300, which increases p53 stability. MDM2 suppresses p53 acetylation by binding to the acetyl transferase CBP/P300 and inactivating the enzyme. ARF abrogates MDM2's suppression on p53 acetylation, thus restores p53 stability and activity (Ito, Lai et al. 2001). Chen et al also demonstrated that MDM2 stimulatesc p53 sumoylation (Chen and Chen 2003). However, instead of antagonizing MDM2's modification on p53, ARF enhances MDM2-mediated p53 sumoylation. Data from this manuscript demonstrated that ARF stimulates MDM2-mediated MDMX ubiquitination and MDM2-independent MDMX sumoylation. Therefore, ARF is a dynamic regulator of MDM2.

Ubiquitination and proteasome degradation review

Cellular protein degradation is a strictly regulated process, affecting all aspects of biological functions. One important mechanism for protein degradation is through ubiquitination-proteasome degradation pathway. Ubiquitin, as its name indicated, is a widely expressed protein present in most of the tissues and organisms, well conserved from yeast to human. It is a 76-amino acid small protein that can be conjugated through

an isopeptide linkage to the lysines in the target protein as a post-translational modification (Pickart 2001; Weissman 2001). Since its initial discovery, ubiquitination has been recognized in a wide variety of biological processes, ranging from signal transduction to endocytosis.

Ubiquitin-mediated proteasome degradation is a stepwise procedure (Haas and Siepmann 1997). The first step involves ubiquitin-activating enzyme E1 activating ubiquitin in an ATP-dependent fashion. Then ubiquitin molecule is transferred to E2, ubiquitin-conjugating enzyme, forming a high-energy thio-ester intermediate of E2-ubiquitin. E3 ubiquitin ligase is a group of enzymes mostly responsible for substrate recognition and specificity. There are two sets of E3s classified by their distinct functional domains. The HECT domain E3s contain a highly conserved 350 amino acid region in their carboxyl termini that was first discovered in E6-AP, the ubiquitin E3 that mediates the ubiquitination and degradation of tumor suppressor p53 by association with E6 viral oncoprotein. HECT domain E3s form intermediates between E2 and the substrate proteins while the RING domain containing E3s directly transfer E2 to the substrate proteins (Haas and Siepmann 1997).

RING finger containing proteins were originally recognized merely for its potential in protein dimerization. Only until recently was it uncovered as a group of enzymes playing important roles in ubiquitination pathway. RING proteins share conserved structure $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CH_{(4-48)}CX_2C$, the cysteines and histidines represent the zinc binding sites. Crystal structures of RING finger proteins indicated that the protein structure of RING domain permits the optimal positioning between E2 and the substrate, ensuring the transfer of ubiquitin to the target protein.

RING finger E3s can be divided into two groups: single and multi subunit E3s (Haas and Siepmann 1997). Single-subunit E3s contain the substrate binding site and the RING finger catalytic domain on one protein. Some of the well-studied single subunit RING finger E3s include p53 regulating oncoprotein MDM2; Parkin, the mutation of which associates with juvenile Parkinson's disease and c-Cbl, a proto-oncoprotein. Multi-subunit E3s have the RING finger-containing subunit and the substrate recognition subunit separated on distinct proteins. Among them are SCF (Skp1/Cul11/F-box protein), APC (anaphase promoting complex) and VHL-CBC (Von Hippel–Lindau–Cul2/elongin B/elongin C complex).

Different numbers of the ubiquitination enzymes involved in each step indicates that the specificity of activity differs among E1, E2 and E3. While only one E1 has been identified in yeast, there are at least 13 E2s in yeast and 25 E2s in mammalians discovered so far. In contrast, the most diversity of the ubiquitination enzymes comes with E3, the ubiquitin ligase. To date, more than a hundred E3s have been recognized, implicating their role in substrate selection. In order to achieve strictly regulated protein degradation, different proteins are recognized by distinct ubiquitin ligases, targeting them to the ubiquitination system. The substrate specificity is reached by the pool of E3s that recognize only certain proteins for ubiquitination and in the case of polyubiquitination, often lead to substrate degradation.

Proteins can be modified by a single ubiquitin conjugated to one or more of its lysine residues, so called monoubiquitination. While the functional significance of protein monoubiquitination is still elusive, it has been implicated in the regulation of biological processes such as endocytosis, lysosomal degradation and chromatin

remodeling. With the presence of an internal lysine in the ubiquitin protein, a polymer of ubiquitin could also be constructed through isopeptide bond between the C-terminus of one ubiquitin and the internal lysine 48 residue of the other (Pickart 1997).

. When conjugated to a target protein, the ubiquitin chain containing no less than four ubiquitin polymers is often recognized as a signal for proteasome destruction (Thrower, Hoffman et al. 2000). The destination of destruction for the polyubiquitinated proteins is the 26S proteasome. It is composed of two major subunits: one is the 20S core particle that carries out the proteolytic activity; the other is the 19S regulatory particle, regulating the functionality of the core particle. Both 20S core particle and 19S regulatory particles contain multiple subunits. The 20S core is a cylindrical barrel-shaped structure composed of four rings stacked upon each other. The two rings on both ends are called α subunits, which gate the entry of the substrates for degradation. Without the regulation by the 19S caps, the multiple-ubiquitinated proteins cannot enter the catalytic core for degradation. The 19S regulatory particle carries out multiple functions: selecting target, processing substrates and leading the entrance of target proteins to the catalytic core. Across species and between human and yeast, up to 40-70% of sequence identity was found in some of the proteasome subunits, indicating ubiquitination system as one of the most conserved systems in eurkaryotes.

Although targeting substrate proteins for proteolysis is a key function of ubiquitination, it is becoming clear that ubiquitination plays much more complicated roles in myriad of biological processes, including DNA damage response, translation regulation, kinase regulation and endocytosis. The functional mechanism of these processes has just started to be appreciated.

Posttranslational modification by ubiquitin is a reversible process. A group of thiol protease enzymes namely DUBs tightly control the removal of ubiquitin by dissembling poly-ubiquitin chains and cleaving ubiquitin off the protein substrates (Wilkinson 1997). This ensures the recycling of the ubiquitin and prevents the accumulation of ubiquitin chains at the proteasome, which will cause disordered proteasome functions. DUBs also add to the layer of ubiquitination regulation by affecting the steady state level of the ubiquitin-conjugated proteins since the deubiquitination of the protein can rescue it from destruction by proteasome degradation.

Sumoylation process

Posttranslational modification is an efficient way to modulate protein-protein interaction, stability, function or localization. Small molecules such as ubiquitin, phosphate, and acetyl conjugate to specific residues on target proteins and modify their localization, stability or activity. Among these small molecules participated in protein posttranslational modifications, the functional significance of sumo is less well understood. Sumo, or small ubiquitin like molecule, is a 101 amino acid protein that bears homology with ubiquitin (Boddy, Howe et al. 1996; Matunis, Coutavas et al. 1996; Okura, Gong et al. 1996; Shen, Pardington-Purtymun et al. 1996; Kamitani, Nguyen et al. 1997; Mahajan, Delphin et al. 1997; Tsytsykova, Tsitsikov et al. 1998). Despite the limited 18% overall sequence homology, sumo and ubiquitin share very similar three-dimensional structure and protein folding. Nevertheless, significant differences were

found between sumo and ubiquitin on the N-terminus structure as well as the surface charge of both proteins.

The process of sumoylation parallels that of ubiquitination. Both sumoylation and ubiquitination are processes of stepwise conjugation of the small molecules to the target proteins, involving functional specific enzymes in each step. Aos1/Uba2 in yeast or SAE1/SAE2 in human are E1 activating enzymes, activating the sumo or ubiquitin molecule; E2 conjugates sumo or ubiquitin to E3 or directly to the substrates. Unlike multiple E2s for ubiquitination, sumoylation has a unique E2, Ubc9. Both sumo/ubiquitin activation and conjugation steps are ATP-dependent. In the process of ubiquitination, hundreds of identified E3 ubiquitin ligases provide the substrate specificity needed to determine the fate and on many occasions, the degradation of the target proteins. On the other hand, the E3 ligases for sumoylation process have just emerged and await further investigation. To date, two classes of sumo E3s have been discovered. The first class consists only one member, the RanBP2, which is a component of the nuclear pore complex. The other class includes siz1 and siz2 in the *S. cerevisiae* as well as the PIAS family, which was first recognized as co-regulators of the JAK-STAT pathway.

Sumoylation functions

A consensus sumoylation site has been identified. The presence of consensus sequence ψKXE, where ψ represents a large hydrophobic amino acid and K stands for the sumo conjugating lysine residue indicates potential modification by sumo conjugation. Although the number of identified sumo substrates is limited, they seem to

play important roles in diverse fields such as maintenance of genomic stability, DNA repair, gene transcription, signal transduction and nuclear pore function.

In 1999, two labs simultaneously discovered that p53 is sumoylated at lysine 386 and this modification activates p53 transcriptional activity (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999). The requirement for sumo E3 ligase in p53 sumoylation is controversial. Original findings claimed that p53 *in vitro* sumoylation requires only sumo-1, E1 and E2. However, it was later discovered that PIAS directly binds both p53 and Ubc9 and stimulates p53 sumoylation *in vitro* (Schmidt and Muller 2002). MDM2 was also proved sumoylated and the process is stimulated by ARF (Xirodimas, Chisholm et al. 2002). The functional significance of MDM2 sumoylation remains unclear.

RanGAP1 is a regulator of nuclear pore transportation. Unmodified RanGAP1 localizes in the cytoplasm while sumoylated RanGAP1 relocalizes to the nuclear pore complex and associates with a nuclear pore complex subunit, RanBP2, facilitating nuclear import (Matunis, Coutavas et al. 1996). PML, promyelocytic leukemia protein is another example of sumoylation modifying protein subcellular localization (Sternsdorf, Jensen et al. 1997; Kamitani, Nguyen et al. 1998; Muller, Matunis et al. 1998). Upon sumo conjugation, PML relocalizes to the nuclear bodies where it recruits Daxx, inhibiting Daxx-mediated transcription repression of target genes (Kim, Park et al. 2003).

Sumoylation can also regulate transcription by recruiting coactivators or corepressors to sumo-modified transcription factors. For example, the sumoylation of the transcription coactivator p300 leads to the recruitment of HDAC6, the histone deacetylase (Girdwood, Bumpass et al. 2003).

Various proteins participating in the maintenance of genomic stability are posttranslational modified by sumo. These include topoisomerase I and II, thymine-DNA glycosylase enzyme (TDG) as well as the proliferating cell nuclear antigen (PCNA) (Mao, Desai et al. 2000; Mao, Sun et al. 2000; Saitoh and Hinchey 2000; Hardeland, Steinacher et al. 2002; Stelter and Ulrich 2003). Sumo modification affects their protein-protein interaction, subcellular localization or activity.

There are cross talks between sumoylation, ubiquitination, phosphorylation and acetylation. For example, the phosphorylation of NF-kB stimulates its ubiquitination while antagonizing sumo conjugation (Huang, Wuerzberger-Davis et al. 2003). Noticeably, all acetylation, ubiquitination and sumoylation take place solely on lysine residues. However, modifications on common lysine residues have had both antagonistic and synergistic functions on certain substrates.

We identified MDMX as another substrate of sumo conjugation. Similar to MDM2, the sumoylation of MDMX can also be stimulated by ARF. Unlike MDM2 however, MDMX is sumoylated in an ARF independent fashion. In vitro assay revealed that the presence of sumo-1, E1 and E2 are sufficient for MDMX sumoylation. Major sumoylation sites of MDMX were identified to two lysines. However, the sumoylation status of MDMX does not affect its association or function with either p53 or MDM2. Thus, the physiological significance of MDMX sumoylation remains elusive.

Sumoylation is a reversible process

Sumoylation process is dynamic and reversible. Sumo-specific proteases participate in both the process of sumo maturation and the cleavage of sumo from the

substrates. Sumo is synthesized as precursors and processed at the carboxy-terminus before entering the cycle of sumo activation and conjugation by sumo-specific proteases. In yeast, the Ulp proteases are crucial for viability. The Ulp homologs in mammalian cells are a family of enzymes termed SENP (Sentrin-specific protease). To date, four SENP family members have been characterized (Gong, Millas et al. 2000; Kim, Baek et al. 2000; Nishida, Tanaka et al. 2000; Nishida, Kaneko et al. 2001). Even though the substrate specificity of different SENPs are not known, the fact that these SENP family members distribute in different subcellular compartments indicates that there might be functional specificity of these sumo proteases. SENP1 localizes in nuceloplasm and nuclear bodies; SENP2 is concentrated at nuclear pore; SENP3 is contained in nucleolus and SENP6 resides in cytoplasm (Gong, Millas et al. 2000; Kim, Baek et al. 2000; Nishida, Tanaka et al. 2000; Nishida, Kaneko et al. 2001; Bailey and O'Hare 2002; Hang and Dasso 2002). .

Materials and Methods

Cell Lines

MDM2/p53 double-null mouse embryo fibroblast 174.1 is a kind gift from Dr. Guillermina Lozano (McMasters, Montes de Oca Luna et al. 1996). ARF/p53 double-null mouse embryo fibroblast is a kind gift from Dr. Gerry Zambetti. All cell lines including H1299 (non-small cell lung carcinoma, p53-null), U2OS (osteosarcoma, p53 wild type, ARF-deficient), HFF (passage 20 human primary foreskin fibroblast) and MCF7 (breast cancer, p53 wild type) cells were maintained in DMEM medium with 10% fetal bovine serum and 100 units/ml penicillin and streptomycin as well as 1% non-essential amino acids (cellgro).

Plasmids and Reagents

Human MDMX cDNA (myc-MDMX, MDMX-myc-His6) were kindly provided by Dr. Donna George (Sharp, Kratowicz et al. 1999). The myc epitope tag fused to the MDMX cDNA was removed by subcloning to generate a non-tagged MDMX. A Flag epitope tag was added to the C terminus of myc-MDMX by PCR to create double-tagged myc-MDMX-Flag. His6-ubiquitin expression plasmid was kindly provided by Dr. David Lane

(Xirodimas, Chisholm et al. 2002). ARF deletion mutants were kind gifts from Dr. Yue Xiong (Zhang and Xiong 1999). MDM2 deletion mutants were described previously (Chen, Marechal et al. 1993; Zhang and Xiong 1999). MDMX deletion mutants were generated by PCR and subcloned into pcDNA3.1 (+) vector. Point mutants of MDM2 and MDMX were created using the Quick Change kit (Stratagene). All p53, MDM2, MDMX and ARF constructs used in this study were of human origin. Monoclonal antibody 8C6 against human MDMX was generated in our laboratory and reacts specifically with MDMX in a region between residues 101-393 (Li, Chen et al. 2002). Adenovirus expressing human ARF was kindly provided by Dr. Yue Xiong and used as described previously (Lu, Lin et al. 2002). U2OS stable cell line expressing tetracycline-repressible human MDM2 was created by cloning MDM2 cDNA into the pUHD15.1 vector and cotransfecting with pUHG10.3 (Gossen and Bujard 1992).

Western Blot

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF], centrifuged for 5 mins at 10,000 g, and the insoluble debris were discarded. Cell lysate (10-50 μg protein) was fractionated by SDS-PAGE and transferred to Immobilon P filters (Millipore) using a semi-dry electro-blotting apparatus for 1 hr. 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 2 hrs with primary antibodies diluted in washing buffer. MDM2 was

detected by 3G9 with 1:40 dilution (Chen, Marechal et al. 1993); p53 was detected by DO-1 with 1:10,000 dilution (Pharmingen); ARF was detected by 14PO2 (Neomarkers) with 1:2000 dilution and MDMX was detected by 8C6 with 1:40 dilution. The filter was then washed four times (5 min each) with PBS containing 0.1 % Tween-20. Next, bound primary antibodies were conjugated with secondary antibody IgG goat-anti-mouse HRP by incubating the filter with the secondary antibody for two hrs in the washing buffer containing phosphate-buffered saline (PBS), 5% non-fat dry milk and 0.1 % Tween-20. The filter was washed and developed using ECL-plus reagent (Amersham).

Immunoprecipitation

Cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF], centrifuged for 5 mins at 10,000 g, and the insoluble debris were discarded. To detect the binding between MDM2 and MDMX, two mg of total cellular protein was immunoprecipitated using 100 µl of anti-MDM2 monoclonal antibody 3G9 and 20 µl packed protein A-Sepharose beads (Sigma) for 3 hours at 4^{o}C. The beads were then spinned down and washed three times with lysis buffer, boiled in SDS sample buffer and the immunoprecipitate was fractionated by SDS PAGE followed by western blot.

Transfection

Calcium phosphate transfection was performed in H1299 and U2OS cells for their high transfection efficiency. In transient transfection, $2x10^6$ cells were seeded into 10 cm tissue culture dishes for 24 hrs. For each transfection, a total amount of 40μg of plasmid DNA was mixed with 450μl of H_2O, 125 mM calcium chloride and 500 μl HEPES (0.28 M NaCl, 0.05 M HEPES, 1.5mM $CaCl_2$). Immediately after mixing, the mixture was added to the cells and incubated for 16 hours. Transfected cells were washed 5 times with PBS, refed with complete medium and incubated for another 24 hours before harvest. In a stable transfection, 48 hours after transfection, the cells were drug selected by complete medium containing 750 μg/ml G418 for two weeks. Drug-resistant colonies were either pooled or cloned.

Lipofectamine transfection was performed in MEF cells for their low transfection efficiency with calcium phosphate transfection (invitrogene). $2x10^5$ cells were seeded into 6 cm tissue culture dishes for 24 hrs, washed with 3 ml and refed with 2 ml of serum-free medium. For each transfection, a total amount of 4μg 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. Pre-mix 10 μl of lipofectamine with 250 μl serum-free medium and mix with the DNA solution. The mixture was incubated for another 15 min before added to the cells. After 3 hrs incubation, the cells were refed with complete medium.

Immunofluoresence Staining

Approximately 50,000 cells were plated on chamber slides and incubated for 18 hours before staining. The cells were fixed by 1:1 ratio acetone/methanol for 3 minutes at RT. The slides were immediately rinsed two times with washing buffer (PBS with 0.1% Triton-X 100) and then incubated in washing buffer twice for 5 minutes each. The slides were blocked with blocking buffer (10% normal goat serum in PBS) for 20 minutes. Primary antibody 8C6 is diluted 1:40 in the blocking buffer and incubated with the cells for 2 hrs at RT. Then the slides were rinsed and incubated in washing buffer twice for 5 minutes each. The secondary antibody goat anti-mouse FITC was diluted 1:400 in blocking buffer and incubated with the cells in dark for 2 hrs. The slides were then washed and mounted.

