Oncogene (2015) **34,** 2943–2948

www.nature.com/onc

SHORT COMMUNICATION

Mice engineered for an obligatory Mdm4 exon skipping express higher levels of the Mdm4-S isoform but exhibit increased p53 activity

B Bardot[1,2,3], R Bouarich-Bourimi[1,2,3,5], J Leemput[1,2,3,5], V Lejour[1,2,3], A Hamon[1,2,3], L Plancke[1,2,3], AG Jochemsen[4], I Simeonova[1,2,3], M Fang[1,2,3] and F Toledo[1,2,3]

Mdm4, a protein related to the ubiquitin-ligase Mdm2, is an essential inhibitor of tumor suppressor protein p53. In both human and mouse cells, the *Mdm4* gene encodes two major transcripts: one encodes the full-length oncoprotein (designated below as Mdm4-FL), whereas the other, resulting from a variant splicing that skips exon 6, encodes the shorter isoform Mdm4-S. Importantly, increased Mdm4-S mRNA levels were observed in several human cancers, and correlated with poor survival. However, the role of Mdm4-S in cancer progression remains controversial, because the Mdm4-S protein appeared to be a potent p53 inhibitor when overexpressed, but the splice variant also leads to a decrease in Mdm4-FL expression. To unambiguously determine the physiological impact of the Mdm4-S splice variant, we generated a mouse model with a targeted deletion of the *Mdm4* exon 6, thereby creating an obligatory exon skipping. The mutant allele ($Mdm4^{\Delta E6}$) prevented the expression of Mdm4-FL, but also led to increased Mdm4-S mRNA levels. Mice homozygous for this allele died during embryonic development, but were rescued by a concomitant p53 deficiency. Furthermore in a hypomorphic $p53^{\Delta P/\Delta P}$ context, the $Mdm4^{\Delta E6}$ allele led to p53 activation and delayed the growth of oncogene-induced tumors. We next determined the effect of $Mdm4^{+/\Delta E6}$ heterozygosity in a hypermorphic $p53^{+/\Delta 31}$ genetic background, recently shown to be extremely sensitive to Mdm4 activity. $Mdm4^{+/\Delta E6}$ $p53^{+/\Delta 31}$ pups were born, but suffered from aplastic anemia and died before weaning, again indicating an increased p53 activity. Our results demonstrate that the main effect of a skipping of *Mdm4* exon 6 is not the synthesis of the Mdm4-S protein, but rather a decrease in Mdm4-FL expression. These and other data suggest that increased Mdm4-S mRNA levels might correlate with more aggressive cancers without encoding significant amounts of a potential oncoprotein. Hypotheses that may account for this apparent paradox are discussed.

Oncogene (2015) **34,** 2943–2948; doi:10.1038/onc.2014.230; published online 4 August 2014

INTRODUCTION

The transcription factor p53 has a critical role in maintaining genomic stability and preventing tumor development. Mutations in *TP53*, the gene encoding p53, are found in about 50% of human cancers, and at least 20% of the remaining tumors present an overexpression or amplification of *MDM2* or *MDM4* (the latter also known as *MDMX* or *HDMX*), the major negative regulators of p53.[1,2] In recent years, MDM4 emerged as a promising target in anti-cancer therapeutic strategies.[3–8] Thus, understanding the mechanisms that control MDM4 function is of major clinical importance.

The *MDM4* gene may encode several isoforms, due to alternative mRNA splicing.[9,10] Among these, two transcripts are most abundant: one encodes the full-length protein (MDM4-FL), and the other (an evolutionary conserved splicing variant resulting from the skipping of exon 6) encodes MDM4-S (also known as HDMX-S)[11] (Supplementary Figure 1a). Importantly, although MDM4-FL is a demonstrated oncoprotein, the role of the MDM4-S variant remains controversial. MDM4-S (with an S for short) encodes only one of the four domains of MDM4-FL (that is, the p53-binding domain), followed by 26 amino acids of unrelated sequence resulting from the frameshift caused by exon skipping (Supplementary Figure 1b). High expression of the MDM4-S splice variant correlated with faster progression and poor survival in several cancers, including glioblastomas, osteosarcomas, soft-tissue sarcomas and thyroid carcinomas.[12–15] In overexpression studies, the MDM4-S protein was found to be a stronger p53 inhibitor than MDM4-FL, in part because this short protein localized into the nucleus more efficiently than its full-length counterpart,[11,16] but possibly also because it lacks an autoinhibitory sequence contained in MDM4-FL.[17] Together, these observations suggested that MDM4-S could be a potent oncoprotein. Importantly however, other studies suggested that a high expression of the MDM4-S splice variant might not lead to massive increases in MDM4-S protein levels, because the MDM4-S mRNA or its encoded protein are unstable.[18,19] According to this view, the alternative MDM4 mRNA splicing would mainly serve as a mechanism to reduce MDM4-FL expression, and would rather correlate with tumors that express a mutant p53.[15]

These conflicting hypotheses, together with the fact that the same splice variant was reported in mouse cells,[11] prompted us to

[1]Institut Curie, Centre de recherche, Genetics of Tumor Suppression (Equipe Labellisée Ligue 2014), Paris, France; [2]UPMC Univ Paris 06, Paris, France; [3]CNRS UMR 3244, Paris, France and [4]Leiden University Medical Center, Leiden, The Netherlands. Correspondence: Professor F Toledo, Genetics of Tumor Suppression, UMR 3244, Institut Curie, Centre de recherche, 26, rue d'Ulm, 75248, Cedex 05, Paris, France.
E-mail: franck.toledo@curie.fr
[5]These authors contributed equally to this work.
Received 21 January 2014; revised 15 May 2014; accepted 24 June 2014; published online 4 August 2014

