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Widespread Overexpression of Epitope-Tagged Mdm4 Does Not Accelerate Tumor Formation In Vivo

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Mdm2 and Mdm4 are critical negative regulators of p53. A large body of evidence indicates that elevated expression of either Mdm2 or Mdm4 may favor tumor formation by inhibiting p53 tumor suppression function. To explore this possibility in vivo, we generated conditional Mdm2 and Mdm4 transgenic mice. We show that although both transgenes are designed to be expressed ubiquitously and at comparable levels, only the Mdm4 transgenic protein is produced at high levels in vivo. In contrast, exogenous Mdm2 is constitutively degraded in a proteasome-dependent manner, indicating that cells are equipped with efficient mechanisms that prevent Mdm2 accumulation in vivo. Mice that are homozygous for the Mdm4 transgene die during embryogenesis owing to severe vascular maturation defects. Importantly, this lethality is not rescued on a p53-null background, indicating that high levels of Mdm4 impact on a pathway(s) other than p53 that controls vascular and embryonic development. Mice expressing a single copy of the Mdm4 transgene are viable and, surprisingly, are not prone to spontaneous, radiation-induced or Eμ-myc-induced tumor formation. The findings have clear implications for cancer etiology as well as for cancer therapy.

The p53 tumor suppressor pathway is altered in virtually all human cancers (26). While the p53 gene itself is mutated or deleted in roughly 50% of cases, other mechanisms that disable p53 function are selected for in tumors with wild-type p53. Overexpression of MDM2, a critical negative regulator of p53, represents one way by which tumor cells escape p53-mediated tumor suppression (49). MDM2 inhibits p53 mainly, if not exclusively, by promoting its proteasome-dependent degradation through its E3 ubiquitin ligase activity (17, 19, 24, 29).

Mdm2 is overexpressed in one third of all human sarcomas (37), 10% of glioblastomas and astrocytomas (41), 40% of oral squamous cell carcinomas (30), and 53% of myeloid (4) and 28% of B-cell (56) lymphomas. In addition, high levels of MDM2 are associated with poor prognosis in patients with non-Hodgkin's lymphoma (33). It is believed that overexpression of MDM2 is, at least in part, a direct consequence of gene amplification (34). However, high MDM2 levels have also been seen in many tumors that do not harbor gene amplification, indicating that there are additional mechanisms leading to MDM2 overexpression in human tumors.

Ubiquitous overexpression of an Mdm2 transgene, under the control of its own promoter and regulatory regions, predisposes mice to spontaneous tumor formation (21). Tissue-specific Mdm2 overexpression in the basal layer of the epidermis induces hyperplasia and predisposes the epidermis to the formation of premalignant lesions and squamous cell carcinomas (11). These data establish a clear causal link between Mdm2 overexpression and increased tumor susceptibility in vivo. However, this effect is not clear-cut in all tissues: overexpression of Mdm2 in the granular layer of the epidermis does not promote tumorigenesis but rather affects its differentiation in a p53-independent manner (12). Mdm2 may therefore function as an oncogene only in a restricted number of cell types and tissues. Moreover, ubiquitous overexpression of Mdm2 also created a predisposition to sarcoma formation in mice deficient for p53, thus highlighting a clear p53-independent role for Mdm2 in tumorigenesis, the molecular mechanism of which remains to be elucidated (21).

p53 activity is also antagonized by Mdm4, which is structurally related to Mdm2 but which regulates p53 transcriptional activity rather than its stability (28). Mdm4 and Mdm2 form stable heterodimers through their respective RING finger domains (43, 48). Mdm4 binding to Mdm2 interferes with Mdm2 autoubiquitylation and subsequent degradation (46), whereas in specific conditions, such as in response to DNA damage, Mdm2 promotes Mdm4 ubiquitylation and degradation (6, 23, 38). DNA damage-induced degradation of Mdm4 is regulated by phosphorylation and is critical for effective p53-mediated radiation responses and tumor suppression in vivo (55).

A clear link between Mdm4 and p53 has also been highlighted by genetic experiments in mice. Mdm4-deficient mice die during embryonic development, a phenotype that is completely rescued on a p53-null background (32, 39). Perhaps not so surprisingly, then, an increasing body of evidence indicates that overexpression of Mdm4 could also contribute to tumorigenesis through inhibition of p53 tumor suppression function (27). Elevated levels of MDM4 are seen in about 15% of all human cancers (49). We have for instance shown that MDM4 is overexpressed in about 20% of breast carcinomas, a subset of which harbor MDM4 gene amplifications but wild-type p53 (5).
MDM4 is also overexpressed in 19% of colon and 18% of lung cancers (5), in 80% of pre-B lymphoblastic leukemias (16), and in 40% of head and neck squamous carcinomas (52). Amplification and overexpression of MDM4 were also found in 65% of retinoblastomas, all of which retain wild-type p53 (25). Further evidence of an oncogenic function of MDM4 is provided by tissue culture experiments and analysis of tumor xenografts in mice (5, 25). We have previously shown that Mdm4 transforms mouse embryonic fibroblasts (MEFs) when coexpressed with oncogenic RasV12 (5).

