

mdmx Is a Negative Regulator of p53 Activity *in Vivo*

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ABSTRACT

Regulation of p53 protein activity is required for normal embryogenesis, tumor suppression, and cellular response to DNA damage. Here we report that loss of *mdmx*, a p53-binding protein, results in midgestational embryo lethality, a phenotype that is completely rescued by the absence of p53. Mice homozygous for both *mdmx* and *p53* null mutations are viable and appear developmentally normal. Fibroblasts derived from embryos with reduced *mdmx* expression demonstrate a decreased growth rate and increased UV-induced apoptosis compared with wild-type cells and contain elevated levels of p53 and several p53 target proteins including the proapoptotic bax protein. These observations demonstrate that *mdmx* functions as a critical negative regulator of p53 *in vivo*.

INTRODUCTION

mdmx (also known as *mdm4*) is a p53-binding protein with structural similarity to the proto-oncoprotein *mdm2* (1). p53 binding by *mdm2* results in a masking of the p53 transactivation domain (2–4) and the subsequent targeting of p53 for proteasome-dependent degradation (5, 6). Thus, *mdm2* is a major negative regulator of p53 function (see Ref. 7 for a recent review). *mdm2* null embryos die *in utero*; however, this embryonic lethality can be overcome by deletion of the functional p53 gene product (8, 9). *mdmx* also inhibits p53 function through suppression of p53-dependent transactivation of target genes; yet *mdmx* has been shown to protect p53 from *mdm2*-mediated degradation (10, 11). In contrast to *mdm2*, the levels of *mdmx* expression do not change in cultured cells exposed to UV irradiation (1), suggesting that the activation of these two p53 regulators is controlled by different pathways. Given the central role of p53 in human cancer, a better understanding of the regulation of the p53 pathway may uncover new cancer therapeutic targets and strategies.

MATERIALS AND METHODS

Genotyping. In some studies, determination of the genotype of mice at the *mdmx* locus was performed by using a Southern blotting method to compare the intensities on autoradiograms of 1.5-kb *SacI* fragments hybridizing to a neomycin phosphotransferase-specific probe. This strategy enabled discrimination of zero, one, or two gene disruptions, representing (+/+), (+/-), and (-/-) mice, respectively, at any trapped genetic locus. The *c-src* tyrosine kinase gene was probed as a normalization control.

A Southern blotting RFLP strategy was used for analysis of the *mdmx* gene status in the context of disrupted *p53*. A probe directed to the genomic sequence between the *mdmx* coding sequence in the 5' region of the trapped sequence tag and the 3' end of the 3'-long terminal repeat in the trapping vector was generated using PCR. This 500-bp probe recognizes *SacI* fragments of approximately 10 and 5.5 kb in wild-type and disrupted *mdmx* alleles, respectively.

p53 genotyping was performed using a multiplex PCR-based strategy developed by Timme and Thompson (12). A 320-bp PCR product is produced

from the wild-type *p53* allele, and a 150-bp PCR product is produced from the disrupted *p53* allele.

Northern Blot Analysis of *mdmx*. Total cellular RNA was isolated from cultured cells using TRIzol reagent (Life Sciences, Gaithersburg, MD). An [α^{32} P]dCTP random primer-labeled probe corresponding to nucleotides 1–298 of the mouse *mdmx* cDNA was used for Northern hybridization.

Apoptosis Assay. Apoptosis was quantified by two methods: (a) using a Cell Death Detection ELISA kit (Roche Diagnostics Corp., Indianapolis, IN), which is a photometric enzyme-immunoassay for quantitation of histone-associated DNA fragments (nucleosomal DNA) in the cytoplasm after apoptosis induction; and (b) assessment of the morphological appearance of acridine orange/ethidium bromide-stained cells using fluorescence microscopy (13, 14).

