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Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3

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Regulation of MDMX Nuclear Import and Degradation by Chk2 and 14-3-3

by

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DEDICATION

I would like to dedicate this dissertation to all my family and friends who have supported me throughout my college career and my decision to complete my doctorates degree. Most notably: my parents, sister and brother. I owe all my successes to them and will forever be grateful for their love and guidance. I also feel it necessary to acknowledge my grandparents, aunts, uncles and cousins who all lost their fight against cancer. I pledge to them a career focused on pursuing the cure and prevention of cancer.

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LIST OF ABBREVIATIONS

NLS- nuclear localization sequence

NES- nuclear export sequence

DNA- Deoxyribose nucleic acid

CPT- Campthothecin

IR- Ionizing radiation

GFP- Green fluorescence protein

NPC- nuclear pore complex

Ub: ubiquitin

UV: Ultraviolet radiation

Rb: Retinoblastoma gene

REGULATION OF MDMX NUCLEAR IMPORT AND DEGRADATION BY CHK2 AND 14-3-3

Cynthia LeBron

ABSTRACT

The MDM2 homolog MDMX is an important regulator of p53 during mouse embryonic development. DNA damage promotes MDMX phosphorylation, nuclear translocation, and degradation by MDM2. Here we show that MDMX copurifies with 14-3-3, and DNA damage stimulates MDMX binding to 14-3-3. Chk2-mediated phosphorylation of MDMX on S367 is important for stimulating 14-3-3 binding, MDMX nuclear import by a cryptic NLS, and degradation by MDM2. Mutation of MDMX S367 inhibits ubiquitination and degradation by MDM2, and prevents MDMX nuclear import. Expression of 14-3-3 stimulates the degradation of phosphorylated MDMX. Chk2 and 14-3-3 cooperatively stimulate MDMX ubiquitination and overcome the inhibition of p53 by MDMX. These results suggest that MDMX-14-3-3 interaction plays a role in p53 response to DNA damage by regulating MDMX localization and stability. We also show the identification of a cryptic nuclear localization sequence within the C-terminus RING finger domain MDMX. Mutation of MDMX on one lysine residue at position 468 to glutamic acid completely abrogates the nuclear import after DNA damage. This mutation had no effect on MDM2-mediated nuclear import of MDMX in cotransfection assays,

suggesting that it is specifically required for the MDM2-independent nuclear import. Interestingly, the MDMX- K468E mutant induces the expression of p21 more efficiently than the wild-type MDMX after ionizing radiation (IR). Furthermore, the K468E mutant induction of p21 is associated with enhanced G1 arrest after DNA damage. These results indicate an important function of MDMX nuclear import in regulating p53 activity after DNA damage.

INTRODUCTION

Carcinogenesis

The development of cancer is a complex, multi-step process that usually initiates with changes in the activation of proto-oncogenes and inactivation of tumor suppressors. Oncogenes are cellular or viral (inserted by virus into a cell) genes that can promote the development of neoplasm. Proto-oncogenes are normal genes involved in cell proliferation, but amplification or modifications can result into the conversion of oncogenes. Tumor suppressor or anti-oncogenes are cellular genes that when inactivated increase the probability of cancer formation, whereas reactivation of their function can be growth suppressive (Kopnin 2000). While oncogenes promote cell proliferation, tumor suppressors play important roles in restraining proliferation. The accumulation of genetic mutations eventually leads to genomic instability. However, for this process to develop into cancer, these mutations must arise in cells with the potential to proliferate and survive. Carcinogenesis can be divided into three steps: initiation, promotion, and progression. Modifications to the structure of DNA by chemical carcinogens or epigenetic changes initiate the process. These genetic changes are thought to lead to abnormal proliferation of a single cell. Tumor promotion is composed of the selective expansion of these abnormal cells, which develop into a larger population of cells that are extremely unstable and are at risk for acquiring more mutations at the end of each round of cell division. Tumor progression is the process that results in transformation of pre-

malignant cells to those that display malignant phenotypes. The expansion of the cells that acquire this malignant phenotype facilitates the development of more aggressive characteristics and confers a selective advantage. The majority of cancer cells display the following traits: uncontrolled cell growth, evasion from cell death, invasion and metastasis of other tissues/organs and reduced requirements for extracellular growth factors (Friend, Bernards et al. 1986; Hanahan and Weinberg 2000). Aforementioned, carcinogenesis is a process that destabilizes the genome resulting in changes to gene activities and stability (Vogelstein, Lane et al. 2000). Many of the genes affected, are involved in cell cycle control, DNA damage/checkpoint responses and DNA repair. One such example is the TP53 tumor suppressor gene, currently termed the guardian of the genome (Lane 1992). The function of p53 is to maintain cellular homeostasis and provide critical steps to ensure an effective tumor response. Understandably, p53 is mutated or deregulated in over 50% of human cancers (Hollstein, Sidransky et al. 1991; Levine, Momand et al. 1991).

P53

History of p53

P53 belongs to a family of transcription factors including p53, p63 and p73. Although p63 and p73 are required for normal development, only p53 prevents tumor formation (Yang and McKeon 2000). The P53 gene was first discovered in 1979 and was thought to play a role as an oncogene, due to the observation that it bound to the Simian Virus (SV40) large T-antigen and accumulated in cancer cells (DeLeo, Jay et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979; Linzer, Maltzman et al. 1979). In the late 1980's researchers were studying a mutant form of p53 and not the wild type. These mutant forms contain missense mutations that acquire gain of function activities, which explain why p53 was acting in an oncogenic manner, thus accumulating in the nuclei of cancer cells. Mutated p53, unlike wild-type p53, is very stable due to its interaction with HSP90 that inhibits the ubiquitination by MDM2 (Peng, Chen et al. 2001; Peng, Chen et al. 2001). It was also found to form tetramers with wild-type p53, thereby acting like a dominant negative to suppress wild-type p53 functions (Sigal and Rotter 2000). Interestingly, in 1990, *Scheffner et al.* found that DNA viruses, such as HPV, evolved a mechanism to inactivate p53 (Scheffner, Werness et al. 1990). More specifically they identified the viral E6 oncoprotein that could promote the ubiquitination and proteolytic degradation of p53. P53 is now widely accepted as a tumor suppressor that continuously monitors the integrity of DNA. It responds to a wide variety of stress signals and

becomes a rate-limiting factor in certain steps of tumor development.

p53 structure and function

P53 is a 393 amino acid transcription factor that binds to defined consensus sites within DNA as a tetramer (el-Deiry 1998). P53 can be divided into four functional domains: a transactivation domain in the N-terminus, a PxxP domain, a sequence specific DNA binding domain in the central region, and a tetramerization domain in the C-terminus. The transactivation domain (residues 1-42) can interact with transcriptional machinery to regulate the expression of p53 target genes. The proline XX proline domain (residues 61-94) can regulate p53 induced cell cycle arrest and apoptosis, however mutation of this domain has no effect on the transactivation of p53. The central core domain (residues 102-292) is required for sequence specific DNA binding, and the tetramerization domain (residues 324-355) is required for the efficient oligomerization of p53 tetramers. In addition the carboxyl terminus (residues 311-393) contains a nuclear localization and nuclear export sequence respectively (Cho, Gorina et al. 1994; Hainaut, Soussi et al. 1997) (**Figure 1**). Over 80% of human cancer contains missense mutations with in the DNA binding domain (Hainaut, Hernandez et al. 1998). Thirty percent of missense mutation in the DNA binding domain occur in six codons: 175, 245, 248, 249, 273 and 282 (Greenblatt, Bennett et al. 1994).

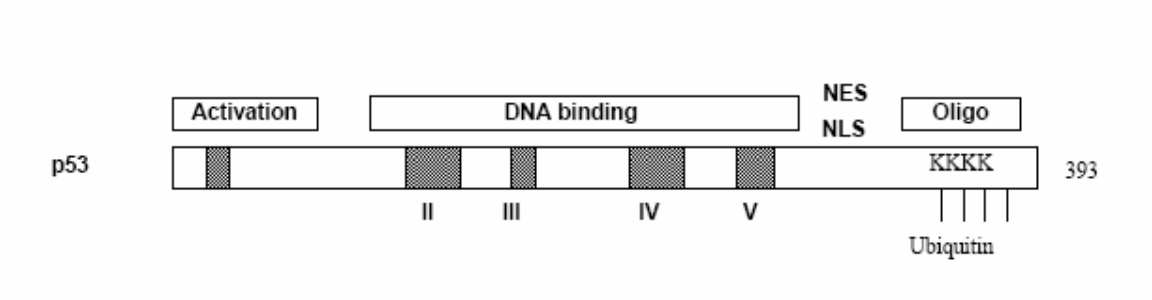


Figure 1: Structure of p53

In the late 1980's several important discoveries defined the normal function of p53 as anti-oncogenic. Introduction of wild type p53 into cells was found to be growth suppressive and more striking was the effect of p53 ablation. Genetic models of mice homozygous null for p53 were viable and appeared healthy. This indicated that p53 was indispensable for mouse development. However, after six months of age the mice were clearly more predisposed to spontaneous tumor development than control littermates. Mice heterozygous for p53 were also more prone to tumor development, however at a more modest rate. The development of cancer in these mice was due to somatic loss of the wild-type allele; also referred to as loss of heterozygosity (Donehower, Harvey et al. 1992; Donehower, Harvey et al. 1995). Not surprising, germline mutations of p53 occur in individuals with Li-Fraumeni syndrome who are predisposed to cancer formation (Malkin, Li et al. 1990; Srivastava, Zou et al. 1990).

P53 is a transcriptional activator, regulating the expression of many genes involved in growth arrest, DNA repair and apoptosis. Through these mechanisms p53

facilitates the repair and survival of damaged cells or promotes the removal of severely damaged cells by initiating apoptosis. P53 has also been shown to be capable of transcriptional repression of its target genes. The inhibition may be in part due to p53 association with basal transcription machinery, needed to initiate transcription (Seto, Usheva et al. 1992).

Mammalian cell division is controlled by two tumor suppressors proteins, retinoblastoma protein (Rb) and p53 (Tao and Levine 1999). Regulating the cell cycle is a very important step in ensuring that only normal genetic material is passed on to daughter cells. Mistakes during DNA synthesis or division can all lead to the development of cancer. The cell cycle consists of four distinct phases: G1- phase, S-phase, G2-phase and M-phase. Rb regulates the exit from G1 phase and p53 serves to maintain genomic integrity. Each of these distinct phases is monitored by checkpoints. The molecular events that control the cell cycle are ordered and directional. All these stages have regulatory molecules that determine the cells progress through the cell cycle. Passage through the cell cycle requires activation of cyclin dependent kinases (CDKs) and the transient association with regulatory subunits: cyclins and cyclin dependent inhibitors. As their name implies, these kinases are directly dependent on cyclins for there activity. In order for the cell to move from G1 phase into the replication phase (S), the intactness of DNA is required. If the cell is exposed to carcinogens or factors known to mutate DNA such as ultraviolet light or ionizing radiation, the G1 checkpoint will stop the progression into S-phase. DNA damage can directly induce the cell cycle checkpoints by the activation of p53, which stimulates the transcription of p21, a cell

cycle inhibitor that binds to Cdks to block the transition of G1 to S and from G2 to M phase (el-Deiry, Tokino et al. 1993). Another transcriptional target of p53 is 14-3-3 sigma gene (Hermeking, Lengauer et al. 1997). 14-3-3 proteins function at several key points in the G1/S and G2/M transition to regulate the cell cycle progression. Induction of 14-3-3 sigma by p53 results in the cytoplasmic sequestration of CDC2-cyclin B complexes that are essential for mitosis. The number of divisions for most normal cells is limited, excluding stem cells. The progressive shortening of telomeres and incomplete replication of the entire chromosome maintains this mechanism. Notably many cancers have been found to overexpress telomerase, the enzyme required to maintain telomeres. Proto-oncogenes such as Myc, activated Ras and MDM2 have all been implicated in controlling telomerase activity (Fukasawa and Vande Woude 1997). These factors provide cancer cells with the potential to become immortalized and grow indefinitely.

DNA repair is the process whereby the cell identifies and corrects damage to the DNA molecules that encode its genome. Although not required, p53 facilitates multiple types of DNA repair such as nucleotide excision repair, base excision repair and correction of double stranded breaks (Gatz and Wiesmuller 2006). P53 can also function to promote the transcription of genes involved in DNA repair including GADD45 and p53R2. P53R2 encodes a ribonucleotide reductase, which is important for both DNA replication and repair.

The manner in which p53 initiates apoptosis is a matter of intensive study since it was first demonstrated (Yonish-Rouach, Resnitzky et al. 1991). Apoptosis can be

initiated by two distinct mechanisms: intrinsic and extrinsic pathways. The intrinsic pathway is governed by Bcl-2 family of proteins that regulate the release of cytochrome C from the mitochondria (Cory and Adams 2002; Kuwana, Mackey et al. 2002). This family consist of pro-apoptotic members: Bax, Noxa, PUMA and anti-apoptotic members: Bcl-2, Bcl-X_L (Vousden and Lu 2002). Bax was the first identified member of pro-apoptotic proteins that can be induced by p53. In response to stress, Bax forms homodimers, which are thought to bind to the mitochondria creating pores. These pores allow the release of cytochrome C from the mitochondria, resulting in caspase 9 activation and the formation of the apoptosome leading to cell death. P53 can also induce PUMA and Noxa, which also promote mitochondrial depolarization leading to cytochrome c release (Miyashita, Krajewski et al. 1994; Oda, Ohki et al. 2000; Nakano and Vousden 2001). Another group of p53-induced genes belongs to the death-receptor or tumor necrosis factor receptor family (TNF-R) including DR5, Fas and PERP (Owen-Schaub, Zhang et al. 1995; Wu, Burns et al. 1997; Muller, Wilder et al. 1998; Lin, Ma et al. 2000). The extrinsic pathway involves the engagement of death receptors at the cell membrane and the consequent induction of caspase 8 and caspase 3 activities. Fas is a cell surface receptor that is activated by Fas ligand binding. P53 can induce the transcription of Fas mRNA expression and DR5 in response to DNA damage (Wu, Burns et al. 1997; Bouvard, Zaitchouk et al. 2000) (**Figure 2**).

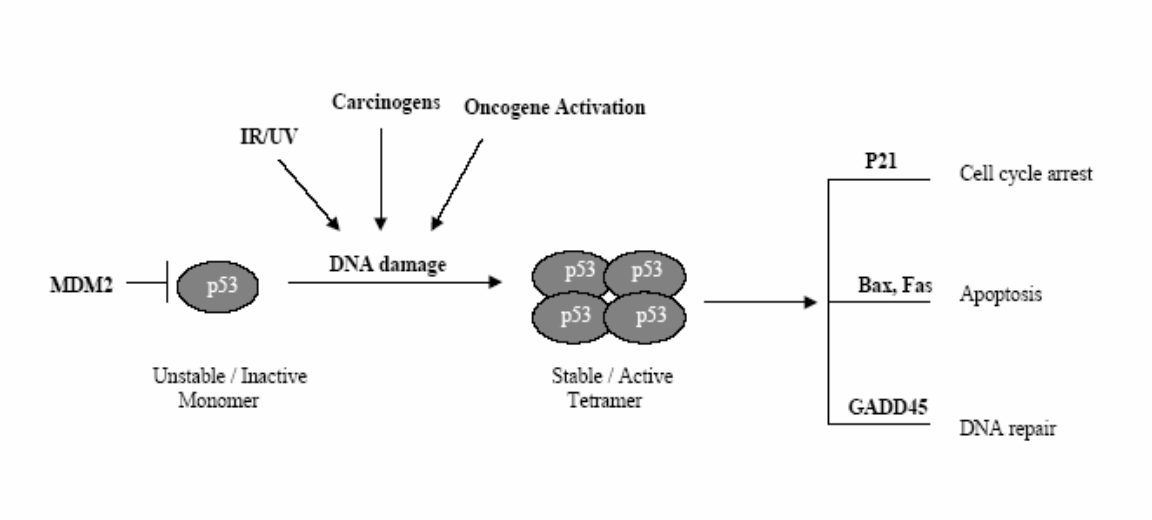


Figure 2: Functions of p53

Regulation of p53

The p53 protein is subject to tight regulation on three major levels: protein stability, protein activity and sub-cellular distribution. Each of these levels of control is achieved by positive and negative feedback loops (**Figure 3)**. Under normal conditions, resting cells maintain low levels of p53 by continuous ubiquitination and degradation by the 26S proteasome. A variety of different stresses can activate and stabilize p53. Stress signals are transmitted to p53 by multiple posttranslational modifications that allow for the activation and stabilization of p53. Posttranslational modifications are covalent additions of functional groups to p53, such as, ubiquitination, phosphorylation, acetylation, and sumoylation (Appella and Anderson 2001).

P53 phosphorylation is pivotal for its activation after DNA damage. Phosphorylation of p53 generally results in its stabilization. So far there are 18 serine

and threonine sites that are reported to be phosphorylated on p53 (Bode and Dong 2004). Of particular importance are the phosphorylations of serine 15 and serine 20, which leads 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). MDM2 can also be phosphorylated on serine 395, promoting the disassociation from p53 (Mayo, Turchi et al. 1997).

In addition, several lysines residues located in the C-terminal region have been identified as acetylation sites on p53. The role of acetylation is believed to enhance the affinity to the p53 responsive element, increase protein activity and promote an apoptotic response (Luo, Su et al. 2000; Rodriguez, Desterro et al. 2000; Ito, Lai et al. 2001). Phosphorylation of the N-terminus has been implicated in facilitating the acetylation of p53 and enhancing the recruitment of transcriptional co-activators such as CBP/p300 and PCAF (Avantaggiati, Ogryzko et al. 1997; Gu, Shi et al. 1997; Lill, Grossman et al. 1997; Dumaz and Meek 1999) . Posttranslational modifications such as sumoylation and neddylation can also be stimulated by MDM2. Interestingly, these modifications have very different effects on p53 activity. It has been shown that p53 sumoylation can enhance its transcriptional activity, while p53 neddylation leads to the transcriptional inhibition of p53 activity (Melchior and Hengst 2002; Harper 2004; Xirodimas, Saville et al. 2004). MDM2 can also render p53 more susceptible to degradation by interfering with CBP/P300 acetylation of p53 (Kobet, Zeng et al. 2000; Ito, Lai et al. 2001).

P53 stability and activity can also be regulated by a variety of interacting proteins.

The short half-life of p53 is primarily maintained by the interaction with MDM2, an E3 ubiquitin ligase. However, other proteins can mediate the ubiquitination and degradation of p53 including Pirh2 and COP1. Both these genes, like MDM2, are p53 inducible genes, thus creating a negative feedback loop (Leng, Lin et al. 2003; Dornan, Wertz et al. 2004). Pirh2 interacts with p53 and promotes its ubiquitination independent of MDM2 (Leng, Lin et al. 2003). Cop1 also can increase p53 turnover by promoting its degradation through the proteasome (Dornan, Wertz et al. 2004). Because MDM2 is involved in so many of the autoregulatory loops that regulate p53 function, it is likely that it plays a central role in regulating p53 activity.

MDMX, a homolog of MDM2 has recently been implicated in the negative regulation of p53. Unlike its relative, MDMX does not have any intrinsic E3 ligase activity and thus does not promote the ubiquitination or degradation of p53 (Jackson and Berberich 2000; Stad, Ramos et al. 2000; Stad, Little et al. 2001; Migliorini, Danovi et al. 2002). Instead, MDMX can bind to the transactivation domain of p53, thereby inhibiting its transcriptional activation (Finch, Donoviel et al. 2002). The inhibition of p53 by MDMX is mainly mediated through the interference of co-activator bindings and transcriptional machinery. MDM2 and MDMX can also interact with each other forming heterodimers in the RING domains. This interaction was shown to protect p53 from MDM2-mediated degradation (Jackson and Berberich 2000). This appears to be achieved by MDMX preventing nuclear export of p53 (Stad et al. 2001). Our laboratory identified a link between MDMX downregulation and p53 activation following ribosomal

stress. Studies showed that tumor cells expressing high levels of endogenous MDMX had less efficient p53 activation and growth arrest during ribosomal stress and were less sensitive to agents that induce ribosomal stress. Interestingly, down regulation of MDMX by siRNA knockdown enhanced p53 response to ribosomal stress by promoting the ubiquitination of MDMX, mediated by MDM2 and L11 binding (Gilkes, Chen et al. 2006). Furthermore, they suggested that MDMX inhibits the DNA binding activity of p53 and possibly the recruitment of basal transcription factors to the promoter.

The Alternate Reading Frame tumor suppressor or ARF is another interacting protein that can positively regulate p53 stability. ARF was identified as an alternative transcript of the INK4a/ARF locus. The other transcript is the p16 ^{INK4a,} a checkpoint gene that inhibits cyclin-dependent kinase 4 (Hatakeyama and Weinberg 1995; Sherr 2001). ARF is completely unrelated to p16, by virtue of unique first exons, generating transcripts translated by alternative reading frames (Duro, Bernard et al. 1995). Hereditary human cancers commonly contain alterations to both p16 and ARF (Sharpless 2005). Mitogenic signals such as: E1A, E2F, myc, ras and v-abl induce the expression of ARF (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998). The induction of ARF can stabilize p53 by virtue of ARF's nucleolar localization signal within the C-terminus (Kamijo, Weber et al. 1998; Pomerantz, Schreiber-Agus et al. 1998; Stott, Bates et al. 1998; Zhang, Xiong et al. 1998). By separating MDM2 from p53, ARF can stabilize p53, however recent findings indicate that the activation of p53 is a result of nucleolar disruption, instead of ARF

physically sequestering MDM2 in the nucleolus (Korgaonkar, Hagen et al. 2005). ARF has also been shown to stabilize MDM2 self E3 ligase activity (Weber, Taylor et al. 1999; Zhang and Xiong 1999; Ito, Lai et al. 2001).

Interestingly, other components of the nucleolus have been shown to affect p53 activity, including ribosomal proteins L5, L11, L23 (Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003; Bhat, Itahana et al. 2004; Dai and Lu 2004; Dai, Zeng et al. 2004; Jin, Itahana et al. 2004; Dai, Shi et al. 2006). These proteins are released from the nucleolus after ribosomal stress and have been shown to stabilize p53 by negatively regulating both MDM2 and MDMX. The L-proteins can bind the acidic domain of MDM2 to inhibit the E3 ligase activity towards p53 (Dai, Shi et al. 2006). The inhibition of MDM2's repression of p53 can induce target gene expression and a cell cycle arrest (Bhat, Itahana et al. 2004). It has been suggested that the mechanism of p53 protection by L-proteins is the interference with the transfer of ubiquitin from the E2 to p53 (Zhang, Wolf et al. 2003). Nucleophosmin/B23 has been shown to activate p53 when overexpressed and can affect p53 stability by interacting with ARF (Colombo, Marine et al. 2002; Korgaonkar, Hagen et al. 2005).

Preventing degradation or reducing the interaction with MDM2 are ways to stabilize p53; however, removing the ubiquitin moieties from p53 is another mechanism. The interaction between HAUSP (herpesvirus protein associated cellular) and p53 add another level of regulation. HAUSP has been shown to bind the C-terminus of p53 and deubiquitinate, thus stabilizing p53. The infection of HAUSP into p53 positive cells,

indicated a stabilization of p53, measured by p21 induction and a G1 arrest (Li, Chen et al. 2002). Interestingly, HAUSP can indirectly affect p53 stability and activity by associating and deubiquitinating MDM2 (Li, Brooks et al. 2004). In addition, loss of HAUSP resulted in a destabilization of MDM2 that lead to an increase in p53 levels and p53 dependent G1 arrest (Cummins and Vogelstein 2004). Understanding how HAUSP balances these two proteins in response to cellular stresses are currently being investigated.

Sub-cellular localization is another important mechanism in regulating p53. Compartmentalization provides an opportunity for regulation and in eukaryotes is tightly controlled. Macromolecules can move back and forth between the nucleus and cytoplasm through structures called nuclear pore complexes (NPC's) (Lyman and Gerace 2001). Cargo proteins that are destined to be imported, carry nuclear localization sequences whereas a nuclear export sequence (NES) is used for export. These sequences are recognized by carriers: karyopherins α and β also referred to as: importins, exportins or transportins. During the process of nuclear import, carrier proteins bind to cargo proteins in the cytoplasm. These complexes are than transported through the nuclear pore complex where they are dissociated by Ran-GTP (Stewart 2003). As a transcription factor, p53 must be in the nucleus to respond to stress signals and perform its normal functions in growth inhibition and apoptosis (Tan, Wallis et al. 1986; Yew and Berk 1992; Ueda, Ullrich et al. 1995). Regulation of p53 localization occurs on two levels by direct modifications to the NLS or NES sites on p53 or the interaction with other protein that could limit or interfere with NLS or NES accessibility.