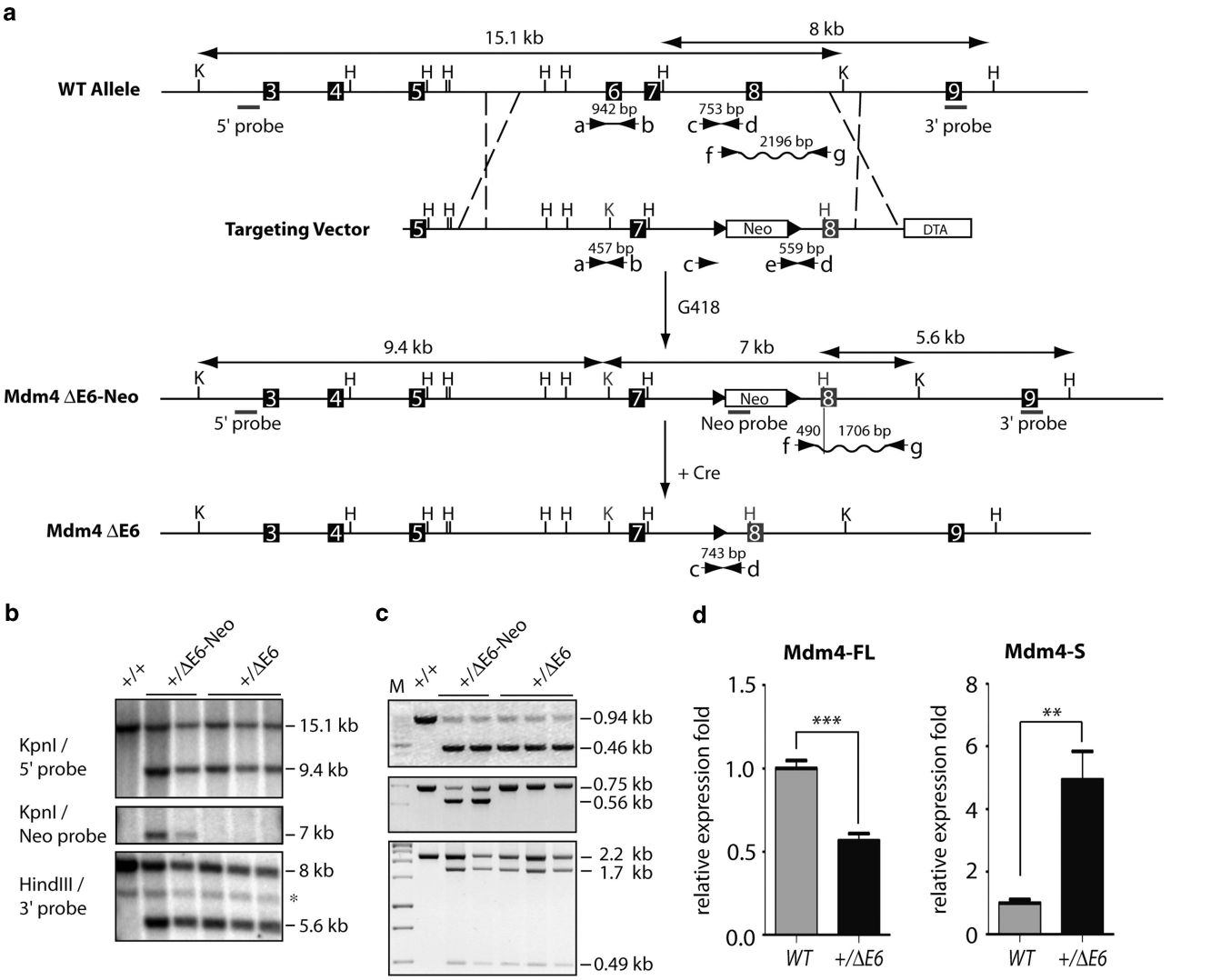


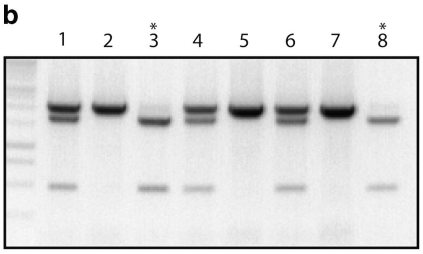
Figure 1. Generation of mice with a specific deletion of *Mdm4* exon 6. (**a**) Targeting and screening strategy for the generation of the $Mdm4^{\Delta E6}$ allele. On top, a portion of the WT allele is shown. Below, the targeting vector was generated by recombineering from the RP23-365M5 BAC clone containing the entire mouse *Mdm4* locus of C57Bl6/J origin. A loxP-flanked neomycin cassette (Neo) and a diphtheria toxin A gene (DTA) were inserted, respectively, in introns 7 and 8 for positive and negative selections. Exon 6 and its flanking conserved sequences were deleted and a KpnI site (K) was created. A single-nucleotide deletion, generating a *Hin*dIII site (H), was also created in exon 8, to prevent a putative splicing event between exons 5 and 8 to encode for a Mdm4 protein with an internal deletion. The targeting vector was sequenced, then electroporated into W4/129 mouse ES cells (Taconic), and G418-resistant clones were analyzed by Southern blot and PCR. Two independent clones were expanded and injected into C57Bl/6 blastocysts to generate $Mdm4^{+/\Delta E6\text{-}Neo}$ chimeras. Neo excision was performed by breeding $Mdm4^{+/\Delta E6\text{-}Neo}$ mice with C57Bl6/J PGK-Cre mice, and two additional backcrosses with C57Bl6/J mice were performed before phenotype analyses. (**b**) Southern blots with 5', Neo and 3' probes, performed on *Kpn*I- or *Hin*dIII-digested DNA from $Mdm4^{+/\Delta E6\text{-}Neo}$ and $Mdm4^{+/\Delta E6}$ mice confirmed the targeted recombination and correct excision of the Neomycin cassette in $Mdm4^{+/\Delta E6}$ mice. Asterisk (*) indicates a non-specific band. (**c**) Genotyping of the alleles by PCR with primer sets a and b (top); c, d and e (middle), and f and g followed by *Hin*dIII digestion (bottom). See Supplementary Table 1 for primer sequences. (**d**) $Mdm4^{+/\Delta E6}$ thymocytes exhibit decreased Mdm4-FL and increased Mdm4-S mRNA expression levels. Thymi were recovered from 6-week-old male mice of indicated genotypes, then total RNAs were extracted from thymocytes using nucleospin RNA II (Macherey-Nagel), reverse-transcribed using superscript III (Invitrogen), and real-time quantitative PCRs were performed on an ABI PRISM 7500 using Power SYBR Green (Applied Biosystems) as recently described.[35] Mdm4-S and Mdm4-FL mRNAs were quantified using real-time PCR with boundary primers: Mdm4-S transcripts with a forward primer encompassing the boundary between exons 4 and 5, and a reverse primer encompassing the boundary between exons 5 and 7, whereas Mdm4-FL transcripts were amplified with the same forward primer and a reverse primer located in exon 6 (see Supplementary Table 2 for primer sequences). Results were normalized to control mRNAs, then the amount in cells from WT mice were assigned a value of 1. Means+s.e.m. from $\geqslant 3$ experiments are shown; $^{***}P \leqslant 0.001$, $^{**}P \leqslant 001$ by Student's *t*-test.