Nevertheless, the consequences of overexpression of wild-type Mdm4 in vivo for the functionality of the p53 pathway in response to DNA damage remain to be investigated. It also remains to be clearly established whether overexpression of Mdm4 is sufficient to induce the formation of a broad range of tumor types in vivo. By analogy with previous reports for Mdm2, it is of interest to establish whether, upon its overexpression, Mdm4 also possesses functions that are p53 independent in vivo. In order to explore these possibilities, we generated the first Mdm4 transgenic mouse model using a sophisticated approach previously described by us (36). This approach allows efficient generation of conditional transgenic mice by targeting a single copy of the transgene into the ROSA26 locus. To further dissect the oncogenic properties of Mdm2 in vivo, we also used the same approach to generate a novel Mdm2 mouse model. We show that although the levels of overexpression of both transgenes are comparable, exogenous protein expression is detected only in Mdm4 transgenic mice. Contrary to expectations, these mice are not prone to spontaneous or radiation-induced tumor formation. Neither does the increased Mdm4 expression accelerate Myc-induced B-cell lymphomagenesis. In addition, expression of exogenous Mdm4 from two transgenic alleles leads to a further 2-fold increase in expression that is incompatible with life. These Mdm4 overexpressing mice die during embryonic development owing to severe vascular defects. Importantly, this lethality is not rescued on a p53-deficient background, indicating that at high levels Mdm4 exhibits p53-independent activities.

MATERIALS AND METHODS

Generation of Mdm2 and Mdm4 conditional transgenic mice. The Mdm2 and Mdm4 conditional transgenic mice were generated using the Gateway-compatible ROSA26 locus targeting vector as previously described (36). PCR-based screening of targeted ROSA26 ES cell clones was performed using external primer d, 5'-TAGTGAAGGATGGGAGAGGC-3' and the internal primer e, 5'-GGGCATCGACTTCAAGGAGGACGG-3', to generate a 1.5-kb fragment (Fig. 1A and B). PCR-positive clones were confirmed by Southern blotting for 5' integration and single-copy insertion as described previously (36). A PCR-based strategy was developed to distinguish between the floxed targeted allele and the Cre-excised allele of both Mdm2 and Mdm4 transgenes. The primers are as follows: a, 5'-TCTGATACATTACCTTGTTTGC-3'; b, 5'-CTCGCTACACCAT GTCTGAC-3'; c for Mdm2 transgene, 5'-GGCATCGATAGAAAGACAGG G-3'; c for Mdm4 transgene, 5'-GTACCGTCTGTTAGTATAC-3' (Fig. 1A). An 800-bp fragment indicates the presence of the Cre-excised allele, whereas a 2.5-kb fragment is amplified from the floxed targeted allele.

Mice and PCR genotyping. To distinguish between the wild-type and the targeted ROSA26 alleles, for both Mdm2 and Mdm4 transgenes, we used the following primers: Fwd 5' arm ROSA26, 5'-AAAGTGCCTCGTGAAGGTGATA- T3'; Rev 3' arm ROSA26, 5'-AAAGTGCCTCGTGAAGGTGATA- C-3'; and Rev eGFP, 5'-GGCATCGACCTTGAAGGAGGAGG-3'. To detect the presence of the Cre recombinase (Sos2-Cre and Nestin-Cre), the following primers were designed: Fwd, 5'-CCTGGAAGAAACTCGTTGCTTCC-3' and Rev, 5'-CAGG TGTTTAAAGACATCC-3'. Genotyping of the Mdm2 floxed, p53-null, Mdm2-null, Mdm4-null, and Eμ-myc alleles was done as previously described (1, 7, 14, 32, 35). MdmT17+ mice were bred with Eμ-myc transgenic mice, and their offspring (all in a 129/Sv-C57BL/6 mixed background) were monitored once a week for signs of morbidity and tumor development.

Histology and IHC. Embryos were fixed overnight in 4% paraformaldehyde, dehydrated, paraffin embedded, sectioned (6 μm) and stained with hematoxylin and eosin (H&E). For immunohistochemistry (IHC), slides were stained with antibodies against Nestin (mouse, clone rat 401, 1:1,000; Becton Dickinson), p53 (mouse, clone 1C12, 1:3,000; Cell Signaling), CD31 (PECAM-1) (rat, clone MECI3.13, 1:50; Becton Dickinson), and the cleaved form of caspase-3 (rabbit, 1:1,000; Cell Signaling). Detection was performed with the Envision kit (Dako) according to the manufacturer's instructions except for the CD31 IHC, where the TSA biotin system (PerkinElmer) was used by following the manufacturer's protocol. Sections were counterstained with hematoxylin.