Western Blotting. Total cell lysates were prepared in Laemmli buffer and subjected to Western blot analysis using p53-, bax-1-, *mdm2*-, p21-, and thrombospondin I-specific antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

RESULTS AND DISCUSSION

We now report the use of a large-scale reverse genetic approach based on genome-wide gene trapping technology (15, 16) to demonstrate the role of the *mdmx* gene in the regulation of the p53 tumor suppressor protein *in vivo*. We identified an ES² cell clone containing a single gene trap insertion in the *mdmx* gene using a bioinformatic screen of the sequence-tagged ES cell clones in the OmniBank library. This allowed for the rapid development and phenotypic analysis of a knockout mouse line. As judged by sequence alignment, the retroviral insertion is in the forward direction near the 5'-end of the coding region of the *mdmx* cDNA, 1–2 codons downstream of the ATG start site. Mice heterozygous for the *mdmx* disruption (*mdmx*¹) were mated, and their offspring were genotyped at the *mdmx* locus. Of 61 offspring from nine litters representing five separate crosses, 24 were wild-type, 37 were *mdmx*¹/+, and none were homozygous for *mdmx* disruption, indicating that expression of *mdmx* is required for normal embryo development. *mdmx*¹/*mdmx*¹ embryos died between E10 and E12 and were resorbed by E13.5. The lethality may be attributable to cardiovascular defects or partial chorioallantoic fusion, as suggested by the pooling of blood in the pericardial cavity or collapsed allantois, respectively (not shown). *mdmx*¹/*mdmx*¹ embryos exhibited cephalic structures that were reduced in size, most notably the telencephalon (Fig. 1, compare A and B). Although the hindbrain/midbrain and forebrain/midbrain boundaries appear to form, these looked abnormal in some of the embryos (Fig. 1, C and D).

To study the effects of *mdmx* on cell proliferation, apoptosis, and gene expression, we generated mouse embryonic fibroblasts from E14 embryos produced by crossing *mdmx*¹/+ mice (17). We were unable to recover any lines of *mdmx*¹/*mdmx*¹ fibroblasts from early embryos because of a severe defect in the ability of cells that are homozygous for the mutation to proliferate in culture. However, we were successful in recovering a large number of embryonic fibroblast lines that were heterozygous for the *mdmx*¹ allele as well as developmentally matched, wild-type control fibroblasts. *mdmx* mRNA was not abundantly expressed in embryonic fibroblasts and therefore did not produce a strong signal by Northern hybridization. Even so, Northern blot

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²The abbreviation used is: ES, embryonic stem.

