MdmX Promotes Bipolar Mitosis To Suppress Transformation and Tumorigenesis in p53-Deficient Cells and Mice

Zdenka Matijasevic, Heather A. Steinman, Kathleen Hoover, and Stephen N. Jones

Departments of Cell Biology and Cancer Biology, University of Massachusetts Medical School, 55 Lake Avenue North, Worcester, Massachusetts 01655

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Mdm2 and MdmX are structurally related p53-binding proteins that function as critical negative regulators of p53 activity in embryonic and adult tissue. The overexpression of Mdm2 or MdmX inhibits p53 tumor suppressor functions in vitro, and the amplification of Mdm2 or MdmX is observed in human cancers retaining wild-type p53. We now demonstrate a surprising role for MdmX in suppressing tumorigenesis that is distinct from its oncogenic ability to inhibit p53. The deletion of MdmX induces multipolar mitotic spindle formation and the loss of chromosomes from hyperploid p53-null cells. This reduction in chromosome number, not observed in p53-null cells with Mdm2 deleted, correlates with increased cell proliferation and the spontaneous transformation of MdmX/p53-null mouse embryonic fibroblasts in vitro and with an increased rate of spontaneous tumorigenesis in MdmX/p53-null mice in vivo. These results indicate that MdmX has a p53-independent role in suppressing oncogenic cell transformation, proliferation, and tumorigenesis by promoting centrosome clustering and bipolar mitosis.

Although genetic and biochemical studies clearly indicate that Mdm2 and MdmX are key regulators of p53 activity, there are distinct differences in their mechanisms of p53 inhibition. Mdm2 forms a complex with p53 and functions as an E3 ligase to target p53 for ubiquitination and proteosomal degradation (47). MdmX complexes with p53 and inhibits p53 transactivation without altering p53 stability (11, 46), and in contrast to that of Mdm2, MdmX expression is not regulated by p53. Regardless of these differences, Mdm2 and MdmX act as critical negative regulators of p53 function in development. The developmental block imposed by the loss of Mdm2 or MdmX can be relieved by the deletion of p53 (9, 21, 28, 30, 32) or by Mdm2 amplification (20, 22) or, in the case of MdmX, partially rescued by the deletion of the p53 downstream effector p21 (43). These data indicate that the primary role of Mdm2 or MdmX in development is to regulate p53.

The amplification and overexpression of either Mdm2 (31) or MdmX (7, 36) have been observed in a variety of human cancers, including sarcoma, glioma, and, in the case of MdmX, retinoblastoma (24), suggesting that either Mdm2 or MdmX can function as an oncogene to inhibit p53 activity and promote tumorigenesis. Since many of these Mdm-overexpressing tumors retain wild-type p53 alleles, the reactivation of p53 by small-molecule inhibition of Mdm2 or MdmX-p53 interaction is an attractive strategy for treating these cancers (26, 27).