Western blotting analysis. Mouse embryonic fibroblasts (MEFs), B220- cells, and adult tissue samples were dissolved in Giordano buffer (50 mM Tris-HCl, pH 7.4, 250 mM NaCl, 0.1% Triton X-100, 5 mM EDTA) containing phosphatase and protease inhibitors (Sigma). The adult tissue samples were sonicated three times for 10 s each. The protein concentration (expressed as optical density at 595 nm [OD595]) was determined by Bradford assay, and 30 μg of each sample was fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Amer sham). The primary antibodies used were anti-myc (mouse, clone 9E10, 1:1,000; Abcam), anti-Mdm4 (mouse, clone MX-82, 1:650; Sigma), anti-Mdm2 (mouse, clone 2A10, 1:10; mixed with mouse, clone 4B2, 1:10,000), antivinculin (mouse, clone IVN-1, 1:10,000; Sigma), anti-green flou rescent protein (anti-GFP) (American Type Culture Collection, 1:100,000; Clontech), anti-β- tubulin (rabbit, 1:10,000; Sigma), anti-CD45R (B220) (rat, clone RA3-682, 1:100; Santa Cruz Biotechnology), anti-phospho-histone H2AX (yH2AX) (rabbit, 1:1,000; Cell Signaling), and anti-p53 (mouse, clone 1C12, 1:800; Cell Signaling). The secondary antibodies used were anti-rabbit antibody–horseradish peroxidase (HRP) (donkey polyclonal, 1:10,000; Amersham), anti-mouse antibody–HRP (sheep, 1:10,000; Amersham), and anti-rat antibody–HRP (goat, 1:10,000; Amersham). For the detection, ECL Western blotting detection reagents (Amersham) or SuperSignal West Femto maximum sensitivity substrate (Thermo Scientific) was used.

Ta qMan real-time quantitative RT-PCR assays. Total RNA was extracted from MEFs or adult tissues using the RNeasy minikit (Qiagen) according to the manufacturer's protocol. One microgram of total RNA of each sample was reverse transcribed with a high-capacity cDNA archive kit (Applied Biosystems). Quantitative reverse transcriptase PCR (RT-qPCR) assays were performed by following the manufacturer's instructions (Applied Biosystems). Primer pairs and TaqMan probes were designed by Applied Biosystems (assays on demand).

Tissue culture assays. Mouse embryonic fibroblasts (MEFs) were prepared from embryos from embryonic day 12.5 (E12.5) and cultured as described previously (32). The procedure followed for the colony-forming assay was previously described (32). The life span of MEFs was assayed by plating 3 × 105 cells on 6-cm dishes and passaging them on a 3T3 protocol (5), p53 activity was induced as follows: MEFs were treated with a 1-h pulse of doxorubicin (0.4 μg/ml) or with Nutlin-3α (5 μM). Proteasome inhibitor MG132 (Calbiochem) was dissolved in dimethyl sulfoxide (DMSO), and the MEFs were treated with 20 μM MG132 for 6 h prior to harvesting the cells.

Magnetic sorting of B cells, flow cytometry, and Caspase-Glo assay. Spleen and bone marrow were harvested from 6- to 7-week-old mice. Single-cell suspensions were obtained by pressing the spleen and the bone marrow through nylon cell strainers followed by hypotonic lysis of red blood cells. To isolate B cells, we incubated single-cell suspensions from bone marrow or spleen with B220 MicroBeads (Miltenyi Biotech) and enriched them by magnetic cell sorting (MACS) according to the manufacturer's instructions (Miltenyi Biotech). We measured the proliferation rates of B cells from spleen and bone marrow using a BrdU flow kit (BD Pharmingen) as described by the manufacturer. Briefly, animals were injected intraperitoneally with 5-bromodeoxyuridine (BrdU) in sterile phosphate-buffered saline (PBS) (80 μg per g body weight), and spleen and bone marrow were harvested 1 h later. We incubated 106 B220+ cells, isolated by MACS, with antibodies against B220 (fluorescein isothiocyanate [FITC] conjugated) and BrdU (al phosphoeycuan conjugated). All samples were analyzed by FACSCalibur (Becton Dickinson). To determine the level of apoptosis, the B cells from bone marrow were used the Caspase-Glo 3/7 luminescence assay (Promega). B220+ cells (1.75 × 105), isolated by MACS, were resuspended in 70 μl of RPMI 1640 medium supplemented with 10% fetal calf serum. Then, 70 μl of Caspase-Glo 3/7 reagent was added to each sample and the measurement of luminescence was performed 2 h later with a Glomax luminometer (Promega) according to the manufacturer's protocol.
RESULTS

Generation of Mdm2 and Mdm4 conditional transgenic mouse lines. We generated Mdm2 and Mdm4 conditional transgenic mouse lines using a previously described approach (36). In brief, to avoid positional effects and allow reliable and comparable expression levels, we targeted a single copy of both transgenes into the ROSA26 locus (Fig. 1A). Expression of the transgenes is under the control of the artificial pCAGG promoter to ensure robust and ubiquitous expression. To allow spatiotemporal control of transgene expression, a floxed β-geo STOP element was placed downstream of the promoter. A myc tag and an IRES-eGFP element were inserted at the 5' and 3' ends of both transgenes, respectively, for comparison of their expression levels and GFP tagging of the transgenic cells. ROSA26 locus targeting was initially screened using a PCR-based strategy to identify 5' targeted clones (primers d and e; Fig. 1A and B). The positive clones were verified by Southern blot analysis using a 5' external probe and an internal probe complementary to the eGFP sequence to confirm 5' integration and single-copy insertion (Fig. 1C). Correctly targeted clones harboring the Mdm4 and Mdm2 transgenes were used to generate one conditional transgenic line for each transgene.

