Loss of *Mdm4* Results in *p53*-Dependent Dilated Cardiomyopathy

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Background—Although several loci for familial dilated cardiomyopathy (DCM) have been mapped, the origin of a large percentage of DCM remains unclear. Mdm2, a *p53*-negative regulator, protects cardiomyocytes from ischemic and reperfusion-induced cell death. Mdm4, a homolog of Mdm2, inhibits *p53* activity in numerous cell types. It is unknown whether Mdm4 plays a role in the inhibition of p53 in fully differentiated tissues such as adult cardiomyocytes and whether this role is associated with DCM.

Methods and Results—The conditional knockout of *Mdm4* in the heart by use of cardiomyocyte-specific *Cre* (*αMyHC-Cre*) allele does not result in any developmental defects. With time, however, mice with deletion of *Mdm4* in the adult heart developed DCM and had a median survival of 234 days. More interestingly, the onset of DCM occurs significantly earlier in male mice than in female mice, which mimics human DCM disease. DCM in *Mdm4* mutant mice was caused by loss of cardiomyocytes by apoptosis, and it was *p53*-dose dependent.

Conclusion—Activity of p53 was inhibited by Mdm4 even in the fully differentiated cardiomyocyte. Elevated apoptosis mediated by the *p53* pathway in cardiomyocytes may be a mechanism for DCM. **(*Circulation*. 2007;115:2925-2930.)**

Key Words: apoptosis ■ myocytes, cardiac ■ cardiomyopathy ■ genetics ■ survival

Dilated cardiomyopathy (DCM) is one of the leading causes of heart failure in the United States, with an annual incidence estimated to be 5 to 8 cases per 100 000 population. DCM is characterized by ventricular chamber dilation with normal or decreased wall thickness and impaired systolic function.[1–3] DCM has both hereditary and acquired forms. Two sarcomere structural genes have been identified to be involved with DCM, the cardiac actin and phospholamban genes.[4,5] Additionally, several loci for familial DCM have been mapped to 1p1−1q1,[6] 3p25−3p22,[7] 1q32,[8] and 9q13−9q22,[9] which suggests genetic heterogeneity in DCM.[10] Despite efforts over many years to identify the causative genes in DCM, the underlying mechanism(s) of a large percentage of DCM remains elusive. Mice have also been used as models for different types of cardiomyopathies, such as DCM.[5,11] Mdm2, a negative regulator of the p53 tumor suppressor, was recently shown to protect murine cardiomyocytes from ischemic/reperfusion-induced cell death, which implicates the p53 pathway in cardiac cell survival[12] and cardiomyopathy diseases.

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Mdm2 is an E3 ubiquitin ligase and negative regulator of p53. Mdm4 is a homolog of Mdm2, which also inhibits p53 activity by masking its transcriptional activation domain.[13] During embryonic development, p53 activity is suppressed by both Mdm2 and Mdm4. Loss of *Mdm2* in mice results in embryonic lethality by *p53*-dependent apoptosis.[14,15] Loss of *Mdm4* also causes p53-dependent embryonic lethality by initiation of cell cycle arrest and apoptosis.[16–18] Additionally, Mdm2 and Mdm4 synergize to inhibit p53 in the developing central nervous system during embryogenesis.[19,20] These data demonstrate that p53 activity is inhibited by Mdm2 and Mdm4 in proliferating cells. In postmitotic cells in the central nervous system, both Mdm2 and Mdm4 are also required to inhibit p53.[20] However, when *Mdm2* and *Mdm4* are deleted in the adult smooth muscle cells, loss of *Mdm2*-induced *p53*-dependent apoptosis, but acute loss of *Mdm4* does not show obvious defects,[21] which suggests that, in quiescent or fully differentiated cells, Mdm4 is not required to inhibit p53 activity. It remains unclear whether Mdm4-mediated p53 inhibition in differentiated cells is tissue specific.

