CHARACTERIZATION OF SYNTHETIC LETHALITY BETWEEN MDM2 OVEREXPRESSION AND GENOTOXIC AGENTS AND IDENTIFICATION OF A NOVEL FUNCTION OF THE MDMX ONCOGENE IN DNA REPAIR

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Dedication

To my mother Lisa, my grandmother Carla,
and my dad Andres for always believing in and supporting me.

To my wonderful husband Carlos for always encouraging me.

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LIST OF ABBREVIATIONS

ATM	Ataxia Telangiectasia mutated
BRCA1	breast cancer associated protein 1
BRCA2	breast cancer associated protein 2
CC3	Cleaved caspase 3
^{137}Cs	cesium-137
CSR	class switch recombination
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	dimethyl sulfoxide
DN	double null
DNA	deoxyribonucleic acid
FHA	forkhead-associated domain
Foxo3a	forkhead box O3a
GFP	green fluorescent protein
H2AX	Histone 2A variant X
γH2AX	gamma H2AX; phosphorylated H2AX
HR	homologous recombination
γIR	gamma irradiation
IP	immunoprecipitation
kDa	kilodalton
Mdc1	mediator of DNA damage checkpoint protein 1
Mdm2	murine double minute 2

Mdm4	murine double minute 4
Mdmx	murine double minute x
MEF	murine embryonic fibroblast
MG132	carbobenzoxy-Leu-Leu-leucinal
Mre11	mitosis recombination 11
mRNA	messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MSCV	murine stem cell virus
NBS1	Nijmegen Breakage Syndrome 1
NES	nuclear export signal
NHEJ	non-homologous end joining
NLS	nuclear localization signal
p21	CIP1/WAF1; CDK-interacting protein 1
p53	protein 53kDa
pS/T-Q	phosphorylated serine-glutamine or threonine-glutamine
PUMA	p53 upregulated modulator of apoptosis
qRT-PCR	quantitative real time polymerase chain reaction
Rad50	radiosensitive mutant 50
Rad51	radiosensitive mutant 51
Rb	retinoblastoma protein
RING	Really Interesting New Gene
RNA	ribonucleic acid
SNP	single nucleotide polymorhpism

S/T-Q	serine/glutamine or threonine/glutamine
TCGA	The Cancer Genome Atlas
YFP	yellow fluorescent protein
WT	wild type
Zn+	zinc

Chapter 1

INTRODUCTION

DNA repair, genome integrity and cancer

Genome instability and cancer

The integrity of our genome is constantly being challenged by various endogenous sources, such as reactive oxygen species, and exogenous sources, such as gamma-irradiation (γIR).[95,170] If not properly repaired, these alterations to the DNA can result in genome instability.[20,58,118,152] Genome instability, also commonly referred to as chromosome instability, is an established hallmark of cancer and postulated to contribute to the development and progression of malignancies.[57,118] Genome instability can manifest as structural and/or numerical chromosome aberrations, such as chromosome and chromatid breaks, insertions, deletions, translocations, amplifications, aneuploidy, and polyploidy.[48,66] Chromosome aberrations are linked to tumorigenesis, as translocations resulting in the activation of oncogenes, such as Myc,[32] or the deletion/mutation and subsequent loss of function of tumor suppressors, such as p53 or Rb,[103,140] lead to cancer development. Cells acquiring the necessary genomic alterations permitting uncontrolled proliferation and evasion of apoptosis are predisposed to becoming transformed into a cancer cell.[57,140,153] Therefore, elucidating the cellular processes contributing to genome instability is essential for understanding tumorigenesis.

The DNA damage response

Although the causes of genome instability are incompletely understood, data show DNA damage and the inability to properly repair this damage contribute to genome instability, with double-strand DNA breaks being the most detrimental.[1,9,153] Double-strand DNA breaks can occur from endogenous sources, such as a collapsed replication fork, or exogenous sources, such as ionizing radiation.[65,80] Since these lesions are extremely detrimental to the integrity of the genome, cells have evolved sophisticated DNA damage signaling and repair pathways to repair these lesions and maintain genome integrity. Following DNA damage, DNA damage response (DDR) proteins must detect the DNA lesion, signal to other proteins that damage has occurred, and coordinate repair of the damaged DNA to maintain genome integrity.[179]

MRN complex and ATM

DNA double-strand breaks are sensed by the Mre11-Rad50-Nbs1 (MRN) DNA repair complex (Figure 1).[153] The Nbs1 subunit of the MRN complex is involved in the activation of the central DNA damage-induced kinase ATM through a direct interaction.[89,128] ATM then phosphorylates hundreds of proteins involved in the DNA damage response, including Nbs1, Mdm2, Mdmx, p53, histone H2AX, and others (Figure 1).[107] The function of these specific phosphorylation events is not entirely clear, but phosphorylation can alter protein stability, protein:protein interactions, and localization, which could allow ATM to be a master regulator of the DNA damage response. This is supported by the finding that ATM has hundreds of potential substrates, many of which have been implicated in DNA repair.[107] For example,

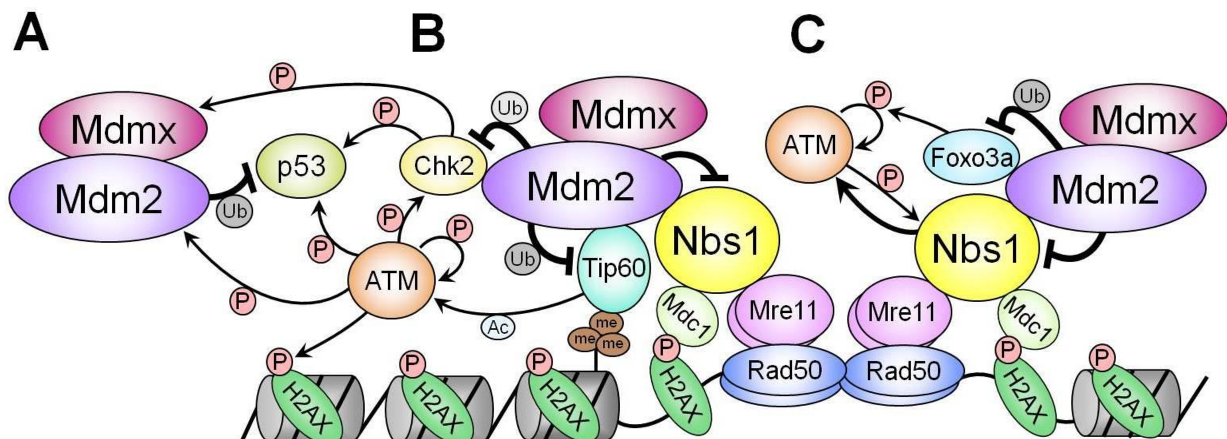

Figure 1. Mdm2 interacts with and inhibits multiple proteins in the DNA damage response. (A) Mdm2 inhibits p53 partially thorough the ubiquitin (Ub) ligase activity of Mdm2. The inhibition of p53 by Mdm2 is relieved, in part, through phosphorylation of Mdm2 and p53 following DNA damage. (B, C) After DNA damage, such as a double-strand break, Mdm2 binds and inhibits Nbs1, which is retained at damaged DNA through Mdc1 association and is part of the Mre11-Rad50-Nbs1 DNA repair complex. ATM activation is catalyzed by the Mre11-Rad50-Nbs1 complex, acetylation (Ac) by Tip60, which is associated with chromatin by binding tri-methylated (me) histone H3, and Foxo3a, which facilitates ATM autophosphorylation. ATM then phosphorylates (P) hundreds of proteins in the DNA damage response. Mdm2 inhibits Nbs1 and ubiquitinates Tip60 and Foxo3a, each of which would result in suppression of ATM activation. Mdm2 also indirectly leads to Chk2 kinase ubiquitination, unless it is phosphorylated. Mdm2 normally slows DNA break repair through interactions with Nbs1, likely to allow repair to be accomplished with high fidelity. The interaction of Mdm2 with the proteins illustrated above and the influence these have on DNA repair and genome stability is incompletely resolved, but support a larger role for Mdm2 in tumor development and progression beyond p53. (Reproduced from Melo and Eischen 2012) [109]

phosphorylation of histone H2AX by ATM acts as a docking site for DNA repair proteins, including many modified by ATM, to localize to and repair the DNA lesion.[19]

Nbs1 is important in the regulation of the MRN complex activity because it anchors Mre11/Rad50 to the site of damage through phospho-dependent interaction with Mdc1, which is bound to γH2AX.[96] Rad50 is thought to hold together broken DNA ends by its hook and coiled-coil domains (Figure 2).[59,153] The ATPase activity of Rad50 promotes the 3'-5' exonuclease activity of Mre11 which serves to prepare the DNA for repair.[82] The importance of Nbs1 is highlighted when the Mre11-binding domain in Nbs1 is absent, because Mre11 and Rad50 are no longer able to associate with sites of DNA damage.[87,127] These proteins are important for the DNA repair process as deletions or inactivating mutations in Nbs1, Mre11, Rad50, or ATM result in suboptimal DNA repair that translates into patients having increased radiosensitivity and increased cancer incidence.[20,143,152] Therefore, identifying proteins that modulate or inhibit the function of these DNA repair proteins is important for understanding the DNA repair process and the implications in tumorigenesis. From my thesis work that is described in Chapter 3, Mdmx is likely one such protein.

Altered DNA repair and genome instability

Alterations in DNA damage signaling or in the timing or fidelity of DNA repair can result in mild to severe chromosome abnormalities.[134,171,172] For example, DNA breaks can serve as substrates for translocations and fusions, which occur when breaks are not repaired correctly. Cells with irreparable DNA damage or chromosome aberrations incompatible with life undergo apoptosis, which can be mediated by the p53 tumor

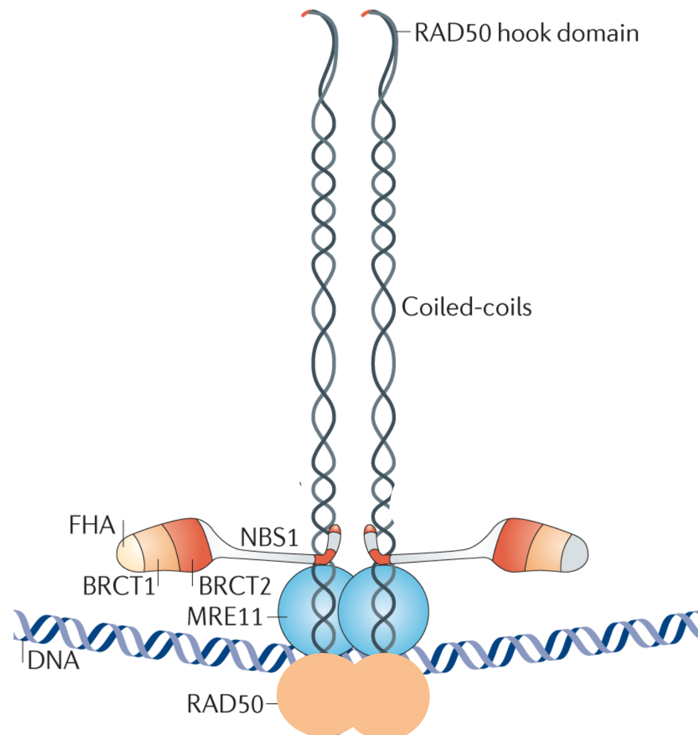

Figure 2. The Mre11-Rad50-Nbs1 complex at the site ofDNA damage. Mre11 interacts with the site of DNA damage. Rad50 is a large flexible protein with a hook domain at one end. This domain in Rad50 is predicted to allow Rad50 to hold broken DNA ends or chromosomes together. Nbs1 binds to Mre11 directly and has various domains available to interact with other DNA repair proteins. (Modified from Stracker and Petrini 2011)[153]

suppressor.[91,119,178]

If a cell acquires the ability to bypass the DNA damage cell cycle checkpoints and apoptosis, such as by inactivating the p53 pathway, then this can lead to transformation and tumorigenesis. This is evident in patients with mutations in various DNA repair proteins (Table 1). Specifically, individuals born with mutations in p53 or other genes in the DNA damage response have increased genome instability and incidence of cancer. For example, humans born with mutations in p53 develop Li-Fraumeni syndrome and have a greatly increased rate of cancer development.[11,91,100] Moreover, people with mutations in any of the three components of the Mre11-Rad50-Nbs1 DNA repair complex or the DNA damage-induced kinase ATM have impaired DNA damage response signaling and DNA repair, which leads to increases in chromosomal abnormalities, genome instability, radiation sensitivity, and cancer incidence.[20,143,152] Therefore, proteins capable of inhibiting p53 and other proteins in the DNA damage response would result in loss of DNA damage-induced cell cycle checkpoints and apoptosis, and increased genome instability. Identifying such proteins is important, as these effects would provide a significant advantage to cells during tumorigenesis. In this dissertation, I describe the identification of a previously uncharacterized function of Mdmx which increases genome instability independent of p53. Therefore, overexpression of Mdmx would provide multiple advantages during tumorigenesis, as it both promotes genome instability and inhibits the p53 tumor suppressor.

Table 1. DNA repair proteins and disease.

Protein	Function	Disease	Symptoms
ATM	Amplification of DNA damage response signal	Ataxia Telangiectasia	Increased cancer predisposition; genome instability; inhibited DNA repair
Brca1	Promotes DNA resection and HR	Hereditary Breast-Ovarian Cancer Syndrome	Inhibition in HR but increase in error-prone NHEJ; Increased cancer predisposition; genome instability
Brca2	Mediates recombination	Hereditary Breast-Ovarian Cancer Syndrome	Inhibition in HR but increase in error-prone NHEJ; Increased cancer predisposition; genome instability
Mre11	Recognizes double-strand DNA break; processes broken DNA ends through endo- and exonuclease activity	Ataxia Telangiectasia-like disorder	Inhibition in double-strand DNA break repair; Increased incidence of cancer
Nbs1	Recognizes double-strand DNA break; activates ATM	Nijmegen Breakage Syndrome (NBS)	Inhibition in double-strand DNA break repair; Increased incidence of cancer
Rad50	Recognizes double-strand DNA breaks; connect DNA ends		Inhibition in double-strand DNA break repair; Increased incidence of cancer
p53	Tumor suppressor; activated following DNA damage; activates cell cycle checkpoint or induces apoptosis	Li-Fraumeni Syndrome	Genome instability; Increased incidence of cancer

The p53 pathway, genome instability and cancer

The p53 pathway

In addition to the DDR, the p53 pathway is a major mechanism used by the cell to prevent genome instability. p53 is a transcription factor activated following various cellular stressors including DNA damage from a variety of sources.[74,91,101] Upon activation, p53 can control the transcription of genes involved in cell cycle arrest, such as p21, and apoptosis, such as PUMA.[40,43] This quality allows p53 to play a crucial role in protecting genome stability and has earned p53 the designation of "guardian of the genome".[83] Because p53 prevents genome instability, it is not surprising that p53 is mutated or deleted in half of all cancers. Furthermore, in virtually all human cancers, p53 is indirectly inactivated.[91,104,166] Inactivation can occur through overexpression of the two major negative regulators of p53: Mdm2 and Mdmx.

Mdm2 and Mdmx in cancer

Mdm2 and Mdmx are overexpressed in many malignancies, but it is unclear whether increased levels of Mdm2 and Mdmx affect the same proteins in the same manner as normal levels.[42,104,165] While many human cancers may select for Mdm2 and/or Mdmx overexpression as a mechanism of p53 inhibition, instances of tumors with mutated or deleted p53 and elevated levels of Mdm2 and/or Mdmx have been reported.[22,42]

It is likely that the frequency of increased levels of Mdm2 in human malignancies is grossly underestimated due to technical issues with Mdm2 specific antibodies and a lack of scoring 2-4 fold increased protein as elevated. Because of the identification of a

single nucleotide polymorphism(SNP309 T/G) in the promoter of *MDM2* that results in an increase in *MDM2* mRNA and protein, it is now appreciated that 2-4 fold increased levels of Mdm2 enhances cancer susceptibility in specific patient populations,[14] and a mouse model of this polymorphism supports this finding.[133] Prior to the SNP309 discovery, 4-fold elevated expression of Mdm2 in *Mdm2* transgenic mice was shown to increase tumor formation. As for Mdmx, two individual *Mdmx* transgenic mouse lines where *Mdmx* expression was driven by the chicken β-actin promoter spontaneously developed malignancies,[174] whereas another mouse model using an artificial promoter in the *Rosa26* locus to express a tagged version of Mdmx did not.[34] The differences in cancer susceptibility among the *Mdmx* transgenic lines are potentially due to differences in the levels of Mdmx expression, but this has not been confirmed. The *in vivo* data could also suggest that the role of Mdmx in tumorigenesis is more complicated than what is known for Mdm2. These and other results indicate Mdm2 and Mdmx overexpression contribute to tumor development and there is likely a p53-independent component. Since the occurrence of Mdm2 or Mdmx overexpression in tumors with mutated or deleted p53 is not as common as with wild-type p53, the p53-independent functions for Mdm2 and Mdmx have not been extensively investigated. Thus for my dissertation, I investigated a novel p53 independent function of Mdmx, and these results are described in Chapter 3.

Regulation of p53 by Mdm2

Mdm2 was discovered as a gene amplified more than 50-fold on double-minute chromosomes in a tumorigenic mouse cell line.[44] Mdm2 is an E3-ubiquitin ligase that

negatively regulates p53 by ubiquitinating it and targeting it for proteosomal degradation, binding to it and preventing its transactivation, or shuttling it out of the nucleus (Figure 3). The importance of this regulation of p53 by Mdm2 was discovered using mouse models. *Mdm2*-null mice are not viable as loss of *Mdm2* is embryonic lethal, but this phenotype is rescued when p53 is also deleted. [71,115,126] In combination with a number of studies focusing on the Mdm2:p53 interaction, these studies have resulted in the regulation of p53 being assigned as the major role of Mdm2. However, additional functions of Mdm2 have been described, by the Eischen lab and others, as described in detail below.

Mdm2 and proliferation

In the absence of p53, transgenic mice overexpressing *Mdm2* developed an altered tumor spectrum compared to *p53*-null mice, suggesting p53-independent effects of Mdm2.[70] Given that Mdm2 inhibits p53, it would be reasonable to assume that cells with wild-type p53 should have a growth/survival advantage when Mdm2 is overexpressed. However, investigators have repeatedly attempted to generate stable cell lines overexpressing Mdm2 in the presence of wild-type p53, but they have been unsuccessful.[18,23,79] These findings were supported by the inability of cells to tolerate high levels of Mdm2 *in vivo*.[34,70] However, cell lines with mutated or deleted p53 can survive prolonged Mdm2 overexpression. Although there are data to suggest that Mdm2 overexpression induces a p53-independent cell cycle arrest that blocks proliferation,[36] others have shown Mdm2 can target for degradation Rb,[144,158] the cell cycle inhibitor p21,[67,176] and Foxo3a,[47,175] a transcriptional regulator of cell cycle

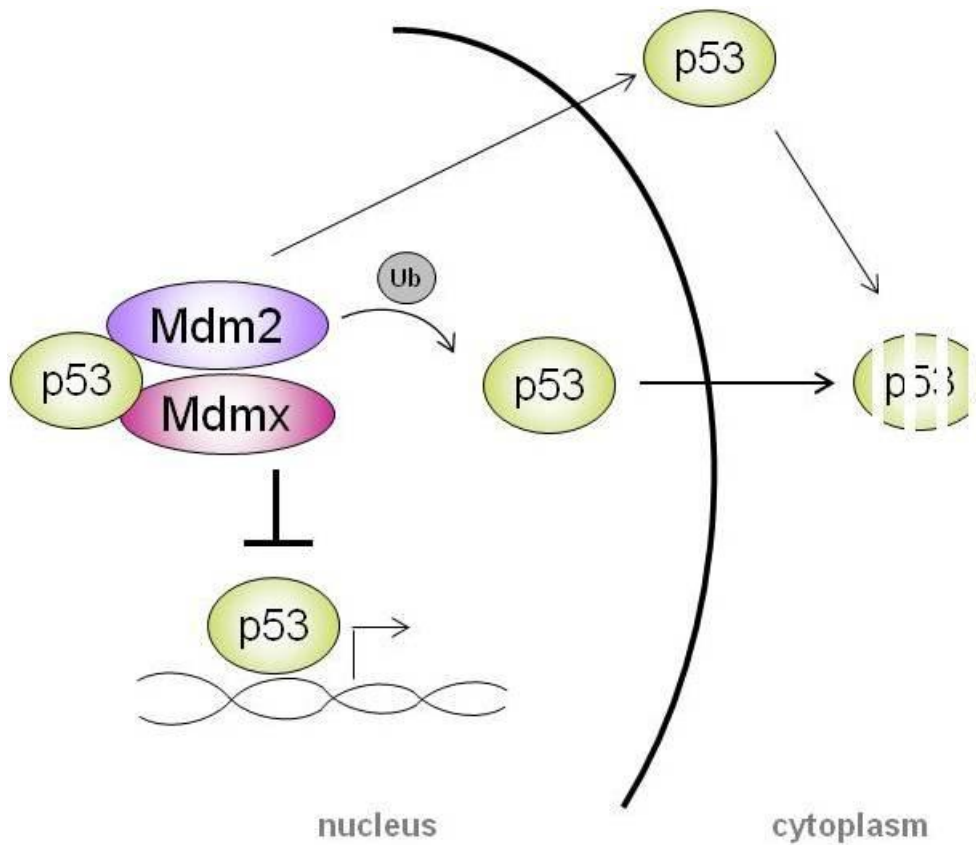

Figure 3. Mdm2 and Mdmx inhibit p53. The heterodimers Mdm2 and Mdmx bind p53 and promote its inactivity and/or degradation. This can be accomplished by Mdm2 and Mdmx preventing p53 transactivation activity. Mdm2 can ubiquitinate p53 to induce proteosomal degradation. Mdm2 can shuttle p53 out of the nucleus to prevent its activity, and this may also lead to proteosomal degradation in the cytoplasm.

inhibitors, for proteosomal degradation, which would increase proliferation.

Mdm2 and genome instability

Importantly for tumorigenesis, Mdm2 overexpression is reported to also induce genome instability. Specifically, Mdm2 overexpression (2-4 fold) in cultured fibroblasts retaining wild-type p53 results in centrosome hyperamplification and chromosome instability.[23] Cells with elevated Mdm2 levels have increased chromosome and chromatid breaks. In addition, the Eischen lab has shown *Mdm2* transgenic mice with ~4-fold chromosome fusions, aneuploidy, and polyploidy *in vivo* prior to the development of cancer with the incidence of these chromosomal alterations increasing with age.[98,169] In contrast, when levels of Mdm2 are decreased, as in *Mdm2* heterozygous primary murine fibroblasts containing wild-type p53, there are decreased chromosome aberrations and increased genome stability.[168] Although these studies did not evaluate whether the genome instability observed resulted from the regulation of p53 by Mdm2 versus a p53-independent function of Mdm2, altering Mdm2 levels appears to have significant consequences on chromosome stability.

Mdm2 and genome instability independent of p53

There is evidence Mdm2 impacts genome stability independent of its interactions with p53. Increased polyploidy was observed *in vivo* in mammary epithelial cells with elevated Mdm2 levels, and this was also evident in Mdm2-overexpressing mammary epithelial cells lacking p53.[23] Notably, as a result of a non-biased screen, the Eischen lab discovered the Mre11-Rad50-Nbs1 (MRN) DNA repair complex as novel proteins

interacting with Mdm2 (Figure 1). Mdm2 overexpression was shown to inhibit DNA double-strand break repair, and this was mediated through a novel, direct interaction between Mdm2 and Nbs1 that was independent of p53.[3,16] Regardless of p53 status, increased levels of Mdm2, but not Mdm2 lacking its Nbs1-binding domain, caused delays in DNA break repair and induced chromosomal abnormalities and genome instability.[16] These data demonstrated Mdm2-induced genome instability can be mediated through Mdm2:Nbs1 interactions and is independent from its association with p53.

Regulation of p53 by Mdmx

Mdmx was discovered as a protein that could interact with p53.[148] Mdm2 and Mdmx are homologous proteins with the highest homology being in the N-terminal p53 binding domain and the C-terminal RING domain (Figure 4).[148] Like Mdm2, loss of Mdmx is embryonic lethal, and this can be rescued with deletion of p53.[126] Similar to Mdm2, Mdmx is important in the regulation of p53 during embryogenesis, but it appears to be in a non-redundant manner. While loss of Mdm2 causes lethality by increasing apoptosis, loss of Mdmx results in a decrease in cellular proliferation.[112,126] Although Mdmx and Mdm2 are family members and both regulate p53, there is evidence from conditional knockouts of each that Mdmx and Mdm2 also have tissue-specific functions in a variety of cell types, including cardiac, neuronal progenitor, mouse embryonic fibroblasts (MEFs), smooth muscle, and hematopoietic.[45,126] [13,46,55,99,106]

Further characterization of the function of Mdmx has provided some insight into possible explanations for the differences between Mdm2 and Mdmx. Despite having a

RING domain, Mdmx has not demonstrated any ubiquitin ligase activity *in vivo*.[64,150] Instead, Mdmx negatively regulates p53 by preventing its transactivation function rather than affecting its stability.[150] Although Mdmx can bind to and regulate p53 independent of Mdm2, the heterodimerization of Mdm2 and Mdmx through their RING domains cooperates to effectively block p53 transcriptional activity (Figure 3).[56,61,94,122,147,150,151,156] Collectively, these findings suggest p53-independent functions of Mdmx, which may or may not require Mdm2. Little is known about Mdmx, especially functions not requiring Mdm2 or centering on its regulation of p53. My thesis work has focused on identifying and describing a novel function of Mdmx independent of both p53 and Mdm2.

p53-independent functions of Mdmx

Due to the negative regulation of p53, overexpression of Mdmx is expected to alter genome integrity, at least somewhat. However, p53-independent effects of Mdmx on genome stability were recently revealed. In contrast to Mdm2, loss of Mdmx is associated with genome instability. Specifically, in *Mdmx/p53*-double null mice, the development of spontaneous tumors occurred earlier than in *p53*-null mice.[106] Murine embryonic fibroblasts (MEFs) from *Mdmx/p53*-double null mice exhibited multipolar mitosis and subsequent loss of chromosomes. These genomic alterations were not observed in *Mdm2/p53*-double null MEFs, indicating the loss of Mdmx promotes genome instability in a non-redundant manner from Mdm2.[106] Interestingly, high levels of Mdmx were shown to be lethal in developing embryos, and although genome instability was not investigated, this could not be rescued with loss of p53.[34] These data

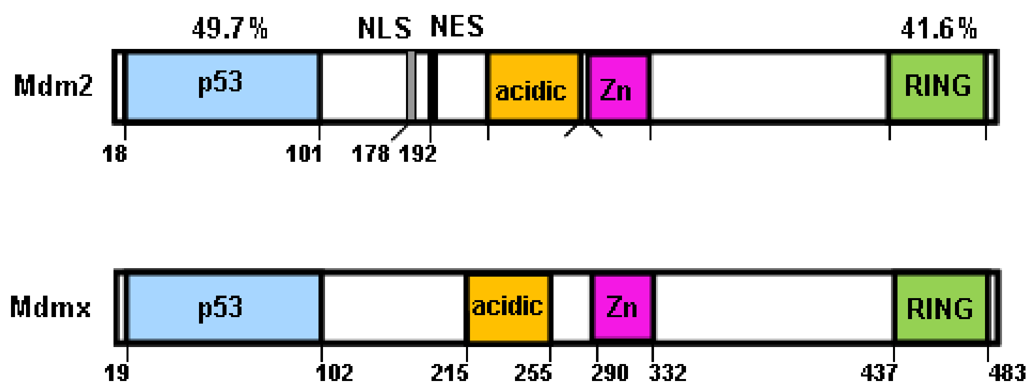

Figure 4. Mdm2 and Mdmx domains. Schematic representation of Mdm2 and Mdmx highlighting the conserved regions between the two. The highest percentage of conservation is found in the p53 binding domain and the RING domain. The p53 binding, and acidic, Zinc (Zn), and RING domains and NLS and NES are shown.

suggest that the levels of Mdmx are important for maintaining genome stability. Combined with the fact that Mdm2 and Mdmx are homologous proteins, these findings led to my hypothesis that Mdmx overexpression promotes genome instability. With relatively little known about Mdmx, it was important to determine the function of Mdmx in genome instability, as this could contribute to tumorigenesis. In the studies highlighted in Chapter 3 of this dissertation, I identify and characterize a novel function of Mdmx that is independent of both p53 and Mdm2 and negatively impacts genome instability.[22]

Mdm2 and Mdmx and the DNA damage response

Mdm2 and Mdmx interact with a number of DNA repair proteins, but the consequences of these interactions are not well characterized, which led me to investigate the influence of Mdmx in the DNA damage response (Figure 1). DNA damage induces complex signaling cascades including many proteins, the identity of which depends on the type and extent of the DNA damage. For example, following double-strand DNA breaks, phosphorylation of Mdm2, Mdmx, and p53 induces Mdm2:Mdmx interaction, inhibits Mdm2:p53 association, and may inhibit or promote Mdmx:p53 binding.[25,27,28,37,110,132] DNA damage also leads to an increase in the E3 ubiquitin ligase activity of Mdm2 that appears to be regulated by phosphorylation and is reported to result in the degradation of both itself and Mdmx.[27,28,121] However, due to commonly used antibodies that do not recognize phosphorylated Mdm2, the destabilization of Mdm2 following DNA damage is controversial.[26,41] Mdmx can, paradoxically, facilitate Mdm2-mediated p53 ubiquitination, while it is also reported to stabilize p53.[25,37,121,124] This difference in p53 regulation between Mdm2 and Mdmx is

postulated to be due to the relative levels of Mdmx and Mdm2 in the cell.[37]

Mdm2 has been associated with the DNA repair process through its regulation of p53. Ultimately, post-translational modifications of Mdm2, Mdmx, and p53 are thought to allow p53 activation following DNA damage. There is evidence the DNA damage-induced kinase ATM phosphorylates Mdm2, Mdmx and p53.[7,27,35,49,76,108,110,116,131,132,142] While the effect of these phosphorylation events on Mdm2 and Mdmx remains to be determined, ATM phosphorylation of p53 is important for its function following DNA damage.[108] It is thought that phosphorylation of Mdm2, Mdmx, and p53 by ATM prevents their interaction following DNA damage thus allowing for the activation of p53 and subsequent induction of cell cycle arrest or apoptosis.[27,49,110]

In addition to affecting the interaction between Mdm2 and Mdmx, DNA damage-induced phosphorylation events and other post-translational modifications also likely promote alterations in protein interactions between Mdm2, Mdmx and other proteins involved in the DNA damage response. For example, Mdm2 has been reported to associate with, as well as alter the function or stability of, proteins other than p53 in the DNA damage response, such as ATM and Nbs1 (Figure 1).[3,35] Specifically, Mdm2 was shown to co-immunoprecipitate with ATM, but it is unresolved as to whether this interaction is direct or mediated through another protein both bind, such as Nbs1.[35] Mdm2 is also reported to bind to the ATM substrate Chk2 and facilitate its ubiquitination and degradation independent of the ubiquitin ligase activity of Mdm2 (Figure 1). Phosphorylation of Chk2 by ATM following DNA damage decreases its ability to bind Mdm2 which presumably prevents Chk2 from being targeted by Mdm2 for degradation, and thus, allows Chk2 to function in the DNA damage response.[73] Indirectly through

ATM, Chk2 also phosphorylates Mdmx, which is thought to promote the degradation of Mdmx following DNA damage; however, through my thesis work, I have described in Chapter 3 that at least a fraction of Mdmx protein is actually associating with chromatin following DNA damage, rather than being degraded.[25,86,131,132]

There are a number of studies linking Mdm2 to the DNA damage response independent from its role as a regulator of p53. Activation of ATM is catalyzed by the Mre11-Rad50-Nbs1 complex.[88,89] The Eischen lab has shown Mdm2 directly binds Nbs1 of the Mre11-Rad50-Nbs1 complex and inhibits DNA double-strand break repair independent of p53 (Figure 5).[3,16] Unexpectedly, this function of Mdm2 did not require its E3 ubiquitin ligase activity or its RING domain.[3,16] Only the region of Mdm2 binding to Nbs1 was necessary and sufficient to delay double-strand DNA break repair. Moreover, the Mdm2 binding domain of Nbs1 was required for Mdm2 to inhibit DNA repair. Mdm2 overexpression results in a delay in phosphorylation of ATM targets. The histone H2AX is a phosphorylation target, which provides an anchor for many DNA damage response proteins, including the MRN DNA repair complex, yet Mdm2 overexpression results in a delay in phosphorylation of H2AX (γH2AX).[16] Therefore, the mechanism for this novel function of Mdm2 appears to be inhibition of the early DNA damage response signal mediated by ATM. Data also show a delay in the appearance of phosphorylated ATM/ATM target protein foci at sites of DNA damage, and this required the Nbs1 binding domain of Mdm2.[16] Therefore, Mdm2 inhibition of DNA break repair is independent of its E3 ubiquitin ligase activity, but dependent on its interaction with Nbs1 and affects ATM signaling.

