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# Mdmx promotes genomic instability independent of p53 and Mdm2

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## Abstract

The oncogene Mdmx is overexpressed in many human malignancies, and together with Mdm2, negatively regulates the p53 tumor suppressor. However, a p53-independent function of Mdmx that impacts genome stability has been described, but this function is not well understood. In the present study, we determined that of the thirteen different cancer types evaluated, 6–90% of those that had elevated levels of Mdmx had concurrent inactivation (mutated or deleted) of p53. We show elevated levels of Mdmx inhibited double-strand DNA break repair and induced chromosome and chromatid breaks independent of p53, leading to genome instability. Mdmx impaired early DNA damage response signaling, such as phosphorylation of the serine/threonine-glutamine motif, mediated by the ATM kinase. Moreover, we identified Mdmx associated with Nbs1 of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex, and this association increased upon DNA damage and was detected at chromatin. Elevated Mdmx levels also increased cellular transformation in a p53-independent manner. Unexpectedly, all Mdmx-mediated phenotypes also occurred in cells lacking Mdm2 and were independent of the Mdm2-binding domain (RING) of Mdmx. Therefore, Mdmx-mediated inhibition of the DNA damage response resulted in delayed DNA repair and increased genome instability and transformation independent of p53 and Mdm2. Our results reveal a novel p53- and Mdm2-independent oncogenic function of Mdmx that provides new insight into the many cancers that overexpress Mdmx.

### Keywords

Mdmx/Mdm4; Mdm2; p53; DNA damage response; genome instability



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**Conflict of interest**

The authors declare no potential conflict of interest.

## Introduction

The E3 ubiquitin ligase Mdm2, a negative regulator of the p53 tumor suppressor, is frequently overexpressed in many human malignancies.[1–3] While it is established that Mdm2 inhibits p53, studies have identified other functions of Mdm2 that impact genome stability that are independent of p53 and contribute to tumorigenesis.[4–7] We, specifically, discovered a novel p53-independent association between Mdm2 and Nbs1 of the Mre11-Rad50-Nbs1 (MRN) DNA repair complex.[5,6,8] 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.[9–13] 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 that can occur independent of its regulation of p53.[6,8]

Genome instability is a hallmark of cancer and is a known contributor to tumorigenesis.[14,15] While the exact causes of genomic instability continue to be elucidated, altered DNA repair has been implicated as a significant contributing factor.[10] 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.[10,16,17]

MDMX, also known as MDM4, is overexpressed or amplified in at least 15% of human cancers.[3,18–20] 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.[21,22] Mdmx was initially described as an Mdm2 homologue with high conservation in the N-terminal p53 binding domain and the C-terminal RING domain;[23] 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.[24,25] Additionally, it has been shown that through their RING domains, Mdmx binds Mdm2 and enhances the ability of Mdm2 to regulate p53.[23,24,26–28] Although Mdmx inhibits p53 function, homozygous Myc-tagged Mdmx transgene expression was embryonic lethal, and this could not be rescued with deletion of *p53*,[29] suggesting a p53-independent function of Mdmx in development. Moreover, human tumors with MDMX overexpression may also have inactivated p53,[3,19,30,31] which indicates a potential oncogenic benefit to the cancer cells from Mdmx overexpression in addition to its inhibition of p53.

Recent studies determined that Mdmx influences genome stability independent of p53.[32,33] However, its function in genome instability remained unclear. In this study, we identified a novel p53-independent function of Mdmx that leads 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.[3,18–22] Studies have also shown MDMX is overexpressed in cancers that have mutated p53, indicating that they are not mutually exclusive events during tumorigenesis.[3,19,30,31] Analysis of the data in The Cancer Genome Atlas provides further evidence that a certain fraction of multiple cancers have overexpressed *MDMX* (amplification or mRNA) concurrently with inactivated (mutated or deleted) p53 (Table 1;[34,35]). 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 1), and 30% (12 of 40 cell lines) of an aggressive form of breast cancer have increased Mdmx protein together with mutated p53.[31] In the tumors that had increased levels of MDMX, 10 of the 13 cancer types evaluated also had alterations in p53 in at least 10% of them (Table 1). 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.[15] Mdmx has been shown to enhance functions of Mdm2 (e.g. negative regulation of p53;[23,24,26–28]); 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 1). 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 1). These results indicate elevated levels of Mdmx were capable of inducing genome instability, and this was independent of p53.

