Chapter 4

Mdm2 protects terminally differentiated SMCs

Mdm2 protects terminally differentiated smooth muscle cells from p53-mediated cell death with a necrotic morphotype

Lianne S.M. Boesten[1,3,*]; A. Susanne M. Zadelaar[2,3,*]; Sarah De Clercq[4,*]; Sarah Francoz[4]; Anita van Nieuwkoop[1,3]; Erik A.L. Biessen[5]; Aart G. Jochemsen[6]; Chris Zurcher[7]; Louis M. Havekes[1,3]; Jean-Christophe Marine[4,†]; Bart J.M. van Vlijmen[8]

[1]Dept. of General Internal Medicine, [2]Dept. of Cardiology, Leiden University Medical Center, Leiden, The Netherlands, [3]TNO-Gaubius Laboratory, Biomedical Research Unit, Leiden, The Netherlands, [4]Laboratory For Molecular Cancer Biology, Flanders Interuniversity Institute For Biotechnology (VIB), Ghent, Belgium, [5]Dept. of Biopharmaceutics, Leiden Amsterdam Center for Drug Research, Leiden, The Netherlands, [6]Dept. of Molecular and Cell Biology, Leiden University Medical Center, Leiden, The Netherlands, [7]Dept. of Veterinary Pathology, Utrecht University, Utrecht, The Netherlands, [8]Hemostasis and Thrombosis Research Center, Dept. of Hematology, Leiden University Medical Center, Leiden, The Netherlands.

*These authors contributed equally to this study.

ABSTRACT

p53 is a potent inhibitor of cell growth and an inducer of apoptosis. During embryonic development and in adult homeostatic tissues, Mdm2 inhibits the growth suppressive activities of p53. However, whether tight surveillance of p53 activity is required in quiescent cells is unknown. To test this, conditional inactivation of *mdm2* was carried out in smooth muscle cells (SMCs) *in vivo*. Upon SMC-specific *mdm2* inactivation, mice rapidly became ill and died. Necropsy showed small intestinal dilation, and histological analyses indicated a severe reduction in the number of intestinal SMCs. Increased p53 levels and activity was detected in the remaining SMCs, and the phenotype was completely rescued on a p53-null background. Surprisingly, SMCs did not exhibit signs of apoptotic cell death but had a necrotic morphotype. These results show that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*.

The p53 tumor suppressor protein plays a critical role in suppressing tumor formation by inducing two types of anti-proliferative responses: cell-cycle arrest and apoptotic cell death.[1] Cell cycle arrest is mediated by transcriptional induction of genes whose products inhibit cell cycle progression, such as $p21^{Waf1/Cip12}$ or Ptprv.[3] The apoptotic function of p53 depends on both transcription-dependent and independent mechanisms.[4]

The importance of p53 in tumor suppression is highlighted by the observation that virtually all human cancers display an impaired p53 response. This is achieved either through direct inactivating mutations within the *p53* gene, or through aberrant expression of proteins acting in the p53 pathway. For instance, Mdm2 is overexpressed in human tumors of diverse origin.[5,6] The *mdm2* gene was originally identified as an amplified and overexpressed gene in a spontaneously transformed mouse BALB/c cell line.[7] Its transformation potential was later demonstrated and explained by the ability of Mdm2 to inactivate p53.[8,9]

Lack of functional *mdm2* is lethal in early mouse embryos, which die before implantation. This dramatic phenotype is completely rescued by concomitant deletion of *p53*.[10,11] In addition, mice with a hypomorphic *mdm2* allele have lower body weight and higher rates of p53-dependent apoptosis in various tissues. Notably, this phenotype is only observed in a subset of actively dividing cells, such as lymphocytes, and in the crypts of the small intestine, which could indicate that, in adult mice, Mdm2 restraints p53 activity only in homeostatic tissues.[12] Together, clear genetic evidence highlights the importance of the p53/Mdm2 interaction. However, limitations of the existing mouse models, such as early embryonic lethality of the constitutive null mutation, preclude analysis of the function of Mdm2 in a spatial and temporal specific manner.