The results of experiments in vitro or in vivo involving the forced overexpression of Mdm proteins suggest that Mdm2 and MdmX may also have p53-independent roles in promoting cell growth (13, 23, 29, 38, 41). However, molecular targets for Mdm2 or MdmX activity other than p53 have yet to be confirmed. Furthermore, it remains unclear if physiologic levels of either Mdm2 or MdmX expression can exert functions that are distinct from their abilities to downregulate p53 activity. We have previously characterized mice and primary mouse embryonic fibroblasts (MEFs) with p53 or both Mdm2 and p53 deleted. These cells display equivalent rates of cell proliferation and cell transformation in culture, and Mdm2/p53 double null mice and p53-null mice present with the same rate and tissue spectrum of spontaneous tumor formation (22), indicating that normal cellular levels of Mdm2 function primarily to regulate p53 activity. In contrast, MEFs generated from Mdm2-transgenic mice with intact MdmX proliferated slower than MEFs lacking MdmX (42), suggesting that MdmX might have anti-proliferative properties when p53 functions are inhibited. To determine if physiologic levels of MdmX can regulate cell proliferation in a p53-independent manner, we have generated and analyzed the growth and transformation of MEFs derived from mice with p53 or both p53 and MdmX deleted. Unlike our previous results with Mdm2, the deletion of MdmX increases the spontaneous transformation and proliferation of immortalized p53-null MEFs. Also, in contrast to what occurs with Mdm2, the loss of MdmX seems to have a profound impact on chromosome stability. Although both Mdm2/p53 double null cells and MdmX/p53 double null cells initially display the chromosomal hyperploidy characteristic of p53-null cells, MdmX-deficient cells undergo a reduction in chromosome number that correlates with increased cell proliferation and transformation during their growth in culture. Furthermore, MdmX/p53 double null mice display increased rates of spontaneous tumorigenesis relative to those of p53-null mice. Similarly to MEFs, tumor cells isolated from MdmX/p53-null mice proliferate faster and have fewer chromosomes than p53-null tumor...
cells, corroborating the role for MdmX in the maintenance of genome stability. Hyperploid, p53-deficient MEFs and tumor cells that lack MdmX display reduced centrosome clustering and high levels of multipolar mitotic spindle formations, likely accounting for the aberrant chromosome segregation and loss of chromosomes during mitosis. The reintroduction of MdmX into MdmX/p53-null tumor cells increased the ploidy, reduced the incidence of multipolar spindles, and decreased the proliferation rate in these cells. These data reveal that MdmX has a p53-independent role in suppressing cell proliferation, transformation, and tumorigenesis by promoting bipolar mitosis and preventing chromosome loss in polyploid p53-deficient cells.

RESULTS

The loss of MdmX increases the growth potential of p53-null MEFs. To analyze the growth characteristics of p53-null MEFs in the presence or absence of MdmX, MEFs were generated from embryos obtained from crosses between MdmX+/− p53−/− and MdmX+/− p53+/− mice harvested at embryonic day 13.5. In keeping with an earlier report indicating that the growth rate of p53-null MEFs was not altered by the deletion of MdmX (28), we found that early-passage p53-null and MdmX/p53-null MEFs exhibited similar rates of cell proliferation (Fig. 1A, left panel). However, an increase in the growth rate of the double null MEFs was observed repeatedly following serial passage of the primary cells (Fig. 1A, right panel).

This finding prompted us to compare long-term growth characteristics of these cells under the standard conditions of an immortalization assay (15) (Fig. 1B). As expected, the control wild-type MEFs entered senescence around passage 7 and failed to immortalize. In contrast, all p53-deficient cell lines immortalized and continued to divide throughout the course of the assay. However, there was a striking difference in the growth patterns of p53-null and MdmX/p53-null MEFs at higher passages. After passage 7, the loss of MdmX conferred a distinct growth advantage to p53-deficient cells. Calculation of the accumulated PDLs (Fig. 1B, right panel) revealed that, by passage 17, the MdmX/p53-null cells had undergone almost 10 more doublings than the p53-null cells, and the deletion of MdmX allowed p53-deficient cells to proliferate much faster and reach drastically higher saturation densities (Fig. 1C). The estimated population doubling time from the proliferation curve in Fig. 1C is 15 h for MdmX/p53-null cells versus 23 h for p53-null cells, and the estimated number of times the population doubled from day 1 to day 3 is 2.9 for MdmX/p53-null cells versus 2.1 for p53-null cells (see Table S1 in the supplemental material).

The increased growth capacity of p53-null cells relative to that of wild-type cells is also reflected in their ability to grow when plated at a low cell density (15). However, p53-null cells with MdmX deleted not only retain the same plating efficiency and ability to grow at a low density (see Table S1 in the supplemental material) but also form much larger colonies than p53-null cells with intact MdmX (Fig. 1D). Eighty percent of all MdmX/p53 double null colonies exceeded 200 cells per colony at day 5 postplating, whereas only 30% of the p53-null colonies reached this size. These data further demonstrate an increase in the rate of p53 colony proliferation when MdmX is deleted. The ability of the MdmX/p53-null cells to reach a much higher saturation density than the p53-null cells (Fig. 1C) suggested that the loss of MdmX further compromises the regulation of cell growth and might induce the transformation of immortalized p53-deficient cells. Therefore, we performed a cell transformation assay by allowing the MEFs to grow to saturation density and assaying for the formation of trans-
formed foci at 2 weeks postplating. Primary cells deficient for p53 do not form foci under these conditions, unless they are transduced with an activated oncogene such as ras (10). However, the results of the transformation assay (Fig. 1E) revealed that MdmX/p53-null cells formed numerous foci in culture. Counts of triplicate 10-cm plates of MdmX/p53-null cells documented 188 (±53 standard deviations) foci larger than 1 mm in diameter per plate. In contrast, p53-null MEFs did not form any foci in culture. These results confirm that MdmX inhibits the spontaneous transformation of immortalized p53-deficient cells.