In vivo Ubiquitination Assay

H1299 and U2OS cells in 9 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 expression plasmids using conventional calcium phosphate precipitation method. 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 Na2HPO4/NaH2PO4, 0.01 M Tris-Cl pH8.0, 5 mM imidazole, 10 mM β-mecaptoethanol) and incubated with Ni^{2+}-NTA beads (Qiagen) for 4 hours at room temperature. The beads were washed with buffer A, B (8 M urea, 0.1 M $_{Na2PO4}$/NaH_2PO_4, 0.01 M Tris-Cl pH8.0, 10 mM β-mecaptoethanol), C (8 M urea, 0.1 M Na_2PO_4/NaH_2PO_4, 0.01 M Tris-Cl pH6.3, 10 mM β-mecaptoethanol), 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.

In vitro **Ubiquitination Assay**

GST-MDM2 full-length and deletion mutants were expressed in ***E. coli*** and purified by binding to glutathione-agarose beads. The substrate MDMX was produced by *in vitro* translation in rabbit reticulocyte lysate using the TNT system (Promega) in the presence of 35S-methionine. 15 µl packed beads loaded with ~2 µg GST fusion proteins were incubated with 4 µl of MDMX *in vitro* translation product, 250 ng GST-Ubc5Hb (Boston Biochem), 250 ng purified rabbit E1 (AG Scientific), 2 µg His6-ubiquitin (AG Scientific), and 20 µl reaction buffer (50 mM Tris pH7.5, 2.5 mM MgCl2, 15 mM KCl, 1 mM DTT, 0.01% Triton X-100, 1% glycerol, 4 mM ATP). The mixture was incubated at

37˚C for 1 hr with shaking, boiled in SDS sample buffer and fractionated by SDS-PAGE. The gel was dried and MDMX was detected by autoradiography.

MDM2 Promotes Ubiquitination and Degradation of MDMX

Abstract

The p53 tumor suppressor is regulated by MDM2-mediated ubiquitination and degradation. Mitogenic signals activate p53 by induction of ARF expression, which inhibits p53 ubiquitination by MDM2. Recent studies showed that the MDM2 homolog MDMX is also an important regulator of p53. We present evidence that MDM2 promotes MDMX ubiquitination and degradation by the proteasomes. This effect is stimulated by ARF and correlates with the ability of ARF to bind MDM2. Promotion of MDM2-mediated MDMX ubiquitination requires the N-terminal domain of ARF, which normally inhibits MDM2 ubiquitination of p53. An intact RING domain of MDM2 is also required, both to interact with MDMX and to provide E3 ligase function. Increase of MDM2 and ARF levels by DNA damage, recombinant ARF adenovirus infection, or inducible MDM2 expression leads to proteasome-mediated down-regulation of MDMX levels. Therefore, MDMX and MDM2 are coordinately regulated by stress signals. The ARF tumor suppressor differentially regulates the ability of MDM2 to promote p53 and MDMX ubiquitination and activates p53 by targeting both members of the MDM2 family.

Results

MDM2 stimulates MDMX ubiquitination and degradation

MDM2 is rapidly degraded by the proteasome due to self-ubiquitination (Fang, Jensen et al. 2000). In contrast, little is known about the regulation of MDMX turnover. We tested whether it is subjected to ubiquitination *in vivo*. To detect MDMX ubiquitination, we coexpressed MDMX and His6-ubiquitin by transient transfection into H1299 cells. MDMX conjugated to His6-ubiquitin was purified by Ni^{2+}-NTA beads under denaturing conditions and detected by western blot with MDMX-specific monoclonal antibody 8C6 (Li, Chen et al. 2002). The result showed that a small portion of MDMX was conjugated to ubiquitin *in vivo* (mainly mono-ubiquitination based on slightly slower mobility than unmodified MDMX). Transfection of MDM2 strongly stimulated MDMX poly-ubiquitination (Figure 4).

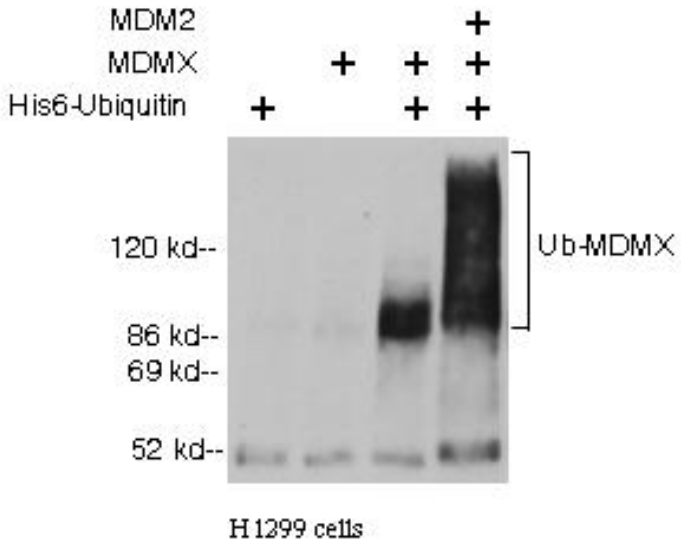


Figure4. MDM2 stimulates MDMX polyubiquitination. MDMX was coexpressed with His6 -ubiquitin in H1299 cells by transient transfection. His6-ubiquitinated proteins were purified, and MDMX was detected by Western blotting with 8C6 antibody.

Interestingly, cotransfection of MDM2 and ARF did not inhibit but rather stimulated MDMX polyubiquitination (Figure 5). MDM2 expression level was also increased in the presence of ARF (due to stabilization) and may account for the increase in MDMX ubiquitination.

Direct western blot also showed that MDMX expression level was significantly reduced when coexpressed with MDM2 (Figure 5), suggesting that ubiquitination of MDMX led to subsequent degradation by the proteasomes.

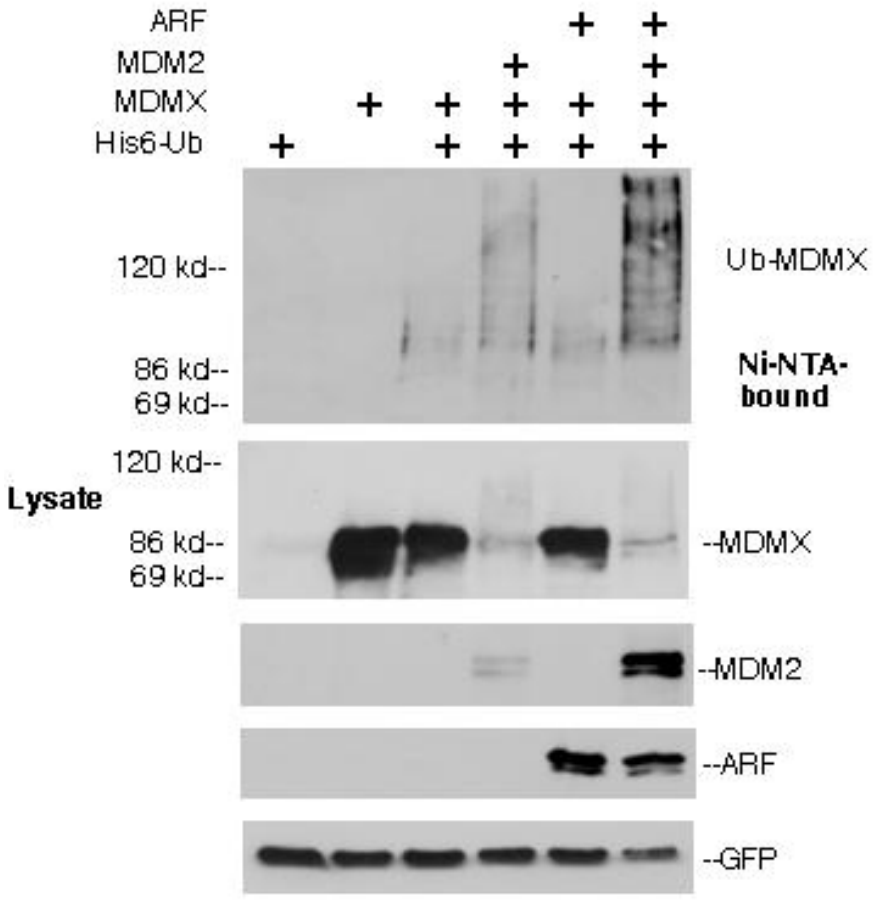


Figure 5. MDM2 and ARF synergistically stimulate the polyubiquitination and degradation of MDMX. MDMX was coexpressed with His6 -ubiquitin in H1299 cells by transient transfection. His6 -ubiquitinated proteins were purified, and MDMX was detected by Western blotting with 8C6 antibody (top panel). The lower panels are control Western blots of nonpurified cell lysate (10% of the amount used for Ni-NTA purification).

Consistent with this hypothesis, treatment with proteasome inhibitor MG132 for 4 hours before cell harvest partially prevented MDMX down-regulation by MDM2 cotransfection (Figure 6). In the absence of cotransfected MDM2, MDMX was relatively stable and did not accumulate in the presence of MG132.

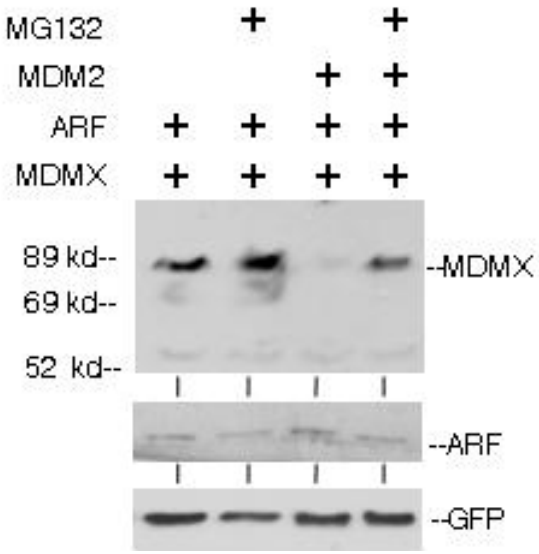


Figure 6. MDM2 stimulates MDMX degradation by proteasome pathway. H1299 cells transfected with the indicated plasmids were treated with 30 μM MG132 for 4 hours prior to Western blot analysis for MDMX. Transfection efficiency was verified by expression of cotransfected GFP plasmid.

To determine whether MDM2 and ARF are required for the basal ubiquitination of MDMX, the assay was repeated using MDM2/p53-null or ARF/p53-null mouse fibroblasts. The results showed that mono-ubiquitination of MDMX occurred in the absence of endogenous MDM2 (Figure 7) and ARF (data not shown). Therefore, MDMX can undergo ARF and MDM2-independent mono-ubiquitination. Cotransfection of MDM2 or MDM2 and ARF also stimulated MDMX poly-ubiquitination in mouse cells.

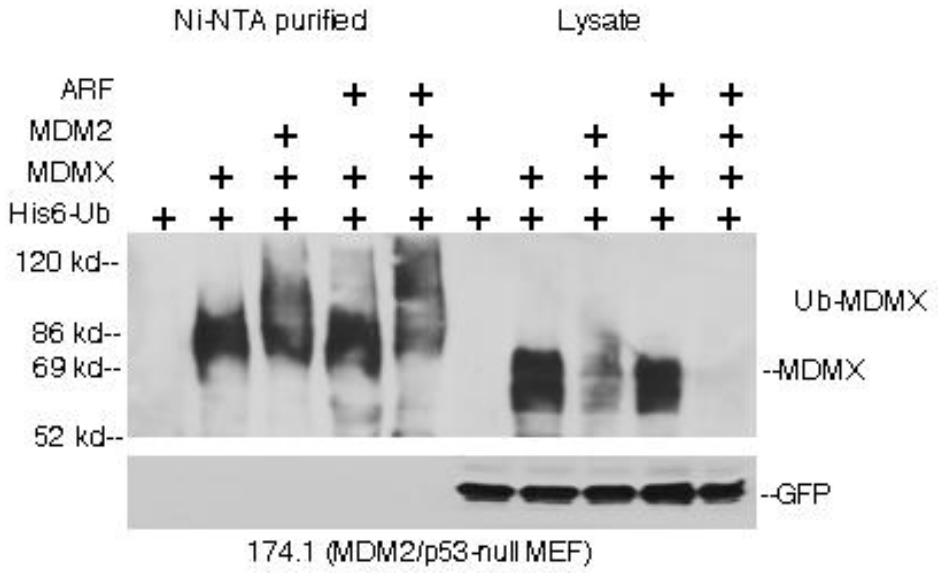


Figure 7. MDMX can be ubiquitinated independent of MDM2. MDM2/p53-null 174.1 cells were transfected with the indicated plasmids. Ni-NTA-purified ubiquitinated MDMX and cell lysate were run on the same gel to show the molecular weight shift due to MDM2-independent monoubiquitination.

MDM2 RING domain is sufficient for promoting MDMX ubiquitination

Next, a panel of MDM2 mutants was tested for ubiquitination of MDMX. The results showed that the RING domain of MDM2 was important for promoting MDMX ubiquitination (representative mutants are shown in Figure 8 & 10). MDM2 deletion mutants without the p53-binding or ARF-binding domains were still capable of stimulating MDMX ubiquitination, indicating that these regions were not essential for this function. An MDM2 NLS point mutant (182R) that prevents nuclear translocation was also able to ubiquitinate and degrade MDMX. Furthermore, the C terminal 130 amino acid of MDM2 containing the RING domain was sufficient to ubiquitinate MDMX (Figure 8 & 10). Deletion of MDMX C terminal RING domain (Δ394-490) also prevented poly-ubiquitination by MDM2 (Figure 9 & 10). Therefore, these results showed that poly-ubiquitination of MDMX by MDM2 required formation of MDMX-MDM2 complex through RING domain-mediated heterodimerization and possibly the E3 function of MDM2.

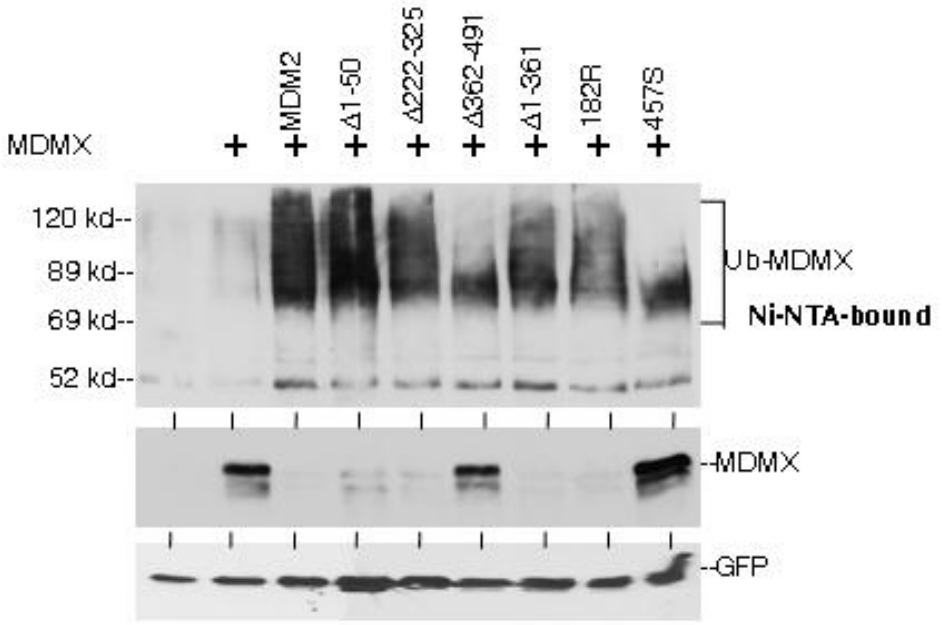


Figure 8. MDM2 RING domain is required for stimulating MDMX polyubiquitination. H1299 cells were transfected with His6 -ubiquitin, MDMX, and representative MDM2 mutants. MDMX ubiquitination and degradation were detected by Ni-NTA purification and Western blotting.

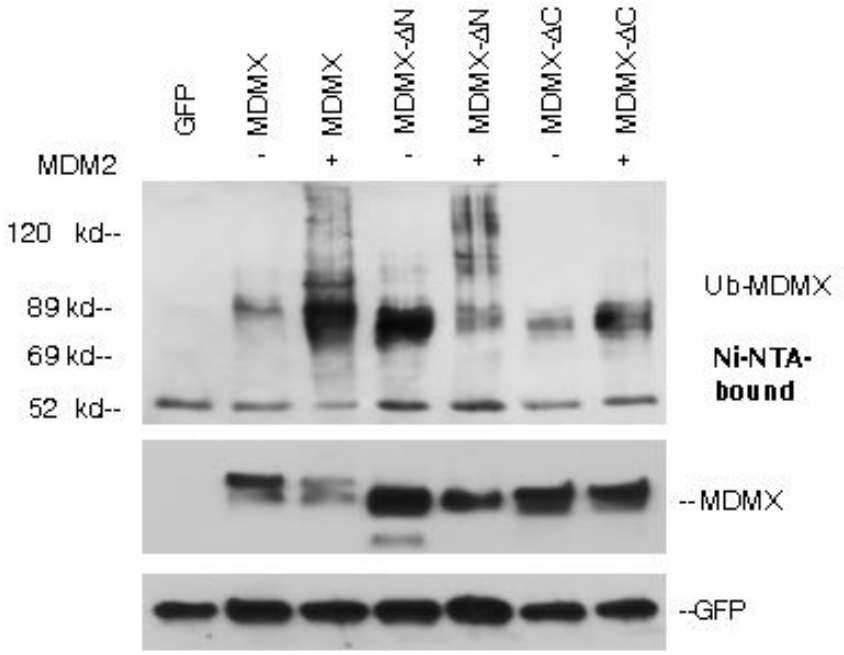


Figure 9. MDMX RING domain is required for its polyubiquitination and degradation by MDM2. H1299 cells were transfected with His6 -ubiquitin, MDM2, and MDMX mutants. MDMX ubiquitination was detected by Ni-NTA purification and Western blotting.