In addition, these models do not allow firm establishment of the role of Mdm2 in the regulation of p53 stability. It was indeed shown that, beside the ability of Mdm2 to bind p53 in its transactivation domain and to interfere with p53-transcriptional activity, Mdm2 acts as an E3 ubiquitin ligase responsible for the ubiquitination of p53 and itself.[13-16] It was later proposed that Mdm2 mediates monomeric p53 ubiquitination on multiple lysine residues, rather than polyubiquitination, as previously thought.[17] Because chains of multiple ubiquitin molecules are necessary for efficient protein degradation, the data suggested that the enzymatic activity of Mdm2 might not be sufficient for optimal degradation of p53, and that other proteins must aid in polyubiquitination and degradation of p53 *in vivo*. More recent data indicated that Mdm2 differentially catalyzes either monoubiquitination or polyubiquitination of p53 in a dosage-dependent manner.[18] The authors proposed that Mdm2-mediated polyubiquitination and nuclear degradation occurs only in specific contexts, such as when Mdm2 is malignantly overexpressed. On the other hand, Mdm2-mediated monoubiquitination and subsequent cytoplasmic translocation of p53 may represent an important means of p53 regulation in unstressed cells, in which Mdm2 is maintained at low/physiological levels. In addition, in mice with the hypomorphic *mdm2* allele, the level of p53 protein was not coordinately increased, suggesting that Mdm2 can inhibit p53 function in a manner independent of degradation.[12] Moreover, other cellular ubiquitin ligases, such as Pirh2, Cop-1, yin-yang and ARF-BP1, were reported to also promote p53 ubiquitination and degradation.[19-22] Thus,

while Mdm2 is a key regulator of p53 function *in vivo*, p53 degradation may be mediated through both Mdm2-dependent and Mdm2-independent pathways *in vivo*.

Here, we show that specific inactivation of *mdm2* in terminally differentiated smooth muscle cells (SMCs) results, concomitantly with severe cell loss, in increased p53 protein levels and transcriptional activity. Interestingly, SMCs undergoing cell death did not show evidence of caspase-3 activation and DNA fragmentation, but displayed hallmarks of necrosis. Together, Mdm2 is critical for the regulation of p53 steady state levels and activity in quiescent cells *in vivo*. Moreover, the data indicate that increased p53 activity *in vivo* can lead to cell death with a necrotic morphotype, and consequently can go undetected when using apoptosis-specific methodology.

MATERIALS AND METHODS

Transgenic Mice

To achieve SMC-specific *mdm2* deletion we combined mice that carry a tamoxifen-inducible Cre-recombinase under control of the SMC specific SM22 promoter (SM-CreER^{T2}(ki) mice)[23] and mice carrying the *mdm2* gene modified by flanking exons 5 and 6 with two loxP sites (*mdm2*$^{FM/FM}$)[24] to create SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice. To determine p53 dependent effects of *mdm2* deletion SM-CreER^{T2}(ki); *mdm2*$^{FM/FM}$ mice were crossed with p53 knock out (p53$^{-/-}$) mice[25] resulting in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice. In addition, to facilitate the monitoring of Cre activity *in vivo*, we combined the SM-CreER^{T2}(ki) mice and the *Rosa*26 reporter mouse line[26] to generate SM-CreER^{T2}(ki);*Rosa*26 mice. The SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$, SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$, SM-CreER^{T2}(ki);R*osa*26 and their control littermates mice were born at the expected Mendelian frequency, developed normally and were genotyped by PCR, as described previously.[23,24,26]

Conditional deletion of *mdm2* and quantification of recombination

Mice, aged 8-10 weeks, were injected intra-peritoneally with 100 μl of 20mg/ml tamoxifen (TMX, Sigma) or vehicle (peanut oil) for 0, 2, 5, and 7 continuous days. Intra-peritoneal TMX injections did not result in liver toxicity as measured by serum alanine aminotransferase (ALAT) levels (40.6±12.7 for *mdm2*$^{FM/FM}$ mice vs. 36.3±16.0U/l for SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice; P=0.754, ALT, Roche). Recombination of the FM allele (226bp) was assessed by PCR.[24] Recombination in SM-CreER^{T2}(ki);*Rosa*26 mice was quantified by counting β-galactosidase positive (β-gal^{+}) cells and was expressed as a percentage of the total number of cells (Figure 1C).