generate a mutant mouse expressing altered Mdm4-S/Mdm4-FL mRNAs ratios, to unambiguously address the functional impact of the Mdm4-S splice variant. Mouse models with a genetic ablation of *Mdm4* were previously used to demonstrate that Mdm4 is an essential negative regulator of p53.[20–22] More recently, the use of *Mdm4* knockin mouse mutants highlighted the importance of phosphorylation and heterodimerization in the regulation of Mdm4 activity.[23–25] However, these mutant mice could not address the role of Mdm4-S, due to the loss of both Mdm4-FL and Mdm4-S expression, or to the concomitant expression of a mutant Mdm4 protein. Here we targeted a specific deletion of the *Mdm4* exon 6, thereby mimicking an obligatory skipping of this exon to generate Mdm4-S, and determined the phenotypes of animals and cells carrying one or two copies of the mutant allele.

a

Mdm4+/ΔE6 X Mdm4+/ΔE6			
	Mdm4+/+	Mdm4+/ΔE6	Mdm4ΔE6/ΔE6
Expected	14.25	28.5	14.25
Observed	12	45	0

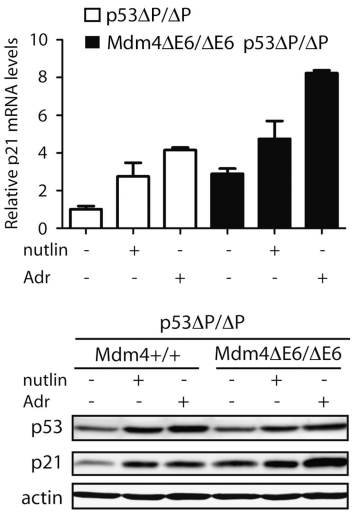
b



c

Mdm4+/ΔE6 p53+/- X Mdm4+/ΔE6 p53+/-		
	Expected	Observed
Mdm4ΔE6/ΔE6 p53-/-	1/16	3/51

d



e

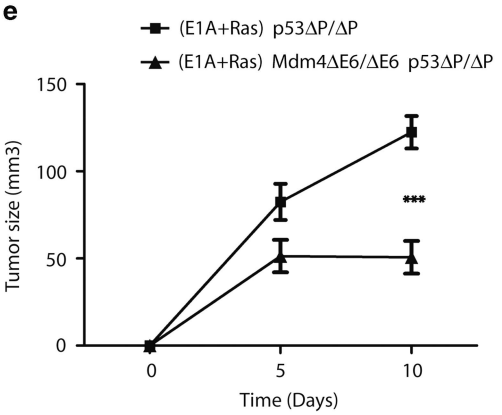


Figure 2. *Mdm4*$^{\Delta E6/\Delta E6}$ mutants exhibit increased p53 activity. (**a**) Expected and observed distributions of mice from Mdm4$^{+/\Delta E6}$ intercrosses. (**b**) PCR genotyping analyses with primers f and g followed by *Hind*III digestion (see Figure 1) on DNA extracted from E9.5 embryos (lanes 1, 2, 4–7) or fetal placentas (lanes 3 and 8) resulting from *Mdm4*$^{+/\Delta E6}$ intercrosses. Asterisks (*) indicate *Mdm4*$^{\Delta E6/\Delta E6}$ offspring, as genotyped from fetal placentas and corresponding to empty deciduas. (**c**) Frequencies of *Mdm4*$^{\Delta E6/\Delta E6}$ *p53*$^{-/-}$ mice from Mdm4$^{+/\Delta E6}$ *p53*$^{+/-}$ intercrosses, expected and observed at weaning. Importantly, RNA prepared from *Mdm4*$^{\Delta E6/\Delta E6}$ *p53*$^{-/-}$ cells were used to verify that the mutant *Mdm4*$^{\Delta E6}$ allele encodes a Mdm4-S isoform with a wild-type sequence. (**d**) *Mdm4*$^{\Delta E6/\Delta E6}$ *p53*$^{\Delta P/\Delta P}$ MEFs exhibit increased p53 activity. *p53*$^{\Delta P/\Delta P}$ and *Mdm4*$^{\Delta E6/\Delta E6}$ *p53*$^{\Delta P/\Delta P}$ MEFs were left untreated, or treated with 10 μM Nutlin 3a or 0.5 μg/ml adriamycin (Adr) for 24 h before RNA or protein extractions. Top: real-time PCR quantification of mRNAs for the p53 transcriptional target p21. Results were normalized to control mRNAs, then the amount in untreated *p53*$^{\Delta P/\Delta P}$ MEFs was assigned a value of 1. Bottom: western blot analysis. Cells were lysed in Giordano's buffer (50 mM Tris-HCl pH 7.4, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) with a cocktail of protease inhibitors (Roche) and 1 mM PMSF (Sigma). Protein concentration was determined by BCA assay (Thermo Scientific) and 50 μg of each lysate was fractionated by SDS–PAGE on a 12% polyacrylamide gel and transferred onto PDVF membrane (Amersham). The membrane was incubated with specific antibodies to detect p53 (rabbit CM5, 1/1000, Novocastra), p21 (mouse sc-6246, 1/250, Santa Cruz) and actin (actin-HRP sc47778, 1/10 000, Santa Cruz), then SuperSignal West femto detection reagent (Thermo Scientific). (**e**) The growth of oncogene-induced tumor xenografts is delayed in the *Mdm4*$^{\Delta E6/\Delta E6}$ genetic context. *p53*$^{\Delta P/\Delta P}$ and *Mdm4*$^{\Delta E6/\Delta E6}$ *p53*$^{\Delta P/\Delta P}$ MEFs were sequentially infected with pWZL-E1A12S and pBABE-Hrasv12 viruses as previously described.[3] In total, 5×10^6 E1A- and Ras- (E1A+Ras) expressing MEFs of each genotype were injected subcutaneously into the rear flank of 7-week-old female athymic nude mice and tumor volume was determined 5 and 10 days after injection. Importantly, populations of (E1A+Ras)-expressing cells were used to minimize potential differences in expression levels that could result from independant viral insertion sites. Results are from seven mice for each genotype. Error bars represent s.d. ***$P = 0.0001$ at 10 days. All experiments were performed according to IACUC regulations.