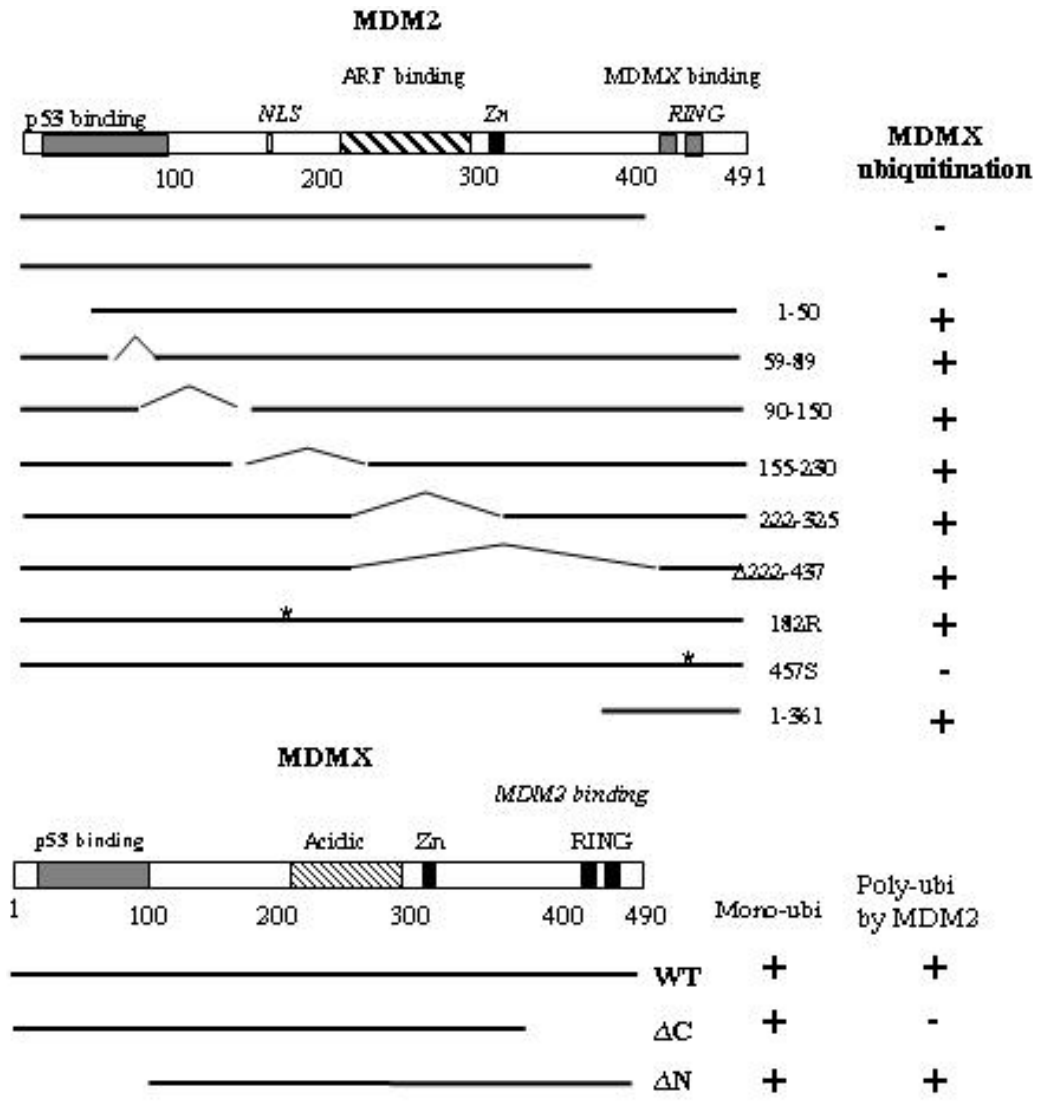


Figure 10. Diagram of MDM2 and MDMX mutants and summary of results. MDMX±N has a deletion of residues 1 to 100. MDMX±C has a deletion of residues 394 to 490.

Ubiquitination of MDMX requires MDM2 ubiquitin ligase function

To test whether the E3 function of MDM2 is required for poly-ubiquitination of MDMX, we mutated three key cysteine and histidine residues in the RING domain of MDM2 that a previous study found to be important for E3 function (438C-L, 457H-S, 478C-S, Figure 11 & 12) (Fang, Jensen et al. 2000). These mutations prevented poly-ubiquitination of MDMX in the presence of ARF (Figure 11 & 12).

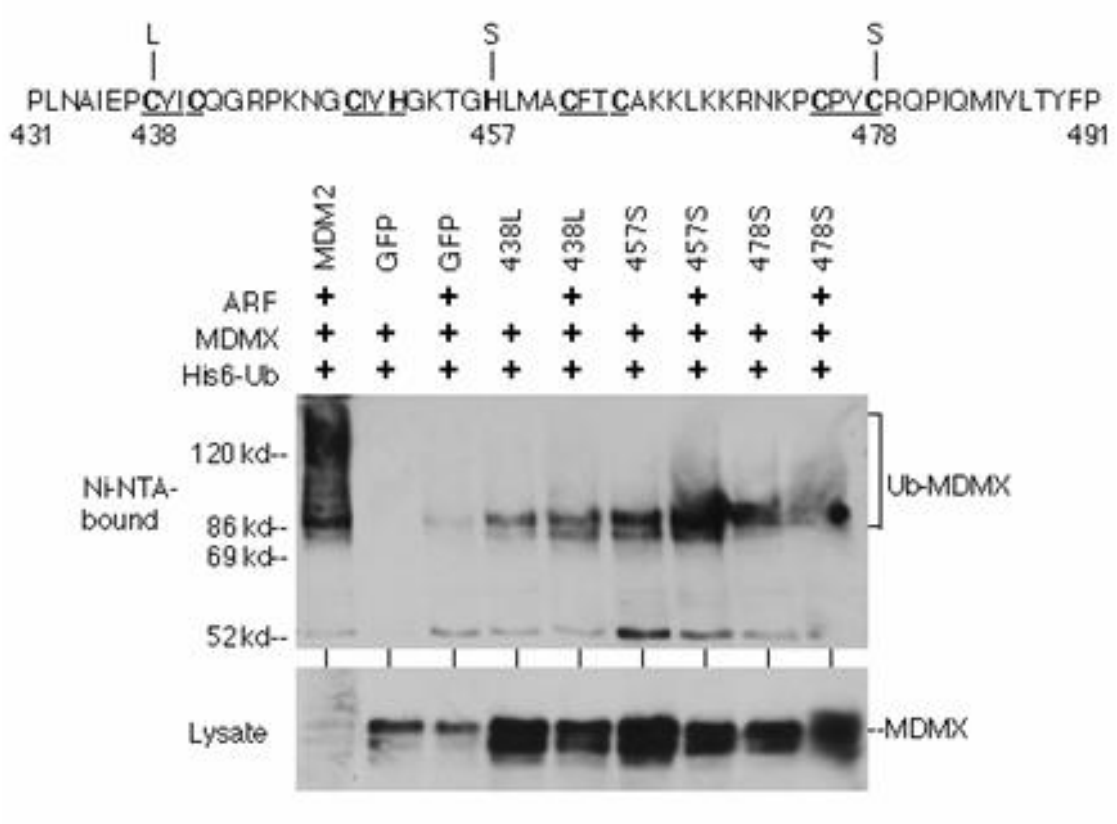


Figure 11. Ubiquitination of MDMX requires MDM2 E3 function.
(Upper panel) Position of point mutations in the MDM2 RING domain. C and H residues important for zinc coordination are underlined.
(Lower panel) Ubiquitination of MDMX by MDM2 mutants was determined by transfection of H1299 cells.

When analyzed by MDM2 immunoprecipitation and MDMX western blot, the 438L and 478S mutants lost most or all of MDMX binding, indicating that these mutations affected both E3 and MDMX binding functions (Figure 12). The 457S mutant was still capable of binding to MDMX, but the efficiency was significantly lower than wild type MDM2 considering the higher expression levels of both proteins in the transfected cells due to lack of degradation (Figure 12). Since the absolute amount of 457S-MDMX complex was comparable to MDM2-MDMX, yet MDMX ubiquitination was much weaker in the 457S-MDMX combination, we concluded that the E3 function of MDM2 was critical for ubiquitination of MDMX. However, it was clear from these results that MDMX-binding and E3 functions were both dependent on the integrity of MDM2 RING domain and could not be completely separated by these mutations.

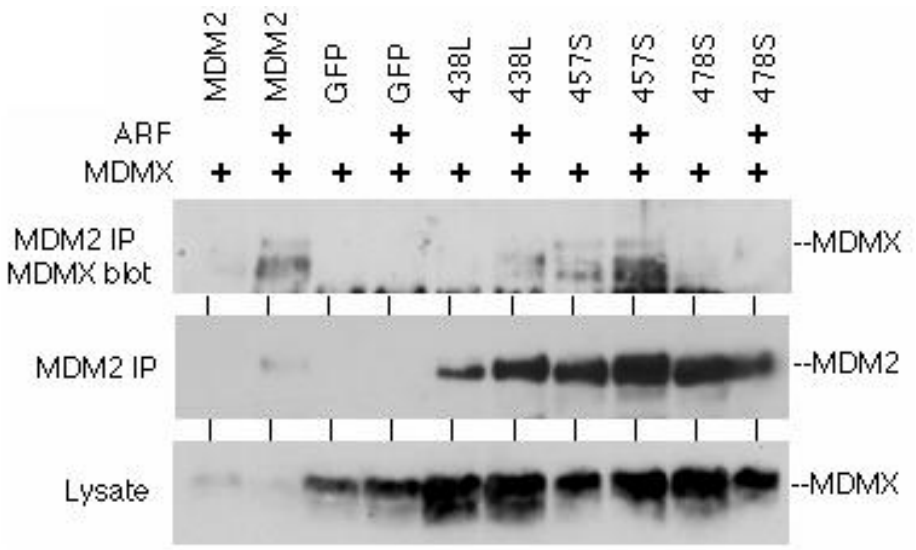


Figure 12. Binding of MDMX to MDM2 point mutants. MDMX and MDM2 mutants were cotransfected into H1299 cells and analyzed by MDM2 immunoprecipitation followed by MDMX Western blotting.

ARF N terminal domain is required to cooperate with MDM2

It is well established that ARF is an inhibitor of MDM2 E3 ligase function on p53 (Zhang and Xiong 2001). The results in Figure 5 showed that its effect on MDM2-mediated MDMX ubiquitination was opposite to that of p53. Therefore, we analyzed a panel of ARF deletion mutants to identify the domain required for stimulating MDMX ubiquitination. The results showed that the N terminal domain of ARF containing the MDM2-binding region was required for stimulating MDMX ubiquitination in the presence of MDM2 (Figure 13). Deleting the nucleolar targeting signal between 82-101 did not prevent stimulation of MDMX ubiquitination (Zhang and Xiong 1999). Interestingly, the N terminal region of ARF contains the MDM2 binding site and is sufficient to inhibit p53 ubiquitination by MDM2 (Midgley, Desterro et al. 2000). Therefore, ARF binding to MDM2 differentially regulates its ability to ubiquitinate p53 and MDMX.

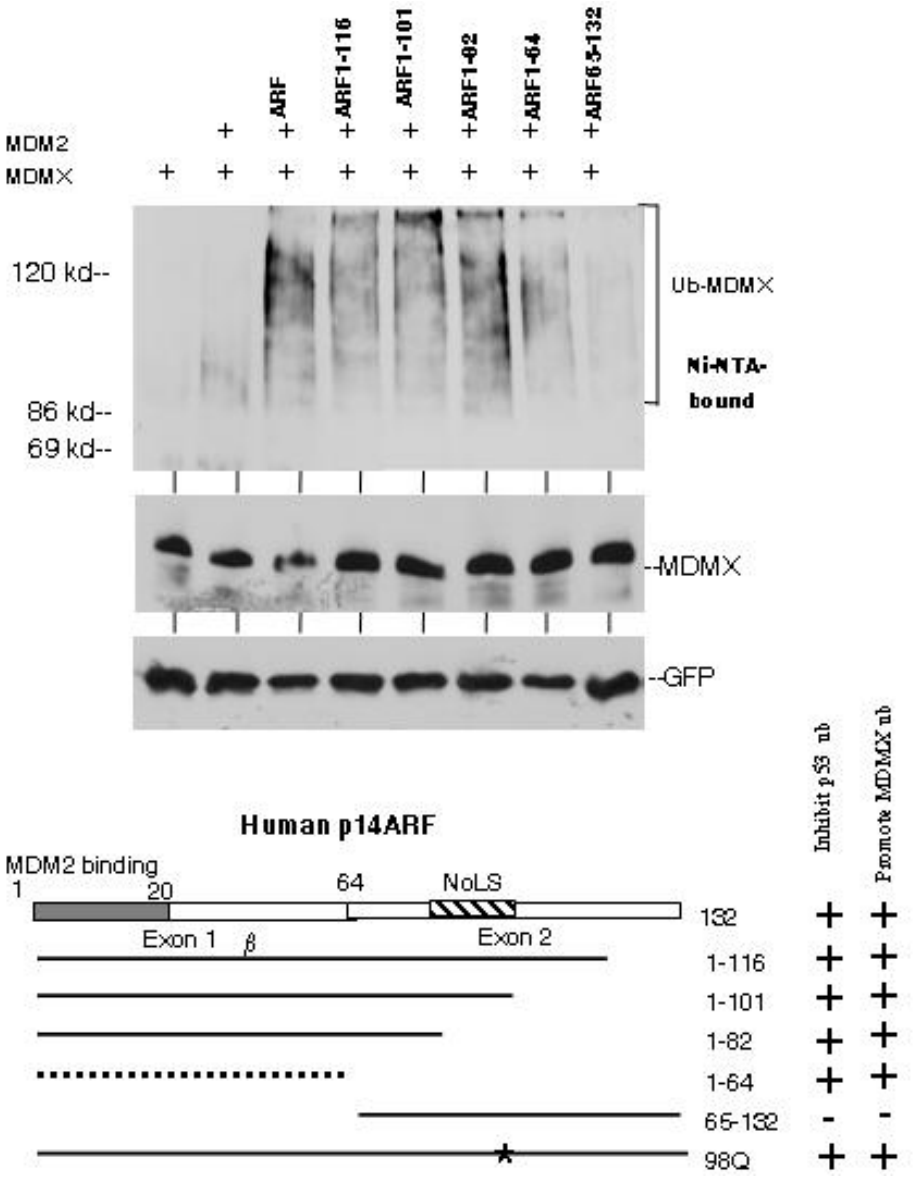


Figure 13. The ARF N-terminal domain is sufficient to cooperate with MDM2. (Upper panel) H1299 cells were transfected with MDMX, a suboptimal amount of MDM2, and different ARF mutants. His6 -ubiquitin-conjugated MDMX was purified and detected by Western blotting.
(Lower panel) Diagram of ARF mutants and summary of results.

Although ARF stimulated MDMX ubiquitination, MDM2 alone was sufficient to induce significant degradation of MDMX even in ARF-deficient cells (data not shown), indicating that ARF is not essential for this process. To observe stimulation of MDMX degradation by ARF in H1299 cells requires careful titration to limit the amount of MDM2 plasmid (data not shown). The effect of ARF on MDMX degradation was more evident and reproducible in mouse cells (Figure 7), suggesting that cell type may affect the effect of ARF in this transfection assay.

Regulation of endogenous MDMX level by ARF and MDM2

To determine whether endogenous MDMX expression level was regulated by ARF, ARF-deficient U2OS cells were infected with ARF adenovirus to restore ARF expression. As expected, expression of ARF resulted in strong induction of p53 and MDM2 levels. This was accompanied by reduction of MDMX expression (Figure 14).

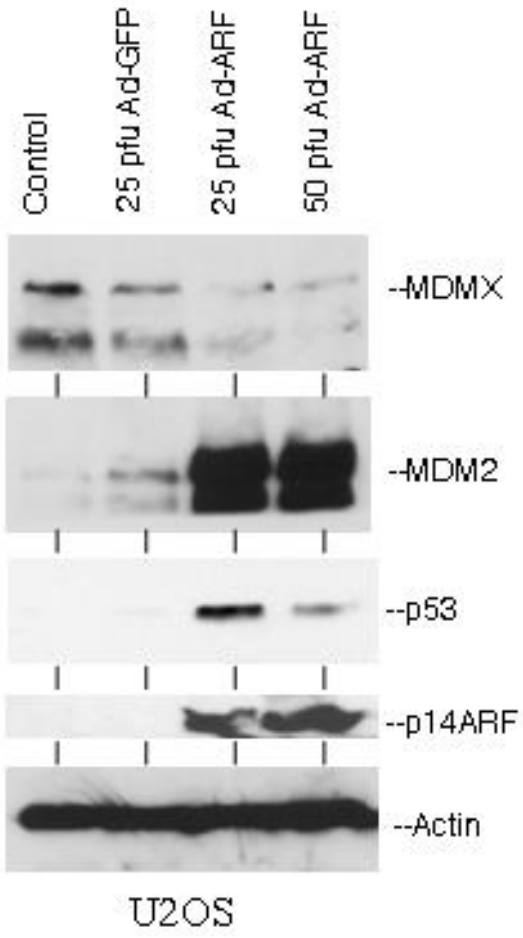


Figure 14. Overexpression of ARF down regulates MDMX level. U2OS cells were infected with ARF adenovirus at the indicated multiplicity for 24 hours and analyzed for MDMX, MDM2, and p53 expression levels by direct Western blotting.

In the second experiment, primary human foreskin fibroblasts (HFF) were treated with ionizing radiation to induce MDM2 expression. DNA damage resulted in induction of p53 and MDM2 expression levels in HFF and corresponding reduction of MDMX expression (Figure 15). Reduction of MDMX level was also observed after induction of MDM2 expression by irradiation of U2OS cells, or induction of MDM2 by activating a temperature-sensitive p53 mutant in H1299 cells (data not shown).

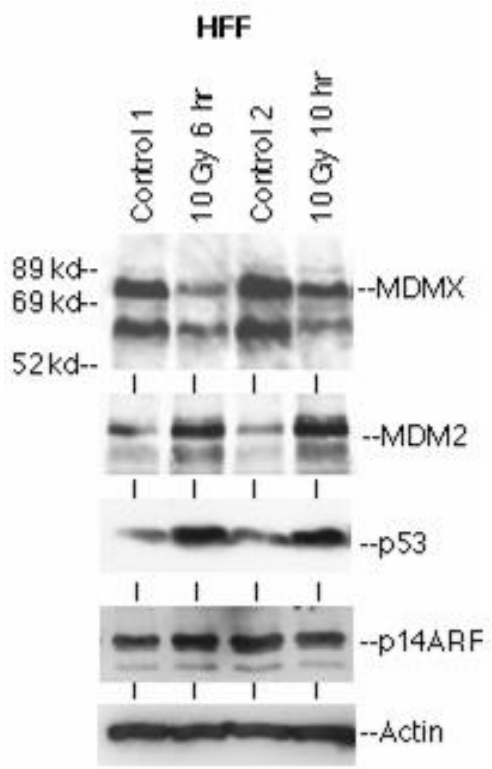


Figure 15. DNA damage induces MDMX degradation. HFF cells were treated with 10 Gy of gamma radiation for 6 hours and 10 hours, respectively before collection. The levels of MDMX, MDM2, p53, ARF and actin were determined by Western blotting.

