Mdm4 loss in the intestinal epithelium leads to compartmentalized cell death but no tissue abnormalities

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Abstract

Mdm4 is a critical inhibitor of the p53 tumor suppressor. *Mdm4* null mice die early during embryogenesis due to increased p53 activity. In this study, we explore the role that Mdm4 plays in the intestinal epithelium by crossing mice carrying the *Mdm4* floxed allele to mice with the *Villin Cre* transgene. Our data show that loss of *Mdm4* (*Mdm4^{int}*) in this tissue resulted in viable animals with no obvious morphological abnormalities. However, these mutants displayed increased p53 levels and apoptosis exclusively in the proliferative compartment of the intestinal epithelium. This phenotype was completely rescued in a *p53* null background. Notably, the observed compartmentalized apoptosis in proliferative intestinal epithelial cells was not due to restricted Mdm4 expression in this region. Thus, in this specific cellular context, p53 is negatively regulated by Mdm4 exclusively in highly proliferative cells.

Keywords

p53 stability; cell cycle arrest; apoptosis; Mdmx

Introduction

The p53 tumor suppressor plays an essential role in inhibiting tumorigenesis by transcriptionally activating genes involved in cell cycle arrest, apoptosis and senescence in abnormal cells (Vousden 2000). However, p53 activity must be kept under control in healthy cells to avoid detrimental effects to the organism such as unnecessary cellular death. Two major players involved in the regulation of p53 activity are Mdm2 and Mdm4.

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The Mdm2 oncogene inhibits p53 activity principally by acting as an E3 ubiquitin ligase to target p53 for 26S proteasome-dependent protein degradation (Honda et al 1997, Kubbutat et al 1997). The importance of Mdm2 in regulating p53 function *in vivo* was demonstrated with the generation of *Mdm2* null mice. These mutants die before implantation due to increased apoptosis, while concomitant loss of *Mdm2* and *p53* completely rescued this phenotype (Chavez-Reyes et al 2003, Jones et al 1995, Montes de Oca Luna et al 1995). Interestingly, Mdm2 is also a transcriptional target of p53 itself and, thus, it participates in a negative feedback loop with p53 (Barak et al 1993, Wu et al 1993). On the other hand, Mdm4 inhibits p53 mainly by physically binding to p53 and interfering with its transcriptional activity (Shvarts et al 1996). Although Mdm4 possesses a RING finger domain in its structure, it cannot ubiquitinate and target p53 for protein degradation (Jackson & Berberich 2000, Sharp et al 1999). Notably, loss of *Mdm4* leads to embryonic lethality that can be completely rescued in a *p53* null background (Finch et al 2002, Migliorini et al 2002, Parant et al 2001). Together, these data indicate that both Mdm2 and Mdm4 are critical inhibitor of p53 *in vivo*.

The importance of these genes in specific cell types *in vivo* has been further demonstrated with the use of *Mdm2* and *Mdm4* conditional mouse alleles (Grier et al 2006, Grier et al 2002). For example, deletion of *Mdm2* or *Mdm4* in cells of the central nervous system leads to p53-dependent embryonic lethal phenotypes (Francoz et al 2006, Xiong et al 2006). Simultaneous deletion of both genes in proliferative and quiescent cells of the central nervous system yields a more dramatic phenotype, indicating that these two proteins synergize to inhibit p53 activity in both dividing and non-dividing cells (Francoz et al 2006, Xiong et al 2006). Furthermore, *Mdm2* deletion in embryonic cardyomyocytes or in quiescent adult smooth muscle cells also results in lethal phenotypes due to increased p53-dependent apoptosis (Boesten et al 2006, Grier et al 2006), whereas loss of *Mdm4* in these two cell types leads to a much weaker or complete absence of cellular defects, respectively (Boesten et al 2006, Grier et al 2006, Xiong et al 2007). Finally, in contrast to the aforementioned studies, deletion of *Mdm2* in the intestinal epithelium results in multiple abnormalities in this tissue early in life, yet mice survive due to the ability of the tissue to compensate for cellular death mediated by p53 activation (Valentin-Vega et al 2008). Thus far, these studies together suggest that Mdm2 inhibits p53-activity in both proliferative and fully differentiated cells in all tissues analyzed. However, it appears that Mdm4 is required to restrain p53 activity specifically depending on the cellular context.

To obtain more insights about the role of Mdm4 toward p53 regulation in cells with different differentiation potentials, we sought to delete this oncogene in the intestinal epithelium. The architecture of this organ provides an excellent model to study the function of genes in cells at various stages of differentiation (Sancho et al 2004). The intestinal epithelial-specific *Villin Cre* mouse model was used in this study as this transgene targets both highly proliferative and differentiated intestinal epithelial cells (Madison et al 2002). Our data show that Mdm4 regulates p53 levels and activity exclusively in the highly proliferative compartment of this tissue. Interestingly, this compartmentalized regulation is not due to a restricted expression of Mdm4 in proliferative intestinal epithelial cells.