Exogenous Mdm4 protein, but not Mdm2, is highly expressed in vivo. We first intercrossed the Mdm2 and Mdm4 conditional transgenic mice with a general deleter Sox2-Cre strain (18). Sox2-CreT/+; ROSA26-pCAGG-Mdm4T/+ (hereinafter called Mdm4T/+) and Sox2-CreT/+; ROSA26-pCAGG-Mdm2T/+ mice (hereinafter called Mdm2T/+) were viable and fertile. Complete excision of the β-geo STOP element was observed in all organs analyzed by a genomic PCR-based approach (primers c and b in Fig. 1A) (Fig. 2A). Conveniently, both Mdm4T/+ and Mdm2T/+ mice displayed green fluorescence in the toes, for instance, making them easily distinguishable from Cre-negative mice (Fig. 2B). A robust and comparable increase in Mdm4 and Mdm2 expression at the

FIG. 1. Generation of conditional ROSA26-pCAGG-Mdm2/Mdm4 mice. (A) Schematic representation of the wild-type and targeted ROSA26 alleles. Before Cre-mediated excision the pCAGG promoter drives the expression of the β-geo STOP cassette only. Upon Cre expression the floxed β-geo STOP cassette is excised and Myc-Mdm2/4-IRES-eGFP is transcribed. Exons are indicated by black boxes. Arrowheads represent loxP sites. (B) PCR analysis of correctly targeted ROSA26-pCAGG-Mdm2/Mdm4 ES cell clones using primers d and e. (C) Southern blot analysis of the positive ROSA26-pCAGG-Mdm2 and ROSA26-pCAGG-Mdm4 ES cell clones with external (left) and internal (right) probes.
FIG. 2. Characterization of the Sox2-Cre;ROSA26-pCAGG-Mdm2/Mdm4T/H11001 mice. (A) PCR analysis of genomic DNA isolated from control non-Cre transgenic (Cre −) tail detecting presence of the floxed nonrecombined allele (primers a and b) but not the excised allele (primers c and b). In contrast, efficient Cre-mediated recombination is detected in the samples isolated from Sox2-Cre;Mdm2T/H11001/Mdm4T/H11001 (Cre +) mice showing only the excised allele and not the floxed allele. The three primers a, b, and c are indicated in Fig. 1A. B, brain; E, eye; S, skin; Th, thymus; Sp, spleen; t, tail. (B) Green fluorescence is detected in the toes of both Sox2-Cre;Mdm2T/H11001/Mdm4T/H11001 (Cre +) mice. (C and D) The relative values are set to zero to accommodate the lack of eGFP- and myc-tagged Mdm4 or myc-Mdm2 in the absence of cre-mediated deletion of the floxed β-geo STOP cassette. (C) RT-qPCR analysis of eGFP and myc-Mdm2 or myc-Mdm4 mRNA expression levels in parental non-Cre-excised (Cre −) ES clones compared to enhanced levels of Cre-excised (Cre +) pCAGG-promoter-driven samples. (D) RT-qPCR analysis of pCAGG-driven transgene expression in selected organs upon Cre-mediated recombination. Robust and comparable increases in Mdm4 and Mdm2 expression are observed at the transcriptional level in all organs analyzed. The data represent the means (± standard deviation [SD]) of results of two independent experiments. Bars: T, Sox2-Cre; ROSA26-pCAGG-Mdm2/Mdm4T/H11001; WT, wild type. (E and F) Endogenous and Myc-tagged exogenous Mdm4/Mdm2 proteins are detected by Western blot analysis in total extracts from different organs isolated from wild-type (WT [in panel E]; W [in panel F]) or Sox2-Cre; Mdm2T/H11001/Mdm4T/H11001 (T) mice. Vinculin (vinc.) was used as a loading control. H, heart; other tissue abbreviations are as for panel A. * indicates tissues in WT mice in which endogenous Mdm4 is not detectable. Fold inc., fold increase of exogenous Mdm4/Mdm2 compared to endogenous expression levels.
to only marginally elevated in others (i.e., eye and skin) (Fig. 2F).

Transgene mRNA and protein expression levels were also analyzed in primary fibroblasts (MEFs) derived from the Sox2-Cre-positive transgenic mice. RT-qPCR analysis confirmed comparable increases in Mdm4 and Mdm2 expression at the transcriptional level. Moreover, expression levels were correlated with the number of transgenic alleles present (Fig. 3A). Exogenous Mdm2 protein was detected only in the presence of the proteasome inhibitor MG132, suggesting that Mdm2 transgenic protein is extremely unstable and rapidly undergoes constitutive proteasome-dependent degradation in vivo (Fig. 3B).