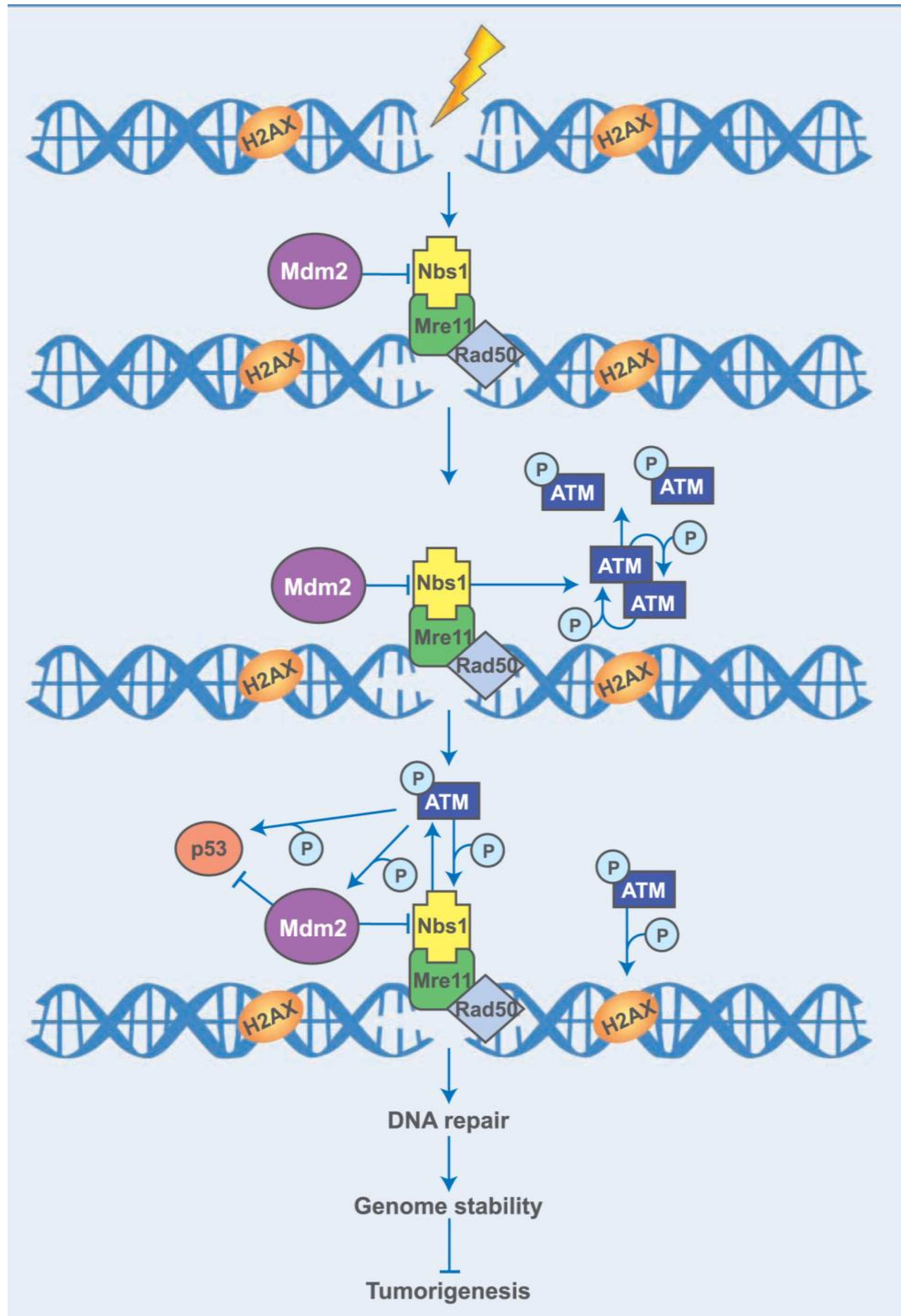

Figure 5. Mdm2 inhibits DNA repair through Nbs1. Mdm2 binds to Nbs1 of the MRN DNA repair complex. Nbs1 is involved in the activation of ATM promoting the phosphorylation of ATM. Through interacting with and inhibiting Nbs1, Mdm2 inhibits DNA repair and genome instability to promote transformation. (Reproduced from Bouska and Eischen 2009)[15]

In addition to altering protein interactions, DNA damage changes the cellular localization of both Mdm2 and Mdmx. While under homeostatic conditions Mdm2 is primarily a nuclear protein, appearing diffusely nuclear by immunofluorescence, but following gamma irradiation (γIR), shows a punctate staining pattern partially co-localizing with sites of DNA damage and the Mre11-Rad50-Nbs1 complex.[3] Mdm2 has also been reported to associate with and be stabilized by Mdc1, a scaffold protein mediating the interaction between Nbs1 and phosphorylated H2AX to retain Nbs1 at sites of DNA damage (Figure 1).[62,96] Moreover, recent biochemical data from the Eischen lab show increased levels of Mdm2 at chromatin and bound to Nbs1 after γIR (unpublished data).

As for Mdmx, it primarily resides in the cytoplasm and moves into the nucleus after DNA damage. Phosphorylation of Mdmx promotes its nuclear localization, and this is thought to result in its degradation mediated by ubiquitination from Mdm2.[86,92,121] Although it has been shown to participate, Mdm2 is not required for the nuclear localization of Mdmx, as it can move to the nucleus in cells lacking Mdm2.[86,92] The consequences of Mdmx nuclear localization remain unresolved, but my findings described in Chapter 3 would implicate Mdmx has a role at chromatin following DNA damage, providing a partial explanation as to why Mdmx may be localized to the nucleus. Additionally, Mdmx was recently shown to bind p53 and facilitate its binding to the Mdm2 promoter following DNA damage.[12] While Mdmx may function with or without Mdm2 to regulate p53, it is also likely able to regulate other proteins independent of p53 and Mdm2 in the nucleus, such as the MRN complex, which is further described in this dissertation (Chapter 3). Therefore, elucidating the function of Mdm2 and Mdmx

localization after DNA damage, and the proteins with which they associate, will provide important insight into their genomic functions and reveal further links to the processes involved in maintaining genome stability. While the Eischen lab had started to reveal the role of Mdm2 in the DNA damage response, little was known about the function(s) of Mdmx at the start of my thesis work. Due the ability of Mdmx to enhance other functions of Mdm2, I hypothesized Mdmx could affect the DNA damage response through influencing Mdm2. Therefore, I investigated the possible contribution of Mdmx to the DNA damage response and genome instability (described in Chapter 3).

Targeting Mdm2 and Mdmx in cancer

Because Mdm2 and/or Mdmx are overexpressed in cancer, the development of pharmacological approaches to prevent Mdm2 and Mdmx from binding to p53, and subsequently activating p53 to induce tumor cell apoptosis or cell cycle arrest, has been a recent focus in cancer therapeutics.[29,146] Nutlin is a small molecule inhibitor which specifically binds to Mdm2 and prevents binding of Mdm2 to p53 (Figure 6).[162] As anticipated, reactivation of wild-type p53 in cancer cells by Nutlin treatment resulted in apoptosis. MI-63 is another small molecule inhibitor of Mdm2 that has yielded similar results as Nutlin.[39] While proof of principle of the therapeutic promise of these inhibitors has been achieved *in vitro* and in mouse models, it has not yet translated into clinical success. Although these inhibitors hold promise for treating cancers with wild-type p53, half of human cancers lack functional p53 at the time of diagnosis.[91] An additional result of Nutlin is an increase in Mdm2 protein levels that does not depend on p53. Because the Eischen lab had previously shown that increased Mdm2 inhibited double-

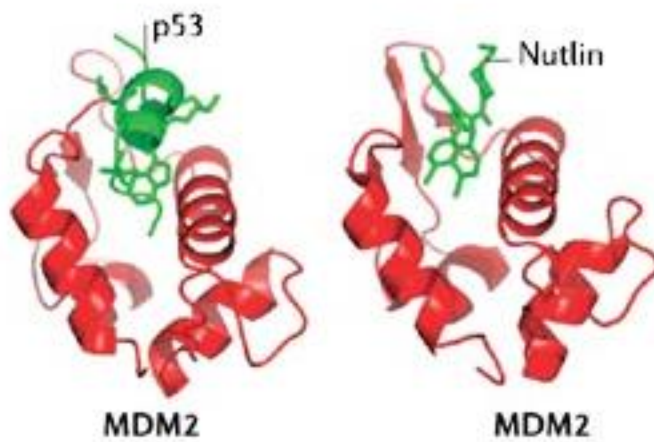

Figure 6. Nutlin prevents the interaction between Mdm2 and p53. In the p53 binding domain of Mdm2, p53 binds in a specific hydrophobic pocket. The small molecule inhibitor Nutlin was designed to mimic p53 and bind to this same hydrophobic pocket to prevent Mdm2 from binding to p53. (Reproduced from Nalepa *et al* 2006)[117]

strand DNA break repair, this led us to hypothesize that increasing Mdm2 levels with a small-molecule (e.g., Nutlin and MI-63) and combining this with genotoxic chemotherapeutics may be an effective therapy in cancers with inactivated p53. Studies have demonstrated promise in therapies designed to capitalize on the p53-independent functions of Mdm2 in genome instability. For example, γIR of sarcoma cells with inactive p53 overexpressing Mdm2 showed a decrease in colony formation.[27] There are numerous studies highlighting the cooperative effect of Nutlin and genotoxic agents in cancer cells with wild-type p53.[5,8,30,52,113,120,139,167] A few studies have also demonstrated the cooperation of Nutlin and genotoxic agents in cells with inactivated p53.[4,31,120,155,167] For example, Nutlin radiosensitized p53 mutant or null prostate cancer cells, but the mechanism behind this effect of Nutlin is not entirely understood.[155]

In pancreatic cancer, Nutlin has also been shown to cooperate with topoisomerase II inhibitors, which cause double-strand DNA breaks.[31] Nutlin also increased cell death in sarcoma and cutaneous T cell lymphoma cells with or without functional p53 in the presence of the DNA-damaging agents Doxorubicin or Cisplatin.[102,120] Recently, it was shown Gemcitabine, which causes DNA replication stress and subsequent DNA breaks, cooperated with MI-63 and increased tumor cell death.[68] These and other data suggest a therapeutic benefit of combining DNA damaging agents with small molecules/compounds that increase the levels of Mdm2 to kill cancer cells expressing or lacking functional p53, and a similar approach could be taken with Mdmx. While evidence supports the benefit of this cooperation, the mechanism behind the effect of Nutlin in the absence of p53 is not clearly established. Being able to elucidate this mechanism provides valuable knowledge for the

development of future therapeutic regimens. In Chapter 4, I describe my data demonstrating a novel p53-independent mechanism of Nutlin that contributes to cooperativity with genotoxic agents.

It is well established that Mdm2 and Mdmx are oncogenes that negatively regulate p53, but the p53-independent functions of these proteins remains to be completely understood. Studies from our lab and others, the p53-independent functions of Mdm2 and Mdmx likely affect genome instability, and targeting proteins that negatively affect genome stability are potential chemotherapeutic targets. While some p53-independent functions of Mdm2 have been determined, very little is known about Mdmx. It has been observed that Mdmx can enhance the p53-regulatory function of Mdm2. Therefore, my hypothesis was that Mdmx inhibits double-strand DNA break repair and promotes genome instability independent of p53, and this genome instability can cooperate with genotoxic agents to promote cell death in cancers with inactivated p53. In Chapter 2 of this dissertation, I will explain the materials and methods used for my studies. Chapter 3 describes my data characterizing a novel role for Mdmx in genome instability through inhibition of double-strand DNA break repair. The potential for this family of proteins being a therapeutic target in cancer is described in Chapter 4, with a focus on Mdm2, specifically. Finally, in Chapter 5, I summarize my findings and suggest future directions for these studies. Overall, the work described in this dissertation provides significant knowledge for understanding tumorigenesis and developing new cancer therapeutics.

CHAPTER 2

MATERIALS AND METHODS

Cell culture

293T cells, *p53*$^{-/-}$, *p53*$^{-/-}$*Mdm2*$^{-/-}$, and ΔB/ΔB murine embryonic fibroblasts (MEFs) were cultured as previously described.[22] SKOV3, OVCAR5 and OVCAR8 ovarian cancer cell lines were cultured as described previously.[78] *p53*$^{-/-}$, *Arf*$^{-/-}$ and *p53*$^{-/-}$*Mdm2*$^{-/-}$ murine embryonic fibroblasts (MEFs) were isolated and cultured as previously described.[180] *Atm*$^{-/-}$*Arf*$^{-/-}$ were provided by Dr. Michael Kastan (Duke University), and the *p53*$^{-/-}$*Mdmx*$^{-/-}$ MEFs were provided by Dr. Stephen Jones (University of Massachusetts). 293T cells were cultured as described by the American Type Culture Collection (Manassas, VA). Human retinal epithelial cells were provided by Dr. David Cortez (Vanderbilt University) and were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 0.258% sodium bicarbonate and 10% fetal bovine serum.

Chemotherapeutic agents and antibodies

Nutlin (Sigma) was resuspended in DMSO at a concentration of 17.2 mM. Nutlin described refer to the entire mixture, but only half of the total concentration used represents the active enatomer A. Cisplatin (Sigma) was resuspended in 0.9% NaCl at a concentration of 5 mM. Etoposide (Sigma) was resuspended in DMSO (Sigma) at a concentration of 50 mM. The following antibodies were used for Western blot analysis:

cleaved Caspase 3 (Cell Signaling), and β-actin. Mdm2 antibodies previously described.[3,16] Mdmx antibody was from Sigma-Alderich (St Louis, MO). For immunofluorescence, pSer139 H2AX (Millipore) or pSer1981 ATM (Rockland) were used for primary detection and Alexa Fluor 594 (Invitrogen) was used for secondary detection.

Comet assay

Cells were treated with the specified drug, where indicated, 24 hours prior to γ-IR (^{137}Cs source). Neutral comet assays were performed as described previously.[16] MEFs and human retinal epithelial cells infected with a bicistronic retrovirus encoding YFP alone, YFP and Mdmx or MxΔRING, or GFP and Mdm2 were exposed to 5 Gy γIR (^{137}Cs source). Neutral comet assays were performed at intervals as previously described to detect double-strand DNA breaks.[3,16] A minimum of two independent experiments were performed for all analyses. Statistical significance was determined by the student's *t*-test.

Immunofluorescence

Cells were seeded onto coverslips and, where indicated, treated for 24 hours with the specified drug. Following γ-IR, cells were fixed and γH2AX and pS/T-Q foci were detected and analyzed as previously described.[22] γH2AX or pS-T/Q foci were detected by immunofluorescence and analyzed as previously described.[16] The number of γH2AX and pS-T/Q foci per cell for at least 40 cells per individual condition was quantified. A minimum of three independent experiments were performed for all

analyses. Statistical significance was determined using a confidence interval of 95%.

Proliferation and apoptosis assays

For MTT assays, cells ($5x10^3$-$1x10^4$) were plated in a 96-well plate 24 hours before treatment with chemotherapeutic agents (Nutlin, Etoposide, Cisplatin or a combination) for 72 hours. MTT reagent was added to the wells at intervals and processed as previously described.[54] To assess apoptosis, cells were treated with chemotherapeutic agents for 72 hours. Cells were collected and protein lysates were processed as previously described.[22] Cleaved Caspase 3 was subsequently evaluated by Western blot analysis.

Plasmids, viral vector construction, and infection

Wild-type full-length N-terminal FLAG-tagged murine Mdmx in the pBabe retroviral construct was generously provided by Dr. Jean-Christophe Marine (VIB, Belgium). Using PCR and restriction enzyme digests of wild-type full-length Mdmx, deletion mutants of Mdmx containing amino acids 1-345 (MxΔRING) or 346-489 (Mx 346-489) were generated and cloned into the pcDNA3 vector. Wild-type full-length Mdmx and both Mdmx mutants were also subcloned into the MSCV-IRES-YFP retroviral vector. The MSCV-Mdm2-IRES-GFP retroviral vector was previously reported.[3,16] Retroviruses were generated, and MEFs were infected as previously reported.[180] YFP and GFP fluorescence was evaluated by flow cytometry. HA-tagged Mdmx was generated by restriction digest, cloning it into the pJ3H vector and then subcloning the HA-tagged cDNA into the pcDNA3 vector. Vectors encoding FLAG-tagged wild type

and deletion mutants of Nbs1 were generated previously.[16]

Metaphase preparation and analysis

p53$^{-/-}$ and p53$^{-/-}$Mdm2$^{-/-}$ MEFs, which were between passage 8-28 and thus, had a relatively stable tetraploid genome, were infected with retroviral vectors encoding YFP, YFP and full-length Mdmx, or YFP and MxΔRING. Metaphases were prepared approximately 48 hours later and analyzed as previously described.[16,169] A minimum of two independent experiments were performed and the results pooled. Statistical significance was determined using a Fisher's exact test with a p value of <0.05 considered significant.

Transfections and protein analyses

HA-tagged full-length Mdmx or Mdmx deletion mutants or FLAG-tagged full-length Nbs1 or Nbs1 deletion mutant constructs were transiently transfected into 293T cells. Whole cell protein lysates were generated and proteins were immunoprecipitated as previously described.[3,16] For protein analysis of γH2AX, MEFs were exposed to 5 Gy of γIR, and at the indicated intervals, cells were harvested and lysates generated as previously described.[16] For chromatin fractionation, at indicated intervals following exposure to 5 Gy of γIR, the chromatin fraction was isolated from MEFs as previously described.[17] For all lysates, following separation of proteins by SDS-PAGE and transfer to nitrocelluose, proteins were subjected to western blot analysis as previously described.[3,16]

In vitro transformation assays

p53$^{-/-}$ *and p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs infected with bicistronic retroviral vectors encoding YFP, YFP and full-length Mdmx, or YFP and MxΔRING were resuspended in 0.6% agarose/media and placed in 6 well plates in triplicate on 0.8% agarose/media. Approximately 2 weeks later, colonies were counted. At least two independent experiments consisting of three replicates each were performed. A student's *t*-test determined statistical significance.

The Cancer Genome Atlas (TCGA) data analysis

Gene copy number variation, mutations, and mRNA expression in cancer data sets were accessed using cBioPortal (www.cbioportal.org) October 2013. As described on the cBioPortal website, gene copy number variation was determined by TCGA based on the GISTIC 2.0 or RAE algorithm, and mutations were determined by whole exome sequencing. *MDMX* mRNA levels were evaluated from microarray or RNA sequencing by RNASeqV2, and Z-scores of 2, which represent 2 standard deviations from the mRNA levels in a reference tissue (normal or blood), were considered significantly increased.

Chapter 3

MDMX PROMOTES GENOMIC INSTABILITY INDEPENDENT OF p53 AND MDM2

Carrillo AM, Bouska A, Arrate MP, Eischen CM. Mdmx promotes genomic instability independent of p53 and Mdm2. *Oncogene* 2014.

Introduction

The E3 ubiquitin ligase Mdm2, a negative regulator of the p53 tumor suppressor, is frequently overexpressed in many human malignancies.[114,137,165] While it is established Mdm2 inhibits p53, studies have identified other functions of Mdm2 impacting genome stability that are independent of p53 and contribute to tumorigenesis.[3,16,70,97] We, specifically, discovered a novel p53-independent association between Mdm2 and Nbs1 of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex.[3,16,109] The MRN complex is important in sensing and processing double-strand DNA breaks, as well as activating downstream DNA damage response proteins that mediate or signal for DNA repair, such as the DNA damage-induced kinase ATM.[10,38,88,153,159] Through binding Nbs1, elevated levels of Mdm2 delay double-strand DNA break repair independent of its E3 ubiquitin ligase activity and p53 status. Ultimately, Mdm2 overexpression results in increased genome instability occurring independent of its regulation of p53.[16,109]

Genome instability is a hallmark of cancer and is a known contributor to tumorigenesis.[57,118] While the exact causes of genomic instability continue to be elucidated, altered DNA repair has been implicated as a significant contributing factor.[153] DNA breaks must be repaired efficiently and accurately or structural

abnormalities, such as chromosome breaks or fusions, can result. This is evident in patients with congenital mutations in DNA repair proteins, such as NBS1 and ATM. Cells from these patients have increased chromosomal fragility, and the patients themselves have an increased incidence of tumor formation.[20,153,161]

MDMX, also known as MDM4, is overexpressed or amplified in at least 15% of human cancers.[33,90,135,165] Most studies evaluated mRNA, which likely underestimates the frequency of Mdmx protein overexpression. Elevated levels of levels of *MDMX* mRNA are detected in 65% of retinoblastomas and the same percentage of cutaneous melanomas overexpress MDMX protein.[51,85] Mdmx was initially described as an Mdm2 homologue with high conservation in the N-terminal p53 binding domain and the C-terminal RING domain;[148] however, Mdmx appears to differ functionally from Mdm2. While Mdmx negatively regulates p53, it does so through inhibiting p53 transcriptional activity rather than promoting p53 degradation through ubiquitination, as is the case for Mdm2.[104,150] Additionally, it has been shown through their RING domains, Mdmx binds Mdm2 and enhances the ability of Mdm2 to regulate p53.[129,147,148,150,156] Although Mdmx inhibits p53 function, homozygous Myc-tagged Mdmx transgene expression was embryonic lethal, and this could not be rescued with deletion of *p53*,[34] suggesting a p53-independent function of Mdmx in development. Moreover, human tumors with MDMX overexpression may also have inactivated p53,[53,81,135,165] which indicates a potential oncogenic benefit to the cancer cells from Mdmx overexpression in addition to its inhibition of p53.

Recent studies established Mdmx influences genome stability independent of p53.[105,106] However, its function in genome instability remained unclear. In this study,

we identified a novel p53-independent function of Mdmx leading to genome instability. We determined Mdmx associates with Nbs1 of the MRN DNA repair complex, and its overexpression delayed DNA damage response signals and double-strand DNA break repair. Elevated Mdmx levels increased chromosome and chromatid breaks as well as promoted transformation *in vitro.* These effects of Mdmx did not require either p53 or Mdm2 and revealed a novel p53- and Mdm2-independent function of Mdmx that would contribute to tumorigenesis.

Results

A subset of human cancers both overexpress MDMX and inactivate p53.

Elevated MDMX levels through amplification or overexpression have been reported for numerous human malignancies.[33,51,85,90,135,165] Studies have also shown MDMX is overexpressed in cancers with mutated p53, indicating they are not mutually exclusive events during tumorigenesis.[53,81,135,165] Analysis of the data in The Cancer Genome Atlas (TCGA) provides further evidence that a certain fraction of multiple cancers have overexpressed *MDMX* (amplification or mRNA) concurrently with inactivated (mutated or deleted) p53 (Table 2).[24,50] Specific cancers, such as ovarian serous cystadenocarcinoma and lung squamous cell carcinoma show a high frequency (77-90%) of tumors that overexpress *MDMX* also have inactivated p53. For breast cancer, 27% that overexpress *MDMX* showed concurrent p53 inactivation (Table 2), and 30% (12 of 40 cell lines) of an aggressive form of breast cancer have increased Mdmx protein together with mutated p53.[81]

Table 2. Increased *MDMX* co-occurs with mutant/deleted *p53* in multiple cancer types[a]

Cancer Type	Samples	*MDMX* increased[b]	mut/del[c] p53 in *MDMX* increased[b]
Breast, Invasive Carcinoma	825	144/825 (18%)	39/144 (27%)
Colon and Rectum Adenocarcinoma	195	11/195 (6%)	2/11 (18%)
Glioma, Lower Grade	213	15/213 (7%)	6/15 (40%)
Glioblastoma Multiforme	135	18/135 (13%)	7/18 (39%)
Head and Neck Squamous Cell Carcinoma	295	18/295 (6%)	10/18 (56%)
Hepatocellular Carcinoma	110	35/110 (32%)	2/35 (6%)[d]
Lung Adenocarcinoma	129	20/129 (16%)	4/20 (20%)
Lung Squamous Cell Carcinoma	177	13/177 (7%)	10/13 (77%)
Ovarian Serous Cystadenocarcinoma	316	40/316 (13%)	36/40 (90%)
Sarcoma (soft tissue)	149	26/149 (18%)	2/26 (8%)
Skin, cutaneous melanoma	225	34/225 (15%)	3/34 (9%)
Stomach Adenocarcinoma	197	12/197 (6%)	7/12 (58%)
Uterine Corpous Endometroid Carcinoma	233	39/233 (17%)	11/39 (28%)

[a] TCGA data was obtained October 2013 through the cBioPortal for Cancer Genomics

[b] Gene amplification or mRNA levels two standard deviations (Z score ≥2) above control

[c] Mutations include nonsense, missense, insertions, and small deletions; deletions determined by copy number alterations

[d] *p53* deletion only, mutation data not available for these samples

In the tumors with increased levels of MDMX, 10 of the 13 cancer types evaluated also had alterations in p53 in at least 10% of them (Table 2). These data, together with previous studies, suggest certain tumor types or subsets of specific tumor types may select for co-alteration of Mdmx and p53.

Elevated Mdmx increases genome instability independent of p53.

Genome instability is observed in many malignancies and is considered a hallmark of cancer.[57] Mdmx has been shown to enhance functions of Mdm2 (e.g. negative regulation of p53;[129,147,148,150,156]); therefore, we postulated elevated Mdmx levels would enhance the ability of Mdm2 to promote genome instability. Since Mdmx could potentially influence genome stability through its regulation of p53, we utilized *p53*$^{-/-}$ murine embryonic fibroblasts (MEFs) for these studies. To mimic the cancer situation, we overexpressed Mdmx in *p53*$^{-/-}$ MEFs with a bicistronic retrovirus encoding HA-tagged Mdmx and YFP or as a control, YFP alone.

Metaphases were then evaluated for chromosome aberrations. Mdmx overexpression alone significantly increased the prevalence of cells with breaks (chromatid or chromosome; Figure 7). There was a 4.9-fold increase in chromatid breaks and a 2.8-fold increase in chromosome breaks in *p53*$^{-/-}$ MEFs with elevated Mdmx compared to control *p53*$^{-/-}$ MEFs (Figure 7). These results indicate elevated levels of Mdmx were capable of inducing genome instability, and this was independent of p53.