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

Alterations in DNA repair have been linked to genome instability.[10] Previously, we reported Mdm2 overexpression inhibits double-strand DNA break repair independent of its regulation of p53.[5,6] 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 2A). The percentage of cells overexpressing Mdmx that repaired their DNA damage was similar to that of cells overexpressing Mdm2 (Figure 2A). 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.[26,27] 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 1A and 2B). Although it has been well established that Mdm2 and Mdmx bind through their RING domains,[25–27] it was recently postulated that Mdm2 and Mdmx may associate indirectly through the Mdm2-binding protein, Arf.[36] 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 2C). 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 2D). Furthermore, full-length Mdmx and the mutant form of Mdmx (MxΔRING) similarly inhibited DNA break repair in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs (Figure 2E). Also, MxΔRING overexpression in human retinal epithelial cells that have Arf and p53 also resulted in a delay in DNA break repair (Supplementary Figure S1A). 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 and alters the early DNA damage response

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.[9,37] 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 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 (Figure 3A; Supplementary Figures S1B and S2). 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 3A; Supplementary Figure S2). Similar results were obtained in MEFs lacking Mdm2 and in human retinal epithelial cells that express ARF and p53 (Figure 3A and Supplementary Figure S1B). 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 3B). 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 Figures 3A 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 3C). Later (75 min) after γIR, γH2AX protein levels were elevated compared to controls in both *p53*$^{-/-}$ and *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs (Figure 3C). 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.

ATM is a DNA damage response kinase that phosphorylates H2AX and hundreds of other proteins upon double-strand DNA breaks.[38] The phosphorylation site ATM prefers is a serine or threonine followed by a glutamine (S/T-Q).[38,39] 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 3D). 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 3D; Supplementary Figure S1C). 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 3D), 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 3D). 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 (Figures 3D and 3E; Supplementary Figure S1C). 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 MxΔRING expressing MEFs (Figure 3F). 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 2).

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.[40] 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 (Supplementary Figure S3). 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.[10] Additionally, Nbs1 of this complex is critical for activating ATM following double-strand DNA breaks.[9,11–13] Since we observed Mdmx overexpression resulted in an altered kinetics of ATM-mediated phosphorylation events and Mdm2 is known to bind Nbs1,[5,6] we evaluated whether Mdmx associates with the MRN complex. Following transient expression of HA-tagged Mdmx in 293T cells, endogenous Nbs1, Mre11, and Rad50 co-immunoprecipitated with Mdmx (Figure 4A). 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 immunoprecipitations from control MEFs lacking Mdmx (Figure 4B). To narrow the region in Mdmx necessary for its interaction with the MRN complex, we generated and then evaluated deletion mutants of Mdmx (Figure 4C). 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 4D). Thus, the region of Mdmx that associates with the MRN complex lies within amino acids 1–345, the same region that induced genome instability (Figure 1A) and inhibited DNA break repair (Figure 2; Supplementary Figure S1A) and the DNA damage response (Figure 3; Supplementary Figures S1B and S1C).

Mre11 of the MRN complex binds to both Nbs1 and Rad50, but Nbs1 does not bind Rad50.[10] 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 4C;[5,6]). 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 4E). 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 4C). 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). 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 4F). 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[6] 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.[10] Mre11 and Rad50 bind directly to DNA, and Nbs1 binds indirectly through Mdc1-mediated association with γH2AX at sites of DNA damage.[9,10,41,42] Since Mdmx interacts with Nbs1 of the MRN complex (Figure 4A–F) and alters DNA repair (Figures 2 and 3; Supplementary Figures S1–3), 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 4G). 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 4H). 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.

### Mdmx increases genome instability independent of Mdm2

Although Mdmx inhibited DNA repair and DNA damage signaling independent of both p53 and Mdm2, it was unclear whether the genome instability detected (Figure 1) 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 5A). 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 5B). Although rare, we also detected other structural abnormalities, such as radials, in the Mdmx overexpressing MEFs that were not present in the controls (Figure 5C). Moreover, only the Mdmx overexpressing $p53^{-/-}Mdm2^{-/-}$ MEFs had more than one chromosomal aberration in individual cells (Figure 5D). 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.[15] Moreover, it has been reported that elevated Mdmx levels are observed in many tumors.[3,18–22] 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 6A). To determine whether Mdmx promoted transformation independent of

its association with Mdm2, we evaluated colony formation in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs. We observed that Mdmx overexpression resulted in a significant increase in colony formation compared to vector control (Figure 6B). 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,[25,43] 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.[3,19,30–33] 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.[9,37,38] Full activation of ATM following DNA double-strand breaks requires Nbs1 of the MRN complex.[9,11–13] 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,[44–47] 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.[48,49] 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.[26,27] 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*.[50,51] 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.[22] 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.[25,43] 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.[10,15] 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, $Mdmx^{-/-}p53^{-/-}$ MEFs had multipolar spindle formation and altered chromosome number.[32,33] 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.[52] 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.