To determine whether Mdm4-mediated p53 inhibition is important in another differentiated cell type, we chose to delete *Mdm4* in cardiomyocytes in mice. Previously, we crossed the α-myosin heavy chain promoter–driven *Cre* mouse (*αMyHC-Cre*) to a *Mdm4*-conditional allele to generate cardiomyocytes that lack *Mdm4*. Mice with deletion of *Mdm4* in cardiomyocytes did not show obvious defects during development and perinatal stages,[22] which provides an

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excellent opportunity to study whether Mdm4 is required to inhibit p53 activity in the adult cardiomyocytes. Adult mouse cardiomyocytes do not have regenerative capacity,[23,24] although they do proliferate during fetal development. Shortly after birth, positive cell cycle factors such as cyclin A and cdk2 are downregulated and the cell cycle inhibitors p21 and p27 are upregulated, which allows cardiomyocytes to become quiescent.[25] Mice with deletion of *Mdm4* in cardiomyocytes showed severe edema and heart failure as early as 3 months of age. Mutant mice developed DCM and exhibited apoptosis in adult cardiomyocytes, which indicates a role for Mdm4 in differentiated cardiomyocytes.

To test whether the DCM phenotype was p53-dependent, we used *p53*-conditional[26] and null alleles[27] to generate Mdm4 mutant mice with 1 or no *p53* alleles. Survival studies of these mice clearly showed the phenotype was dependent on p53 dose, which demonstrates that elevated p53 activity may be one of the causes for DCM.

Methods

Mice

A conditional allele of *Mdm4*, $Mdm4^{FX}$, has 2 *lox P* sites that surround exon 2, which contains the ATG start codon. Deletion of exon 2 results in a null allele, designated $Mdm4^{\Delta 2}$.[22] $Mdm4^{+/\Delta 2}$*αMyHC-Cre* mice were crossed to $Mdm4^{FX/FX}$ mice to generate the cohorts of $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* and $Mdm4^{+/FX}$ *αMyHC-Cre* mice.[22] The breeding and maintenance of mice were performed in a specific pathogen–free mouse facility under institutional guidelines.

X-Gal Staining of Adult Heart

Frozen cross-sections of *αMyHC-Cre*, *Rosa26-lacZ* adult hearts were stained according to a previous protocol.[28]

TUNEL Assays/Immunohistochemistry Staining

Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assays carried out on paraffin-embedded sections were modified with avidin biotin complex and diaminobenzidine kits from Vector Laboratories (Burlingame, Calif).[19] Mason's trichrome staining was performed by the veterinary pathology laboratory at M.D. Anderson Cancer Center. The immunohistochemistry staining of atrial natriuretic peptide was performed as previously described[19] with atrial natriuretic peptide antibody FL-153 (1:100) from Santa Cruz Biotech (Santa Cruz, Calif).

Statistical Analysis

Two-way ANOVA and Kaplan-Meier survival analysis were performed with Prism 4 software (GraphPad Software, San Diego, Calif). Differences were considered significant at a value of $P<0.05$.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Loss of Mdm4 in the Adult Heart Caused Dilated Cardiomyopathy

A conditional allele of *Mdm4*, $Mdm4^{FX}$ contains lox P sites that surround exon 2, and deletion of exon 2 in the germline results in a null allele designated $Mdm4^{\Delta 2}$.[22] $Mdm4^{+/\Delta 2}$ with the *αMyHC-Cre* transgene[29] were crossed to mice homozygous for the *Mdm4*-conditional allele ($Mdm4^{FX/FX}$) to generate mice with different combinations of alleles, such as those that lack *Mdm4* in cardiomyocytes. Deletion of *Mdm4* in the embryonic heart does not cause any developmental defects.[22] We maintained mice for >1 year to examine the possible role of Mdm4 in fully differentiated adult cardiomyocytes. Cohorts of mutant $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* and control $Mdm4^{+/FX}$ *αMyHC-Cre* mice were monitored. To make possible examination of *Cre*-specific recombination, some mice contained the *ROSA26-lacZ* reporter, as *Cre*-recombination at the *ROSA26* locus allows expression of β-galactosidase (these mice were not part of the cohort study). Robust and specific *LacZ* staining was observed in the adult heart with frozen sections from 5-month-old mice with both *αMyHC-Cre* and *Rosa26-lacZ* transgenes (Figure 1A). Obvious blue staining in the cardiomyocytes was present in both right and left ventricles, as well as in the septum between the 2 ventricles (Figure 1A; other data not shown), which was consistent with previous studies that showed tissue-specific expression of *Cre* from the *αMyHC* promoter.[29] Specific recombination at the *Mdm4* locus was previously observed in the adult mice with PCR primers that distinguish conditional and recombined alleles.[22] Because mice inherit an *Mdm4*-conditional and a null allele, every recombination event leads to a cell that completely lacks *Mdm4*.