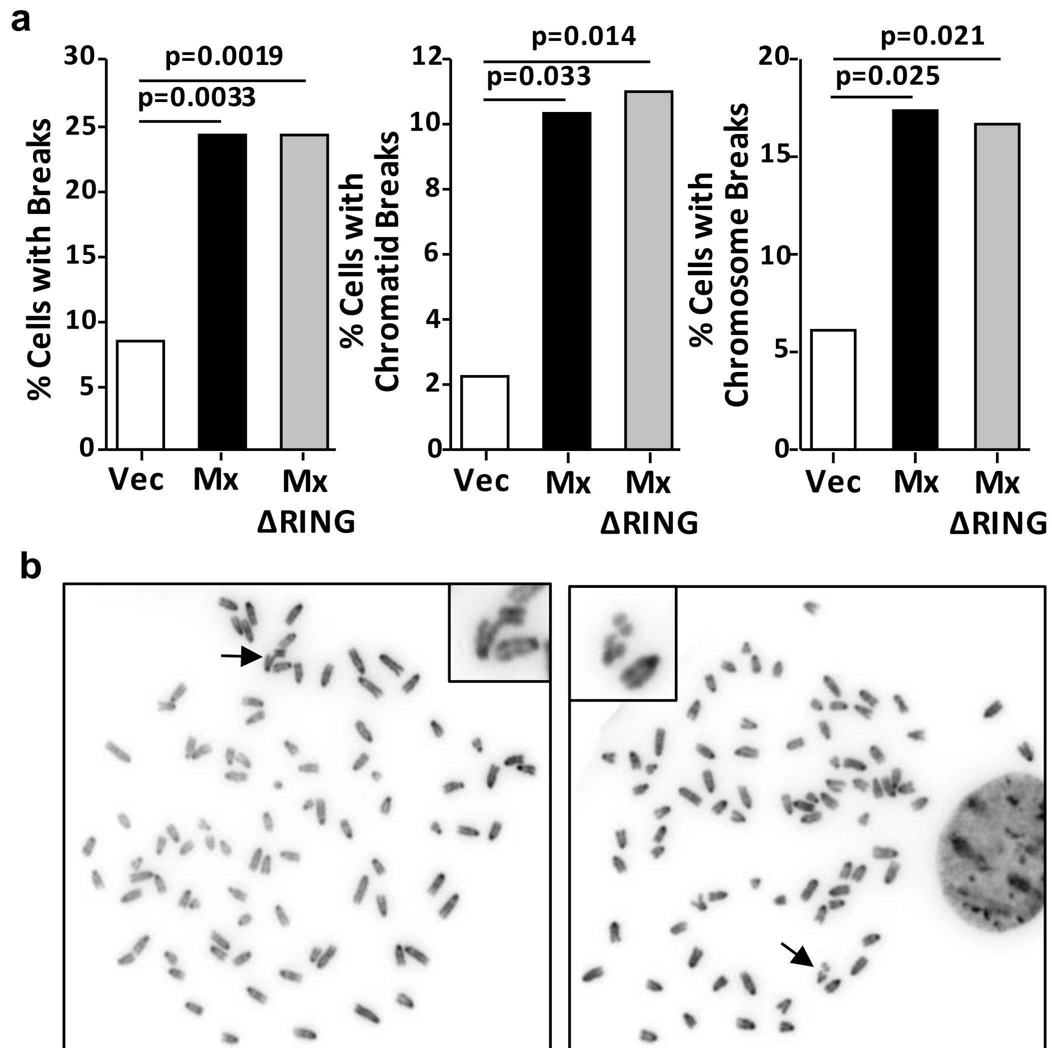

Figure 7. Elevated Mdmx levels promote genome instability independent of p53. *p53*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (Vector; n=96), YFP and HA-Mdmx (n=98), or YFP and HA-Mdmx1-345 (MxΔRING; n=119). a) Metaphases were evaluated for chromosome aberrations, and the percentage of cells with the indicated aberration is graphed. Statistical significance was determined using the Fisher's exact test. b) Representative pictures of a chromatid (left) and a chromosome (right) break in *p53*$^{-/-}$ MEFs overexpressing Mdmx. Inset is an expanded view of the aberration marked by the arrow.

Mdmx inhibits double-strand DNA break repair independent of p53 and Mdm2.

Alterations in DNA repair have been linked to genome instability.[153] Previously, we reported Mdm2 overexpression inhibits double-strand DNA break repair independent of its regulation of p53.[3,16] To determine if Mdmx was modulating genome instability through its interactions with Mdm2, we first needed to determine the effects of elevated Mdmx levels on double-strand DNA break repair. *p53*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding either YFP alone, FLAG-tagged Mdmx and YFP, or Mdm2 and GFP. Following the induction of double-strand DNA breaks using gamma irradiation (γIR), DNA repair was then assessed using neutral comet assays. While 79% of the control *p53*$^{-/-}$ MEFs were able to repair double-strand DNA breaks within 90 minutes post γIR, only 42% of the *p53*$^{-/-}$ MEFs overexpressing Mdmx repaired their DNA breaks (Figure 8). The percentage of cells overexpressing Mdmx that repaired their DNA damage was similar to that of cells overexpressing Mdm2 (Figure 8). Therefore, Mdmx inhibited DNA double-strand break repair independent of p53. To determine if Mdmx was acting through Mdm2, we generated a deletion mutant of Mdmx (1-345 amino acids) that lacked its RING domain (MxΔRING), and thus, was unable to heterodimerize with Mdm2.[147,156] We assessed the ability of this mutant to increase genome instability and inhibit DNA repair. Surprisingly, the Mdmx mutant was also able to promote chromatid and chromosome breaks and inhibit double-strand DNA break repair analogous to full-length Mdmx (Figures 7 and 8). Although it has been well established that Mdm2 and Mdmx bind through their RING domains,[104,147,156] it was recently postulated that Mdm2 and Mdmx may associate indirectly through the Mdm2-binding protein, Arf.[93]

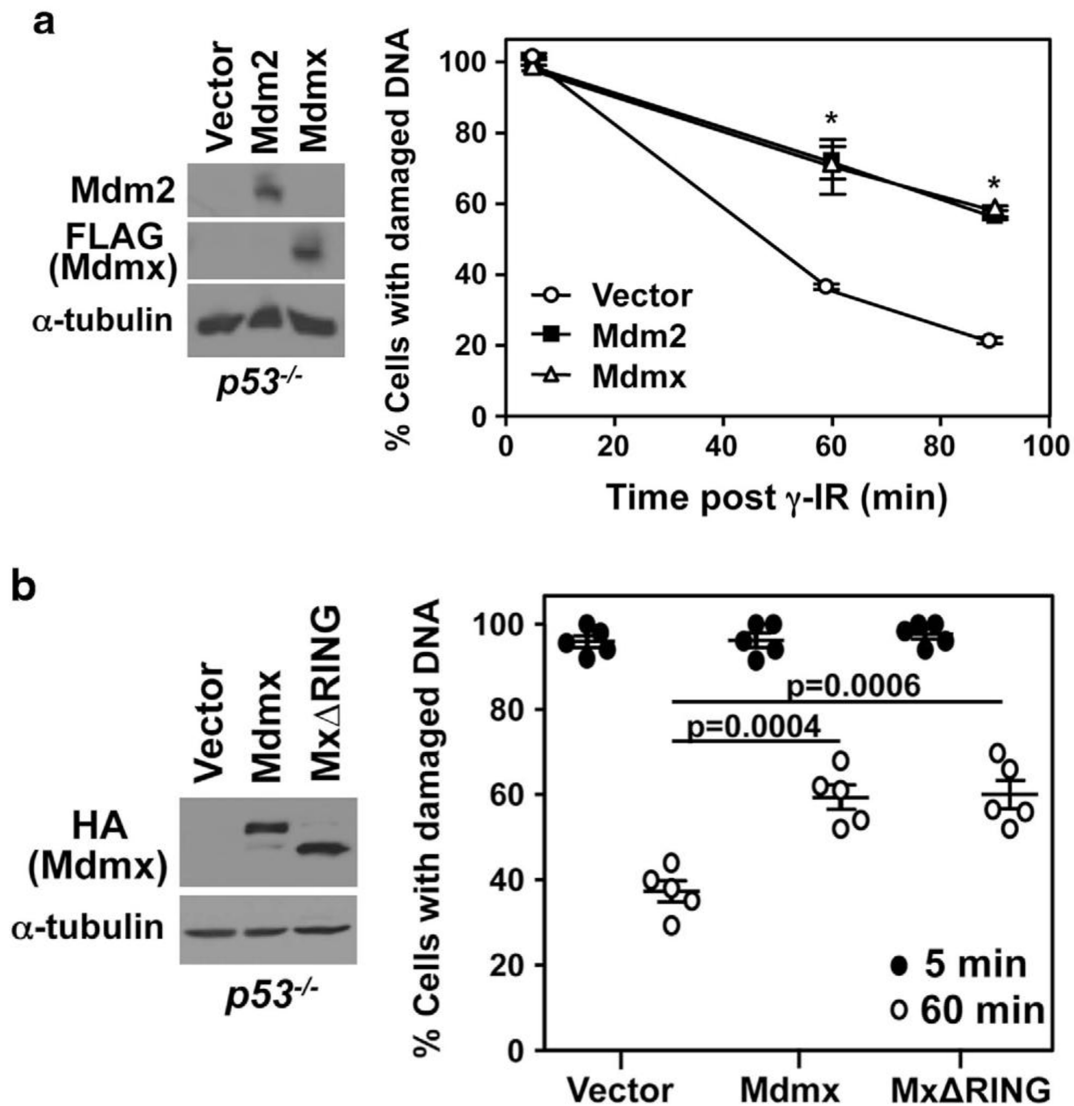

Figure 8. Mdmx overexpression inhibits double-strand DNA break repair independent of p53. *p53*$^{-/-}$ (a,b) MEFs were infected with a bicistronic retrovirus encoding YFP alone (Vector), GFP and HA-Mdm2, YFP and FLAG-Mdmx, YFP and HA-Mdmx, or YFP and HA-MxΔRING. Western blots of the indicated proteins were performed. Following 5 Gy of γIR, neutral comet assays were performed at intervals. All data are a mean of a minimum of three independent experiments. Each symbol in b is the mean of an individual experiment and the line is the mean of all experiments. Error bars represent SEM. *$p \leq 0.01$ in A; each compared to vector control; student's *t*-test

To determine whether Arf could influence the ability of Mdmx to inhibit DNA repair, we evaluated *Arf*$^{-/-}$ MEFs, which contain p53. Both wild-type Mdmx and MxΔRING effectively delayed double-strand DNA break repair in cells lacking Arf (Figure 9).

To definitively address the requirement of Mdm2 for this effect of Mdmx on DNA repair, we utilized MEFs lacking Mdm2. In *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs, Mdmx overexpression resulted in a similar inhibition of DNA break repair as in the *p53*$^{-/-}$ MEFs (Figure 10). Furthermore, full-length Mdmx and the mutant form of Mdmx (MxΔRING) similarly inhibited DNA break repair in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs (Figure 10). Also, MxΔRING overexpression in human retinal epithelial cells that have Arf and p53 also resulted in a delay in DNA break repair (Figure 11). Therefore, Mdmx inhibits double-strand DNA break repair in different cell types, and this can occur independent of p53, Arf, and Mdm2. The data also illustrate the functional domain of Mdmx responsible for the inhibition in DNA repair is within amino acids 1-345.

Mdmx delays the DNA damage response and alters the early DNA damage response signaling.

Following double-strand DNA break repair, the histone variant H2AX is rapidly phosphorylated (γH2AX). The presence of this phosphorylation site indicates activation of the DNA damage response pathway and is a marker of DNA double-strand break sites.[10,141] Therefore, we evaluated γH2AX foci and protein levels immediately following and at later times after induction of DNA breaks to assess initiation of the DNA damage response and subsequent resolution of the DNA damage. Similar to Mdm2 overexpression, *p53*$^{-/-}$ MEFs with elevated Mdmx or the MxΔRING mutant were

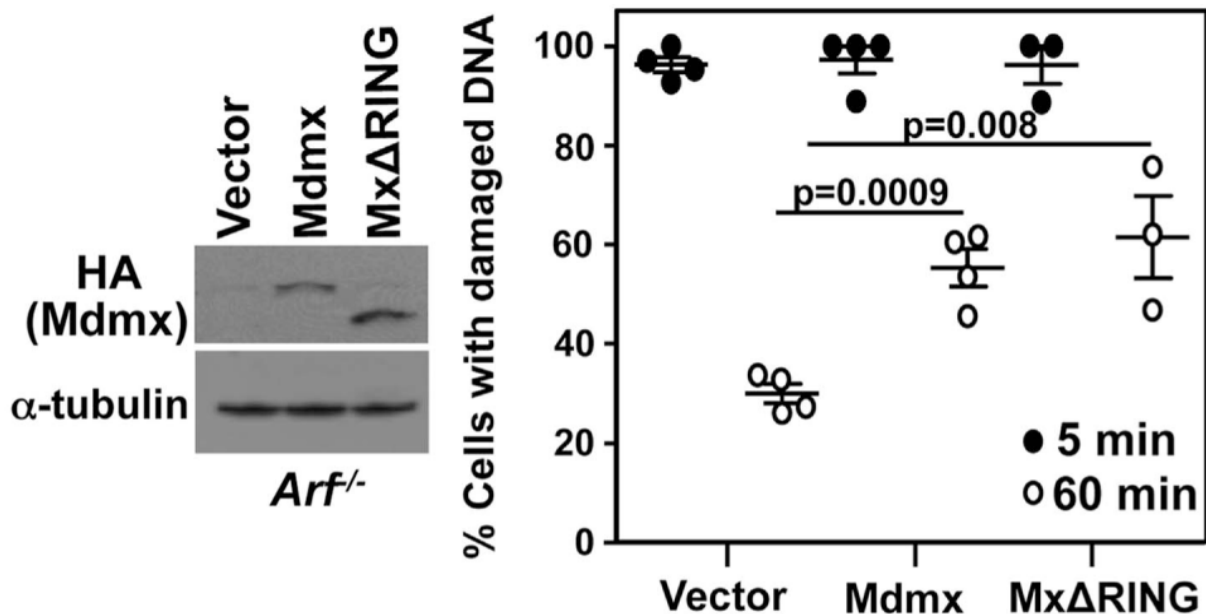

Figure 9. Mdmx overexpression inhibits double-strand DNA break repair independent of ARF. *ARF*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (Vector), GFP and HA-Mdm2, YFP and HA-Mdmx, or YFP and HA-MxΔRING. Western blots of the indicated proteins were performed. Following 5 Gy of γIR, neutral comet assays were performed at intervals. Data are a mean of a minimum of three independent experiments. Each symbol is the mean of an individual experiment and the line is the mean of all experiments. Error bars represent SEM. *student's *t*-test

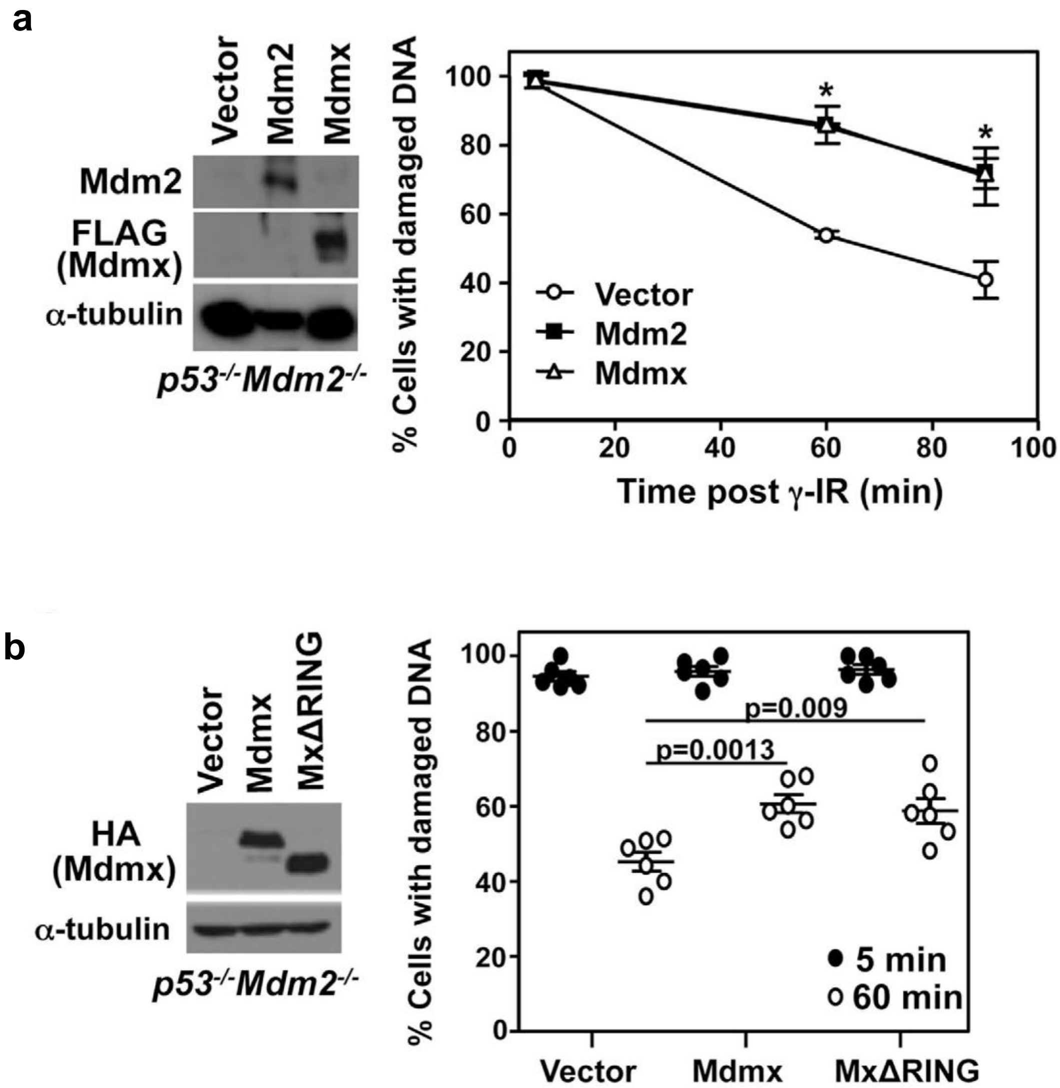

Figure 10. Mdmx overexpression inhibits double-strand DNA break repair independent of p53 and Mdm2. $p53^{-/-}Mdm2^{-/-}$ (a,b) MEFs were infected with a bicistronic retrovirus encoding YFP alone (Vector), GFP and HA-Mdm2, YFP and FLAG-Mdmx, YFP and HA-Mdmx, or YFP and HA-MxΔRING. Western blots of the indicated proteins were performed. Following 5 Gy of γIR, neutral comet assays were performed at intervals. All data are a mean of a minimum of three independent experiments. Each symbol in b is the mean of an individual experiment and the line is the mean of all experiments. Error bars represent SEM. *$p < 0.05$ in A; each compared to vector control; student's t-test

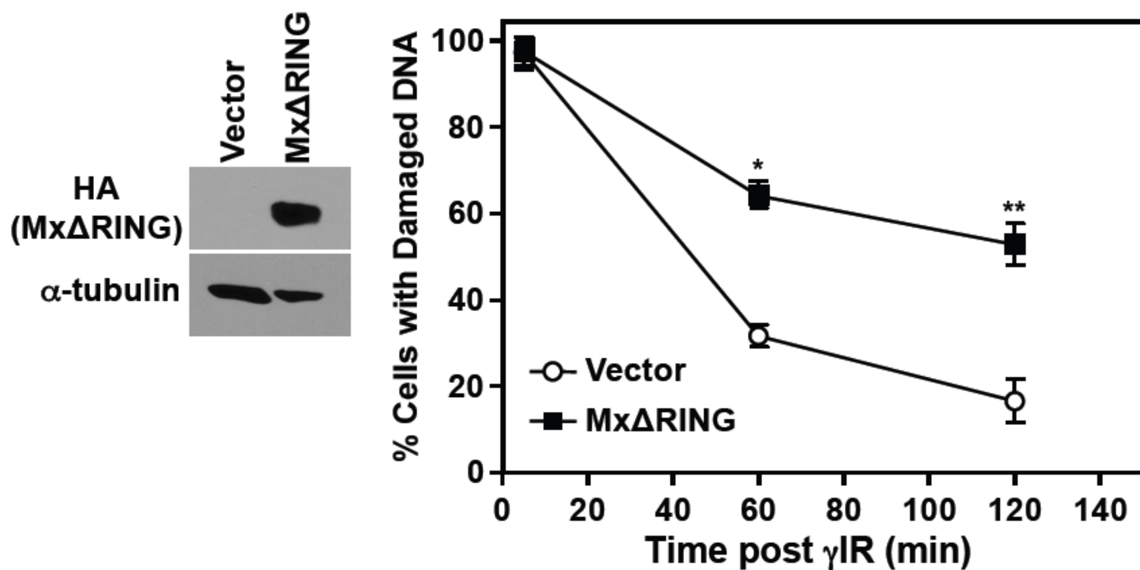

Figure 11. Mdmx overexpression inhibits double-strand DNA break repair in human retinal epithelial cells. Human retinal epithelial cells (HREC) were infected with a bicistronic retrovirus encoding YFP alone (vector) or YFP and HA-MxΔRING. Western blots of the indicated proteins were performed. Following 5 Gy of γIR, neutral comet assays were performed at the indicated intervals. Data are the mean of at least two independent experiments. Error bars are the SEM. *p=0.048, **p=0.007 student's *t*-test.

inhibited in their ability to form γH2AX foci immediately following γIR, as they showed a decreased mean number of foci compared to control 10 minutes post γIR (Figures 12-17). In addition, the resolution of γH2AX foci was also delayed by Mdmx expression. Cells overexpressing Mdmx or MxΔRING had an increased mean number of foci 150 minutes after γIR compared to the vector control cells (Figure 12, Figure 13, Figure 16). Similar results were obtained in MEFs lacking Mdm2 and in human retinal epithelial cells that express ARF and p53 (Figure 12, Figure 14-16).

Ranking of γH2AX foci in individual cells from a representative experiment of $p53^{-/-}$ and $p53^{-/-}Mdm2^{-/-}$ MEFs revealed that there was an overall reduction in foci at 5 minutes and an overall increase in foci at 150 minutes following γIR in the Mdmx and the MxΔRING expressing MEFs (Figure 16). These data further establish the inhibitory effects of Mdmx on the DNA damage signaling response and illustrate that the mean values of the γH2AX foci in Figure 12 were not due to extremes at either end. Moreover, evaluation of γH2AX protein showed that levels were reduced in cells overexpressing either Mdmx or MxΔRING early (10 and 30 min) after γIR compared to control (Figure 17). Later (75 min) after γIR, γH2AX protein levels were elevated compared to controls in both $p53^{-/-}$ and $p53^{-/-}Mdm2^{-/-}$ MEFs (Figure 17). Taken together, these data indicate increased levels of Mdmx inhibit an early DNA damage signaling response, leading to an extension of this response and a delay in the resolution of the DNA breaks.

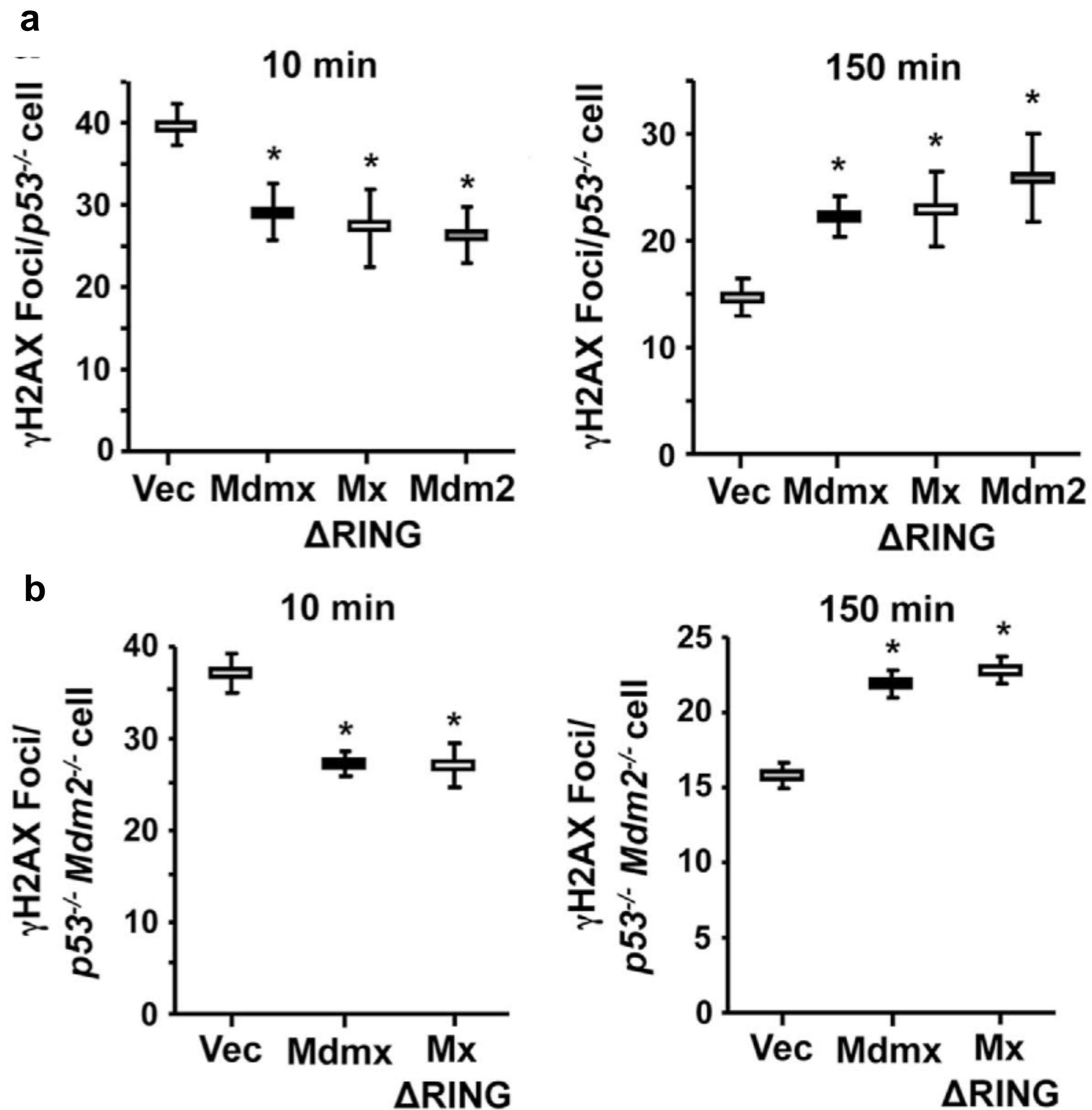

Figure 12. Elevated Mdmx impairs the early DNA damage response signal. *p53*$^{-/-}$ (a) or *p53*$^{-/-}$*Mdm2*$^{-/-}$ (b) MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector), YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING. Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX was performed. The number of foci per cell was quantified. The mean of at least three independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%.

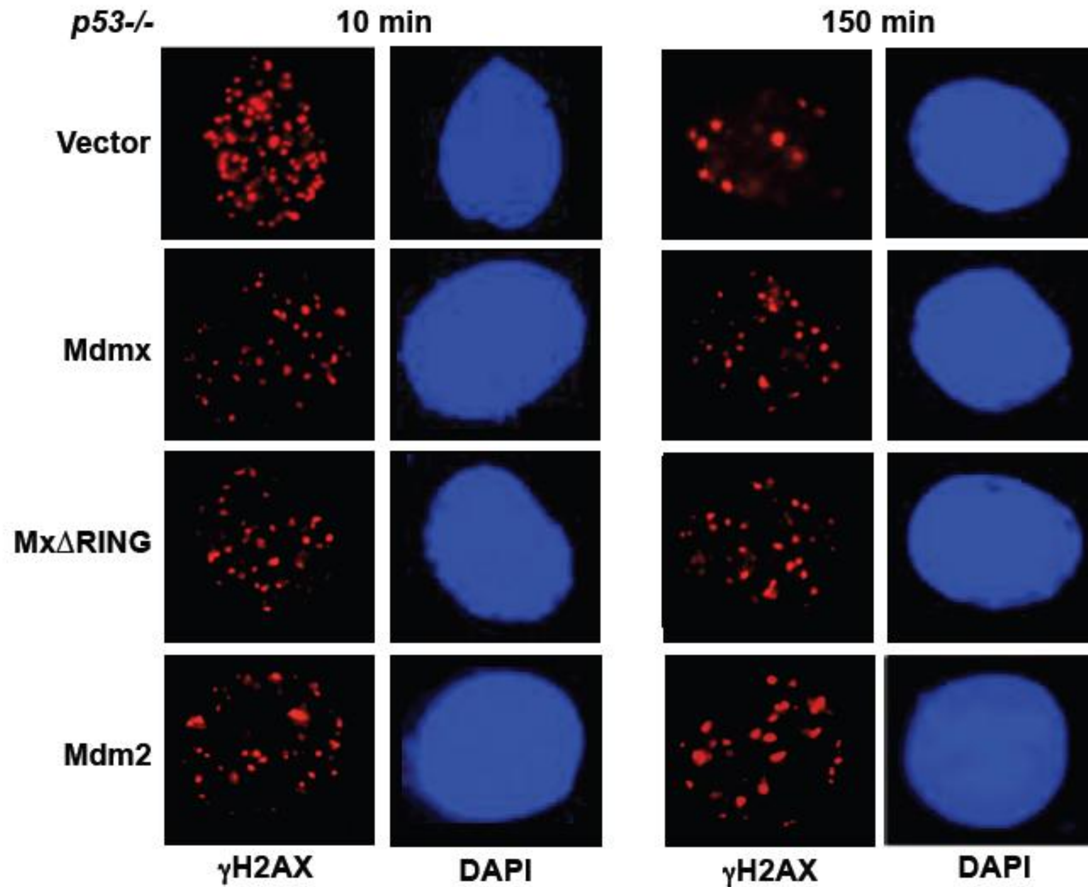

Figure 13. Elevated Mdmx inhibits γH2AX foci formation and resolution independent of p53. *p53*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector, V), GFP and full-length Mdm2, YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX was performed. Representative pictures of γH2AX (red) and DAPI (blue).