RESULTS AND DISCUSSION

We used homologous recombination in mouse embryonic stem (ES) cells to generate an *Mdm4*-mutant allele with a specific deletion of exon 6 (*Mdm4*$^{\Delta E6}$), preventing Mdm4-FL, but not Mdm4-S, expression (Figure 1a,b and c). To determine if the ratio of Mdm4-S/Mdm4-FL mRNAs is altered in the mutant mice, RNAs were isolated from the thymocytes of *Mdm4*$^{+/+}$ and *Mdm4*$^{+/\Delta E6}$ mice, and real-time quantitative PCRs with specific primer sets were performed (Figure 1d). As expected, we measured an ~50% decrease in Mdm4-FL mRNA levels in *Mdm4*$^{+/\Delta E6}$ cells compared with wild-type (WT) cells from littermate controls. Interestingly, a fivefold increase in Mdm4-S mRNA levels was observed in *Mdm4*$^{+/\Delta E6}$ cells, most likely because the exon 5 to exon 7 is an obligatory splicing event in the processing of the mutant Mdm4$^{\Delta E6}$ pre-mRNA. Assuming that in WT cells both *Mdm4* alleles generate equal amounts of each Mdm4 isoform, these results suggested that, compared with a WT allele, the *Mdm4*$^{\Delta E6}$ allele leads to the loss of Mdm4-FL, and a ninefold increase in Mdm4-S mRNA expression (Supplementary Figure 2).

The inactivation of *Mdm4* causes a p53-dependent death in murine embryos around embryonic day (E) 9.5.[20–22] If, as previously proposed,[11,13,16] the Mdm4-S transcript encodes a potent p53 inhibitor, a less severe phenotype was expected in *Mdm4*$^{\Delta E6/\Delta E6}$ mice, because the *Mdm4*$^{\Delta E6}$ allele enables Mdm4-S overexpression. *Mdm4*$^{\Delta E6/\Delta E6}$ mice might thus be viable, or at least *Mdm4*$^{\Delta E6/\Delta E6}$ embryos might be able to develop beyond E9.5. We intercrossed *Mdm4*$^{+/\Delta E6}$ mice to generate *Mdm4*$^{\Delta E6/\Delta E6}$ mice. We did not observe any homozygous mice from multiple crosses, which suggested a recessive embryonic lethal phenotype (Figure 2a). Consistent with this, genotyping analysis on DNA extracted from embryonic tissue did not reveal any homozygous embryos at E9.5. On the opposite, genotyping analysis on DNA extracted from the fetal placenta, which develops from the same blastocyst that forms the embryo, revealed *Mdm4*$^{\Delta E6}$

homozygosity in empty deciduas (Figure 2b). Furthermore, the concomitant deletion of p53 rescued the embryonic lethal phenotype of $Mdm4^{\Delta E6/\Delta E6}$ animals (Figure 2c). Together, these results indicated that two $Mdm4^{\Delta E6}$ alleles are not sufficient to keep p53 in check during mouse development.

Because Mdm4-S overexpression was observed in tumor cells, we next evaluated the impact of the $Mdm4^{\Delta E6}$ allele in response to oncogenic stress. To do so, we used $p53^{\Delta P/\Delta P}$ cells, which express an hypomorphic mutant p53 that lacks the proline-rich domain. Importantly, $p53^{\Delta P}$ is a poor suppressor of oncogene-induced tumors that can be improved by Mdm4 loss.[3] $Mdm4^{+/\Delta E6}$ $p53^{\Delta P/\Delta P}$ mice were intercrossed, and E13.5 $Mdm4^{\Delta E6/\Delta E6}$ $p53^{\Delta P/\Delta P}$ mouse embryonic fibroblasts (MEFs) were prepared: compared with $p53^{\Delta P/\Delta P}$ cells, these MEFs exhibited an increased p53 activity (Figure 2d). We next infected $p53^{\Delta P/\Delta P}$ and $Mdm4^{\Delta E6/\Delta E6}$ $p53^{\Delta P/\Delta P}$ MEFs with viruses allowing the overexpression of the E1A and Ras oncogenes, then injected the resulting E1A- and Ras-expressing (E1A+Ras) MEFs into the flanks of athymic nude mice. Ten days after infection, tumors from (E1A+Ras) $Mdm4^{\Delta E6/\Delta E6}$ $p53^{\Delta P/\Delta P}$ MEFs were significantly smaller than tumors from (E1A+Ras) $p53^{\Delta P/\Delta P}$ MEFs, indicating that the $Mdm4^{\Delta E6}$ allele led to increased p53 activity in response to oncogenic stress (Figure 2e).