Materials and Methods

Mice

All animal protocols were approved by the Institutional Animal Care and Use Committee. All mice were >90% C57BL/6. *VilCre* mice were kindly provided by Dr. Gumucio (Madison et al 2002). *VilCre* mice were crossed with $Mdm4^{FX}$ conditional mice (Grier et al 2006). The following primers were used to distinguish between the $Mdm4^{FX}$ and $Mdm4$ null ($Mdm4^{int}$) alleles: A, 5'-TAGAATCTGGAATTACAGACAG-3'; B, 5'-TGTCTTTAGCATTTACTAAGAGCT-3'; and C, 5'-TATCCAGTGTCCTCTTCTGGCTT-3' (Grier et al 2006). Intestines of postnatal day 3 (P3) pups were dissected and washed in Phosphate Buffered Saline (PBS), rolled, incubated in 10% formalin for up to 48 hours, and embedded in paraffin. Intestines of 8-week old mice were cut into sections (duodenum, jejunum, ileum, and colon), and luminal contents flushed with cold PBS prior to rolling. Tissues were cut in 4μ-thick sections, stained with hematoxylin and eosin (H&E) and analyzed by pathology.

β-galactosidase activity assays

Postnatal intestines were dissected and quickly washed in cold PBS followed by fixation in 4% paraformaldehyde for 2 hours at 4°C. Then, samples were cryopreserved in sucrose and embedded in Optimum Cutting Temperature compound (Sakura). β-galactosidase activity assays were performed as previously described (Valentin-Vega et al 2008).

Immunohistochemistry, immunofluorescence, TUNEL, and BrdU incorporation assays

Immunohistochemistry and immunofluorescence assays were performed as described, respectively (Evans et al 2001, Valentin-Vega et al 2008). TUNEL assay was performed by using the TdtT Fragel DNA fragmentation kit (Oncogene) as indicated by the company except that the Diaminobenzidine (DAB) substrate supplied in the kit was substituted for the DAB distributed by the Vector Laboratories Company. The following antibodies were used for immunohistochemistry and immunofluorescence studies: rabbit anti-p53 (1:200, CM5, Vector Laboratories), rabbit anti-Caspase-3 (1:100, Cell Signaling,), rabbit anti-Ki-67 (1:50, Abcam), and mouse anti-pan cytokeratin (1:200, Sigma). BrdU incorporation analyses were performed as previously described (Parant et al 2001).

Immunoprecipitation and western blot analyses

Protein extracts were isolated from 3-day old whole intestines using NP-40 lysis buffer with proteinase inhibitors (50 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.5% NP-40.50 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 100 mM PMSF). For immunoprecipitation, 500 μg of total protein were pre-cleared with Protein A/G-conjugated beads (Santa Cruz Biotechnology), and then immunoprecipitated with 4 μg of anti-Mdm4 antibody (mouse monoclonal anti-Mdm4, Sigma) overnight at 4°C. Protein A/G-conjugated beads (Santa Cruz Biotechnology) were added next and samples were incubated for 1 hr at 4°C. Samples were then washed with lysis buffer and beads were collected. Proteins were subjected to 8% SDS-polyacrylamide gel electrophoresis. Mdm4 was detected by western blot using mouse anti-Mdm4 from Sigma (overnight, 1:1000 dilution). 50 μg of total protein was separated by

SDS-PAGE to analyze equal loading in gel. Vinculin was used as loading control (mouse anti-Vinculin, 1:2000, Sigma). Protein in film was quantified using the ImageQuant program (Version 5.2, Molecular Dynamics). Mdm4 protein levels were normalized to the value of Vinculin.

In situ hybridization

3-day old pups were sacrificed and intestines removed. Samples were washed in cold PBS, rolled and embedded in OCT. In situ hybridization (ISH) was performed at the Baylor College of Medicine ISH core facility using digoxigenin-labelled Mdm4 probe on 25 μm sections (Yaylaoglu et al 2005).

Quantification of apoptotic and proliferative cells

Quantification of apoptotic bodies in the intervillus compartment of P3 mice was performed by counting the number of apoptotic cells in more than 150 intervillus units per mouse (n = 3 per group). Apoptotic bodies were identified by H&E staining. Similarly, cellular proliferation in P3 mice was quantified by counting the number of Ki-67 positive cells per intervillus unit. At least 200 units were counted per mouse, n   3 per group).

Reverse transcriptase RT-PCR analysis

Reverse transcriptase real time-PCR analysis of the p53 target gene, *p21* was performed as described (Valentin-Vega et al 2008).

Results

Mice lacking Mdm4 in the intestinal epithelium display normal intestinal morphology