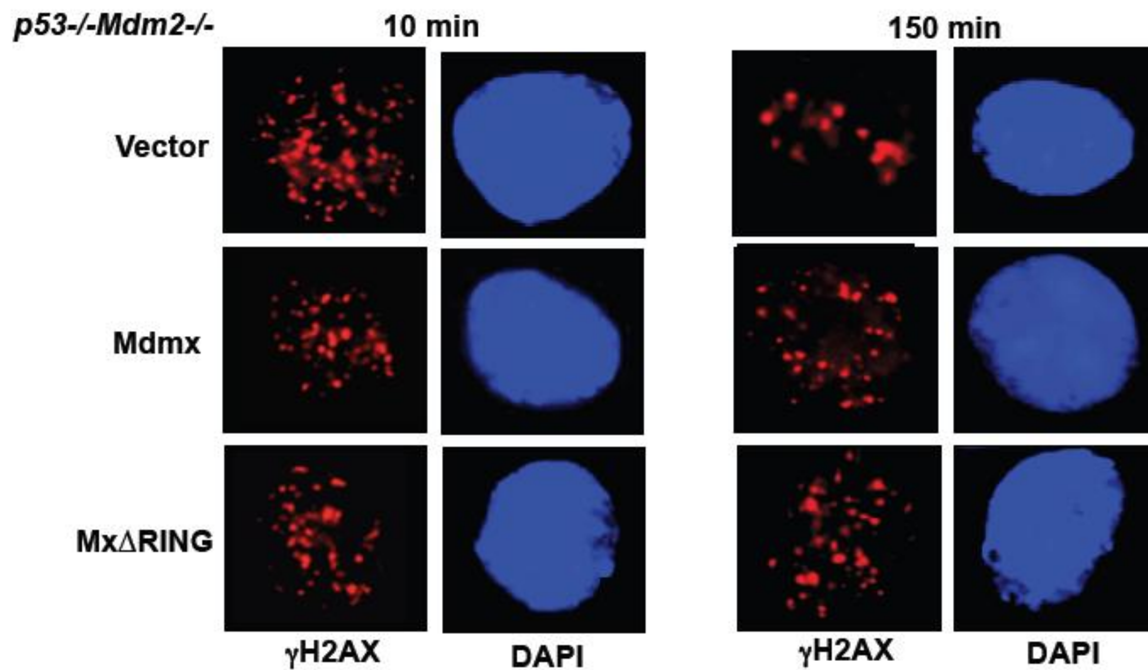

Figure 14. Elevated Mdmx inhibits γH2AX foci formation and resolution independent of p53 and Mdm2. *p53$^{-/-}$Mdm2$^{-/-}$* MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector, YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX was performed. Representative pictures of γH2AX (red) and DAPI (blue).

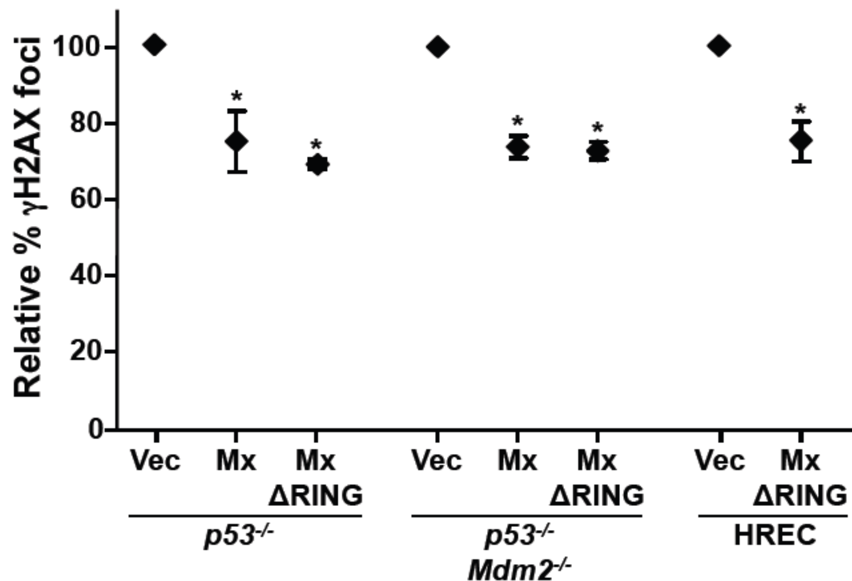

Figure 15. Increased levels of Mdmx impair the early DNA damage response signal in cells with and without p53. *$p53^{-/-}$ MEFs, $p53^{-/-}Mdm2^{-/-}$ MEFs, or* HRECs were infected with a bicistronic retrovirus encoding YFP alone (vector, V) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX was performed. The number of foci per cell was quantified. The mean of at least three independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%.

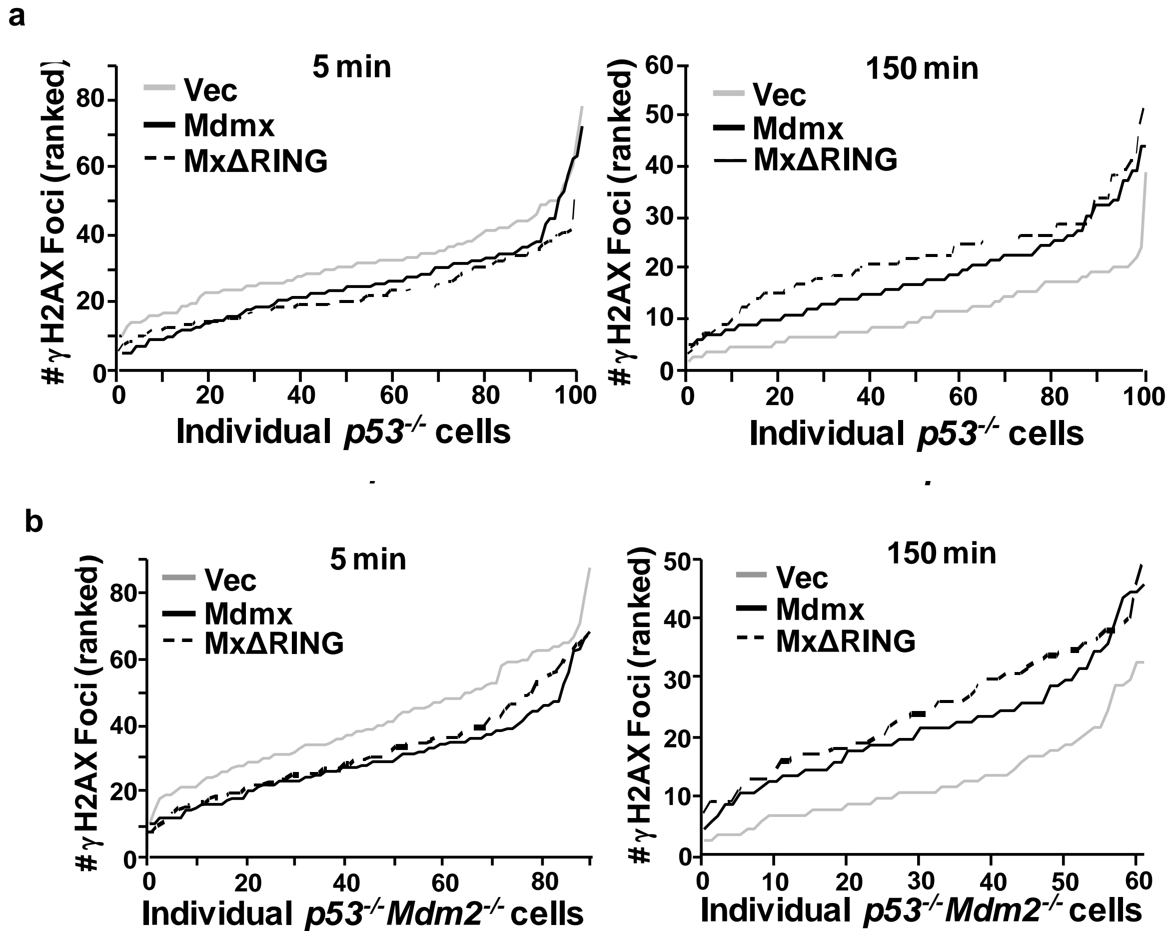

Figure 16. Elevated Mdmx impairs the early DNA damage response signal in the overall cell population. *$p53^{-/-}$(a) or $p53^{-/-}$Mdm2$^{-/-}$(b)* MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector), YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING. Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX was performed. The number of foci per cell was quantified. The ranking of foci in individual cells was graphed for a representative experiment in both MEF genotypes.

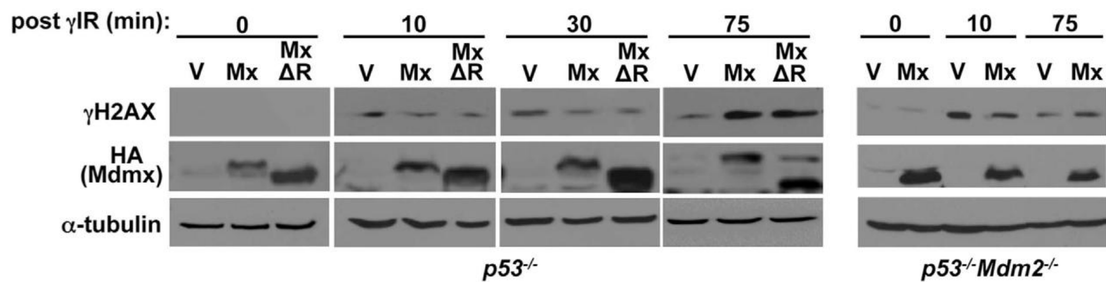

Figure 17. Increased levels of Mdmx alter γH2AX protein levels following γIR. *p53*$^{-/-}$ or *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector, V), YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, cells were harvested, and Western blot analysis of whole cell lysates for the proteins indicated to the left of the panels at the indicated intervals was performed.

ATM is a DNA damage response kinase that phosphorylates H2AX and hundreds of other proteins upon double-strand DNA breaks.[107] The phosphorylation site ATM prefers is a serine or threonine followed by a glutamine (S/T-Q).[77,107] To further investigate the effects of Mdmx on the early DNA damage signaling response, we carefully evaluated ATM-induced phosphorylation events over time by quantifying phosphorylated S/T-Q (pS/T-Q) sites on a per cell basis using immunofluorescence. Following γIR of *p53*$^{-/-}$ MEFs, the number of pS/T-Q foci peak within 20 minutes in vector control cells and decrease over the course of 240 minutes as the DNA is repaired (Figure 18, Figure 20). In contrast, Mdmx overexpression inhibits the formation of pS/T-Q foci with an approximate 35% reduction in the number of foci as compared to vector control cells within the initial 20 minutes following DNA damage (Figure 18, Figure 19-20). Moreover, over the 240 minutes analyzed, Mdmx overexpressing cells fail to increase the number of pS/T-Q foci to the peak levels observed with vector control MEFs (Figure 18), indicating DNA damage response signaling is significantly impaired by increased levels of Mdmx. In addition, as DNA repair occurs and the number of pS/T-Q foci decreases in control cells, the number of foci in Mdmx overexpressing cells stays elevated (Figure 18). Notably, Mdm2 was not required for Mdmx to exert this effect, as MxΔRING in *p53*$^{-/-}$ MEFs and human retinal epithelial cells and wild-type

Mdmx in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs produced similar results (Figure 18-20). There were a similar number of foci in all cells without γIR. Ranking of pS/T-Q foci in individual cells from a representative experiment for each cell type revealed that there was an overall reduction in foci at 10 minutes, a slightly lower or similar number of foci at 60 minutes, and an overall increase in foci at 240 minutes following γIR in the Mdmx and the

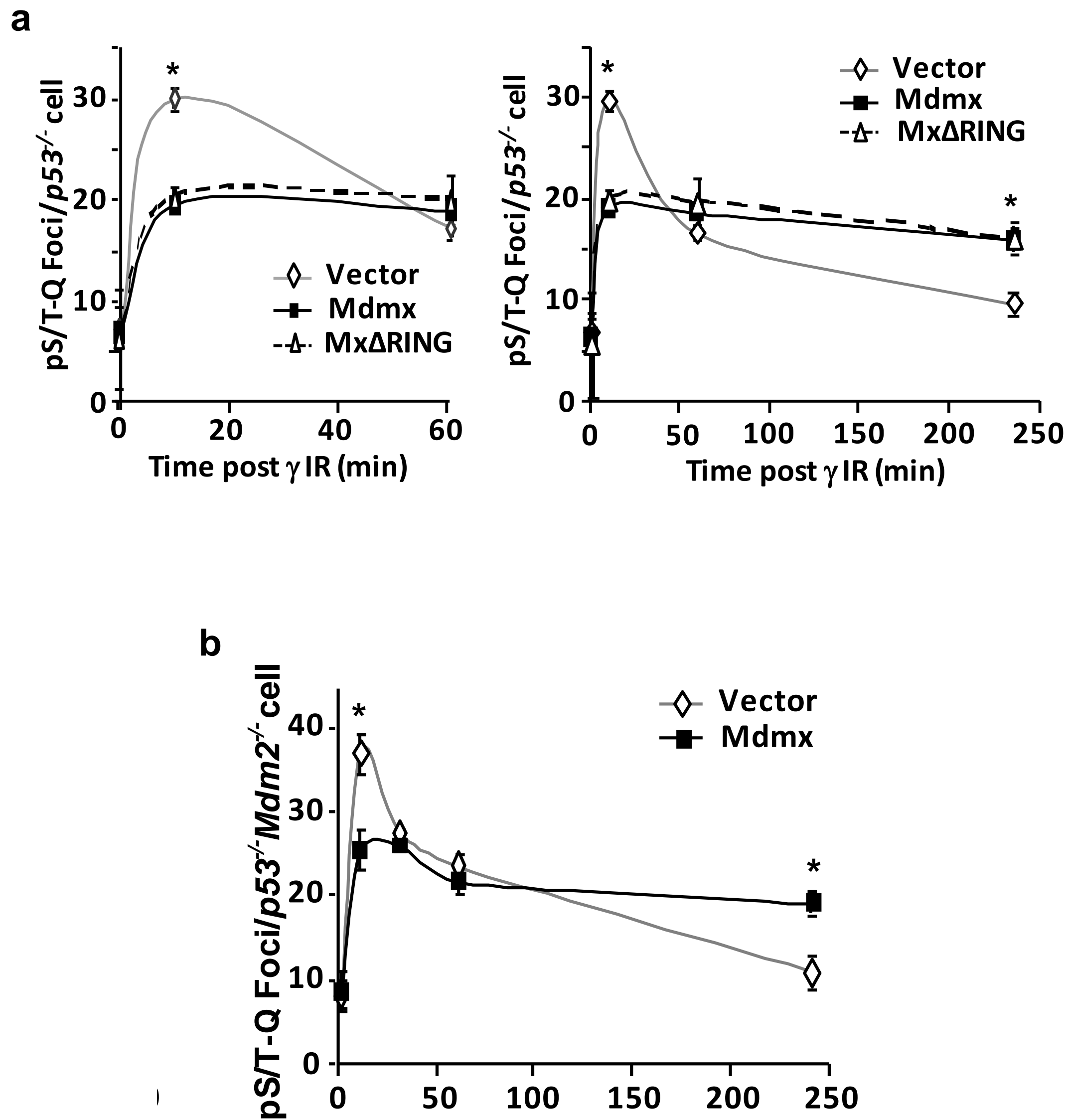

Figure 18. Elevated Mdmx impairs pS/TQ sites. *p53$^{-/-}$* (a) or *p53$^{-/-}$Mdm2$^{-/-}$* (b) MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector, V), YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for pS/T-Q was performed. The number of foci per cell was quantified. The mean of at least three independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%.

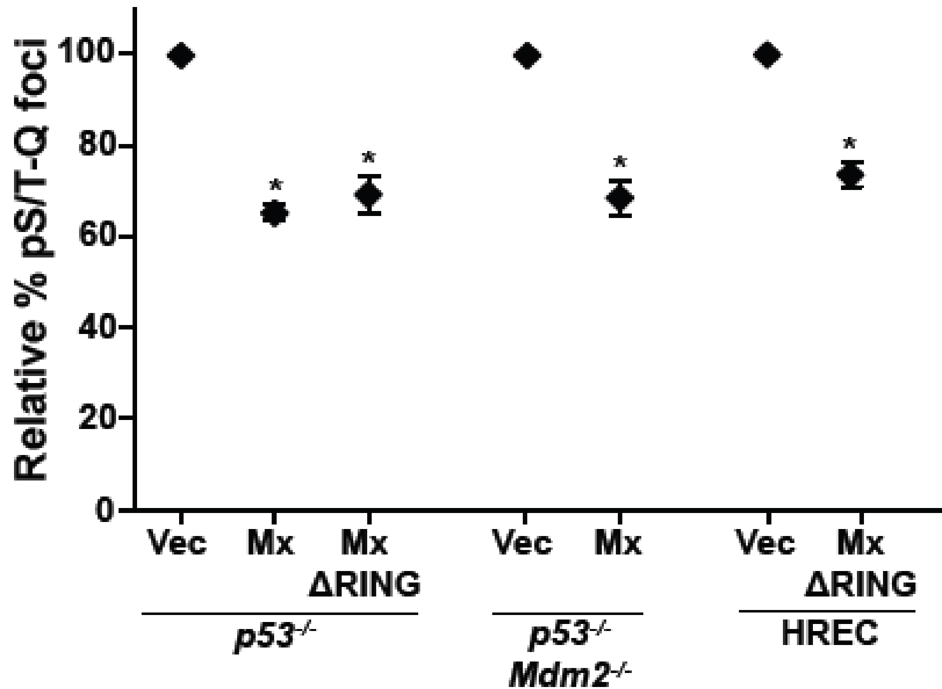

Figure 19. Elevated Mdmx impairs pST/Q foci formation in cells with and without p53. *$p53^{-/-}$ MEFs, $p53^{-/-}Mdm2^{-/-}$ MEFs, or* HRECs were infected with a bicistronic retrovirus encoding YFP alone (vector, V) or YFP and HA-MxΔRING (MxΔR). Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for pST/Q was performed. The number of foci per cell was quantified. The mean of at least three independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%.

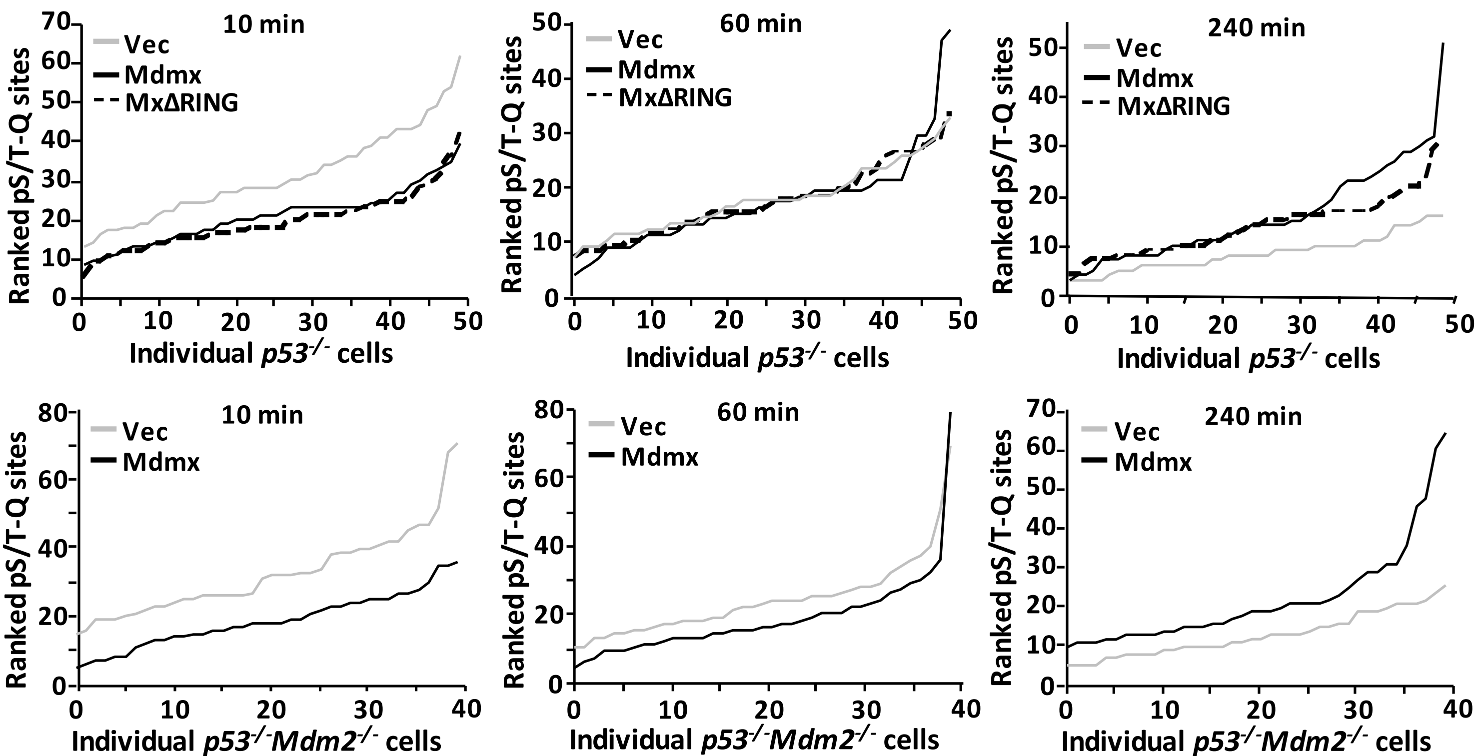

Figure 20. Elevated Mdmx impairs pST/Q foci signals in the overall cell population. *p53*$^{-/-}$ or *p53*$^{-/-}$ *Mdm2*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector), YFP and full-length HA-Mdmx (Mx) or YFP and HA-MxΔRING. Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for pST/Q was performed. The number of foci per cell was quantified. The ranking of foci in individual cells was graphed for a representative experiment in both MEF genotypes.

MxΔRING expressing MEFs (Figure 20). These data illustrate that elevated levels of Mdmx severely blunt the early DNA damage response signal mediated by ATM causing the response to be prolonged, resulting in the delay in DNA break repair observed (Figure 8-11).

To specifically test the contribution of ATM to the effects of Mdmx in DNA break repair, we utilized MEFs that lacked Atm. The MEFs also were *Arf*-null to prevent senescence that rapidly occurs in cells lacking ATM.[72] Overexpression of Mdmx in *Atm*$^{-/-}$*Arf*$^{-/-}$ MEFs, which have an impaired DNA damage response, did not further delay DNA break repair. A similar number of cells with elevated levels of Mdmx had damaged DNA as compared to the vector control cells at each interval evaluated (Figure 21). These results suggest ATM is necessary for Mdmx to inhibit DNA break repair.

Mdmx associates with Nbs1 of the MRN complex.

The Mre11-Rad50-Nbs1 (MRN) complex is necessary for efficient and accurate repair of double-strand DNA breaks.[153] Additionally, Nbs1 of this complex is critical for activating ATM following double-strand DNA breaks.[10,38,88,159] Since we observed Mdmx overexpression resulted in an altered kinetics of ATM-mediated phosphorylation events and Mdm2 is known to bind Nbs1,[3,16] we evaluated whether Mdmx associates with the MRN complex.Following transient expression of HA-tagged Mdmx in 293Tcells, endogenous Nbs1, Mre11, and Rad50 co-immunoprecipitated with Mdmx (Figure 22). This association also occurred with endogenous Mdmx and was not mediated by Mdm2. Specifically, endogenous Nbs1 co-immunoprecipitated with endogenous Mdmx in both *p53*$^{-/-}$ and *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs, whereas Nbs1 was not detected in

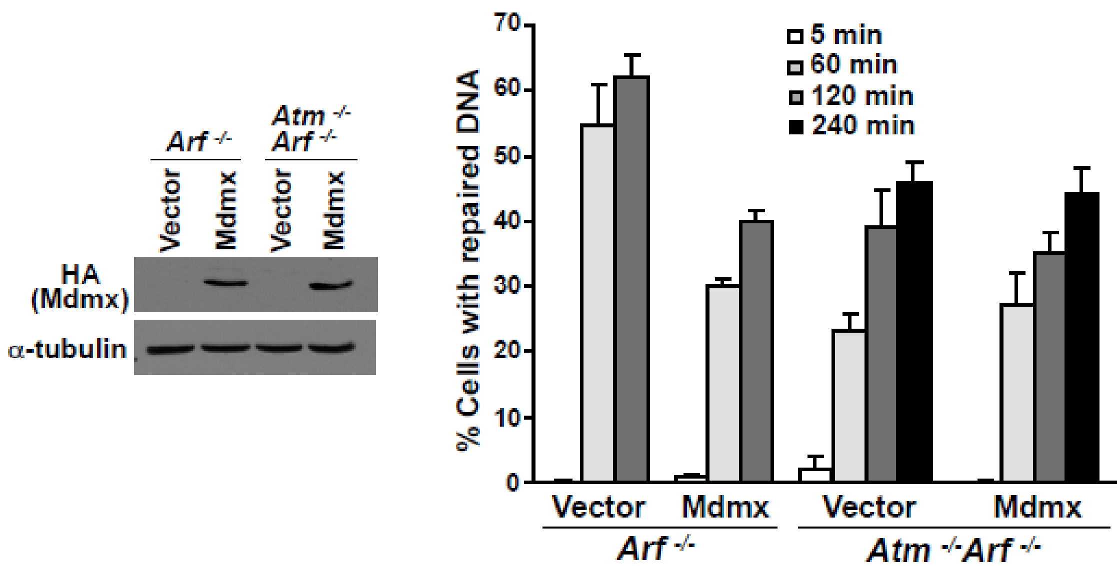

Figure 21. Mdmx cannot inhibit DNA repair in the absence of Atm. *Arf*$^{-/-}$ or *Atm*$^{-/-}$*Arf*$^{-/-}$ MEFs were infected with a bicistronic retrovirus encoding YFP alone (vector) or YFP and HA-Mdmx. Western blots of the indicated proteins were performed. Neutral comet assays were performed at the indicated intervals following exposure to 5 Gy of γIR. Only *Atm*$^{-/-}$*Arf*$^{-/-}$ MEFs were evaluated at 240 min post γIR. Each bar represents a mean of a minimum of 2 independent experiments. Error bars are SEM.

immunoprecipitations from control MEFs lacking Mdmx (Figure 22). To narrow the region in Mdmx necessary for its interaction with the MRN complex, we generated and then evaluated deletion mutants of Mdmx (Figure 23). In 293T cells, we transiently expressed either HA-tagged full-length Mdmx, a deletion mutant of Mdmx lacking the RING domain (MxΔRING; aa 1-345) or a mutant of Mdmx consisting of only amino acids 346-489 and containing the RING domain. Full-length Mdmx and MxΔRING (aa 1-345), but not Mdmx 346-489, co-immunoprecipitated the endogenous MRN complex (Figure 23). Thus, the region of Mdmx that associates with the MRN complex lies within amino acids 1-345, the same region that induced genome instability, inhibited DNA break repair and the DNA damage response (Figures 7-20).

Mre11 of the MRN complex binds to both Nbs1 and Rad50, but Nbs1 does not bind Rad50.[153] To determine with which proteins of the MRN complex Mdmx specifically interacts, we utilized Nbs1 deletion mutants we previously generated that contain (aa 513-754) or lack (aa 1-592) the Mre11-binding domain (Figure 24a;[3,16]). We co-expressed HA-Mdmx and FLAG-tagged full-length Nbs1, Nbs1 1-592, or Nbs1 513-754 in 293T cells. Both full-length Nbs1 and Nbs1 1-592 co-immunoprecipitated with Mdmx, but Nbs1 513-754 did not (Figure 24b). These data indicate Mdmx interacts with Nbs1 and not Mre11 or Rad50. To further narrow the region in Nbs1 responsible for mediating the association with Mdmx, we utilized additional Nbs1 deletion mutants (Figure 24c).

In addition, to eliminate the possibility of Mdm2 mediating the interaction between Mdmx and Nbs1, we utilized Mdmx lacking its RING domain (Mdmx 1-345; MxΔRING).