The NLS and NES of p53 can be modified by posttranslational modifications. Although not clear, phosphorylation is thought to play an important role in regulating p53 sub-cellular localization. It has been reported that insulin-like growth factor I can induce elevated levels of phosphorylation of p53 resulting in elevated cytoplasmic levels of p53 in MCF-7 human breast cancer cells (Takahashi, Sumimoto et al. 1993). In addition, ATM phosphorylation of serine 15 can regulate cellular localization by inhibiting MDM2 binding with p53 (Liang and Clarke 2001). Furthermore, MDM2 regulates p53 localization in two ways. First, MDM2 mediates the nuclear export and degradation of p53, by shuttling it into the cytoplasm (Roth, Dobbelstein et al. 1998; Tao and Levine 1999). Alternatively, MDM2 can ubiquitinate p53 in the nucleus by unmasking p53 NES (Inoue, Wu et al. 2005). Studies have indicated that knockdown of MDM2 or inhibition by p19 (ARF) tumor suppressor results in nuclear import and retention. Then again, it has been reported that the nuclear export of p53 is an MDM2 independent event (Stommel, Marchenko et al. 1999).

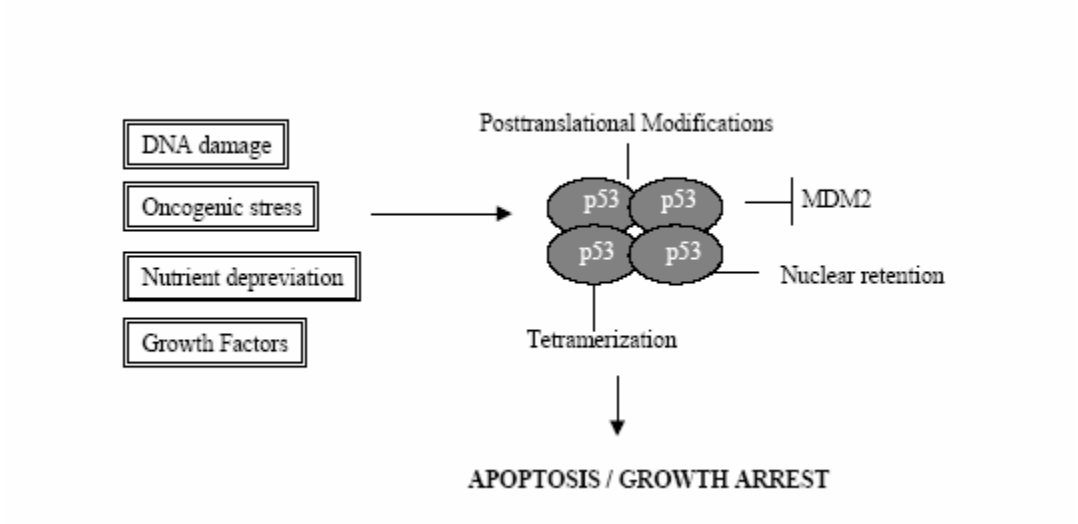


Figure 3: Regulation of p53

Activation of p53 in response to DNA damage

It appears that one of the most important functions of p53 in suppressing tumorigenesis is the ability to respond to damage DNA. By initiating cell cycle arrest or apoptosis, p53 prevents the propagation of cells that may have acquired cancer promoting mutations or an altered genome. Ionizing radiation (IR), ultraviolet radiation (UV) and chemical carcinogens cause different types of DNA lesions. One of the more potent damages inflicted on the genome is DNA double stranded breaks, induced by IR. Cells possess a complex set of signaling pathways for recognizing DNA damage. Several kinases have been identified that detect genotoxic stress and initiate signaling to p53. The ATM gene is one of the main sensors of DNA damage. ATM belongs to a conserved family of proteins, PI3 kinase like protein kinases (PIKK), and is also one of the main

kinases that target p53. ATM deficiency leads to the autosomal recessive genetic disorder called Ataxia telangeictasia in humans. This genetic disorder is featured by immunodeficiency, radiation sensitivity, neurodegneration and cancer predisposition (Crawford 1998; Becker-Catania and Gatti 2001). Most of the PIKK family proteins possess serine/threonine kinase activity. Phosphorylation is an important mechanism for the activation of p53 in response to DNA damage. ATM/A-T related (ATR) kinase plays a fundamental role as the first activator of the DNA damage response, through the phosphorylation of a variety of target proteins with the consensus sequence characterized by serine or threonine residue followed by glutamic acid (Banin, Moyal et al. 1998; Canman, Lim et al. 1998). Furthermore, ATM can phosphorylate p53 directly on serine 15 and concomitantly mediates additional phosphorylation and posttranscriptional modification that enhance p53 stability and transcriptional activity (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Khanna, Keating et al. 1998; Meek 2004). In addition, ATM also phosphorylates Chk1 and Chk2, checkpoint kinases needed to propagate the damage signal. While ATM and Chk2 activate p53 in response to ionizing radiation, ATR and Chk1 appear to be required for response to ultraviolet radiation (Chehab, Malikzay et al. 1999; Hirao, Kong et al. 2000). The phosphorylation of Chk2 on threonine 68 by ATM facilitates the dimerization of Chk2 homodimers (Ahn, Schwarz et al. 2000). Both Chk1 and Chk2 have been reported to phosphorylate p53 on Serine 37 or 20, respectively (Bartek, Falck et al. 2001; McGowan 2002). Chk2 is an important regulator of both the stability and activation of p53 in cells exposed to IR and also functions as a tumor suppressor (Hirao, Cheung et al. 2002). P53 phosphorylation of

serine 15 and 20 promotes its stabilization and activation, through the disruption of its interaction with MDM2 and transcriptional activation of target genes important for cell cycle arrest and apoptosis. The precise mechanism by which Chk2 regulates p53 apoptosis remains unclear. Chk2 deficient mice are viable, fertile and do not show a tumor prone phenotype, except when exposed to IR (Hirao, Kong et al. 2000; Takai, Naka et al. 2002). The phenotype of these mice included increased resistance to IR, cellular defects in p53 function and checkpoint responses, and apoptosis.

Furthermore, ATM can directly target phosphorylation of MDM2 on serine 395, enhancing its self-ubiquitination and degradation (Stommel and Wahl 2005). Other newly identified PIKK family proteins such as ATX can carry out similar functions in phosphorylating p53, however mechanisms remain elusive (Kastan and Lim 2000; Abraham 2001; Hammond, Denko et al. 2002; Heffernan, Simpson et al. 2002; Shiloh 2003). ATX can collaborate with both ATM and ATR in response to other genotoxic stress such as hypoxia. Thus, the DNA-damage response of p53 is crucial for homeostasis and for avoiding neoplasia generated from DNA lesions.

Targeting p53 in the clinics

The current treatment for cancer includes radiation therapy and a variety of chemotherapeutic reagents, which all carry numerous physical and emotional side effects. Radiation therapy is considered the most effective form of cancer treatment for a variety

of malignant tumors of all stages. However, the limitation of this treatment is the non-selectivity of action between normal and tumor cells. P53 is mutated in about 50% of human tumors or its functionality compromised by means of loss of positive regulators or overexpression of negative regulators. Current research has focused on three approaches to treat these types of cancers. The first approach would be to selectively target cancer cells that possess the mutated form of p53, from those that are normal. Using a genetic engineering approach, researchers have designed retroviral and adenoviruses that are modified to selectively replicate in cells deficient for p53 (Bischoff, Kirn et al. 1996; Roth 1996; Roth, Konig et al. 1998). For example, an adenovirus that encodes E1b can only replicate in mammalian cells by inactivating p53, however engineering a virus with this exact defect could selectively target cells with missense mutations or allelic loss of p53. The hypothesis is that the introduction of this virus into cells would have no effect on normal cells, but tumor cells with mutant p53 are killed by the productive lytic infection. Preliminary research in mice using the virus ONYX-015, showed promising results; however, preclinical trials have been less effective (Heise, Sampson-Johannes et al. 1997). Better results were obtained when the virus was combined with conventional chemotherapy (Yang, You et al. 2001). Although promising, the use of viruses in the treatment of cancer is very preliminary and requires further testing for improvement. Another approach to restoring the normal function to mutant p53 is identifying pharmacological agents. PRIMA-1 a chemical reagent was designed to eliminate p53 mutant bearing cells. This compound was shown to induce cytotoxic effects and apoptosis in human tumor cells bearing mutant p53. The selective nature of this

compound provided an excellent proof of concept for targeting mutant cells and providing a strong anti-tumor response and no apparent toxicity. Researchers found an increase in serine 15 phosphorylation of p53 and well as restored DNA binding activity to pro-apoptotic promoters such as BAX and PUMA (Bykov, Issaeva et al. 2002; Chipuk, Maurer et al. 2003; Wang, Lee et al. 2007).

The second approach would be to rescue the functionality of wild-type p53 that was been compromised by the loss or overexpression of key regulators. The negative effect of MDM2 inflicted on p53 is not without control. As the inhibition of MDM2 will favor p53 function, MDM2 inhibitor might possess tumor suppressor characteristics. Extensive work has been put into developing drugs towards selective targets that affect the function of p53. High throughput screening identified Nutlins, which are cis-imidazoline analogs that inhibit p53-MDM2 binding with a low IC50 (Bottger, Bottger et al. 1996). Nutlin 3 showed the ability to activate p53 in cell culture and inhibit tumor growth in vivo, by inhibiting its association of MDM2 with wild-type p53 and promoting self-ubiquitination of MDM2 with a low IC50 in the 100-300nM range (Vassilev 2004; Vassilev, Vu et al. 2004; Vassilev 2005). This treatment would be ideal in cancers, such as sarcomas that overexpress MDM2, but retain wild-type p53, however no clinical trial data is available as of yet. A limitation of using this drug would be in targeting tumor cells overexpressing MDMX, another key negative regulator of p53. We found that although MDMX and MDM2 are homologous and bind to the same region on p53, Nutlin

does not disrupt MDMX-p53 interaction (Hu, Gilkes et al. 2006). This finding supports the need for developing small molecule inhibitors that are specific for MDMX.

RITA is another compound identified to inhibit the p53-MDM2 interaction; however its mechanism of action is quite different from that of Nutlins. RITA binds the N-terminus of p53 and leads to accumulation of transcriptionally active p53. One advantage of this compound is its ability to activate the apoptosis inducing functions of p53 (Baker, Markowitz et al. 1990; D'Orazi, Marchetti et al. 2000) .

The third approach is in its infancy stages; however the concept may lead to new chemotherapeutic agents. Lai et al. identified compounds that inhibit the E3 ligase activity of MDM2 (Lai, Yang et al. 2002). These compounds are selective for MDM2 and show no off target effects on other E3's. Interestingly, these compounds can differentiate between MDM2 ubiquitination of p53 and self-ubiquitination with a low IC50. Another group has also identified similar compounds that target MDM2 activity. These compounds indicate good cellular permeability, however they show some p53-independent cellular toxicity (Yang, Ludwig et al. 2005).

MDM2

MDM2 history:

MDM2 or Murine Double Minute Clone 2 was first discovered through its amplification on murine double-minute chromosomes (Cahilly-Snyder, Yang-Feng et al. 1987). These abnormal chromosomes called double minute frequently harbor amplified genes, leading to uncontrolled cell proliferation and tumorigenesis (Fakharzadeh, Trusko et al. 1991). Mice bearing an amplification of MDM2 developed a high risk of tumor formation. Later, MDM2 was co-purified with p53 and found to negatively regulate p53 stability and transcriptional activity (Momand, Zambetti et al. 1992). This gave the first indication that MDM2 was an oncogene due to its negative regulation of p53. It was later found that overexpression of MDM2 can immortalize primary cultures of rodent fibroblast (Finlay 1993). In humans, over 30-40% of sarcomas and leukemic cells overexpress MDM2 (Oliner, Kinzler et al. 1992; Bueso-Ramos, Yang et al. 1993). These findings all implicated MDM2 as a typical proto-oncogene.

MDM2 structure:

Full length human MDM2 is composed of 491 amino acids (Oliner, Kinzler et al. 1992). MDM2 belongs to a family of RING finger E3 ubiquitin ligases that promote the ubiquitination of itself and other substrate proteins (**Figure 4**). MDM2 contains three major conserved regions, including the N-terminal composed of 90 amino acids in length (Region I). The second region is the central and highly acidic domain (Region II). Also

present in the last domain is the nuclear localization and export sequences (Fakharzadeh, Trusko et al. 1991; Boddy, Freemont et al. 1994). Region I has been closely mapped due the finding that this region mediates the binding to p53. In the human protein amino acids 19-102 were the amino acids that interacted with p53 in vitro (Chen, Marechal et al. 1993; Oliner, Pietenpol et al. 1993). It was later revealed by using a synthetic peptide of Xenopus laevis that MDM2 bound to the 15-residue transactivation domain of p53. In addition, it was reported that the central acidic region of MDM2 has a second p53-binding domain that is regulated by phosphorylation (Kulikov, Winter et al. 2006). The central acidic domain is also essential for MDM2 interaction with ARF, L11 and p300 (Kulikov, Winter et al. 2006). MDM2 contains a hydrophobic cleft which p53 binds as an amphipathic alpha helix (Kussie, Gorina et al. 1996). The amino acid contacts essential for p53 binding to the MDM2 cleft includes: phenylalanine 19, tryptophan 23 and lysine 26 (Lin, Chen et al. 1994). Region III or the RING finger domain features a consensus motif characterized by $CX_2CX_{(9-39)}CX_{(1-3)}HX_{(2-3)}C/HX_2CX_{(4-48)}CX_2C$.

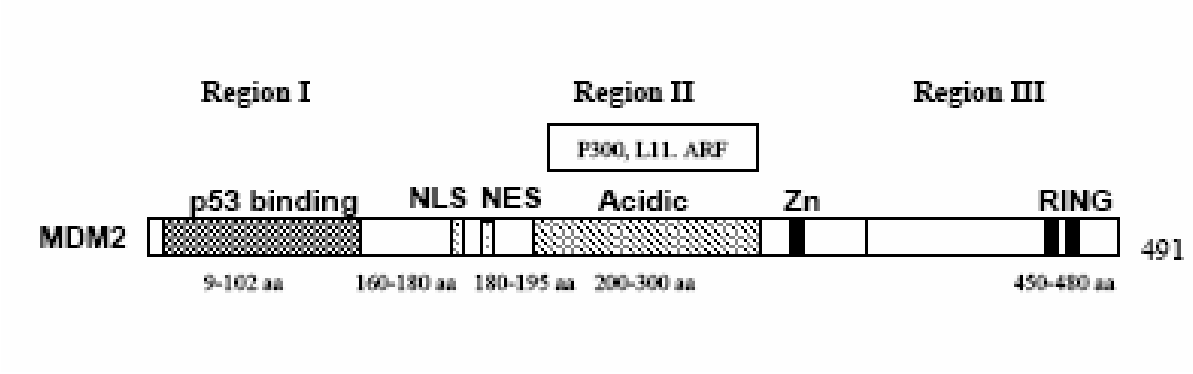


Figure 4: Structure of MDM2

MDM2 functions

The co-purification of MDM2 and p53 began the task of understanding its biological significance. Initial studies were performed in cells containing wildtype p53 and identified the transforming capacity of MDM2; while other studies later revealed that the oncogenic properties of MDM2 could be attributed to inhibition of p53's growth suppression properties (Fakharzadeh, Trusko et al. 1991; Finlay 1993). After the discovery that MDM2 could inhibit p53 function, future studies identified one third of human sarcomas retained wildtype p53, but harbor MDM2 amplifications (Oliner, Kinzler et al. 1992).

The observations that the MDM2 gene is a transcriptional target of p53 and MDM2 is a negative regulator of p53 transcription, led to the proposed model of the p53-MDM2 auto-regulatory feedback loop (Momand, Zambetti et al. 1992; Barak, Juven et al. 1993; Wu, Bayle et al. 1993) . The hypothesis was that in order for cell cycle to progress, p53 must be inhibited by the activation MDM2. In support of these results, was the observation that the lethality of MDM2 null mice could be rescued by the simultaneous knockout of p53 alleles (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). The ability of the cell to regulate and maintain p53 at low levels is crucial for its survival. MDM2 acts as the major negative regulator of p53 by binding to the protein, masking its transactivation domain and keeping it functionally inactive (Momand, Zambetti et al. 1992). Biochemically, MDM2 functions as an E3 ligase that can ubiquitinate p53 on several lysine residues, in addition to itself (Fang, Jensen et al. 2000; Honda and Yasuda 2000; Nakamura, Roth et al. 2000; Rodriguez, Desterro et al. 2000).

MDM2 can transcriptionally inactivate p53 simply by binding to its transactivation domain and blocking the recruitment of coactivators or transcriptional machinery. MDM2 promotes p53 turnover through the ubiquitin proteasome system (Jackson and Berberich 2000). The extent of ubiquitination can also determine the fate of the protein. Polyubiquitination is believed to promote degradation while mono-ubiquitination of p53 assist in the nuclear export (Yang and Yu 2003). Furthermore, MDM2 can shuttle p53 from the cytoplasm to the nucleus thereby regulating its localization and its function as a transcription factor (Freedman and Levine 1998; Roth, Dobbelstein et al. 1998).

As mentioned previously, MDM2 can promote the degradation and nuclear export of p53, thereby reducing the induction of target genes including p21, Bax and Gadd45 necessary for proper growth arrest and apoptosis. Alternatively, MDM2 has been reported to also have p53 independent functions. MDM2 affects the cell cycle regulation by inhibiting the interaction with other key cell cycle proteins. *Martin et al.* identified that the N-terminus of MDM2 can stimulate the transactivation of reporter genes for E2F1/DP1 (Martin, Trouche et al. 1995). In addition, MDM2 can modulate the activity of Rb by forming a complex and perturbing Rb-mediated G1 arrest (Xiao, Chen et al. 1995).

Cancers with mutant p53, as well as MDM2 amplifications, show a poorer prognosis compared to cancers possessing a single mutation or amplification (Cordon-Cardo, Latres et al. 1994). In the absence of p53, mice overexpressing MDM2 are predisposed to developing spontaneous tumors (Jones, Hancock et al. 1998). Although limited, additional studies suggest that MDM2 also plays roles in cell cycle control, DNA

repair, DNA repair and differentiation, in the absence of p53.

M*DM2 mouse models*

The physiological importance of MDM2 as the major negative regulator of p53 was also revealed in mouse models. Homozygous knockouts of MDM2 resulted in 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 in vivo model illustrated the importance of maintaining p53 level under tight control. In order to elucidate the extent of MDM2 levels in regulating p53 in normal tissues; conditional knockout mice were generated. These mice were engineered using the Cre-Lox recombination mechanism ,by inserting two loxP flanking sites into the coding regions of exons 7-9, and a puromycin resistance cassette in the non-coding region of MDM2 . They crossed these mice to p53 wild-type mice to generate animals with one wild-type allele and one null allele of MDM2 (hypomorphic allele), thus avoiding the embryonic lethality of a complete MDM2 knockout (Mendrysa, O'Leary et al. 2006). Unexpectedly, these mice had dampened levels of the wild-type MDM2 allele due to the introduction of the puro-cassette in the non-coding region of MDM2. These mice had reduced body size, deficiencies in B-cell development and lymphoid lineage cells, due to

increase in p53 dependent apoptosis. In addition, all tissues showed increase in p53 transcription, however this increase did not overlap with nuclear accumulation or phosphorylation on serine 18. As expected, these mice were hypersensitive to ionizing radiation.

An alternative approach to studying the functions of MDM2 regulation of p53 was to generate mice overexpressing MDM2. *Lundgren et al,* generated animals that overexpressed MDM2 in the mammary epithelium. These mice were prone to tumor formation and increased hypertrophy, although at a low penetrance (Lundgren, Montes de Oca Luna et al. 1997). Expression of extra copies of MDM2 by an endogenous promoter, supported the findings by Lundgren et al. They observed that higher levels of MDM2 reduced the anti-tumorsuppressor functions of p53 (Jones, Hancock et al. 1998).

MDMX

MDMX history

In 1996, *Shvarts et al.* set out to identify new p53 interacting proteins. Using a 16-day-old mouse embryo cDNA expression library, they isolated a p53 binding protein that shared homology at the DNA and protein level with MDM2. The protein was named MDMX, due to this structural similarity to MDM2. The human ortholog was identified a year later and termed HDMX (Shvarts, Bazuine et al. 1997). Both the mouse and human ortholog interacted and inhibited p53 transcriptional activation (Shvarts, Steegenga et al. 1996).

MDMX structure

MDMX and MDM2 share significant homology in several functional domains, such as p53 binding domain, Zn finger and the RING domain. While MDM2 is 491 amino acids long, MDMX is 490 amino acids in length. The p53 binding region which compromises amino acids 42-94 share 53.6% homology with MDM2. Due to the conservation of the p53-binding domain, MDMX was found to bind the N-terminus of p53 and inhibit its transactivation (Jackson and Berberich 2000; Stad, Little et al. 2001). The Zinc finger region spans amino acids 301-329, however the function of this domain remains unclear. Recent results indicate that this region is needed for the interaction of CKI-alpha and MDMX (Chen, Li et al. 2005). The RING domain located between amino acids 444-483 shares 53.2% homology with MDM2. This region allows for

heterodimerization of MDM2 and MDMX (Tanimura, Ohtsuka et al. 1999). The RING finger domain of MDM2 is essential for its E3 ligase function, however MDMX does not possess any intrinsic E3 ligase activity (Momand, Zambetti et al. 1992; Jackson, Lindstrom et al. 2001; Migliorini, Danovi et al. 2002; Linares, Hengstermann et al. 2003) (**Figure 5**).

The protein homology of MDM2 and MDMX can be explained by the conservation of exons 4-12 (Parant, Chavez-Reyes et al. 2001). Alternatively, there is little sequence homology in the central region of MDMX compared to MDM2, although both regions are highly acidic. This region of MDM2 is required for the interaction with ARF and L-proteins, however the differences in homology could explain the inability of MDMX to interact with ARF (Jackson, Lindstrom et al. 2001; Wang, Arooz et al. 2001). MDM2 is transcriptionally activated by p53 due to the presence of two p53-responsive elements in its promoter located in exon 1 (Wu and Yan 2003). Conversely, MDMX does not contain a p53 responsive element on its promoter, and is not transcriptional activated by p53. As noted in **Figure 5**, other discrepancies between MDM2 and MDMX include the presence of a NLS and NES in MDMX. However data in this manuscript reveals the presence of a cryptic NLS in MDMX that is only functional after DNA damage induced phosphorylation and 14-3-3 binding.