Tissue preparation and histology

Mice were sacrificed and a complete gross necropsy was performed. Organs and selected tissues, including oesophagus, stomach, jejunum, proximal and distal ileum, colon, aorta, urinary bladder and liver were sampled for further investigation. Sampled organs were either directly snap frozen in sterile eppendorf tubes and stored at -80°C or fixed in phosphate buffered formalin pH 7.4 and embedded in

paraffin. Of the tissues sampled and fixed in formalin microscopical analysis was performed of 5 μm routinely stained hematoxylin-phloxin-saphron (HPS), hematoxylin-eosin (HE) or 4'-6-Diamidino-2-phenylindole (DAPI) sections. In addition, slides were stained with antibodies against SM-α-actin (clone 1A4, dilution 1:1500, DAKO), pro-caspase-3 (1:1000, Cell Signaling), cleaved form of caspase-3 (1:1000, Cell Signaling), p53 (CM5, 1:1000, Novocastra Lab Ltd.). DNA fragmentation (ISEL staining) was assessed with the FragEL kit (Oncogene Research Products) according to the manufacturer's directions and sections were counterstained with methyl green (Vector Laboratories).

LCM and Q-PCR

Laser Capture Microdissection (LCM) samples were prepared from frozen sections of three control and three SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice and pooled. Total RNA was extracted using the PicoPure RNA isolation kit and amplified using the RiboAmp RNA Amplification Kit according to manufacturer's instructions (Acturus Bioscience). 1 μg of total RNA from each pool was reverse-transcribed using a SuperScript kit (Invitrogen). These assays were performed following the manufacturer's specifications (PE Applied Biosystems). Primer pairs and TaqMan probes were designed by Applied Biosystems (Assays on demand).

Electron Microscopy

Tissue samples were immersed in a fixative solution of 2 % paraformaldehyde and 2.5 % glutaraldehyde and postfixed in 1% OsO4 with 1.5% K3Fe(CN)6 in 0.1 M NaCacodylate buffer, pH 7.2. Samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step followed by embedding in Spurr's resin. Ultra thin sections, made on a Ultracut E microtome (Reichert-Jung), were post-stained in an ultrostainer (Leica,Herburgg, Switzerland) with uranyl acetate and lead citrate. Sections were viewed with a transmission electron microscope 1010 (JEOL,Tokyo, Japan).

Statistical Analysis

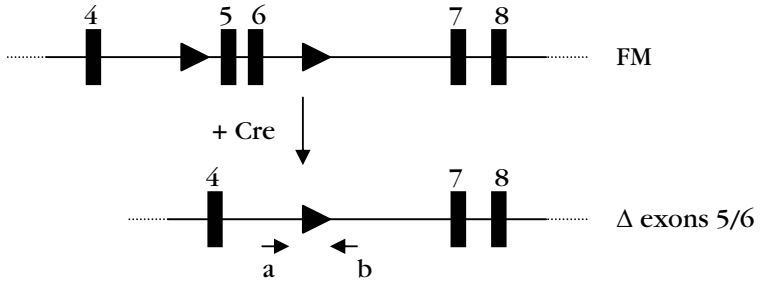
All data are represented as mean±SD. Data were analysed using the non-parametric Mann-Whitney rank sum test. P-values less than 0.05 were regarded as statistically significant.

RESULTS

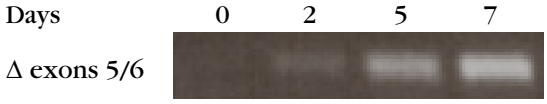
Strategy for conditional bi-allelic inactivation of *mdm2* in quiescent smooth muscle cells *in vivo*

To test whether Mdm2 is required for regulating p53 stability and activity in quiescent cells *in vivo*, we specifically inactivated *mdm2* in G_0 smooth muscle cells (SMCs). To this end, conditional inactivation of *mdm2* was carried out in mice harbouring *mdm2* floxed alleles and a tamoxifen (TMX)-inducible Cre-recombinase under control of the SM22 promoter (SM-CreER^{T2}(ki) mice).[23] The *mdm2* floxed allele (FM) had been previously described.[24] It carries a *lox*P recombination site in

A.



B.



C.

Oesophagus	Stomach	Proximal Ileum	Distal Ileum	Colon	Bladder	Aorta
7.5±2.5	61.5±8.8	55.3±4.2	39.8±3.1	47.4±5.5	92.5±4.2	6.7±3.1

D.