To determine whether reduced MDMX level was due to degradation by the proteasomes, irradiated HFF cells were treated with MG132 for 4 hours prior to analysis. The decrease in MDMX level after irradiation was blocked by MG132 (Figure 16), suggesting it was mediated by proteasomes. In contrast, the p53-null H1299 cells did not express elevated MDM2 after irradiation and MDMX level was not reduced.

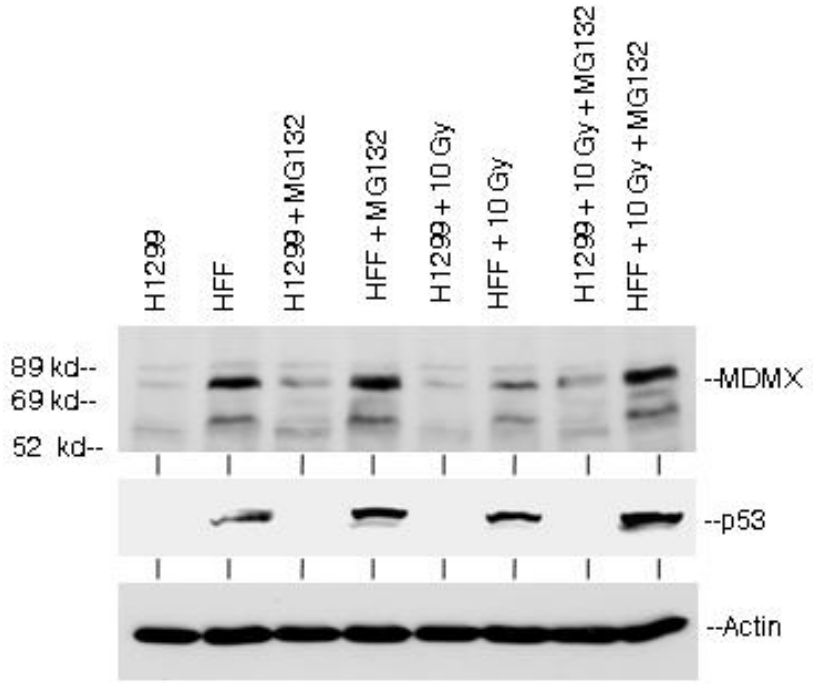


Figure 16. Induction of MDM2 results in MDMX degradation by proteasomes. HFFs and H1299 cells were treated with 10 Gy of gamma radiation for 6 hours. MG132 (30 μM) was added immediately after radiation. The MDMX level was determined by Western blotting.

To determine if the reduction of MDMX protein level after DNA damage is caused by the change in MDMX mRNA level, we performed Northen blot. The analysis using several cell lines confirmed that MDMX mRNA level was not reduced by radiation (Figure 17). These results suggested that ionizing radiation induced proteasome-mediated degradation of MDMX, which may be due to p53 activation and induction of MDM2 expression.

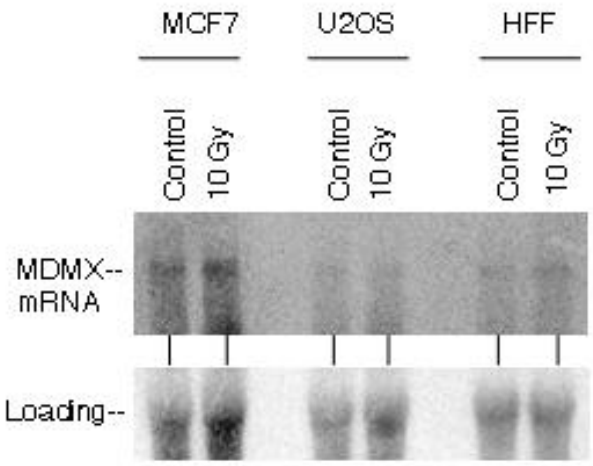


Figure 17. Gamma irradiation does not reduce the MDMX mRNA level. Cells were treated with gamma radiation, and 6 hours later total RNA was isolated and hybridized with an MDMX cDNA probe in a Northern blotting analysis.

To test whether change in MDM2 level independent of p53 activation or radiation was sufficient to regulate MDMX level, we created a U2OS cell line expressing tetracycline-repressible MDM2. Induction of MDM2 expression by removal of tetracycline correlated with decrease of MDMX level (Figure 18, lanes 5 and 7), which was restored by a 4 hour MG132 treatment. Therefore, increase of MDM2 expression alone was sufficient to promote MDMX degradation. However, the moderate decrease of MDMX was not proportional to the strong induction of MDM2, suggesting that other factors may limit the efficiency of MDMX degradation by MDM2.

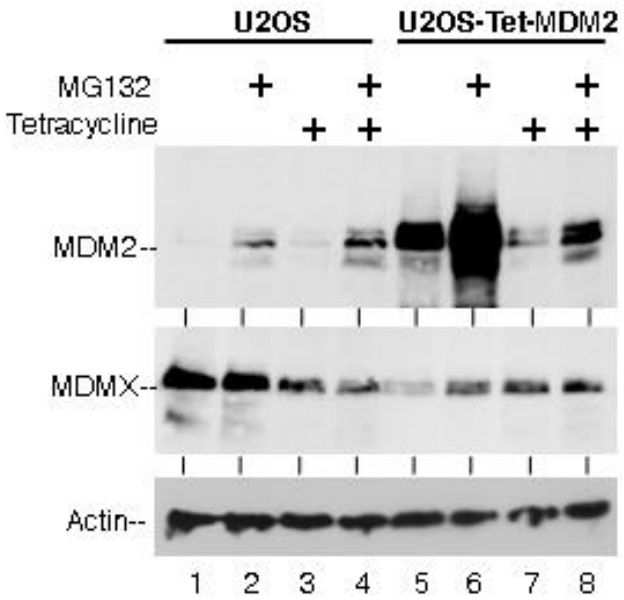


Figure18. Induction of MDM2 correlates with MDMX degradation through proteasome pathway. U2OS cells stably expressing tetracycline-repressible MDM2 were cultured in the presence or absence of 0.5 μg of tetracycline/ml for 20 hours. MG132 was added for the last 4 hours. MDMX and MDM2 levels were determined by Western blotting.

Epitope tagging prevents MDMX degradation by MDM2

Previous studies using epitope-tagged MDMX did not indicate an effect of MDM2 on MDMX degradation (Sharp, Kratowicz et al. 1999; Jackson, Lindstrom et al. 2001). We also had inconsistent results in early experiments when tagged and non-tagged versions of MDMX were used interchangeably. A direct comparison of non-tagged MDMX with several versions of epitope tagged MDMX revealed that addition of c-myc or Flag epitopes to the C terminus of MDMX significantly inhibited poly-ubiquitination by

MDM2 and completely prevented degradation (Figure 19). Therefore addition of epitopes to the RING domain of MDMX interferes with ubiquitination and degradation by MDM2. Because of this finding, all of the results described above were from repeat experiments using the non-tagged version of MDMX.

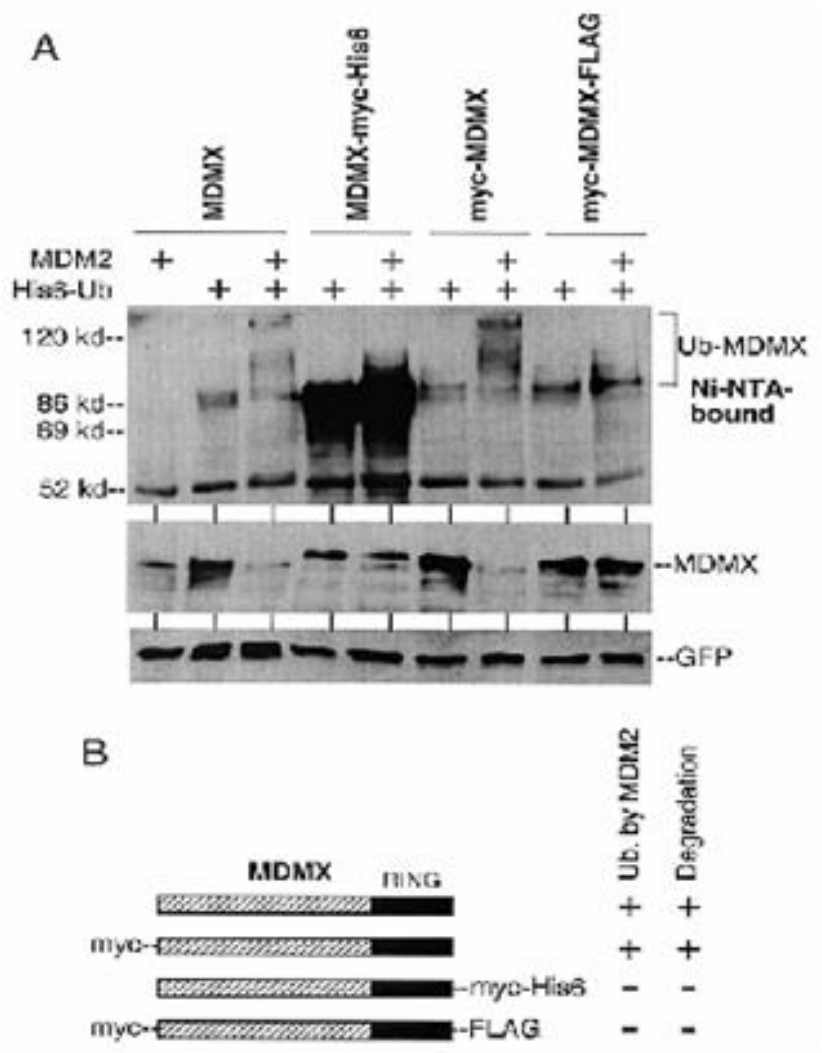


Figure 19. MDMX degradation is inhibited by C-terminal epitope tags.
(A) H1299 cells were transfected with MDMX with different epitope tags at the N or C terminus and subjected to Ni-NTA purifi-cation and Western blotting. The MDMX-myc-His6 protein was com-pletely recovered by Ni-NTA beads as expected.
(B) Diagrams of epitope-tagged MDMX and summary of results. C-terminal epitope tags block polyubiquitination and degradation by MDM2.

MDM2 promotes MDMX poly-ubiquitination in vitro

In order to confirm the role of MDM2 as an E3 for the poly-ubiquitination of MDMX, we established an *in vitro* ubiquitination assay for MDMX. In this assay, GST-MDM2 was able to stimulate the poly-ubiquitination of *in vitro* translated MDMX in the presence of purified E1 and E2 (GST-UbcH5b) (Figure 20). Furthermore, the C terminal fragment of MDM2 containing the RING domain was sufficient to promote MDMX ubiquitination, whereas an MDM2 N-terminal fragment had no effect. These results corroborate the *in vivo* ubiquitination results of Figure 8 and demonstrate that MDM2 functions as an ubiquitin E3 ligase for MDMX.

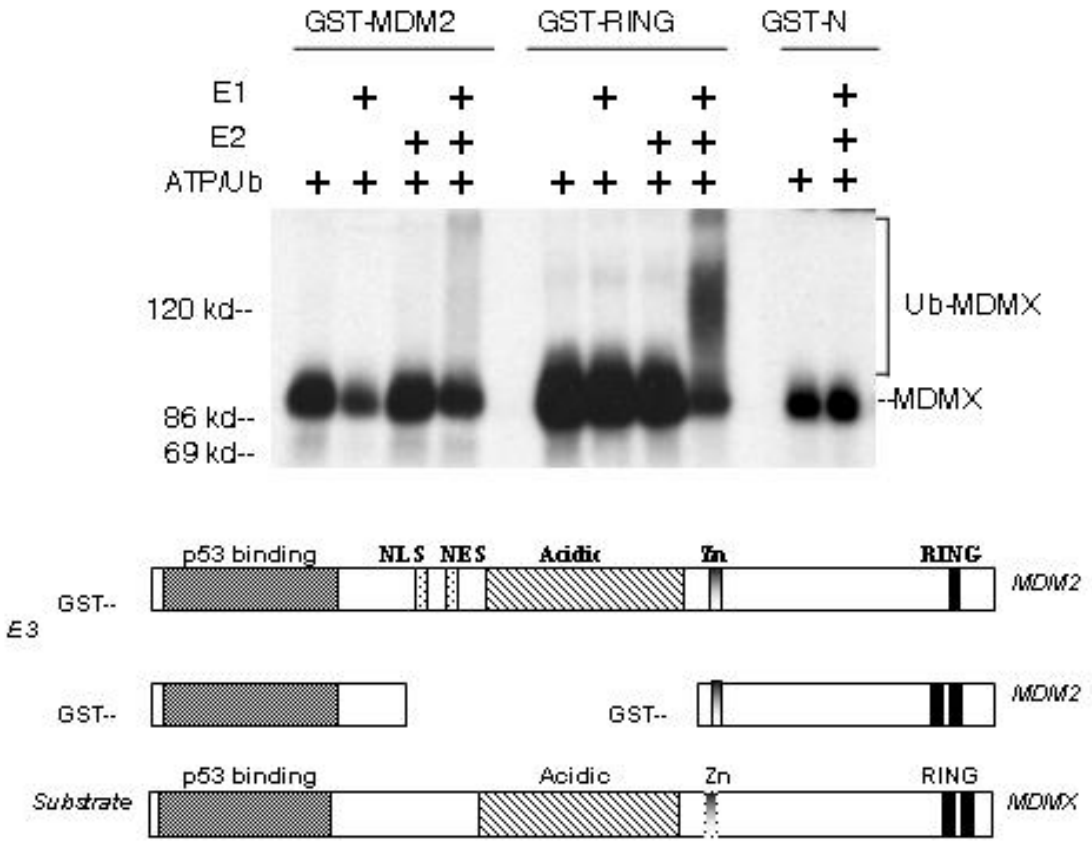


Figure 20. MDM2 promotes MDMX polyubiquitination in vitro. GST-MDM2, GST-RING (containing MDM2 residues 290 to 491), and GST-N (containing MDM2 residues 1 to 150) were purified using glutathione agarose beads. Loaded beads were incubated with in vitro-translated MDMX in the presence or absence of E1 and E2 in an ubiquitination reaction as described in Materials and Methods. Poly-ubiquitinated MDMX appears as a high-molecular-weight smear above the unmodified MDMX band.

Discussion

The results described above show that MDMX is subjected to modification by ubiquitin conjugation. Interaction with MDM2 leads to strong stimulation of MDMX poly-ubiquitination and degradation by proteasomes. MDM2 appears to function as an ubiquitin E3 ligase in this process, since the MDM2^{457S} RING domain mutant that prevents p53 or self-ubiquitination also abrogates ubiquitination of MDMX while still partially retaining MDMX binding. Furthermore, purified recombinant GST-MDM2 is able to stimulate MDMX poly-ubiquitination in cell-free conditions. MDMX also undergoes basal mono-ubiquitination independent of MDM2, suggesting interaction with other ubiquitination factors. It is noteworthy that previous studies using epitope-tagged MDMX did not observe an effect of MDM2 on MDMX degradation (Sharp, Kratowicz et al. 1999; Jackson, Lindstrom et al. 2001).We found that MDMX tagged with c-myc or FLAG epitopes at the C terminus were resistant to ubiquitination and degradation by MDM2, possibly due to altered RING domain conformation. Therefore, use of non-tagged or N terminally tagged MDMX appears to be critical for detecting its efficient ubiquitination and degradation by MDM2.

Surprisingly, ARF stimulates MDMX ubiquitination by MDM2, which is opposite to its effect on p53 ubiquitination. The stimulating effect of ARF is carried out by the N terminal domain that normally inhibits MDM2 ubiquitination of p53. Therefore, ARF interaction with MDM2 differentially affects its E3 function toward different

substrates rather than inactivating its E3 function in general. This effect is plausible because ARF does not directly interact with the RING domain of MDM2, which may be involved in recruiting E2. Binding of ARF to the acidic domain of MDM2 may simply alter its ability to properly orient E2 for the transfer of ubiquitin to certain substrates. In the case of p53, ubiquitin conjugation is blocked. However, in the case of MDMX, ubiquitination is not affected or even stimulated. ARF increases MDM2 expression level by stabilization, which may account for increased MDMX ubiquitination. However, ARF may also qualitatively stimulate the E3 activity of MDM2 toward MDMX.

These results suggest that ARF is a more effective and multi-functional regulator of the p53 pathway than previously appreciated. By blocking the ability of MDM2 to ubiquitinate p53, ARF is sufficient to induce p53 stabilization and activation. Additionally, by increasing MDMX ubiquitination, ARF also simultaneously promotes the elimination of another important inhibitor of p53. The combination of these two functions should result in more potent activation of p53. Because p53 activation induces MDM2 expression, higher MDM2 level should further cooperate with ARF to degrade MDMX, resulting in a positive feedback effect. Activation of p53 by other stress signals such as DNA damage also leads to strong induction of MDM2 expression. Increased MDM2 level may in fact facilitate p53 activation by degrading MDMX, while its own interaction with p53 is temporarily blocked by phosphorylation (Figure 14, 15 & 16).

The MDM2 negative feedback loop is an important mechanism in p53 regulation. However, knock out experiments showed that MDMX is also critical for regulating p53 (Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002). Recent studies suggested that MDMX and MDM2 depend on each other for

efficient inhibition of p53. MDM2 stimulates MDMX by targeting it to the nucleus, whereas MDMX enhances MDM2's ability to degrade p53 in a highly dose-dependent manner (Gu, Kawai et al. 2002; Migliorini, Danovi et al. 2002). Therefore, MDMX may be part of a more complicated MDM2 feedback loop by regulating MDM2 function and acting as a receiver of other signals. The ability of MDM2 to degrade MDMX ensures that it also exists in a balance with MDM2 and p53. Although MDMX can inhibit p53 function, the dependence on a suicidal interaction with MDM2 enables MDMX to be regulated by other signals. The MDMX degradation mechanism also ensures that stress signals can efficiently activate p53 by coordinately regulating both p53 inhibitors (Figure 21).