## Materials and Methods

### Cell culture

$p53^{-/-}$, $Arf^{-/-}$ and $p53^{-/-}Mdm2^{-/-}$ murine embryonic fibroblasts (MEFs) were isolated and cultured as previously described.[53] $Atm^{-/-}Arf^{-/-}$ 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.

### 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.[5,6] Retroviruses were generated, and MEFs were infected as previously reported.[53] 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.[6]

### 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.[6,54] 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.

### Comet Assay and Immunofluorescence

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[5,6] to detect double-strand DNA breaks. γH2AX or pS-T/Q foci were detected by immunofluorescence and analyzed as previously described.[6] The number of γH2AX and pS-T/Q foci per cell for at least 40 and 39 cells, respectively, per individual condition was quantified. A minimum of three independent experiments were performed for all analyses. Statistical significance was determined by the student's t-test for the comet assay data and using a confidence interval of 95% for all foci analyses.

### 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.[5,6] 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.[6] For

chromatin fractionation, at indicated intervals following exposure to 5 Gy of γIR, the chromatin fraction was isolated from MEFs as previously described.[55] For all lysates, following separation of proteins by SDS-PAGE and transfer to nitrocelluose, proteins were subjected to western blot analysis as previously described.[5,6] Antibodies previously described[5,6] except anti-Mdmx, which was from Sigma-Alderich (St Louis, MO).

### *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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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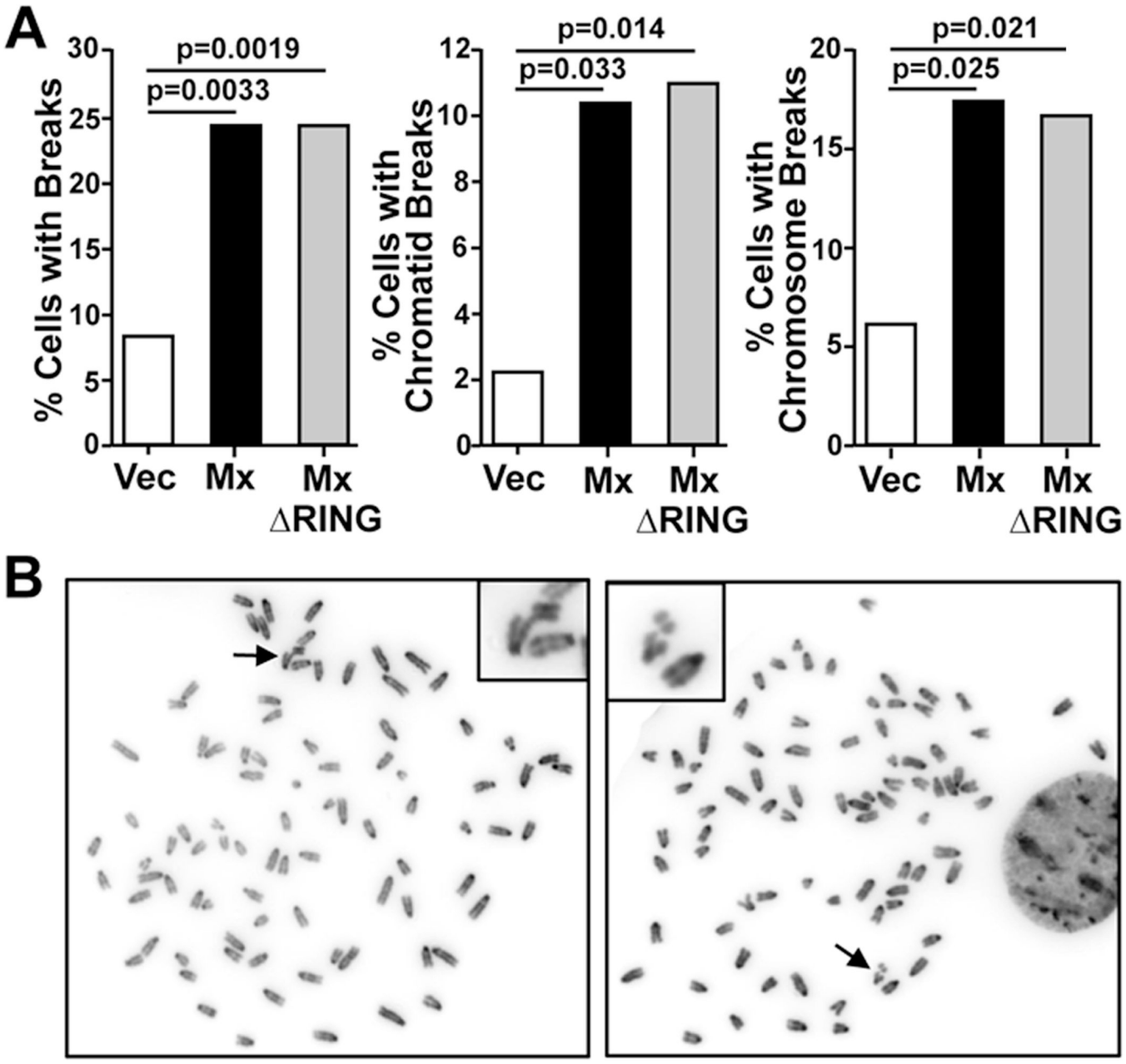