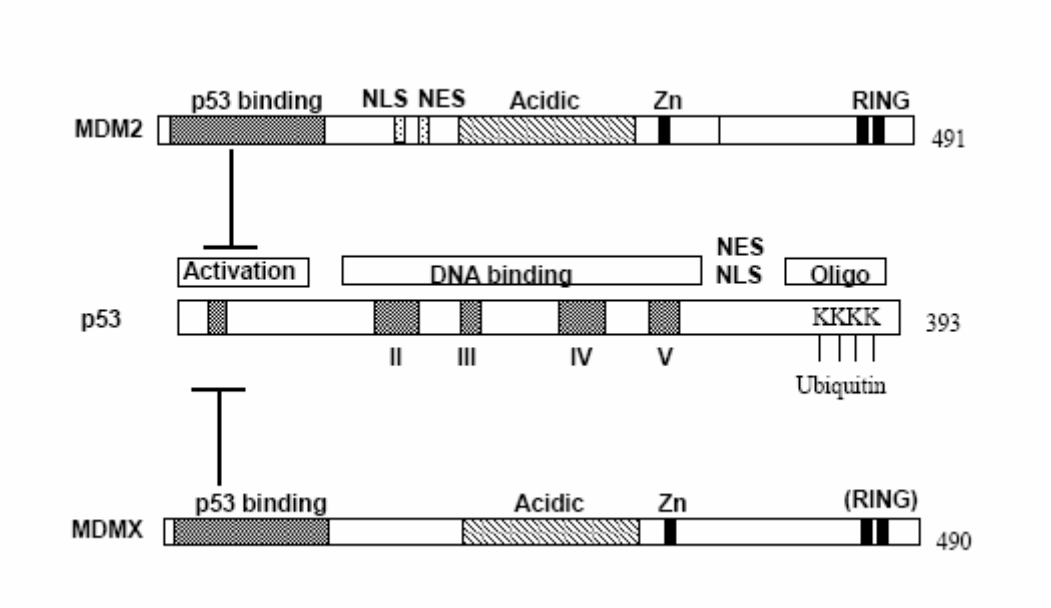


Figure 5: Structure homology of MDMX and MDM2

MDMX mouse models

The similarities in structure between MDMX and MDM2 suggested that MDMX was also a negative regulator of p53. Loss of function mouse models supported this hypothesis. MDMX mutant mice lacking the p53-binding domain were generated using homologous recombination in stem cells. MDMX knockout mice result in embryo lethality at embryonic day 7.5 due to severe proliferation deficiency, in contrast of massive apoptosis as in the case of MDM2 knockout mice (Parant, Chavez-Reyes et al. 2001). The functional relationship between MDM2 and MDMX null mice was demonstrated by the rescue of embryonic lethality by nullifying P53 simultaneously (Parant, Chavez-Reyes et al. 2001; Finch, Donoviel et al. 2002; Migliorini, Danovi et al. 2002). Furthermore the loss of MDMX could not be compensated by MDM2, arguing in

favor for non-redundant roles for each protein in regulating p53.

Regulation of MDMX

Human tumor cell lines with wild-type p53 often overexpress MDMX, suggesting that MDMX may contribute to the p53 inactivation during tumorgenesis (Ramos, Stad et al. 2001). Understanding the regulation of MDMX expression is equally as important in ensuring a proper p53 response. In the presence of DNA damage, there are vital steps needed for the activation of p53. These include inhibition of MDM2 mediated degradation of p53, MDM2 –MDMX binding and activation of key posttranslational modifications. Although MDMX alone does not promote p53 ubiquitination or degradation (Stad, Little et al. 2001), recent evidence showed that MDMX cooperates with MDM2 to promote p53 degradation (Gu, Kawai et al. 2002). Many *in-vivo* and *in-vitro* studies elucidated the functional role of MDMX in the inactivation of p53, however only recently has insight into the mechanism of MDMX regulation been explored. The instability of MDM2 is due to its self-ubiquitination, unlike that of the enhanced protein stability of MDMX, which lacks E3 ligase functions. The differences in stability also appear to be regulated not only at the protein level but also through the interaction with MDM2. The heterodimerization of MDMX-MDM2 is mediated through the C-terminal Zn and RING finger domains (Tanimura, Ohtsuka et al. 1999). The interaction of these two proteins was shown to stabilize the E3 ligase function of MDM2 towards p53, although the other studies indicated that the ratios of the two proteins determine whether

p53 is more efficiently ubiquitinated or stabilized (Sharp, Kratowicz et al. 1999; Gu, Kawai et al. 2002; Linares, Hengstermann et al. 2003). Early studies found that MDMX is constitutively expressed at both the mRNA and protein levels during cell proliferation, differentiation and stress (Jackson and Berberich 1999). Key to elucidating the role of MDMX is understanding its regulation after DNA damage. Unlike MDM2, which is mainly nuclear, but also shuttles from the cytoplasm, MDMX is localized primarily in the cytoplasm, depending on the cell type (Rallapalli et al. 1999; Migliorini et al. 2002). The ability of MDMX to inhibit p53 is regulated by MDM2 mediated nuclear import and MDM2 independent mechanisms, demonstrated by the overexpression of MDM2 promoting MDMX nuclear entry and by p53 null/MDM2 null mouse embryo fibroblast translocation to the nucleus after DNA damage (Rallapalli, Strachan et al. 1999; Migliorini, Danovi et al. 2002). Similar to MDM2 effects on p53, MDMX is also ubiquitinated and degraded by MDM2, however the main difference is that MDMX degradation is stimulated under cellular stress conditions such as DNA damage or by the overexpression of ARF (de Graaf, Little et al. 2003; Kawai, Wiederschain et al. 2003; Pan and Chen 2003). The destabilization of both MDMX and MDM2 are required for a proper p53 response to DNA damage and aberrant mitogenic signaling. Recent evidence from our laboratory and others indicates the importance of MDMX phosphorylation for efficient degradation in response to DNA damage. Evidence indicated that degradation of MDMX requires ATM dependent phosphorylation on serine 342 and serine 367 by Chk2 and serine 403 by ATM, following DNA damage (Chen, Gilkes et al. 2005; Okamoto, Kashima et al. 2005; Pereg, Shkedy et al. 2005). Another report observed that

ultraviolet treatment results in Chk1-mediated phosphorylation of serine 367 (Jin, Dai et al. 2006). Phosphorylation of MDMX enhanced the binding and ubiquitination by MDM2 (Chen, Gilkes et al. 2005). More importantly was the role of Chk2 phosphorylation in MDMX degradation, as demonstrated by HCT116-Chk2 -/- cells having an impaired DNA damage induced phosphorylation and degradation of MDMX after ionizing radiation as compared to HCT116-Chk2 wild-type cells (Chen, Gilkes et al. 2005). To further elucidate this mechanism, the findings in this manuscript provide another mechanism for the enhanced degradation of MDMX. We and others provide evidence that the phosphorylation of MDMX on serine 367 after ionizing radiation creates a 14-3-3 binding site (Okamoto, Kashima et al. 2005; LeBron, Chen et al. 2006). 14-3-3 binding stimulated the nuclear import of MDMX by exposing a cryptic NLS region and subsequent degradation of phosphorylated MDMX. However the exact mechanism on how 14-3-3 promotes MDMX degradation is not clear. Additionally, it was reported that HAUSP can bind and deubiquitinate MDMX, thus counteracting MDM2 mediated degradation (Meulmeester, Pereg et al. 2005). Interestingly, recent reports by *Pereg et al* suggest the displacement of HAUSP from MDMX by 14-3-3, as a possible mechanism for MDMX destabilization after DNA damage (Pereg, Lam et al. 2006).

Mitogenic stimulation can also regulate MDMX protein levels, however phosphorylation does not appear to be involved. Studies indicated that MDMX degradation was induced by ARF overexpression. ARF binding to MDM2 selectively blocked p53 ubiquitination, while enhancing MDMX ubiquitination (Pan and Chen

2003).

P53 response to ribosomal stress is another important factor in tumor suppression. A recent connection between ribosomal stress and p53-dependent cell cycle arrest was suggested due to aberrant rRNA being sensed by p53 (Marechal, Elenbaas et al. 1994; Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003). *Gilkes et al. 2006* determined the effect on MDMX stability after the addition of Actinomycin D (5nM) to induce ribosomal stress. They found that HCT116 p53+/+, HCT116 p53-/- and U2OS cells treated for 8-20 hours with Act.D, resulted in induction of p21 and MDM2. In contrast, MDMX levels decrease, due to an increase in L11 stimulating MDMX ubiquitination by MDM2.

MDMX expression in tumors

Disruption of the p53 pathway has been well established in human cancers. Over 50% of human cancers have mutations to the p53 locus and the other 50% of tumors, which retain wild-type p53, have disruptions in p53 regulating proteins. Several studies implicate MDMX functions as oncogenic and implicate it in tumor formation. HDMX localizes on chromosome 1q32, which happens to be frequently aberrant in cervical and ovarian carcinomas (Danovi, Meulmeester et al. 2004). Human gliomas and more recently 65% of retinoblastomas show amplification of HDMX (Danovi, Meulmeester et al. 2004; Laurie, Donovan et al. 2006). In all cases, HDMX amplifications correlated with wild-type p53 and few HDM2 amplifications. In addition, MDMX overexpression can block oncogenic ras-induced premature senescence, allowing formation of tumors in

nude mice (Danovi, Meulmeester et al. 2004; Laurie, Donovan et al. 2006). MDMX overexpression provides a mechanism in which, the mutation of p53 is not necessary to promote tumorgenesis.

MDMX-MDM2-P53 circuit

There are several lines of published evidence supporting the importance of maintaining the p53-MDM2-p53 interaction, most notably, the mouse models experiments supporting MDMX as another major negative regulator of p53 function in vivo (Parant, Chavez-Reyes et al. 2001; Migliorini, Danovi et al. 2002; Chavez-Reyes, Parant et al. 2003). The role of MDMX in control of p53 and MDM2 stability remains unclear, however the negative effect of MDMX on p53 transcription is becoming clearer. The negative feedback loop between p53 and MDM2 has been well established. 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; Wu, Bayle et al. 1993; Barak, Gottlieb et al. 1994). 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; Ramos, Stad et al. 2001). 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. Aforementioned, MDM2 can induce the transcriptional inactivation, ubiquitination and degradation of p53 (Haupt, Maya et al. 1997; Kubbutat,

Jones et al. 1997; Thut, Goodrich et al. 1997), however MDMX overexpression in cells, does not seem to affect p53 ubiquitination and degradation directly (Jackson and Berberich 2000; Stad, Ramos et al. 2000; Migliorini, Danovi et al. 2002). Instead MDMX seems to work along side MDM2 to maintain the transcriptional activation and stability of p53. This is evident in genetic knockout models for both MDMX and MDM2, in which neither protein can compensate for the other in regulating p53 function. In vivo studies in mice null for MDM2 and MDMX in the central nervous system seem to indicate a more dramatic phenotype then seen with a single mutation (Xiong, Van Pelt et al. 2006). However the situation becomes a little for obscure when conditional inactivation studies were performed. Studies were conducted in cardiomyocytes and GI tract that indicated that the conditional loss of MDM2 showed more apparent phenotypes than that of loss of MDMX alone (Boesten, Zadelaar et al. 2006; Grier, Xiong et al. 2006). The investigators concluded that MDMX is only required for the inhibition of p53, in a subset of cell types and physiological conditions. Another study further complicated the genetic knockout studies. It was found that the overexpression of the MDM2 transgene could compensate for the embryonic lethal phenotype associated with loss of MDMX (Steinman, Hoover et al. 2005), indicating that high levels of MDM2 can compensate for loss of MDMX.

Furthermore, the model for regulation of p53 by both negative inhibitors proposes that in the absence of DNA damage or other stresses, the primary function of MDM2 is to maintain p53 at low levels, whereas the function of MDMX is to contribute to the overall transcriptional inhibition of p53. However in the presence of stress, p53 becomes

posttranslationally modified which inhibits the interaction with MDM2, thus stabilizing the protein. Although MDM2 is a direct transcriptional target of p53, the cell maintains a lag in the induction of MDM2 after DNA damage. Another important factor is the interaction of MDM2 with ARF. All these factors allow for a proper and efficient p53 response. On the other hand, MDMX levels are very stable under non-stressed conditions, but after DNA damage becomes very unstable. This is in part due to phosphorylation, enhanced degradation of MDMX after DNA damage, and it not being transcriptionally induced by p53. Conversely, *Gilkes et al. 2006* suggested that after ribosomal stress, the overexpression of MDMX could maintain both p53 and MDM2 in inactive complexes.

Ubiquitination and deubiquitination process

The attachment of ubiquitin and ubiquitin like proteins has emerged as an important mechanism to regulate diverse cellular and biological processes. These regulatory processes include cell cycle progression, signal transduction, transcriptional regulation and growth control (Hochstrasser 1996; Pickart 2001). Ubiquitin is a protein composed of 76 amino acids and is activated by an ATP dependent mechanism. The conjugation of ubiquitin requires three different enzymes termed: E1, E2, E3. The first enzyme E1 activates the ubiquitin by activation of the C-terminus, forming a covalently bound ubiquitin to which the terminal glycine residue of ubiquitin is linked to the thiol group of the cysteine residue of the E1 active site. Afterwards the ubiquitin is transferred to the carrier, ubiquitin conjugating enzyme or E2. The E2 enzyme then tags the

ubiquitin to a lysine residue on the substrate protein with the aid of the E3 enzyme or ubiquitin protein. E2 enzymes have the ability to interact with multiple E3 enzymes, however the interaction is very specific. Genome analysis reveals a small number of E1 encoding genes, while E2 enzymes have 10-fold higher amount of encoding genes. Interestingly, there are over hundreds of E3 encoding ligases (Semple 2003). There are two sets of E3s classified by their distinct functional domains. The E3 HECT domains contain a highly conserved 350 amino acid region in their carboxyl termini. HECT domain E3s form intermediates between E2 and the substrate proteins. The second functional domain is the RING finger containing proteins, which were thought only to mediate protein dimerization. Until recently, the 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.

The extent of the number of ubiquitin attachments also dictates the fate of the tagged substrate protein. Chains of four or more ubiquitin(s), also referred to as polyubiquitination, promotes efficient binding to the 26S proteasome and degradation (Pickart 2001). Alternatively attachment of short linked ubiquitin chains to a protein can have a variety of consequences. Take for example p53; monoubiquitination can act as a mechanism for nuclear export to limit the potential of this transcription factor to activate target genes. However, the functional significance of protein monoubiquitination is still elusive.

The final apparatus that regulates disposal is the proteasome. The proteasomes is very abundant that constitutes nearly 1% of cellular proteins. In mammalian cells, proteasomes are primarily in the cytosol, however also display associations with the nucleus, endoplasmic reticulum, plasma membrane and cytoskeletal elements depending on cell type (Wojcik and DeMartino 2003). The proteasome consist of a central hollow cylinder formed from multiple subunits (20S). These cylinders associate and stack up four heptameric rings. Each end of the cylinder is capped with large protein complexes termed 19S subunit. The 19S subunit contains over six proteins that hydrolyze ATP, and are thought to unfold proteins to be digested (Baumeister, Walz et al. 1998). Protein degradation is an important mechanism in controlling homeostasis and the functional mechanism of these processes has just started to be appreciated.

The process of ubiquitination is also reversible and carried out by a large subset of deubiquitin enzymes (DUBs) or thiol proteases. The classical deubiquitinating enzymes belong to ubiquitin processing (UBP) and ubiquitin carboxy-terminal hydrolazes (UBH) families. UBP enzymes remove polyubiquitinated proteins whereas UBH remove small adducts of ubiquitins, which creates free monomeric ubiquitins that can be used later for recycling. The process of deubiquitination also has a plethora of functions.

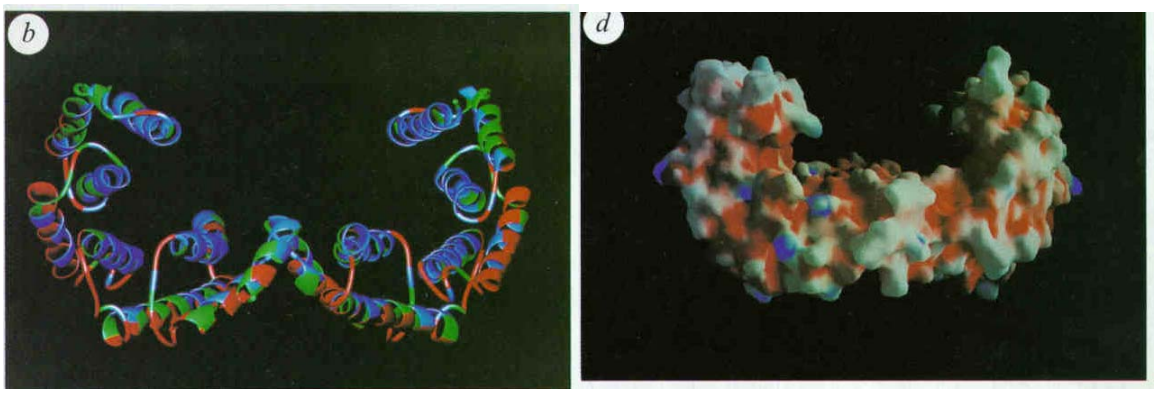
14-3-3

14-3-3 proteins

The name 14-3-3 is derived from the combination of its fraction number on a DEAE-cellulose chromatography and it migration position in the starch-gel electrophoresis (Zdrojewski, Dubois et al. 1967). The family consists of highly conserved group of small, acidic proteins that are nearly ubiquitous in one form or another in all cells of every organism. Multiple genes encode different isoforms of 14-3-3. 14-3-3 proteins were first isolated in brain tissue, however have been isolated and described in all tissues. The 14-3-3 protein family have been found to participate in numerous signaling pathways that regulate cell proliferation and apoptosis, often binding to phosphorylated serine or threonine residues (Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). The family consist of seven genes which each encode a highly conserved isoforms: β, γ, ε, η, σ, θ , ζ̃ 14-3-3 proteins are expressed in all eukaryotic cells and are highly conserved in protein sequence form yeast to mammals, although 14-3-3 sigma is restricted to epithelial cells (Hermeking 2003). All 14-3-3 proteins form dimers that provide two binding sites for phosphorylation motifs in ligand proteins. They can therefore act as adaptor proteins, bringing two proteins that would not otherwise associate into close proximity (Hermeking 2003) **(Figure 6)**.

14-3-3 Structure:

14-3-3 proteins exist as monomers that can form homo and heterodimers. Each monomer consists of nine alpha helices organized in an anti-parallel fashion, resembling a U-shape structure. Each monomer contains a highly conserved amphipathic groove. The interior of the groove consist of four alpha helices: Helix 3and Helix 5 contain charged and polar amino acids, while Helix 7 and Helix 9 are composed of hydrophobic amino acids. In particular, amino acids K49, R59 and R127 of the amphipathic groove are postulated to mediate interactions with phosphoamino acids in the ligand. This interaction is mediated through the formation of salt bridges between the ligand and the 14-3-3 groove (Yaffe, Rittinger et al. 1997; Petosa, Masters et al. 1998). As previously mentioned, 14-3-3 proteins can form dimers. The dimerization occurs through the amino terminal of helix 1 of one monomer and the helix 3 and 4 of the other monomer (Aitken, Jones et al. 1995; Jones, Ley et al. 1995; Jones, Martin et al. 1995). The dimeric structure of 14-3-3 allows for the simultaneous binding of two ligands. The extreme N- and C termini of 14-3-3 proteins are highly variable and are missing in the crystal structure, indicating the regions are disordered.



(Wilker and Yaffe 2004)

Figure 6: Secondary and tertiary structure of 14-3-3. Hydrophobic residues in green, acidic residues in red and basic residues in blue.

It has been observed that some 14-3-3 isoforms preferentially form homodimers, such as 14-3-3 sigma due to the makeup of their structure (Jones, Ley et al. 1995; Chaudhri, Scarabel et al. 2003; Wilker and Yaffe 2004). In the case of 14-3-3 sigma, homodimers form due to seven unique hydrophobic amino acids in helix1 and helix 7. In nature, many 14-3-3 isoforms are found as heterodimers, which may suggest a mechanism for specific protein ligand interactions and the promotion of ligand interactions.

Figure 7: Primary 14-3-3 structure.

Modes of regulation by 14-3-3 proteins

14-3-3 are abundant and stable proteins that play key roles in regulating homeostasis within the all eukaryotic organisms (Aitken, Collinge et al. 1992; Aitken, Jones et al. 1995; Wang, Grammatikakis et al. 2003).Modes of regulation include alteration of DNA binding activity. For example, 14-3-3 can bind p53 at the C-terminus and increase its DNA binding affinity (Waterman, Stavridi et al. 1998). 14-3-3 protein binding have also been implicated in masking nuclear localization and export sequences, inhibiting and promoting protein interactions as well as altering enzymatic activities of proteins. 14-3-3 facilitates the interactions of RAF1 proteins, which are bridged together through 14-3-3 dimers, on phosphorylation of 14-3-3 binding motifs (Tzivion, Luo et al. 1998). Although highly homologous in sequence and structure, different 14-3-3 isoforms have distinct biological functions, as demonstrated by the phenotypes of 14-3-3 epsilon and zeta disruption in Drosophila (Su, Parry et al. 2001). There is also extensive evidence that 14-3-3 sigma play significant roles in regulating cell differentiation and cancer development (Hermeking 2003).

Diseases associated with 14-3-3 proteins:

There are many reports that implicate the deregulation of 14-3-3 protein expressions as indicators of many human diseases. Reports have indicated elevated levels of 14-3-3 proteins in cerebrospinal fluid of patients with various neurodegenerative disorders. Several 14-3-3 isoforms are expressed at unregulated levels in Alzheimer's disease and recent evidence suggest 14-3-3 plays a causative role in the development of

this disease. 14-3-3 has been shown to retain the Tau protein in a hyperphosphorylated form. Hyperphosphorylated Tau generates neurofibral tangles associated in patients with Alzheimer's disease. Alterations of expression of many 14-3-3 isotypes have been associated with several human cancers. For example, 14-3-3 sigma is detected in normal breast epithelial, however is undetectable in breast cancer tissue (Ferguson, Evron et al. 2000). In humans, deletion of 14-3-3 epsilon indicates a more severe form of lissencephaly grade seen in patients with Miller-Dicker syndrome (MDS) (Dalal, Yaffe et al. 2004; Nguyen, Rothman et al. 2004). Until recently, only indirect evidence has accumulated which directly implicates 14-3-3 proteins as the cause of disease. Some labs have recently shown that the overexpression of 14-3-3 zeta promotes tumor survival, and the abrogation of this overexpression results in efficient apoptosis after DNA damage (Tzivion, Gupta et al. 2006). 14-3-3 proteins ability to interact and promote so many different responses could be a clear indicator of why its deregulation could cause so many problems for the cell. It could also be a reason why cancers target such proteins for modification, due to the benefits associated with protection from apoptosis and increase proliferation.

14-3-3 Functions:

The precise function of 14-3-3 proteins in cell biology has been very difficult to describe, partially due to 14-3-3 proteins ability to interact with so many different proteins. However, there are proposed models for the function of 14-3-3 including:

1. Modulating enzymatic activity

2. Alter localization

3. Regulate protein stability

4. Inhibit and/or promote protein interactions

1. Modulating Enzymatic Activity: The activation of tyrosine and tryptophan hydroxylases was the first reported function of 14-3-3 proteins. 14-3-3 can modulate the enzymatic activity of the kinase; however the exact mechanism of this kinase dependent activation is unknown. The enzymatic activity of AANAT (two-serotonin-N-acetyl transferase) and its affinity for substrates has been shown to increase after 14-3-3 zeta binding. This interaction has been shown to be dependent on phosphorylation of AANAT by PKA (protein kinase A) (Obsil, Ghirlando et al. 2001).

2. Altering Localization: 14-3-3 function to modulate protein localization through sequestering the protein in cellular compartments or simply masking a proteins nuclear export/import sequences. Binding of 14-3-3 proteins in most cases sequesters the protein and release of the protein allows for the relocation. For example, 14-3-3 can bind to the human telomerase protein (Tert1) and promote the nuclear import of the protein to the nucleus, thus promoting expression of telomerase (Seimiya, Sawada et al. 2000; Wang, Liu et al. 2004). 14-3-3 proteins have also been shown to bind the kinase Chk1 a kinase important for the induction of the DNA damage response (Jiang, Pereira et al. 2003). In addition, 14-3-3 can also affect the cytoplasmic-mitochondrial shuttling of Bad and Bax

(Wang, Pathan et al. 1999; Samuel, Weber et al. 2001; Nomura, Shimizu et al. 2003).