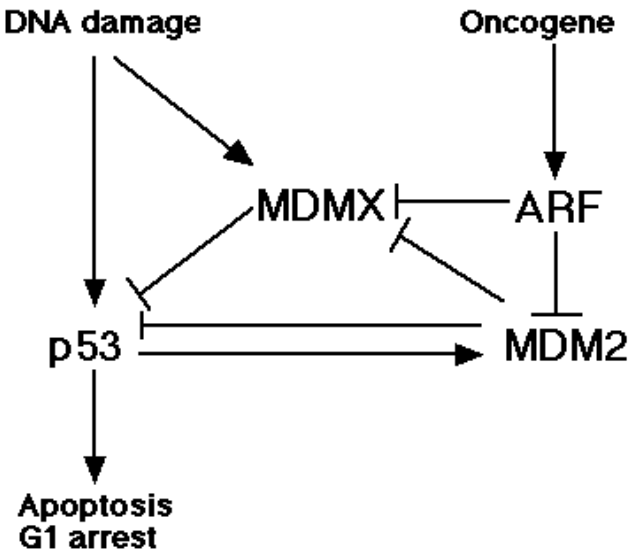


Figure 21. MDMX is regulated by stress signals. MDM2 and MDMX cooperate to inhibit p53 activity in the absence of stress. Stress signals activate p53 by targeting both MDM2 and MDMX. Oncogenes such as E2F1 and c-*myc* induce ARF expression, which activates p53 by inhibiting p53 ubiquitination and promoting MDMX ubiquitination by MDM2. DNA damage activates p53 and induces MDM2 expression, which in turn degrades MDMX and leads to further activation of p53. DNA damage also promotes MDMX nuclear translocation, which may expose it to degradation by MDM2 but also enables it to inhibit p53 more efficiently at certain stages of the damage response.

Recent studies showed that ectopically expressed MDMX is mainly localized in the cytoplasm and undergoes nuclear translocation by both MDM2-dependent and –independent mechanisms (Gu, Kawai et al. 2002; Li, Chen et al. 2002; Migliorini, Danovi et al. 2002). Nuclear entry of MDMX is critical for its ability to inhibit p53 (Gu, Kawai et al. 2002; Migliorini, Danovi et al. 2002), suggesting that MDMX plays an active role in limiting p53 activation during DNA damage response. However, results presented in

this report show that DNA damage can induce degradation of MDMX, which would facilitate p53 activation. Therefore, the net outcome of these regulatory events on the function of MDMX may depend on the level of damage and different stages of the stress response. Further work will be required to elucidate the role of MDMX in p53 regulation during DNA damage and other stress conditions.

In summary, our findings reveal that MDMX expression level is directly regulated by MDM2-mediated ubiquitination. The results also identify ARF as a more dynamic regulator of MDM2 ubiquitin ligase function and suggest a potential mechanism of MDMX regulation by a variety of stress signals through MDM2 and ARF. Elucidating how MDMX ubiquitination and degradation is regulated should lead to better understanding of its role in the p53 pathway and tumorigenesis.

MDMX forms trimeric complex with MDM2 and ARF

Abstract

ARF is a tumor suppressor that functions mainly through inhibition of MDM2, which is the major negative regulator of p53. ARF directly binds to MDM2 and inhibits its E3 ubiquitin ligase activity toward p53 and MDM2 itself, thus stabilizes both proteins. Furthermore, ARF relocates MDM2 from nucleus to nucleolus, physically separates MDM2 from p53 and activates p53 transcriptional activity. Previous studies showed that ARF stimulates p53 and MDM2 sumoylation. Our studies in this manuscript demonstrated that ARF stimulates both ubiquitination and sumoylation of MDMX while the ubiquitination of MDMX led to its degradation in an MDM2-dependent fashion. In this study, we showed that ARF stimulates MDMX ubiquitination while stabilizing MDM2, which may account for the stimulation of MDMX ubiquitination by ARF. On the other hand, the binding between MDMX and ARF has been controversial. Here we demonstrated that MDMX binds to ARF in an MDM2-dependent fashion. Our previous data showed that ARF N-terminus stimulates MDMX ubiquitination and sumoylation. We found that ARF N terminus is also required for binding to MDMX in the presence of exogenous or endogenous MDM2.

Introduction

Retinoblastoma protein (Rb) and p53 are two tumor suppressors that have been found to exhibit loss of function in large percentage of human tumors. The main function of Rb lies in its regulation of E2Fs, which promote cell cycle progression (Weinberg 1995; Dyson 1998). Oncogenic signals such as Ras, induce the phosphorylation of Rb through cyclinD-Cdks and free E2F from the sequestration of Rb, promoting entry into S phase (Sherr 2001). In this pathway, there is another tumor suppressor working in the upstream of this Rb-E2F pathway: $p16INK^{4a}$ is a Cdk inhibitor that inhibits cyclin-dependent kinase 4 and disrupts the cyclin-Cdk complexes, thus maintaining Rb in the hypophosphorylated and active state in inhibiting the cell cycle progression promoted by E2Fs (Sherr and Roberts 1995; Weinberg 1995; Sherr 2001). The gene locus of $p16^{INK}$ on chromosome 9 is mutated at high frequency in human tumors (Kamb, Gruis et al. 1994; Nobori, Miura et al. 1994). Later it was found that the necessity in tumor cells to mutate p16 locus lies in the fact that this locus encodes a second gene transcribed with an alternative reading frame, namely ARF (Serrano, Hannon et al. 1993; Quelle, Zindy et al. 1995).

Although ARF is completely unrelated to p16, the mutation of ARF also caused high frequency of tumor genesis independent of p16, supporting ARF as a tumor suppressor (Foulkes, Flanders et al. 1997; Ruas and Peters 1998; Sharpless and DePinho 1999). The mechanism of ARF induced cell cycle arrest is distinct from that of $p16^{INK}$.

A myriad of genetic stress including E1A, E2F, myc, ras and v-abl induce the expression of ARF, which activates the function of tumor suppressor p53 by harnessing p53 inhibitor, MDM2 (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998).

Thus, ARF plays an important role in linking the Rb-E2F with p53-MDM2 signal transduction pathways. When mitogenic signal induced E2F activity reaches a critical threshold, ARF is induced and activates another checkpoint protein: p53, which executes cell cycle arrest or apoptosis.

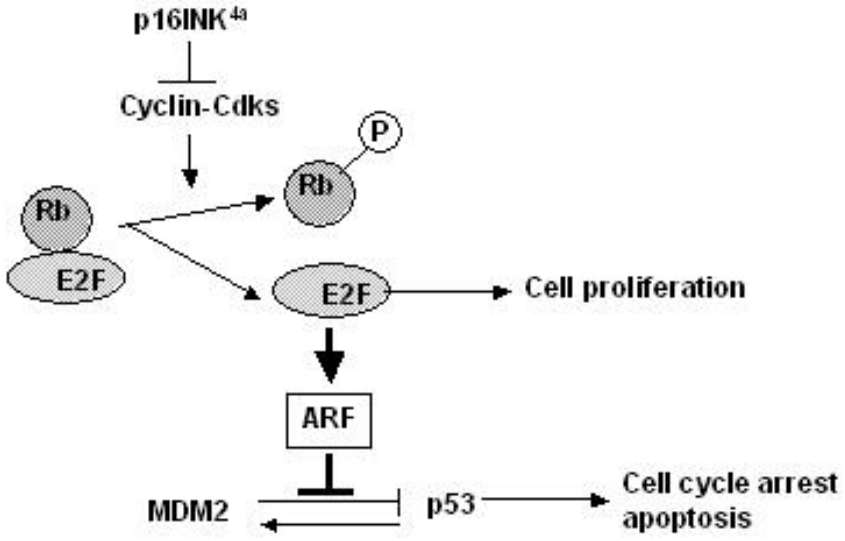


Figure 22. ARF connects Rb-E2F pathways to p53-MDM2 pathway

Results

MDMX forms trimeric complex with MDM2 and ARF

It is well recognized that ARF binds to MDM2 in the acidic domain and inhibits MDM2's regulation of p53. However, the binding between MDMX and ARF has been controversial. In order to compare the binding efficiency between MDMX-ARF to that of MDM2-ARF, FLAG-tagged MDMX or FLAG-tagged MDM2 were cotransfected with myc-tagged ARF in H1299 cells. The FLAG-tagged proteins were immunoprecipitated by M2 beads and co-precipitated ARF protein was detected by Western blot using monoclonal antibody 14P02 against ARF (Fig 23). The result showed that ARF binds to MDM2. MDMX overexpression does not change the binding efficiency between MDM2 and ARF. No significant MDMX-ARF binding was detected in the absence of overexpressed MDM2. However, in the presence of overexpressed non-tagged MDM2, significant amount of ARF was pulled down by FLAG-MDMX, indicating that MDM2 mediated the binding between MDMX and ARF. MDM2 lacking the RING domain, which is the MDMX binding region, did not mediate the binding between FLAG-MDMX and ARF; neither did the two MDM2 mutants lacking ARF binding regions. This experiment suggests that MDM2 bridges the binding between MDMX and ARF and that a tertiary complex can be formed among MDM2, MDMX and ARF.

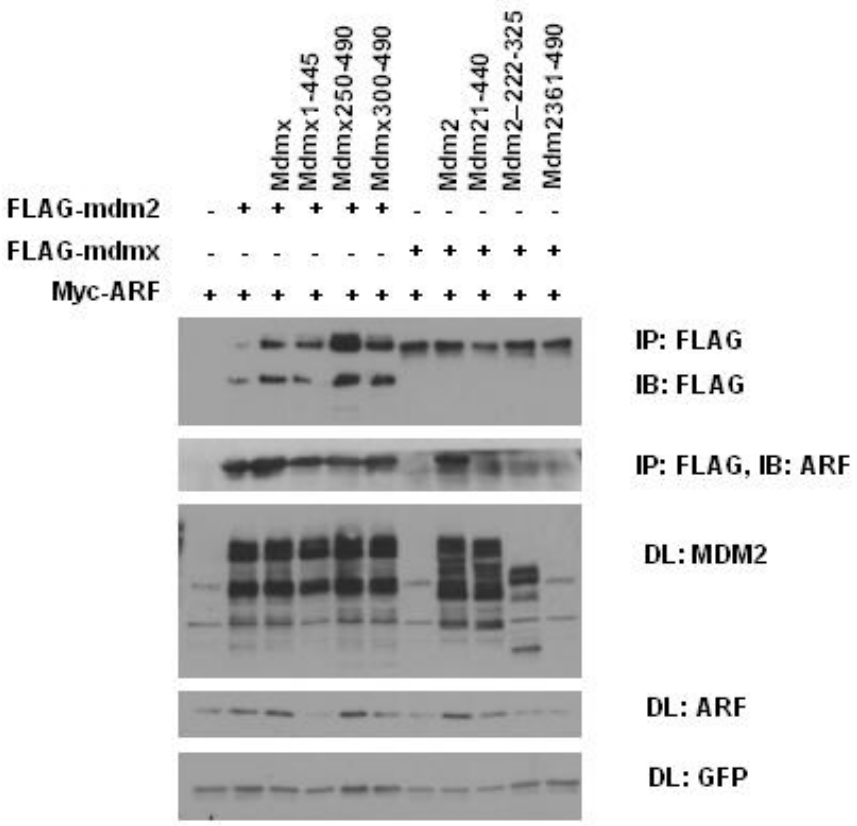


Figure 23. **MDMX forms trimeric complex with MDM2 and ARF.** FLAG-MDMX and FLAG-MDM2 were cotransfected with ARF expression plasmids in H1299 cells in the presence or absence of non-tagged MDMX or MDM2 mutants. FLAG-MDMX or FLAG-MDM2 was immunoprecipitated by M2 beads followed by western blot against FLAG-tagged proteins with rabbit-anti-FLAG antibody to show the level of immunoprecipitated MDMX and MDM2 (top panel). Co-immunoprecipitated ARF was detected by Western blot using mouse monoclonal antibody 14P02 against ARF (2nd panel). Expression level of MDM2 was confirmed by western blot with rabbit-anti-MDM2 antibody (3rd panel). The level of ARF expressed was verified by direct western blot against ARF in the whole cell lysate (4th panel). Transfection efficiency was verified by expression of cotransfected GFP plasmid (bottom panel).

To further confirm the binding between MDMX and ARF, the experiment was repeated in H1299 cells with FLAG-p53 as another positive control. FLAG-MDMX and MDM2 were cotransfected in the presence or absence of overexpressed ARF. Previous studies showed that ARF binds directly to both MDM2 and p53 (Kamijo, Weber et al. 1998). Indeed, we observed that ARF was co-precipitated with either FLAG-p53 or FLAG-MDM2. However, significant ARF-MDMX binding was only detected when MDM2 was coexpressed, verifying MDM2's recruitment of ARF to MDMX (Fig 24).

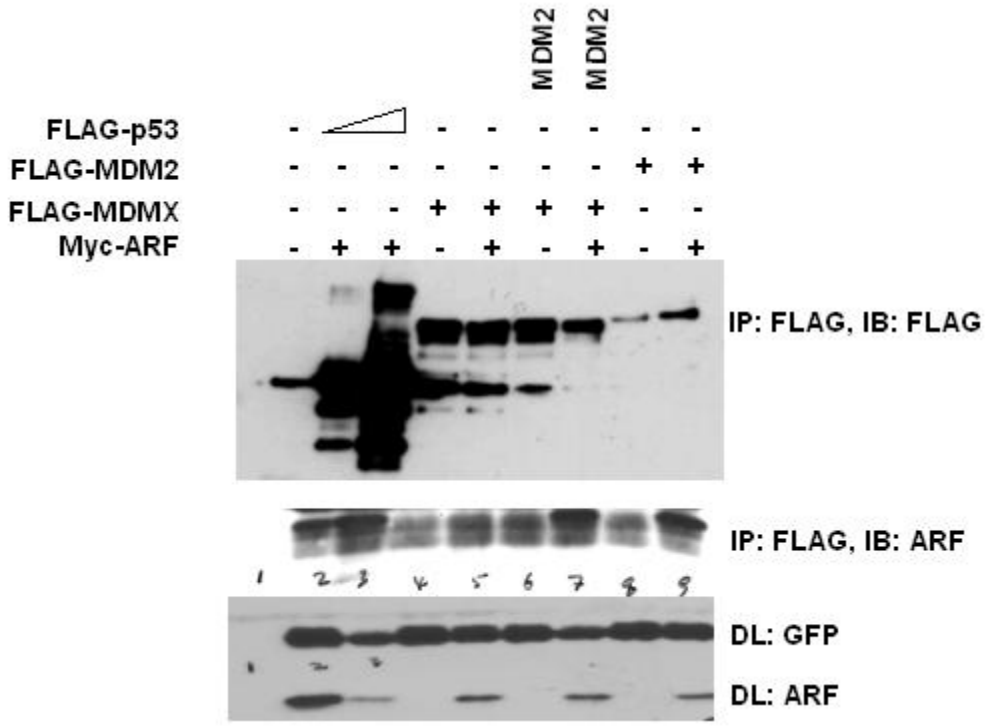
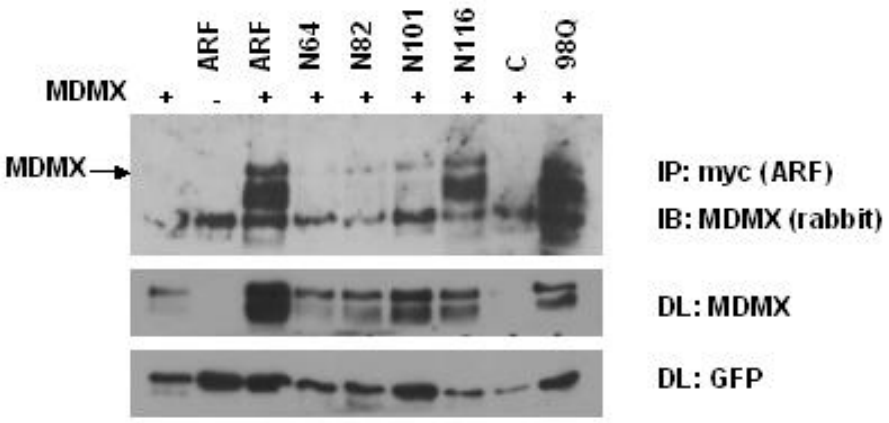


Figure 24. **MDM2 enhances the binding between MDMX and ARF.** FLAG-p53, FLAG-MDMX and FLAG-MDM2were cotransfected in the presence or absence of ARF in H1299 cells. FLAG-tagged proteins were immunoprecipitated by M2 beads followed by western blot against FLAG-tagged proteins with rabbit-a-FLAG antibody to show the level of immunoprecipitated p53, MDMX and MDM2 (top panel). Co-immunoprecipitated ARF was detected by Western blot using mouse monoclonal antibody against ARF (2nd panel). Transfection efficiency was verified by expression of cotransfected GFP plasmid (3rd panel). The level of ARF expressed was verified by direct western blot against ARF in the whole cell lysate (3rd panel)

MDM2 mediates the binding between MDMX and ARF

In order to verify the binding between MDMX and ARF, we used UO2S cells, which lacks endogenous ARF expression. MDMX was cotransfected with myc-tagged wild type ARF and ARF deletion mutants. Immunoprecipitation was performed using protein A beads and mouse monoclonal antibody against myc-tag. Co-precipitated MDMX was detected in western blot by rabbit polyclonal antibody against MDMX. The result showed that MDMX was co-precipitated with wild type as well as the N-terminal mutants of ARF. ARF C terminus cannot bind to MDMX. ARF98Q is mutated at the NoLS sequence and retains the capability to bind MDMX (Fig 25). To see if MDMX-ARF binding is cell-type specific, we performed the experiment in H1299 cells and observed similar result (data not shown). We speculated that the binding is mediated by endogenous MDM2 in both cell lines. To find out if MDM2 is required for MDMX-ARF binding, we performed the same experiment in MEF 174.1, which is a p53/-/MDM2-/- cell line that lacks endogenous MDM2. Result showed that only in the presence of MDM2 does MDMX bind to ARF, indicating that the previously observed MDMX and ARF binding in U2OS and H1299 cells was possibly mediated by endogenous MDM2 in those cell lines (Fig 25). Thus, MDM2 bridges the binding between MDMX and ARF and the MDM2-binding region in ARF N-terminus is required for the indirect binding between MDMX and ARF.