Mice lacking *Mdm4* in the whole body die during early embryogenesis, a phenotype that is completely rescued by loss of *p53* (Finch et al 2002, Migliorini et al 2002, Parant et al 2001). In order to understand the function of Mdm4 in non-embryonic tissues, an *Mdm4* conditional allele (or *FX* allele) has been generated (Fig. 1A) (Grier et al 2006). Disruption of this conditional *Mdm4^{FX}* allele in the whole mouse phenocopies the phenotypes observed in the original *Mdm4*-null mouse (Grier et al 2006). To delete *Mdm4* in the intestinal epithelium, the intestinal epithelial-specific *Villin Cre* (*VilCre*) transgenic mouse was used in this study (Madison et al 2002). A series of genetic crosses were performed to generate *Mdm4$^{FX/ 2}$* and *Mdm4$^{FX/FX}$* mice with or without *VilCre*. Since *Mdm4* mutants of both genotypes showed equivalent phenotypes, we labeled *Mdm4$^{FX/FX}$ VilCre+* or *Mdm4$^{FX/ 2}$ VilCre+* mice as *Mdm4^{int}* to simplify the nomenclature. Analysis of progeny at weaning indicates that *Mdm4^{int}* mice are born at the expected Mendelian ratio (Table 1). To investigate if homologous recombination occurred in the intestine of *Mdm4^{int}* mice, we first performed a PCR-based analysis using a set of primers that recognize the non-recombined (*FX*) or the recombined (*int*) alleles (Fig. 1A). As expected, recombination was observed exclusively in the intestine of both young and adult *Mdm4^{int}* mice (Fig. 1B). The observed non-recombined alleles may originate from cells carrying the non-recombined allele or from contaminated non-epithelial cells present in the sample. Recombination efficiency was also analyzed by breeding *Mdm4^{int}* mice to the *ROSA26R* reporter mouse (Soriano 1999) followed by-galactosidase activity assay. High frequency of recombination

was observed in both, highly proliferative and differentiated compartments of the small intestine of 3-day and 8-week old *Mdm4^{FX/FX} R26R VilCre+* mice (Fig. 1C). Finally, to investigate whether Mdm4 protein levels were lost in the intestine of *Mdm4^{int}* mice, we performed IP-western blot analysis against Mdm4 protein. Intestines of *Mdm4^{int}* mice showed a significant decrease in the protein levels of Mdm4 as compared to control intestines (Fig. 1D). The remaining Mdm4 protein observed in *Mdm4^{int}* mice could be due to contamination with non-epithelial cells in the sample as whole intestine was used in this experiment. Together, these data indicate that *Mdm4* is lost in both proliferative and differentiated intestinal epithelial cells of *Mdm4^{int}* mice and, thus, loss of *Mdm4* in this tissue yields viable animals.

As *Mdm4^{int}* mice survive, we next examined the intestinal morphology of these mutants. We did not detect obvious morphological defects in the intestine of 3-day old *Mdm4^{int}* pups (Fig. 1E). Similarly, analysis of the intestinal epithelium of adult (8-week old) mice also showed no significant morphological changes in the intestine of *Mdm4^{int}* mutants (Fig. 1E). Furthermore, we aged *Mdm4^{int}* mice and observed that these mutants display a normal lifespan as compared to control littermates (data not shown). Collectively, depletion of *Mdm4* in the intestinal epithelium does not significantly alter the architecture of this tissue.

Absence of Mdm4 results in increased p53-dependent apoptosis exclusively in highly proliferative cells of the small intestine

Since Mdm2 is an E3 ubiquitin ligase that targets p53 for 26S proteosome-dependent protein degradation (Honda et al 1997, Kubbutat et al 1997), loss of *Mdm2* in cells always results in high p53 protein levels (Boesten et al 2006, Francoz et al 2006, Grier et al 2006, Xiong et al 2006). On the other hand, Mdm4 does not directly affect p53 degradation but interacts with Mdm2 to regulate p53 function (Sharp et al 1999, Stad et al 2000). To elucidate the role of Mdm4 in regulating p53 activity in the intestinal epithelium, we first investigated if absence of *Mdm4* affects p53 protein levels in this tissue. p53 immunohistochemistry revealed that *Mdm4^{int}* mice showed increased p53 levels exclusively in the proliferative compartment (Fig. 2A). High levels of p53 in the absence of *Mdm4* have been previously reported only in cells of the central nervous system (Xiong et al 2006). The apoptotic response was then examined in the intestines of *Mdm4^{int}* mice. 3-day old *Mdm4^{int}* mice displayed a significant increased in apoptotic response in the intestinal epithelium as compared to control animals (control, 0.053 ± 0.029; *Mdm4^{int}*, 0.573 ± 0.22; *t*-Test, P = 0.01; 200 intervillus units scored per mouse; n = 3 per group) (Fig. 2B–D). Like 3-day old mutants, adult *Mdm4^{int}* mice also showed a comparable increase in apoptosis in all regions of the intestinal epithelium (duodenum, jejunum, etc.) (data not shown). Interestingly, in contrast to 3-day old *Mdm2^{int}* mice that exhibit apoptosis in both profieartive and differentiated cells of the intestinal epithelium (Valentin-Vega et al 2008), 3-day old *Mdm4^{int}* mice showed a restricted apoptotic response in the proliferative compartment of this tissue (Fig. 2B–C). Collectively, loss of *Mdm4* in the intestinal epithelium results in increased apoptotic response exclusively in highly proliferative cells of the intestinal epithelium.

To investigate if the increased apoptotic response observed in *Mdm4^{int}* mice is p53-dependent, we bred these mutants into a *p53*-null background. As expected, a complete

rescue of the apoptotic response was observed in neonatal $Mdm4^{int}$ mice that simultaneously lack $p53$ ($Mdm4^{int}$ $p53^{-/-}$) (Fig. 2D). These data indicate that the increased apoptotic response observed in the absence of $Mdm4$ in the intestinal epithelium is dependent on p53 activity.