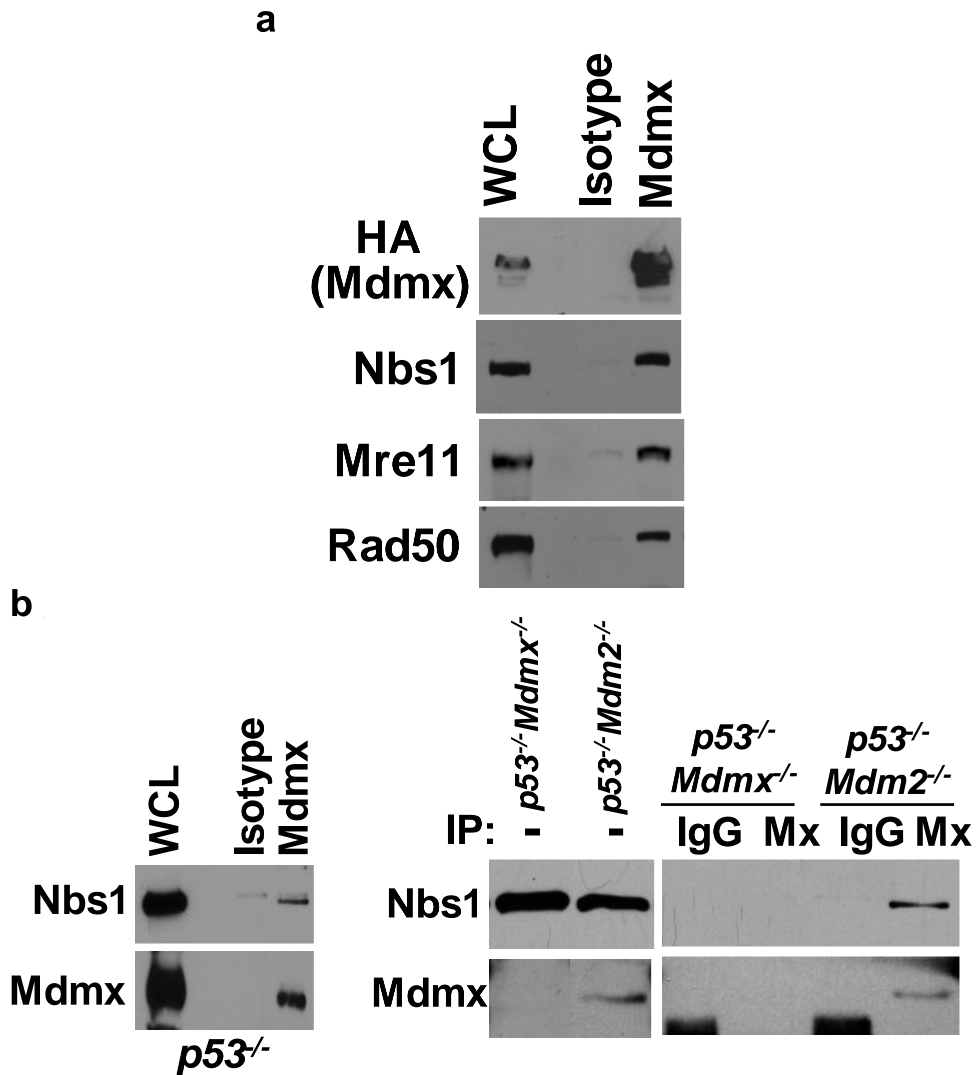

Figure 22. Mdmx associates with Nbs1 independent of Mdm2. a) Whole-cell lysates from HA-Mdmx expressing 293T ells were immunoprecipitated with anti-HA or isotype control antibody and western blotted. b) Whole-cell lysates from *p53*$^{-/-}$, *p53*$^{-/-}$*Mdm2*$^{-/-}$ or *p53*$^{-/-}$*Mdmx*$^{-/-}$ MEFs were immunoprecipitated with anti-Mdmx or isotype control antibody and western blotted for the indicated proteins.

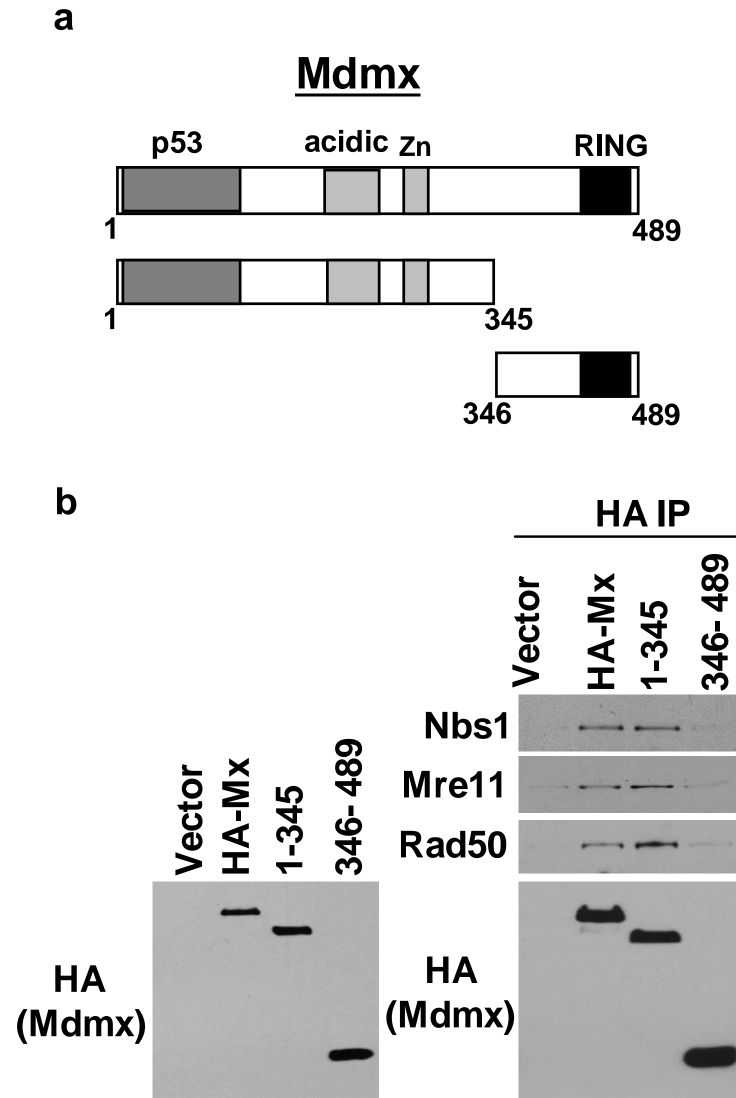

Figure 23. The RING domain of Mdmx is not required to mediate the interaction with Nbs1. a) Schematic representation of full length Mdmx. Binding domains for p53, Mdm2, and the acidic, Zinc (Zn), and RING domains are shown. b) 293T cells transfected with empty vector or vectors encoding protein tagged full-length or deletion mutants of Mdmx. Whole-cell lysates (left panel) and anti-HA immunoprecipitations (right panel) were western blotted for the indicated proteins.

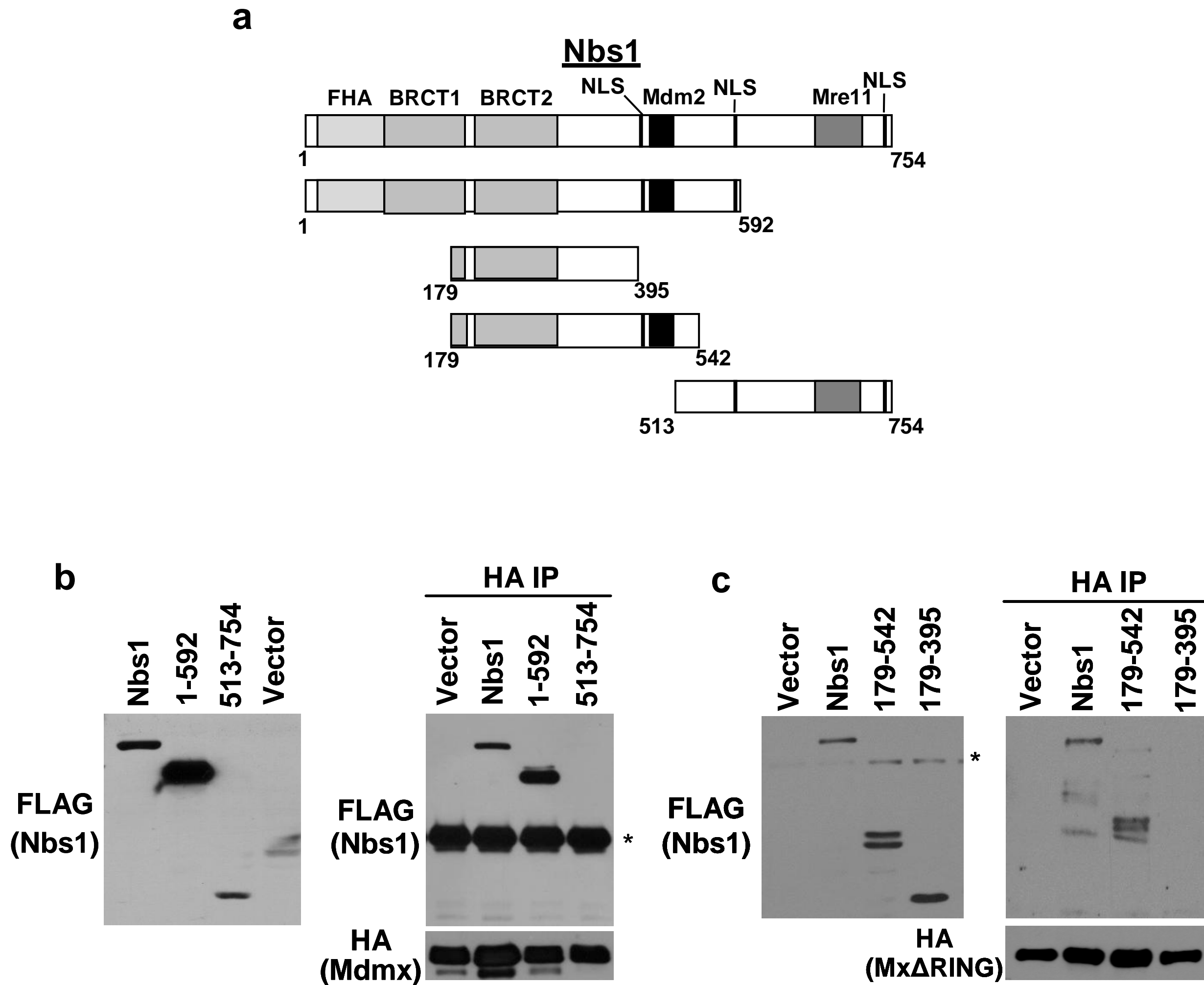

Figure 24. The region mediating the interaction between Mdmx and Nbs1 is within amino acids 396-512. a) Schematic representation of full length Nbs1 and deletion mutants. Binding domains for FHA, BRCT1/2, Mdm2, and Mre11, and NLS domains are shown. b,c) 293T cells transfected with empty vector or vectors encoding protein tagged full-length Mdmx and either protein-tagged full length Nbs1 or deletion mutants of Nbs1. Whole-cell lysates (left panel) and anti-HA immunoprecipitations (right panel) were western blotted for the indicated proteins. * denotes immunoglobulin heavy chain and non-specific band in B and C respectively.

HA-MxΔRING was co-expressed with FLAG tagged Nbs1 179-542, Nbs1 179-395, or full-length Nbs1 in 293T cells. Nbs1 179-542, but not Nbs1 179-395, co-immunoprecipitated with MxΔRING (Figure 24c). Similar results were obtained when full-length Mdmx was overexpressed (data not shown). The data indicate the region of Nbs1 mediating the interaction with Mdmx is within amino acids 396-512. We compared the 31 amino acid Nbs1-binding domain we previously identified in Mdm2 to the entire length of Mdmx and determined that this sequence was not conserved in Mdmx. Collectively, the data show the RING domain of Mdmx is dispensable for its interaction with Nbs1 of the MRN complex, and a specific region of Nbs1 is required for this association. Additionally, the Mdmx:Nbs1 association was independent of the interaction of either with Mdm2.

Interaction of Mdmx and Nbs1 at chromatin following DNA damage.

The MRN complex serves as a sensor of double-strand DNA breaks and mediator of the repair process.[153] Mre11 and Rad50 bind directly to DNA, and Nbs1 binds indirectly through Mdc1-mediated association with γH2AX at sites of DNA damage.[10,96,153,154] Since Mdmx interacts with Nbs1 of the MRN complex (Figure 24) and alters DNA repair, we evaluated whether Mdmx was recruited to chromatin after DNA damage. To address this, we isolated chromatin-bound proteins with cellular fractionation following γIR and evaluated the levels of Mdmx. Rapidly after DNA damage, there were increased levels of Mdmx in the chromatin-bound protein fraction of cells, with the levels of Mdmx associating with chromatin appearing to decrease with time as DNA was repaired (Figure 25a). We next tested whether Mdmx and Nbs1

interacted at chromatin after DNA damage. Following γIR of *p53$^{-/-}$Mdm2$^{-/-}$* MEFs, immunoprecipitation of Mdmx from the chromatin-bound protein fraction showed increased levels of Nbs1 co-immunoprecipitating with Mdmx (Figure 25b). Specifically, there was increased association between Mdmx and Nbs1 within 5 minutes after DNA damage that stayed elevated over the 60 minutes of analysis. Therefore, DNA double-strand breaks cause an increase in Mdmx:Nbs1 interaction at chromatin.

Because Mdmx interacted with Nbs1 and this interaction increased at chromatin following DNA damage, we evaluated if Nbs1 was required for Mdmx to inhibit DNA repair. To analyze this, we used ΔB/ΔB MEFs, which have a point mutation resulting in a truncated form of Nbs1 that is unstable and quickly degraded, or MEFs retaining wild-type Nbs1.[173] We infected these cells with bicistronic retrovirus encoding YFP or HA-Mdmx and YFP, and YFP positivity was confirmed by flow cytometry. Following γIR, we used neutral comet assays to assess DNA damage. We observed in the cells with wild-type Nbs1, only 37.1% (+/-4.11%) of Mdmx overexpressing cells repaired their DNA compared to 55.9% (+/-1.72%) of the vector control cells up to 120 minutes following γIR (Figure 26). In contrast, when cells were lacking Nbs1 (ΔB/ΔB), vector control and Mdmx overexpressing cells had a similar percent of cells repair DNA damage within 180 minutes after γIR (34.2% (+/-5.2%) and 32.4% (+/-4.6%), respectively) (Figure 26). These results suggest Nbs1 is required for Mdmx to mediate its inhibition of double-strand DNA break repair.

Mdmx increases genome instability independent of Mdm2.

Although Mdmx inhibited DNA repair and DNA damage signaling independent of

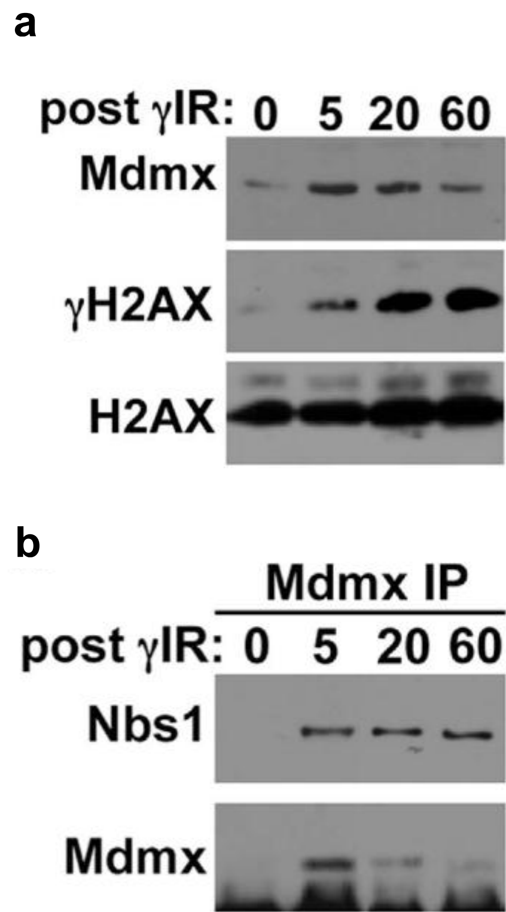

Figure 25. Nbs1 associates with Mdmx at chromatin. a) At intervals (minutes) after 5 Gy of γIR, Western blots were performed on the chromatin-bound protein fraction of *p53*$^{-/-}$ MEFs. b) Following 5 Gy of γIR for the indicated intervals (minutes), the chromatin-bound protein fraction of *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs was immunoprecipitated with anti-Mdmx and Western blots were performed.

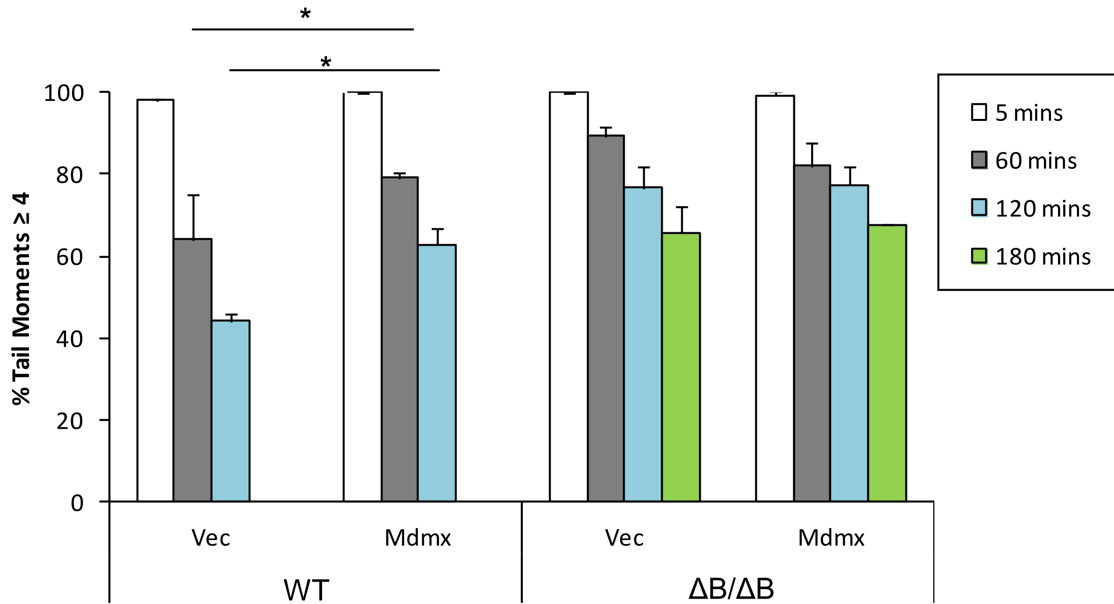

Figure 26. Mdmx cannot inhibit DNA repair in the absence of Nbs1. Wild-type or ΔB/ΔB MEFs were infected with a bicistronic retrovirus encoding either YFP alone or HA-Mdmx and YFP. Following γIR, neutral comet assays were performed. Each bar represents a minimum of 2 independent experiments evaluating at least 50 cells per experiment per sample. *p value <0.05; student's *t* test.

both p53 and Mdm2, it was unclear whether the genome instability detected (Figure 7) was dependent on the presence of Mdm2. To address this, we analyzed metaphases from *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs expressing either empty vector or HA-tagged Mdmx. Mdmx overexpression resulted in a significant increase in the frequency of cells containing breaks (chromatid and chromosome) (Figure 27). Specifically, there was a 3-fold increase of cells with either chromatid or chromosome breaks. Chromosome fusions, which result from chromosome breaks, showed a 5-fold increase in Mdmx overexpressing *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs compared to controls (Figure 27). Although rare, we also detected other structural abnormalities, such as radials, in the Mdmx overexpressing MEFs that were not present in the controls (Figure 28). Moreover, only the Mdmx overexpressing *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs had more than one chromosomal aberration in individual cells (Figure 28). These results demonstrate that elevated levels of Mdmx increase genome instability independent of Mdm2.

Increased transformation potential with elevated levels of Mdmx.

We have determined that Mdmx promotes genome instability independent of p53, and genome instability is linked to tumorigenesis.[57] Moreover, it has been reported that elevated Mdmx levels are observed in many tumors.[33,51,85,90,135,165] Therefore, we tested whether elevated Mdmx levels would promote transformation independent of its regulation of p53, using *in vitro* soft agar colony formation assays. Overexpression of either Mdmx or MxΔRING in *p53*$^{-/-}$ MEFs resulted in a significant increase in the number of colonies compared to vector control (Figure 29). To determine whether Mdmx promoted transformation independent of its association with Mdm2, we evaluated

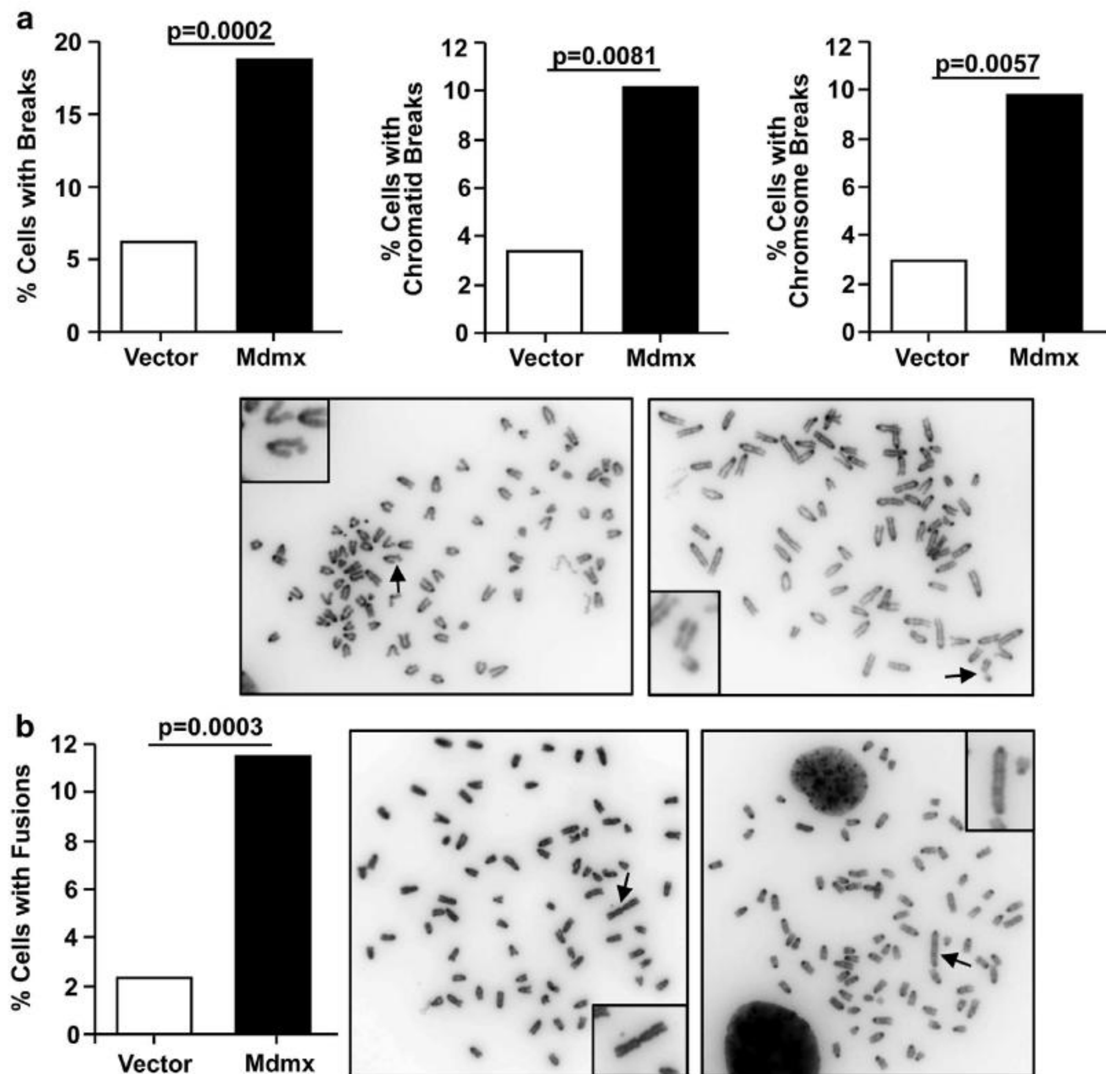

Figure 27. Mdmx promotes chromosome instability independent of Mdm2. Metaphases from vector control (n=176) or Mdmx overexpressing (n=246) *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were evaluated for chromosome aberrations. a) The percentage of cells with one or more breaks of either kind (chromatid or chromosome) or that have the specified break is graphed. Representative pictures of a chromatid (left) and a chromosome (right) break in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs overexpressing Mdmx are shown. b) The percentage of cells with one or more chromosome fusions is graphed. Representative pictures of a centromere-centromere (left) and a telomere-telomere (right) fusion in *p53*$^{-/-}$ *Mdm2*$^{-/-}$ MEFs overexpressing Mdmx are shown. Expanded views of each aberration marked by an arrow. Statistical significance was determined using the Fisher's exact test.

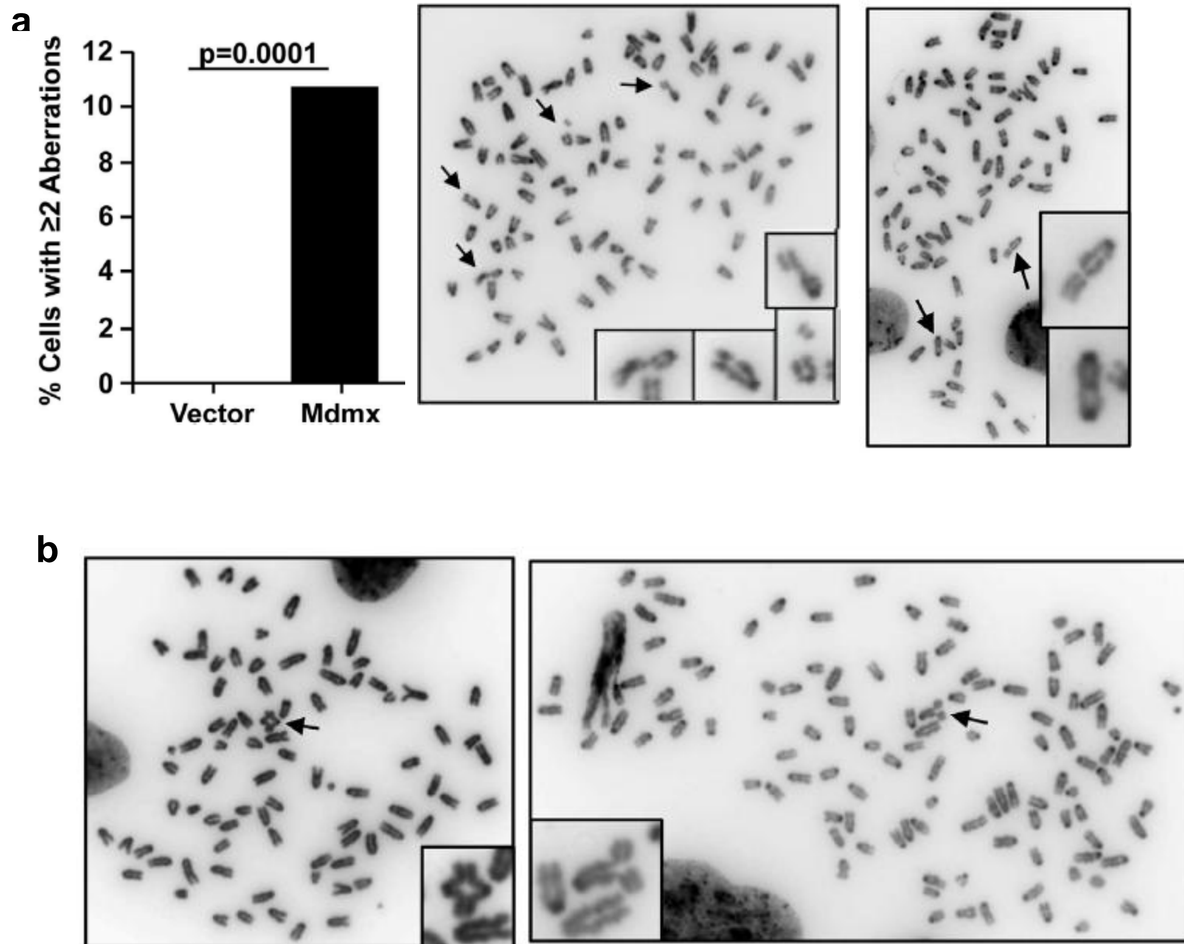

Figure 28. Mdmx promotes structural aberrations and increases the frequency of more than one aberration per cell independent of Mdm2. Metaphases from vector control (n=176) or Mdmx overexpressing (n=246) *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were evaluated for chromosome aberrations. a) Representative pictures of structural aberrations. b) The percentage of cells with more than one chromosomal aberration is graphed. Representative pictures of metaphases with multiple aberrations are shown. Insets are expanded views of each aberration marked by an arrow. Statistical significance was determined using the Fisher's exact test.