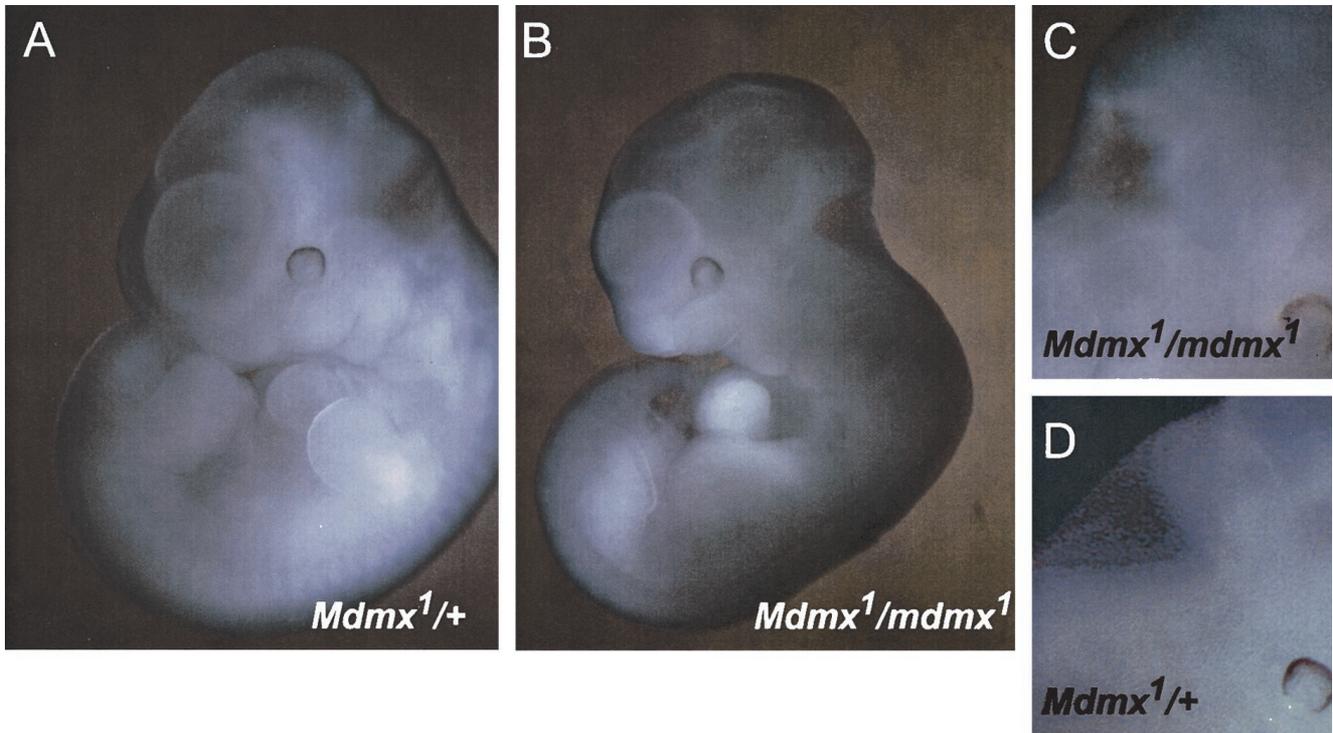


Fig. 1. Cephalic abnormalities in *mdmx¹/mdmx¹* embryos. *A*, wild-type E11.5 embryo (littermate of embryo shown in *B*). *B*, *mdmx¹/mdmx¹* embryo exhibiting a telencephalon that is reduced in size. *C*, close-up of the head of the embryo shown in *B*, showing an abnormal hindbrain-midbrain boundary. *D*, close-up of the head of the embryo shown in *A*.

analysis did confirm a lower level of *mdmx* mRNA in *mdmx¹/+* embryonic fibroblasts than in wild-type fibroblasts (Fig. 2*A*). This decrease in *mdmx* expression was correlated with a markedly reduced cell proliferation rate (Fig. 2*B*). We also observed occurrence of replicative senescence in the *mdmx¹/+* cells after fewer passages in culture compared with wild-type cells. Thus, further comparisons of wild-type and *mdmx¹/+* fibroblasts were made using cells that had undergone fewer than five passages in culture.

To confirm the influence of the *mdmx* deficiency on p53-dependent processes, we investigated the induction of apoptosis in these fibroblasts after exposure to low dosages of UV irradiation. Sixteen h after exposure to UV irradiation, *mdmx¹/+* fibroblasts with reduced *mdmx* expression exhibited an increased occurrence of apoptosis compared

with wild-type cells as determined by the increased number of cytoplasmic histone-associated DNA fragments (Fig. 3*A*). Fluorescence microscopic assessment of the morphology of acridine orange/ethidium bromide-stained fibroblasts showed that nonirradiated wild-type and *mdmx¹/+* cultures contained few apoptotic cells ($1.0 \pm 0.6\%$ and $1.0 \pm 0.5\%$, respectively). Consistent with the results shown in Fig. 3*A*, after irradiation, a greater percentage of apoptotic cells was observed in the *mdmx¹/+* cultures ($11.1 \pm 0.9\%$) than in the wild-type cultures ($5.9 \pm 1.8\%$).

We also found that p53 protein levels were notably higher in nonirradiated *mdmx¹/+* fibroblasts than in wild-type cells (Fig. 3*B*), indicating that the presence of *mdmx* reduces p53 protein levels. It has been shown that proapoptotic mediators such as *bax* and *bak* are