**Elevated Mdm4 levels do not compensate for Mdm2 loss.**

Mice deficient for Mdm4 die at around E10.5 due to ectopic activation of p53 resulting in cell proliferation defects and activation of apoptotic cell death (9, 32, 39). To ascertain the functionality of the Mdm4 transgene, Mdm4<sup>T/T</sup>; Mdm2<sup>T/T</sup> mice were intercrossed and offspring were genotyped. Viable Mdm4<sup>T/T</sup>; Mdm2<sup>T/T</sup> mice were found at the expected Mendelian ratio (Table 1). We therefore concluded that the Mdm4 transgene is functional as it rescues the Mdm4-null lethality.

It has been reported that overexpression of Mdm2 rescues the embryonic lethality associated with Mdm4 deficiency, indicating that high levels of Mdm2 compensate for Mdm4 loss (47). We therefore tested whether overexpression of Mdm4 can compensate for Mdm2 loss. We first attempted to rescue germ line Mdm2 loss, which results in embryonic lethality at around E3.5 to E5.5 (22, 35). Mdm2<sup>Lox/Lox</sup>; Mdm4<sup>T/T</sup> mice were intercrossed. No viable Mdm2<sup>Lox/Lox</sup>; Mdm4<sup>T/T</sup> animals were found, suggesting that Mdm4 overexpression cannot compensate for complete Mdm2 loss (data not shown).

We next tested whether elevated Mdm4 could compensate for tissue-specific Mdm2 loss. Conditional loss of Mdm2 in neuronal progenitors leads to p53 stabilization and activation of function resulting in widespread apoptosis throughout the neuroepithelium (10, 57). We intercrossed the ROSA26-pCAGG-Mdm4<sup>T/T</sup> transgenic mice with the Nestin-Cre mice (50) and mice harboring the previously described Mdm2 conditional allele (14). As observed in the Nestin-Cre; Mdm4<sup>Lox/Lox</sup> embryos, the neuroepithelia of Nestin-Cre; Mdm2<sup>Lox/Lox</sup>; Mdm4<sup>T/T</sup> or Nestin-Cre; Mdm2<sup>Lox/Lox</sup>; Mdm4<sup>T/T</sup> E12.5 embryos were severely disrupted due to activation of apoptosis (as measured by IHC with an antibody specific to the cleaved form

### Table 1. The Mdm4 transgene rescues the Mdm4-null lethality

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Mdm4 type</th>
<th>No. of pups obtained</th>
<th>No. of pups expected</th>
<th>Ratio expected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>+/+</td>
<td>16</td>
<td>7.125</td>
<td>6.25</td>
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<tr>
<td>WT</td>
<td>+/-</td>
<td>16</td>
<td>14.25</td>
<td>12.5</td>
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<tr>
<td>WT</td>
<td>+/-</td>
<td>0</td>
<td>7.125</td>
<td>6.25</td>
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<tr>
<td>Mdm4&lt;sup&gt;T/T&lt;/sup&gt;</td>
<td>+/-</td>
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<td>14.25</td>
<td>12.5</td>
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<td>25</td>
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<tr>
<td>Mdm4&lt;sup&gt;T/T&lt;/sup&gt;</td>
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<tr>
<td>Mdm4&lt;sup&gt;T/T&lt;/sup&gt;</td>
<td>+/-</td>
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<tr>
<td>Mdm4&lt;sup&gt;T/T&lt;/sup&gt;</td>
<td>+/-</td>
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<td>+/-</td>
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| Total     | 114       | 114                  | 100                  | 100               |

*a Mdm4<sup>T/T</sup>; Mdm4<sup>T/T</sup> mice intercrossed.
*b WT, wild type.
of caspase-3*) (Fig. 4A and B). Levels of p53 accumulation in the neuroepithelia of embryos of both of these genotypes were comparable (Fig. 4A and B). In contrast, p53 expression and caspase-3 activation were not detected in the intact neuroepithelia of control embryos (Fig. 4B). The presence of one (T/+ or two (T/T) alleles of the Mdm4 transgene does not rescue p53 stabilization and caspase-3 activation upon Mdm2 inactivation in the central nervous system. (B) Histogram of p53- and cleaved caspase-3 (Casp-3*)-immunoreactive cells as the percentage of Nestin-positive cells analyzed per field. Data represent the means (± SD) of the analyses of five different fields from two different sections from three different embryos per genotype. (C) PCR amplification of genomic DNA from head and spinal cord of E12.5 embryos, allowing discrimination between the floxed (primers a and b) and excised allele (primers c and b) of the Mdm4 transgene. Efficient excision of the β-geo STOP cassette is detected in the Nestin-Cre-positive (Cre+) embryos. The three primers a, b, and c are indicated in Fig. 1A. B, blank; Ctrls, PCR controls.