U2OS



174.1(p53-/- MDM2-/-)

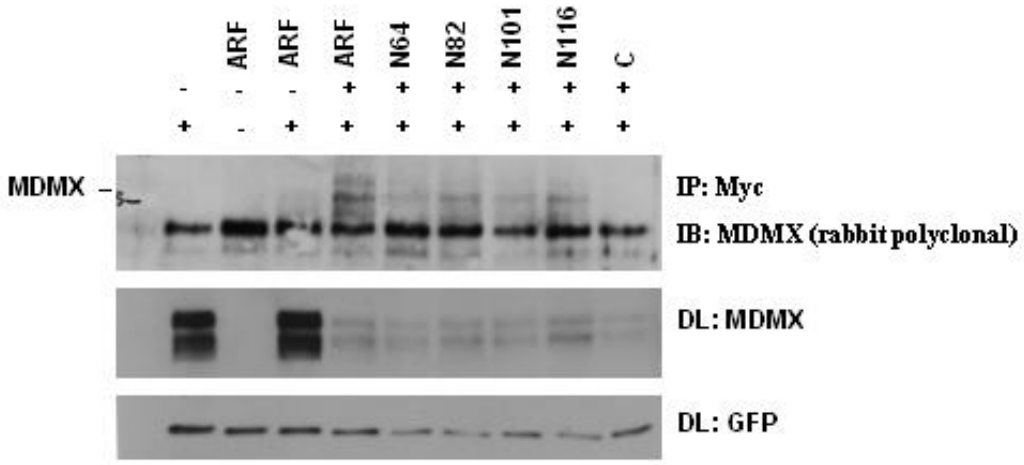


Figure 25. **ARF N-terminus is required for binding MDMX.** MDMX was cotransfected with myc-tagged wild type ARF or ARF deletion mutants as well as ARF nucleolus localization-deficient mutant into U2OS and MEF174.1(p53-/-MDM2-/-), respectively. Myc-ARF was immunoprecipitated by protein A beads and anti-myc-tag antibody. Co-precipitated MDMX was detected by western blot using rabbit polyclonal antibody against MDMX. The MDMX expression level was shown by western blot against MDMX using mouse monoclonal antibody 8C6 against MDMX from the whole cell lysate. Transfection efficiency was verified by expression of cotransfected GFP plasmid.

ARF does not affect the binding efficiency between MDM2 and MDMX

Since we observed that MDM2 enhanced the binding between MDMX and ARF (Fig 23-25), we speculated that the tertiary complex formation results in the increased binding affinity among the three proteins. To find out if MDMX and MDM2 binding efficiency can be changed by overexpression of ARF, H1299 cells were transiently transfected with fixed amount of MDMX and increasing amount of MDM2 and ARF. MDMX was immunoprecipitated with mouse monoclonal antibody 8C6 and the co-precipitated MDM2 was detected by rabbit polyclonal antibody against MDM2. The result showed that the expression of ARF did not affect the binding efficiency between MDMX and MDM2 (Fig 26).

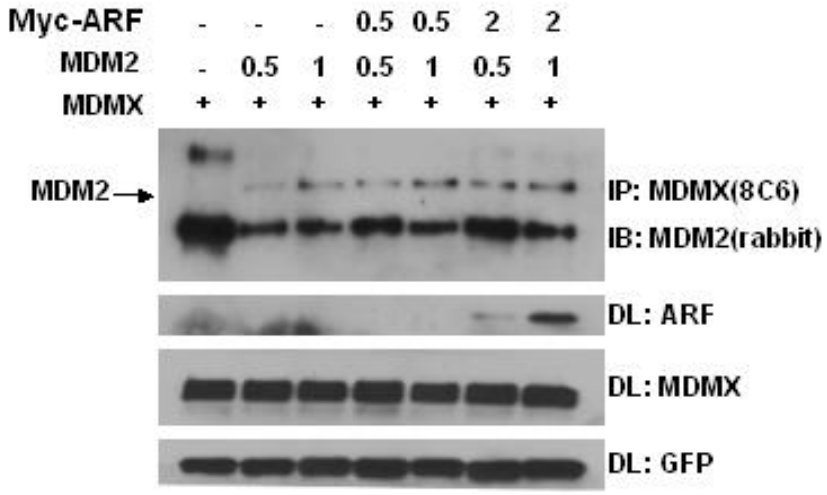


Figure 26. **ARF does not affect the binding efficiency between MDM2 and MDMX.** MDMX was cotransfected with increasing amount of MDM2 and ARF and was immunoprecipitated by protein A beads and mouse anti-MDMX antibody 8C6. Co-precipitated MDM2 was detected by western blot using rabbit polyclonal antibody against MDM2 (top panel). The MDMX and ARF expression levels were shown by western blot from the whole cell lysate (middle panels). Transfection efficiency was verified by expression of cotransfected GFP plasmid (bottom panel).

ARF stimulates MDM2-mediated MDMX ubiquitination by stabilizing MDM2

Previously, we showed that MDM2 promotes the ubiquitination and degradation of MDMX; additionally, ARF enhances ubiquitination of MDMX by MDM2. However, it remains unclear whether ARF stimulates MDMX ubiquitination through stabilizing MDM2 or through other unknown mechanisms. To find out if ARF can stimulate MDMX ubiquitination without stabilizing MDM2, we performed the ubiquitination assay by titrating the amount of MDM2 and ARF. H1299 cells were transiently transfected with suboptimal amount of MDM2 and increasing amount of ARF in the presence of MDMX and his6-ubiquitin. The result showed that MDMX ubiquitination is enhanced with increasing amount of ARF but in accordance with increasing amount of MDM2 stabilized by ARF overexpression (Fig 27). In concert with the finding that MDM2 mediates the binding between MDMX and ARF, our data suggests that ARF stimulates MDM2-mediated MDMX ubiquitination most likely by enhancing the steady state level of MDM2, which directly ubiquitinates MDMX.

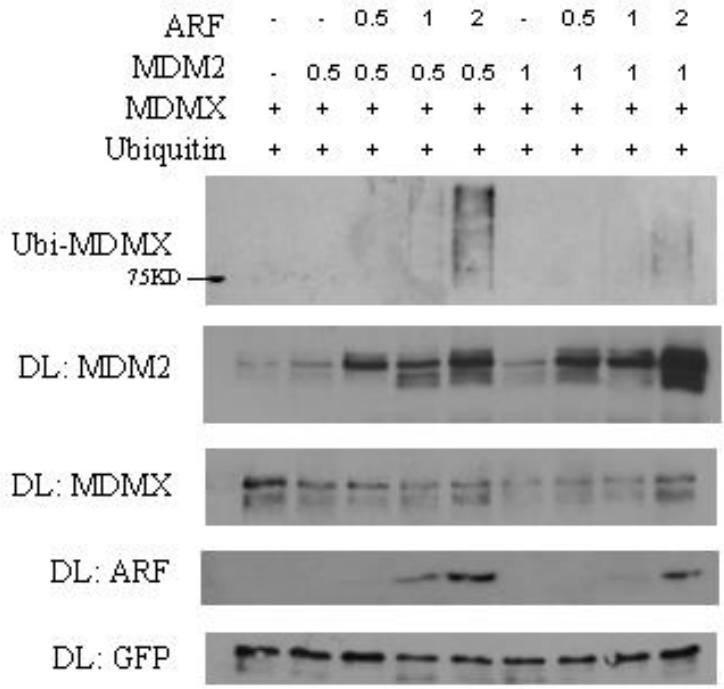


Figure 27. ARF stimulates MDMX ubiquitination while stabilizing MDM2. (Upper panel) H1299 cells were transfected with MDMX, suboptimal amounts of MDM2, and increasing amount of ARF. His6-ubiquitin-conjugated MDMX was purified and detected by Western blotting. (lower panels) The levels of MDM2, MDMX, ARF were detected by western blot from cell lysate. GFP level indicates transfection efficiency.

Discussion

ARF directly binds p53 and MDM2 and regulates their functions (Kamijo, Weber et al. 1998). However, the binding between MDMX and ARF has been controversial with one group showing no binding (Wang, Arooz et al. 2001) and the other group showed the otherwise (Jackson, Lindstrom et al. 2001). In this study, we confirmed the binding between MDMX and ARF in vivo, which is mediated by MDM2. However, the tertiary complex composed of MDMX, MDM2 and ARF failed in enhancing the binding affinity between MDMX and MDM2. This may be accounted for by the tight complex of the heterodimer between MDMX and MDM2 and the binding of which do not need further augmentation.

ARF is not a stereotyped regulator on MDM2 and the interplay between the two proteins is still being explored. Nevertheless, previous studies showed that ARF works on all aspects of MDM2-mediated posttranslational modifications. MDM2 ubiquitination of the substrates including self-ubiquitination is inhibited by ARF, resulting in the stabilization of the MDM2 E3 substrates (Kamijo, Weber et al. 1998). In addition, in response to stress signals, p53 is acetylated by CBP/P300, which increases p53 stability. MDM2 suppresses p53 acetylation by binding to the acetyl transferase CBP/P300 and inactivating the enzymes. ARF abrogates MDM2's suppression on p53 acetylation, thus restores p53 stability and activity (Ito, Lai et al. 2001). Chen et al also demonstrated that MDM2 stimulates p53 sumoylation (Chen and Chen 2003). However, instead of

antagonizing MDM2's modification on p53, ARF enhances MDM2-mediated p53 sumoylation. Data from this manuscript demonstrated that ARF stimulates MDM2-mediated MDMX ubiquitination and MDM2-independent MDMX sumoylation. Therefore, ARF is a dynamic regulator of MDM2 and the relationship between p53, MDM2, MDMX and ARF awaits further exploration.

Modification of MDMX by Sumoylation

Abstract

MDMX is a homolog of MDM2 and is critical for regulating p53 function during mouse embryonic development. MDMX level is regulated by MDM2-mediated poly-ubiquitination, which results in accelerated degradation after DNA damage or expression of ARF. In this report, we demonstrated that MDMX can be modified by conjugation to SUMO-1 both *in vivo* and *in vitro*. We found that double mutation of two lysine residues; K254 and K379 abrogated MDMX sumoylation *in vivo*. Experiments using the sumoylation-deficient MDMX mutant showed that it undergoes normal ubiquitination and degradation by MDM2, normal nuclear translocation and degradation after DNA damage, and inhibits p53 with wild type efficiency. Therefore, sumoylation is not required for several activities of MDMX under our assay conditions.

Introduction

The p53 pathway is regulated by multiple mechanisms, which is critical for its ability to respond to stress and suppress tumor formation. P53 turnover is regulated by MDM2, which functions as an ubiquitin E3 ligase to promote p53 ubiquitination and degradation by the proteasome. Stress signals such as DNA damage induce p53 accumulation by phosphorylation of p53 and MDM2 (Prives and Hall 1999). Mitogenic signals activate p53 by induction of the ARF tumor suppressor encoded by an alternative open reading frame in the p16INK4a locus, which inhibits the E3 ligase function of MDM2 (Kamijo, Weber et al. 1998; Zhang and Xiong 2001). MDM2 also regulates the acetylation of p53 by preventing binding of p300/CBP or recruitment of HDAC1 (Kobet, Zeng et al. 2000; Ito, Lai et al. 2001; Ito, Kawaguchi et al. 2002).

Recent studies revealed that p53 is also regulated by the MDM2 homolog MDMX (Shvarts, Steegenga et al. 1996). MDMX shares strong homology to MDM2 at the amino acid sequence level and can bind to p53 and inhibit its transcription function in transient transfection assays. MDMX alone does not promote p53 ubiquitination or degradation *in vivo* (Stad, Little et al. 2001). However, formation of MDM2-MDMX heterodimer stimulates the ubiquitin E3 ligase activity of MDM2 for itself and p53 (Badciong and Haas 2002; Linares, Hengstermann et al. 2003), suggesting that MDMX may function as a regulator or cofactor of MDM2. Knockout of MDM2 in mice results in embryonic lethality due to hyper-activation of p53 (Montes de Oca Luna, Wagner et al. 1995).

Several studies showed that MDMX-null mice also die in uterus in a p53-dependent fashion, which can be rescued by crossing into the p53-null background (Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Denchi et al. 2002). Therefore, MDMX is also an important regulator of p53 during embryonic development. MDMX overexpression can lead to transformation in cell culture; MDMX gene amplification and overexpression has been observed in certain tumors with wild type p53, suggesting that it may contribute to p53 inactivation in cancer (Ramos, Stad et al. 2001; Danovi, Meulmeester et al. 2004).

Recently, p53 and MDM2 have been found to be modified by SUMO-1 conjugation (Small ubiquitin-like modifier) (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999; Xirodimas, Chisholm et al. 2002). Sumoylation of p53 and MDM2 *in vitro* are carried out by a universal SUMO activating E1 enzyme (SEA1/SEA2 heterodimer) and SUMO conjugating enzyme E2 (ubc9) (Hochstrasser 2002). SUMO is activated in an ATP-dependent manner by E1, transferred to E2, and subsequently attached to the ε-amino group of lysines on target proteins (Hochstrasser 2002). The sumoylated lysine residue in many substrates often locates within a consensus sequence of ψKxE where ψ is an aliphatic residue (Rodriguez, Dargemont et al. 2001). Lysine 386 is the major sumoylation site for p53 (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999), whereas the sumoylation site on MDM2 has not been positively identified (Xirodimas, Chisholm et al. 2002; Chen and Chen 2003).

The functional role of sumoylation is diverse among different substrates. It has been proposed to be important for regulating the localization, activity, and degradation of target proteins (Hochstrasser 2002). Initial studies using p53 386 lysine-to-arginine

mutant resistant to sumoylation suggested that sumoylation moderately enhances p53 transcription activity (Gostissa, Hengstermann et al. 1999; Rodriguez, Desterro et al. 1999). Muller et al. found that the p53 386K mutant has slightly impaired apoptotic activity (Muller, Berger et al. 2000). Other studies did not observe such an effect (Minty, Dumont et al. 2000; Kwek, Derry et al. 2001), possibly due to different assay conditions. MDM2 sumoylation has been shown to be stimulated by ARF through nucleolar targeting of MDM2 (Xirodimas, Chisholm et al. 2002; Chen and Chen 2003). MDM2 and ARF cooperatively stimulate p53 sumoylation (Chen and Chen 2003), suggesting a potential mechanism that contributes to ARF activation of p53. Despite efforts by several laboratories, the sumoylation site on MDM2 has not been determined, possible due to presence of multiple alternative sites. The lack of sumoylation-deficient MDM2 mutants prevents further investigation of the functional of this modification.

Recent work from several laboratories showed that MDMX is regulated by MDM2-mediated ubiquitination and degradation (Kawai, Wiederschain et al. 2003; Linares, Hengstermann et al. 2003; Pan and Chen 2003). Ubiquitination of MDMX leads to increased degradation after DNA damage and expression of ARF (Kawai, Wiederschain et al. 2003; Pan and Chen 2003), suggesting that ubiquitination is an important modification on MDMX. In this report, we present evidence that similar to p53 and MDM2, MDMX is also modified by sumoylation. Two lysine residues critical for MDMX sumoylation have been identified. MDMX point mutants that cannot be modified by sumoylation have similar properties as wild type MDMX in several functional assays, suggesting that sumoylation are not required for these MDMX functions.

Results

MDMX is posttranslationally modified by sumo-1

MDM2 has been demonstrated to be modified by sumo-1. Since MDMX and MDM2 share significant sequence homology, we speculated that MDMX might also be sumoylated *in vivo*. To detect MDMX sumoylation, we coexpressed MDMX and His6-sumo-1 by transient transfection into H1299 cells. MDMX conjugated to His6-sumo-1 was purified by Ni^{2+}-NTA beads under denaturing conditions and detected by western blot with MDMX-specific monoclonal antibody 8C6. The result showed that coexpression of MDMX and his6-sumo-1 resulted in the appearance of MDMX species at 90 kd molecular weight, suggesting that MDMX was sumoylated *in vivo* (Figure 28). Expression of the desumoylating enzyme SENP1 completely eliminated the 90 kd MDMX band (Gong, Millas et al. 2000), consistent with it being a sumoylated form of MDMX.

It has been demonstrated that the sumoylation of MDM2 can be significantly enhanced by ARF (Xirodimas, Chisholm et al. 2002; Chen and Chen 2003). To test if ARF also stimulates the sumoylation of MDMX, ARF was cotransfected together with MDMX and His6-sumo-1. In analogy to MDM2 sumoylation, MDMX sumoylation was also enhanced by the overexpression of ARF (Figure 28). The significance and mechanism of this effect remains to be determined.

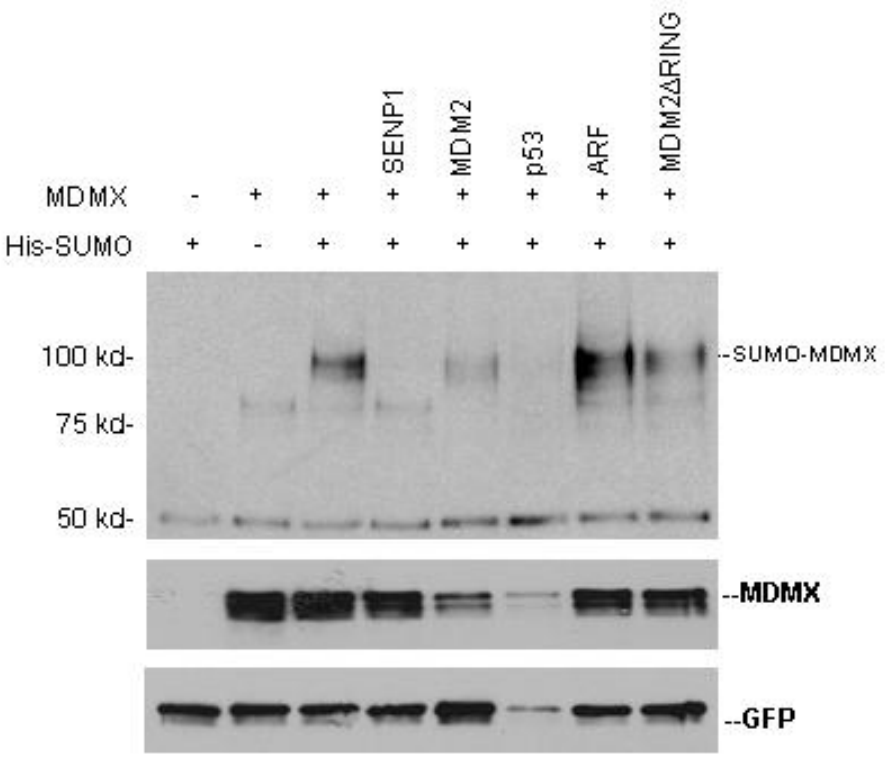


Figure 28. ARF stimulates MDMX sumoylation and the sumo-specific protease SENP1 desumoylates MDMX.
MDMX was coexpressed with His6 -sumo in H1299 cells by transient transfection. His6-sumoylated proteins were purified, and MDMX was detected by Western blotting with 8C6 antibody.
The lower panels are control Western blots of nonpurified cell lysate (10% of the amount used for Ni-NTA purification).Transfection efficiency was verified by expression of cotransfected GFP plasmid.