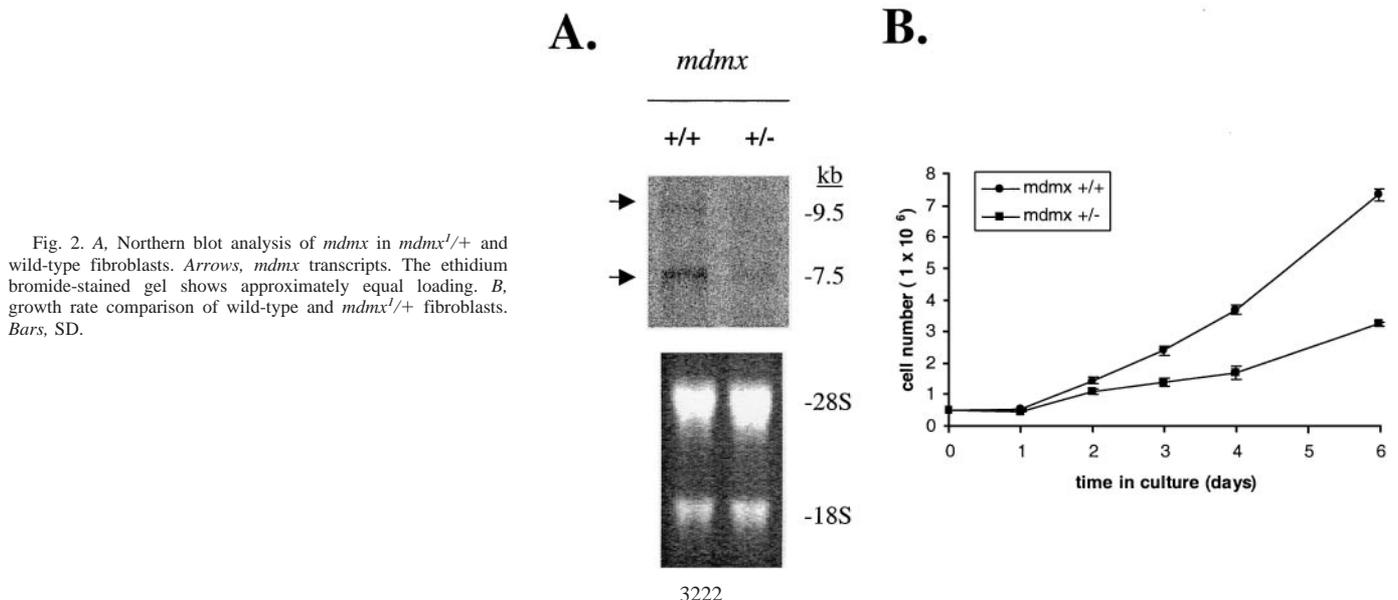


Fig. 2. *A*, Northern blot analysis of *mdmx* in *mdmx¹/+* and wild-type fibroblasts. Arrows, *mdmx* transcripts. The ethidium bromide-stained gel shows approximately equal loading. *B*, growth rate comparison of wild-type and *mdmx¹/+* fibroblasts. Bars, SD.