Homozygous Mdm4 transgenic mice die during embryogenesis. Surprisingly, intercrosses of Mdm4+/– mice produced no viable Sox2-Cre; homozygous Mdm4 transgenic animals (Mdm4TT) indicating embryonic lethality (Table 1). We therefore analyzed a panel of embryos at various stages of embryonic development (data not shown). About 60% of the Sox2-Cre; Mdm4TT embryos showed hemorrhaging as early as E12.5, indicating vascular defects (Fig. 5A). By E16.5, 90% of the Sox2-Cre; Mdm4TT embryos were either resorbing (dead; no heartbeat) or resorbed. To characterize this lethality we performed sagittal sectioning and hematoxylin and eosin staining of several E12.5 embryos. Severe hemorrhaging around the veins connected to the heart, as well as free red blood cells and edema in subcutaneous regions, was observed in Sox2-Cre; Mdm4TTembryos (Fig. 5B and data not shown). To further analyze the structure of the blood vessels, E13 embryos were sectioned transversely and immunostained with a PECAM-1 antibody. We observed distended jugular veins (Fig. 5C) as well as defects in the mural cell coverage of the aorta in Mdm4TT embryos (Fig. 5D). Together, these observations indicate that the Sox2-Cre; Mdm4TT embryos have structurally abnormal vessels, a defect that likely causes the observed embryonic lethality.

We next addressed whether this embryonic lethality was dependent on the presence of functional p53. Sox2-Cre; Mdm4TT mice were mated with p53-null animals (20) to obtain Sox2-Cre; Mdm4TT;p53–/– offspring, which were subsequently intercrossed. No viable Sox2-Cre; Mdm4TT;p53–/– mice were found, indicating that loss of p53 expression does not rescue the lethality associated with a high level of Mdm4 expression (data not shown). Moreover, E13.5 Sox2-Cre; Mdm4TT;p53–/– embryos also showed hemorrhaging, indicating that the vascular defects observed in Sox2-Cre; Mdm4TT embryos are independent of p53 (Fig. 5A). These results indicate a p53-independent function of Mdm4 when ubiquitously expressed at high levels.

Exogenous Mdm4 inhibits basal, but not DNA damage-induced, p53 activity. Wild-type MEFs in culture see their proliferative potential decrease with passages and eventually undergo permanent cell cycle arrest, a phenotype known as replicative senescence. A similar phenotype can be induced by plating early-passage MEFs at very low density in a so-called colony formation assay. Both of these phenotypes are caused by activation of the p19ARF-p53 pathway (44). Accordingly, p53-deficient MEFs are immortal; they do not undergo replicative senescence in culture and form colonies when plated at low density. Similarly, retrovirus-mediated Mdm4 overexpression immortalizes MEFs (5). To test the ability of the Mdm4
transgene to interfere with p53 function, we first investigated whether Sox2-Cre-positive Mdm4 transgenic MEFs also exhibit an immortal phenotype. We observed that Sox2-Cre; Mdm4T/+, Mdm4T+/H11001 and Sox2-Cre; Mdm4T/T MEFs were able to form colonies when plated at very low density (Fig. 6A). Moreover, they were able to proliferate indefinitely in culture on a 3T3 schedule protocol while, as expected, wild-type cells senesced after 8 passages (Fig. 6B). We conclude that p53-induced permanent cell cycle arrest is compromised in Mdm4 transgenic MEFs. Interestingly, basal p53 steady-state levels were not significantly affected in cells expressing high Mdm4 levels (Fig. 6C). In contrast, its basal transcriptional activity was attenuated, as measured by a significant decrease in p21 mRNA levels in P3 and P4 Mdm4T+/H11001 MEFs (Fig. 6C).

We also assessed the ability of exogenous Mdm4 to interfere with acute DNA damage-induced stabilization and activation of p53 function. Mdm4 levels rapidly decline in an Mdm2-dependent manner following DNA damage (6, 23, 38). Accordingly, exogenous Mdm4 is degraded upon transient exposure to the DNA-damaging agent doxorubicin. In contrast, p53

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**FIG. 5.** Homozygous Mdm4 transgenic mice die during embryogenesis in a p53-independent manner. (A) External views of Sox2-Cre; Mdm4T/+, Sox2-Cre; Mdm4T+/H11001 and Sox2-Cre; Mdm4T/T; p53−/− embryos at E12.5 and E13.5. (B) Severe hemorrhage around the jugular veins is visible on H&E staining of Sox2-Cre; Mdm4T/− and Sox2-Cre; Mdm4T/T; p53−/− embryos. Magnifications are indicated. (C) PECAM-1 IHC counterstained with H&E staining on transverse sections at the neck level of E13 embryos. The jugular veins are distended in Sox2-Cre; Mdm4T/− embryos. Magnifications are indicated. (D) E13 heterozygous and homozygous Mdm4 transgenic embryos sectioned transversely at the thoracic level and immunostained with PECAM-1. The mural cell coverage of the aorta (red bracket) appears thinner in Sox2-Cre; Mdm4T/+ embryos (magnification, ×20).
protein levels increased in Mdm4 transgenic cells in a way comparable to that in wild-type MEFs (Fig. 6D). In order to assess activation of p53 function, we measured the induction of expression of a selected set of endogenous p53 target genes by RT-qPCR. Data for p21 and NoxA are shown in the right panel of Fig. 6D. Importantly, the induction in wild-type cells was comparable to that in Mdm4 transgenic cells. However, consistent with the data presented in Fig. 6C, the basal level of
expression was significantly reduced in Mdm4T/+ MEFs. Together these data indicate that increased levels of the Mdm4 transgenic protein interfere with basal p53 transcriptional activity but not with p53 stabilization and activation of function in response to acute DNA damage.