Previous studies have shown that unlike $Mdm2$, loss of $Mdm4$ in the whole mouse or in the central nervous system results in p53-mediated cell cycle arrest (Francoz et al 2006, Migliorini et al 2002, Parant et al 2001, Xiong et al 2006). To investigate if p53 also triggers cell cycle arrest in the intestinal epithelium in the absence of $Mdm4$, we first performed immunofluorescence against the proliferative marker, Ki-67. Our data showed no difference in Ki-67 positive cells between $Mdm4^{int}$ and control mice (control, 10.4 ± 0.189; $Mdm4^{int}$, 9.72 ± 0.858; t-Test, P = 0.231; 200 intervillus units scored per mouse; n = 3 per group) (Fig. 3A). Additionally, we performed BrdU incorporation analyses in 8-week old mouse intestines and again observed no significant difference in the number of BrdU labeled cells between both groups (control, 13.21 ± 0.799; $Mdm4^{int}$, 15.5 ± 0.14; t-Test, P = 0.06; 100 intervillus units scored per mouse; n = 3 per group) (Fig. 3B).

We also analyzed the mRNA levels of the p53 target gene, $p21$, which is involved in cell cycle arrest. Interestingly, even though the proliferation capacity of the intestinal epithelium in $Mdm4^{int}$ mice was intact, we detected a significant increase in p21 mRNA levels in the mutant intestines (control, 1.05 ± 0.086; $Mdm4^{int}$, 2.38 ± 0.19; t-Test, P = 0.03; n = 5 per group (Fig. 3C). In summary, in contrast to embryonic tissues and cells of the central nervous system, loss of $Mdm4$ in the intestinal epithelium did not lead to defects in the proliferation capacity of these cells despite the increase in p21 mRNA levels. Thus, Mdm4 does not regulate p53-dependent cell cycle arrest in this cellular context.

Increase in p53-dependent apoptosis exclusively in the proliferative compartment of 3-day old $Mdm4^{int}$ mice correlated with the expression pattern of p53 protein in these mutants (compare Fig. 2A with Fig. 2B–C). A possible explanation for this phenomenon is that in the small intestine, Mdm4 is expressed only in highly proliferative cells. To investigate this possibility, we performed *in situ* hybridization against Mdm4 mRNA in 3-day old wild-type intestine given that Mdm4 is not detectable at the protein level *in vivo*. Unexpectedly, Mdm4 mRNA was detected in both proliferative and differentiated compartments of the intestinal epithelium (Fig. 4). This finding indicates that the expression pattern of Mdm4 is not responsible for the regulation of p53 exclusively in the intervillus compartment.

Discussion

The data presented in this study indicate that like Mdm2, Mdm4 is also an important modulator of the p53 tumor suppressor in the intestinal epithelium. Importantly, while Mdm2 regulates p53 in both highly proliferative and differentiated intestinal epithelial cells (Valentin-Vega et al 2008), Mdm4 regulates p53 exclusively in the proliferative compartment of this tissue. Interestingly, our results demonstrate that this compartmentalized regulation is not due to a restricted expression of Mdm4 in the intestinal epithelium as Mdm4 RNA was clearly detected in both highly proliferative and differentiated cells of the tissue. Perhaps, posttranslational modifications of Mdm4 are

necessary to achieve its full activation and inhibition of p53 in specific cell populations of the intestine.

An interesting observation from this study is that, in contrast to mice lacking *Mdm2* in the intestinal epithelium (Valentin-Vega et al 2008), *Mdm4^int^* mice did not display morphological defects in this organ despite the increase in p53-dependent apoptosis. The intestinal epithelium is one of the most dynamic and highly proliferative organs in the body (Sancho et al 2004). Therefore, it is likely that newly formed cells are replacing the dying ones. Consequently, the morphology of the tissue is preserved in *Mdm4^int^* mice. However, mice lacking *Mdm2* in the intestine display a drastic morphological defect probably because the resultant apoptotic index is much higher than in the absence of *Mdm4*. In these mutants, the intestinal epithelium needs to increase its proliferation potential to compensate for cellular loss and guarantee organismal survival (Valentin-Vega et al 2008).

Noticeably our data, together with previous reports, indicate that Mdm2 is not only a stronger p53 inhibitor than Mdm4 in all cell types analyzed (Boesten et al 2006, Francoz et al 2006, Grier et al 2006, Xiong et al 2007, Xiong et al 2006), but it also regulates p53 activity in both proliferative and differentiated cells whereas Mdm4-mediated p53 regulation appears to be tissue specific. For example, while Mdm4 is required to modulate p53 activity in both proliferative and quiescent cells of the central nervous system (Francoz et al 2006, Xiong et al 2006), it is dispensable for regulating p53 in quiescent smooth muscle cells and adult red blood cells (Boesten et al 2006, Maetens et al 2007). In contrast, mice with loss of *Mdm4* in cardyomyocytes do not show developmental defects, but acquire heart defects during adulthood indicating that Mdm4 is important in fully differentiated cardyomyocytes (Xiong et al 2007). In summary, these data suggest that Mdm2 is the major inhibitor of p53 function *in vivo*, while Mdm4 may only be required to regulate p53 in certain cell types at specific time points.