3. Regulate protein stability: It is reasonable to conceive why 14-3-3 could promote protein stability. 14-3-3 binding could affect the interaction of a protein and its corresponding E3 ligase. For example, recently reported was the induced stabilization of E2F1 by 14-3-3 tau binding. Binding prevented the ubiquitination of E2F1 thereby promoting its stability. The vital mechanism was the blocking degradation mediated by the p45 protein an E3 ligase. This report also provided evidence of the specificity of 14-3-3 isoforms. RNA interference against 14-3-3 tau abrogated the protein stability effect conferred by 14-3-3 binding. This report also indicated that other isoforms could not perform a redundant function as the tau isoform (Wang, Das et al. 2004; Milton, Khaire et al. 2006). Recently our lab has shown that 14-3-3 binding to Mdmx can promote the degradation of this protein by through phosphorylation of MDMX and unmasking of a cryptic NLS region. Mutating the 14-3-3 binding site of this protein confers an increase in protein stability as well as abrogating the phosphorylation of this protein (Chen, Gilkes et al. 2005; Okamoto, Kashima et al. 2005; LeBron, Chen et al. 2006)

4. Inhibit / promote protein interactions: The activation of AKT protein kinase by survival signals leads to the phosphorylation of Bad on serine 136, which in turn promotes 14-3-3 binding (Zha, Harada et al. 1996; Datta, Katsov et al. 2000). Upon 14-3-3 binding to Bad, allows the release of the BCL2 family proteins, which can then activate anti-apoptotic responses in the cell (Brunet, Bonni et al. 1999; Brunet, Kanai et

al. 2002). 14-3-3 can also reduce the ability of CBL an E3 ligase to degrade IRS1, thereby blocking the activation of CDC2 (Craparo, Freund et al. 1997; Liu, Stanton et al. 1997; Forrest and Gabrielli 2001). Alternatively, 14-3-3 binding could promote protein interactions. The oncogene kinase BCR-ABL has also been shown to interact with 14-3-3. This interaction can aid in the transforming capabilities of this kinase and promote aberrant interactions with other signaling pathways. 14-3-3 proteins also play a role in the DNA damage response. 14-3-3 sigma has been shown to bind p53 and is also a direct p53 target gene (Hermeking 2003). In the presence of IR, p53 dephosphorylation of serine 376 occurs and promotes 14-3-3 binding. Binding between p53 and 14-3-3 is a mechanism for increased affinity of p53 for sequence-specific DNA (Waterman, Stavridi et al. 1998). It is also suggested that 14-3-3 is important for the G2/M checkpoint maintenance. Targeted deletion of 14-3-3 by homologous recombination in HCT116 cells fails to maintain the G2/M checkpoint following exposure to Adriamycin (Chang, Chen et al. 1999).

14-3-3 regulation of p53:

There are several lines of evidence that 14-3-3 proteins also play a role in regulating the p53 pathway during DNA damage response. 14-3-3 proteins are scaffold proteins that regulate many cellular functions by interacting with other proteins, often binding to phosphorylated serine or threonine residues (Fu, Subramanian et al. 2000; Tzivion, Shen et al. 2001). There are seven 14-3-3 isoforms in humans (beta, gamma,

epsilon, eta, sigma, tau, and zeta). Most of the isoforms are expressed in all tissues, although 14-3-3 sigma is restricted to epithelial cells (Hermeking 2003). Although highly homologous in sequence and structure, different 14-3-3 isoforms have distinct biological functions, as demonstrated by the phenotypes of 14-3-3 epsilon and zeta disruption in Drosophila (Su, Parry et al. 2001). There is also extensive evidence that 14-3-3 sigma play significant roles in regulating cell differentiation and cancer development (Hermeking 2003).

Involvement of 14-3-3 in the p53 pathway was first revealed by the ability of p53 to transcriptionally activate expression of 14-3-3 sigma, which can lead to G2/M cell cycle arrest (Hermeking, Lengauer et al. 1997). Furthermore, 14-3-3 sigma, gamma, epsilon, and tau isoforms can bind to p53 C terminus and activate p53 transcriptional activity (Stavridi, Chehab et al. 2001; Yang, Wen et al. 2003). P53-14-3-3 interaction requires phosphorylation of S378, and is stimulated by dephosphorylation of the adjacent S376 after DNA damage through an ATM-dependent mechanism (Waterman, Stavridi et al. 1998). Mutation of S376 compromises the ability of p53 to induce p21 expression and G1 arrest after ionizing irradiation (Stavridi, Chehab et al. 2001). 14-3-3 sigma interaction with p53 also inhibits p53 ubiquitination and degradation by MDM2 (Yang, Wen et al. 2003), possibly contributing to stabilization of p53 after DNA damage.

MATERIALS AND METHODS

Cell lines and plasmids. H1299, U2OS, MCF-7, and 293T cells were maintained in DMEM medium with 10% fetal bovine serum. HCT116 and HCT116-Chk2-/- cells were kindly provided by Dr. Bert Vogelstein and maintained in McCoy 5A medium with 10% fetal bovine serum. Human MDMX cDNA was kindly provided by Dr. Donna George (Sharp, Kratowicz et al. 1999). FLAG tagged Chk2 wild type and A347 mutant were provided by Dr. Thanos Halazonetis (Chehab, Malikzay et al. 2000). Expression plasmid for FLAG-14-3-3 tau was generated by PCR amplification of 14-3-3 tau cDNA from a Hela cDNA library and subcloning into pcDNA3. Expression plasmids for 14-3-3 beta, epsilon and eta were provided by Dr. Haian Fu. 14-3-3 gamma was provided by Dr. Hua Lu, 14-3-3 sigma was provided by Dr. Mong-Hong Lee, and 14-3-3 zeta was provided by Dr. Anthony J. Muslin. GST-importin α constructs were provided by Dr. Nancy Reich. For affinity purification and mass spectrometric analysis, a FLAG epitope tag was added to the C terminus of myc-MDMX by PCR to create double-tagged myc-MDMX-Flag. All MDM2 and MDMX constructs used in this study were based on human cDNA clones.

Affinity purification of MDMX-associated protein. Hela-S cells stably transfected with FLAG-tagged MDMX were grown as a suspension culture. Cells from 500 ml culture (~$2x10^8$ cells) were lysed in 10 ml lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA,

150 mM NaCl, 0.5% NP40, 1 mM PMSF], centrifuged for 5 minutes at 10,000 g, and the insoluble debris were discarded. The lysate was precleared with 100 µl bed volume of protein A Sepharose beads for 30 minutes, and then incubated with 50 µl bed volume of M2-agarose bead (Sigma) for 4 hours at 4˚C. The beads were washed extensively with lysis buffer and MDMX and its associated proteins were eluted with 70 µl of 20 mM Tris pH8.0, 2% SDS, 200 µg/ml FLAG epitope peptide (Sigma) for 15 minutes. The eluted proteins were fractionated on SDS-PAGE and stained with Coomassie Blue. Proteins co-purified with MDMX were excised from the gel and subjected to protease digestion and peptide sequencing by mass spectrometry at the Harvard Microchemistry Laboratory.

Site directed mutagenesis: The point mutation of MDMX nuclear localization region on lysine 468 to glutamic acid was performed using Quickchange kit (Stratagene) according to the manufacturers instructions.

Forward primer: 5' CACTGTGCCAGAAGACTA**GAG**AAGGCTGGGGCTTCATGC 3'

RNA interference: Purified and annealed duplex siRNA oligonucleotides targeting the 14-3-3 sequence: AACCGGTACCTTGCTGAAGTT were generated commercially by Dharmacon (Lafayette,Co.). The sense sequence was: 5' CCGGTACCTTGCTGAAGTT 3'. Transfection of 100-300nM RNA was performed using Oligofectamine (Invitrogen) according to manufacturers instructions for 24-48hrs. After 48hrs of transfection, cells were treated with 10Gy ionizing radiation for various time points and analyzed.

FACS analysis: U20S-stable pools were treated in the absence or presence of 10Gy IR and 100ng nocodazole for 18 hours. The cells were then harvested by trypsinization, fixed in 70% ethanol and stained with propidium iodide (50mg/ml) containing 50mg/ml RNase A. The cells were then analyzed for cell cycle distribution.

Protein analysis. To detect proteins by 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, 200 nM Okadaic acid], 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 using a gradient gel and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 hr with phosphate-buffered saline (PBS) containing 5% non-fat dry milk, 0.1 % Tween-20. The following monoclonal antibodies were used: 3G9 for MDM2 (Chen, Marechal et al. 1993); DO-1 (Pharmingen) for p53 western blot; 8C6 monoclonal or a rabbit polyclonal serum for MDMX western blot and IP (Li, Chen et al. 2002), PS357 antibody for phosphorylated MDMX (Chen, Gilkes et al. 2005). The filter was developed using ECL-plus reagent (Amersham).

Immunoprecipitation assay. 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 minutes at 10,000 g, and the insoluble debris were discarded. Cell lysate (500-1000 µg protein) was immunoprecipitated using 100 µl 8C6 hybridoma supernatant against MDMX and protein G agarose beads for 4 hours at 4˚C. The beads were washed extensively with lysis

buffer, boiled in SDS sample buffer, fractionated by SDS-PAGE, and analyzed by anti-14-3-3τ western blot using an isoform-specific rabbit polyclonal antibodies (Santa Cruz Biotechnology).

Capture of 14-3-3 using MDMX phosphopeptides Thirty micrograms of MDMX peptides S342 (HSLSTSDIT), PS342 [HSL(pS)TSDIT], S367 (RTISAPVVR) and PS367 [RTI(pS)APVVR] were crosslinked to 30 µl of CarboxyLink beads (Pierce) according to instructions from the manufacturer. The beads (15 µl) were incubated with 200 µg lysate of MCF7 cells stably transfected with FLAG-14-3-3τ (expressed at a level similar to endogenous 14-3-3τ) for 3 hours at 4˚C, washed with lysis buffer, and analyzed by anti-FLAG western blot.

In vivo **ubiquitination assay**. HCT116-Chk2-/- cells in 6 cm plates were transfected with combinations of 0.5 µg His6-ubiquitin expression plasmid, 1 µg MDMX, 0.5 µg MDM2 and 1 µg Chk2 expression plasmids using Lipofectamine Plus reagents (Life Technologies). For detection of 14-3-3 and Chk2 cooperation, 0.5 µg His6-ubiquitin, 1 µg MDMX, 0.1 µg Chk2, and 0.05 µg 14-3-3τ plasmids were cotransfected. Twenty-four hours after transfection, cells were lysed in buffer A (6 M guanidinium-HCl, 0.1 M Na2HPO4/NaH2PO4, 0.01 M Tris-HCl 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/NaH2PO4, 0.01 M Tris-Cl pH8.0, 10 mM β-mecaptoethanol), C (8 M urea, 0.1 M Na2PO4/NaH2PO4, 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 using 8C6 antibody.

Luciferase assay. HCT116-Chk2-/- cells were plated in 24-well plates (50,000/well) for 24 hours and transfected with a mixture containing 20 ng p53-responsive BP100-luciferase, 40 ng MDMX, 5 ng CMV-lacZ, 2 ng 14-3-3τ, and 5 ng Chk2 plasmids. Transfection was achieved using Lipofectamine PLUS reagents (Invitrogen) and cells were analyzed for luciferase and beta galactosidase expression after 24 hours. The ratio of luciferase/beta galactosidase activity was used as indicator of p53 transcriptional activity.

Immunofluorescence staining. Cells cultured on chamber slides were transfected with indicated combinations of MDMX and 14-3-3τ expression plasmids using Lipofectamine PLUS reagents. Twenty-four hours after transfection, cells were fixed with acetone-methanol (1:1) for 3 minutes at room temperature, blocked with PBS+10% normal goat serum (NGS) for 20 minutes, and incubated with a mixture of anti-MDMX 8C6 hybridoma supernatant and rabbit-anti-PS367 antibody in PBS+10% NGS for 2 hrs. The slides were washed with PBS+0.1% Triton X-100, incubated with rhodamine-goat-anti-rabbit IgG and

FITC-goat-anti-mouse IgG in PBS+10% NGS for 1 hr, washed with PBS+0.1% TritonX-100 and mounted.

Nuclear cytoplasmic fractionation Approximately $1x10^7$ cells were pelleted and suspended in 500 µl of 1X Hypotonic buffer (40 mM Hepes pH7.9, 2 mM EDTA, 2 mM EGTA, 20 mM NaF, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4) containing protease inhibitor cocktail (Sigma). After incubation on ice for 2 min, cells were homogenized with ten strokes in a Dounce homogenizer. Samples were centrifuged at 4˚C at 1,000 g for 5 min. Supernantant was collected (Cytosolic fraction) and the pellet was washed with Nuclear Wash Buffer (50 mM NaCl, 10 mM Hepes pH 8.0, 25% glycerol, 0.1 mM EDTA, 1 mM NaF, 20 mM Na_3VO_4) and centrifuged briefly at 4˚C. The pellet was then lysed on a rotating wheel at 4˚C for 20 min in 100 µl of High Salt Buffer (840 mM KCl, 40 mM Hepes, 2 mM EDTA, 2 mM EGTA, 40% glycerol, 1 mM DTT) containing protease inhibitors and phosphatase inhibitors. After centrifugation at 10,000 g for 20 min, supernatant was collected (Nuclear fraction). Protein concentrations in the cytoplasmic and nuclear fractions were determined, and identical amount of protein were subjected to western blot analyses.

***In vivo* phospholabeling and phosphopeptide analysis** To detect MDMX phosphorylation in vivo, 293T cells in 10 cm plates were transiently transfected with 10 µg MDMX expression plasmids using the calcium phosphate precipitation method. Forty

hours after transfection, cells were washed with DMEM without phosphate and incubated with ^{32}P-orthophosphate (0.2 mCi/ml) in DMEM without phosphate for 4 hrs. Cell lysate was immunoprecipitated with 8C6 and analyzed by SDS-PAGE and autoradiography. Nylon membrane containing radio-labeled MDMX bands were excised and incubated with 50 ng endoproteinase Asp-N (Sigma) for 16 hrs in 50 mM ammonium bicarbonate at 37 °C. MDMX peptides were oxidized with performic acid and resolved by electrophoresis on a thin layer cellulose plate for 30 min at 1.0 kV in formic acid/glacial acetic acid/water (1:3.1:35.9; pH 1.9) using the HTLE-7002 apparatus. This was followed by chromatography in the second dimension in n-butyl alcohol/pyridine/glacial acetic acid/water (5:3.3:1:4) for 16 hrs. The phosphopeptides were visualized by autoradiography.

RESULTS

MDMX interacts with 14-3-3

In order to determine whether MDMX interacts with novel cellular proteins, Hela cells were stably transfected with FLAG-tagged MDMX. When FLAG-MDMX was purified by immunoprecipitation, two proteins reproducibly copurified with MDMX (**Figure 8)**. Peptide sequencing by mass spectrometry determined the 34 kd protein as casine kinase 1 alpha, and the 24 kd protein as 14-3-3τ (tau) isoform. In a separate report, we showed that CK1α interaction with the central region of MDMX promotes phosphorylation of S289 and cooperates with MDMX to inactivate p53 (Chen, Li et al. 2005). MDMX binding to 14-3-3 appeared to be independent of CK1αand is the focus of this study.

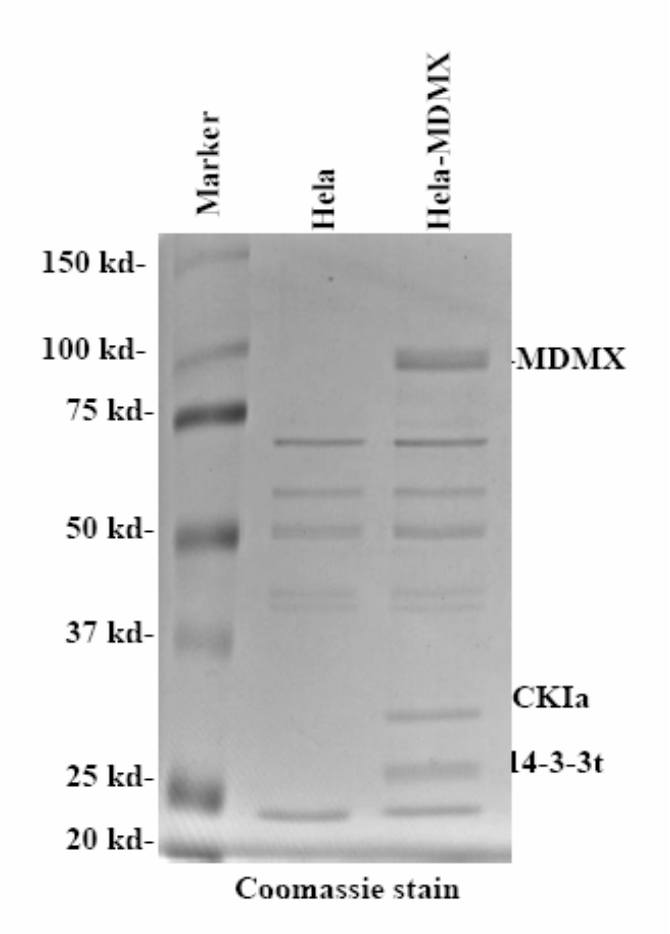


Figure 8: Copurification of 14-3-3 with MDMX: Hela cells stably transfected with FLAG-MDMX were immunoprecipitated using anti-FLAG-M2 antibody, the purified MDMX and associated proteins were detected by Coomassie stain. Two marked bands were identified as CKIα and 14-3-3 tau my mass spectrometric peptide sequencing.

To confirm that endogenous MDMX interacts with 14-3-3, MCF7 cells were immunoprecipitated with MDMX monoclonal antibody and probed for 14-3-3 using a 14-3-3τ specific antibody. Endogenous MDMX interaction with 14-3-3 was nearly below our detection limit in the absence of stress. However, DNA-damaging treatments with camptothecin or ionizing irradiation significantly stimulated MDMX-14-3-3τ binding (**Figure 9**). Furthermore, treatment with the proteasome inhibitor MG132 also increased MDMX-14-3-3 binding, suggesting that a population of unstable MDMX may preferentially bind to 14-3-3. DNA damage stimulation of MDMX-14-3-3 binding has also been reported in a recent publication (Okamoto, Kashima et al. 2005).

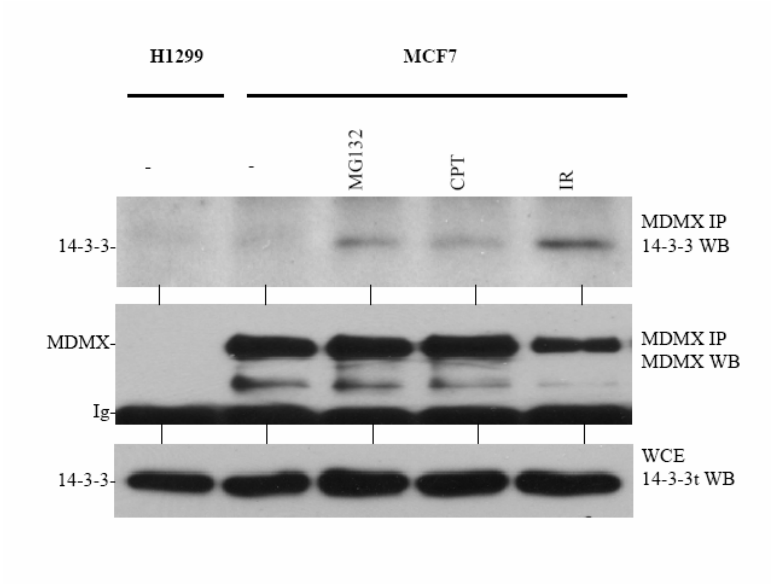


Figure 9: Endogenous interaction between MDMX and 14-3-3 tau: MCF7 cells were treated with 30uM MG132 for 4 hrs, 0.5uM Camptothecin for 16 hours, or irradiated with 10Gy gamma irradiation for 4 hours. Cell lysates were immunoprecipitated with MDMX antibody (8C6) followed by anti-14-3-3 tau western blot.

One of the dominant 14-3-3 isoform expressed in Hela is 14-3-3τ (Nomura, Shimizu et al. 2003). This may be the reason that 14-3-3τ was found in the MDMX complex from this cell line. To determine whether MDMX also interacts with other 14-3-3 isoforms, MDMX was cotransfected with the entire 14-3-3 panel (tau, sigma, beta, gamma, zeta, epsilon, eta). The epitope tagged 14-3-3 was immunoprecipitated and the coprecipitated MDMX was detected by western blot. The results showed absence of binding to 14-3-3 sigma, weak binding to gamma, and strong binding to tau, beta, zeta, epsilon, and eta (**Figure 10).** This result suggested that MDMX interacts with multiple 14-3-3 isoforms.

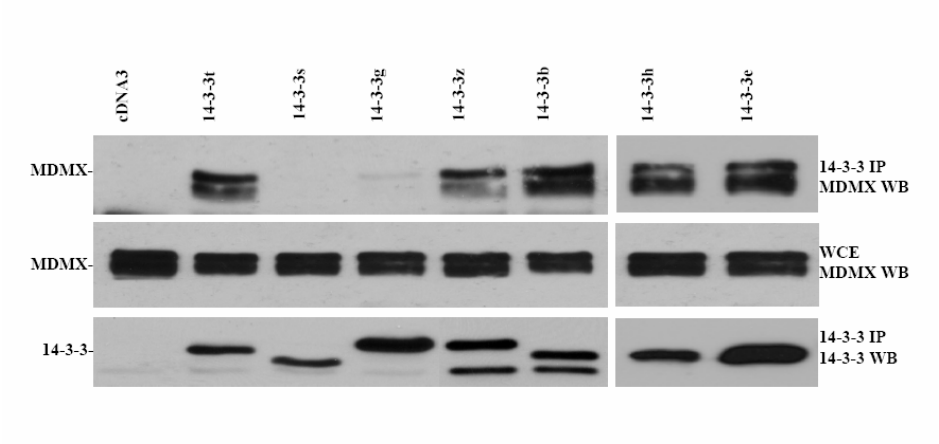


Figure 10: MDMX interacts with other 14-3-3 isoforms: H1299 cells transiently cotransfected with MDMX and epitope-tagged 14-3-3 plasmids were immunoprecipitated using anti-FLAG (for τ, σ, γ), and anti-myc (for ζ, β) and anti-His6 (for ε, η) antibodies. Coprecipitations of MDMX with 14-3-3 isoforms were detected by western blot.

To_confirm the specificity of 14-3-3 isoforms binding to p53, we cotransfected p53 with the entire 14-3-3 panel (tau, sigma, beta, gamma, zeta, epsilon, eta). The epitope tagged 14-3-3 was immunoprecipitated and the coprecipitated p53 was detected by western blot. The results showed that all 14-3-3 isoforms bound to p53 with similar affinities (**Figure 11**).

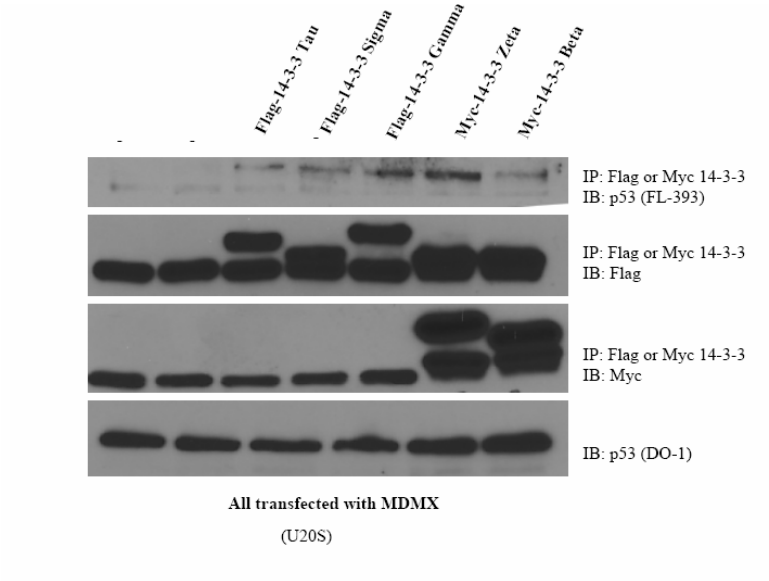
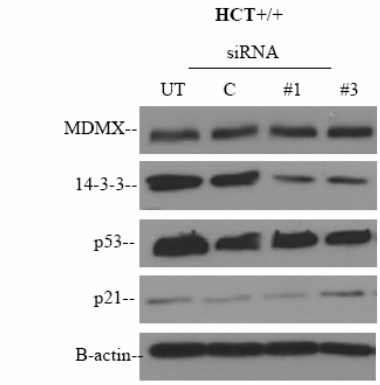


Figure 11: p53 interacts with all 14-3-3 isoforms. U20S cells transiently cotransfected with MDMX and epitope-tagged 14-3-3 plasmids were immunoprecipitated using anti-FLAG (for τ, σ, γ), and anti-myc (for ζ, β) antibodies. Coprecipitation of p53 (DO-1 antibody) with 14-3-3 isoforms were detected by western blot.