Although these results do not support the hypothesis that Mdm4 exon 6 skipping leads to p53 inhibition, it remained possible that Mdm4-S required the co-expression of Mdm4-FL to promote p53 negative regulation. Thus, we next analyzed the potential regulatory role of Mdm4-S in an $Mdm4^{+/\Delta E6}$ context. Because $Mdm4^{+/\Delta E6}$ mice did not present any detectable phenotype in the absence of stress, we evaluated p53 activity in their thymus, a radiosensitive tissue that undergoes p53-dependent apoptosis upon exposure to ionizing radiation.[26] In thymocytes from unirradiated and irradiated WT or $Mdm4^{+/-}$ mice, Mdm4-S was 4–5 times less abundant than Mdm4-FL at the mRNA level, whereas it was 1.5–2.5 more abundant in $Mdm4^{+/\Delta E6}$ cells (Supplementary Figure 3a). Nevertheless, we observed little if any difference in p53 transcriptional activity in $Mdm4^{+/\Delta E6}$ cells compared with WT or $Mdm4^{+/-}$ cells (Supplementary Figure 3b) and apoptotic responses were similar in thymocytes of all genotypes (Supplementary Figure 3c). However, the sensitivity of these assays might be limited by the fact that they only evaluate short-term cellular responses to stress, and that Mdm4 may play a more modest role in p53 regulation in some adult tissues.[23]

To evaluate the physiological impact of $Mdm4^{+/\Delta E6}$ heterozygosity, we next used another *in vivo* approach, recently shown to be extremely sensitive to Mdm4 levels. Our lab recently characterized mutant mice expressing $p53^{\Delta 31}$, a p53 lacking its carboxy-terminal domain. $p53^{\Delta 31/\Delta 31}$ mice exhibited increased p53 activity, and most died shortly after birth from pancytopenia or consecutive heart failure. By contrast, $p53^{+/\Delta 31}$ mice were mildly affected, and most stayed alive for over a year. However, in $Mdm4^{+/-}$ $p53^{+/\Delta 31}$ compound heterozygotes, the decrease in Mdm4 levels caused bone marrow failure and premature death.[27] Thus, the $p53^{+/\Delta 31}$ genetic background appeared as a model of choice to evaluate the consequences of a $Mdm4^{+/\Delta E6}$ heterozygosity. $Mdm4^{+/\Delta E6}$ mice were mated with $p53^{+/\Delta 31}$ mice to generate $Mdm4^{+/\Delta E6}$ $p53^{+/\Delta 31}$ animals. We never observed any $Mdm4^{+/\Delta E6}$ $p53^{+/\Delta 31}$ mice at weaning (Figure 3a). Furthermore, the genotyping of dead or moribund pups 10–12 days after birth allowed the identification of $Mdm4^{+/\Delta E6}$ $p53^{+/\Delta 31}$ mice. In these animals, a dramatic decrease in bone marrow cellularity was a likely cause of death (Figure 3b). Interestingly, p21 levels impact on hematopoiesis,[28] and higher p21 levels were observed in $Mdm4^{+/\Delta E6}$ bone marrow cells compared with WT cells (Supplementary Figure 4). Together, these results clearly indicate that, even in the presence of Mdm4-FL, the Mdm4-S encoded by the $Mdm4^{\Delta E6}$ allele does not act as a potent p53 inhibitor.

a

Mdm4+/ΔE6 X p53+/Δ31				
	Mdm4 +/+		Mdm4+/ΔE6	
	p53+/+	p53+/Δ31	p53+/+	p53+/Δ31
Expected	16.25	16.25	16.25	16.25
Observed	31	14	20	0

b

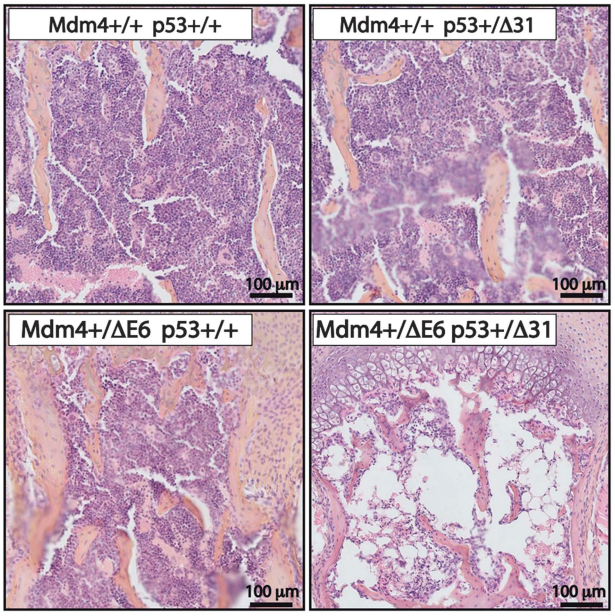


Figure 3. $Mdm4^{+/\Delta E6}$ mice are not viable on $p53^{+/\Delta 31}$ background. (**a**) Expected and observed distributions of mice from $Mdm4^{+/\Delta E6} \times p53^{+/\Delta 31}$ crosses genotyped at weaning. The genotyping of p53 alleles was performed as recently described.[27] (**b**) Comparative analysis of sternum sections from 12 days old littermates, showing a decreased cellularity in the bone marrow of the $Mdm4^{+/\Delta E6}$ $p53^{+/\Delta 31}$ double heterozygote. For three mice of each genotype, organs were fixed in formaldehyde 4% for 24 h, then ethanol 70%, and embedded in paraffin wax. Serial sections were stained with hematoxylin and eosin using standard procedures.[36]