An important observation from our study is that loss of *Mdm4* in the intestinal epithelium leads to increased p53 levels in the proliferative compartment exclusively, yet Mdm4 is not an E3 ubiquitin ligase (Jackson & Berberich 2000, Sharp et al 1999). It has been demonstrated *in vitro* that Mdm4 and Mdm2 interact through their RING finger domains, which results in Mdm2 stabilization due to the inability of Mdm2 to self-ubiquitinate (Sharp et al 1999, Stad et al 2000). In this system, we could not detect Mdm2 protein levels (data not shown) and could not therefore address this possibility. However, Mdm2 haplo-insufficiency (a 2-fold difference) affects p53-dependent apoptosis and transformation (Terzian et al 2007). Thus, it is possible that loss of *Mdm4* causes self-ubiquitination and degradation of Mdm2 and subsequently results in p53 stabilization in this cell type.

Elucidating the tissue-specific functions of the p53/Mdm2/Mdm4 pathway has important therapeutic implications. Tumor cells that retain wild type p53 might be treated by interfering with the Mdm2/p53 or Mdm4/p53 complexes to activate p53 in such cells. Recently, molecules such as Nutlin-3 and Rita have been identified and shown to disrupt the association between Mdm2 and p53 resulting in death of cancer cell lines and xenografts (Issaeva et al 2004, Sarek et al 2007, Tovar et al 2006, Vassilev et al 2004). However, given that Mdm2 affects a wide variety of cell types, the administration of such therapeutic

molecules in cancer patients might be detrimental for the organism since it might target normal cells as well. Administration of Mdm4 inhibitors instead might be more beneficial for the individual since Mdm4 is a less potent p53 inhibitor in normal cells *in vivo*. A recent study shows that although retinoblastoma tumors generally lack *p53* mutations, the activity of wild type p53 is frequently abrogated by the ability of the tumor to select for cells with high Mdm4 expression (Laurie et al 2006). This study suggests that indeed Mdm4 might serve as a good chemotherapeutic target for retinoblastoma patients. As Mdm4 inhibitors have not yet been identified, it would be worth to put more efforts on the development of these novel anti-cancer drugs.

Acknowledgments

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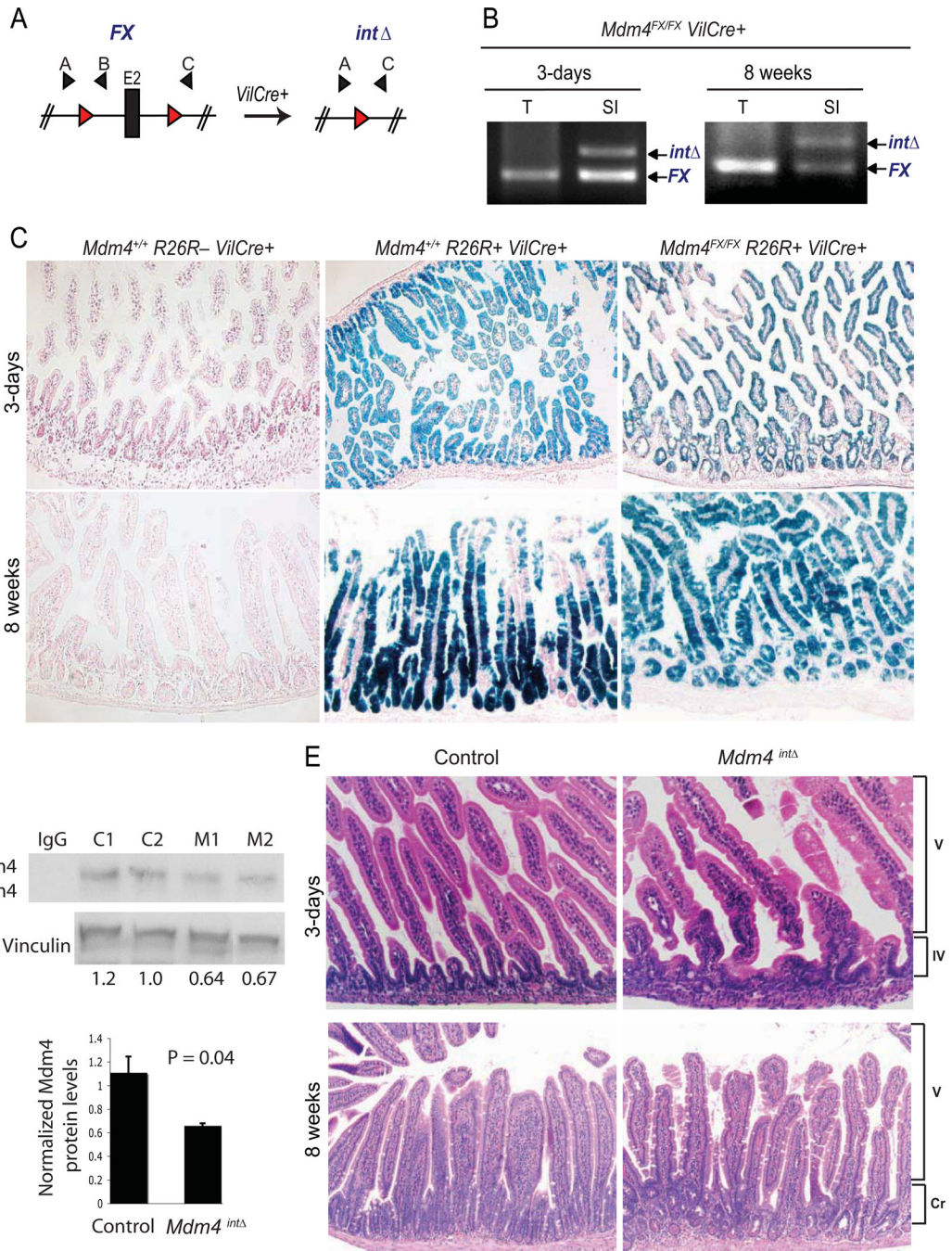
Figure 1. Mice survive with loss of *Mdm4* in the intestinal epithelium and display normal intestinal morphology