To determine the functional consequence of MDMX-14-3-3 interaction, HCT (p53+/+), were transfected 14-3-3 siRNA oligonucleotides and scramble siRNA as a negative control. Although knockdown of 14-3-3 tau was significant, there was no change in p53 levels and a small increase in p21 for siRNA construct #3 (**Figure 12a**). Since the interaction between 14-3-3 and MDMX increased after DNA damage, we next tested whether the knockdown of 14-3-3 would have any effect on MDMX stability and p53 induction after DNA damage. HCT p53 +/+ cells were transiently knockdown with 14-3-3 siRNA for 48hrs followed by 10Gy of ionizing radiation for various time points. Unexpectedly, knockdown of 14-3-3 had no effect on MDMX degradation after DNA damage, as well as induction of p21 (**Figure 12b**)

(a)



(b)

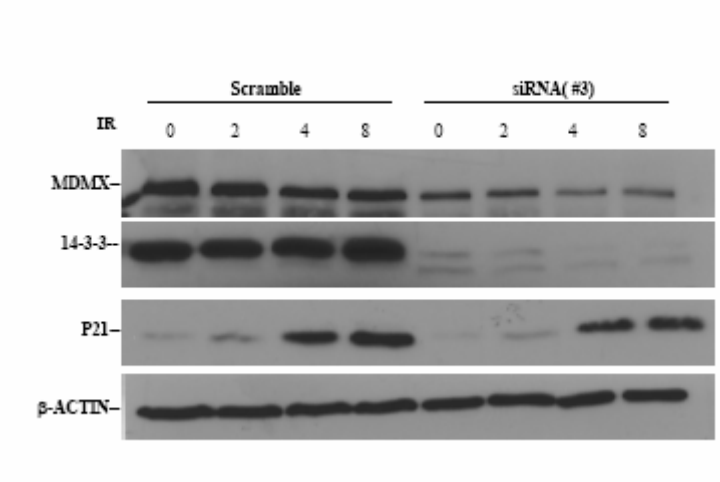


Figure: 12: Knockdown of 14-3-3 has no effect on MDMX stability: (a) HCT p53+/+ were transiently transfected with 14-3-3 siRNA oligonucleotides (100ng(#1) and 300ng (#3). Control siRNA (C) was used as a non-specific control. Using oligofectamine, cells were transfected for 48 hrs. Cell lysate was collected and western blots for endogenous 14-3-3 tau, p53, p21 and b-actin levels were compared. **(b**) HCT p53 +/+ cells were transfected in a 10cm dish with 300ng of 14-3-3 siRNA construct #3 for 48 hours. Each plate was split into 4 6-cm dishes and treated and treated for various time points after DNA damage.

We reasoned that knockdown of only one isoform of 14-3-3 would not show any obvious effects on MDMX levels, since many other isoforms also interacted. However, it has been shown that knockdown of 14-3-3 gamma after treatment with UV, induced p53 levels and G1 arrest (Jin el al. 2006). Since ablation of 14-3-3 tau had no significant effect on p53 levels, we next tested the possibility that overexpression of 14-3-3 may have some detectable effect. Again, in order to determine the role of 14-3-3 binding to MDMX, we generated 14-3-3 overexpression stable clones in MCF7 cell lines. The pCDNA pool was used as a negative control, while clones 1, 2, 3 were compared to pCDNA3 due to their varying expression levels. Selected clone 1 had no expression of FLAG-14-3-3, clone 2 has a lower expression, while clone 3 had the highest expression. To examine whether different levels of 14-3-3 overexpression had an effect on MDMX stability after DNA damage, we treated each pool with 10Gy gamma irradiation at various time points. We expected that overexpression of 14-3-3 could perhaps more efficiently promote the degradation of MDMX, since binding correlated with a decrease in MDMX binding after genotoxic stresses. However, overexpression alone or with IR had no effect on MDMX levels as well the induction of p53 target gene p21 (**Figure 13**).

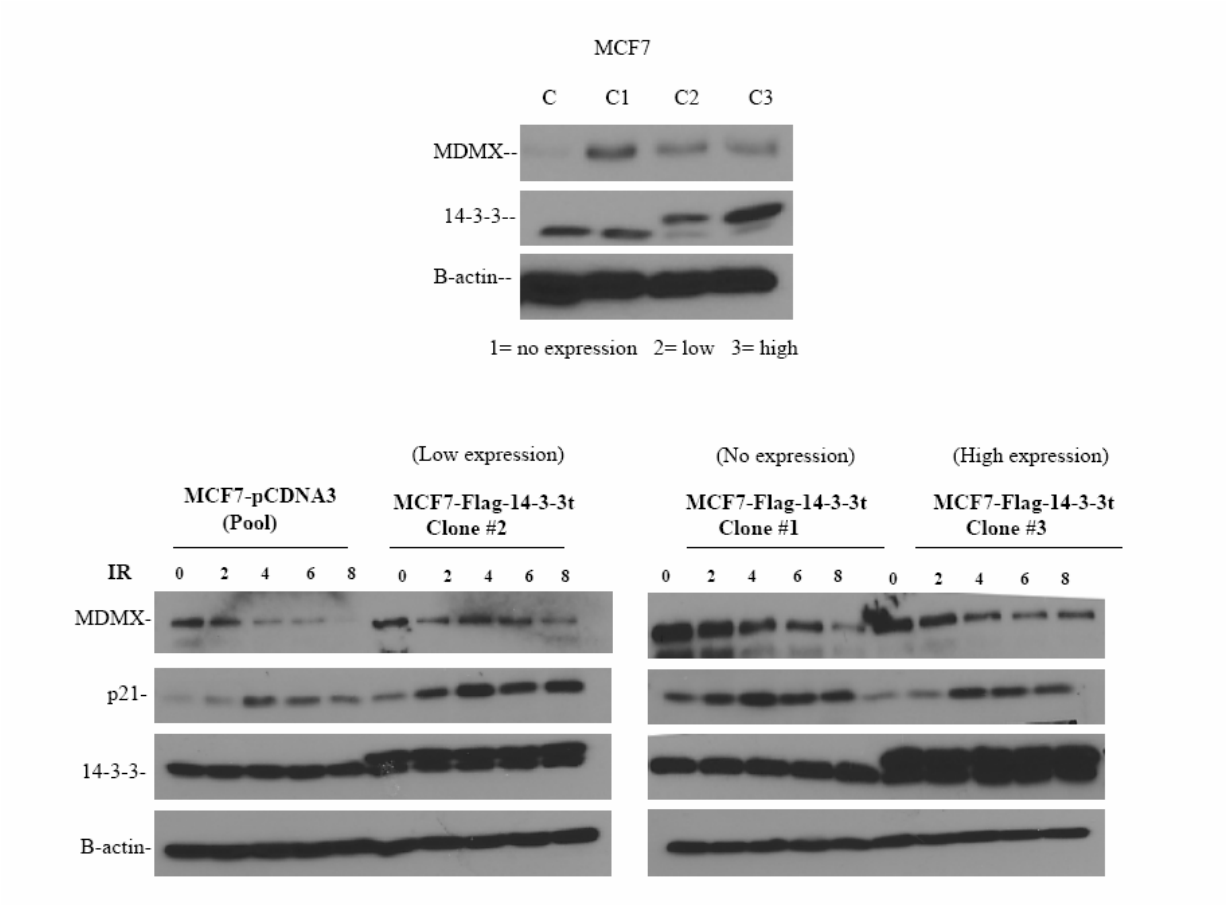


Figure 13 : Overexpression of 14-3-3 has no effect on p53 target gene induction. (a) MCF7 cells were stable transfected with FLAG-14-3-3 using 750ug/ml of G418. Single clones were selected that expressed high, low and no detectable levels of FLAG-14-3-3. (b) Cells were treated with 10Gy IR for 0,2,4,6,8 hrs and collected for analysis. Cell lysate was were used to western blot for MDMX (8C6), p21, and 14-3-3. B-actin was used as a loading control.

Identification of 14-3-3 binding site on MDMX

A major function of 14-3-3 is to bind to proteins with phosphorylated serine and threonine residues. The increased MDMX binding to 14-3-3 after DNA damaging treatment suggested that MDMX phosphorylation is involved in regulating 14-3-3 binding. We recently found that DNA damage induces the appearance of MDMX with reduced mobility on SDS-PAGE. Inhibition of proteasome using MG132 can further preserve this labile population. Mass spectrometric analysis identified several phosphorylation sites on MDMX after DNA damage by gamma irradiation, including S342, S367, S391, and S403 (Chen, Gilkes et al. 2005) (**Figure 14**). A recent report by Pereg et al also identified phosphorylation on S342, S367 and S403, and showed that S403 is a target for ATM after DNA damage (Pereg, Shkedy et al. 2005).

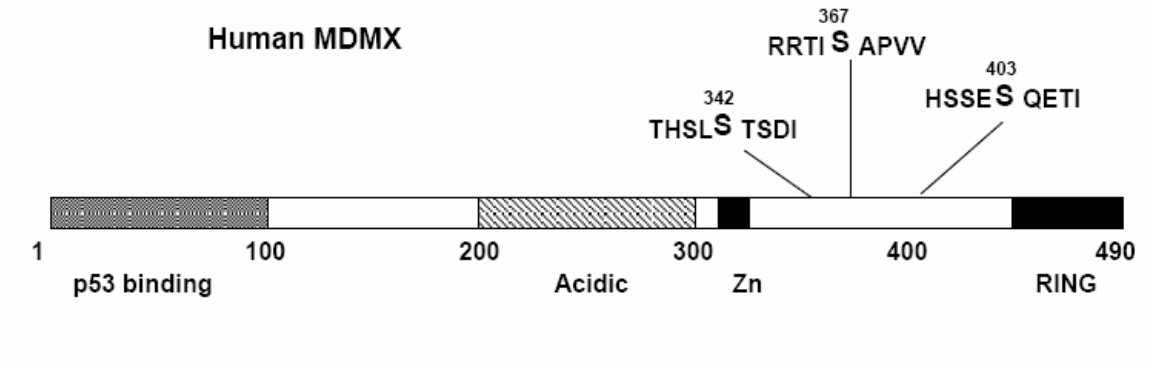


Figure 14: Identification of phosphorylation sites on MDMX by mass spectrometry
Location of phosphorylation sites on MDMX. Phosphorylation of S342, S367 and S403 were detected by mass spectrometry and verified by phosphorylation-specific antibody

analyses in previous studies.

Of particular interest, S367 is present in a sequence context favorable for interaction with 14-3-3 after phosphorylation (xSxPx) (Yaffe, Rittinger et al. 1997). We tested this possibility by generating S367A and P369S mutations to target the phosphorylation site and the adjacent proline residue important for providing the optimal sequence context. Mutants of adjacent phosphorylated sites S342A and S403A were also tested. The MDMX mutants were co-expressed with FLAG-14-3-3τ in H1299 cells and analyzed by anti-FLAG IP followed by MDMX western blot. The results showed that MDMX binding to 14-3-3 was abrogated by the S367A mutation and significantly reduced by P369S mutation (**Figure 15**). Furthermore, S342A and S403A mutations had no effect on MDMX-14-3-3 binding (data not shown). These results suggested that S367 is the major binding site for 14-3-3.

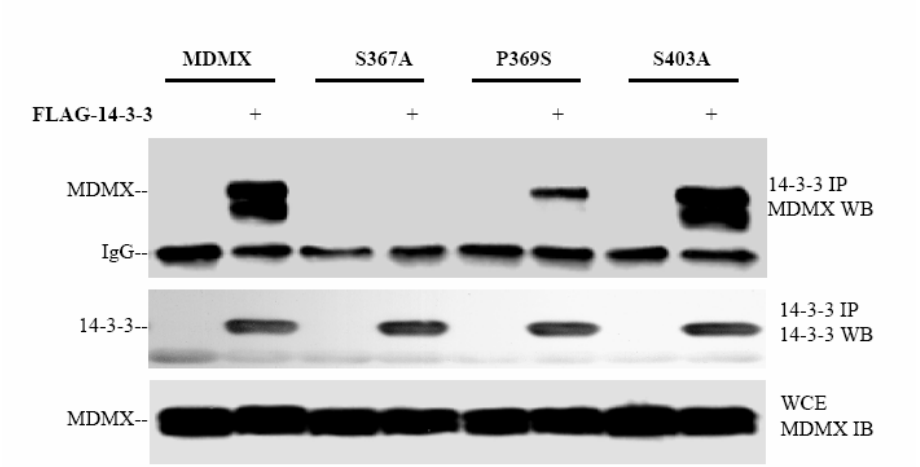


Figure 15: S367 is the major MDMX-14-3-3 binding site. H1299 cells were transiently cotransfected with MDMX mutant and FLAG-14-3-3τ plasmids for 48 hours, followed by anti-FLAG IP and MDMX western blot.

To confirm that phosphorylated S367 is directly involved in binding to 14-3-3, peptides with phosphorylated or un-phosphorylated S367 were conjugated to agarose beads. Whole cell extract was incubated with peptide-loaded beads and the amount of 14-3-3τ captured was determined by western blot. The result showed that peptide with phosphorylated S367 specifically captured 14-3-3τ from the extract. Un-phosphorylated S367 peptide, phosphorylated or un-phosphorylated S342 peptides showed no binding above background noise (**Figure 16**). These results further demonstrated that phosphorylated S367 is the 14-3-3 binding site on MDMX.

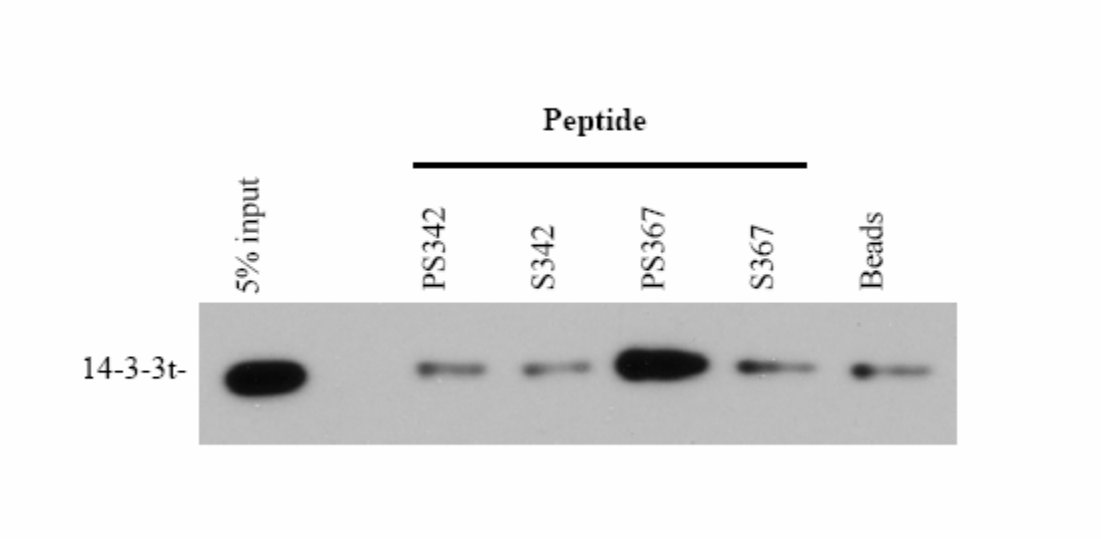


Figure 16: Phosphorylated S367 binds specifically to 14-3-3: 14-3-3 binding by phosphorylated S367 peptide. MDMX peptides with or without phosphorylation on S342 and S367 were chemically cross-linked to agarose beads. The beads were incubated with whole cell extract, washed and bound 14-3-3τ was detected by western blot.

S367 is a major phosphorylation site in vivo

If S367 plays a significant role in the regulation of MDMX after DNA damage, we would expect that this site should be strongly phosphorylated. Therefore, we determined the relative modification levels of several MDMX phosphorylation sites in vivo by metabolic labeling and 2-dimensional phosphopeptide analysis. 293T cells were transiently transfected with MDMX mutants with serine to alanine mutations at each phosphorylation site and labeled with ^{32}P-orthophosphate. The labeled MDMX was immunoprecipitated, gel purified, digested with Asp-N endoproteinase, and the peptides were analyzed by 2D peptide mapping. The labeling of wild type and MDMX point mutant enabled us to assign some of the phosphopeptide spots on the 2D peptide map to particular serine residues **(Figure 17).** The results showed that the most intensely labeled MDMX peptide was eliminated by the S367A mutation, suggesting that S367 was the major phosphorylation site on MDMX under the labeling condition **(Figure 17a).** Although phosphorylation of S403 was detectable by mass spectrometry and phosphorylation specific antibody, we were not able to definitively assign this site to a particular spot on the 2D peptide map of Asp-N digestion. This may be due to weak signal or poor solubility of the phosphopeptide containing S403.

We also tested whether metabolic labeling and 2D peptide mapping can be used to detect gamma irradiation stimulated phosphorylation of MDMX. The results indicated that treatment of cells with 10 Gy gamma irradiation immediately before the 4 hour labeling with 0.2 mCi/ml ^{32}P-orthophosphate did not have an effect on the labeling level

of S367 relative to other spots, suggesting that the amount of irradiation received from the ^{32}P-orthophosphate incubation alone was sufficient to induce high level modification of S367 (**Figure 17d**).

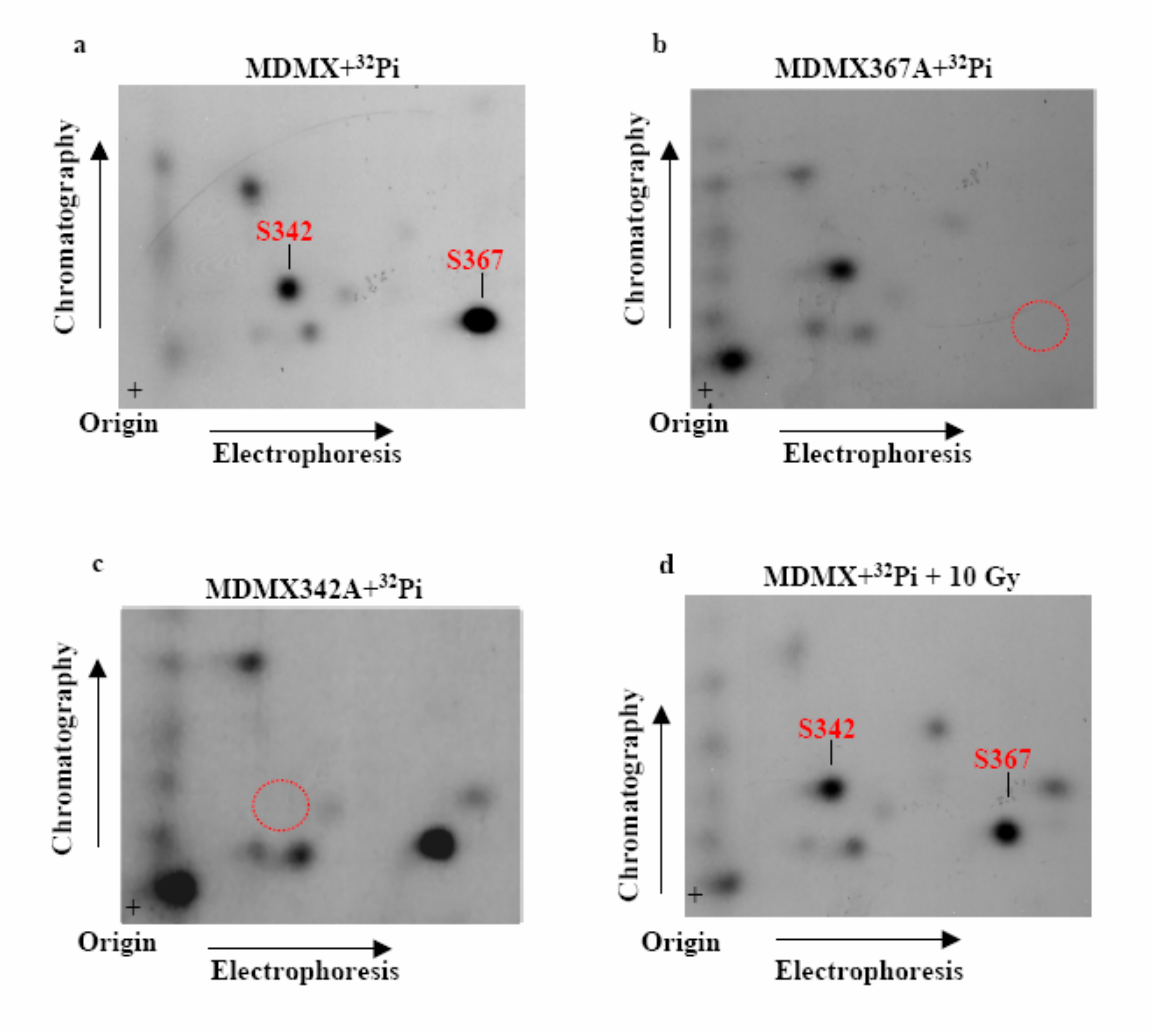


Figure 17: S367 is a major MDMX phosphorylation site *in vivo*. 293T cells were transiently transfected with MDMX mutants with serine-to-alanine mutations at the indicated phosphorylation site and labeled with ^{32}P-orthophosphate. The labeled MDMX was immunoprecipitated, digested with Asp-N endoproteinase, and analyzed by 2D peptide mapping. As an additional control, one plate of wild-type MDMX was treated with 10Gy IR for 4 hours.

Chk2 phosphorylation of S367 is important for stimulating 14-3-3 binding

The sequence context of S367 (RRTIS^{367}APVV) fits the consensus substrate motif LxRxxS/T for Chk2 kinase as identified by oriented peptide libraries in vitro (O'Neill, Giarratani et al. 2002). In order to study the regulation of MDMX by phosphorylation, we recently generated phosphorylation-specific antibody against S367. Using this antibody, we found that S367 was phosphorylated at a low basal level in undamaged cells, but significantly phosphorylated after DNA damage by gamma irradiation. Furthermore, in vitro kinase reaction showed that purified Chk2 kinase preferentially phosphorylates S367 of recombinant MDMX (Chen, Gilkes et al. 2005). As expected, DNA damage by gamma irradiation or UV induced S367 phosphorylation in wild type HCT116 cells, but not in HCT116-Chk2-/- cells (**Figure 18a**) (Jallepalli, Lengauer et al. 2003). Therefore, Chk2 kinase is important for both ionizing irradiation and UV-induced phosphorylation of S367. Phosphorylation of MDMX on at least some of the other sites (possibly on S403 by ATM) was still evident after gamma irradiation in Chk2-/- cells, generating a visible shift in MDMX mobility as in wild type HCT116 cells (**Figure 18a**). To test the role of Chk2 in DNA damage-induced MDMX-14-3-3 binding, HCT116-Chk-/- cells were infected with lentivirus expressing MDMX to obtain a level necessary for detection of endogenous 14-3-3 binding. The cells were treated with 10 Gy irradiation in the presence of MG132 (to prevent degradation of phosphorylated MDMX), MDMX was immunoprecipitated and analyzed for the coprecipitation of 14-3-3. The results showed that loss of Chk2 expression completely abrogated the stimulation of 14-3-3 binding by DNA damage (**Figure 18b**). These results showed that Chk2

phosphorylation of S367 is required for stimulation of MDMX-14-3-3 binding after DNA damage.

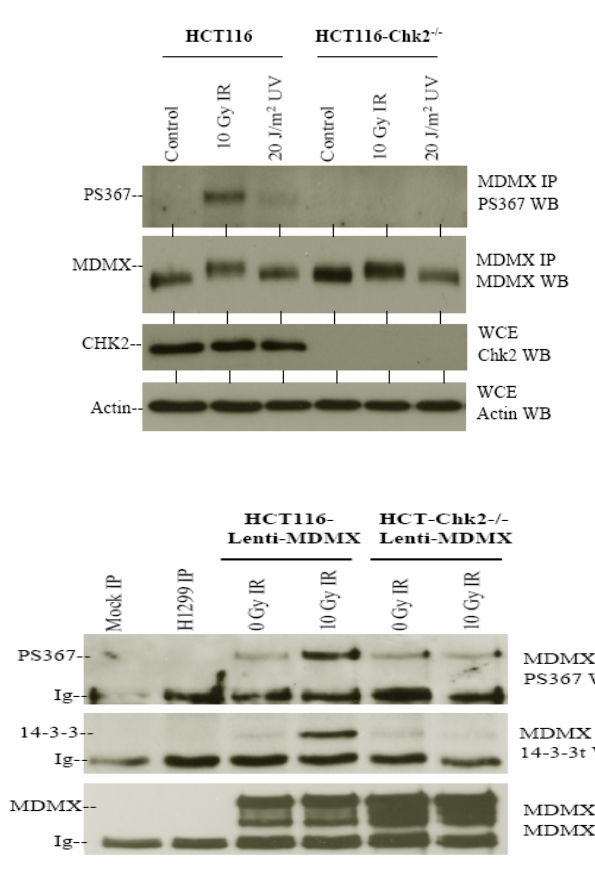


Figure 18: Chk2 is required for MDMX and 14-3-3 interaction. (a) HCT116 and HCT-Chk2 null cells were treated with 10 Gy gamma irradiation for 4 hours in the presence of 30 μM MG132 to preserve phosphorylated MDMX. MDMX was immunoprecipitated with 8C6 and analyzed using anti-PS367 antibody by western blot. The membrane was reprobed with 8C6 to confirm the level of total MDMX. **(b)** HCT116 cells were stably infected with MDMX lentivirus to facilitate detection of MDMX binding to endogenous 14-3-3. Cells were treated with 10 Gy gamma irradiation for 4 hours in the presence of 30 μM MG132. MDMX was immunoprecipitated and analyzed using anti-PS367 antibody. H1299 cells with very low MDMX level were used as negative control. MDMX immunoprecipitate was analyzed for the coprecipitation of 14-3-3τ.