Altogether, these experiments suggested that a $Mdm4^{\Delta E6}$ allele and a *Mdm4* knockout allele have rather similar phenotypic consequences. These results implied that the increased Mdm4-S mRNAs resulting from a $Mdm4^{\Delta E6}$-mutant allele, in both homozygotes and heterozygotes, appeared to have little if any impact on p53 regulation. This was best explained if the overexpression of Mdm4-S at the mRNA level did not lead to high levels of the Mdm4-S protein, due for example, to a nonsense-mediated decay or low translation efficiency of the Mdm4-S mRNA,[19] and/or due to an increased sensitivity to proteasomal degradation of the Mdm4-S protein.[18] In order to unambiguously identify the Mdm4-S protein, we first cloned the Mdm4-S cDNA expressed from a $Mdm4^{\Delta E6}$ allele into a pSV expression plasmid. Transfection of the pSV-MDM4-S vector into $p53^{-/-}$ MEFs resulted in overexpression of a 14-kD protein, the expected molecular mass for mouse Mdm4-S (Supplementary Figure 5a). Importantly, the co-transfection of Mdm4-S and p53 expression plasmids in these cells indicated that, when strongly overexpressed, the encoded Mdm4-S protein can interact with p53 and inhibit its transactivation capacity (Supplementary Figure 5b and c). We next immunoprecipitated endogenous Mdm4 proteins from the thymocytes of WT and $Mdm4^{+/\Delta E6}$ littermate animals. As expected, we detected a decrease in Mdm4-FL protein levels in $Mdm4^{+/\Delta E6}$ cells compared with WT cells (Figure 4a and Supplementary

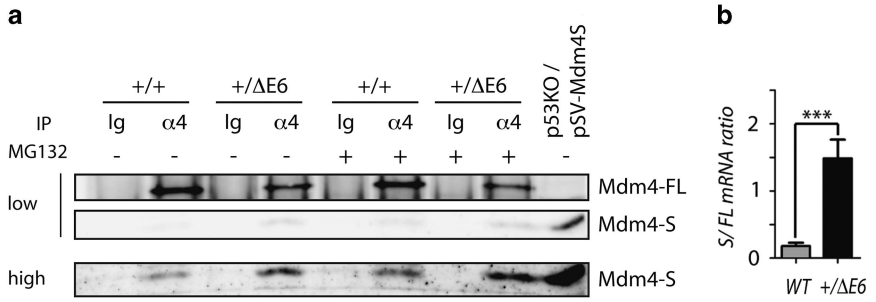


Figure 4. Mdm4-S protein levels are increased in *Mdm4*$^{+/\Delta E6}$ cells, but remain low compared with Mdm4-FL protein levels. (**a**) Thymi, recovered from 6-week-old mice, were filtered through a 70-μm filter in DMEM Glutamax (Gibco) completed with 50% fetal bovine serum (PAN Biotech), 0.01 mM non-essential amino acids, 100 μM 2-mercapto-ethanol and penicillin/streptomycin (Gibco). Thymocytes were incubated for 2 h at 37 °C with or without 10 μM of MG132, then lysed in Giordano's buffer. Whole-cell extracts were sonicated three times for 10 s and centrifuged at 13 000 r.p.m. for 30 min to remove cell debris. Immunoprecipitations were performed by incubating 900 μg of pre-cleared whole-cell extracts with a rabbit polyclonal anti-Mdm4 antibody mixture (α4: p55+1327+1328) under gentle rocking ON at 4 °C. Antibody p55 was previously decribed.[37] The 1327 and 1328 antibodies were raised against bacterially produced human Mdm4-S protein (de Graaf and Jochemsen, unpublished results). Rabbit immunoglobulins (Ig) were used for control immunoprecipitation (see also Supplementary Figure 6 for input controls in this experiment). After incubation with protein A/G Ultralink resin (Thermo Scientific), immunoprecipitates were washed seven times with Giordano's buffer, resuspended in 40 μl of 2× Laemmli sample buffer, boiled, resolved by SDS–PAGE on a 12% gel, and transferred onto a PVDF membrane. On the right, extracts from *p53*$^{-/-}$ (p53 knockout (KO)) fibroblasts transfected with the pSV-MDM4-S expression plasmid (see Supplementary Figure 5a) were loaded as a control. Mdm4 proteins were detected with Mouse MX-82 antibody (1/250, Sigma) and SuperSignal West femto detection reagent (Thermo Scientific). Low or high exposures are shown. (**b**) Total RNAs were extracted from thymocytes, and Mdm4-S and Mdm4-FL mRNAs were quantified by real-time PCR as previously described,[38] by using boundary-spanning primers and comparing expression levels with a standard curve created by serial dilution of a plasmid containing both alternative transcripts (plasmid sequence available upon request). Means+s.e.m. from ⩾3 experiments are shown; ***$P \leqslant 0.001$ by Student's *t*-test.

Figure 6). Importantly, we detected a faint 14-kD band in WT thymocytes, expressed at a very low level compared with the Mdm4-FL protein. This 14-kD band was stronger in *Mdm4*$^{+/\Delta E6}$ thymocytes, again indicating that it corresponds to the endogenous Mdm4-S protein. Importantly, although Mdm4-S was more abundant than Mdm4-FL at the mRNA level in *Mdm4*$^{+/\Delta E6}$ thymocytes (Figure 4b), Mdm4-FL was much more abundant than Mdm4-S at the protein level (Figure 4a), consistent with notions of decreased translation of the Mdm4-S mRNA and/or decreased stability of the Mdm4-S protein. Indeed, a treatment with the proteasome inhibitor MG132 for only 2 h was sufficient to significantly increase Mdm4-S levels, indicating that this protein is very efficiently degraded by the proteasome (Figure 4a). Altogether, these results confirmed that the *Mdm4*$^{\Delta E6}$ allele leads to the loss of Mdm4-FL, and an increase in Mdm4-S expression at both mRNA and protein levels, but indicated that Mdm4-S protein levels remain extremely low in the mutant cells.