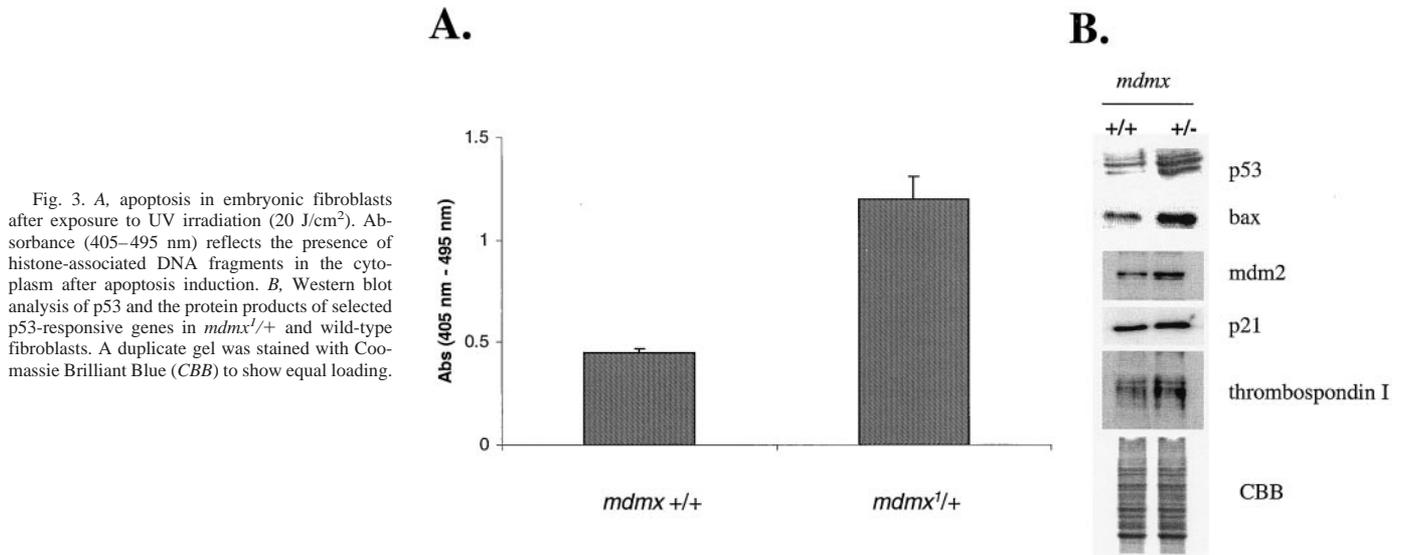


Fig. 3. *A*, apoptosis in embryonic fibroblasts after exposure to UV irradiation (20 J/cm²). Absorbance (405–495 nm) reflects the presence of histone-associated DNA fragments in the cytoplasm after apoptosis induction. *B*, Western blot analysis of p53 and the protein products of selected p53-responsive genes in *mdmx*^{1/+} and wild-type fibroblasts. A duplicate gel was stained with Coomassie Brilliant Blue (CBB) to show equal loading.

up-regulated when p53 is overexpressed in cancer cells (18), and that p53-induced apoptosis requires activation of the expression of downstream target genes such as *bax* (19). Consistent with the increased p53 levels, *mdmx*^{1/+} fibroblasts were found to contain elevated levels of the protein products of several p53-responsive genes including *bax* (Fig. 3*B*). The levels of all p53-responsive gene products examined (*e.g.*, p21) were not notably increased in *mdmx*^{1/+} fibroblasts, however. Interestingly, the amount of mdm2, a mediator of p53 degradation, was increased in the *mdmx*-deficient cells. It is possible that *mdmx* is required to cooperate with mdm2 to control levels of p53 protein and prevent excessive activation of p53-dependent signaling. Taken together, these results support the hypothesis that the presence of *mdmx* can reduce p53-mediated apoptotic responses as well as modulate downstream targets of p53.

The increased steady-state level of p53 protein in *mdmx*^{1/+} fibroblasts was not attributable to stabilization of p53 protein with reduction in *mdmx*. Laser densitometric scanning of p53 Western blots produced from *mdmx*^{1/+} and wild-type cells incubated with cycloheximide showed no significant change in p53 protein half-life (Fig. 4*A*). Northern analysis revealed increased steady-state *p53* mRNA in

the *mdmx*^{1/+} fibroblasts compared with wild type (Fig. 4*B*), suggesting that the increased p53 protein level in the *mdmx*^{1/+} cells results from increased *p53* mRNA, either directly or indirectly as a result of lowered *mdmx* levels.

To determine whether *mdmx* regulates p53 *in vivo*, we investigated the consequences of *mdmx* loss in mice null for *p53*. We crossed *mdmx*^{1/+} mice with mice heterozygous for a *p53* mutation to generate mice heterozygous for both *p53* and *mdmx*. Of 99 viable offspring from matings of these “double heterozygotes,” 6 were confirmed by Southern blot analysis and PCR to be homozygous null for both *mdmx* and *p53* (Fig. 5*A* and Table 1). Northern blot hybridization was used to demonstrate the loss of *mdmx* expression in the viable *p53* null/*mdmx* null animals (Fig. 5*B*). The rescue of the *mdmx*^{1/*mdmx*¹ embryonic lethality in the absence of p53 constitutes strong genetic evidence that *mdmx* is, in fact, a potent negative regulator of p53 function and confirms the results of an important recent report by Parant *et al.* (20). The double-null animals appeared normal but were susceptible to early tumor development, similar to *p53* null animals (21). The five *p53/mdmx* double null animals that were monitored for}