**Figure 1. Elevated Mdmx levels promote genome instability independent of p53**

*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.

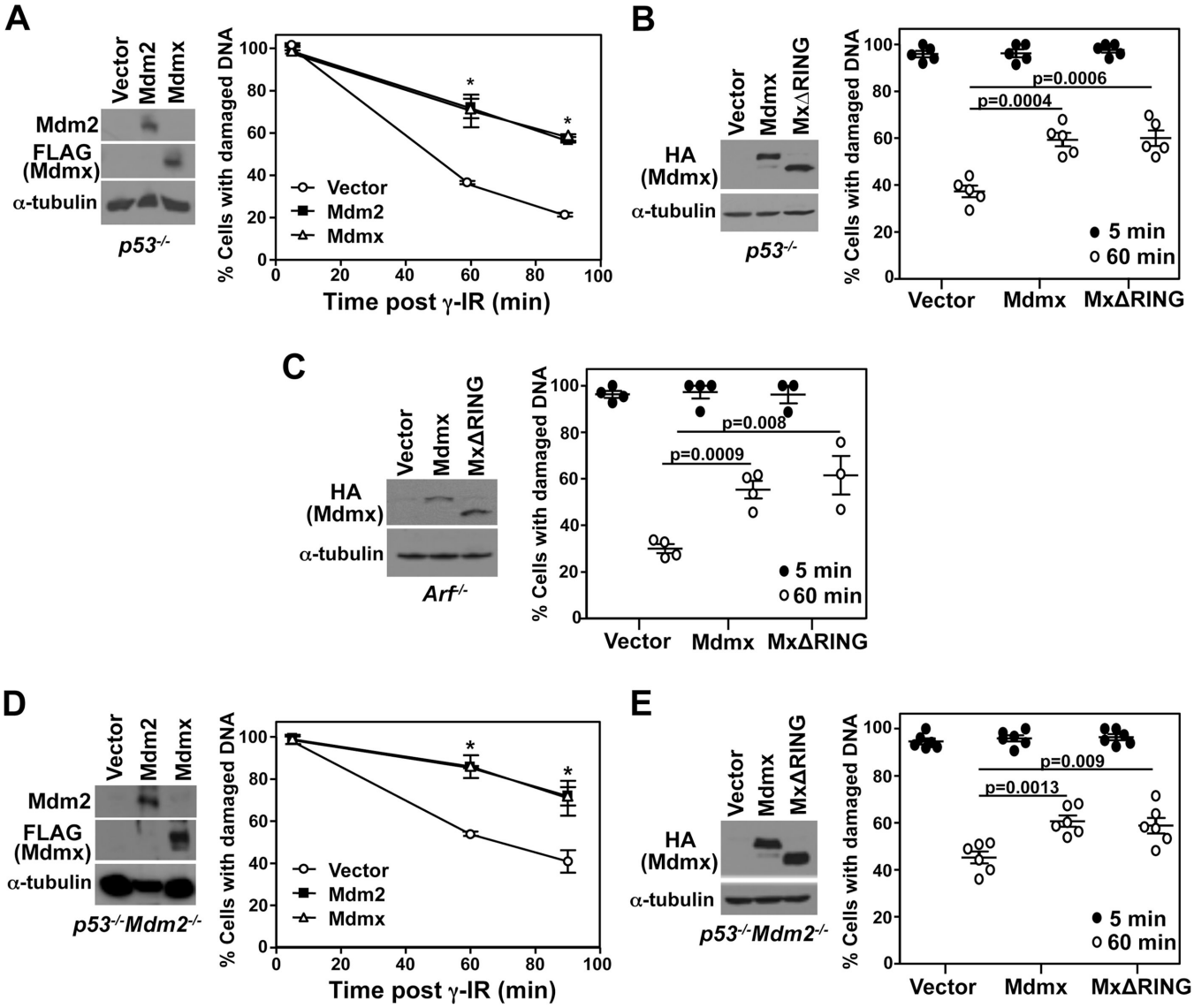


**Figure 2. Mdmx overexpression inhibits double-strand DNA break repair independent of p53 and Mdm2**

*p53*$^{-/-}$ (A,B), *Arf*$^{-/-}$ (C), or *p53*$^{-/-}$*Mdm2*$^{-/-}$ (D,E) 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, C, and E is the mean of an individual experiment and the line is the mean of all experiments. Error bars represent SEM. *p ≤ 0.01 in A; *p < 0.05 in D; each compared to vector control; student's *t*-test.