We have recently reported that MDM2 functions as an E3 ligase for MDMX polyubiquitination (Pan and Chen 2003). Furthermore, MDM2 expression also stimulates p53 sumoylation (Chen and Chen 2003). Therefore, we tested whether MDM2 and p53 regulate MDMX sumoylation. The results showed that expression of MDM2 led to degradation of MDMX in a RING finger-dependent fashion as expected. The level of MDMX sumoylation was also reduced accordingly, suggesting that MDM2 does not stimulate MDMX sumoylation (Figure 28). Coexpression of wild type p53 significantly reduced transfection efficiency and MDMX expression level due to squelching effects, the corresponding reduction of sumoylated MDMX level also ruled out a stimulatory effect.

Next, we tested whether MDMX sumoylation can be recapitulated in cell-free conditions. In vitro translated MDMX was incubated with Hela cell E1 fraction, purified E2, and ATP. Significant modification of MDMX was observed after addition of purified sumo-1 (Figure 29). Addition of in vitro translated ARF had no effect on MDMX sumoylation level in this assay, possibly due to insufficient amount and poor binding to MDMX under the assay condition, or that the *in vivo* stimulatory effect involved additional factors.

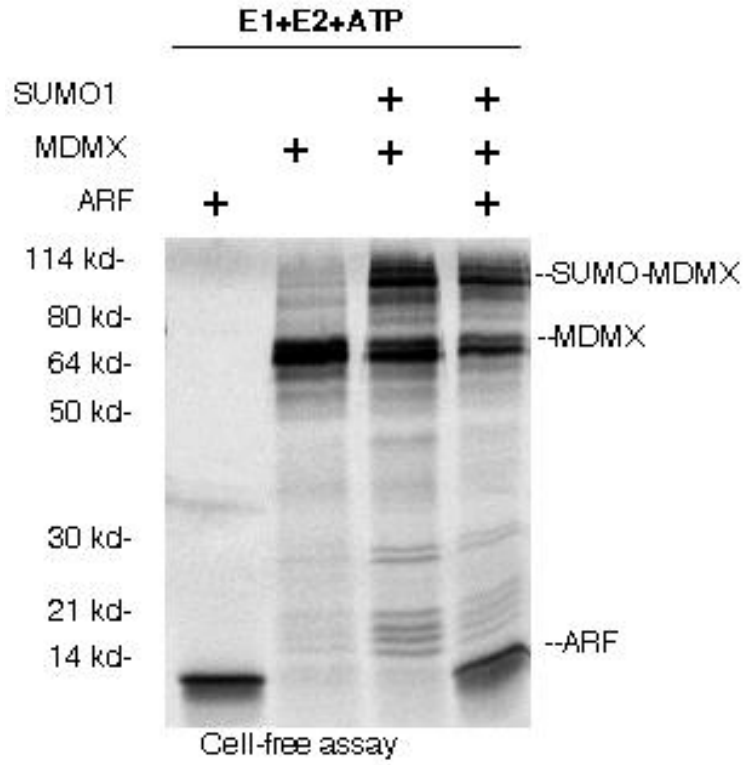


Figure 29. *In vitro* sumoylation of MDMX.
In vitro translated proteins were incubated in the presence or absence of E1 fraction, E2, ATP or SUMO1. ARF did not stimulate MDMX sumoylation in vitro.

Identification of MDMX sumoylation sites

The consensus sequence for sumoylation has been identified as ψKXE, where ψ represents a large hydrophobic amino acid and K stands for the sumo conjugating lysine residue. However, despite the presence of such consensus site, previous studies were not able to definitively identify the sumoylation site on MDM2 (Xirodimas, Chisholm et al. 2002; Chen and Chen 2003). To determine the region containing sumoylation sites on

MDMX, a panel of MDMX deletion mutants was generated with N or C terminal truncations (Figure 30). The MDMX deletion mutants were coexpressed with His6-sumo-1 by transient transfection into H1299 cells, purified by Ni^{2+}-NTA beads under denaturing conditions and detected by western blot with MDMX polyclonal antibody. The result showed that major sumoylation sites are contained within the region 250-490 (Figure 31).

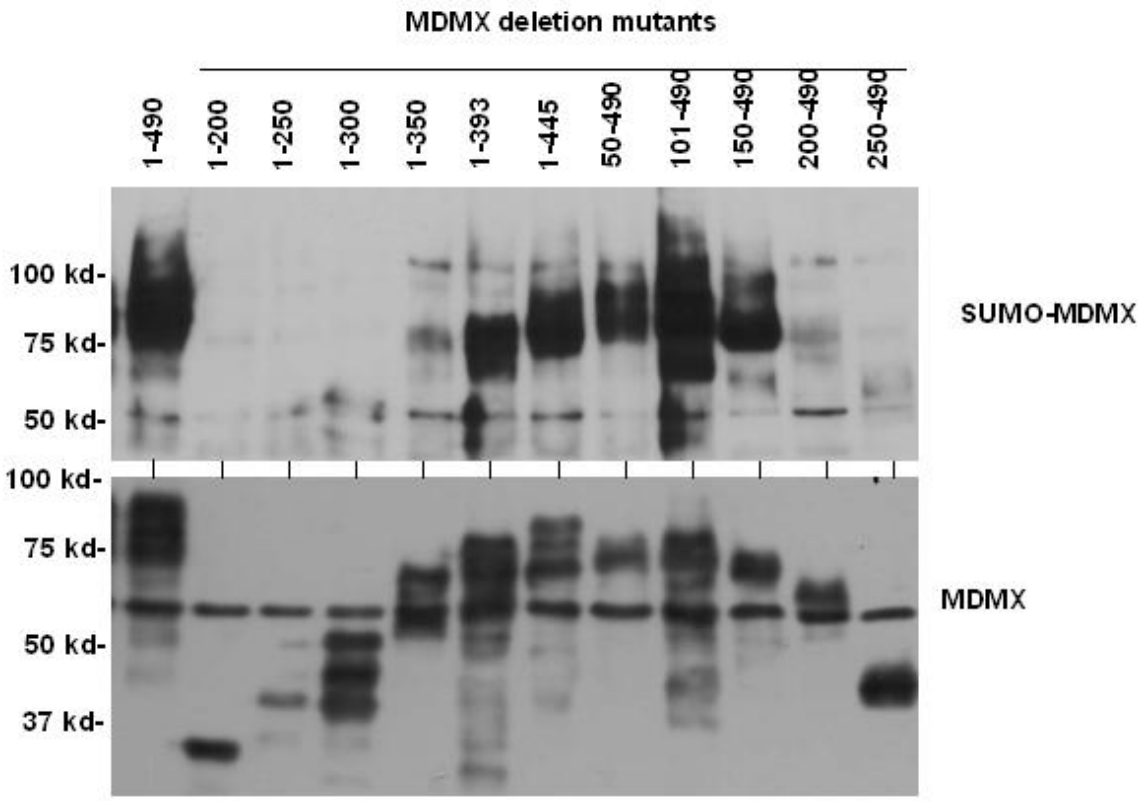


Figure 30. The major sumoylation sites of MDMX are contained within the region 250-490.

A panel of MDMX deletion mutants was generated for their sumoylation status. MDMX deletion mutants were coexpressed with His6 -sumo in H1299 cells by transient transfection. His6-sumoylated proteins were purified, and MDMX deletion mutants were detected by Western blotting with polyclonal rabbit-anti-MDMX antibody.

The lower panels are control Western blots of nonpurified cell lysate, showing the expression of the MDMX deletion mutants (10% of the amount used for Ni-NTA purification).

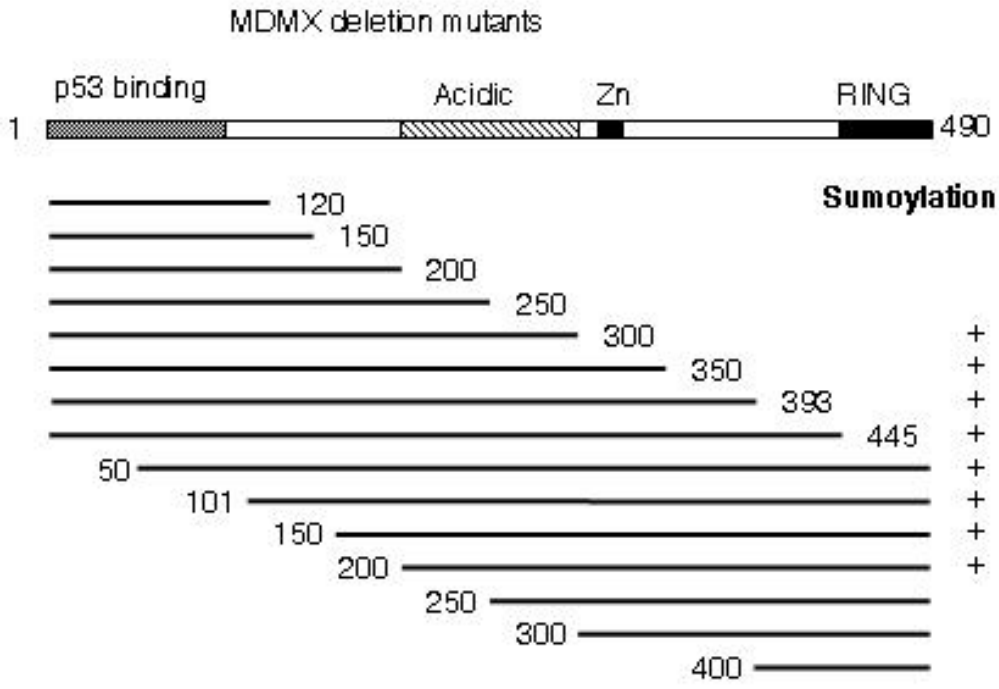


Figure 31. Diagram of MDMX mutants and summary of results.
Major MDMX sumoylation sites are contained within the region 250-490.

The MDMX sequence contains three sites that conform to the ψKXE consensus, lysine 254 (IKVE), 379 (IKKE) and 478 (CKKE). To directly test whether these sites are required for sumoylation, single or combined site-directed mutagenesis of these three lysines were performed. The mutants were tested for their sumoylation efficiency by transient transfection of H1299 cells. MDMX sumoylation was not affected by the mutation of any single lysines. However, double-mutation of K254 and K379 to arginine

abrogated the sumoylation of MDMX (Figure 32). Therefore, K254 and K379 are likely to be the major sumoylation sites, which is consistent with the deletion mutant results.

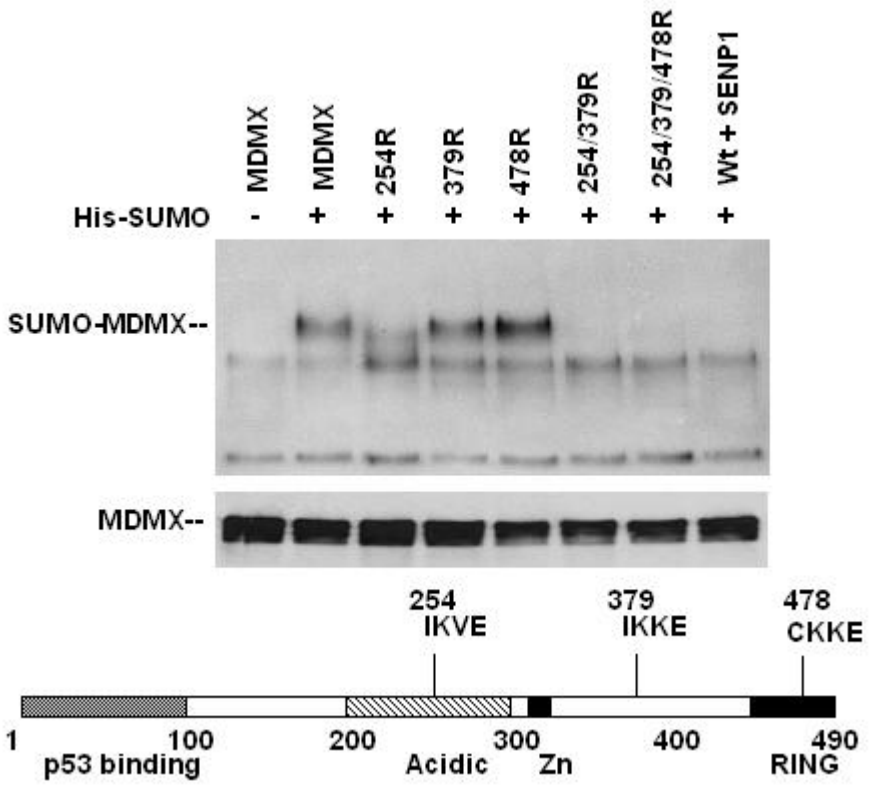


Figure 32. Characterization of MDMX sumoylation sites on serine 254 and serine 379.
Point mutants serine 254, 379 and 478 were generated for identification of MDMX sumoylation sites. Sumoylated wild-type MDMX and MDMX point mutants K254, K379, K478 and the double mutant of K254 and K379 (DM) as well as the triple mutant of all three serines in the consensus sequence were purified by Ni-NTA purification and detected by Western blot with 8C6 antibody. The lower panels are control Western blots of nonpurified cell lysate (10% of the amount used for Ni-NTA purification).

SENP1 has been identified as a sumo-specific protease. In order to further verify the modification of MDMX by sumo, we cotransfected His6-sumo-1 with wild type MDMX, MDMX sumo-deficient single mutants, double mutant and triple mutant in H1299 cells in the presence or absence of SENP1 expression plasmid. Sumoylated MDMX was purified by Ni^{2+}-NTA purification and detected by Western blot. The result showed that SENP1 reversed the sumoylation of wild type MDMX and all three MDMX point mutants but had no effect on sumoylation-deficient MDMX double or triple mutants, confirming the characterization of MDMX sumoylation sites (Figure 33).

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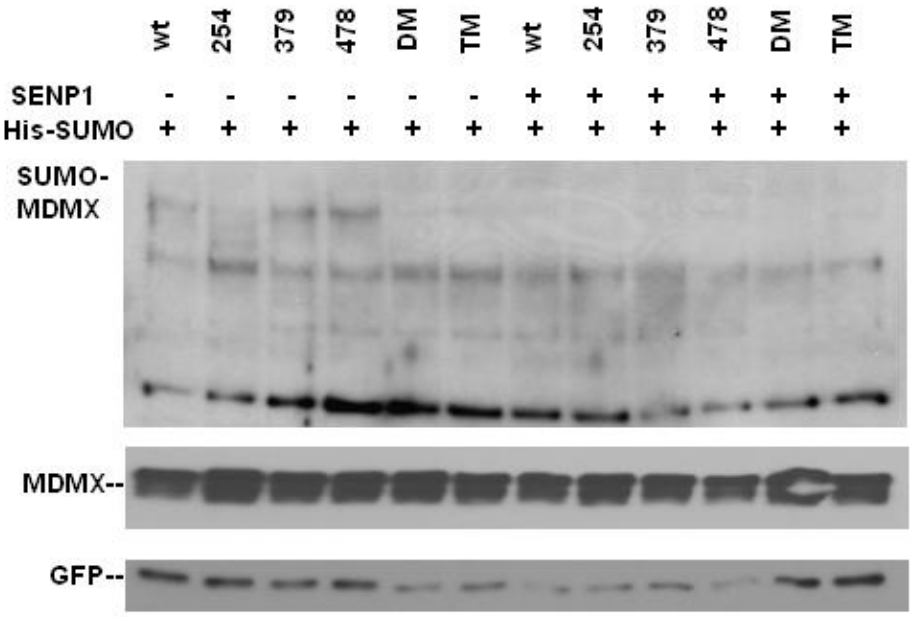

Figure 33. Desumoylation of MDMX by SENP1.
H1299 cells were transfected with His6-SUMO1 and indicated plasmids. Sumoylated MDMX was detected by Ni-NTA purification and Western blot. Expression of SENP1 abrogated MDMX sumoylation.

MDMX sumoylation does not affect its stability and regulation by DNA damage

MDMX forms a heterodimer with MDM2 and is poly-ubiquitinated and degraded by MDM2. MDMX is also modified by mono-ubiquitination in the absence of MDM2 expression through unknown mechanism (Pan and Chen 2003). To test if blocking MDMX sumoylation affects its ubiquitination, we coexpressed MDMX and His6-

ubiquitin by transient transfection into H1299 cells. MDMX conjugated to His6-ub was purified by Ni^{2+}-NTA beads under denaturing conditions and detected by western blot with MDMX-specific monoclonal antibody 8C6. The result showed that K254 and K379 single or double mutations had little effect on MDMX mono-ubiquitination (Figure 34). Interestingly, mutating K478 led to significantly higher mono-ubiquitination either alone or in the triple mutant background. Since K478 is located immediately after the last cysteine residue of the RING finger, its mutation to arginine may have stimulated the weak self-ubiquitination of MDMX (Badciong and Haas 2002). Poly-ubiquitination of the MDMX mutants by MDM2 was similar to wild type MDMX (Figure 34), suggesting that lack of sumoylation does not affect MDMX ubiquitination by MDM2. Surprisingly, the K478R mutant was also poly-ubiquitinated and degraded at similar efficiency compared to wild type MDMX, suggesting that increased basal mono-ubiquitination does not promote poly-ubiquitination and degradation by MDM2.

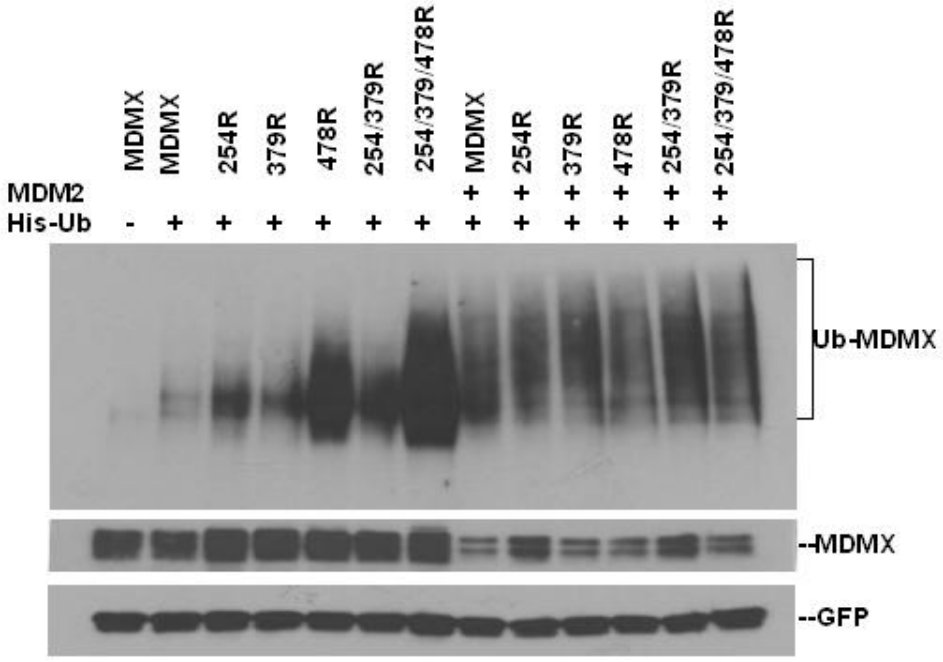

Figure 34. Lack of sumoylation does not affect MDMX mono-ubiquitination or polyubiquitination by MDM2.
MDMX and His6-ubiquitin were coexpresssed by transient transfection into H1299 cells. MDMX conjugated to His6-ub was purified by Ni^{2+}-NTA beads under denaturing conditions and detected by western blot with MDMX-specific monoclonal antibody 8C6.
MDMX sumoylation status has little effect on the stability of MDMX. The lower panels are control Western blots of non-purified cell lysate (10% of the amount used for Ni-NTA purification). Transfection efficiency was verified by expression of cotransfected GFP plasmid.