(A) Schematic diagram of the non-recombined (*FX*) and recombined (*int*Δ) *Mdm4* alleles. The *Mdm4* conditional allele targets exon 2 (E2), which contains the translation start site. Red triangles represent *loxP* sites flanking exon 2; Black triangles represent primer sets used to identify the *FX* and intΔ alleles (primers A and B amplify the non-recombined (*FX*) allele while primers A and C amplify the recombined (*int*Δ) allele). (B) PCR analysis in the intestine of 3-day old *Mdm4^FX/FX^ VilCre+* mice using primers A, B, and C. SI, small

intestine; T, tail. (C) β-galactosidase activity assays in the small intestine of 3-day and 8-week old mice. Blue denotes cells with β-galactosidase activity (original magnification X100). *Mdm4$^{+/+}$ R26R− VilCre+,* negative control; *Mdm4$^{+/+}$ R26R+ VilCre+,* positive control; *Mdm4$^{FX/FX}$ R26R+ VilCre+, Mdm4^{int}* mutant. (D) Mdm4 protein expression in 3-day old whole intestines. Mdm4 protein was immunoprecipitated and then detected by western blot analysis. Mdm4 protein levels were quantified and normalized to the value of Vinculin (loading control). Normalized Mdm4 protein levels were graphed and the p-value was calculated using the Student's *t*-test. IgG, Immunoglobulin G (negative control); C1, control #1; C2, control #2; M1, *Mdm4^{int}* mutant #1; M2, *Mdm4^{int}* mutant #2. (E) H&E staining of the small intestine of 3-day old pups or intestinal jejunum region of 8-week old mice (original magnification X200 or X100, respectively). V, villus region, IV, intervillus region, Cr, crypt compartment. Intervillus and crypt compartments are regions of undifferentiated cells in young and adult intestines, respectively. The villus compartment contains differentiated intestinal epithelial cells.
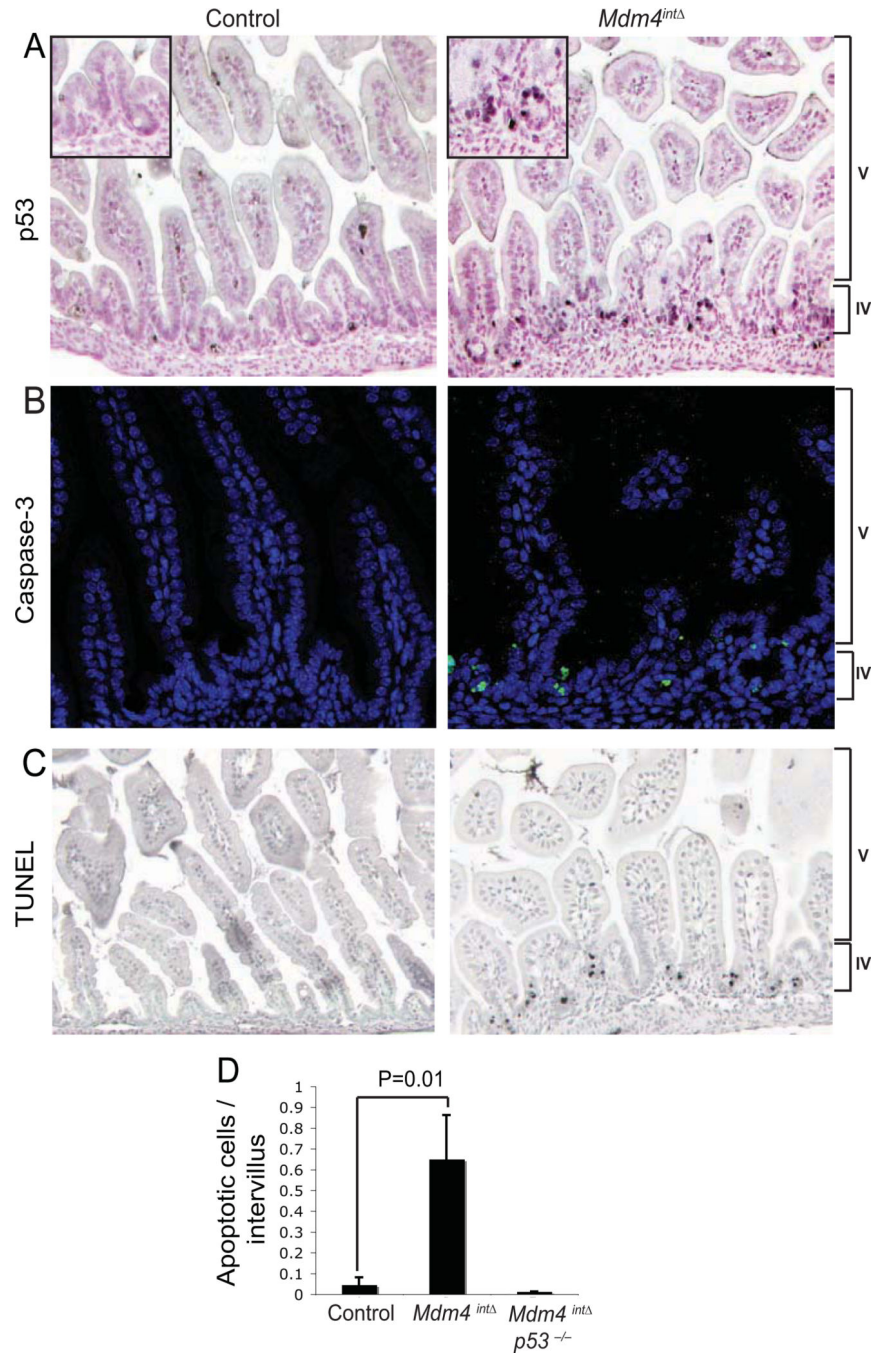
Figure 2. Mdm4 regulates p53 specifically in highly proliferative cells of the small intestine
(A) Immunohistochemistry against p53 in the small intestine of 3-day old mice (original magnification X200). Box shows higher magnification of the intervillus (proliferative) region. (B) Caspase-3 immunofluorescence in 3-day old small intestines. Green staining shows caspase-3 positive cells (original magnification X400). (C) TUNEL assay performed in 3-day old small intestines. Black staining illustrates TUNEL positive cells (original magnification X200). (D) Quantification of apoptotic cells in the intervillus pockets of 3-day old small intestines scored by H&E (n = 3 per group). $Mdm4^{int\Delta}$ $p53^{-/-}$ genotype represents