In conclusion, we generated a mouse model with a specific deletion of *Mdm4* exon 6 to address the potential role of the Mdm4-S splice variant, a variant transcript frequently overexpressed in a variety of human cancers. Previous studies led to opposing views on the significance of this splice variant, with some authors concluding that the Mdm4-S mRNA encodes a potent oncoprotein acting as a p53 inhibitor,[11–14,16] while others proposed that the main effect of this splice variant would be to reduce Mdm4-FL expression in tumor cells with mutant p53 or amplified *Mdm2*.[15,18] Although our transfection experiments indicate that the Mdm4-S protein can act as a p53 inhibitor when massively overexpressed, our *in vivo* data clearly demonstrate that the skipping of *Mdm4* exon 6, even when obligatory, is not sufficient to ensure a strong expression of the Mdm4-S protein. Thus, in tumors that retain a functional p53 pathway, additional mechanisms enabling Mdm4-S mRNA and/or protein stabilization would have to operate for Mdm4 alternative splicing to effectively promote tumorigenesis. For example, alternative polyadenylation may stabilize Mdm4-FL mRNA in some cancers[29] and conceivably, it might similarly stabilize Mdm4-S mRNA. Importantly, however, evidence that the Mdm4-S protein is overexpressed in cancer cells remains controversial.[15]

Our data rather support the alternative proposal that the main effect of the Mdm4 splice variant is to reduce Mdm4-FL expression. In cells with an intact p53 pathway, decreasing Mdm4-FL levels could be a way to increase p53 activity,[19] but why would cancer cells with a mutant p53 or amplified *Mdm2* select for a decrease in Mdm4-FL? A possible explanation, suggested by a few reports,[30–32] might be that Mdm4-FL would exhibit tumor suppressive functions in cells with alterations in the p53 pathway, either through p53-independent mechanisms,[32] or by inhibiting oncogenic functions acquired by mutant p53.[33] Alternatively, because aberrant splicing is a common feature of cancer cells,[34] an increase in Mdm4-S mRNAs might simply result from alterations in the splicing regulatory machinery that frequently occur during tumor progression, and only tumor cells with an inactive p53 pathway would tolerate a switch in splicing that leads to decreased Mdm4-FL expression. Importantly, both hypotheses could explain why increased Mdm4-S mRNA levels, without encoding significant amounts of a potential oncoprotein, may nevertheless correlate with more aggressive cancers and serve as a prognostic biomarker.

CONFLICT OF INTEREST

The authors declare no conflict of interests.

ACKNOWLEDGEMENTS

We thank members of the Curie technological platforms for their contribution to this study: I Grandjean, C Daviaud and M Verlhac from the Animal facility, C Alberti, E Belloir and N Mebirouk from the transgenesis platform, M Richardson, A Nicolas, R Leclere and M Huerre from the pathology service. Our lab received funding as an Equipe Labellisée by the Ligue Nationale contre le Cancer. The project was initiated by grants from the Fondation de France (Comité Tumeurs), the Ligue Nationale contre le Cancer (Comité Ile de France) and the Fondation ARC. JL was supported by a post-doctoral fellowship from the Cancéropôle Ile de France.

REFERENCES

1 Toledo F, Wahl GM. Regulating the p53 pathway: *in vitro* hypotheses, *in vivo* veritas. *Nat Rev Cancer* 2006; **6**: 909–923.