MdmX suppresses chromosome loss in p53-null cells. To investigate the possible effects of MdmX on genome stability, we compared the cell ploidy, measured as the DNA content per cell, of propidium iodide-stained p53-null cells with that of MdmX/p53-null cells at different passages using wild-type MEFs at passage 2 as a diploid control (Fig. 2A). Very low passage number (passage 2) MEFs with either p53 or both MdmX and p53 deleted displayed normal, diploid FACS profiles similar to that of the wild-type MEFs. It was reported previously (6, 12, 15) that MEFs with inactivated p53 genes are genetically unstable and contain supernumerary chromosome

FIG. 1. The loss of MdmX increases the growth potential of p53-null cells in culture. (A) At passage 4 (Pass 4), growth rates of p53-null and MdmX/p53-null cells are equivalent and much higher than that of wild-type (WT) cells. At passage 9, MdmX/p53-null cells proliferate faster than p53-null cells. Error bars represent standard errors of the mean from two independent cell lines. (B) The loss of MdmX confers a growth advantage to p53-deficient cells during long-term growth in culture. A 3T3 immortalization assay was performed with two or three cell lines of each genotype. The actual cell number at each passage (left panel) and accumulated PDLs (right panel) are shown. By passage 17, MdmX/p53-null populations underwent almost 10 more doublings than the p53-null population. PD, number of population doublings. (C) The loss of MdmX allows immortalized p53-deficient cells to proliferate much faster and to reach much higher saturation densities than the wild-type cells. A proliferation assay was performed with passage 17 immortalized cells. (D) The loss of MdmX stimulates growth within the individual clones at a low plating density. Colony formation was evaluated for crystal violet-stained cells at passage 18. (E) The loss of MdmX triggers the spontaneous transformation of p53-deficient cells. In contrast to p53-null cells, which failed to form foci, MdmX/p53-null cells formed numerous dark-blue foci on Giemsa-stained plates. Counts of triplicate 10-cm plates revealed 188 ± 53 foci larger than 1 mm in diameter per plate.
numbers after repeated passages in culture. Indeed, at passage 9, both MdmX/p53-null MEFs and p53-null MEFs displayed very small 2N cell subpopulations, and the majority of the cells had 4N or >4N DNA content. However, while the p53-null cells maintained a significant hyperploid subpopulation for more than 20 additional passages, MEFs with both MdmX and p53 deleted underwent a drastic reduction in ploidy at higher passages.

To confirm that the difference in DNA content reflects differences in chromosome numbers, mitotic spreads were prepared from multiple lines of MEFs at low and high passage numbers, and chromosome numbers per cell were determined in DAPI-stained spreads (Fig. 2B). Wild-type MEFs at a low passage number contained mostly diploid numbers of chromosomes ($n = 40$), whereas MEFs lacking p53 showed supernu-