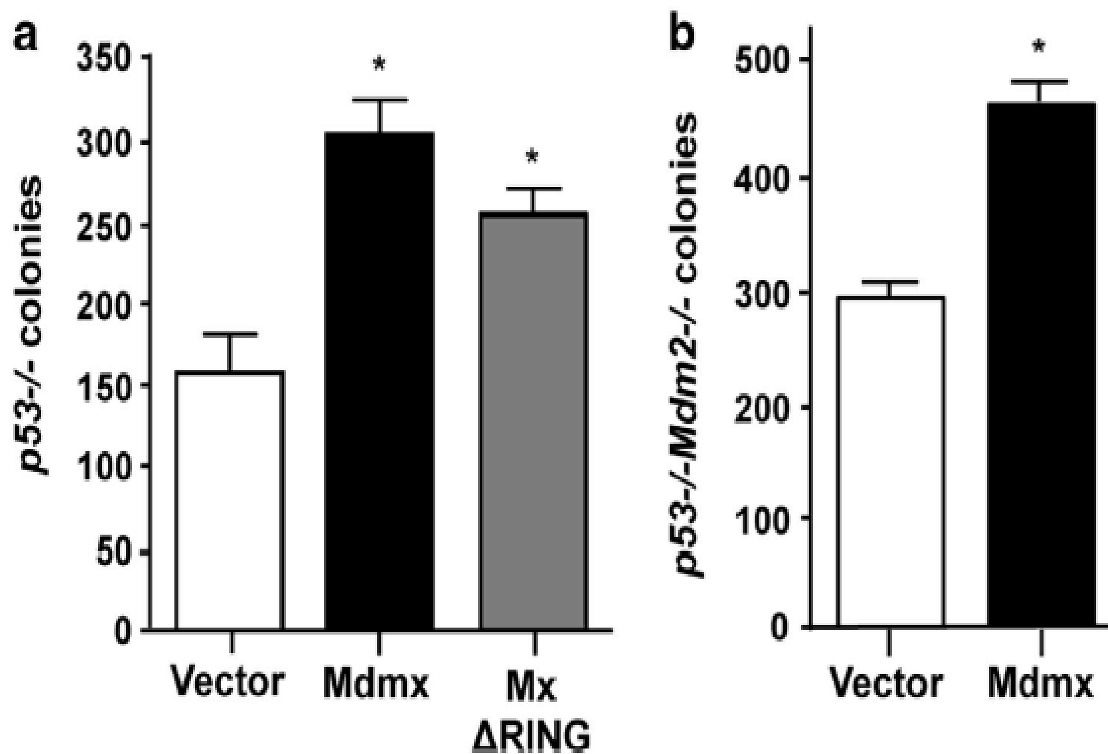

Figure 29. Increased levels of Mdmx promote transformation independent of p53 or Mdm2. Soft agar assays of *p53*$^{-/-}$ (a) or *p53*$^{-/-}$*Mdm2*$^{-/-}$ (b) MEFs infected with bicistronic retrovirus expressing YFP alone, HA-tagged full-length Mdmx and YFP, or HA-tagged MxΔRING and YFP were performed. Each graph represents the average colonies per well from at least 2 independent experiments with each experiment consisting of three replicates. *$p<0.0001$ student's t-test.

colony formation in *p53$^{-/-}$Mdm2$^{-/-}$* MEFs. We observed that Mdmx overexpression resulted in a significant increase in colony formation compared to vector control (Figure 29). Therefore, Mdmx overexpression promoted transformation, and this was independent of its regulation of p53 and did not require Mdm2.

Discussion

While it is clear Mdmx regulates p53,[104,166] the identification of Mdmx overexpression in tumors with mutated or deleted p53 and effects of Mdmx on genome instability in cells lacking p53 indicate p53-independent functions of Mdmx.[53,81,105,106,135,165] Yet, identification of the p53-independent functions of Mdmx remained elusive. Here, we describe a novel protein interaction and function of Mdmx that neither required p53 nor surprisingly, Mdm2. We determined Mdmx interacts with the MRN complex and delays the early DNA damage signaling response, resulting in reduced DNA repair and increased genome instability and cellular transformation. Our results provide new and unexpected insight into Mdmx function and its oncogenic contributions to tumorigenesis.

Our data show Mdmx overexpression delayed the early DNA damage response that is mediated by the DNA damage-induced kinase ATM. Phosphorylation of H2AX and S/T-Q sites, both of which are targets of ATM, occurs rapidly after DNA breaks.[10,107,141] Full activation of ATM following DNA double-strand breaks requires Nbs1 of the MRN complex.[10,38,88,159] We detected Mdmx and Nbs1 interaction that increased following DNA double-strand breaks. While an overall reduction in Mdmx protein following genotoxic stress has been reported,[25,75,124,132] we observed increased

levels of Mdmx protein localizing to chromatin and associating with Nbs1 following DNA damage. Our measurements of the effects of elevated levels of Mdmx on the early DNA damage response through phosphorylation of S/T-Q sites and H2AX revealed three important findings. Firstly, Mdmx slowed the initial kinetics of S/T-Q and H2AX phosphorylation. Secondly, Mdmx decreased the amplitude of the initial DNA damage response, as the peak of S/T-Q phosphorylation in Mdmx overexpressing MEFs never reached the peak detected in control MEFs. Finally, Mdmx delayed the resolution of pS/T-Q and γH2AX foci leading to a higher number of foci at later times after DNA damage, prolonging the DNA damage response. These data indicate Mdmx blunts the initial DNA damage response allowing DNA damage to persist, and this should result in a delay in DNA repair, which is what we detected. Consistent with our data, transient inhibition of ATM for one hour led to increased chromosome aberrations following γIR.[171,172] Our results indicate Mdmx inhibits phosphorylation of ATM targets resulting in a delay in DNA repair and an increase in chromosome and chromatid breaks. Moreover, the inhibition of DNA repair signals by Mdmx, which results in severe consequences for the ability of cells to repair damaged DNA, ultimately promotes transformation.

The RING domain of Mdmx is required to bind to Mdm2 through its RING domain.[147,156] Mice engineered to express Mdmx lacking its RING domain or containing a mutant RING domain die *in utero*, and this phenotype was rescued with deletion of *p53*.[61,125] These results indicate Mdmx:Mdm2 interactions are critical to control p53 during development, but since DNA repair was not investigated, it may also indicate that simultaneously the more stable mutant Mdmx was also inhibiting DNA break repair,

which contributed to the death of the embryo. We show that Mdmx lacking its RING domain was as capable as wild-type Mdmx of interacting with Nbs1, inhibiting the DNA damage signal, delaying DNA repair, and inducing genome instability and transformation. Furthermore, this mutant form of Mdmx effectively inhibited DNA break repair in human retinal epithelial cells, which retain p53 and Arf and frequently overexpress *MDMX* when transformed, as in retinoblastoma.[85] Full-length Mdmx had similar effects in MEFs lacking Mdm2. We were initially surprised that Mdmx could have negative effects on DNA damage signaling and repair independent of its interaction with Mdm2, but it is known that Mdmx can regulate p53 independent of Mdm2.[104,166] Our data also reveal the regulation of the DNA break repair response by Mdmx and Mdm2 is a conserved function of this family of proteins, akin to their regulation of p53, and is present in multiple cell types.

Genome instability can be a precursor to tumor formation, is a hallmark of malignant cells, and is connected with impairments in the DNA damage response.[57,153] When Mdmx levels were elevated, which occurs during tumorigenesis, we observed a significant increase in genome instability and transformation that are attributed to an inhibition in the DNA damage response. These effects of Mdmx were independent of both p53 and Mdm2. Mdmx overexpression led to an increase in both chromatid and chromosome breaks, fusions (an indicator of chromosome breaks), and the appearance of other structural abnormalities. Loss of Mdmx also led to genome instability in MEFs lacking p53. Specifically, $p53^{-/-}Mdmx^{-/-}$ MEFs had multipolar spindle formation and altered chromosome number.[105,106] Thus, the data indicate that either gain or loss of Mdmx negatively impacts genome integrity, highlighting the critical role Mdmx levels

have in contributing to genome instability.

Recently, it was reported that transgenic mice overexpressing Mdmx had an increased incidence of malignancies.[174] Although the p53-independent contribution of Mdmx to tumorigenesis was not evaluated in this study, our results provide new insight into the oncogenic effects of Mdmx. Our data demonstrate Mdmx overexpression was sufficient to induce transformation of *p53*-null cells. These findings indicate a role for Mdmx in tumorigenesis independent of p53 that is supported with data from patient samples. Specifically, in a subset of human tumors that have overexpressed or amplified Mdmx, p53 is inactivated (deleted or mutated), suggesting that there is an advantage for some cancer cells to both overexpress Mdmx and inactivate p53. In addition, Mdmx promoted transformation independent of Mdm2, indicating that although they bind, Mdmx can function as an oncogene without Mdm2. Taken together, these data further establish the oncogenic nature of Mdmx and provide insight into a new function of Mdmx in the DNA damage response that contributes to cellular transformation. Additionally, since Mdmx is capable of oncogenic activity independent of p53 or Mdm2, this emphasizes the importance of future studies focused on targeting Mdmx in tumors that lack functional p53.

CHAPTER 4

DRUG-MEDIATED MDM2 ELEVATION INHIBITS DNA REPAIR AND COOPERATES WITH GENOTOXIC AGENTS INDEPENDENT OF p53

Introduction

The transcription factor p53 is an important tumor suppressor that can regulate cell cycle progression and apoptosis following cellular stress, such as oncogene-driven proliferation or DNA damage.[91,163] Because of these functions, p53 is mutated or deleted in approximately 50% of all of human cancers. Some cancers have even higher p53 mutation rates, such as ovarian cancer, where >90% of the samples have p53 mutated.[2,91,123,149] In addition to mutations in p53 itself, the p53 pathway can be altered to ultimately render p53 inactive.[91] Mdm2 is an E3 ubiquitin ligase that targets p53 for proteosomal degradation. Mdm2 is often overexpressed in tumors with functional p53 as a way to inhibit p53 activity, which contributes to cancer progression.[42] However, Mdm2 is also overexpressed in tumors with inactivated p53, suggesting p53-independent functions of Mdm2 contribute to tumorigenesis.[42] Due to its contribution to tumorigenesis in potentially various p53-dependent and -independent mechanisms, understanding the function of Mdm2 in these processes is crucial.

Because p53 is inactivated in many human cancers through alterations in upstream proteins which alter p53 activity, it has been a major focus of chemotherapeutic efforts to promote activation of wild-type p53 in order to induce cell cycle arrest or apoptosis in cancer cells.[29] In recent years, the drug Nutlin-3 (Nutlin) was developed to prevent the interaction between Mdm2 and p53.[162] Nutlin prevented

the negative regulation of p53 by Mdm2, thus reactivating p53.[162] In cancer cells with wild-type p53, Nutlin effectively induced p53-mediated apoptosis.[162] While this approach shows promise for tumors retaining wild-type p53, this mechanism of action is not useful for the 50% of cancers with mutated or deleted p53. Therefore, understanding and establishing therapeutics that can act independent of p53 are needed for treating these cancers.

Nutlin has been used in combination with genotoxic agents to induce apoptosis of cancer cells retaining functional p53. In these cases, it is clear Nutlin prevents Mdm2 from inhibiting p53 and allows p53 to be activated by the DNA damage elicited by the genotoxic agents. This cooperation between Nutlin and genotoxic agents has primarily been evaluated in tumors with functional p53; however, some studies have also observed this cooperative effect in cells with inactivated p53. Functions of Nutlin independent of p53 are not well understood. Even though the original Nutlin is not clinically viable, it can be used as a tool to investigate future treatment options that are independent of p53. Elucidating the p53 independent functions by which Nutlin is effective would reveal novel potential therapeutic avenues for the many cancers with inactivated p53.

It has been reported that an additional effect of Nutlin treatment is elevated Mdm2 protein levels, and this occurs both in cells with and without p53.[157] While it is clear Mdm2 negatively regulates p53, Mdm2 also has p53-independent functions, including those influencing genome instability.[3,16] We have previously shown elevated Mdm2 acts through Nbs1 of the Mre11/Rad50/Nbs1 DNA repair complex to inhibit double-strand DNA break repair, impair proper DNA damage response signaling,

promote genome instability and confer transformation potential independent of p53 (Figure 1).[3,16] Therefore, we postulated the increased levels of Mdm2 caused by Nutlin in the absence of p53 are inhibiting the early DNA damage response signal leading to an overall inhibition in DNA repair (Figure 30). Since this function of Nutlin would act independent of p53, this highlights an alternative mechanism of action by which Nutlin can affect tumor cells.

In this study, we show Nutlin inhibits double-strand DNA break repair and DNA damage response signaling independent of p53, and this is mediated by Mdm2. Furthermore, when ovarian cancer cells with inactive p53 received combined treatments of Nutlin and genotoxic agents inducing double-strand breaks, there was a cooperative effect resulting in apoptosis (Figure 30). This study is still ongoing, but the results already reveal a novel mechanism by which Nutlin acts independent of p53. This allows Nutlin to cooperate with genotoxic agents to provide therapeutic potential in cancers with inactivated p53. These studies signify the potential therapeutic benefit of increasing Mdm2 pharmacologically.

Results

Nutlin inhibits double-strand DNA break repair and the DNA damage response through Mdm2 and independent of p53.

Previous studies from the Eischen lab have shown increased levels of Mdm2 inhibit double-strand DNA break repair independent of p53.[3,16] Therefore, we hypothesized the increase in Mdm2 levels by Nutlin would result in an inhibition in

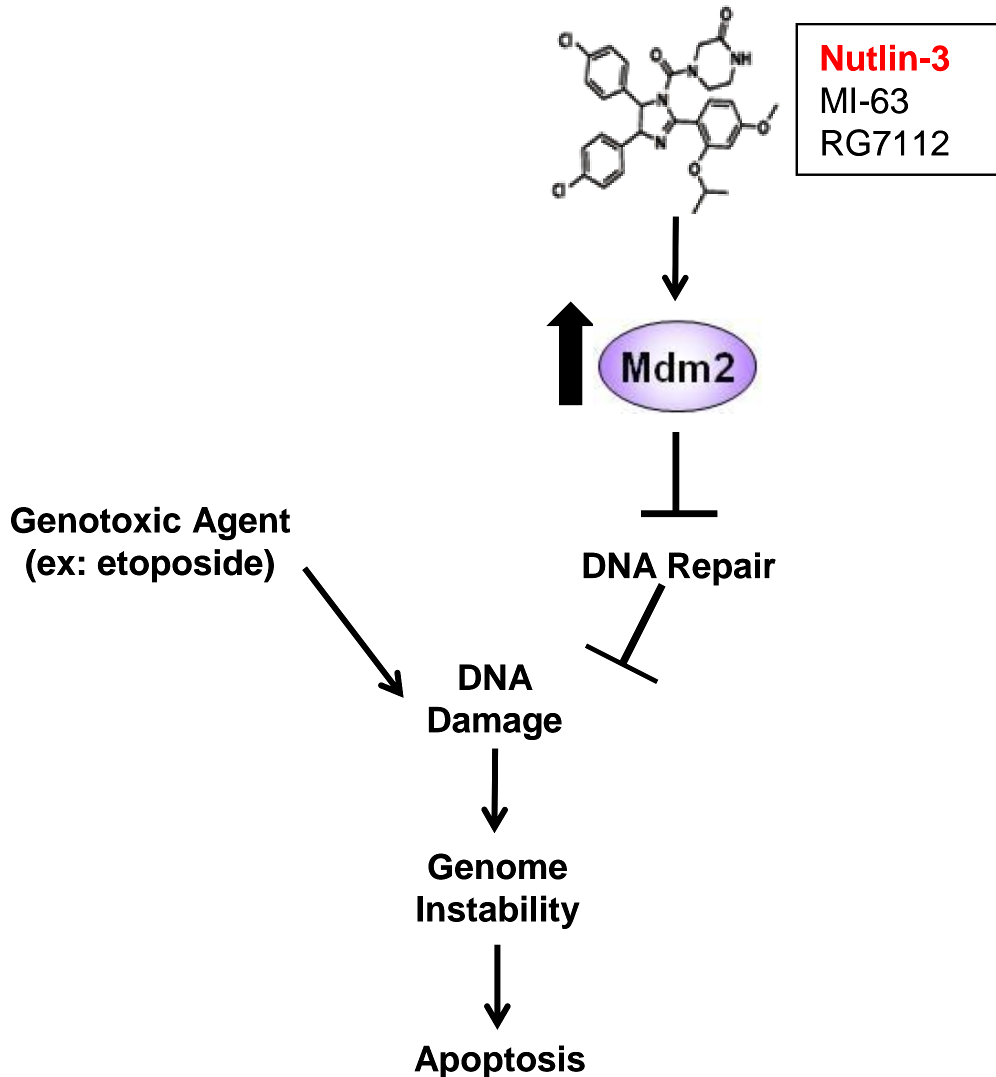

Figure 30. Synthetic induction of Mdm2 cooperates with genotoxic agents in cancers with inactivated p53. Genotoxic agents, such as Etoposide, cause DNA damage. Damaged DNA activates the DNA damage response to initiate the repair process. Improper DNA repair can contribute to genome instability which has the potential to contribute to tumorigenesis. Therefore, it is important that DNA repair occur accurately and efficiently. Nutlin increases levels of Mdm2, which subsequently inhibits DNA repair. The combination of increased Mdm2 and a genotoxic agent results in excessive DNA damage with an inhibited ability to repair that damage. This combination proved to have a cooperative effect and induced apoptosis in cells with inactivated p53.

double-strand DNA break repair. First, we tested this in 293T cells because they have inactivated p53. We observed 58.96% (+/-1.56%) of the cells pre-treated with Nutlin for 24 hours had DNA damage remaining compared to only 31.74% (+/-3.01%) of DMSO treated cells (Figure 31a). The inhibition of DNA repair observed with Nutlin treatment was comparable to that seen with Mdm2 overexpression (49.2% +/-5.2%). To truly assess if this effect of Nutlin is independent of p53, we next evaluated DNA repair in *p53*$^{-/-}$ murine embryonic fibroblasts (MEFs) treated with Nutlin or DMSO, or infected with a bicistronic retrovirus encoding Mdm2, as a positive control. Following treatment with Nutlin or DMSO, cells were exposed to 5 Gy of γIR and DNA repair was evaluated using the neutral comet assay. Nutlin alone inhibited the repair of double-strand DNA breaks (57.7% +/-4.88%) compared to DMSO control (31.27% +/-3.17%) to an extent comparable to retrovirally overexpressed Mdm2 (60.25% +/-6.12%), and this was independent of p53 (Figure 31b). To assess the requirement of Mdm2 for this effect of Nutlin, we treated *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs with DMSO or Nutlin and overexpressed Mdm2 as a control. We then evaluated DNA repair using comet assays. We observed within 90 minutes following γIR, Nutlin treated cells repaired their DNA to a similar extent as DMSO control (22.16% +/- 2.34% vs 20.18% +/- 1.49%, respectively) and no longer had the ability to inhibit double-strand DNA break repair in the absence of Mdm2 (Figure 31c). These results demonstrate that Nutlin alters double-strand DNA break repair in an p53-independent manner that is mediated by Mdm2.

Previously, the Eischen lab determined Mdm2 overexpression inhibited the formation of γH2AX foci immediately following γIR.[16] Because we hypothesize that Nutlin affects DNA repair due to its ability to increase Mdm2 protein levels, we

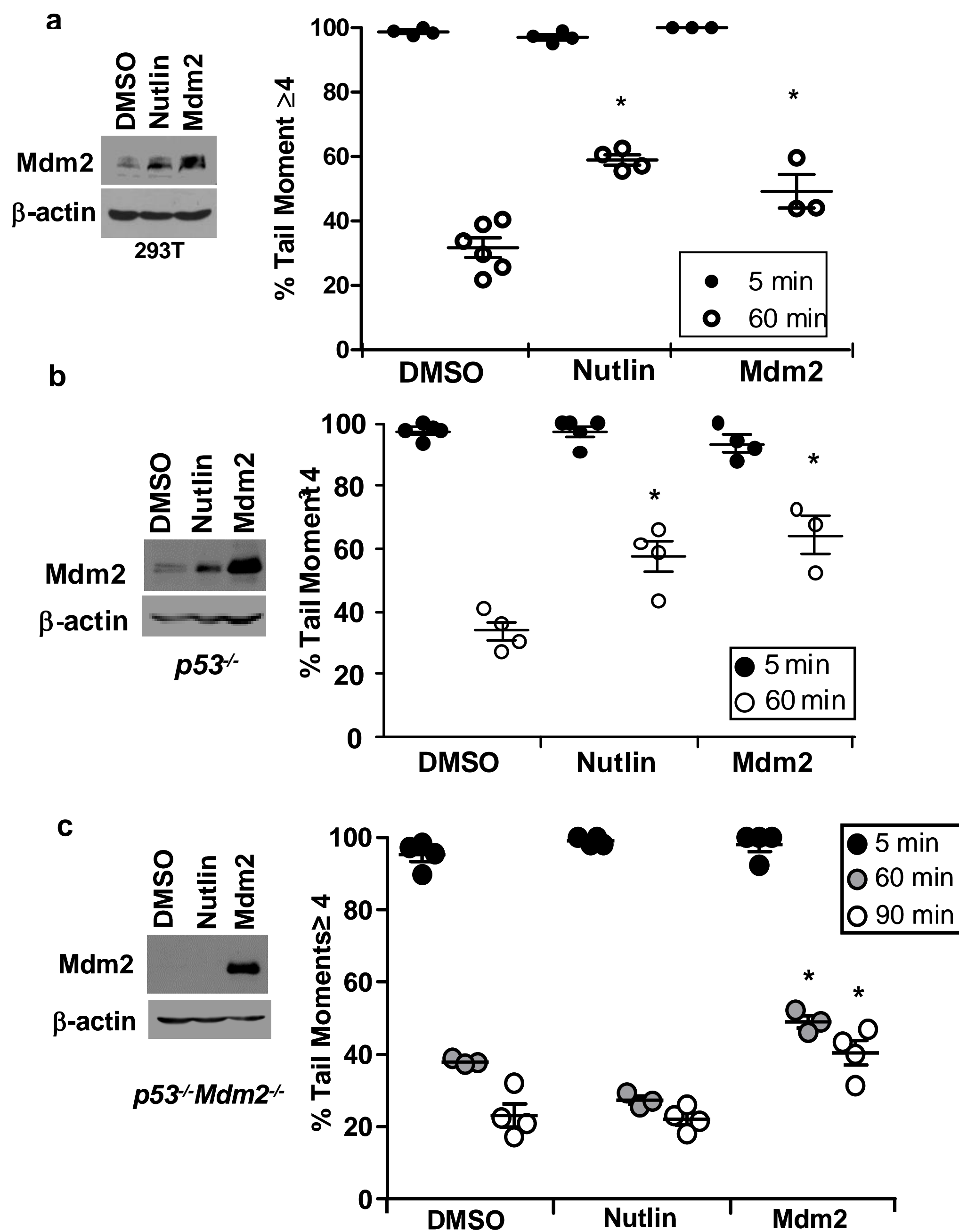

Figure 31. Nutlin requires Mdm2 to inhibit DNA break repair independent of p53. 293T cells (a), *p53*$^{-/-}$ MEFs (b), or *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs (c) were treated with DMSO, Nutlin (10μM), or overexpressed Mdm2. Following γIR, comet assays were used to evaluate the percent of cells with a tail moment ≥4 (damaged DNA). All graphs representative of a minimum of three independent experiments. The circles (b,c) indicate one experimental sample consisting of a minimum of 40 cells analyzed. *p value <0.05

evaluated γH2AX foci formation. Following treatment with DMSO or Nutlin for 24 hours, *p53*$^{-/-}$ MEFs were exposed to γIR, and after 10 minutes, γH2AX foci were quantified using immunofluorescence. Nutlin treated cells formed 22.5% +/- 6.6% fewer foci than DMSO treated cells (Figure 32A). This effect was comparable to the 30.77% +/- 2.3% reduction in foci caused by Mdm2 overexpression, which has been published to inhibit γH2AX foci formation.[16] To determine the role of Mdm2 in the inhibition caused by Nutlin, we used *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs. When Mdm2 was absent, Nutlin no longer inhibited the formation of γH2AX foci per cell compared to DMSO treated cells (33.26% +/-0.85% vs 33.93% +/-1.2%, respectively; Figure 32B). These results indicate Nutlin treatment has a comparable inhibitory effect on γH2AX foci formation as Mdm2 overexpression, and this effect is independent of p53 but requires Mdm2.

Nutlin inhibits DNA repair in ovarian cancer cells with inactive p53.

Ovarian cancer is one of the most deadly cancers among women.[63] Due to typically being diagnosed in late stages, it can be very challenging to treat. This is compounded by the >90% of ovarian cancers that have mutated or deleted p53.[2,63] Current chemotherapy involves the use of various genotoxic agents, such as Cisplatin or Carboplatin.[63] Typically, platinum resistance occurs, requiring the further combinatorial use of other genotoxic agents, such as Etoposide. We postulated the inhibition of double-strand break DNA repair resulting from Nutlin-induced elevation of Mdm2 levels could cooperate with genotoxic agents, like Cisplatin or Etoposide, to promote severe genome instability which would result in apoptosis.

Various doses of Nutlin have been reported to increase Mdm2 levels. To

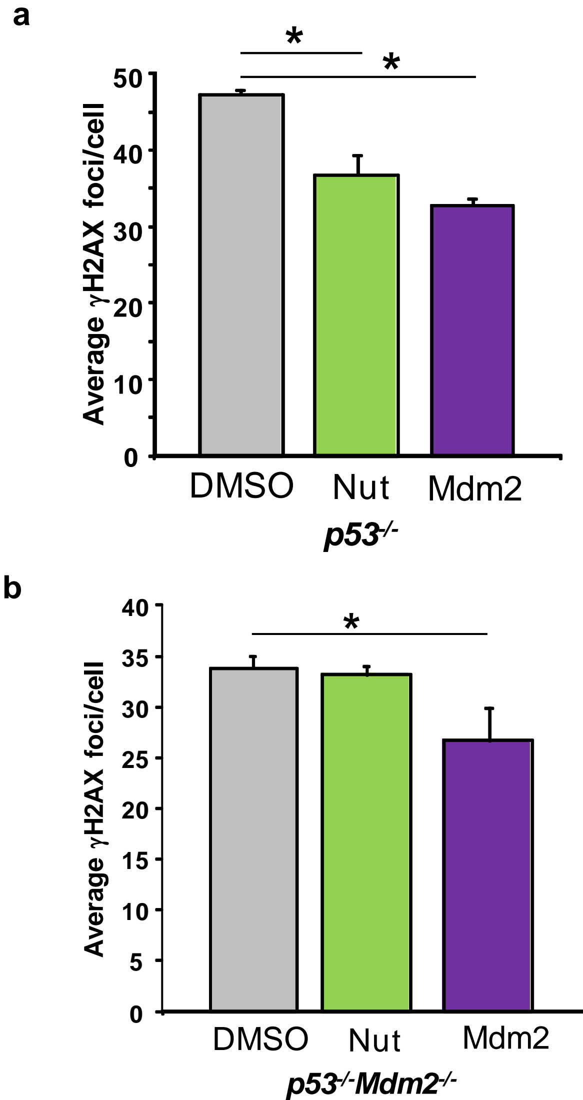

Figure 32. Nutlin requires Mdm2 to inhibit γH2AX foci formation. (a) *p53*$^{-/-}$ MEFs and (b) *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs were treated with DMSO, Nutlin (10μM), or infected with a bicistronic retrovirus encoding Mdm2. Following exposure to 5 Gy of γIR, MEFs were fixed after 10 minutes and immunofluorescence for γH2AX was performed. The number of foci per cell was quantified. The mean of at least two independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%.

determine which dose was sufficient to increase Mdm2 levels in these ovarian cancer cells, we exposed cells to either 10μM or 20μM of Nutlin (5μm and 10μM of the active enantiomer) and evaluated Mdm2 protein levels using Western blot analysis. Mdm2 protein was increased by both doses of Nutlin to a similar extent (Figure 33); therefore, we used 10μM (5μM of the active enantiomer Nutlin-a) for our studies. To assess the p53-independent effect of Nutlin, OVCAR8 ovarian cancer cells, with mutated p53, were used. Following treatment with Nutlin, Mdm2 levels were indeed elevated in the OVCAR8 cells (Figure 34). We then assessed the effect of Nutlin on the cells' ability to repair damaged DNA. OVCAR8 cells were pre-treated with Nutlin or vehicle control (DMSO), and comet assays were used to evaluate the repair of double-strand breaks following γIR. While only 26.43% +/-3.92% of the DMSO treated cells had detectable DNA damage, 42.33% +/- 4.48% of the Nutlin treated cells had DNA damage remaining (Figure 34). Next we evaluated if this effect could be observed in the complete absence of p53 by using SKOV3 cells which have p53 deleted. Only 25.46% +/- 5.27% of the cells treated with Nutlin were able to repair the DNA damage within 90 minutes, compared to 42.38%+ 1.22% of cells treated with DMSO (Figure 35). These results indicate Nutlin treatment increases Mdm2 and results in an inhibition of double-strand DNA break repair in ovarian cancer cells with inactive p53.

To further assess the p53-independent effects of Nutlin on efficient double-strand DNA break repair, we evaluated γH2AX foci formation and resolution. SKOV3 cells were treated with DMSO or Nutlin for 24 hours, and then exposed to 5 Gy of γIR. Immediately following γIR, Nutlin treated SKOV3 cells had 28% +/- 4% fewer γH2AX foci formed and at later times, they had 46% +/- 5 more foci remaining than DMSO

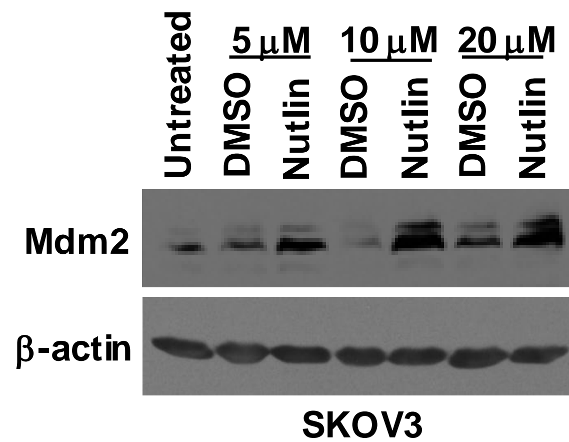

Figure 33. Different doses of Nutlin have similar effects on Mdm2 levels. SKOV3 cells were treated with the indicated doses of Nutlin for 24 hours. Mdm2 levels were subsequently evaluated by Western blot analysis.