2 Marine JC. MDM2 and MDMX in cancer and development. *Curr Top Dev Biol* 2011; **94**: 45–75.
3 Toledo F, Krummel KA, Lee CJ, Liu CW, Rodewald LW, Tang M *et al.* A mouse p53 mutant lacking the proline rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. *Cancer Cell* 2006; **9**: 273–285.
4 Laurie NA, Donovan SL, Shih CS, Zhang J, Mills N, Fuller C *et al.* Inactivation of the p53 pathway in retinoblastoma. *Nature* 2006; **444**: 61–66.
5 Bernal F, Wade M, Godes M, Davis TN, Whitehead DG, Kung AL *et al.* A stapled p53 helix overcomes HDMX-mediated suppression of p53. *Cancer Cell* 2010; **18**: 411–422.
6 Garcia D, Warr MR, Martins CP, Brown Swigart L, Passegue E, Evan GI *et al.* Validation of MdmX as a therapeutic target for reactivating p53 in tumors. *Genes Dev* 2011; **25**: 1746–1757.
7 Gembarska A, Luciani F, Fedele C, Russell EA, Dewaele M, Villar S *et al.* MDM4 is a key therapeutic target in cutaneous melanoma. *Nat Med* 2012; **18**: 1239–1247.
8 Fang M, Simeonova I, Bardot B, Lejour V, Jaber S, Bouarich-Bourimi R *et al.* Mdm4 loss in mice expressing a p53 hypomorph alters tumor spectrum without improving survival. *Oncogene* 2014; **33**: 1336–1339.
9 Mancini F, Di Conza G, Moretti F. MDM4 (MDMX) and its transcript variants. *Curr Genomics* 2009; **10**: 42–50.
10 Phillips A, Teunisse A, Lam S, Lodder K, Darley M, Emaduddin M *et al.* HDMX-L is expressed from a functional p53-responsive promoter in the first intron of the HDMX gene and participates in an autoregulatory feedback loop to control p53 activity. *J Biol Chem* 2010; **285**: 29111–29127.
11 Rallapalli R, Strachan G, Cho B, Mercer WE, Hall DJ. A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. *J Biol Chem* 1999; **274**: 8299–8308.
12 Riemenschneider MJ, Knobbe CB, Reifenberger G. Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int J Cancer* 2003; **104**: 752–757.
13 Bartel F, Schulz J, Bohnke A, Blumke K, Kappler M, Bache M *et al.* Significance of HDMX-S (or MDM4) mRNA splice variant overexpression and HDMX gene amplification on primary soft tissue sarcoma prognosis. *Int J Cancer* 2005; **117**: 469–475.
14 Prodosmo A, Giglio S, Moretti S, Mancini F, Barbi F, Avenia N *et al.* Analysis of human MDM4 variants in papillary thyroid carcinomas reveals new potential markers of cancer properties. *J Mol Med* 2008; **86**: 585–596.
15 Lenos K, Grawenda AM, Lodder K, Kuijjer ML, Teunisse AF, Repapi E *et al.* Alternate splicing of the p53 inhibitor HDMX offers a superior prognostic biomarker than p53 mutation in human cancer. *Cancer Res* 2012; **72**: 4074–4084.
16 Rallapalli R, Strachan G, Tuan RS, Hall DJ. Identification of a domain within MDMX-S that is responsible for its high affinity interaction with p53 and high-level expression in mammalian cells. *J Cell Biochem* 2003; **89**: 563–575.
17 Bista M, Petrovich M, Fersht AR. MDMX contains an autoinhibitory sequence element. *Proc Natl Acad Sci USA* 2013; **110**: 17814–17819.
18 Lenos K, Jochemsen AG. Functions of MDMX in the modulation of the p53-response. *J Biomed Biotechnol* 2011; **2011**: 876173.
19 Bezzi M, Teo SX, Muller J, Mok WC, Sahu SK, Vardy LA *et al.* Regulation of constitutive and alternative splicing by PRMT5 reveals a role for Mdm4 pre-mRNA in sensing defects in the spliceosomal machinery. *Genes Dev* 2013; **27**: 1903–1916.
20 Parant J, Chavez-Reyes A, Little NA, Yan W, Reinke V, Jochemsen AG *et al.* Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a non-overlapping pathway with MDM2 to regulate p53. *Nat Genet* 2001; **29**: 92–95.
21 Migliorini D, Denchi EL, Danovi D, Jochemsen A, Capillo M, Gobbi A *et al.* Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. *Mol Cell Biol* 2002; **22**: 5527–5538.
22 Finch RA, Donoviel DB, Potter D, Shi M, Fan A, Freed DD *et al.* mdmx is a negative regulator of p53 activity *in vivo*. *Cancer Res* 2002; **62**: 3221–3225.
23 Wang YV, Leblanc M, Wade M, Jochemsen AG, Wahl GM. Increased radioresistance and accelerated B cell lymphomas in mice with Mdmx mutations that prevent modifications by DNA-damage-activated kinases. *Cancer Cell* 2009; **16**: 33–43.
24 Pant V, Xiong S, Iwakuma T, Quintas-Cardama A, Lozano G. Heterodimerization of Mdm2 and Mdm4 is critical for regulating p53 activity during embryogenesis but dispensable for p53 and Mdm2 stability. *Proc Natl Acad Sci USA* 2011; **108**: 11995–12000.
25 Huang L, Yan Z, Liao X, Li Y, Yang J, Wang ZG *et al.* The p53 inhibitors MDM2/MDMX complex is required for control of p53 activity *in vivo*. *Proc Natl Acad Sci USA* 2011; **108**: 12001–12006.
26 Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; **74**: 957–967.
27 Simeonova I, Jaber S, Draskovic I, Bardot B, Fang M, Bouarich-Bourimi R *et al.* Mutant mice lacking the p53 C-terminal domain model telomere syndromes. *Cell Rep* 2013; **3**: 2046–2058.
28 Wang YV, Leblanc M, Fox N, Mao JH, Tinkum KL, Krummel K *et al.* Fine-tuning p53 activity through C-terminal modification significantly contributes to HSC homeostasis and mouse radiosensitivity. *Genes Dev* 2011; **25**: 1426–1438.
29 Okada N, Lin CP, Ribeiro MC, Biton A, Lai G, He X *et al.* A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. *Genes Dev* 2014; **28**: 438–450.
30 Steinman HA, Sluss HK, Sands AT, Pihan G, Jones SN. Absence of p21 partially rescues Mdm4 loss and uncovers an antiproliferative effect of Mdm4 on cell growth. *Oncogene* 2004; **23**: 303–306.
31 Steinman HA, Hoover KM, Keeler ML, Sands AT, Jones SN. Rescue of Mdm4-deficient mice by Mdm2 reveals functional overlap of Mdm2 and Mdm4 in development. *Oncogene* 2005; **24**: 7935–7940.
32 Matijasevic Z, Steinman HA, Hoover K, Jones SN. MdmX promotes bipolar mitosis to suppress transformation and tumorigenesis in p53-deficient cells and mice. *Mol Cell Biol* 2008; **28**: 1265–1273.
33 Muller PA, Vousden KH. p53 mutations in cancer. *Nat Cell Biol* 2013; **15**: 2–8.
34 Pajares MJ, Ezponda T, Catena R, Calvo A, Pio R, Montuenga LM *et al.* Alternative splicing: an emerging topic in molecular and clinical oncology. *Lancet Oncol* 2007; **8**: 349–357.
35 Simeonova I, Lejour V, Bardot B, Bouarich-Bourimi R, Morin A, Fang M *et al.* Fuzzy tandem repeats containing p53 response elements may define species-specific p53 target genes. *PLoS Genet* 2012; **8**: e1002731.
36 Prophet E, Mills B, Arrington J, Sobin L. *Afip - Laboratory Methods in Histotechnology*. American Registry of Pathology: Washington, DC, USA, 1992.
37 Ramos YF, Stad R, Attema J, Peltenburg LT, van der Eb AJ, Jochemsen AG. *et al.* Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53. *Cancer Res* 2001; **61**: 1839–1842.
38 Vandenbroucke II, Vandesompele J, Paepe AD, Messiaen L. Quantification of splice variants using real-time PCR. *Nucleic Acids Res* 2001; **29**: E68–E68.

Supplementary Information accompanies this paper on the Oncogene website (http://www.nature.com/onc)