FIG. 2. MdmX suppresses the reduction in ploidy and loss of chromosomes in immortalized p53-null cells. (A) Flow cytometry profiles of PI-stained MEFs. Histograms of DNA content in MdmX/p53-null cells (second row, red) and in p53-null cells (third row, red) are overlayered with a histogram of passage 2 (p2) wild-type (WT) MEFs (blue) shown as a diploid control. At passage 9, the majority of the cells of both genotypes show 4N or >4N DNA content. While p53-null cells maintain hyperploid DNA contents at high passage numbers, MdmX/p53 double null cells display drastic reductions in hyperploid subpopulations at high passage numbers. (B) Chromosome number distribution in low- and high-passage-number MEFs. For chromosome number determination, chromosomes in at least 50 DAPI-stained metaphase spreads for each cell line and each passage shown were counted. The deletion of MdmX, but not of Mdm2, caused a drastic reduction in the chromosome number of high-passage-number p53-null cells. (C) Percentages of cells with greater-than-triploid chromosome contents (more than 60 chromosomes per cell) in populations of MdmX/p53-null (three cell lines) and p53-null (four cell lines) MEFs. (D) The deletion of MdmX also leads to a reduction in chromosome numbers in immortalized p21-null cells.
merary numbers of chromosomes, as expected. This hyperploidy was seen in p53-null MEFs at passages 7 and 8, regardless of MdmX or Mdm2 status. Wild-type MEFs that undergo a spontaneous loss of p53 or p19ARF are capable of spontaneous immortalization (15, 19), and very high passage number (passage 45) wild-type MEFs displayed hyperploidy, similar to that observed in p53-null MEFs at passage 23 or in Mdm2/p53-null MEFs at passage 27, with a large subpopulation of cells containing more than 80 chromosomes per cell. In contrast, MEFs lacking both p53 and MdmX had far fewer chromosomes per cell at passage 24 than did p53-null MEFs or Mdm2/p53 double null MEFs at similar passages. In agreement with the FACS data, chromosome counts from mitotic spreads from low and high passage numbers clearly revealed that MdmX/p53-null cells go through the radical reduction in chromosome number during prolonged growth in culture. The majority of the MdmX/p53-null cells at passage 24 contained between 50 and 60 chromosomes, and only a few cells had more than 70 chromosomes per cell. Similar decreases in chromosome number were observed in all MdmX/p53-null lines tested. The results of a statistical analysis of chromosome number distribution in populations of high-passage-number MdmX/p53-null MEFs (three cell lines) and p53-null MEFs (four cell lines) are shown in Fig. 2C. Almost 60% of the p53-null cells and only 12% of the MdmX/p53-null cells presented with a greater-than-triploid chromosome number.

In contrast to MdmX, the presence or absence of Mdm2 does not seem to affect chromosome numbers of p53-null cells (Fig. 2B). In order to examine whether the loss of MdmX affects chromosome number in polyploid cells of a different background, we examined MEFs previously generated from MdmX-null mice with the p53 target gene, p21/WAF1, deleted (43). The loss of p21 function in MEFs is known to induce endoreduplication, resulting in polyploid cells (44). The results in Fig. 2C show that MdmX/p21 double null MEFs have much lower chromosome numbers per cell than p21-null MEFs at similar passage numbers, suggesting that the MdmX-mediated maintenance of the chromosome number is not unique to p53-deficient cells.

**MdmX suppresses spontaneous tumorigenesis in p53-deficient mice.** Spontaneous transformation of p53-null cells in the absence of MdmX coupled with the increased rate of chromosomal loss in the MdmX/p53-null cells prompted us to investigate whether the antiproliferative growth properties of MdmX could alter spontaneous tumorigenesis in p53-null mice. Cohorts of p53-deficient mice that were wild type, heterozygous, or null for MdmX were generated, and a tumor assay was performed. We have previously demonstrated that Mdm2 status does not affect the onset of tumorigenesis in p53-deficient mice, and mice with both Mdm2 and p53 deleted present with the same tumor types as p53-null mice (22). In contrast, the deletion of one or both MdmX alleles significantly altered tumor formation in p53-heterozygous or p53-null mice (Fig. 3A and B). The tumor spectrum and tumor burden of p53-heterozygous mice or p53-null mice in the presence or absence of MdmX remained unchanged, with thymic lymphomas and sarcomas being the predominant tumor types observed (data not shown); however, the rate of tumorigenesis was accelerated in the absence of MdmX. Fifty percent of the p53-null mice present with spontaneous tumors by 25 weeks of age. On average, mice lacking both p53 and MdmX develop tumors 5 weeks earlier. These results reveal that MdmX suppresses oncogenesis when p53 is deleted and confirm in vivo that MdmX has a p53-independent role in regulating cell transformation.