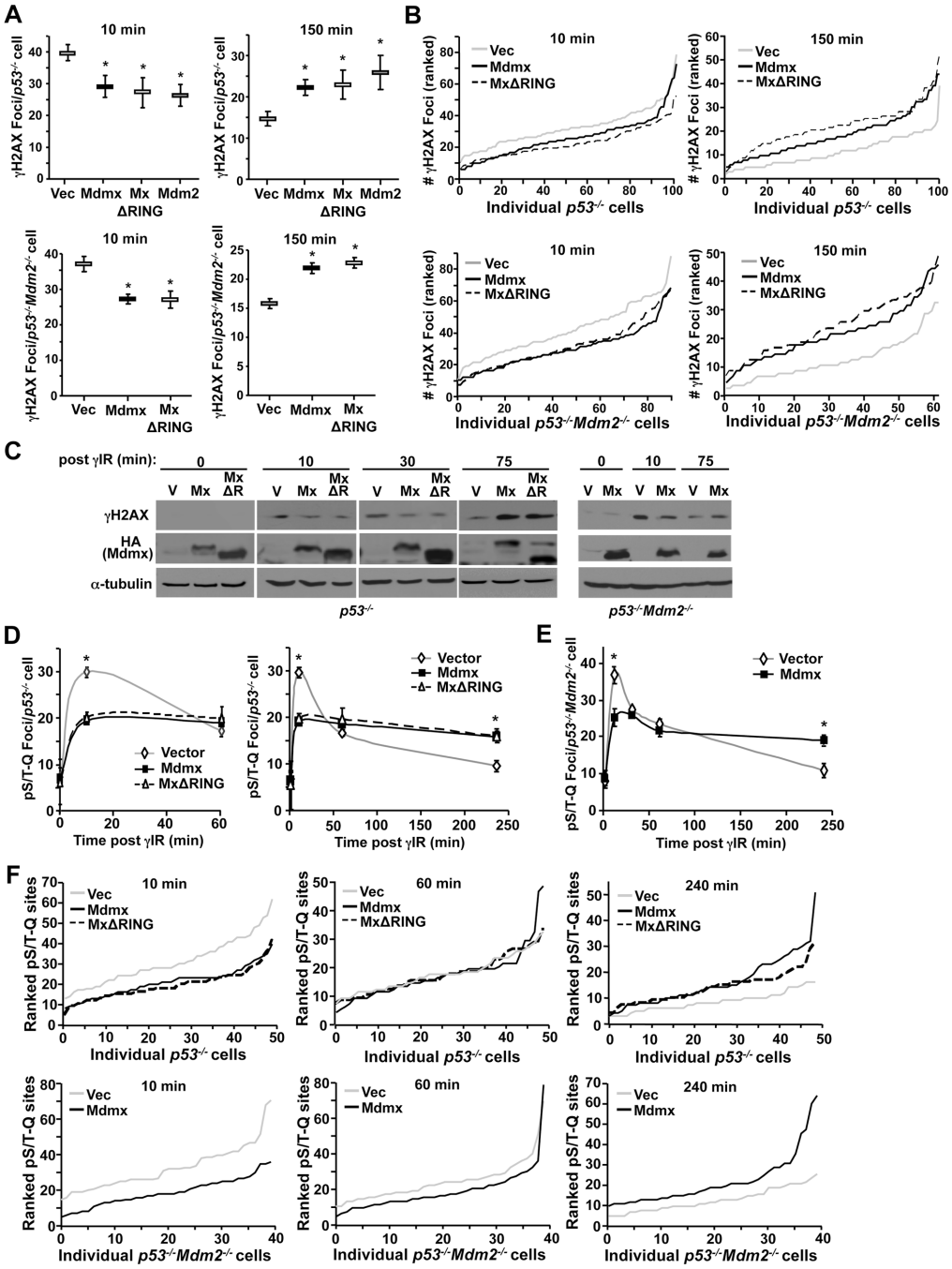


**Figure 3. Elevated Mdmx impairs the early DNA damage response signal**

*p53*$^{-/-}$ (A–D, F) or *p53*$^{-/-}$*Mdm2*$^{-/-}$ (A–C, E, F) 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). A, B, D–F) Following exposure to 5 Gy of γIR, MEFs were fixed at the indicated intervals and immunofluorescence for γH2AX (A, B) or pS/T-Q (D–F) was performed. The number of foci per cell was quantified. (A, D, E) The mean of at least three independent experiments is graphed. Error bars represent SEM, and significance determined using a confidence interval of 95%. (B, F) Ranking of γH2AX (B) and pS/T-Q