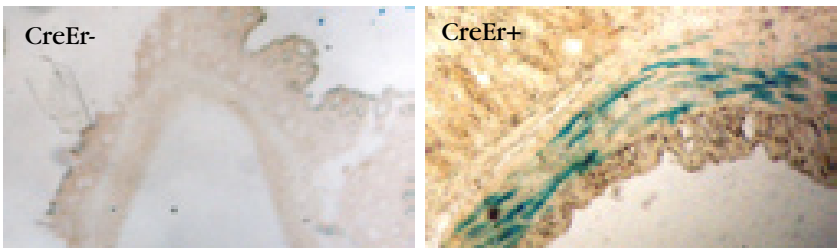


Figure 1. (A.) Schematic representation of part of the *mdm2* floxed allele (FM) and the Cre-mediated recombination event. The a and b arrows designated the position of the primers used to detect the Cre-mediated recombination by PCR. (B.) PCR analysis showing increased detection of the Cre-mediated recombination event, using primers a and b, in the intestine of CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice treated for 0, 2, 5 and 7 days with TMX. (C.) Percentage of recombination of SMC-rich SM-CreERT2(ki);*Rosa*26 organs/tissues after 7 days of intraperitonal TMX administration. No recombination was observed in TMX or vehicle-treated control *Rosa*26 mice. (D.) Detection of *lacZ* reporter gene expression in the stomach of SM-CreERT2(ki);*Rosa*26 mice and *Rosa*26;CreER-negative control mice after 7 days of TMX administration.

intron 4 and another in intron 6 (Figure 1A). Cre-mediated recombination therefore yields an *mdm2* allele lacking exons 5 and 6, which encode for most of the p53-binding domain. Mice homozygous for the FM allele (or *mdm2*$^{FM/FM}$) appear normal; however, ubiquitous deletion of exons 5 and 6 *in vivo* results in an embryonic lethality similar to the *mdm2* null allele.[24]

We first examined by PCR the extent of recombination at the *mdm2* locus in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (Figure 1B) and compared it with the Cre activity at the *Rosa26* locus in SM-CreER^{T2}(ki);*Rosa*26 mice at various sites containing SMCs (Figure 1C). SMCs were identified both morphologically and immunohistochemically by SM-α-actin staining (Figure 2). In order to induce the latent CreER fusion protein, mice were injected daily with TMX for seven days, and then analyzed. Upon