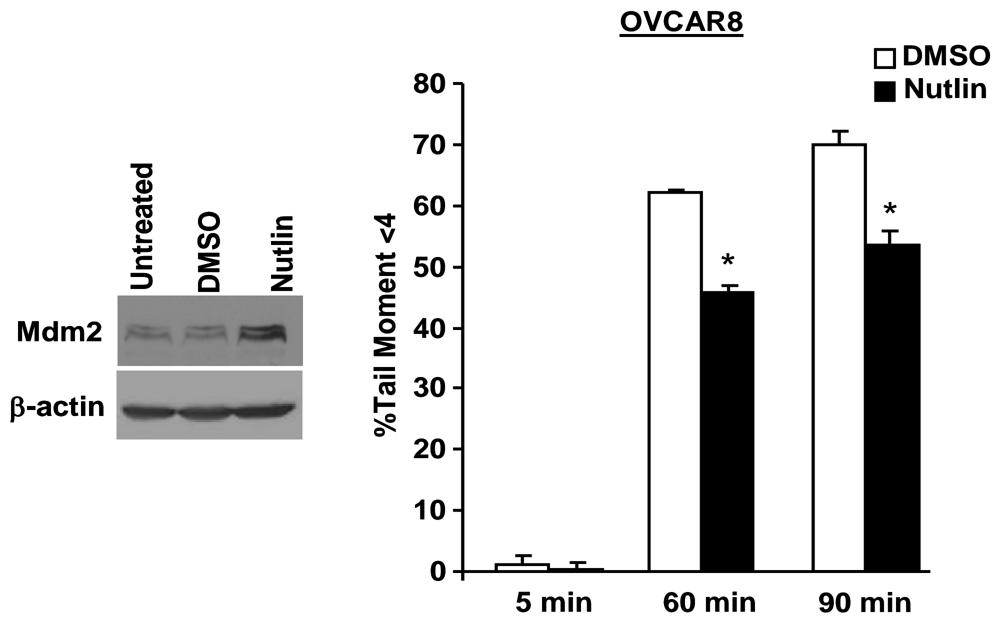

Figure 34. Nutlin inhibits DNA break repair in ovarian cancer cells with mutated p53. OVCAR8 cells with mutated p53 were treated with either DMSO or Nutlin (10μM). Following γIR, comet assays were used to evaluate DNA damage. Each bar represents the average of at least two independent experiments with a minimum of 40 cells analyzed for each sample. *p value ≤ 0.05; student's t test.

treated cells (Figure 35b). These results indicate Nutlin treatment inhibits DNA repair and DNA damage response signals in ovarian cancer cells with inactivated p53, andthis was similar to what we observed in MEFs (Figure 31).

Cooperation between Nutlin and genotoxic agents in ovarian cancer cells with inactive p53

Cisplatin is a genotoxic agent that results in DNA double-strand breaks and is used in first-line therapy for ovarian cancer. To determine if inhibition in DNA repair caused by Nutlin could cooperate with genotoxic chemotherapeutic agents, we evaluated the combinationatorial effect of Nutlin and Cisplatin. We treated SKOV3 cells with DMSO alone, Nutlin alone, Cisplatin alone, and Nutlin and Cisplatin in combination, and MTT assays were used to evaluate expansion of the cells over time. SKOV3 cells treated with both Nutlin and 1μM Cisplatin resulted in a 26.91% +/- 0.62% reduction in cell growth compared to DMSO treated cells, whereas either drug alone only resulted in a maximum of 12.27% +/- 0.86% reduction (Figure 36). This cooperative effect was also observed with Nutlin and 5μM Cisplatin, which inhibited growth by 51.48% +/- 0.49% together, while 5μm Cisplatin alone only inhibited 34.55% +/- 1.08%. Nutlin enhanced the effect of 5μM Cisplatin to more than 10μm Cisplatin alone (51.4% +/-0.49% and 46.28% +/-1.19% respectively). Collectively, these studies indicate pharmacologically increasing Mdm2, such as with Nutlin, enhanced the lethality of Cisplatin in ovarian cancer cells with inactivated p53.

Etoposide also results in double-strand DNA breaks and is often used to treat relapsed ovarian cancer, following platinum-resistance. To evaluate the combination

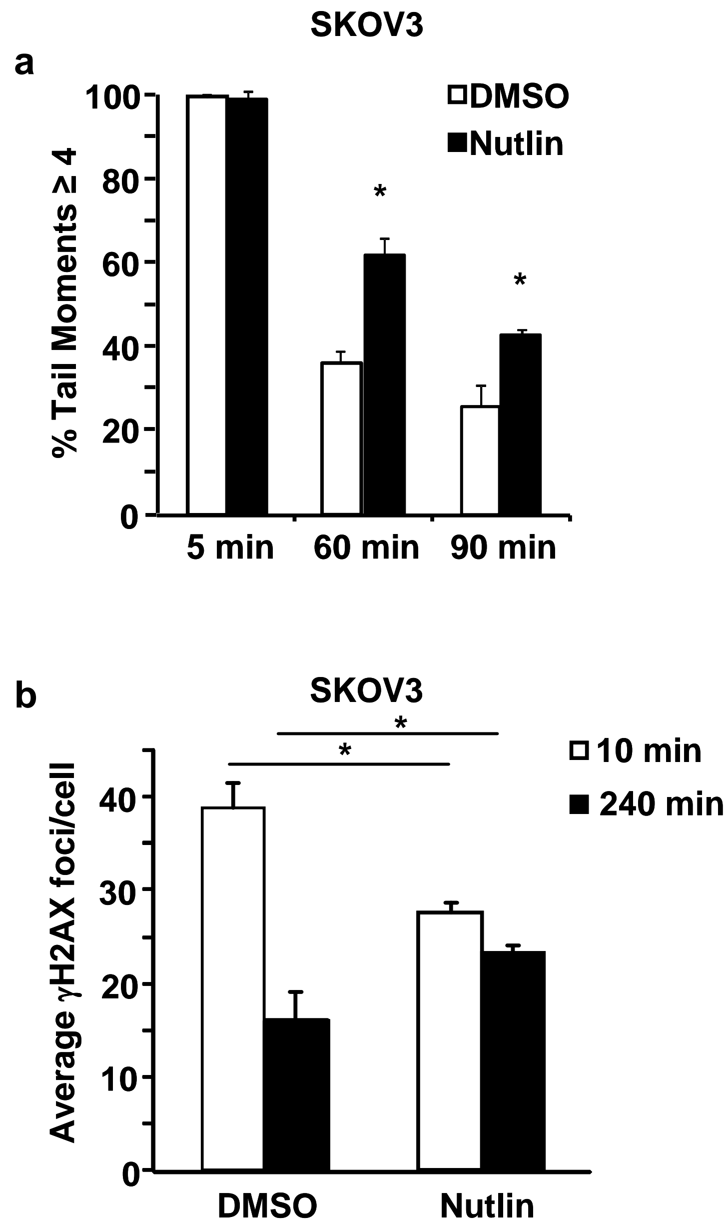

Figure 35. Nutlin inhibits DNA break repair in ovarian cancer cells with deleted p53. SKOV3 (ovarian) cells with deleted p53 were treated with either DMSO or Nutlin (10μM). Following γIR, a) comet assays were used to evaluate DNA repair and b) γH2AX foci were quantified at the indicated times. a,b) Each bar represents the average of at least two independent experiments with a minimum of 40 cells analyzed for each sample. a) *p value ≤ 0.05; student's t test. b) * CI>95%

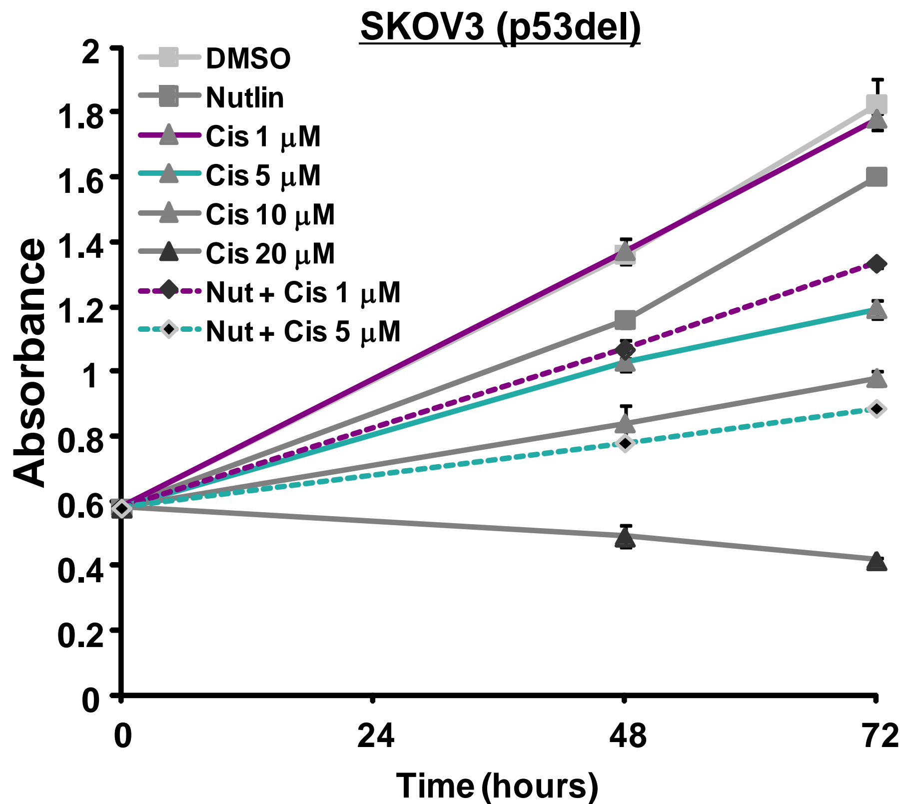

Figure 36. Nutlin cooperates with Cisplatin to reduce cell growth in ovarian cancer cells with p53 deleted. SKOV3 cells were treated with DMSO, Nutlin (10µm), Cisplatin (1-20µM), or a combination of Nutlin (10µM) and Cisplatin (1µm or 5µm). MTT was used to measure cell growth at 48 and 72 hours. Representative graph of 2 independent experiments. Error bars are SEM.

effect of Nutlin with Etoposide, two ovarian cancer cell lines (OVCAR5 and OVCAR8) with mutant p53 were used. Cells were treated with DMSO, Nutlin, Etoposide, or Nutlin and Etoposide in combination, and growth of the cells was determined by an MTT assay. OVCAR5 cells treated with either Nutlin or 1μm Etoposide had a very slight reduction on the expansion of the cells (8.04% +/- 1.7% and 10.81 +/-0.58%, respectively), but when combined, cells were inhibited by 44.08% +/- 1.29% (Figure 37). Similarly, when treated with 5 μM Etoposide and Nutlin, cells were inhibited by 75.31% +/- 0.79% compared to only 52.91% +/-1.14% when 5 μM Etoposide alone was used (Figure 37). A similar effect was observed in the OVCAR8 cells (Figure 37). In SKOV3 cells that have p53 deleted, we observed a similar effect. Nutlin alone and 1μm Etoposide had a very slight effect on the growth of the cells (-8.63% +/-1.34% and -7.01%+/2.08%-, respectively), but when combined, cells were inhibited by 34.80% +/- 0.74 (Figure 38). Similarly, when treated with 5μM Etoposide alone, cells were inhibited 47.42% +/-1.29%, but when combined with Nutlin, cells were inhibited by 61.74% +/- 0.63%. which is similar to treating the cells with 4 times the amount of Etoposide (64.70% +/-0.89%) (Figure 38). These results indicate when ovarian cells were treated with both Nutlin and Etoposide, they were significantly impaired in their ability to expand, and this was more than the effect of either drug alone, reflecting cooperation.

To determine if apoptosis caused the observed reduction in cell growth, the cells were visually evaluated. We observed there were fewer cells in addition to an increase in dead and dying cells when exposed to both Nutlin and Etoposide (Figure 39a). To truly assess if more apoptosis was occurring upon combination treatment, we evaluated

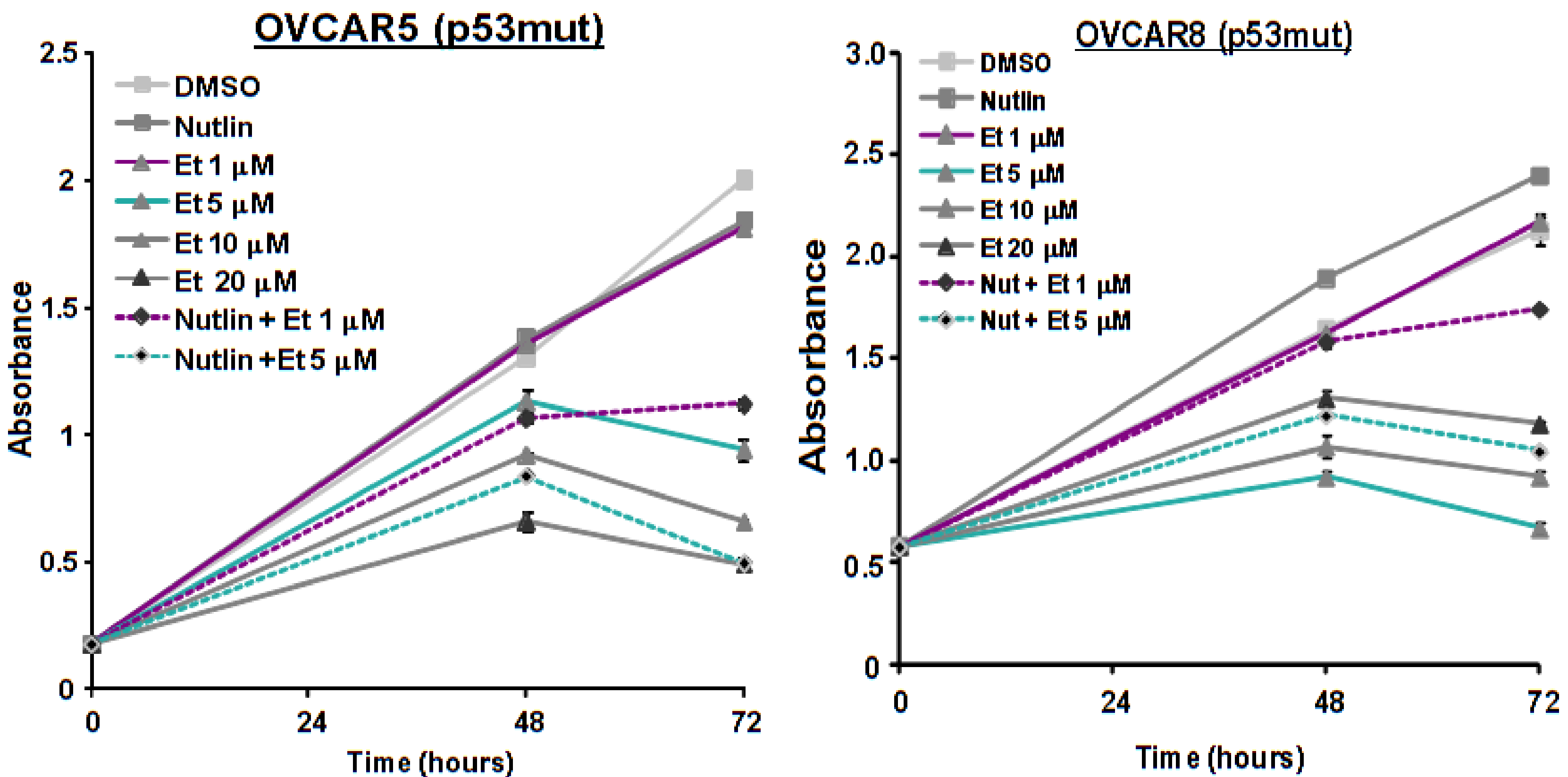

Figure 37. Nutlin cooperates with Etoposide to reduce cell growth in ovarian cancer cells with mutated p53. OVCAR8 and OVCAR5cells were treated with DMSO, Nutlin (10μm), Etoposide (1-20μM), or a combination of Nutlin (10μM) and Etoposide (1μ/M or 5μM). MTT was used to measure cell growth at 48 and 72 hours. Representative graph from 3 independent experiments. Error bars are SEM.
* Indicate combination treatments

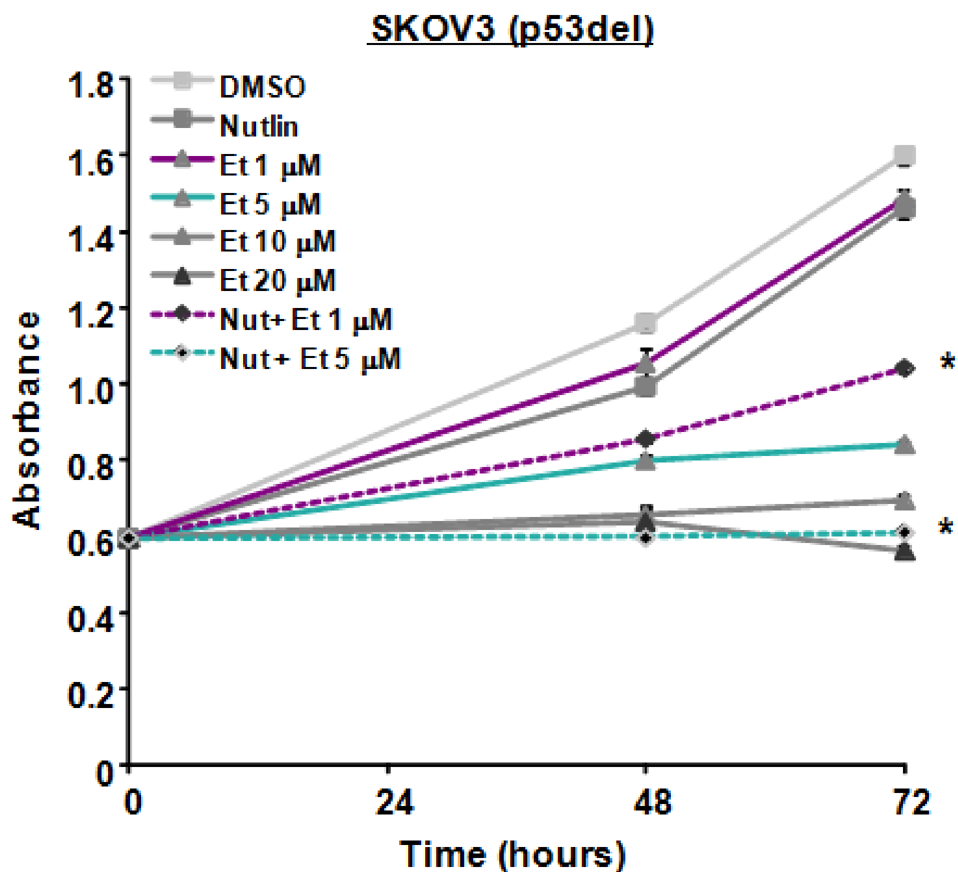

Figure 38. Nutlin cooperates with Etoposide to reduce cell growth in ovarian cancer cells with deleted p53. SKOV3 cells were treated with DMSO, Nutlin (10μm), Etoposide (1-20μM), or a combination of Nutlin (10μM) and Etoposide (1μ/M or 5μM). MTT was used to measure cell growth at 48 and 72 hours. Representative graph from 3 independent experiments. Error bars are SEM. *Indicate combination treatments

little or no cleaved Caspase 3 was detected in DMSO or Nutlin treated cells; however, levels of cleaved Caspase 3 by western blot analysis. Similar to the MTT assay, very there was an increased amount observed in cells treated with Nutlin and Etoposide 5μM together (Figure 39b). This was similar to the amount of apoptosis from Etoposide 10μM alone. Comparable results were observed in OVCAR8 cells, which contain mutant p53 (Figure 39b). These results further corroborate the MTT data suggesting Nutlin has a cooperative effect with Etoposide, which results in apoptosis.

Discussion

It is important to understand the mechanism of action of potential chemotherapeutic drugs, as this knowledge can provide understanding for the cellular pathways affected by the drug and also influence the development of new drugs that have similar but improved properties. Since the development of Nutlin was reported a decade ago, researchers have been using Nutlin as a research tool for elucidating the involvement of the p53 pathway in various settings and for revealing the chemotherapeutic potential for reactivating p53 to treat cancer.[146,162] Studies have clearly demonstrated the p53-dependent properties of Nutlin,[162] yet little is known about the p53-independent effects of Nutlin. The potential benefit of combining Nutlin with genotoxic agents for the treatment of cancers with inactivated p53 had been reported previously, but the mechanism behind the p53-independent effect of Nutlin was unknown.

One observed p53-independent effect of Nutlin is the increase in Mdm2 protein levels. We have previously shown Mdm2 overexpression inhibits DNA break repair and

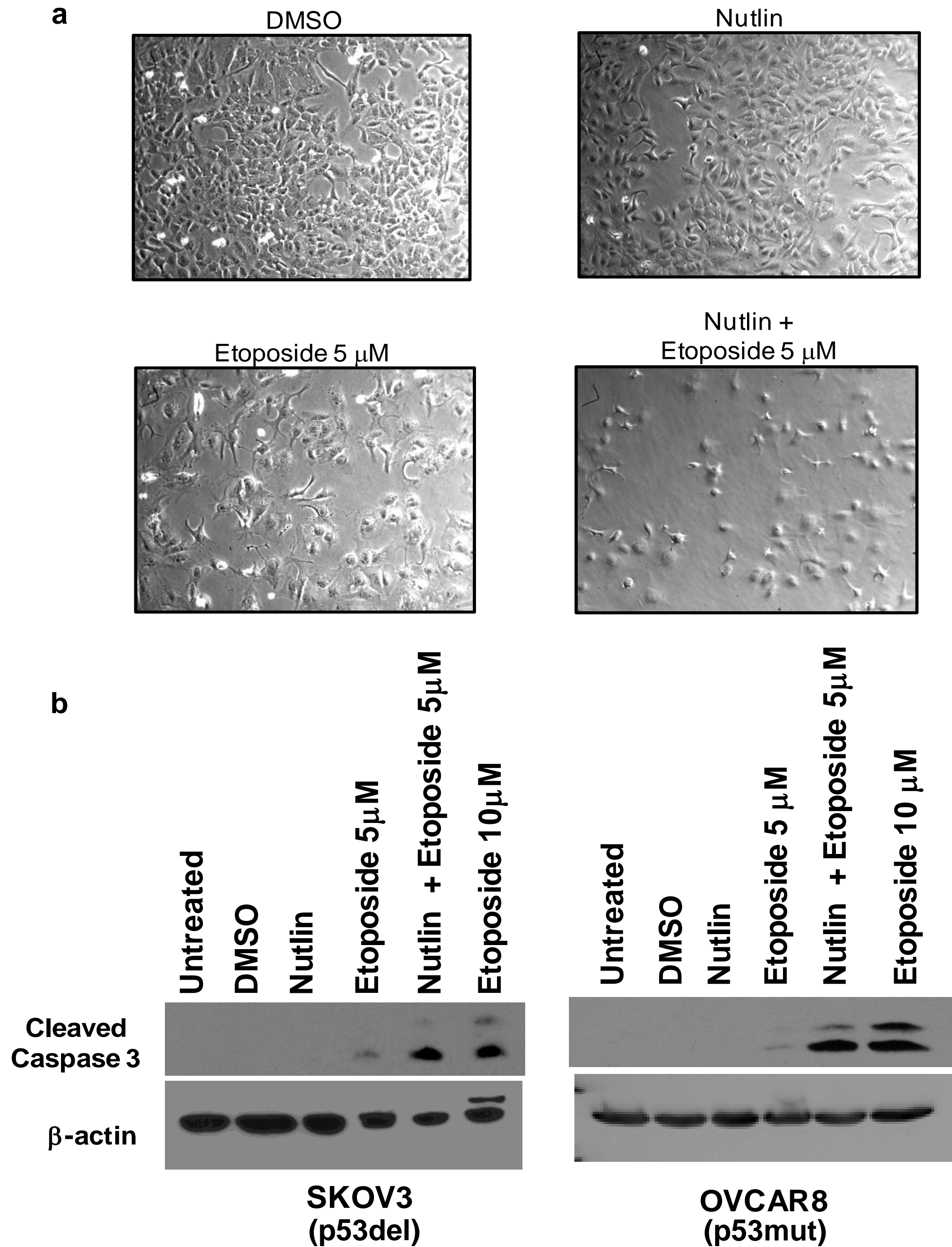

Figure 39. Nutlin and Etoposide cooperate to induce apoptosis in ovarian cancer cells with p53 inactivated. SKOV3 (p53del) and OVCAR8 (p53mut) cells were treated with DMSO, Nutlin (10μM), Etoposide 5μM, Nutlin and Etoposide 5μM, and Etoposide 10μM, as indicated. After 48 hours, A) representative pictures were taken and B) protein lysates were evaluated by Western blot analysis for cleaved Caspase 3 and β-actin.

promotes genome instability independent of p53.[3,15] Therefore, we predicted that the induction of Mdm2 by Nutlin was resulting in an inhibition in DNA repair that could be combined with genotoxic insults to cause genome instability. This instability could induce apoptosis in cancer cells independent of p53 status. Here we demonstrate the increase in Mdm2 caused by Nutlin inhibited DNA break repair and impaired DNA damage response signals independent of p53. These results provide insight into a novel p53-independent mechanism for Nutlin, which had not been thoroughly explored. Importantly, our findings illustrate the therapeutic benefit of pharmacologically increasing Mdm2 in conjunction with genotoxic agents as a means of inducing synthetic lethality in cancers with inactivated p53, such as ovarian cancer.

Numerous studies have described a cooperative effect between Nutlin and genotoxic agents, but most are in the context of wild-type p53. A few studies explore this effect in cells with mutated or deleted p53, but the mechanism for this observed cooperation between Nutlin and genotoxic agents has been poorly understood. For example, Conradt *et al* 2012 described a cooperative effect between Nutlin and topoisomerase II inhibitors in pancreatic cells with inactivated p53.[31] While they demonstrated Nutlin results in an increase in DNA damage response signals in response to genotoxic insults, the mechanism responsible for this observation still remained unclear. Consistent with Conradt et al, we observed an increase in DNA damage response signals 4 hours after γ-IR, and this is likely due to the increased Mdm2 levels caused by Nutlin. This would cause an inhibition in early DNA damage response signaling leading to an impaired ability to repair DNA breaks overtime, manifesting as increased DNA damage response signals. Our studies reveal this is

likely due to the DNA breaks remaining as a result of the inhibition of the early DNA damage response signals and DNA repair through elevated Mdm2 levels caused by Nutlin.

Another proposed p53-independent mechanism of Nutlin cooperation with genotoxic agents is through activating p73 and E2F1 to induce apoptosis.[4,84,130,136] Both p73 and E2F1 can be activated following DNA damage. Because our studies here demonstrate Nutlin inhibits DNA repair through Mdm2, it could be that excessive DNA damage and an inhibited ability to repair it results in subsequent activation of p73 and E2F1, rather than Nutlin directly affecting these proteins. As this was not excluded or explored in these studies, this is a possibility that should be considered.

While reactivating p53 in cancer is an ongoing effort for chemotherapy, selection for p53 inactivation often occurs.[6,11] Therefore, novel chemotherapeutic options are imperative for cancers with inactivated p53, such as ovarian cancer. The data described here provide evidence that increasing Mdm2 pharmacologically and combining this with genotoxic agents has therapeutic potential for cancers with inactivated p53. Current standard chemotherapy treatment involves the platinum-based genotoxic agents Cisplatin or Carboplatin.[63] Our results indicate Nutlin used in combination with Cisplatin can increase the efficacy of Cisplatin in ovarian cancer cells with inactivated p53. Relapse in ovarian cancer often results from platinum-resistance and requires the need to use combination therapy with other genotoxic agents, such as Etoposide. Our results demonstrate that Nutlin also cooperates with Etoposide to increase its efficacy. Our findings have significant therapeutic implications, as they provide insight into the benefit of drugs which increase levels of Mdm2, such as Nutlin,

in combination therapy with genotoxic agents in cancers with inactivated p53. Although Nutlin is not clinically viable due to poor bioavailability, second generation Nutlins are currently being tested in clinical trials.[21,145] The data described in this study provides important information that can be used in establishing additional trials to evaluate the benefit of Nutlins, and other small molecules causing increased Mdm2 levels, in cancers with inactivated p53. This study emphasizes the importance of further assessing drugs that can increase Mdm2 levels as a novel chemotherapeutic method.

Chapter 5

SUMMARY AND FUTURE DIRECTIONS

The oncogenes Mdm2 and Mdmx are overexpressed in a variety of human cancers. The major function of Mdm2 and Mdmx has been primarily associated with negative regulation of the tumor suppressor p53; however, evidence has suggested they have p53-independent functions as well. In particular, we and others have observed that elevated Mdm2 and/or Mdmx levels can occur in tumors with inactivated p53.[22] Despite this evidence, little was known about the p53-independent functions of Mdm2 and Mdmx and the therapeutic implications of these functions.