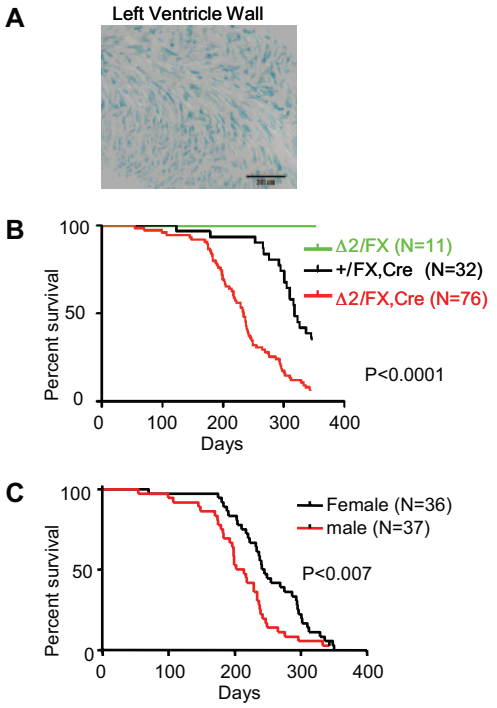

Figure 1. Survival of mice with loss of *Mdm4* in cardiomyocytes. A, LacZ staining of hearts from $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice that inherited the *Rosa 26-LacZ* locus at 5 months of age. Scale bar=201 μm. B, Kaplan-Meier survival curve of $Mdm4^{\Delta 2/FX}$, $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant and $Mdm4^{+/FX}$ *αMyHC-Cre* control mice. C, Survival differences between male and female $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice.

The earliest abnormality was observed in $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice at 3 months of age. By 8 to 10 months of age, most of the mutant mice had swollen bodies, showed difficulty moving, and were out of breath. Mutant mice eventually died as a result of heart failure. The survival of $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice was significantly shorter than

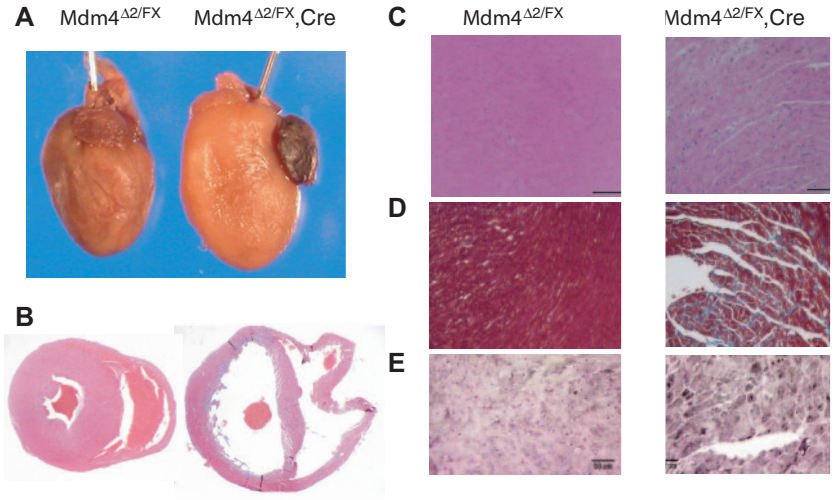

Figure 2. *Mdm4*-deficient mice exhibited dilated cardiomyopathy. A, Gross observation of an *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant and *Mdm4*$^{+/FX}$ *αMyHC-Cre* control hearts at 8 months of age. B, Cross-sections with trichrome staining of normal and mutant heart at 7 months. C, Hematoxylin and eosin staining of heart samples at 7 months. Scale bar=100 μm. D, Trichrome staining at higher magnification (×200) at 7 months. E, Immunohistochemistry staining of atrial natriuretic peptide. Scale bar=50 μm.