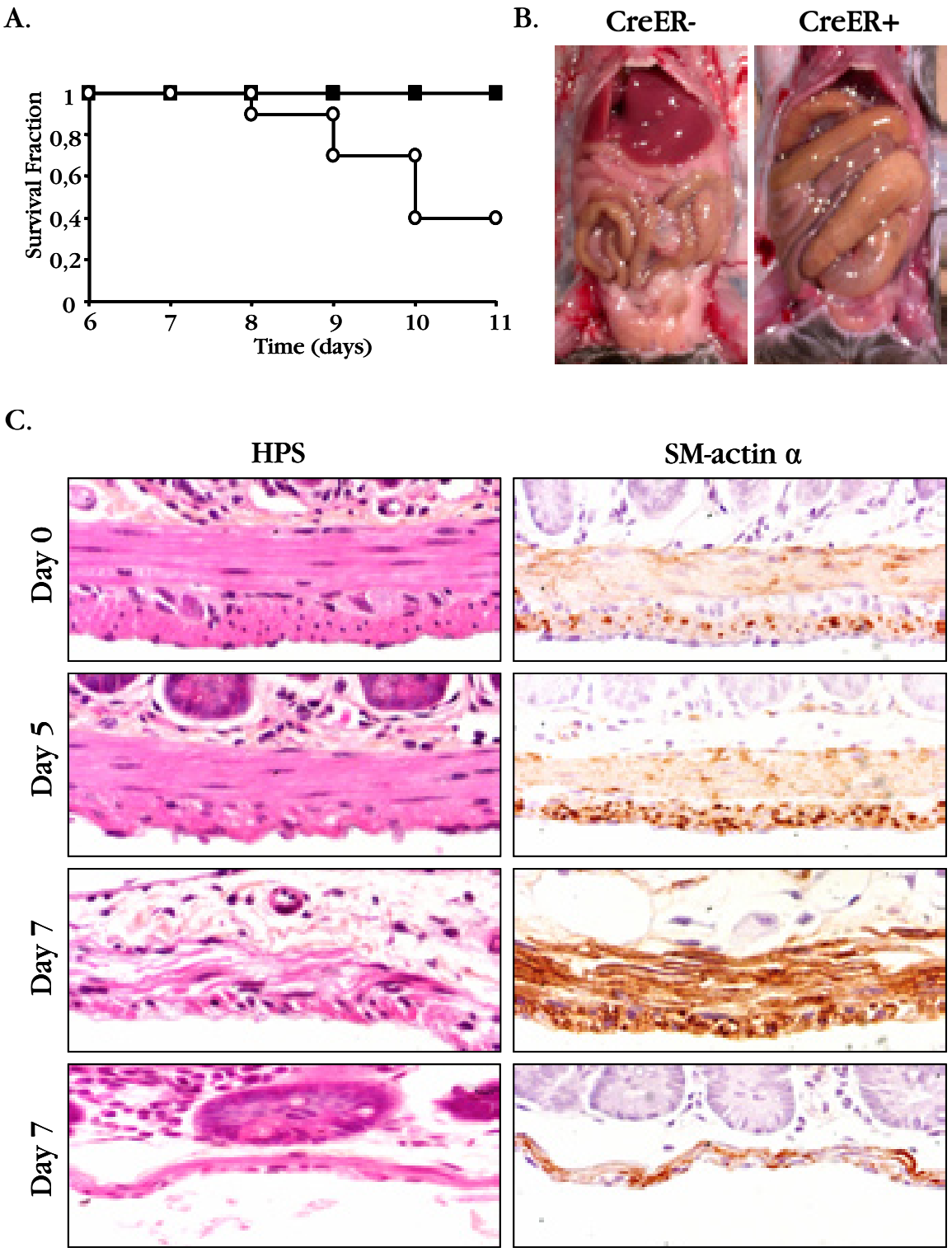


Figure 2. (A.) Kaplan-Meier curves of age-matched CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (open circles, n=21) and control CreER-negative mice (filled squares, n=15). Mice were TMX-treated for 7 consecutive days and followed thereafter. (B.) Gross appearance of control (left panel) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (right panel) after 7 days of intra-peritoneal tamoxifen administration. (C.) HPS (left panels) and IHC for SM-α-actin (right panels) staining of the small intestine of CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice 0, 5, 7 days following TMX treatment (magn. 200x).

TMX injection, we found that SMCs from the gastroinstestinal (GI) tract, particularly the stomach (Figure 1D) and proximal ileum, were stably marked (β-gal$^+$), whereas little reporter activity was found in cells of the cardiovascular system such as in the aorta. Since efficient recombination of the *mdm2* locus was observed in the SMCs of the small intestines (proximal ileum), we concentrated our studies at this site.

TMX-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice exhibit severe lesions in the SMC-containing layers of the intestinal wall and eventually die

Following 7 days of TMX administrations, the body weight of SM-CreER^{T2}(ki); *mdm2*$^{FM/FM}$ mice decreased as compared to TMX-treated control Cre-ER-negative mice. Moreover, SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice were not responsive to stimuli and were hunchbacked with ruffled coat. Strikingly, illness proceeded to death from day 8 on (Figure 2A). In contrast, TMX-treated *mdm2*$^{FM/FM}$ CreER-negative mice appeared normal and did not differ from vehicle-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ and vehicle treated *mdm2*$^{FM/FM}$ CreER-negative mice. SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice were sacrificed for gross necropsy and histopathological analysis.

The stomach and small intestine of TMX-treated SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice adhered to spleen and liver, appeared vulnerable and friable, were filled with soft materials, and were loose. The consistently abnormal findings included liver and spleen atrophy and dilation of the small intestinal, which varied between mice, but could be considerable (Figure 2B). This dilation was associated with a decreased length of the small intestine (from pylorus to ileo-cecal junction: 44.0±3.5 for control mice, 32.0±2.0 cm for SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice; P=0.007).

The lesions in the dilated small intestine of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice treated with TMX were limited to the lamina interna and externa of the muscularis (M.) propria of the intestinal wall, consistent with a specific activity of the Cre in the SMCs. The architecture of the lamina M. interna was disturbed due to a decrease in cell layers and irregular alignment of the SMCs. The number of SMC layers was reduced from 6-8 to 3-5, with multifocal irregular increase of intercellular spaces, tapering of SMCs with wavy ends and, irregular, often shortened hyperchromatic nuclei (Figure 2C). In some extreme cases, the SMCs were simply either missing or unrecognizable.