mice with loss of *Mdm4* in the intestine that also lack *p53* in the whole body. V, villus (differentiated) region, IV, intervillus (proliferative) region.
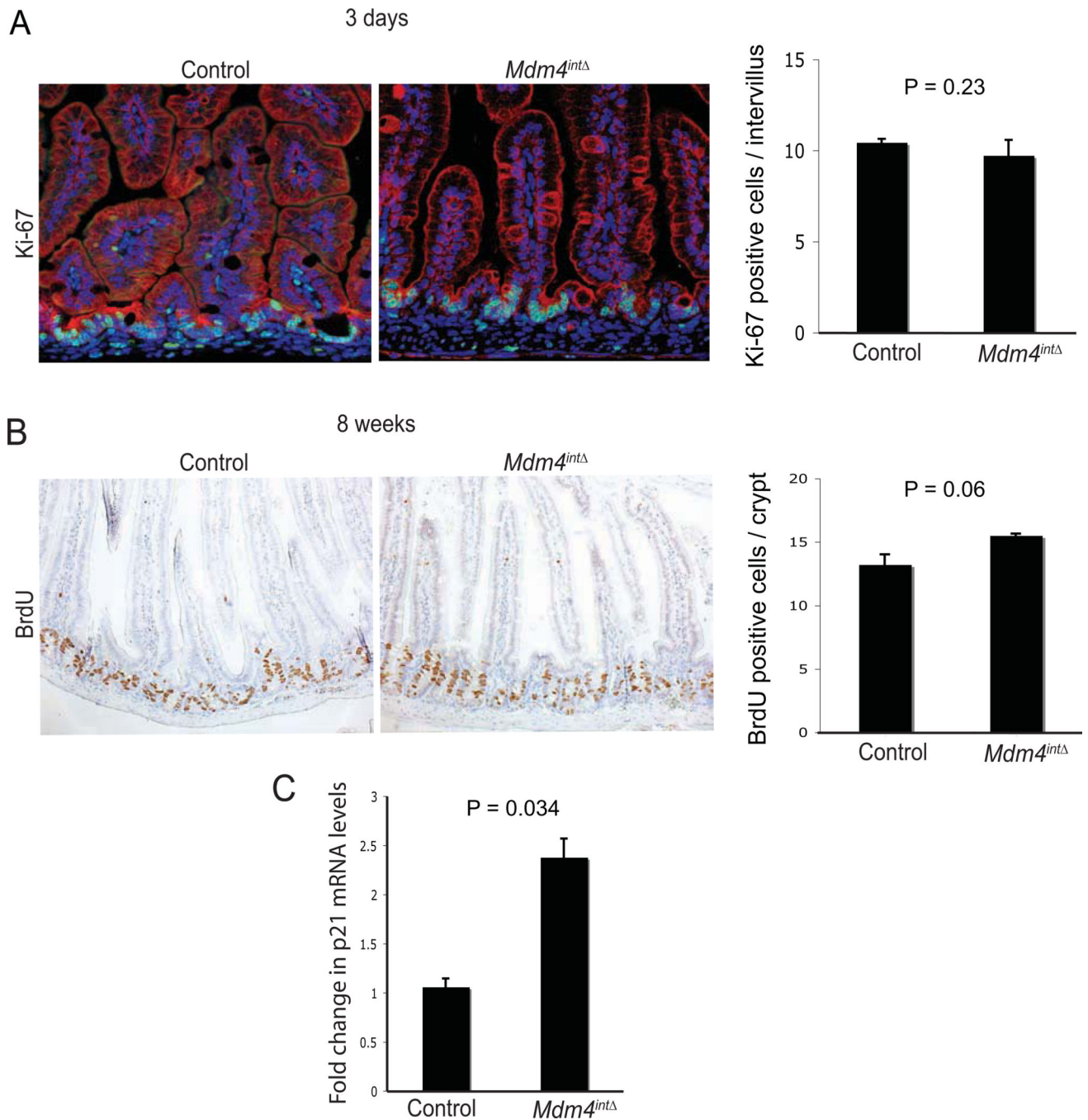
Figure 3. Cellular proliferation is intact in the small intestine of *Mdm4^int^* mice
(A) Ki-67 immunofluorescence in 3-day old small intestines. Orange fluorescence, cytokeratin (epithelial marker); turquoise fluorescence, Ki-67 (proliferation marker); blue fluorescence, TOPRO-3 (nuclear counterstain); original magnification X400. Graph represents the quantification of the Ki-67 positive staining. (B) Analysis of BrdU incorporation in 8-week old mouse intestines. Mice were injected with 100 μg BrdU/g body weight and sacrificed 2 hr later. BrdU labeled cells (brown) were quantified and values were graphed. The p-value was obtained using Student's *t*-test (n = 3 per group); original