Mdm4$^{+/FX}$ *αMyHC-Cre* control mice, with median survival of 234 and 318 days, respectively ($P<0.0001$) (Figure 1B). *αMyHC-Cre* mice survived up to 1 year of age,[30] and *Mdm4*$^{\Delta 2/FX}$ mice survived even up to 2 years of age, although some *Mdm4*$^{+/FX}$ *αMyHC-Cre* mice died before 1 year of age, perhaps as a result of toxicity of *Cre* expression plus the loss of 1 allele of *Mdm4*. Interestingly, the mutant male mice died significantly earlier than the female mice, with median survival at 208 and 243 days, respectively ($P<0.007$) (Figure 1C). To examine the cause of the death in more detail, *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice were euthanized and dissected when they were moribund. The mutant mice had obvious edema in the lung and/or abdomen. The mutant hearts were enlarged, and all 4 chambers were dilated and paler than the hearts of control mice (Figure 2A). Cross-sections of the heart showed that the ventricular walls in mutant hearts were thinner and obviously hypertrophic (Figure 2B and 2C). These observations indicated severe dilated cardiomyopathy in the mutant mice. Mason trichrome staining was also performed to detect collagen deposition, a marker of fibrosis in the heart. Positive blue staining, which indicated fibrosis, was clearly evident in mutant hearts in comparison to hearts from control mice (Figure 2B and 2D). Atrial natriuretic peptide is a molecular marker widely used to characterize cardiomyocyte hypertrophy and heart failure.[31] Immunohistochemistry staining showed atrial natriuretic peptide was also prominent in mutant but not control hearts (Figure 2E). Together, these data indicated that loss of *Mdm4* induced dilated cardiomyopathy, which led to heart fibrosis and eventually heart failure in the mutant mice.

Loss of Adult Cardiomyocytes in *Mdm4* Mutant Mice

Hearts from *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mice showed thinner walls in the both left and right ventricles and were positive for atrial natriuretic peptide and Mason trichrome staining as compared with controls, which suggests loss of cardiomyocytes in the mutant hearts. To test whether loss of *Mdm4* in the adult heart caused loss of fully differentiated cardiomyocytes, the cell number from cross-sections of both mutant and control hearts at 3 and 8 months of age was determined. Although the number of cardiomyocytes at 3 months of age was similar in the control and mutant hearts ($P=0.14$), strikingly, the hearts of *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mice had half the number of cardiomyocytes at 8 months of age as compared with *Mdm4*$^{+/FX}$ *αMyHC-Cre* control mice (Figure 3A and 3B) ($P=0.015$). To investigate whether loss of cardiomyocytes in the heart was caused by the apoptosis, TUNEL assays were performed. TUNEL-positive cells were clearly evident only in mutant mice at 3 months of age when the cardiomyocytes are terminally differentiated (Figure 3B). In line with the differentiated and quiescent nature of adult cardiomyocytes, bromodeoxyuridine labeling indicated a lack of proliferation in both mutant and control mice (data not shown). These experiments demonstrated that loss of *Mdm4*

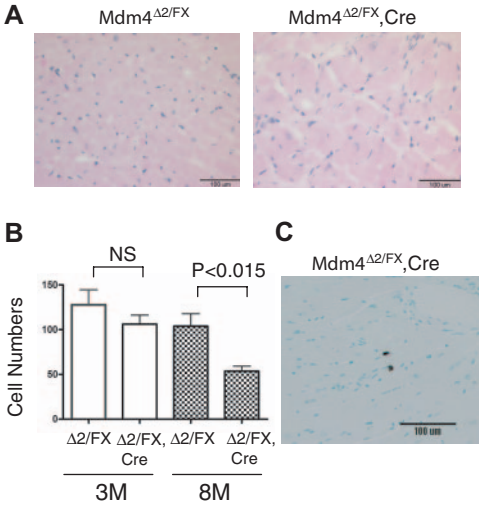

Figure 3. Loss of cardiomyocytes in adult *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant heart. A, Representative hematoxylin and eosin staining of a heart at 8 months from an *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant mouse (×200). B, Cardiomyocyte cell number in the left ventricular walls of *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice ($n=3$) compared with the *Mdm4*$^{\Delta 2/FX}$ control mice ($n=4$) was similar at 3 months of age ($P=0.14$) but significantly decreased at 8 months of age ($P=0.015$). NS indicates not significant; M, months. The cell numbers were averaged by a count of 5 random fields in sections from each mouse. C, TUNEL assay in the *Mdm4*$^{\Delta 2/FX}$ *αMyHC-Cre* mutant heart at 3 months.

in adult cardiomyocytes induced apoptosis, which indicates that *Mdm4* is still required in these differentiated and quiescent cells.