Chk2 phosphorylation of MDMX is required for nuclear import and degradation

Previous studies revealed that transfected MDMX showed predominantly cytoplasmic distribution and can be induced to accumulate in the nucleus by binding to MDM2, or through an MDM2-independent mechanism after DNA damage (Stad, Little et al. 2001; Li, Chen et al. 2002; Migliorini, Danovi et al. 2002). The increased MDMX-14-3-3 binding after DNA damage suggested that Chk2-regulated 14-3-3 binding plays a role in MDMX nuclear translocation. To test this hypothesis, we generated a stable U2OS cell line overexpressing the S367A mutant. Immunofluorescence staining showed that after gamma irradiation, wild type MDMX underwent nuclear translocation as reported previously, whereas the S367A mutant showed significant defect in nuclear translocation (**Figure 19**). However, both wild type MDMX and S367A were targeted into the nucleus when cotransfected with MDM2 (data not shown), indicating that S367A mutation does not affect MDM2-mediated nuclear import. Therefore, phosphorylation of S367 is required for the DNA damage-induced, MDM2-independent nuclear import, possibly mediated by 14-3-3 binding.

U20S

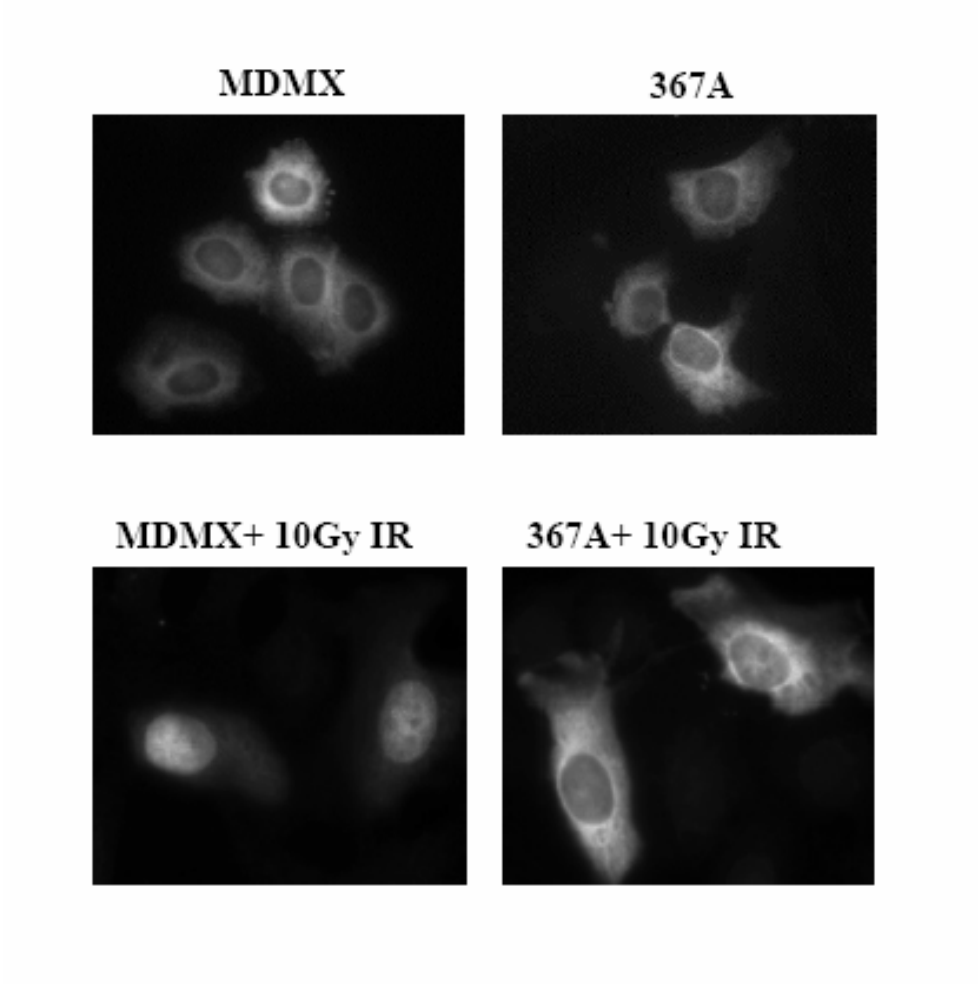


Figure 19: S367 mutant is defective in nuclear translocation after DNA damage: S367A mutant does not undergo nuclear translocation after DNA damage. U2OS cells stably transfected with wild type MDMX or S367A mutant were treated with 0.5 μM CPT for 18 hours and stained using 8C6.

The ability of S367A mutation to block MDMX nuclear translocation suggested that phosphorylation by Chk2 is required for DNA damage-regulated MDMX nuclear import. To test this prediction, wild type and HCT116-Chk2-/- cells were stably infected with lentivirus vector expressing MDMX and treated with gamma irradiation. Immunofluorescence staining of MDMX revealed that DNA damage induced MDMX nuclear import in wild type HCT116 cells, but not in HCT116-Chk2-/- cells (**Figure 20).** Therefore, Chk2 phosphorylation of MDMX on S367 is important for its nuclear translocation after DNA damage.

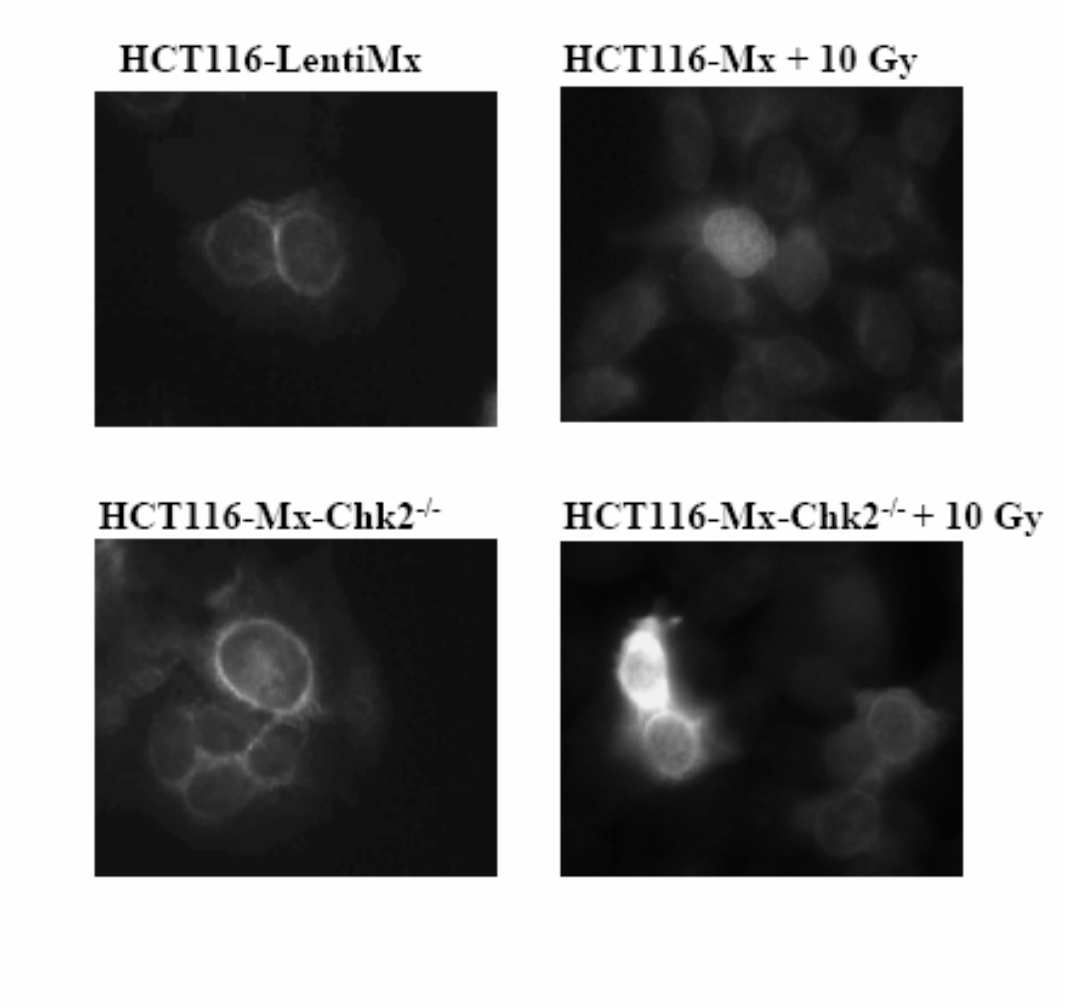


Figure 19: **Chk2 deficiency effects MDMX nuclear translocation:** DNA damage does not induce MDMX nuclear import in Chk2-null cells. HCT116 wild type and Chk2-null cells stably infected with lentivirus expressing MDMX were treated with 10 Gy irradiation for 18 hours and stained with 8C6 antibody.

To further test the role of Chk2 in MDMX nuclear import, we examined the localization of endogenous MDMX by nuclear/cytoplasmic fractionation. The results showed that MDMX in wild type HCT116 cells were distributed in both cytoplasm and nucleus, with moderate preference for the nucleus (**Figure 21).** Nuclear distribution became prominent 4 hours after gamma irradiation when MG132 was also added to prevent degradation of phosphorylated MDMX. Without MG132 treatment, gamma irradiation actually reduced the amount of nuclear MDMX due to degradation (**Figure 21**). The nuclear MDMX in wild type HCT116 cells showed reduced gel mobility after irradiation, suggesting that the phosphorylated form was preferentially transferred to the nucleus (**Figure 20, second panel**). In contrast, MDMX was almost equally distributed between the cytoplasm and nucleus in Chk2-null cells, and this pattern was not significantly altered by gamma irradiation and MG132 co-treatment (**Figure 21**). For comparison, p53 in the same experiment was predominantly localized in the nucleus irrespective of the treatments.

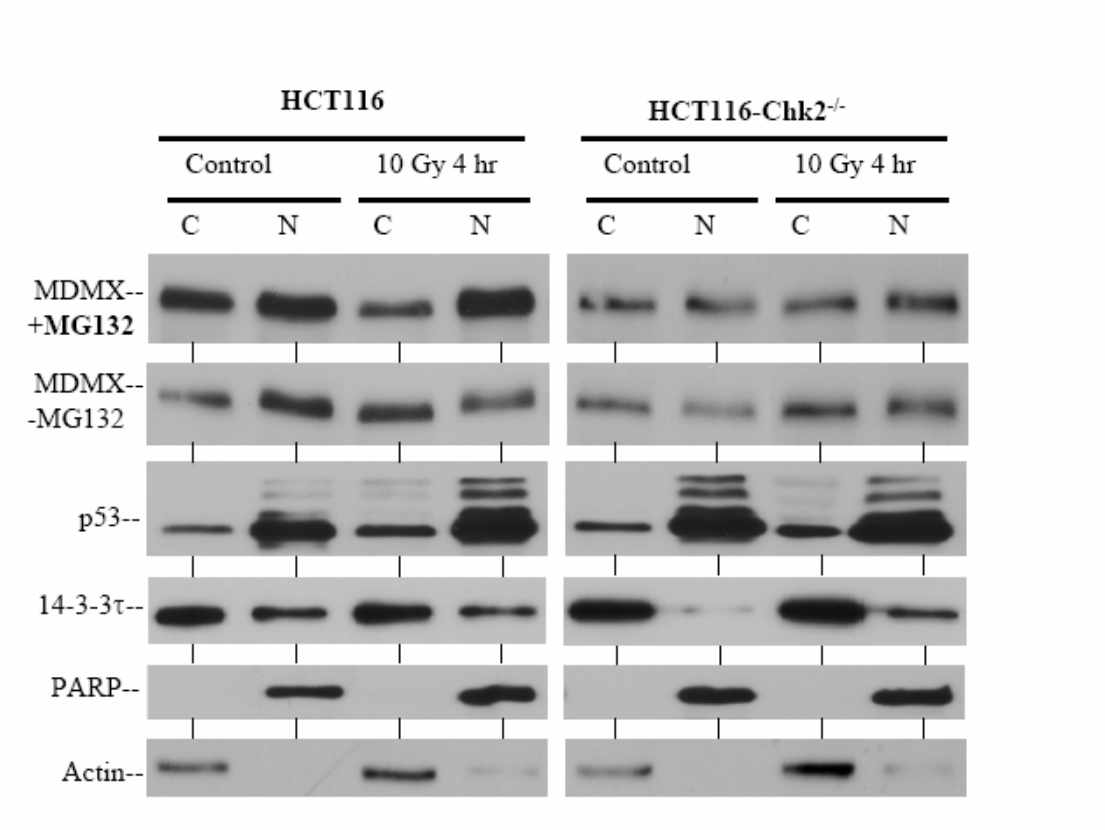


Figure 21: Analysis of endogenous MDMX by subcellular fractionation: Analysis of endogenous MDMX distribution by sub-cellular fractionation. HCT116 cells were treated with 10Gy gamma irradiation in the presence or absence of 30 μM MG132 for 4 hours. Nuclear and cytoplasmic fractions were analyzed by western blot. To facilitate comparison of ratio, loading of IR-treated C/N pair was empirically increased to obtain MDMX signal similar to the untreated pair.

Additional time course experiments showed that in the absence of MG132, MDMX level in both compartments of wild type HCT116 cells decreased rapidly after irradiation. However, in the presence of MG132, cytoplasmic MDMX decreased whereas nuclear MDMX remained the same or increased after irradiation **(Figure 22).** These results showed that nuclear translocation and degradation of MDMX after DNA damage requires Chk2 activity. Inhibition of the proteasome caused accumulation of the phosphorylated MDMX in the nucleus.

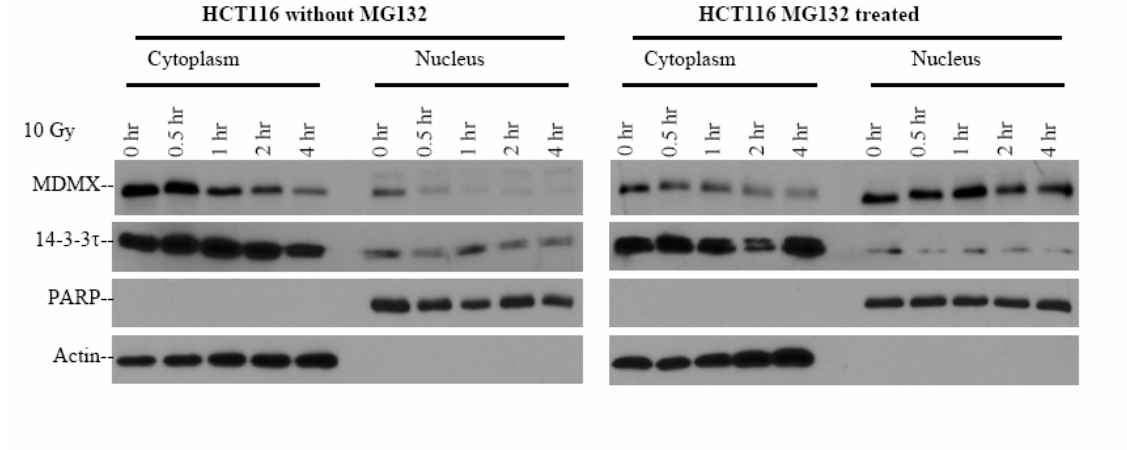


Figure 22: Subcellular distribution of MDMX at early time points after IR: HCT116 cells were treated with 10 Gy gamma irradiation in the presence or absence of 30 μM MG132. Cells harvested at indicated time points were analyzed by nuclear /cytoplasmic fractionation. Samples representing identical cell number were analyzed by western blot. In the absence of MG132, MDMX level in both compartment decreased after irradiation. In the presence of MG132, cytoplasmic MDMX decreased whereas nuclear MDMX remained the same or increased after irradiation, consistent with nuclear import and degradation.

Direct comparison of total MDMX levels in HCT116 with and without Chk2 also showed that loss of Chk2 prevented efficient degradation of MDMX after gamma irradiation. This was associated with moderately reduced stabilization of p53 and weaker induction of p21 expression **(Figure 23**). These results suggested that Chk2 is critical for efficient degradation of MDMX and full activation of p53 after DNA damage, possibly in part through regulation of MDMX-14-3-3 interaction and promoting MDMX nuclear import.

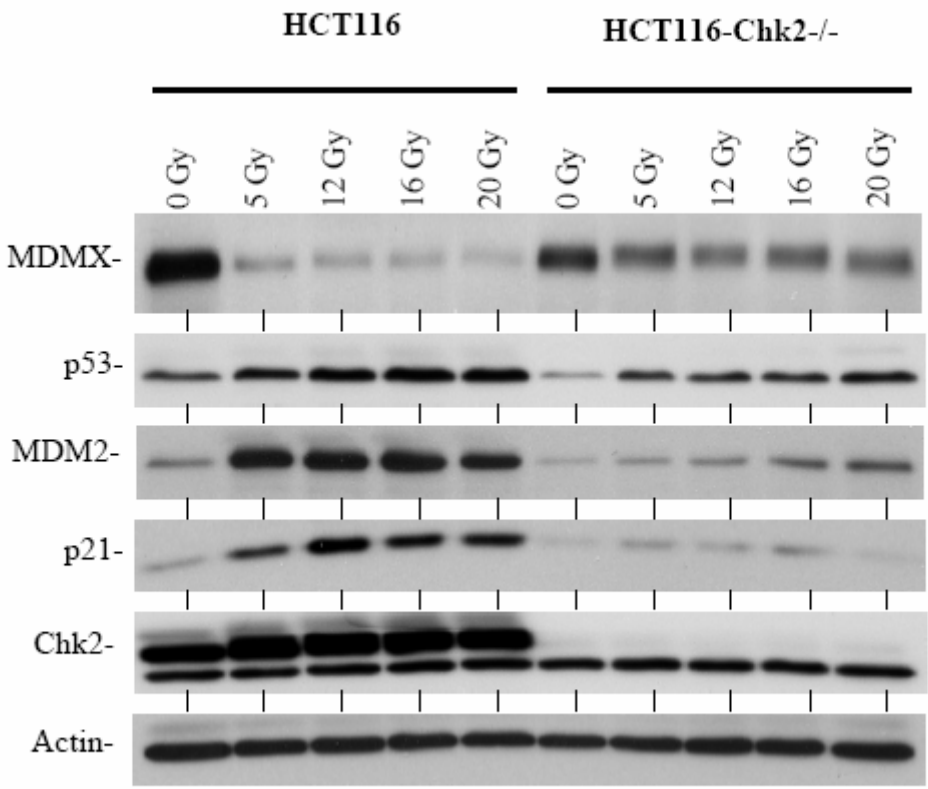


Figure 23 : Chk2-null cells were partially defective for MDMX degradation. Chk2-null cells were partially defective for MDMX degradation. HCT116 cells were treated with indicated doses of gamma irradiation for 4 hours and analyzed by western blot for different markers.

S367 phosphorylated MDMX preferentially localizes to the nucleus

The correlation between Chk2-mediated S367 phosphorylation and MDMX nuclear import suggested that phosphorylated MDMX should preferentially accumulate in the nucleus. To test this prediction, Hela cells stably expressing high level of MDMX were stained with MDMX 8C6 antibody and PS367 antibody. As expected, cytoplasmic MDMX prior to DNA damage was not stained by the PS367 antibody. After gamma irradiation or treatment with camptothecin, the majority of MDMX accumulated in the nucleus and displayed a strong nuclear staining by the PS367 antibody (**Figure 24).**

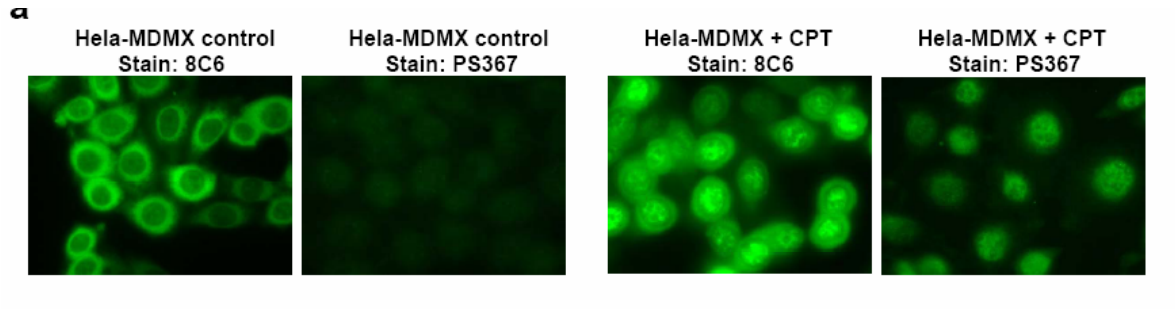


Figure 24: Specificity of P367 antibody. Hela cells stably transfected with MDMX were treated with 0.5 μM CPT for 18 hours and stained using 8C6 or PS367 antibody.

Transient transfection of MDMX into U2OS cells resulted in a subpopulation of cells showing diffused cytoplasmic and nuclear distribution. Double IF staining of these cells also showed that PS367 antibody preferentially stained the nucleus (**Figure 25).** These results are consistent with the cell fractionation experiment showing that nuclear MDMX has a slower electrophoretic mobility after DNA damage (**Figure 21).**

Phosphorylated MDMX is rapidly degraded after DNA damage, whereas mutation of S367 increases MDMX resistance to ubiquitination and degradation by MDM2 (Chen, Gilkes et al. 2005). These observations, together with the results in Figure 5c suggested that 14-3-3 binding help to promote degradation of nuclear MDMX. To test this hypothesis, MDMX was transiently co-transfected with 14-3-3 into U2OS cells, which express relatively high levels of endogenous MDM2. Staining of MDMX by 8C6 antibody showed that 14-3-3 overexpression promoted the elimination of nuclear MDMX, resulted in more prominent cytoplasmic distribution (**Figure 25).**

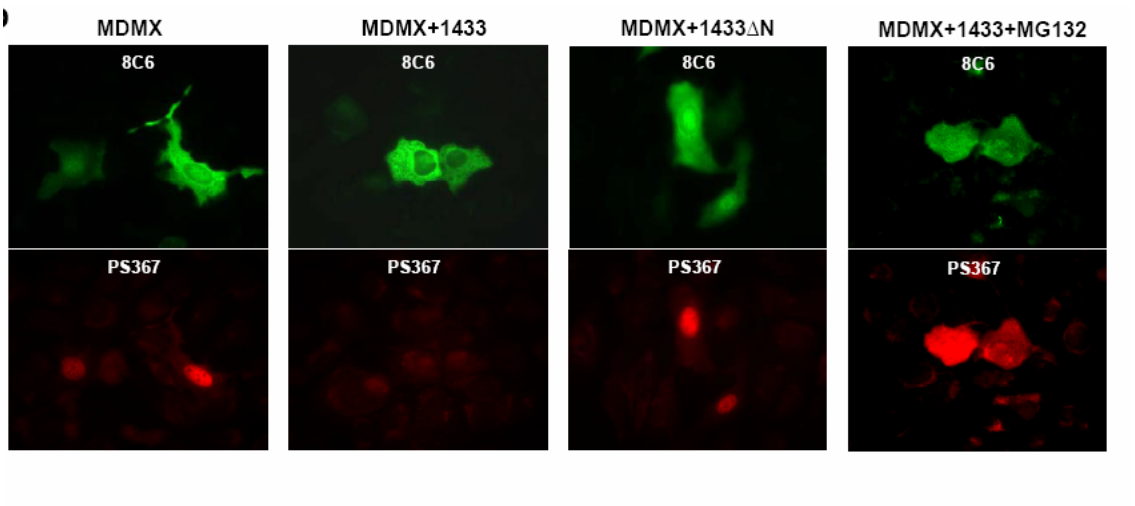


Figure 25: 14-3-3 promotes the elimination of phosphorylated MDMX from the nucleus. 14-3-3 expression promotes elimination of phosphorylated MDMX from the nucleus. U2OS cells were transiently cotransfected with MDMX and 14-3-3τ plasmids for 24 hours and treated with 30 μM MG132 for 4 hours. Cells were double stained using 8C6 for total MDMX (green) and PS367 antibody for phosphorylated MDMX (red).