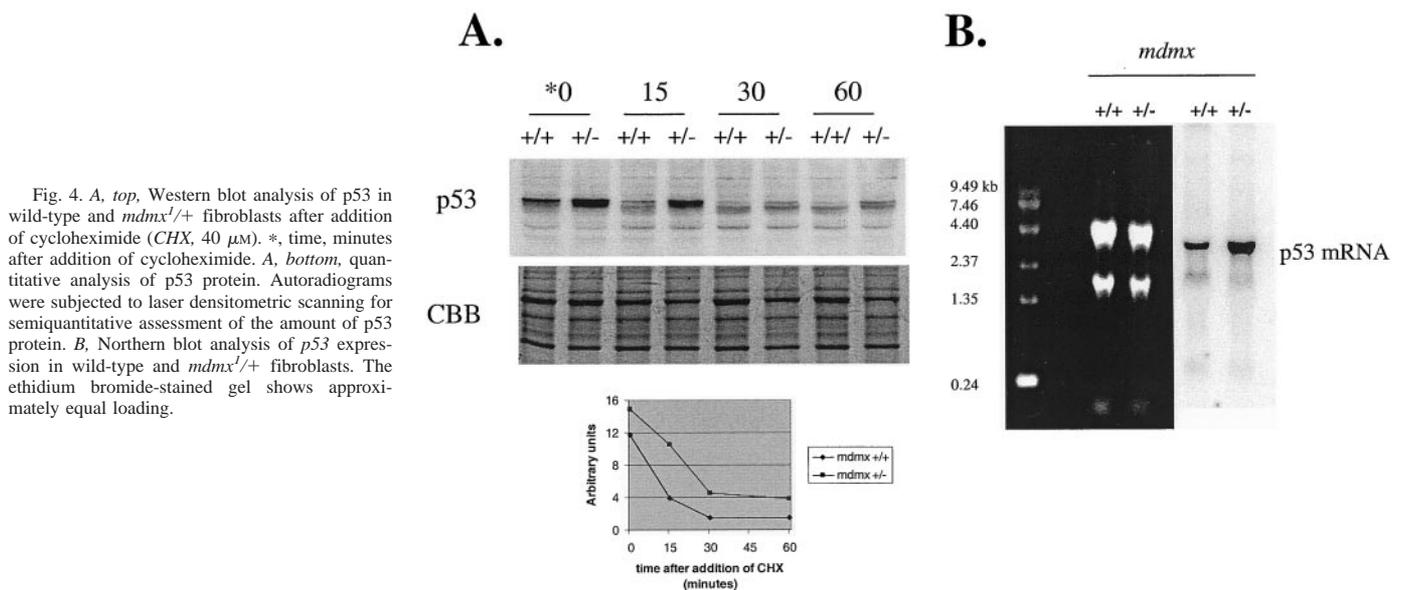


Fig. 4. *A*, *top*, Western blot analysis of p53 in wild-type and *mdmx*^{1/+} fibroblasts after addition of cycloheximide (CHX, 40 μM). *, time, minutes after addition of cycloheximide. *A*, *bottom*, quantitative analysis of p53 protein. Autoradiograms were subjected to laser densitometric scanning for semiquantitative assessment of the amount of p53 protein. *B*, Northern blot analysis of *p53* expression in wild-type and *mdmx*^{1/+} fibroblasts. The ethidium bromide-stained gel shows approximately equal loading.