Next, the MDMX sumo-deficient double point mutants were cotransfected with increasing amount of MDM2 in H1299 cells. To further compare the sensitivity of

MDMX sumoylation mutants to MDM2-mediated degradation, a titration experiment was performed with fixed amount of MDMX plasmid and increasing amount of MDM2 plasmid in the transfection. The dose-dependent degradation by MDM2 was also similar between wild type and mutant MDMX, suggesting that MDMX sumoylation status does not directly affect its stability regulated by MDM2 (Figure 35).

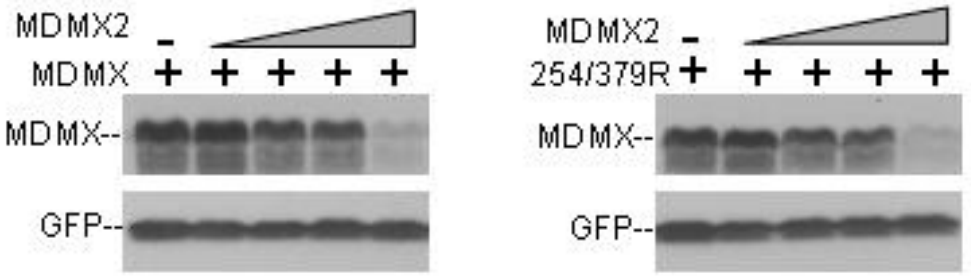

Figure 35. MDMX sumoylation status does not affect its MDM2-mediated degradation.
MDMX and MDMX sumoylation-deficient double mutant were cotransfected with increasing amount of MDM2. The level of MDMX was detected by Western blot with 8C6 antibody.

Previous studies showed that MDMX is destabilized upon DNA damage in an MDM2-dependent fashion (Kawai, Wiederschain et al. 2003; Pan and Chen 2003). Recent work in our lab suggested that DNA damage-induced phosphorylation of MDMX might play a role in accelerating MDMX degradation (manuscript in preparation). To test if blocking MDMX sumoylation affects its regulation by DNA damage, U2OS cells were stably transfected with control vector, wild type MDMX and MDMX sumo-

deficient mutants. Cells were treated with ionizing radiation and collected at different time points. We found that both wild type and sumo-deficient MDMX were degraded at similar efficiency after DNA damage (Figure 36).

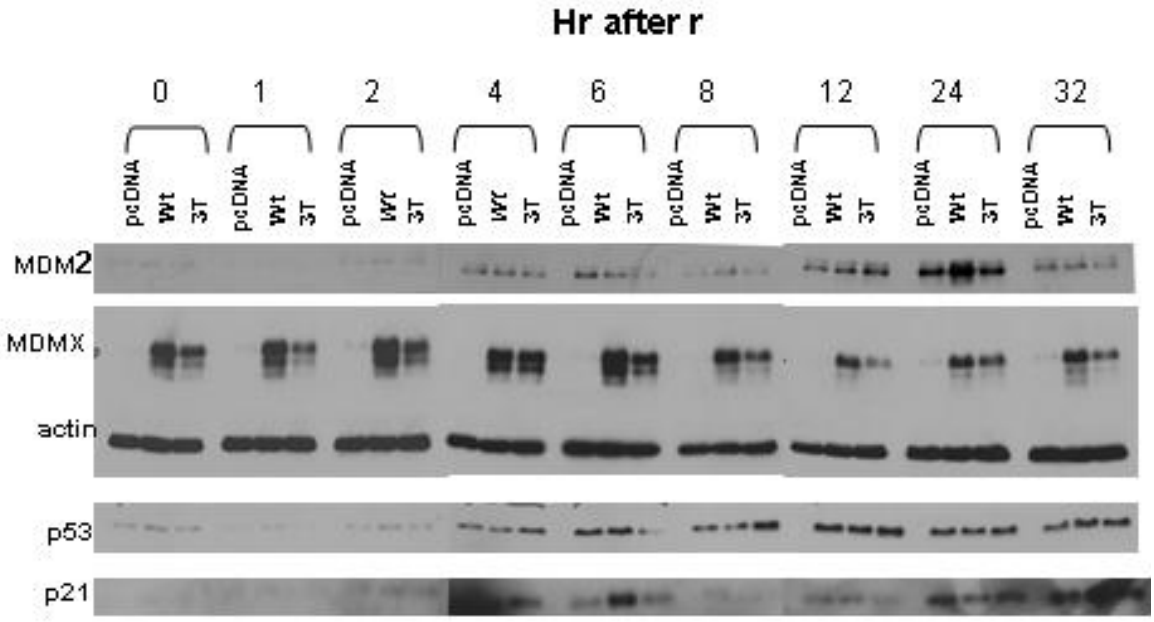

Figure 36. MDMX sumoylation status does not affect its DNA damage induced destabilization.
U2OS cells were stably transfected with control vector, wild type MDMX and MDMX sumo-deficient mutants. Cells were treated with ionizing radiation and collected at different time points. The levels of MDMX, MDM2, p53, p21 and actin were detected by Western blot.

MDMX is mainly localized in the cytoplasm of cells when expressed at high levels and undergoes nuclear translocation after DNA damage through both MDM2-mediated and MDM2-independent mechanisms (Li, Chen et al. 2002)

. When compared to wild type MDMX, the sumoylation-deficient MDMX mutants also translocated into the nucleus after ionizing radiation (Figure 37). Therefore, we conclude that sumoylation is not required for the regulation of MDMX localization and stability after DNA damage.

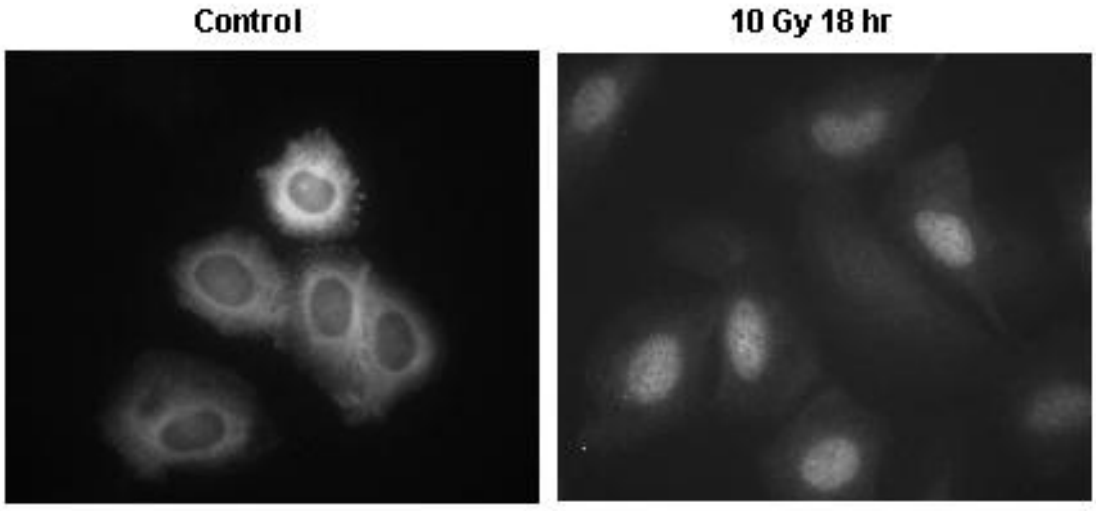

Figure 37. Sumoylation-deficient MDMX mutants translocated into the nucleus after ionizing radiation in the same fashion as wild type MDMX. MDMX mutants were transfected in H1299 cells and r irradiated. Cells were incubated for 18 hrs before collection. MDMX was detected by 8C6 antibody and stained with FITC-goat-anti-mouse IgG.

In further experiments, we compared the ability of MDMX sumoylation mutants to inhibit p53 transcriptional function. The MDMX mutants were cotransfected with p53

expression plasmid and p53-responsive luciferase reporter into p53-null H1299 cells, additionally, MDMX mutants were cotransfected with p53-luciferase reporter into p53-wild type U2OS cells to determine the effect on endogenous p53 transcriptional activity. In both tests, the MDMX sumoylation mutants exhibited similar ability to inhibit p53 function compared to wild type (data not shown). Coimmunoprecipitation experiments with MDM2 and p53 also did not review changes in the ability of the sumoylation mutants to bind MDM2 or p53 (Figure 38). Therefore, we concluded that sumoylation of MDMX is not required for suppression of p53 activity under our experimental conditions.

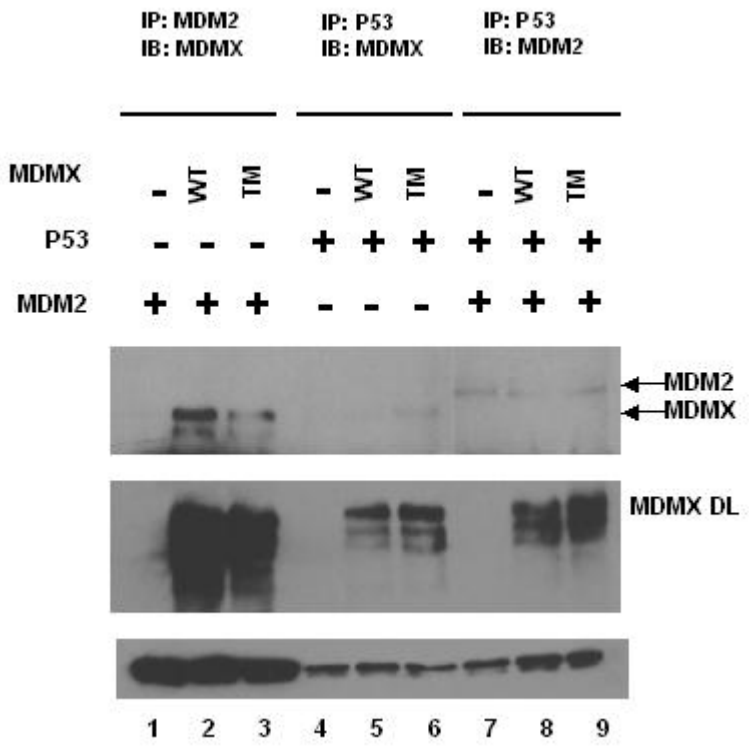

Figure 38. MDMX sumoylation status does not affect protein-protein binding between p53, MDM2 and MDMX.
Wild type MDMX and MDMX sumoylation-deficient mutant were cotransfected with MDM2 (lane 1-3) or p53 (lane 4-6) or both (lane 7-9) in H1299 cells. No change of MDMX co-precipitated with MDM2 (compare lane 2&3) or p53 (compare lane 5&6) was detected by Western blot with anti-MDMX antibody 8C6. No change of MDM2 co-precipitated with p53 in the presence of wild type or mutant MDMX was detected by Western blot with anti-MDM2 antibody 3G9.

Discussion

In this report, we showed that similar to p53 and MDM2, MDMX also undergoes modification by sumoylation. Conjugation to sumo-1 was detected both *in vivo* and *in vitro*. Similar to other sumoylation substrates, MDMX sumoylation in vitro is highly efficient in the presence of E1 and E2 enzymes, suggesting that a specific E3 is not required for this reaction. Previous studies failed to identify the lysine residue important for sumoylation on MDM2, possibly due to presence of multiple alternative sites (Xirodimas, Chisholm et al. 2002; Chen and Chen 2003). Through deletion and point mutagenesis, we were able to determine that K254 and K379 are important for MDMX sumoylation. Single mutations on each site had limited effect on MDMX sumoylation, but the double mutant largely abrogated the modification. Sequence comparison showed that these sites are not conserved on MDM2, suggesting that MDM2 sumoylation involves other lysine residues. Furthermore, mutation of K254 and K379 did not affect MDMX ubiquitination, eliminating the possibility that sumoylation and ubiquitination compete for the same lysines on MDMX.

An unresolved question is the role of sumoylation in the function and regulation of the p53 pathway. Previous studies showed that sumoylation of p53 had very moderate positive effects on its transcription activation function (Melchior and Hengst 2002). Inability to identify the sumoylation site on MDM2 hampered investigation of the role of this modification on MDM2 and p53 function. Generation of MDMX sumoylation-deficient mutants in this study enabled us to test whether sumoylation of MDMX is

involved in several aspects of MDMX function and regulation. At present, the results suggest that MDMX sumoylation is not required for MDMX ubiquitination and degradation by MDM2. Furthermore, regulation of MDMX degradation and nuclear translocation by DNA damage are not affected by lack of sumoylation. The regions of MDMX that contain the sumoylation sites are not directly involved in binding to p53, therefore it is not surprising that p53 binding and regulation by the MDMX mutants are similar to wild type.

Comparison between the level of total MDMX expression in the transfected cells and the amount of sumoylated MDMX recovered by His6-sumo-1 purification indicated that only a small fraction of MDMX (<5%) is conjugated to sumo-1 at any given time. Therefore, it is possible that the modification regulates a specific sub-population of MDMX. Therefore, the impact of this modification on MDMX function may not be evident by the overexpression assays we employed. Although the functional analysis so far have not revealed a requirement for sumoylation in several aspects of MDMX regulation, we cannot rule out a role for sumoylation in regulating other yet to be identified MDMX functions. The findings and reagents described in this report should facilitate future study of the role of sumoylation in regulating MDMX and the p53 pathway.

Scientific Significance

P53 is a tumor suppressor serving as a guardian of the genome, and controls the proliferation and apoptosis of abnormal cells. Under normal condition, p53 needs to be maintained at low level to allow cell growth and proliferation. The two major negative p53-regulators identified to date are MDM2 and MDMX. In mouse models, the abrogation of either MDM2 or MDMX lead to embryonic lethality, which can be rescued by crossing into p53 null background, demonstrating that both MDM2 and MDMX are major regulators of p53.

P53 is a well-known stress sensor for cells. After DNA damage, p53 transcriptionally activates the downstream target genes such as p21 and Bax to stop the cells carrying damaged DNA from proliferating or to eliminate the cells by apoptosis. Hypothetically, full activation of p53 requires the inhibition of both MDM2 and MDMX, which would restrain p53 activity under normal conditions. It is well recognized that p53 binding to MDM2 is weakened after DNA damage due to phosphorylation on the MDM2 binding site. However, the question still remains that if MDMX, the other major negative regulator of p53 can be eliminated after DNA damage to allow the complete activation of p53.

Since MDMX and MDM2 form heterodimers through their RING finger domains and MDM2 contains the intrinsic ubiquitin E3 ligase activity, we speculated that MDMX

might be an E3 substrate of MDM2. Indeed, we found that MDM2 can stimulate the polyubiquitination and degradation of MDMX through the proteasome pathway. From the *in vivo* and *in vitro* experiments, we concluded that MDM2 is the *bona fide* E3 ubiquitin ligase for MDMX and their heterodimerization through the RING domains is required. Based on the fact that MDM2 accumulates due to p53 activation after DNA damage and that MDMX can be degraded in an MDM2-dependent fashion, we speculated that DNA damage might induce the degradation of MDMX partially through the accumulation of MDM2. This was shown to true. After DNA damage, MDMX level significantly reduced, which can be rescued by proteasome inhibitor. The reduction of MDMX protein level is not caused by the mRNA change and is accompanied by the accumulation of MDM2.

Interestingly, we found that ARF overexpression had a synergistic effect with MDM2 in stimulating MDMX polyubiquitination and degradation. This is in contrast to ARF's inhibition of p53 polyubiquitination by MDM2. Additionally, ARF overexpression alone is sufficient in inducing MDMX degradation. This finding changed our understanding of ARF from a stereotypical MDM2 inhibitor into a dynamic MDM2 E3 regulator, which turns on MDM2's E3 function towards MDMX oncogene while suppressing its E3 activity towards p53 tumor suppressor.

Based on the above findings, we proposed the following model: Under normal condition, p53 remains inactive and unstable due to the negative regulation from both MDM2 and MDMX. After DNA damage, MDMX is degraded through a proteasome-dependent pathway. Meanwhile, MDM2-p53 binding is disrupted due to p53 phosphorylation. On the other hand, oncogenic signals such as Ras and E2F1 can induce

the overexpression of ARF, which stimulates MDM2 ubiquitination of MDMX degradation and in the meantime, suppressing the ubiquitination of p53 by MDM2. Thus, both DNA damage and mitogenic signals can activate p53 by abrogating the function of both p53 negative regulators, namely MDM2 and MDMX. Our work established MDMX as an important target of stress signals. Degradation of MDMX by MDM2 connects MDMX to the well-established p53 signaling pathways and provides further insight into the mechanisms of effective p53 response to stress (Figure 21).

Posttranslational modification is an efficient way to modulate protein biological functions. We identified MDMX as an ubiquitin substrate of MDM2. Moreover, we found that MDMX can be conjugated to SUMO-1 and identified the sumoylation sites on MDMX to K254 and K379. It is worth noticing that ARF can stimulate both ubiquitination and sumoylation of MDMX. However, the mechanism through which ARF modulates MDMX posttranslational modification remains elusive. In this manuscript, we found that MDM2 significantly stimulates the binding efficiency between MDMX and ARF. Additionally, ARF stimulates MDMX polyubiquitination in a dose-dependent fashion associated with a stabilization of MDM2. Ongoing research in the lab is focusing on the mechanism by which oncogenes signal through ARF to regulate MDMX function and stability, as well as its implication on p53 activation. In conclusion, data from this manuscript made important contribution to our understanding of the mechanism of MDMX regulation, which is vital in understanding p53 activation upon DNA damage or mitogenic stress.

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