magnification X200. (C) Reverse transcription RT-PCR analysis of the p53 target, *p21*. Five mice per group were analyzed and the fold change in *p21* mRNA levels was graphed. The p-value was obtained using Student's *t*-test.

Mdm4
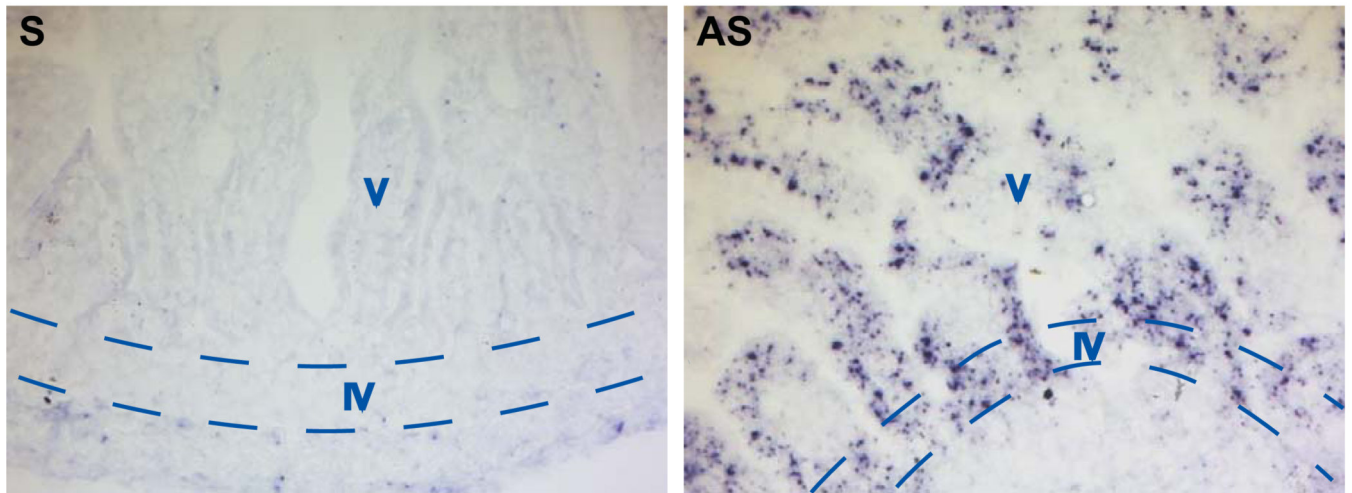
Figure 4. Mdm4 RNA is expressed in the entire intestinal epithelium of 3-day old mice
In situ hybridization against Mdm4 in 3-day old small intestines of wild-type mice. Black dots represent deposition of Mdm4 RNA in the tissue. *S,* sense probe; *AS*, anti-sense probe (original magnification X200). V, villus (differentiated) region, IV, intervillus (proliferative) region.

Table 1

Analysis of the progeny from $Mdm4^{FX/FX} \times Mdm4^{+/\Delta 2}$ VilCre+ cross at weaning

Genotype	Number of mice n=186
$Mdm4^{FX/+}$ VilCre−	47
$Mdm4^{FX/+}$ VilCre+	46
$Mdm4^{FX/\Delta 2}$ VilCre−	49
$Mdm4^{FX/\Delta 2}$ VilCre+	44

Abreviations: $Mdm4^{\Delta 2}$, $Mdm4$-null allele; $Mdm4^{FX}$, $Mdm4$ conditional allele; n, total number of mice analyzed, Chi square, p = 0.969