**MdmX suppresses proliferation and chromosomal loss in p53-null tumor cells.** To further explore the link between MdmX and the maintenance of chromosome ploidy, thymic lymphomas were harvested and cultured from p53-null mice and from MdmX/p53 double null mice. Interestingly, cells derived from the MdmX/p53 double null tumors proliferated faster and reached much higher saturation densities than cells derived from p53-null tumors (Fig. 4A), reminiscent of what we observed in our studies of immortalized MEFs. Furthermore, FACS analysis and mitotic spreads of these cells at four to six passages removed from the tumor tissue revealed that the p53-null tumor cell population had a much larger fraction of cells with 4N and >4N DNA content and with higher chromosome numbers than the MdmX/p53-null tumor cell population (Fig. 4B, upper and middle panels). The presence or absence of Mdm2 does not seem to have an impact on ploidy and the chromosome number of MdmX/p53-null tumor cells (Fig. 4B, bottom panel), indicating that this MdmX function is both p53 and Mdm2 independent. To confirm that MdmX could regulate ploidy and the proliferation of these cells, we
transfected the MdmX/p53-null tumor cells with an expression vector bearing MdmX. Stable, puromycin-resistant MdmX transfectants displayed ploidy and proliferation characteristics similar to those of p53-null tumor cells. Error bars represent standard errors of the means from two independent experiments. (B) Double and triple null tumor cells have reduced ploidy and chromosome numbers compared to those of the p53-null tumor cells. Chromosome number ranges are the same as in Fig. 2. The results represent the average chromosome numbers from two independent experiments for null and double null cells and from one experiment for triple-null cells. (C) DNA content of MdmX/p53-null tumor cells transfected with an MdmX-expressing plasmid. Parental, nontransfected cells (Control), cells transfected with the puromycin vector only (Mock), and stable MdmX transfectants (clones c1 and c2) were analyzed for DNA content. MdmX-transfected clones showed higher DNA contents than those of both controls. (D) The reintroduction of MdmX into the double null tumor cells (clones c1 and c2) significantly reduced the rate of cell proliferation.

FIG. 4. MdmX suppresses cell proliferation and the loss of chromosomes in p53-null tumor cells. Cells were isolated from the thymic tumors of p53-null, MdmX/p53-null, and Mdm2/MdmX/p53-null animals. (A) MdmX/p53-null tumor cells proliferate much faster and reach higher saturation densities than p53-null tumor cells. Error bars represent standard errors of the means from two independent experiments. (B) Double and triple null tumor cells have reduced ploidy and chromosome numbers compared to those of the p53-null tumor cells. Chromosome number ranges are the same as in Fig. 2. The results represent the average chromosome numbers from two independent experiments for null and double null cells and from one experiment for triple-null cells. (C) DNA content of MdmX/p53-null tumor cells transfected with an MdmX-expressing plasmid. Parental, nontransfected cells (Control), cells transfected with the puromycin vector only (Mock), and stable MdmX transfectants (clones c1 and c2) were analyzed for DNA content. MdmX-transfected clones showed higher DNA contents than those of both controls. (D) The reintroduction of MdmX into the double null tumor cells (clones c1 and c2) significantly reduced the rate of cell proliferation.
one-quarter of the mitotic MdmX/p53 double null cells formed symmetrical and asymmetrical multipolar spindles (Fig. 5B). Interestingly, these multipolar spindles permit chromosome segregation, as telophase figures with more than two groups of separated chromosomes are frequently observed in these cells, as evident in the telophase cell (two right-hand sets of images). (B) Multipolar spindles in MdmX/p53-null cells with amplified centrosomes. Symmetrical (left and middle sets of images) and asymmetrical (right set of images) multipolar spindles are common in MdmX/p53-null cells. (C) In cells with multipolar spindles, chromosomes can separate and generate telophase figures with more than two groups of chromosomes. (D) Bipolar spindles in MdmX/p53-null tumor cells transfected with an MdmX-expressing plasmid. (E) The incidence of mitotic cells with multipolar spindles was evaluated by scoring from 100 to 200 mitotic figures for MEFs (M) and tumor cells (T). The histograms show the averages (from left to right) for two p53-null MEF lines, two experiments with p53-null tumor cells, three double null MEF lines, two experiments with double null tumor cells, and two clones of Mdm4-transfected double null tumor cells. Error bars represent standard errors of the means. The loss of MdmX function causes a more-than-twofold increase in spindle multipolarity. Scale bars = 10 μm.