Alterations of the SMC-containing intestinal wall and lethality upon SMC-specific *mdm2* inactivation are entirely p53-dependent

A large body of evidence suggests that Mdm2 can function both dependently and independently of p53. In agreement, Mdm2 binds several proteins involved in the regulation of cell cycle progression and survival other than p53, such as p19/ARF, p63 and p73, Rb, and E2F-1/DP-1.[27] In order to test whether the phenotype observed in the SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice is p53-dependent, they were crossed with p53-null mice (p53$^{-/-}$)[25] to create SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice. Strikingly, as observed in control mice, TMX injection in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice did not cause death (Figure 3A). Gross necropsy did not reveal differences in liver and spleen weight and small intestinal length as compared to control mice. Histological examination did not reveal any obvious lesions, disorganization of intestinal cell alignment or loss of cell viability in SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ (Figure 3B,

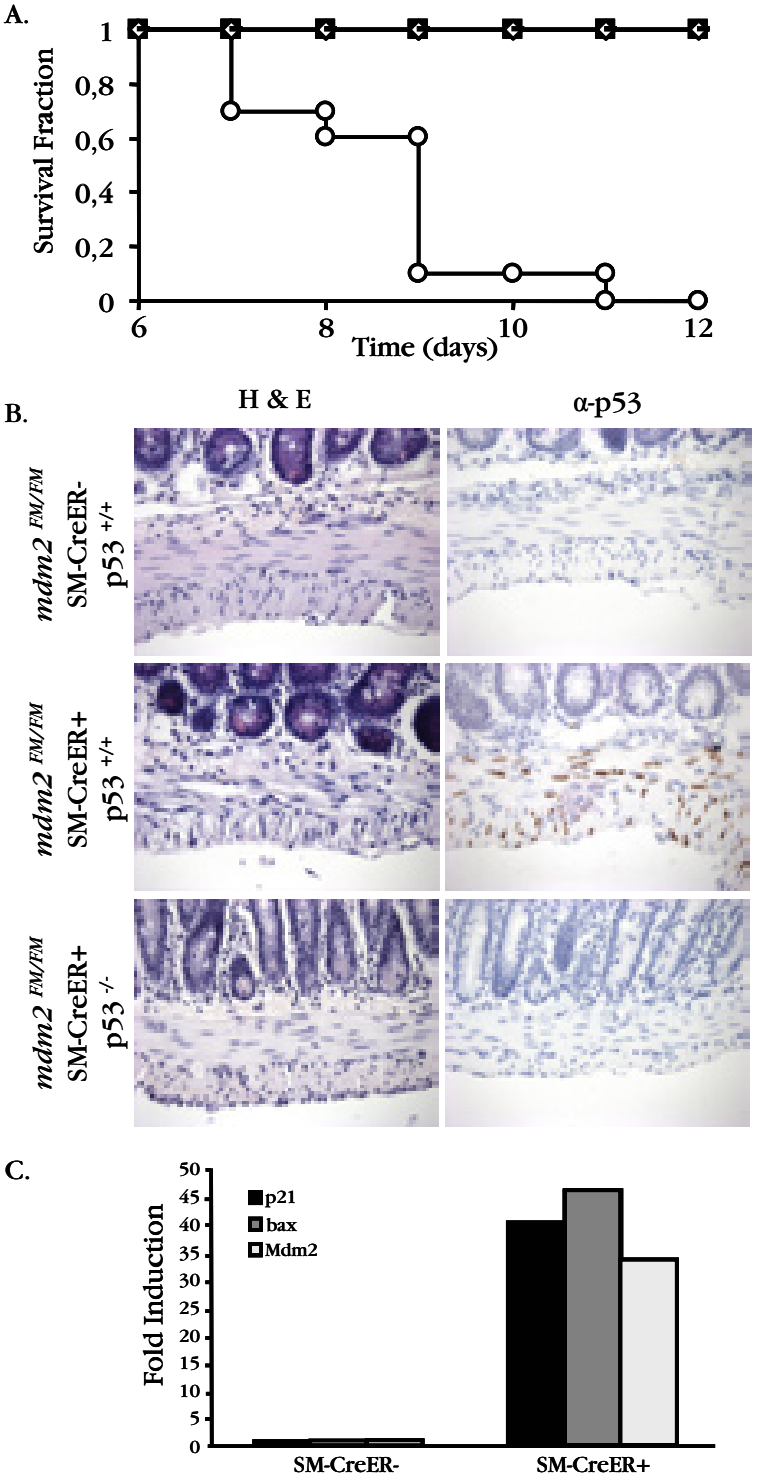


Figure 3. (A.) Kaplan-Meier curves of age-matched CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice (open circles, n=11) and control *mdm2*$^{FM/FM}$;CreER-negative mice (filled squares, n=10) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$;*p53*$^{-/-}$ mice (open diamonds, n=6) following 7 days of intra-peritoneal TMX administration. (B.) H&E staining and p53 immunostaining of the small intestine of mice with the indicated genotypes (magn. 60x). (C.) Q-RT-PCR analysis shows induction of expression of p53 target genes in laser-capture microdissected SMCs of mice with the indicated genotypes. Microdissected samples from three different mice were pooled before the analysis. The data represent therefore the mean expression levels in these three mice treated independently.

left panels). We therefore concluded that loss of *mdm2* in the SMCs of the GI tract causes loss of cell viability and acute lethality in a manner that is completely dependent on the presence of functional p53.

SMC-specific *mdm2* inactivation leads to increased p53 stability and transcriptional activity

To determine whether specific deletion of *mdm2* in the SMCs allows the level of p53 protein to increase, we performed immunostaining for p53. In sections of the proximal ileum from *mdm2*$^{FM/FM}$;CreER-negative mice injected with TMX, no p53 staining could be detected (Figure 3B). In contrast, nuclei of the SMCs of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice showed marked p53 immunoreactivity (Figure 3B, right panels). Importantly, no staining was observed in sections from SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$;p53$^{-/-}$ mice, confirming the specificity of the p53 detection method (Figure 3B). These results suggest that p53 is maintained at low levels in a strict Mdm2-dependent manner in terminally differentiated SMCs. In addition, p53 was not only stabilized but it was also functionally active, as indicated by concomitant upregulation of several p53-target genes such as *p21*$^{Waf1/Cip1}$ and the pro-apoptotic gene *bax* in laser-capture microdissected-SMCs in the proximal ileum of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice, as determined by Q-RT-PCR (Figure 3C). Of note, *mdm2* Δexons5/6 transcripts, were also found upregulated in the SMCs of SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice.

SMC-specific *mdm2* inactivation does not cause apoptotic cell death

Since one of the main p53 antiproliferative activities is induction of caspase-dependent apoptotic cell death, we hypothesized that the hypocellularity observed following *mdm2* inactivation was a consequence of increased p53-mediated apoptosis. To investigate this possibility, we first performed staining using an antibody specifically recognizing the activated form of the main effector caspase, caspase-3. No significant activation of caspase-3 could be observed both in control and SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$TMX-treated mice (Figure 4). Interestingly, the non-activated form of caspase-3 (pro-caspase-3) was not significantly detected in the SMCs of both control (CreER-negative) and SM-CreER^{T2}(ki);*mdm2*$^{FM/FM}$TMX-treated mice (Figure 4). This observation, thus, provides a simple explanation for absence of activated caspase-3 in the SMCs of TMX-treated mice. Absence of caspase-3 protein was also previously reported in rat and mouse adult skeletal muscles, despite the abundant presence of its mRNA.[28] Since other effector caspases might compensate for the lack of caspase-3 in these cells, we explored the presence of other apoptotic signatures. Apoptosis is morphologically defined by several hallmarks including nuclear shrinkage (pyknosis), chromatin condensation, DNA degradation and nuclear fragmentation (karyorrhexis) with formation of apoptotic bodies. Examination of H&E or DAPI staining did not reveal the presence of apoptotic bodies or pyknotic nuclei. Moreover, *in situ* end labeling (ISEL)[29] (Figure 4) and TUNEL assays (data not shown) did not detect DNA fragmentation. To verify that our detection methods could detect both pro-caspase-3 and apoptosis, sections of E15.5 embryos expressing p53 specifically in post-mitotic neurons deficient for mdm2 were analyzed (Francoz *et al.*, submitted 2005). Clear and specific staining for pro-caspase-3, the activated form