One interpretation of this result is that 14-3-3 overexpression promotes MDMX nuclear export. However, PS367 staining also showed a loss of phosphorylated MDMX after 14-3-3 cotransfection, suggesting that 14-3-3 promotes degradation of phosphorylated MDMX. Furthermore, when MDMX and 14-3-3 cotransfected cells were treated with MG132, accumulation of phosphorylated MDMX was observed (**Figure 25**). The diffused localization of phosphorylated MDMX after MG132 treatment may be due to overexpression, which was not seen in the fractionation of endogenous MDMX (**Figure 21**). Overall, the results favor the interpretation that 14-3-3 binding promotes MDMX nuclear import and degradation in the nucleus.

MDMX contains a cryptic NLS that mediates Chk2-regulated nuclear import

To determine how phosphorylation and 14-3-3 binding promote MDMX nuclear import, we tested whether MDMX contains a cryptic nuclear import signal (NLS) that may be activated by 14-3-3 binding. When GFP was fused to the N terminus of full-length MDMX, the fusion protein becomes constitutively cytoplasmic even after DNA damage (data not shown), indicating an interference of nuclear import by the GFP moiety. However, GFP-MDMX300-490 was constitutively nuclear localized (**Figure 26**), suggesting the exposure of a cryptic NLS in this region.

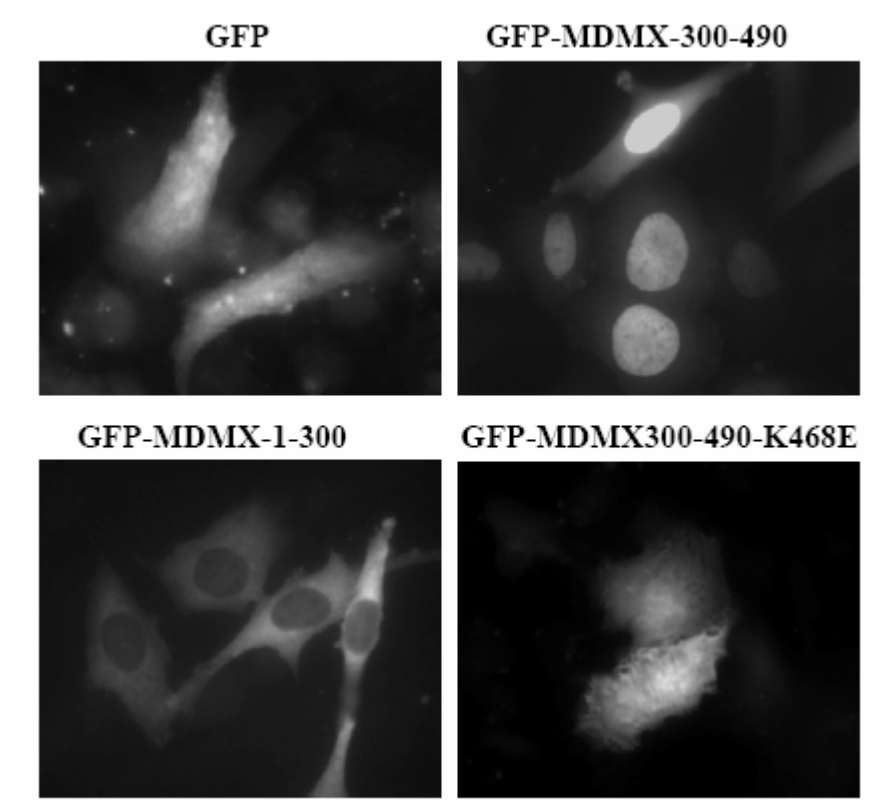


Figure 26: Distribution of fused GFP moiety to full length MDMX and deletion mutants indicates a cryptic NLS region. GFP-MDMX fusions were stably transfected into U2OS cells and photographed for localization using fluorescence from the GFP tag.

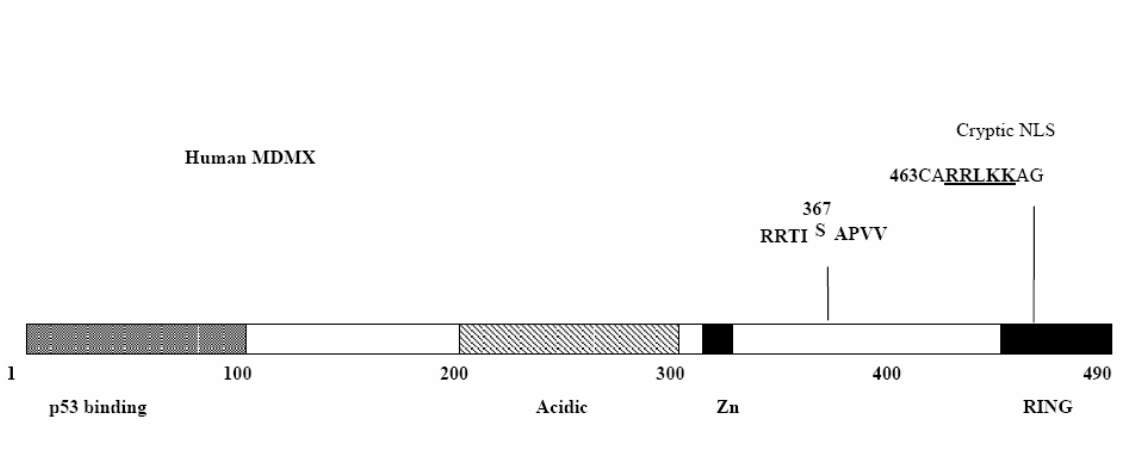


Figure 27 : Diagram of MDMX indicating the location of cryptic NLS

Inspection of MDMX sequence suggested that the only candidate is a stretch of basic residues within the RING domain (465RRLKK), which is similar to the cryptic nucleolar targeting signal on MDM2 (465KKLKK) (Lohrum, Ashcroft et al. 2000) (**Figure 27**). An K468E mutation introduced into GFP-MDMX300-490 resulted in diffused localization (**Figure 26**), demonstrating that it was required for nuclear localization. Furthermore, full-length MDMX with K468E mutation failed to undergo nuclear translocation after DNA damage (**Figure 28**), despite normal levels of S367 phosphorylation and 14-3-3 binding (data not shown). This mutation had no effect on MDM2-mediated nuclear import of MDMX in cotransfection assays (data not shown), suggesting that it is specifically required for the MDM2-independent nuclear import.

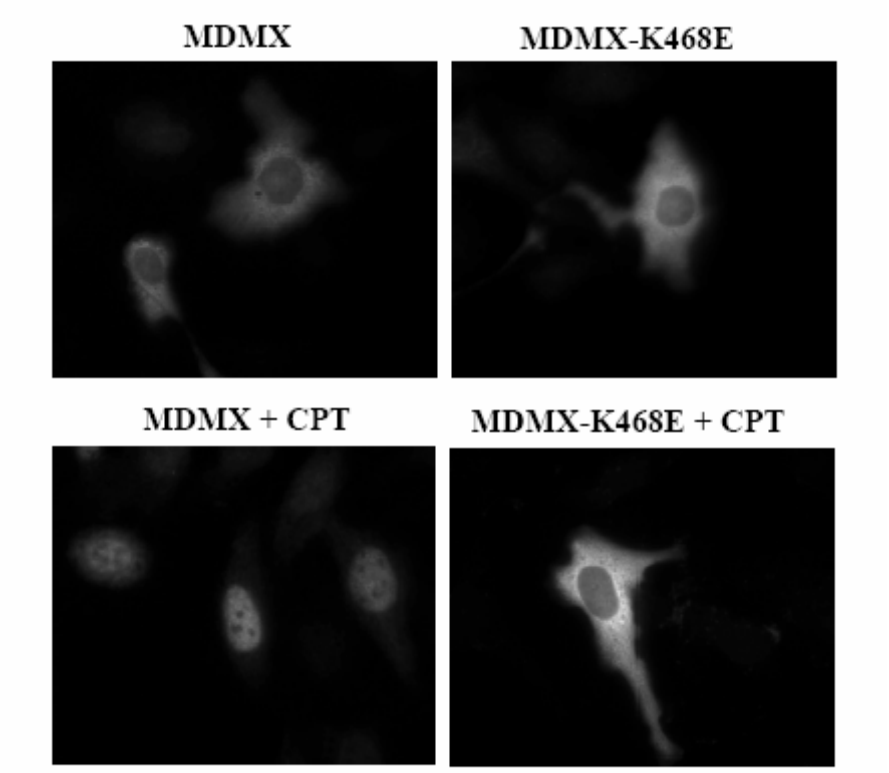


Figure 28: Full length MDMX with K468E mutation failed to undergo nuclear translocation after DNA damage. Full-length MDMX with the K468E mutation was transiently transfected into U2OS cells, treated for 18 hours with 0.5 μM CPT, and stained using 8C6 antibody.

Classic NLS-mediated nuclear import requires binding of the NLS to a family of cytoplasmic receptors (importin α) (Weis 2003). When GST-importin α1, 3, 5, 6, and 7 fusion proteins were incubated with cell lysate containing MDMX, importin α3 showed significant binding to MDMX (Liu, McBride et al. 2005), which was abrogated by the K468E mutation (**Figure 29**).

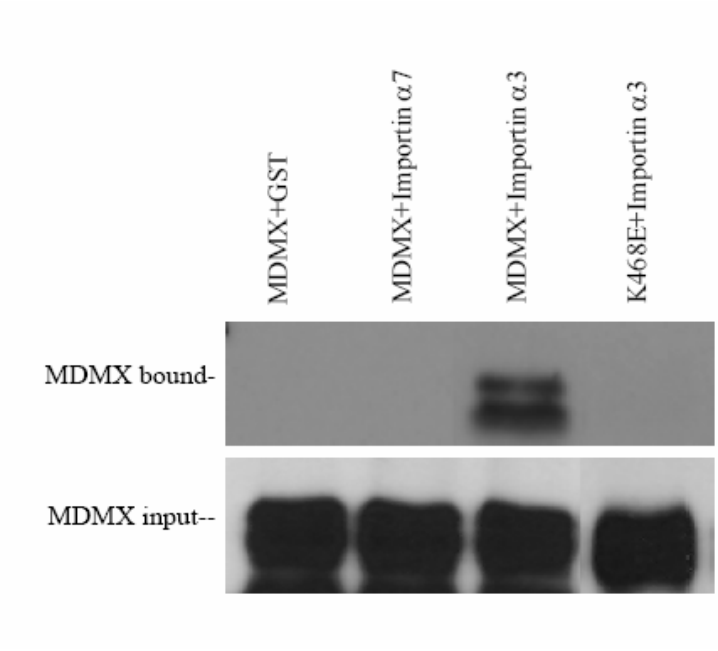


Figure 29: Importin a binds specifically to wildtype MDMX but not K468E mutant. GST-importin loaded beads were incubated with lysate of HCT116 cells transiently transfected with wild type or mutant MDMX. Bound MDMX were detected by western blot.

This result was consistent with a role of the 465RRLKK sequence in mediating MDMX nuclear import. However, GST-importinα3 pull down efficiency was not affected by Chk2 phosphorylation of S367 (data not shown), suggesting that the in vitro binding assay does not recapitulate all aspects of the import process. These results showed that MDMX contains a cryptic NLS sequence that mediates Chk2 and 14-3-3-regulated nuclear import after DNA damage.

14-3-3 cooperates with Chk2 to promote MDMX ubiquitination

To further investigate the role of the 14-3-3 binding site on regulation of MDMX ubiquitination, the effect of Chk2 on wild type MDMX and the S367 mutant was examined. HCT116-Chk2-/- cells were transiently transfected with MDMX, MDM2, His6-ubiquitin, and Chk2 plasmids. MDMX ubiquitination was determined by Ni-NTA purification of ubiquitinated proteins followed by MDMX western blot. The results showed that expression of wild type Chk2, but not the kinase-deficient Chk2-A347 mutant (Chehab, Malikzay et al. 2000), strongly stimulated the poly-ubiquitination and degradation of MDMX by MDM2 (**Figure 30).** In contrast, ubiquitination and degradation of the MDMX-S367A mutant was not stimulated by Chk2 (**Figure 30**). Therefore, phosphorylation of S367 is important for efficient ubiquitination of MDMX and is necessary for the regulation by Chk2.

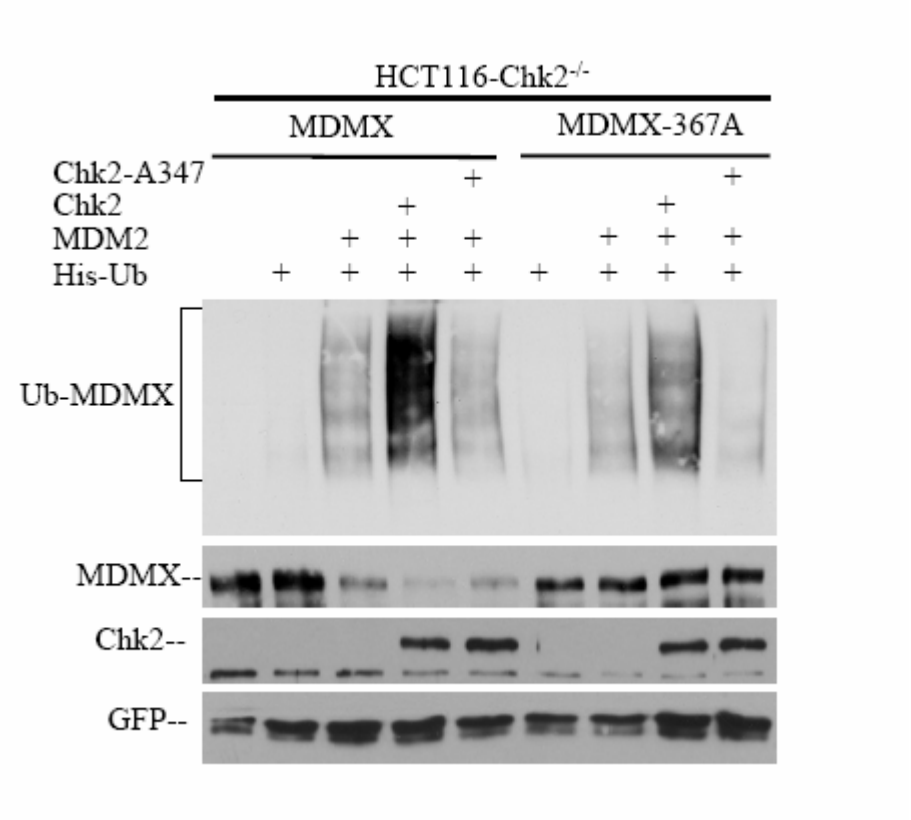


Figure 30: Chk2 promotes ubiquitination of wildtype MDMX and not S367 mutant. HCT116-Chk2-/- cells were transiently transfected with MDMX, MDM2, Chk2, and His6-ubiquitin. MDMX ubiquitination was detected by Ni-NTA purification followed by MDMX western blot. Whole cell extract was analyzed for MDMX level by western blot. Chk2-A347 is a kinase-deficient mutant.

To determine whether 14-3-3 and Chk2-mediated phosphorylation cooperate to stimulate the ubiquitination of MDMX, HCT116-Chk2-/- cells were transfected with His6-ubiquitin, MDMX, 14-3-3 and Chk2 expression plasmids. Under dose-limiting conditions where 14-3-3 and Chk2 alone had negligible effects on MDMX ubiquitination by endogenous MDM2 (50-100 ng plasmid for a 6 cm plate), coexpression of 14-3-3 and Chk2 significantly enhanced MDMX ubiquitination and degradation (**Figure 31, lane 5 vs. 2 and 3**). Furthermore, mutation of S367 abrogated the cooperative effect of 14-3-3 and Chk2 (**Figure 31, lane 11 vs. 9 and 10**). Therefore, Chk2-mediated phosphorylation cooperates with 14-3-3 to stimulate MDMX ubiquitination and degradation.

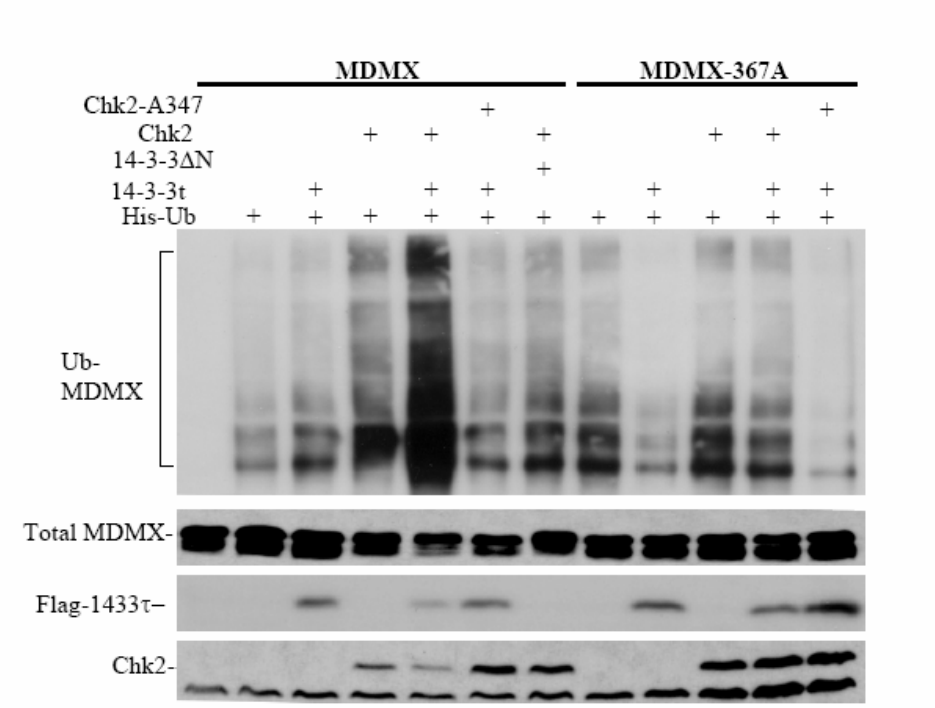


Figure 31: **14-3-3 cooperates with Chk2 to promote MDMX ubiquitination.** HCT116-Chk2-/- cells were transfected with His6-ubiquitin, MDMX, 14-3-3τ and Chk2 plasmids. MDMX ubiquitination was detected by Ni-NTA purification followed by MDMX western blot. 14-3-3ΔN is a 30-245 fragment of 14-3-3τ, lacking the dimerization domain.

To further test whether 14-3-3 stimulates degradation of MDMX phosphorylated on S367, MDMX and MDM2 were cotransfected with 14-3-3 in H1299 cells. The transfection procedure alone induced significant phosphorylation of MDMX on S367 without DNA damaging treatments. The levels of total MDMX level and PS367 MDMX level were determined by IP-western blot. The result showed that when MDM2 level was sub-optimal for significant degradation of total MDMX, 14-3-3 cotransfection selectively

stimulated the degradation of MDMX phosphorylated on S367 (**Figure 32**). Under conditions of MDM2 overexpression, the levels of total MDMX and phosphorylated MDMX were degraded at a similar rate (data not shown), possibly because phosphorylation on S367 was no longer a rate-limiting step. These results showed that 14-3-3 stimulates MDM2 degradation of MDMX phosphorylated at S367.

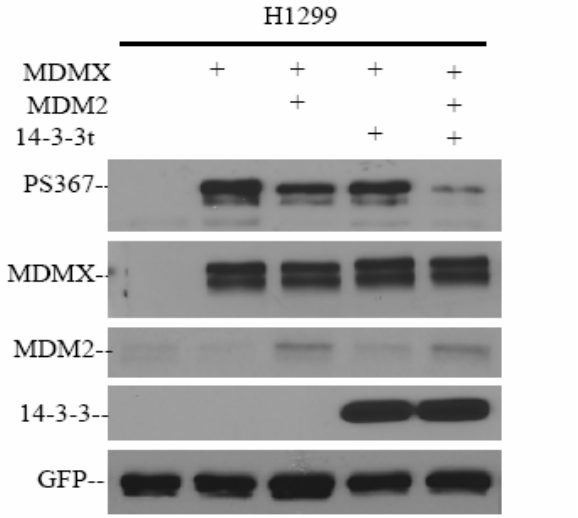


Figure 32: 14-3-3 stimulates degradation of phosphorylated MDMX. 14-3-3 stimulates degradation of PS367 MDMX. H1299 cells were transiently transfected with MDMX, MDM2, and 14-3-3τ for 48 hours. MDMX was immunoprecipitated with 8C6 and probed with PS367 antibody for the phosphorylated form, or with 8C6 for total MDMX level.

To further test the effect of Chk2 and 14-3-3 on the ability of MDMX to inhibit p53 transcriptional activity, HCT116-Chk2-/- cells were transfected with the p53-responsive BP100-luciferase reporter (Freedman, Epstein et al. 1997). Transfected BP100-luc alone was activated by endogenous p53 and produced baseline readout of p53 activity. Cotransfection of MDMX reduced p53 activity by ~2-fold (**Figure 33),** which was typical for MDMX and weaker than the effect of MDM2 in such assay. Coexpression of 14-3-3 and Chk2 partially overcome the inhibition of p53 activity by wild type MDMX, consistent with the ability of this combination in promoting MDMX degradation. This effect required active Chk2 and was not seen using kinase-deficient Chk2-347A mutant (**Figure 33**). Importantly, the MDMX 367A mutant was not regulated by 14-3-3 and Chk2 (**Figure 33)**, confirming a requirement for phosphorylation and 14-3-3 binding. These results demonstrated that Chk2 phosphorylation of MDMX on S367 and recruitment of 14-3-3 cooperates to abrogate its inhibitory effect on p53.

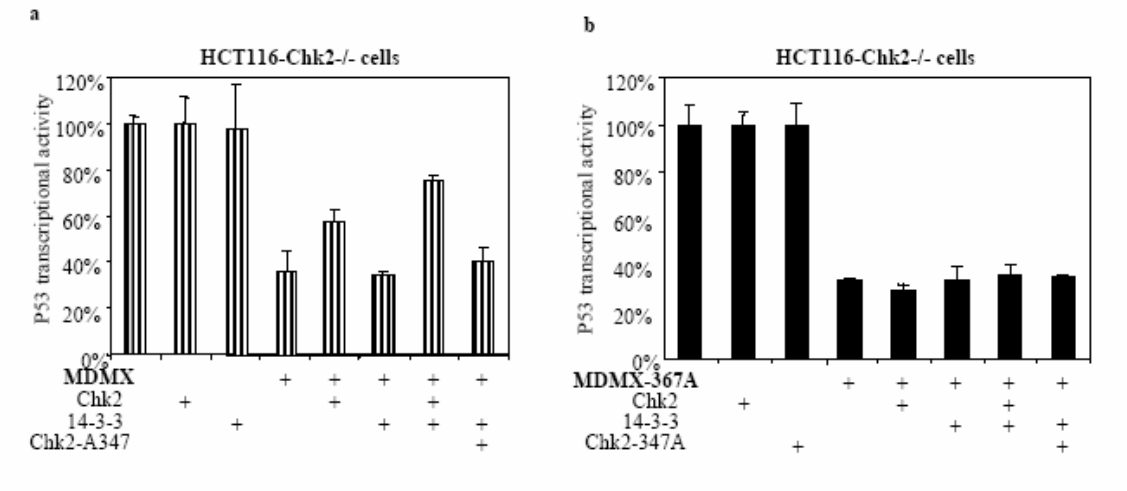


Figure 33: **Chk2 and 14-3-3 cooperate to neutralize MDMX inhibition of p53.** HCT116-Chk2-/- cells were transfected with p53-responsive BP100-luc reporter to detect endogenous p53 transcriptional activity. The effects of wild type MDMX, 14-3-3 and Chk2 coexpression on p53 activity were detected by luciferase assay and normalized to CMV-lacZ transfection efficiency control. (b) A control experiment identical to (a) except that the MDMX 367A mutant was used.