(F) foci in individual cells was graphed for a representative experiment in both MEF genotypes. C) Western blot analysis of whole cell lysates for the proteins indicated to the left of the panels at the indicated intervals following γIR.

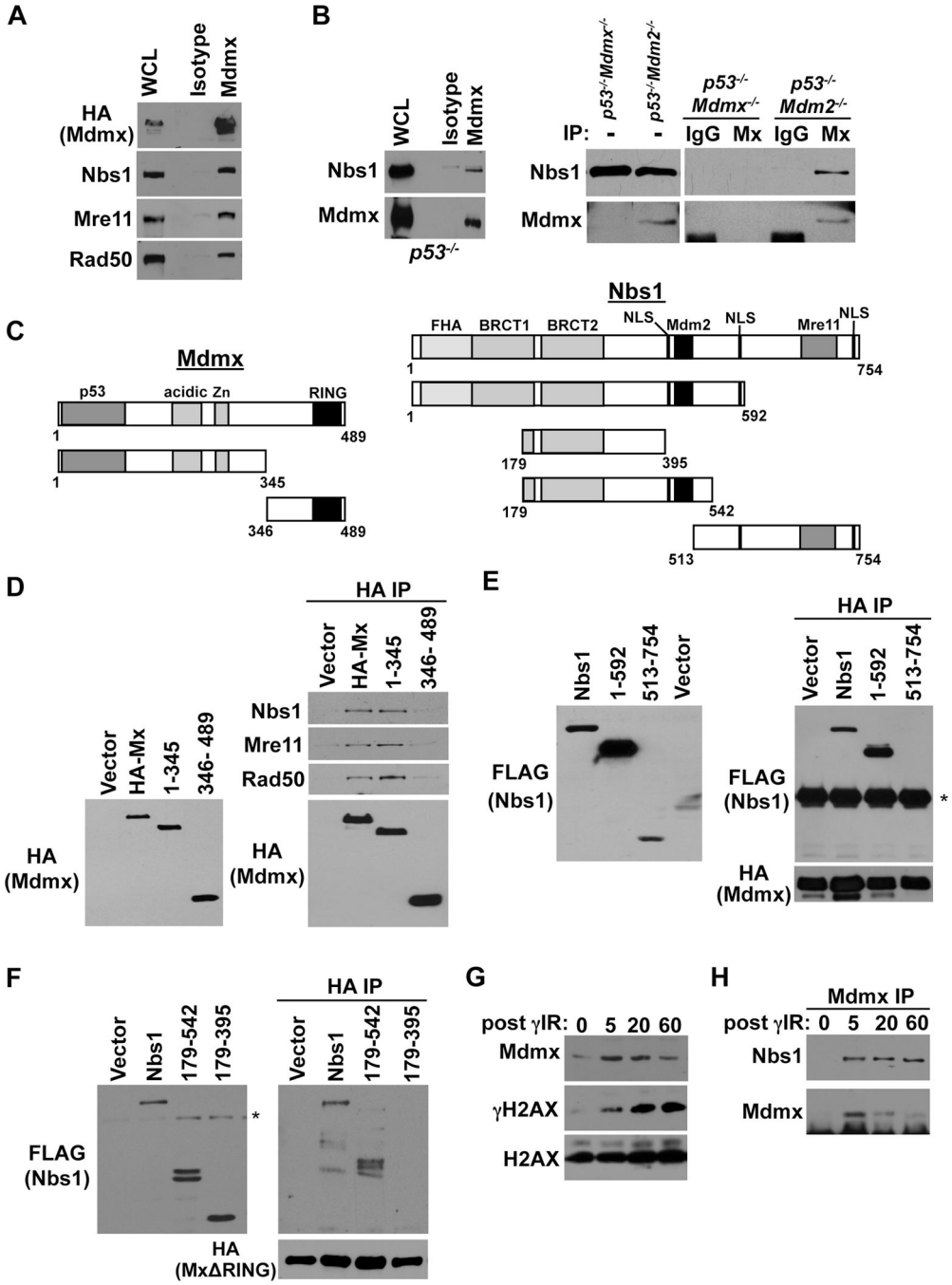


**Figure 4. Mdmx associates with Nbs1 independent of Mdm2**

A) Whole cell lysates (WCL) from HA-Mdmx expressing 293T cells were immunoprecipitated (IP) with anti-HA or isotype control antibody and Western blotted. B) Whole cell lysates from *p53*$^{-/-}$, *p53*$^{-/-}$*Mdm2*$^{-/-}$, *or p53*$^{-/-}$*Mdmx*$^{-/-}$ were immunoprecipitated with anti-Mdmx or isotype control antibody and Western blotted. C) Schematic representation of full-length Mdmx and Nbs1 and deletion mutants of each. Binding domains for p53, Mdm2, Mre11, and the acidic, Zinc (Zn), RING, forkhead-associated (FHA), and BRCT domains, and the nuclear localization sequences (NLS) are