DCM Caused by Loss of *Mdm4* Was Dependent on p53 Dose

To understand whether the DCM phenotype caused by loss of *Mdm4* is *p53*-dependent, the *p53*-null and *p53*-conditional alleles were introduced into $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice. On loss of 1 *p53* allele, $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice showed an extended median survival to 274 days compared with 234 days for $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice with 2 wild-type *p53* alleles ($P<0.0001$). These data indicated that loss of a single *p53* allele alleviated the severity of the phenotype. Because >90% of *p53*-null mice die by 6 months of age as a result of the development of lymphomas,[27,32] we could not examine the survival advantage in $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice null for *p53*. We therefore used a *p53*-conditional allele[26] and combined it with the *Mdm4* mutant alleles to generate $Mdm4^{\Delta 2/FX}$ $p53^{lox/-}$ *αMyHC-Cre* mice to determine whether loss of both *p53* alleles specifically in the heart could actually rescue the DCM phenotype. The median survival for $Mdm4^{\Delta 2/FX}$ $p53^{lox/-}$ *αMyHC-Cre* mice lengthened to 403 days, which was significantly longer ($P>0.0005$) than $Mdm4^{\Delta 2/FX}$ $p53^{+/-}$ *αMyHC-Cre* mice (Figure 4A). Trichrome staining of the hearts of $Mdm4^{\Delta 2/FX}$ $p53^{lox/-}$ *αMyHC-Cre* mice at about 6 months of age showed a significant reduction in staining as compared with the mutant mice with a single *p53* allele (Figure 4B). $Mdm4^{\Delta 2/FX}$ $p53^{lox/-}$ *αMyHC-Cre* mice were not edematous nor out of breath and died as a result of the development of various tumors (data not shown). These data demonstrated that the DCM phenotype caused by loss of *Mdm4* was dependent on *p53* dose.

Discussion

Loss of *Mdm4* in terminally differentiated cardiomyocytes led to a *p53*-dependent lethal DCM. Mice that lacked *Mdm4* in cardiomyocytes gradually lost these cells by apoptosis, and eventually died of heart failure. These data demonstrate that *Mdm4*-mediated inhibition of p53 in adult cardiomyocytes was essential to normal heart function. The rescue of the DCM phenotype by concomitant loss of *p53* indicated that abnormal elevated p53 activity may induce DCM. It will be important to determine whether loss of *Mdm4* and/or elevated *p53* activity actually is a mechanism that leads to heart failure in humans. Another interesting observation in this mouse model is that the phenotype of $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mice recapitulates the gender differences of human heart failure. Women with heart failure survive substantially longer than men.[33,34] This genetically defined mouse model with loss of *Mdm4* in the adult heart may provide a relevant model to understand the gender differences of DCM-induced heart failure.

Recently, the concept of stem cell therapy has attracted many clinicians to test heart repair with a variety of stem cells. Although the cardiac transfer of stem and progenitor cells shows a favorable impact on tissue perfusion and contractile performance of the injured heart, the mechanism of stem cell therapy is still unclear, and it is essential to determine the right stem cell type in the right clinical setting.[35] This genetically defined mouse model with loss of *Mdm4* in the adult heart may provide a good model to test stem cell therapies in DCM.