Dr. Eischen's lab had previously identified and described a novel p53-independent function of Mdm2, whereby elevated levels inhibited double-strand DNA break repair. The p53-independent functions of Mdmx were still very poorly understood. Mdmx and Mdm2 are homologous proteins. Because, Mdmx had been shown to enhance functions of Mdm2, I investigated the potential of a p53-independent function of Mdmx to inhibit DNA repair. Here, I have identified and characterized a novel function of Mdmx where its overexpression alters DNA damage response signaling, inhibits double-strand DNA break repair, and promotes genome instability. This function was independent of p53 and, surprisingly, of Mdm2. This ability to inhibit DNA repair appears to be a previously unknown conserved function of this family of proteins that confers transformation potential independent of p53.

These findings demonstrate a benefit for pharmacologically increasing Mdm2 and Mdmx levels to inhibit DNA repair in the presence of genotoxic agents as a

potential means of inducing synthetic lethality in cancer cells, irrespective of p53 status. Although small molecules that increase Mdmx levels have yet to become commercially available, Nutlin and other small molecules are available that result in elevated Mdm2 levels. While a handful of studies had demonstrated cooperation between Nutlin and genotoxic agents independent of p53, the mechanism behind this cooperation was not understood. Here, I describe how Nutlin inhibits double-strand DNA break repair through Mdm2 and independent of p53. In cooperation with genotoxic agents, Nutlin caused an increase in apoptosis in ovarian cancer cells with mutated or deleted p53. These results demonstrate the importance of pharmacologically inducing the elevation of Mdm2 and Mdmx protein levels as a novel therapeutic avenue for cancers with inactivated p53.

Pharmacological-induction of Mdm2 inhibits DNA repair and cooperates with genotoxic agents to induce apoptosis independent of p53

I determined Nutlin alone could inhibit DNA break repair and delay DNA damage response foci formation and resolution independent of p53. This phenotype mimics what is observed with ectopic overexpression of Mdm2.[3,16] Furthermore, the negative effects of Nutlin on DNA repair were abrogated in cells lacking Mdm2 (Chapter 4). Therefore, these results suggest the increased levels of Mdm2 caused by Nutlin are mediating its negative effects on DNA repair. This provides a novel mechanism of action by which Nutlin can affect cancer cells independent of p53.

Although it has been reported numerous times Nutlin treatment results in an increase in Mdm2 levels, the importance of this has been an underappreciated and

poorly understood effect of Nutlin. The Eischen lab has previously shown Mdm2 overexpression inhibits double-strand DNA break repair.[3,16] In Chapter 4, I describe how the increase in Mdm2 levels caused by Nutlin resulted in an inhibition of DNA break repair, which was independent of p53. Furthermore, γH2AX foci formation and resolution were inhibited. Because γH2AX is at the site of damage, it is often used to signify the DNA break itself; however, it also serves as a beacon for a number of important DNA repair proteins. Alterations in γH2AX signal can be used to reflect the entire DNA damage response signaling cascade. The Eischen lab had previously described Mdm2 mediated its effects on DNA repair through specifically interacting with Nbs1 of the Mre11-Rad50-Nbs1 DNA repair complex.[16] The observed inhibition in DNA repair and γH2AX foci formation and resolution mimics the previously characterized phenotype of Mdm2 overexpression. Because Nutlin increases Mdm2 levels, it is likely Nutlin inhibits DNA repair in an Nbs1-mediated process.

Even though little was known about the p53-independent effects of Nutlin when I began investigating it, two additional studies have reported an observed effect of Nutlin on the DNA damage response independent of p53. In both studies, an increase in the activation of DNA damage response signals, such as γH2AX and phosphorylation of ATM, was observed when Nutlin was combined with genotoxic agents.[31,160] Although both studies noted this increase in DNA damage response signals, neither study was able to explain the mechanism of action as to how exactly Nutlin was causing this alteration in the DNA damage response. The suggested mechanism by *Conradt et al* included Nutlin inducing DNA breaks; however, this mechanism was based on their evaluation of DNA damage response signals three or more hours after genotoxic insult.

Their findings are likely reflective of the same increase in γH2AX foci I observed at 2.5 hours post γ-IR, which is due to an early inhibition in the DNA repair signaling process rather than an induction of DNA breaks. This result also correlates with previous published results by the Eischen lab demonstrating elevated levels of Mdm2 increase DNA damage response signals at later times following DNA damage, which likely results from an earlier inhibition in the formation of DNA repair foci.[3,16] Consistent with this interpretation, Nutlin inhibits early DNA response signals through increasing levels of Mdm2 (Chapter 4).

The studies described in Chapter 4 provide new insight into a novel p53-independent mechanism of Nutlin that can negatively affect DNA repair. The inhibition of DNA repair by Nutlin has potential implications for use as a research tool. Nutlin is also commonly used in the laboratory as a positive control for the activation of the p53 pathway. The results from my studies reveal Nutlin has alternative functions that may need to be considered when interpreting results using Nutlin. Furthermore, the inhibition of DNA repair is a promising tool to use in combination therapy in oncology, because often the combination of genotoxic agents with molecules that inhibit the ability of a cell to properly repair the excessive DNA damage results in synthetic lethality. Since we have shown increased Mdm2 levels promote genome instability and confer transformation potential, there may be some concern of the effects of Nutlins on normal cells. There has been evidence that Nutlins only affect tumor tissue and not normal tissue, and therefore, it would not be expected that Nutlin would promote tumor formation in the normal surrounding tissue.[138,162] Even though Nutlin has poor clinical bioavailability, my findings have important implications in the development of future

chemotherapeutics, such as 2nd generation Nutlins or other Mdm2 inhibitors, and can inform novel combination therapies to treat cancers with inactivated p53.

Pharmacological-induction of Mdm2 provides a novel potential therapeutic option independent of p53

In Chapter 4, I described how Nutlin cooperated with genotoxic agents to induce synthetic lethality in ovarian cancer cells with inactivated p53. This can be beneficial as it allows for the opportunity to decrease the dose of any one drug, avoiding potential side effects, without losing efficacy. An area of focus in the development of new chemotherapeutics and chemotherapeutic regimens is the use of genotoxic agents in combination therapy to induce excessive genome instability, ultimately resulting in apoptosis of cancer cells.[60] My studies provide new information that can direct the development of new therapeutic combinations to include molecules that increase Mdm2 levels.

Because it is often diagnosed at the later stages, ovarian cancer is challenging to treat.[63] A high frequency (>90%) of inactivated p53 through mutations or deletions is very characteristic of ovarian cancer.[2] Of note, when I evaluated Mdm2 expression levels through TCGA analysis, the majority of these samples (270/300) do not have *MDM2* overexpressed. Since these cells have likely not developed compensatory mechanisms to deal with inhibited DNA repair caused by increased levels of Mdm2, these cancers are prime candidates for the combination therapy using molecules to increase Mdm2 and cause genotoxic insults. To this end, I evaluated the combination effect of Nutlin and Etoposide or Cisplatin, because these two clinically relevant

genotoxic agents induce double-strand DNA breaks, of which Mdm2 has been shown to inhibit the repair. I observed a cooperative effect resulting in increased apoptosis independent of p53. In addition to Cisplatin and Etoposide, other DNA damaging drugs are used to treat ovarian cancer, and my data suggest Nutlin will cooperate with these drugs as well. Knowledge of which drugs have a cooperative effect will allow for more flexibility with combination therapies given to an individual patient and those developed in the future. To validate that Nutlin is cooperating with the genotoxic agents through Mdm2 and not an off-target effect, the requirement of Mdm2 for Nutlin to cause a cooperative increase in apoptosis needs to be assessed. One approach could be the use of shRNA to knockdown Mdm2, and subsequently treat with Nutlin to evaluate if the cooperative effect is no longer observed between Nutlin and genotoxic agents. A potential issue with this approach is that shRNA will not result in a 100% knockdown of Mdm2, and therefore, Nutlin will cause an increase in Mdm2 protein levels and continue to cooperate with genotoxic agents. A better approach would be to treat cancer cells with and without Mdm2, and assess cooperation in the absence of Mdm2. To reduce cancer and patient variability, a conditional Mdm2 knockout system would be ideal. Because I determined in MEFs that Nutlin mediates its effects on DNA repair through Mdm2, I predict that Nutlin will no longer have a cooperative effect in the cancer cells without Mdm2.

The ability to induce apoptosis independent of functional p53 has important implications in oncology treatment, as half of all cancers have inactivated p53. This is particularly pertinent for ovarian cancer, since it has a very high frequency of p53 mutations. Novel chemotherapeutic approaches are needed for ovarian cancer

because 70-90% of patients diagnosed in stages III/IV will relapse and need more aggressive therapy.[63] My studies reveal the importance of utilizing or developing chemotherapeutics that increase Mdm2 levels, as these could be used to promote excessive genome instability to induce apoptosis in ovarian cancer, and other cancers with inactivated p53.

The combination of genotoxic agents and Nutlin has been demonstrated numerous times in a variety of cancer types retaining wild-type p53.[8,30,52,113,120,138,167] While these combination effects have been less studied in cancers with inactivated p53, some studies have shown a cooperative effect between Nutlin and genotoxic agents independent of p53, including pancreatic, colon, neuroblastoma, hepatocellular, sarcoma, and retinal cancers.[4,31,120,167,177] Ovarian cancer has a high frequency of p53 mutations,[2] yet the p53-independent effects of Nutlin on this cancer had not yet been investigated. Therefore, I focused my research on this tumor type. I observed that Nutlin cooperated with various genotoxic agents to induce apoptosis of patient-derived ovarian cancer cell lines. These results suggest that Nutlin would be able to cooperate with genotoxic agents *in vivo* as a means of chemotherapy. Animal models, such as patient-derived xenografts, would provide a means of evaluating the overall effect of Nutlin and genotoxic agents on tumor growth *in vivo*. With the knowledge that Nutlin can cooperate with genotoxic agents to promote cancer cell death independent of p53 *in vitro*, it would be expected that inducing Mdm2 pharmacologically *in vivo* would have a cooperative effect with a genotoxic agent, resulting in a reduction in tumor size. This would signify the benefit of developing and using drugs that can increase Mdm2 levels for use in combination chemotherapeutics in a p53-independent setting.

Even though Nutlin has poor bioavailability, derivatives of Nutlin and other small molecules that target Mdm2 are being developed that may be more viable in the clinic. RG7112 and MI-63 both have the ability to cause an increase in Mdm2 protein levels, and therefore, could be used to induce the same synthetic lethal effects as Nutlin on cancer cells with inactivated p53 (Figure 30). RG7112 and another compound generated by Roche are both derivatives of Nutlin currently in clinical trials.[164] Having better bioavailability than Nutlin allows these drugs to be used in the clinic in combination therapy with genotoxic agents as treatment options for cancers with inactivated p53. Furthermore, a noted consequence of using Mdm2 inhibitors is the acquisition of p53 mutations to evade the reactivation of p53 by the drug[6,69,111]; however, my studies suggest Nutlin, or similar acting molecules, would still be useful as a chemotherapeutic agent in the presence of p53 mutations when used in conjunction with a genotoxic agent. In future studies, it would be interesting to evaluate the effects of different Mdm2 inhibitors on the DNA repair process. Because increasing Mdm2 will cause an inhibition in double-strand DNA break repair, other Mdm2 inhibitors causing increases in Mdm2 are predicated to be just as useful as Nutlin. Additionally, small molecules resulting in an increase in Mdmx would be anticipated to have the same outcome, since Mdmx overexpression inhibits DNA repair as well. Keeping this in mind as inhibitors of Mdmx are generated can be useful for developing new therapeutic options.

Mdmx inhibits DNA break repair independent of p53 and Mdm2

Mdmx is overexpressed in many cancers, some with inactivated p53, therefore

elucidating other functions of Mdmx aside from its regulation of p53 is critical. Because p53-independent functions of Mdmx were not clearly understood, I focused a majority of my thesis work identifying and characterizing a novel function of Mdmx that is independent of p53. In this dissertation, I describe a p53- and Mdm2-independent function of Mdmx by which its overexpression inhibits double-strand DNA break repair. Furthermore, I established that Mdmx associates with Nbs1 of the MRN DNA repair complex, and there was an alteration in early and late DNA damage response signaling events (γH2AX and pS/T-Q) occurring after γIR (Figure 40).[22] This novel function of Mdmx has important implications in understanding its poorly elucidated physiologic role in the cell, and how this may be advantageously altered to promote genome instability, which contributes to tumorigenesis.

Little is known about the functions of Mdmx outside of its regulation of p53 and its association with Mdm2; therefore, the ability of Mdmx to inhibit double-strand DNA break repair independent of both p53 and Mdm2 is significant and important for understanding the function of Mdmx. Mdmx acts similarly to Mdm2 in this function of inhibiting DNA repair, yet it can do so in the absence of Mdm2. This begs the question of whether Mdmx is mediating its effects on the DNA damage response through Nbs1, like Mdm2 does, or if Mdmx has a different mechanism to achieve the same end point as Mdm2. This would not be far-fetched considering both Mdm2 and Mdmx inhibit p53, but each negatively regulates p53 in a different manner. To begin evaluating this, determining if Mdmx directly interacts with Nbs1 needs to be assessed. Furthermore, mutations in the regions within Mdmx and Nbs1 mediating their association will provide a means of evaluating the requirement for Nbs1 in order for Mdmx to inhibit DNA repair.

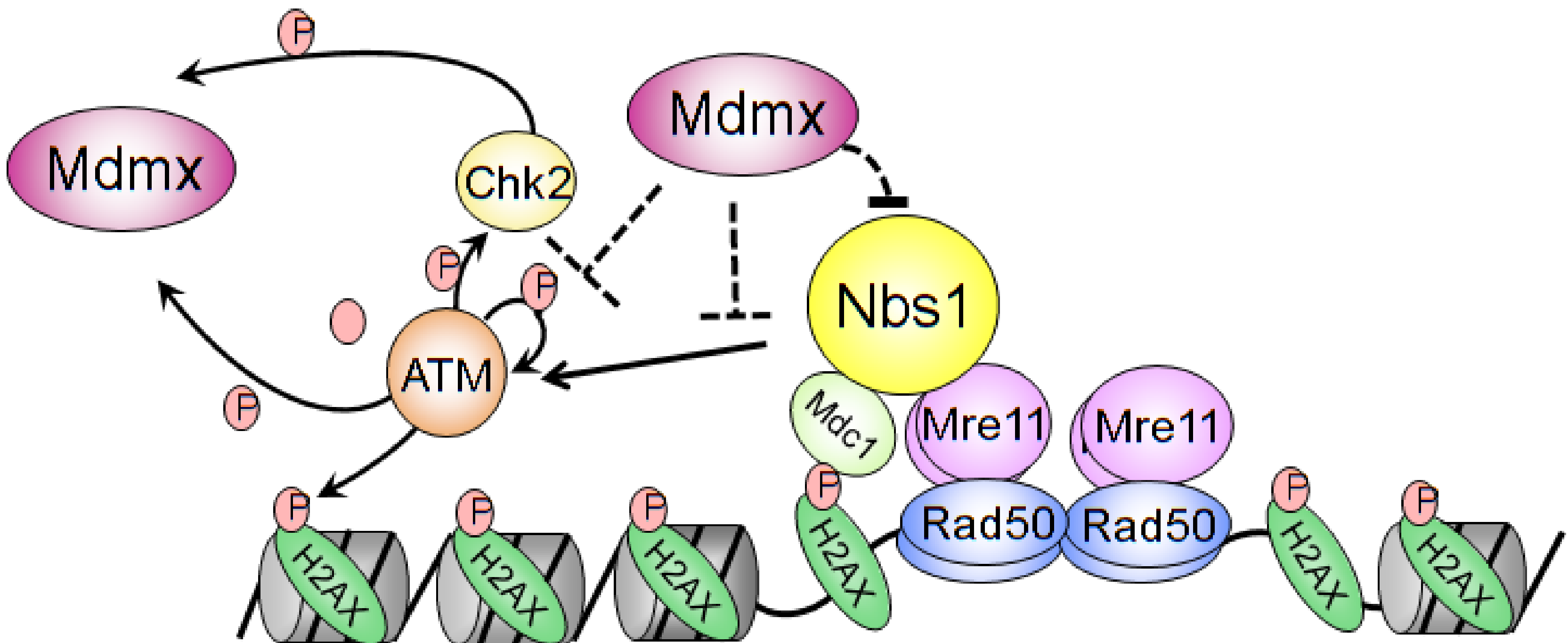

Figure 40. Mdmx interacts with Nbs1 and inhibits multiple proteins in the DNA damage response. Following double-strand DNA breaks, the MRN complex is recruited to the site of damage. Mdmx associates with Nbs1 at chromatin following DNA damage. Mdmx also inhibits the phosphorylation of γH2AX and pST/Q sites. This likely results from an inhibition in Nbs1 or ATM (dashed lines). (Modified from Melo and Eischen 2012)

Because Mdmx inhibits DNA repair and associates with Nbs1, it is likely Mdmx is able to affect the DNA damage response process through Nbs1, but further evaluation will reveal the mechanism Mdmx uses to affect DNA repair.

Upon overexpression of Mdmx, there was a reduction in the formation of γH2AX foci immediately after γ-IR. While γH2AX is often used to mark double-strand DNA break sites, it is also a target of the kinase ATM, which serves as a docking site for many key DNA repair proteins (Figure 40). Both γH2AX and ATM are critical for efficient DNA repair, which correlates with my observed inhibition in overall DNA repair. H2AX phosphorylation is decreased when Mdmx is overexpressed, and this result also indicates Mdmx could potentially be inhibiting ATM signaling. Indeed, Mdmx overexpression dampens the overall ATM signaling response following the induction of double-strand DNA breaks. I observed there was a reduction in pST/Q foci formation immediately following DNA damage As this is reflective of ATM activity, these findings suggest Mdmx is affecting ATM either indirectly or directly. One possibility is Mdmx inhibits the activation of ATM by Nbs1. This would result in a delay in DNA damage response signals, similar to what I observed in Chapter 3. Another possibility is that Mdmx is affecting ATM's kinase activity following activation to cause an overall decrease in phosphorylated ATM targets, which would result in an overall decrease in pS/TQ foci, as I observed. To further elucidate the specific effect Mdmx is having on ATM, analysis of the kinase activity of ATM in the presence of elevated Mdmx levels would be interesting. Although the specific effect of Mdmx on ATM is not clear, my results demonstrate that ATM signaling in the DNA damage response is being impaired by elevated Mdmx, and this is likely resulting in the inhibition in DNA repair.

An additional possible mechanism by which Mdmx is inhibiting DNA repair is through Nbs1. Mdmx could be localized to sites of DNA damage by Nbs1 where it could impair the function of Nbs1 in the repair process and subsequently alter the DNA damage response and overall DNA repair. Since I have shown that Mdmx associates with Nbs1 at chromatin (Figure 25), it is likely that Mdmx is brought to sites of DNA damage by this interaction; however, the possibility remains that Mdmx is brought to sites of DNA damage by other proteins. To ascertain the involvement of Nbs1, it would be important to evaluate the ability of Mdmx to associate with chromatin in cells lacking Nbs1. Further analyses will determine whether Nbs1 is mediating the localization of Mdmx to the sites of breaks.

Nbs1 is involved in the activation of ATM following double-strand breaks (Figure 40). By interacting with Nbs1, Mdmx could be mislocalizing Nbs1 or preventing the interaction between Nbs1 and ATM (Figure 40). We observed that Mdmx associates with Nbs1 at chromatin following DNA damage, and the association between Mdmx and Nbs1 at chromatin was elevated after DNA damage. This demonstrates Mdmx could be associating with Nbs1 following DNA damage and preventing Nbs1 from localizing to sites of damage or altering the ability of Nbs1 to be retained at the breaks sites. As Nbs1 is very important in the repair of double-strand breaks, inhibiting its proper localization to the site of DNA damage would likely result in a delay in DNA repair, which is what we observed using the comet assay. In addition, when I analyzed the DNA damage response signaling after DNA damage, I observed a decrease in the γH2AX and pS/T-Q signals. Another interesting observation was that with time, Mdmx appeared to associate less with chromatin yet retain its association with Nbs1. One

interpretation of this result is that the interaction between Mdmx and Nbs1 increases over time following DNA damage, even as Mdmx appears to decrease its association with chromatin. From these results, it is unclear if Mdmx is decreasing its association with chromatin or if this reflects a technical caveat in this experiment. If the antibody isotope is masked, such as through post-translational modifications or protein:protein interactions, then the antibody would be impaired in its ability to recognize Mdmx. Careful biochemical analyses need to be performed to determine if this is the case. In addition to interacting with Nbs1, Mdmx associating with chromatin after DNA damage allows for the possibility that Mdmx could be affecting other DNA repair proteins at break sites. To assess this, the localization, activation, and activity of other key DNA repair proteins in the presence of increased Mdmx would need to be evaluated. Because I observed an alteration in ATM signaling, key ATM substrates would be ideal initial targets. Determining if other proteins are affected by Mdmx will provide insight into the effects of Mdmx on the global DNA damage response. This may provide further explanation as to the mechanism by which Mdmx is inhibiting DNA repair.

Whereas most studies have focused on the modulation of Mdmx by ATM, the decrease in pST/-Q foci I observed was the first to suggest Mdmx may modulate ATM signaling (Figure 40). Mdmx is phosphorylated by ATM following double-strand DNA breaks. Although it is not entirely clear why ATM phosphorylates Mdmx, one proposed reason is this phosphorylation of Mdmx prevents the interaction with p53. Mdmx is thought to be subsequently degraded to free up p53; however, my thesis work has shown a portion of Mdmx is actually localizing to chromatin following DNA damage. Indeed, others have observed Mdmx localizes to the nucleus from the cytoplasm

following DNA damage. While it has been proposed this translocation serves to allow Mdmx to be degraded, this does not make sense for Mdmx to be first shuttled to the nucleus to be degraded when it could have been degraded in the cytoplasm. Based on my findings described in Chapter 3, Mdmx, or at least a sub-population, is actually going to chromatin following DNA damage and associating with Nbs1. Considering Nbs1 has been shown to localize Mre11/Rad50 to sites of DNA damage, it is possible Mdmx associates with Nbs1 and is being localized to sites of breaks through this association. Overall, my studies shed light onto effects Mdmx has on DNA repair and early DNA damage response signaling.

The interaction between Mdmx, Mdm2, and Nbs1

As described in Chapter 3, I determined endogenous Mdmx and Nbs1 associate in the absence of p53, and Mdmx specifically associates with Nbs1 of the Mre11-Rad50-Nbs1 complex. Since the Eischen lab had previously characterized the specific amino acids required for Mdm2 to interact with Nbs1, I first investigated if the region identified in Mdm2 responsible for interacting with Nbs1 was conserved in Mdmx. The corresponding amino acids in Mdmx have little homology within the same region in Mdm2. The Nbs1-association region could be localized to another area of the Mdmx protein, therefore I aligned the Nbs1-binding sequence of Mdm2 with the entire Mdmx protein sequence, but there was no indication of a conserved region to bind to Nbs1 elsewhere in the protein. This was an analysis of the primary structure, and Mdm2 and Mdmx only share about 40% homology. This does not eliminate the possibility Mdmx directly binds to Nbs1. It remains a possibility that the Nbs1-association region may

reveal itself in the tertiary structure.

Using deletion mutants, the region of Mdmx mediating the interaction with Nbs1 can be identified. Furthermore, determining if Mdmx directly interacts with Nbs1 will reveal a possible mechanism by which Mdmx is mediating its effects on DNA repair. Using deletion mutants of Mdmx, I determined the C-terminal portion of Mdmx containing amino acids 346-489 is not required for association with Nbs1. This correlates with our data showing these amino acids were not required to exert the effect on DNA repair, genome instability, or transformation observed by full-length Mdmx. Once the region mediating this function of Mdmx has been defined, individual amino acids can be mutated to identify the specific amino acids facilitating the interaction with Nbs1. The full structures of Mdm2 and Mdmx are not available, but this knowledge would be insightful to determine binding pockets and surface residues potentially involved in the association between Nbs1 and Mdmx.

In terms of the interaction of Nbs1 with Mdmx, we were able to narrow the region to amino acids 396-512 within Nbs1. It is intriguing that the Mdm2-binding region (a.a. 474-512) of Nbs1 is contained within this region, yet Mdmx associates with Nbs1 in the presence or absence of Mdm2. Although it is yet to be determined if Mdm2 and Mdmx associate with Nbs1 in the same region, the possibility is certainly there. The ability of Mdm2 and Mdmx to associate with Nbs1 in the exact same domain is an interesting scenario. Although Mdmx interacts with Nbs1 independent of Mdm2, Mdm2 and Mdmx may possibly compete for this binding site; however, they have the same functional effect so there is little evidence to suggest Nbs1 associating with Mdm2 or Mdmx would be more beneficial. Mdm2 and Mdmx could have a conserved function where they are

each able to associate with Nbs1 independently as a form of compensation in case either Mdm2 or Mdmx are not able to interact with Nbs1. Because both Mdm2 and Mdmx associate with Nbs1 endogenously, this family of proteins likely has a physiological function involving the subtle regulation of Nbs1 during the DNA damage response allowing proper DNA repair to occur. Future analyses to fully assess the role of Mdm2 and Mdmx in the efficiency and success of the DNA repair process will be important. In cells lacking both Mdm2 and Mdmx, expressing mutants of Mdm2 or Mdmx that cannot bind to Nbs1 and evaluating the DNA repair abilities of these cells could be quite revealing. Mdm2 and Mdmx may have different affinities for Nbs1. In a cancer setting where either or both Mdm2 and Mdmx can be overexpressed, they may have a cooperative effect on the inhibition of DNA repair. This could contribute to genome instability and thus promote tumorigenesis. Therefore, elucidating the associations between Mdm2, Mdmx and Nbs1 is very important for both understanding normal DNA repair mechanisms and how potential alterations in these protein interactions may contribution to cancer formation.

Mdmx promotes genome instability and confers tumorigenic potential independent of p53 and Mdm2

Mdmx overexpression increased chromosomal aberrations, including chromosome breaks, which are reflective of genome instability. Chromosome and chromatid breaks can be caused by unrepaired or poorly repaired DNA breaks. Therefore, our data that Mdmx inhibits DNA repair aligns with our observation of Mdmx promoting genome instability. Genome instability can manifest as breaks and fusions,

such as the ones observed when Mdmx is overexpressed and have the potential to contribute to tumorigenesis.

Since I observed Mdmx inhibits DNA repair and promotes genome instability independent of both p53 and Mdm2, it was likely Mdmx influences cellular transformation. Indeed, I observed Mdmx overexpression alone was sufficient to promote colony formation *in vitro* in untransformed cells, which is an indication of transformation potential. This is the first hint Mdmx can promote tumorigenesis independent of its regulation of p53 and its association with Mdm2. While this does not conclusively say Mdmx will be able to promote tumorigenesis independent of p53, it is certainly suggestive of that. Although the role of p53 was not assessed, *Mdmx* transgenic mice do develop spontaneous tumors indicating Mdmx is indeed an oncogene.[174] To truly evaluate if Mdmx is able to transform cells independent of both p53 and Mdm2, mouse models using subsequent analysis of tumor onset and spectrum will be telling. In previously analyzed *Mdm2* transgenic mice with p53 deleted, the formation of tumors occurred at a similar rate, but the tumor spectrum was different. Therefore, I would expect, in the *Mdmx* transgenic mice lacking *p53*, DNA damage would have to be induced to see an alteration in tumor onset. To tease out what impact inhibiting DNA repair by Mdmx has on tumorigenesis, it would be interesting to generate mice overexpressing Mdmx that no longer retains the ability to inhibit DNA repair and evaluate if the effects on tumorigenesis caused by Mdmx overexpression are altered. This would allow for subsequent analysis of tumor growth focusing on the function of Mdmx in DNA repair.

In the TCGA analysis, I observed Mdmx is overexpressed in many human

cancers, and co-occurs with inactivated p53, supporting my *in vitro* findings that Mdmx has tumorigenic benefits aside from negative regulation of the p53 tumor suppressor. Prior to this analysis, it has been widely accepted Mdmx overexpression primarily occurs in tumors with wild-type p53, which lends to the concept that Mdmx only regulates p53. Although the TCGA information was very informative, Mdmx is likely overexpressed at the protein level in a higher percentage of cancers with inactivated p53 than what was indicated on TCGA which only reports mRNA expression changes and gene amplification. In fact, some studies have shown Mdmx and p53 levels in panels of multiple cancer cell lines revealing Mdmx is overexpressed along with mutated p53 more often than previously imagined.[51,81] Therefore, doing this on a more global scale will allow us to fully understand the frequency at which Mdmx overexpression co-occurs with inactivated p53. The knowledge that Mdmx overexpression occurs with inactivated p53 in patient samples has extremely important implications in understanding the role of Mdmx as an oncogene, ways it may contribute to tumorigenesis, and informing the development of targeted therapies.

The research embodied by this dissertation identified and described a novel p53-independent mechanism of Mdmx independent of Mdm2. These studies provide valuable insight into understanding the role of Mdmx in tumorigenesis independent of its regulation of p53, which was poorly understood. Additionally, my research elucidated a novel mechanism of action for the Mdm2 small molecule inhibitor Nutlin. Even though I evaluated the effects of Nutlin-induced Mdm2 and overexpression of Mdmx on DNA repair, the entire body of work presented here ultimately contributes to the understanding of the DNA repair process, tumorigenesis, and the development of

chemotherapeutics, which can target these pathways to induce synthetic lethality in cancers with inactivated p53.

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