**DISCUSSION**

Our results document a role for MdmX in suppressing cell transformation and tumorigenesis that is distinct from the well-established ability of MdmX to negatively regulate p53. Cells lacking both p53 and MdmX exhibit a higher rate of cell proliferation and higher saturation density than p53-null cells after passage in culture, although they are initially equivalent in their rates of proliferation. In addition, MdmX/p53-null cells form larger colonies when plated at a low density and display a far greater rate of cell growth in a standard 3T3 cell immortalization assay than p53-null cells. These findings are in contrast to what we reported previously for Mdm2, as the deletion of *Mdm2* does not alter the rate of cell proliferation or immortalization of p53-null MEFs (22). Furthermore, unlike p53-null cells, MEFs deficient for MdmX and p53 form numerous foci when plated in a cell transformation assay, confirming that MdmX suppresses the transformation of immortalized p53-null cells. In vivo evidence for a p53-independent
role for MdmX in suppressing transformation is provided by the results of the tumor studies. Mice deficient for both MdmX and p53 develop spontaneous tumors much earlier than p53-null mice. Notably, these differences are not observed in p53-deficient mice lacking Mdm2 (22). As physiologic levels of MdmX are not regulated by p53 (39), these data demonstrate a p53-independent role for native levels of MdmX in tumor suppression.

MEFs deficient for p53 or for both MdmX and p53 display supernumerary centrosomes and hyperploidy at low passage numbers. However, MdmX/p53 double null cells undergo a loss of chromosomes during growth in culture, and reintroduction of MdmX into MdmX/p53-null cells increases their coalescence of centrosomes and promotes bipolar cell division.

Since Hansemann’s (14) and Bovery’s (2) experiments more than a century ago, multipolar mitosis and aneuploidy have been tightly linked to tumorigenesis (35, 45, 48). Centrosomal clustering and bipolar spindle formation have been suggested to be critical for preserving the genomic stability of noncancer cells (34, 37) and for the maintenance and propagation of hyperploid cells with amplified centrosomes (3, 40). The reduction of chromosome numbers observed in polyploid p53-deficient cells after prolonged culturing in vitro has been proposed to give rise to a more stable, yet aneuploid, karyotype that confers a growth advantage to a cell (5). This reduction in ploidy is thought to be analogous to the previously described genomic convergence during tumor progression in vivo (17), and the relatively stable karyotypes found in many advanced tumors and established tumor cell lines may reflect the evolution of a genotype optimized for growth under specific conditions (1).

The increased spindle multipolarity, chromosomal loss, and increased proliferation of p53-null cells lacking MdmX indicate a p53-independent role for MdmX in the suppression of tumorigenesis through the maintenance of genome stability. As the effect of MdmX loss on the proliferation and ploidy of p53-null cells is observed only at late passages or in immortalized cells, it is likely that MdmX plays a p53-independent role in regulating cell transformation and subsequent tumorigenesis. Although the presence or absence of MdmX does not seem to impact the amplification of centrosomes in p53-null cells, the increase in bipolar spindle polarity and chromosome ploidy following the reintroduction of MdmX into MdmX/p53-null cells confirms that MdmX alters the organization of these supernumerary centrosomes. Therefore, we propose that the MdmX-mediated clustering of amplified centrosomes facilitates bipolar mitosis and the propagation of hyperploid genomes. The loss of MdmX increases spindle multipolarity and the rate of chromosomal loss, and the resulting reduction in ploidy should yield a faster-replicating population of double null cells. Conversely, chromosomal clustering and the anti-proliferative effect of exogenous MdmX in p53-null cells suggest that cells with supernumerary chromosomes that undergo bipolar mitosis proliferate slower than cells with multipolar spindles and further support a p53-independent role for MdmX in tumor suppression.

MdmX has recently been recommended as a suitable target for small-molecule inhibition in the treatment of human cancers (24, 46), as the inhibition of the MdmX-p53 interaction in tumors with wild-type p53 likely releases tumor suppressor p53 activity. However, it is unclear what effect this inhibition has on p53-independent MdmX activities, especially in the approximately half of all human tumors that lack functional p53. Therefore, further work is needed to define the precise molecular mechanism of the MdmX-mediated suppression of proliferation and tumorigenesis.

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