Activity of p53 is high in proliferating progenitor cells before and after birth (G.L., unpublished observations, 2006). Loss of *Mdm2* and *Mdm4* in these cells results in *p53*-mediated cell-cycle arrest and apoptosis.[19,20] Additionally, *p53* activity is inhibited by *Mdm2* in differentiated smooth muscle cells in the small intestine, and loss of *Mdm2* in these cells results in *p53*-dependent apoptosis. The role of *Mdm4* in the inhibition of *p53* in smooth muscle cells seems unimportant, as deletion of *Mdm4* does not cause any obvious defects.[21] The difference between our study and loss of *Mdm4* in smooth muscle cells is either a result of tissue specificity of *Mdm4* function, or that *Mdm4* loss requires a longer period to develop a severe phenotype. The second possibility is consistent with the notion that *Mdm4* loss causes less severe phenotypes in all tissues examined thus far in comparison to loss of *Mdm2*.[19–22]

The importance of understanding the role of the *p53* pathway in differentiated tissues is underscored by a strategy to treat cancer patients with molecular drugs to disrupt the binding of *p53* to *Mdm2*.[36,37] This strategy is widely accepted as a treatment option in cancer patients with wild-type *p53*. Recently, Nutlin-3 and Rita, 2 small molecules that disrupt the *p53–Mdm2* interaction, have been shown to be effective in cancer cell lines[36,38–40] and in a xenograft model.[41] Because *Mdm4* is another *p53*-negative regulator, and *Mdm4* is overexpressed in many tumor cell lines and primary tumors such as tumors with wild-type *p53*,[42,43] it is also an attractive choice for the design of drugs to target *Mdm4* interaction with *p53*. Additionally, because *Mdm2* and *Mdm4* bind the same domain of *p53*,[44] it is also possible that future drugs will disrupt both *p53–Mdm2* and *p53–Mdm4* interaction. In consideration of all possibilities for this therapeutic strategy, it is very important to know whether these drugs will affect the normal functions of human tissues. Recent data demonstrate that *p53* activity is inhibited by *Mdm2* even in adult mouse tissues.[45] The present study implicates the importance of the *Mdm4–p53* interaction in normal heart function, which sug-

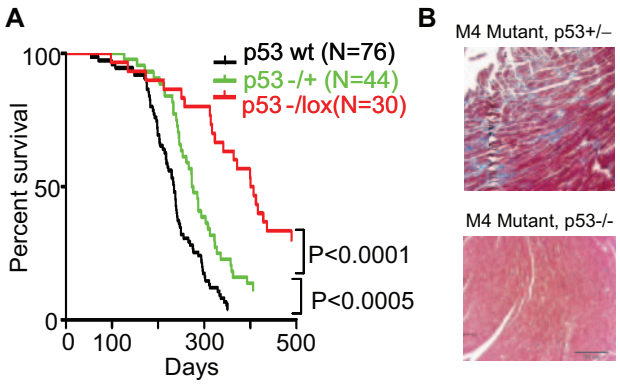

Figure 4. DCM phenotype in $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant heart depends on *p53* dose. A, The survival of $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice with 0, 1, or 2 *p53* alleles. B, Trichrome staining of $Mdm4^{\Delta 2/FX}$ *αMyHC-Cre* mutant mice with or without *p53* at 6 months. Scale bar=100 μm.

gests that drugs that disrupt this interaction in patients may have unwanted side effects.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Although several loci for familial dilated cardiomyopathy have been mapped, the underlying mechanism(s) of a large percentage of dilated cardiomyopathy remains unclear. Mdm4 is an inhibitor of the p53 tumor suppressor, and mice with deletion of *Mdm4* in the adult heart developed dilated cardiomyopathy with significantly earlier onset in male than in female mice, which thus recapitulates the gender differences observed in humans. The cause of dilated cardiomyopathy in this mouse model is a gradual loss of cardiomyocytes by p53-dependent apoptosis. Thus, this genetically defined mouse may provide a good model to test stem cell replacement therapies. The present study also has important implications for cancer treatment with specific drugs to disrupt the interaction between p53 and its negative regulators, Mdm2 and Mdm4. Although 2 small molecules, Nutlin-3 and Rita, have been shown to be effective in cancer cell lines and in a xenograft model, our study suggests the importance of the Mdm4–p53 interaction in normal heart function, which indicates that drugs that disrupt this interaction in patients may have unwanted side effects.

Loss of *Mdm4* Results in *p53*-Dependent Dilated Cardiomyopathy

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