shown. D–F) 293T cells transfected with empty vector or vectors encoding protein tagged full-length or deletion mutants of Mdmx or Nbs1 as indicated. Whole cell lysates (left panels) and anti-HA or anti-FLAG immunoprecipitations (right panels) were Western blotted. Asterisk denotes immunoglobulin heavy chain and non-specific band in E and F, respectively. G) At intervals (minutes) after 5 Gy of γIR, Western blots were performed on the chromatin-bound protein fraction of $p53^{-/-}$ MEFs. H) 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.

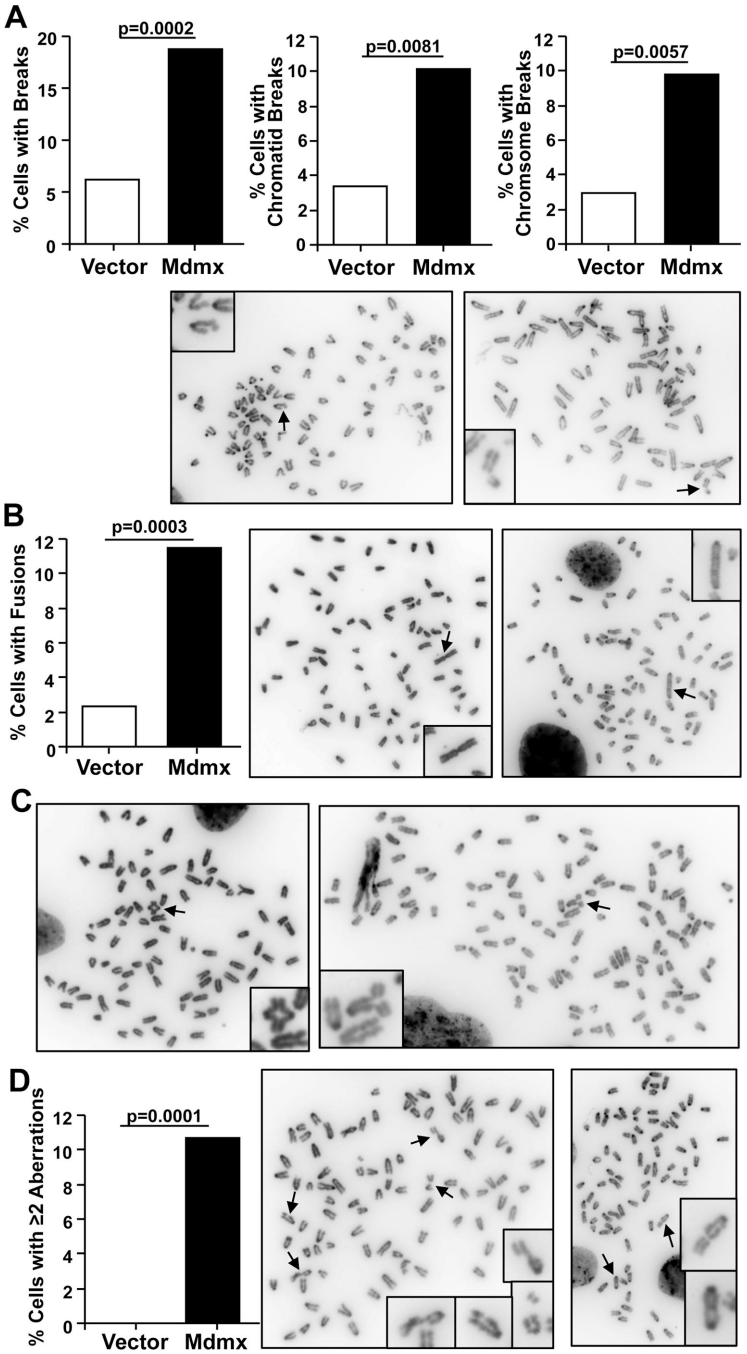


**Figure 5. Mdmx promotes chromosome instability independent of Mdm2**

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. C) Representative pictures of structural aberrations distinct from breaks and fusions detected in *p53*$^{-/-}$*Mdm2*$^{-/-}$ MEFs overexpressing Mdmx are shown. D) 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 (A–D). Statistical significance was determined using the Fisher's exact test (A, B, D)