We also assessed the consequences of Mdm4 overexpression on the p53 response to treatment with Nutlin-3, a small molecule inhibitor of the p53-Mdm2 interaction (53). Nutlin-3 also binds to Mdm4 but with a 40-fold-lower affinity than Mdm2 (25). In contrast to the DNA damage treatment, exposure to Nutlin-3 did not affect transgenic Mdm4 protein levels. Interestingly, p53 accumulation was severely attenuated in Mdm4T/+ MEFs compared to that in wild-type cells (Fig. 6E). Accordingly, induction of different p53 target genes after Nutlin-3 treatment was significantly compromised in Mdm4T/+ cells compared to that in control MEFs (Fig. 6E, right panel). Together, these data indicate that high levels of exogenous Mdm4 interfere with Nutlin-3-induced p53 activation.

**Mdm4-overexpressing mice are not tumor prone.** Unexpectedly, none of the mice ubiquitously overexpressing exogenous Mdm4 (Sox2Cre; Mdm4T/+; n = 20) analyzed developed spontaneous tumors within their first year of life (Fig. 7A). Several of these mice (n = 8) are now more than 2 years old and tumor free. Moreover, the Sox2Cre; Mdm4T/+ mice also did not show any significantly increased susceptibility to whole-body (4 Gy) radiation-induced lymphomagenesis (data not shown).

To test whether Mdm4 overexpression can cooperate with an activated oncogene to induce tumorigenesis in vivo, Sox2Cre; Mdm4T/+ mice were intercrossed with Eu-myc transgenic mice, which overexpress the c-Myc oncogene specifically in the
B-cell lineage and consequently develop spontaneous B-cell lymphoma (1). In contrast to mice with a compromised p53 pathway (42), Mdm4-overexpressing mice did not exhibit accelerated Myc-induced B-cell lymphomagenesis. There was indeed no statistically significant difference in the mean disease-free survival between Eμ-myc and Eμ-myc; Mdm4T+/− mice (Fig. 7A) (P = 0.474). Accordingly, the homeostasis of pre tumoral B cells, including Myc-induced proliferation (Fig. 7B) and apoptosis (Fig. 7C), was not affected in Mdm4 transgenic mice.

It has been previously reported that Myc overexpression causes severe DNA damage in Eμ-myc mice (8, 51). Consequently, p53 function is activated by the classical oncogene-induced p19ARF-dependent mechanism but also by an ATM-dependent pathway. Accordingly, we detected significant induction of phosphorylated γH2AX, a target of ATM, in B220+ splenic cells of 7-week-old Eμ-myc and Eμ-myc; Mdm4T+/− mice (Fig. 7D). As Mdm4 stability decreases in response to acute DNA damage, significant degradation of exogenous Mdm4 protein may account for the lack of cooperativity between Myc and Mdm4 to accelerate lymphomagenesis. However, very high levels of Mdm4 were still detected in Eu- mucy; Mdm4T+/− B220+ cells, indicating that the extent of DNA damage is not sufficient to abolish exogenous Mdm4 expression in these cells (Fig. 7D).

Myc-induced lymphomagenesis proceeds, in part, through inactivation of the ARF-Mdm2-p53 pathway (8). We analyzed Eu-myc; Mdm4T+/− lymphomas by Western blotting to determine the frequency of alterations at the p19ARF-, Mdm2-, and p53-encoding loci (Fig. 7E). Similar alterations were observed in Eμ-myc and Eμ-myc; Mdm4T+/− mice, indicating that high Mdm4 levels do not alleviate the need for inactivation of the p53 pathway on this specific background.

Collectively, these unexpected results indicate that widespread overexpression of Mdm4 alone is insufficient to accelerate tumor formation in vivo.

**DISCUSSION**

One of the main objectives of this study was to explore and compare the oncogenic properties of full-length Mdm2 and Mdm4 in an in vivo setting. To this end, we generated novel Mdm2 and Mdm4 conditional transgenic mouse lines expressing comparable levels of both transgenes. Although both proteins were transcribed at comparable levels, widespread protein overexpression was observed only for Mdm4. In contrast, Mdm2 protein levels were only marginally elevated and only in some tissues in the Mdm2 transgenic mice. These results indicate that in most, if not all, normal tissues there exist mechanisms that prevent accumulation of the Mdm2 protein. We provide evidence that these mechanisms involve the ubiquitin-proteasome system (UPS). Our data confirm that in vivo mechanisms of regulating Mdm2 and Mdm4 protein levels are different. They also raise an important mechanistic question regarding MDM2 protein accumulation in human tumors. It has been proposed that MDM2 is overexpressed in some human cancers as the result of gene amplification (34). Our data, however, indicate that increased MDM2 transcription is per se not sufficient for elevating MDM2 protein levels in normal, nontransformed cells. These data therefore indicate that in addition to increased MDM2 copy number, mechanisms that disrupt proteasome-dependent degradation of MDM2 must be selected for during the process of tumorigenesis.