Investigating the role of MDMX nuclear import after DNA damage

MDMX has been shown to undergo nuclear translocation after DNA damage (Li et al. 2001). Although we now know that MDMX nuclear translocation promotes its degradation, it appears counterintuitive that a negative regulator of p53 would undergo translocation to the nucleus after DNA damage. In order to elucidate the biological function of MDMX nuclear translocation after DNA damage, we investigated the effects that MDMX-K468E deficient in nuclear translocation on p53 and targets gene induction. Stable pools were generated by transiently transfecting U20S cells with wild-type MDMX, K468E mutant as well as a pCDNA3 negative control. The cells were selected in G418 for one month and pooled. In order to determine whether overexpression of the MDMX-K468E mutant provided an advantage in inducing p53 due to its deficiency in nuclear accumulation; cells were exposed to 10Gy of ionizing radiation for 0, 2, and 4 hours, followed by western blots analysis for p53 and target gene induction (**Figure 33**). As expected, DNA damage induced the degradation of MDMX and induction of p53 stability. Degradation of MDMX was most evident after 4 hours of IR treatment. Surprisingly, both wild-type MDMX and MDMX –NLS deficient mutant had similar stabilities after DNA damage, even though wild-type MDMX underwent nuclear translocation and the NLS mutant was cytoplasmic. It is still possible that longer time points would have indicated a difference. The most evident difference amongst wild-type MDMX and the NLS mutant was the levels of p21. The NLS-mutant had higher levels of p21, even before the addition of DNA damage. In addition, MDM2 levels were

somewhat higher after 4 hours of IR. These results suggest that p53 may be more active when MDMX nuclear translocation is blocked **(Figure 34)**.

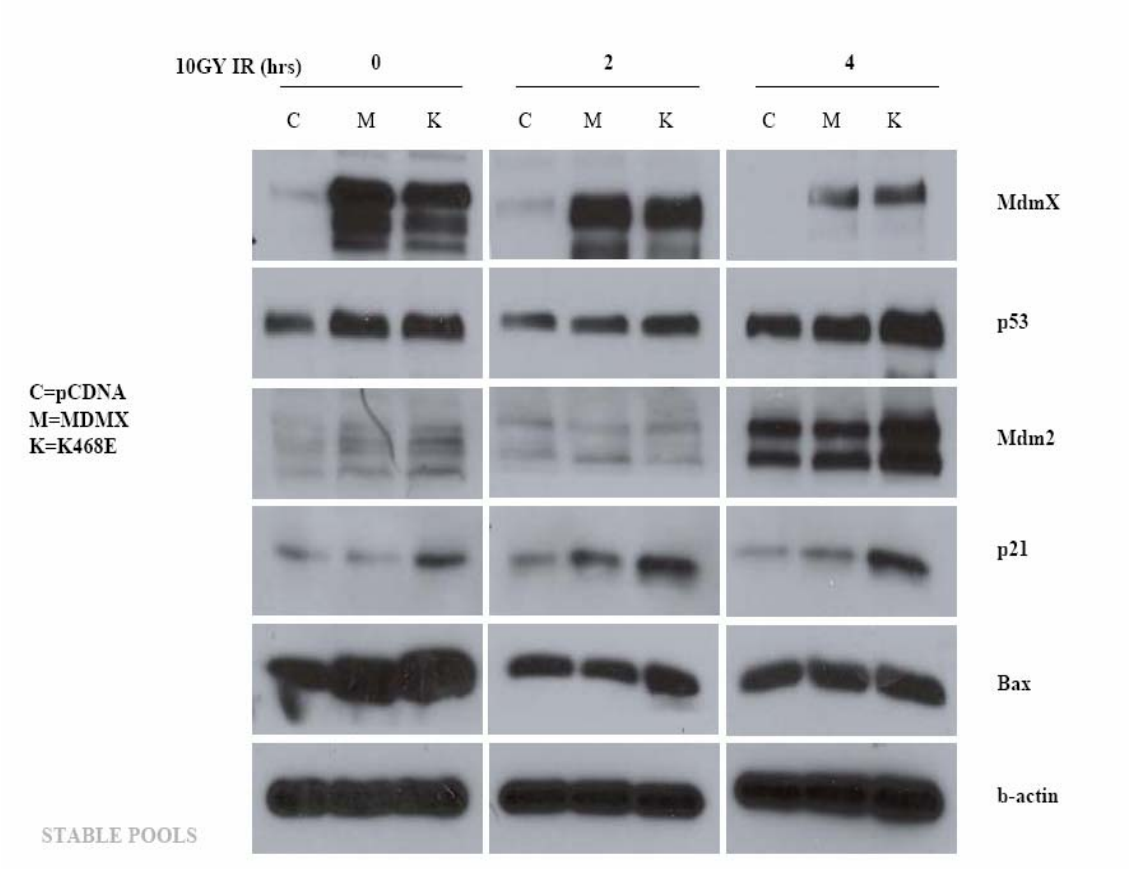


Figure 34: MDMX-K468E NLS mutant increases p21 protein levels after DNA damage more efficiently then wildtype MDMX. U20S stable pools were treated with 10Gy ionizing radiation for indicated time points. Cell lysate was collected and western blots were performed to measure any effects on p53 and p53 target genes.

In order to determine whether the increase in p21, observed in the NLS-mutant would affect p53-dependent cell cycle arrest, the stable pools were treated in the absence or presence of IR and Nocodazole to induce a G1 and G2 arrest, respectively. Cell cycle distribution was determined by flow cytometry analysis (FACS). As shown in (**Figure 34),** stable pools that were untreated had similar distribution profiles. Only after the addition of IR, Nocodazole or combination of IR and Nocodazole, did we see a difference in the ability of cells to induce a G1 arrest after DNA damage. FACS analysis supported the western analysis that the NLS mutant induce more p21 than the wild-type, thus allowing the cells to accumulate in G1 phase more efficiently (**Figure 34**). The population of cells in G1 phase in each stable pool population was compared using bar graphs. Untreated cells were nearly identical, while pools treated with Nocodazole showed a four fold difference between wild-type and NLS mutant. The addition of IR showed a two fold difference, while the combination indicated a three fold difference in cells accumulated in G1-phase (**Figure 36**). It is unclear whether the NLS mutant cells are accumulating faster in the G1 phase or have a delayed response to entering the next cell cycle phase.

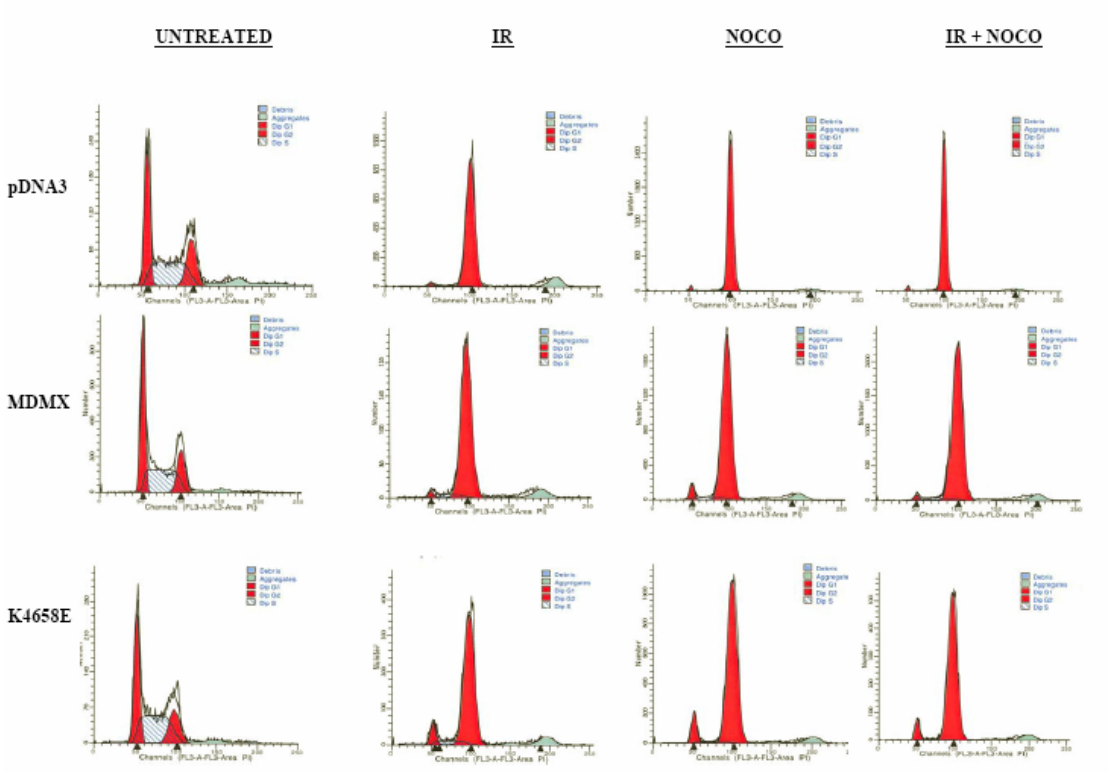


Figure 35 : Cell cycle analysis supports the induction of p21 through enhanced growth arrest in K468E mutant. U20S stable pools were treated in the absence or presence of IR, Nocodazole (100ng/ml) or in combination. Cell cycle distribution characteristics were determined by flow cytometry after 18 hours of treatment. A minimum of 10000 events were analyzed for each pool.

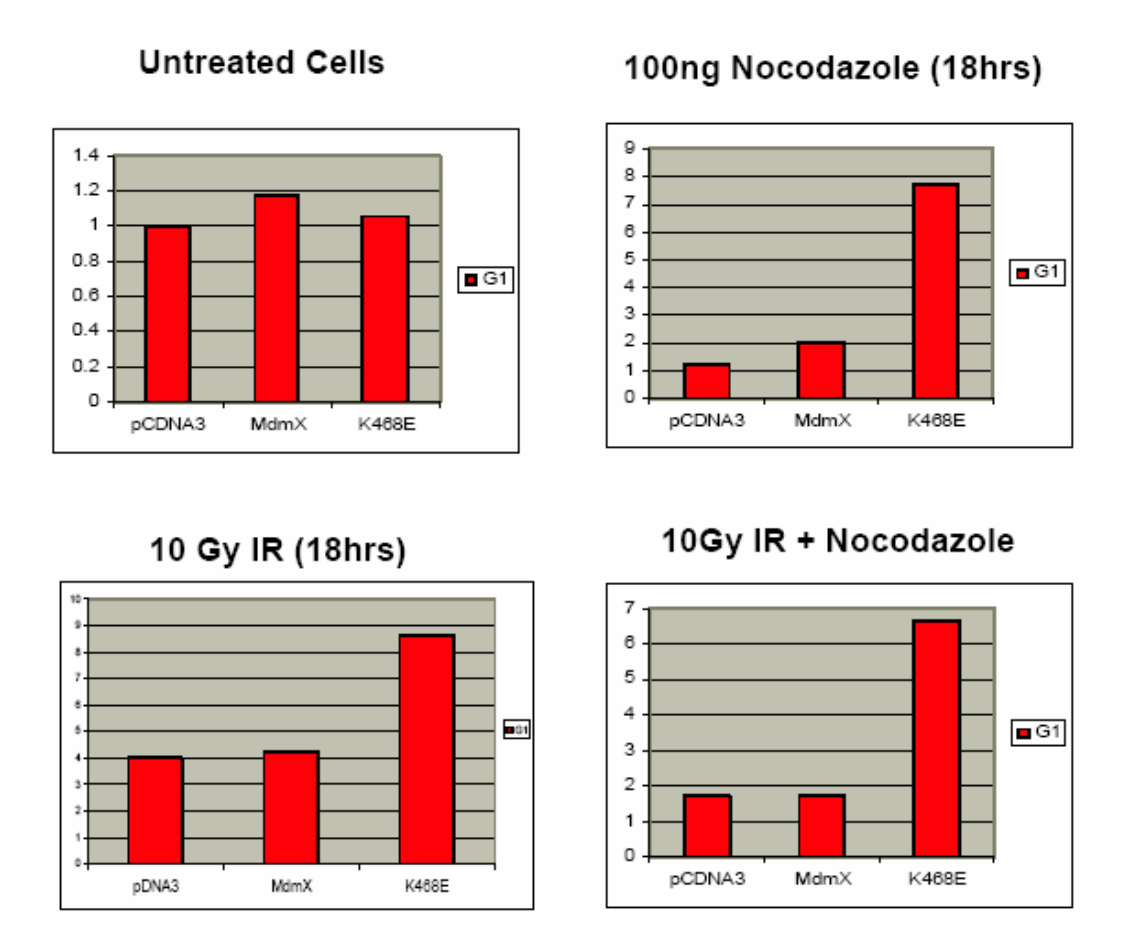


Figure 35 : Percentage of cells in G1. Accumulation of cells in G1-phase of FACS analysis of U20S stable pools was compared using bar graphs. Samples were normalized to the negative pDNA3 control pool.

The correlation between MDMX nuclear import and degradation, suggested that these events are important for the regulation of MDMX levels. To test this hypothesis we compared the ubiquitination status and binding to MDM2 and 14-3-3 between wild-type MDMX and the NLS mutant. H1299 cells were cotransfected with the wild-type and mutant alone and in combination with MDM2 and 14-3-3. The results showed that both wildtype and NLS mutant were ubiquitinated similarly and bound to MDM2 with the same affinity. However, binding to 14-3-3 was reduced for the NLS mutant, possibly because the lack of importin alpha protein binding (**Figure 37**).

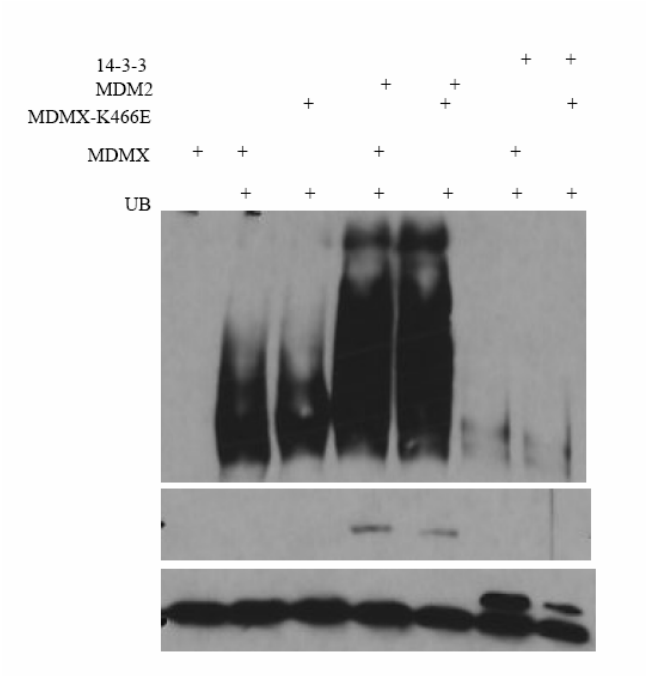


Figure 37: Wildtype and K468E mutant MDMX bound and are degraded equally by MDM2. H1299 cells were transiently transfected with MDMX, MDMX-K468E, MDM2, 14-3-3 and His6-ubiquitin. Cell pellet was collected and divided into two samples. The first pellet was used to determine MDMX ubiquitination by Ni-NTA purification, followed by western blots. The second pellet was lysed and immunoprecipated using MDMX antibody (8C6) followed by anti -MDM2 (2A9) and anti-Flag-14-3-3.

Discussion

Results described above showed that MDMX specifically interacts with 14-3-3. S367 is the major 14-3-3 binding site on MDMX and is significantly phosphorylated after DNA damage in a Chk2-dependent fashion, resulting in increased 14-3-3 binding. S367 phosphorylation and 14-3-3 binding stimulate degradation of MDMX by MDM2. Furthermore, phosphorylation of S367 is required for MDMX nuclear import after DNA damage, possibly by activating a cryptic NLS in the RING domain. These results suggest that 14-3-3 proteins regulate MDMX localization and degradation in response to DNA damage, and this effect may contribute to the efficient activation of p53.

Recent findings showed that after DNA damage, ATM is critical for inducing phosphorylation of MDMX on multiple sites. ATM directly phosphorylates S403, and is also required for phosphorylation of S367 and S342 by activating Chk2. S367 is also the most heavily phosphorylated MDMX residue after ionizing irradiation, and S367 mutation has the most significant impact on MDMX ubiquitination and degradation by MDM2. The recruitment of 14-3-3 by phosphorylated S367 suggests that 14-3-3 is an important regulator of MDMX. It is worth noting that 14-3-3 was initially co-purified with MDMX from Hela cells in the absence of DNA damage, suggesting a basal level of S367 phosphorylation. Chk1 is a likely candidate in this process because it has house keeping functions and is active in un-perturbed cells (Bartek and Lukas 2003). However, ionizing irradiation and UV did not stimulate S367 phosphorylation in Chk2-null cells,

suggesting that Chk2 is critical for the DNA damage response.

Our data showed that phosphorylation of S367 by Chk2 is important for nuclear translocation of MDMX after DNA damage. Mutation of S367 or loss of Chk2 function prevents MDMX nuclear import induced by DNA damage. MDMX has multiple mechanisms for nuclear import. Interaction with MDM2 can target MDMX into the nucleus using the NLS on MDM2. As expected, this effect does not require MDMX S367 phosphorylation or the MDMX cryptic NLS. A second mechanism requires phosphorylation of S367 and possibly involves 14-3-3 recruitment. 14-3-3 binding to phosphorylated S367 may induce conformational change of the RING domain and activate the cryptic NLS. Phosphorylated MDMX also binds to MDM2 with higher affinity (Chen, Gilkes et al. 2005), consistent with conformational change in the RING domain. Therefore, MDMX belongs to a rare group of proteins that are targeted to the nucleus by 14-3-3 binding.

How 14-3-3 binding stimulates MDMX degradation remains to be further investigated. Nuclear translocation should facilitate interaction with MDM2 in the nucleus, resulting in ubiquitination and degradation of MDMX. Conformational change induced by phosphorylation and 14-3-3 binding may increase affinity to MDM2, or increase the ability of MDMX RING domain to activate MDM2 E3 function after forming the heterodimer. A recent report showed that the de-ubiquitinating enzyme HAUSP binds to MDMX and regulates MDMX stability by de-ubiquitination (Meulmeester, Maurice et al. 2005). Interaction between MDMX-HAUSP was reduced after DNA damage and is thought to contribute to MDMX destabilization. It is possible

that 14-3-3 binding displaces HAUSP and contribute to increased MDMX ubiquitination.

The exact function of MDMX nuclear translocation also remains to be elucidated, however our data shed some light on the possible importance of nuclear import in regulating p53 response to DNA damage. We show that mutation of MDMX on one lysine residue at position 468 to glutamic acid, completely abrogates the nuclear import after DNA damage. This mutation had no effect on MDM2-mediated nuclear import of MDMX in cotransfection assays, suggesting that it is specifically required for the MDM2-independent nuclear import. We also show that the pattern of ubiquitination between the wild-type and NLS mutant is unchanged under non-stressed conditions. It is also possible we could have seen differences in ubiquitiantion, if proteasomal degradation was blocked with the addition of MG132. Another important factor to test is the addition of DNA damage, although the use of another cell line besides H1299 would be more beneficial due to the minimal induction of MDMX phosphorylation at serine 367 after DNA damage in this cell line. Another interpretation could be that in the absence of stress, degradation of both MDMX and NLS is unchanged.

We did not test whether there was a change after DNA damage, however we expect there would be a difference in binding due to localization differences. Whether or not the MDMX-NLS mutant can retain more MDM2 within the nucleus after DNA damage, remains to be determined . Interestingly, the MDMX- K468E mutant induces the expression of p21 more efficiently than the wild-type MDMX after ionizing radiation

(IR). This could be explained by MDM2 preferentially binding the nuclear accumulated phosphorylated MDMX . Furthermore, the K468E mutant induction of p21 is associated with enhanced G1 arrest after DNA damage. These results indicate an important function of MDMX nuclear import in regulating p53 activity after DNA damage.

Efficient activation of p53 after DNA damage is likely to be achieved by phosphorylation of multiple targets including p53, MDM2, and MDMX. The results described in this report add another level of complexity to p53 signaling. Further elucidation of the physiological functions of S367 phosphorylation will require a knock-in or gene replacement approach. It will also be important to determine whether other types of stress also target MDMX or p53 by regulating MDMX-14-3-3 interaction.

Proposed Model:

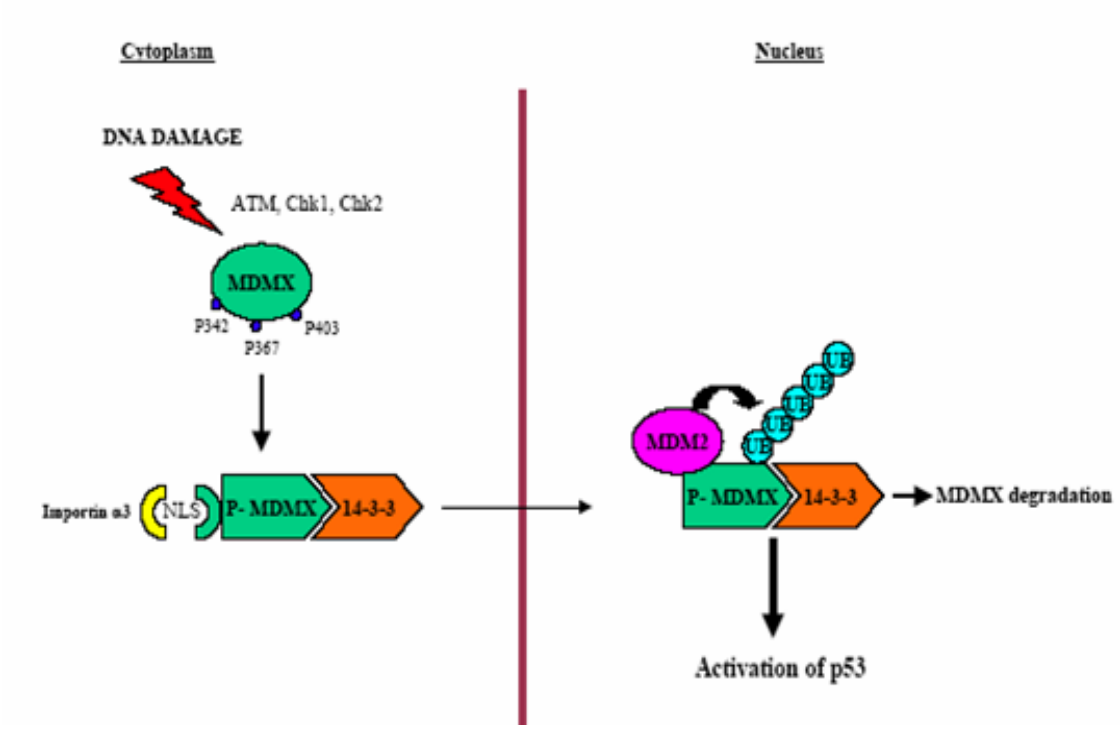


Figure 38: MDMX is a very stable protein, however after the addition of DNA damage, it undergoes rapid degradation. This figure illustrates a possible mechanism for the enhanced degradation. We propose that after IR treatment, MDMX gets phosphorylated on serines 342, 367, 403 in a Chk1, Chk2 and ATM fashion, respectively. Phosphorylation of serine 367 creates a binding site for 14-3-3. Upon binding, 14-3-3 induces a conformational change in MDMX that exposes a cryptic NLS region. Importin alpha 3 proteins bind the NLS region of MDMX to initiate nuclear import. Nuclear translocation of phosphorylated MDMX makes it a better substrate for MDM2 binding. MDM2 binding promotes the polyubiquitination of MDMX and subsequent degradation. The combination of decreased MDM2-p53 association and MDMX degradation allows p53 to become more stable and transcriptional active.

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