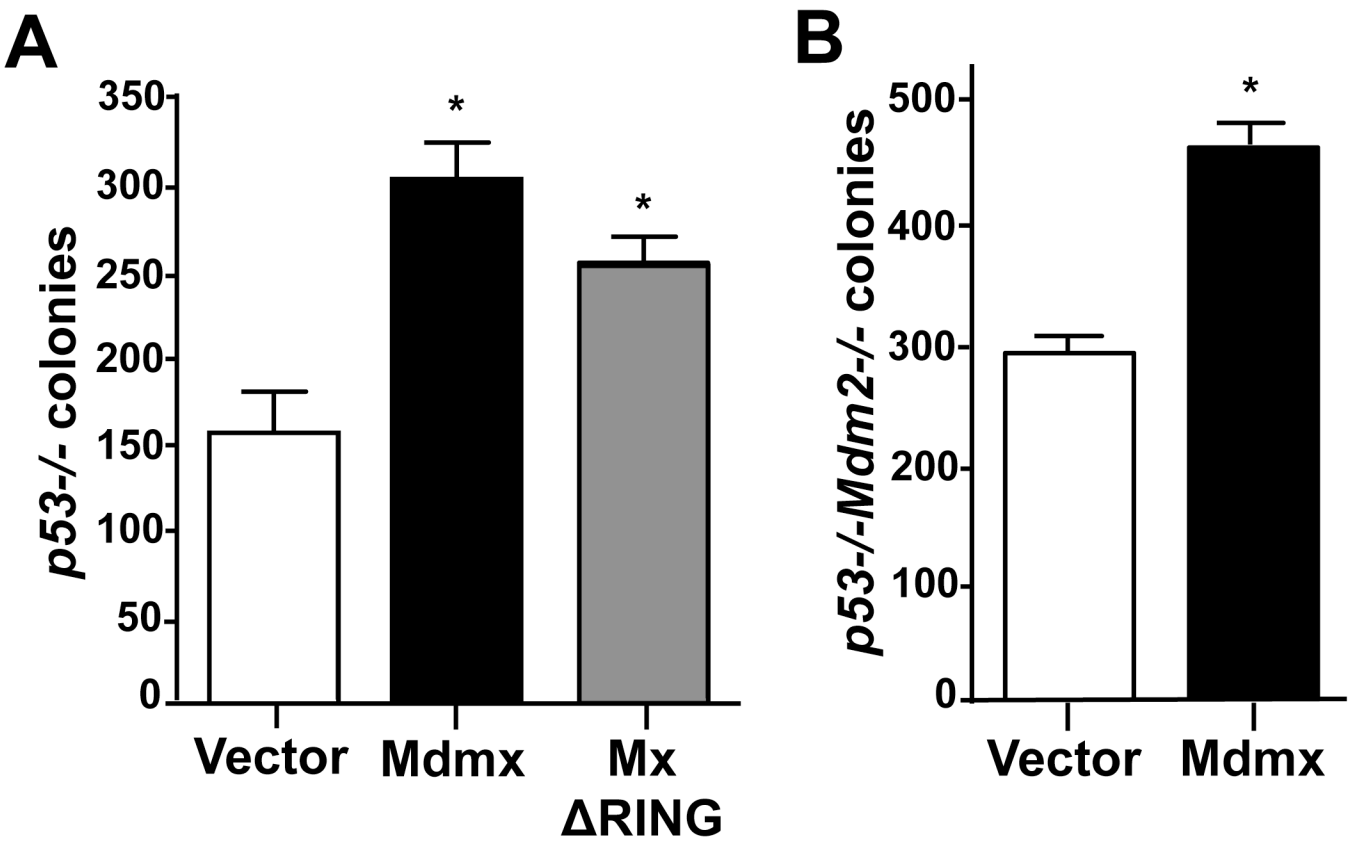


**Figure 6. Increased levels of Mdmx promote transformation independent of p53 or Mdm2**

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.

## Table I

Increased *MDMX* co-occurs with mutant/deleted *p53* in multiple cancer types[1]

| Cancer Type | Samples | *MDMX* increased[2] | mut/del[3] p53 in *MDMX* increased[2] |
|---|---|---|---|
| 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%)[4] |
| 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%) |

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

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

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

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