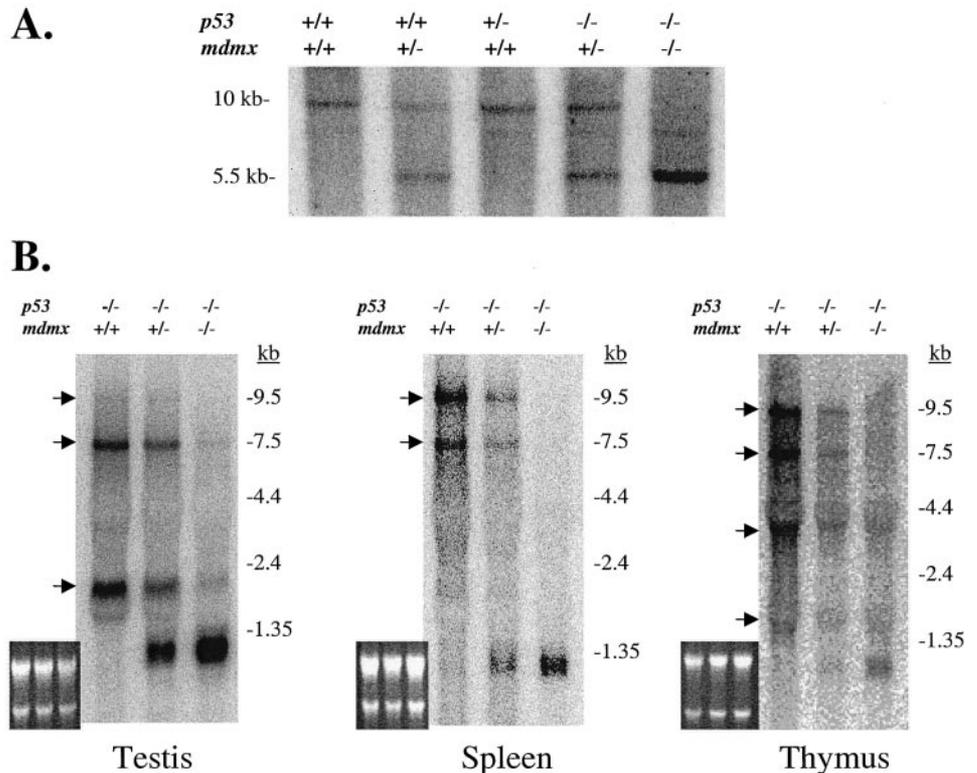


Fig. 5. *A*, *mdmx* genotypic analysis of offspring of *mdmx/p53* double heterozygote matings. *B*, *mdmx* Northern blot analyses of total RNA from selected tissues (that normally express high levels of *mdmx* RNA) from *p53* null, *mdmx* wild-type; *p53* null, *mdmx*^{1/+}; and *p53* null, *mdmx*^{1/mdmx} animals. Arrows, *mdmx* transcripts. This probe recognizes the ~1.3 kb fusion transcript containing the selectable neomycin phosphotransferase marker resulting from the trapping vector insertion as well as endogenous *mdmx* transcripts. The ethidium bromide-stained gels show approximately equal loading.

tumor development died of lymphoid tumors with a mean life span of 26 weeks.

Interestingly, the *mdmx*^{1/mdmx} embryos studied in the present report died on E10–E12, whereas the time of death of homozygous mutant *mdmx* embryos reported previously by Parant *et al.* (20) was E7.5–E8.5. This discrepancy may be attributable to subtle differences in genetic background of the animals and ES cells used to generate the mutant mice.

Previous *in vitro* transient overexpression studies have shown that increased Mdmx inhibits Mdm2-mediated degradation of p53, leading to enhanced p53 levels (10, 11). In contrast, our study shows that reduction of *mdmx* in genetically altered primary fibroblast cell lines leads to increased p53 levels. Our observation of an increased p53 level concomitant with *mdmx* reduction is consistent with the reduced growth and increased apoptosis of *mdmx*^{1/+} fibroblasts as well as with the finding that loss of p53 rescues the lethality of *mdmx*-null mice. An understanding of the mechanistic basis for these apparent contradictions may shed new light on the complex nature of p53 regulation.

The lethality of *mdm2* (8, 9) and *mdmx* null mice shows clearly that these proteins cannot compensate for each other, suggesting that both are required to control the p53 pathway. p53 is an important tumor suppressor gene product, and the correlation between loss of functional p53 and tumor formation in animals and humans is well established. Because *mdmx* functions as a negative regulator of the p53 pathway, it may also be involved in tumorigenesis. This notion is

supported by the report of amplification and overexpression of the *MDMX* gene in a subset of malignant human gliomas containing no *p53* mutations (22), suggesting that excess MDMX may contribute to tumor development by blocking p53 function. Our results suggest that MDMX, similar to MDM2, offers a potential target for antitumor therapies for the roughly 50% of human cancers that retain wild-type p53.

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Table 1 Genotypic analyses of offspring resulting from *mdmx*, *p53* double heterozygote matings

Numbers represent offspring derived from 14 litters from seven separate crosses. Ninety-nine total offspring were analyzed. Observed (expected).

	<i>mdmx</i> +/+	<i>mdmx</i> +/-	<i>mdmx</i> -/-
<i>p53</i> +/+	6 (6.2)	17 (12.4)	0 (6.2)
<i>p53</i> +/-	17 (12.4)	33 (24.8)	0 (12.4)
<i>p53</i> -/-	4 (6.2)	16 (12.4)	6 (6.2)

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