It has previously been shown that the Mdm4-null embryonic lethality is completely rescued by expression of an Mdm2 transgene (47). In contrast, we now show that elevated Mdm4 does not rescue lethality of the Mdm2-null embryos. Consistent with previous genetic studies indicating that Mdm4 does not contribute to the regulation of p53 stability independently of Mdm2 (reviewed in reference 28), Mdm4 overexpression did not prevent p53 stabilization in Mdm2-deficient neuronal cells. Neither did high Mdm4 levels impede induction of p53-dependent apoptosis in these cells, indicating that Mdm4-dependent inhibition of p53 function is at least to some extent dependent on the presence of Mdm2. Mdm2 may, for instance, be required for correct subcellular localization of Mdm4. We and others have shown that MDM2 overexpression facilitates the recruitment of exogenous MDM4 to the nucleus and consequently significantly enhances the ability of MDM4 to inhibit p53 (15, 31). We also show herein that, even at high levels, Mdm4 efficiently interferes only with basal—not DNA damage-induced—p53 transcriptional activity. Collectively, these data indicate that Mdm4’s ability to counteract p53 function requires the presence of Mdm2 and/or only operates in low/basal stress conditions.

Unexpectedly, ubiquitous expression of Mdm4 from two copies of the transgene led to embryonic death at around midgestation, a phenotype likely caused by severe vessel maturation defects. Importantly, this phenotype is not rescued on a p53-null background. Although we cannot formally exclude the possibility that the presence of the Myc tag at the N-terminal part of Mdm4 might contribute to the vascular phenotype, these observations raise the possibility that high Mdm4 levels affect the functionality of a pathway(s), other than the p53 pathway, that controls blood vessel maturation during embryonic development. As Mdm4 is often found at very high levels in human cancers, this observation raises a possibility that MDM4’s contribution to cancer formation and development may not depend only on its ability to inhibit p53 tumor suppression function. The Mdm4 transgenic mouse model will be a valuable tool to further explore the nature of the p53-independent pathway(s) regulated by Mdm4.

Another unexpected result was the finding that widespread overexpression of Mdm4 did not predispose mice to spontaneous, radiation-induced, or oncogene-induced carcinogenesis. A number of possibilities may account for this surprising observation. One potential possibility is that the myc tag in some way affects Mdm4 oncogenic properties. However, we showed that expression of the tagged Mdm4 protein rescues death of the Mdm4-null embryos, indicating that this exogenous, tagged protein is fully functional in vivo. Moreover, we show that this tagged Mdm4 inhibits p53 function in MEFs, as expected. An alternative explanation is that overexpression of Mdm4 alone is not sufficient to promote tumorigenesis in vivo and that the formation of Mdm4/Mdm2 heterodimers might be required to unleash Mdm4 oncogenic activities. However, the observations that high levels of MDM2 or MDM4 gene amplification are mutually exclusive in human gliomas (2) and that many tumors overexpress MDM4 but not MDM2 indicate that...
high MDM2 is, at least in some cases, dispensable for MDM4 oncogenic function.

MDM4 stability is regulated by phosphorylation and ubiqui-
tylation/deubiquitylation events (54). These and other modifi-
cations, such as sumoylation for instance, could also affect MDM4 subcellular localization or its ability to bind p53 and/or other cofactors influencing its oncogenic function.

MDM4 may function as an oncogene only in a restricted number of tissues and cell types. In some cancers, such as retinoblastoma, overexpression of MDM4 is found in the vast majority of cases while in other tumor types, such as prostate cancer, it has never been reported (49). Although this apparent tissue preference can be correlated with the frequency of p53 mutations, it could also be influenced by a tissue-specific presence of essential MDM4 oncogenic cofactors. Analogously, MDM4 may cooperate with only a limited subset of oncogenes. We show that high levels of MDM4 do not cooperate with Myc in the transformation of B cells in vivo. However, Myc is expressed at supraphysiological levels in the Eμ-Myc Burkitt lymphoma model used in this study, and such high levels of oncogene expression lead to robust activation of the DNA damage response in the preneoplastic cells (13), which in turn could interfere with Mdm4 oncogenic activities. Consistently, we show herein that overexpression of MDM4 does not affect p53 stabilization and functional activation in MEFs in response to genotoxic stress. DNA damage is known to activate several mechanisms that interfere with Mdm4-mediated p53 inhibition. p53 is a target of phosphorylation events at its N terminus, and these events have been shown to interfere with the ability of Mdm2, and therefore presumably Mdm4, to bind to p53 (45). Moreover, Mdm4 itself is phosphorylated by DNA damage-activated kinases at conserved C-terminal serines (Ser341, Ser367, Ser402). The physiological relevance of these modifications has recently been demonstrated genetically (55). Mdm4 mutant mice (Mdm4<sup>S341A/S367A</sup>) in which these three conserved serines were replaced by alanines were resistant to "impaired, such as for instance on an ataxia telangiectasia mutated (Atm)-deficient background (3)."

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