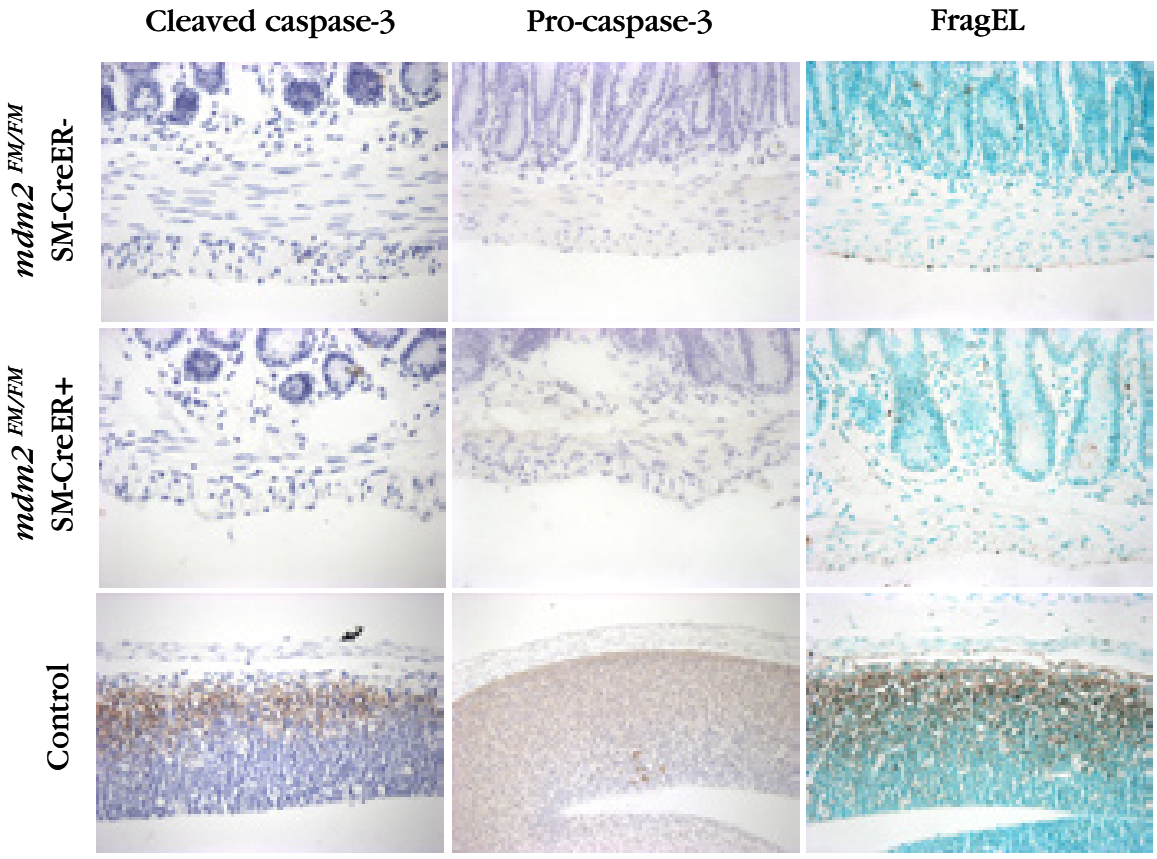


Figure 4. Immunostaining for the activated form of caspase-3 and pro-caspase-3 (non-cleaved caspase-3) and ISEL staining of the small intestine of mice with the indicated genotypes and in the lateral ventricle region of the cerebral cortex of E16.6 embryos expressing p53 specifically in post-mitotic neurons deficient for *mdm2* (control).

of caspase-3 and DNA fragmentation were detected in these embryos (Figure 4), confirming the sensitivity and the specificity of both methods. Together, the observed phenotype and multifocal irregular increase of intercellular spaces with tapering SMCs is not suggested to coincide with an increase in apoptotic cell death.

Evidence for p53-dependent necrotic cell death following SMC-specific *mdm2* inactivation

Electron microscopy (EM) confirmed absence of pyknosis and karyorrhexis with formation of apoptotic bodies. However, EM revealed that many of the remaining SMCs had a morphotype identical to that seen in necrosis[30,31] including a "mottled" nucleus caused by clumped, but not marginalised, and only loosely packed chromatin (Figure 5). Most of the nuclei appeared largely intact, however, in some cases nuclear membrane detachment and rupture were apparent. The affected cells also showed dilated mitochondria and cytoplasmic vacuoles (not shown). External membrane rupture and swelling of cytoplasmic organelles was also evident (Figure 5). In agreement, clusters of roughly intact nuclei, which are no longer surrounded by cytoplasmic membranes, were also observed (Figure 5). Various intermediate aspects could be observed, which ranged from apparently intact cells that had a mottled nucleus, to cells that had a mottled nucleus and gross membrane alterations. Finally,

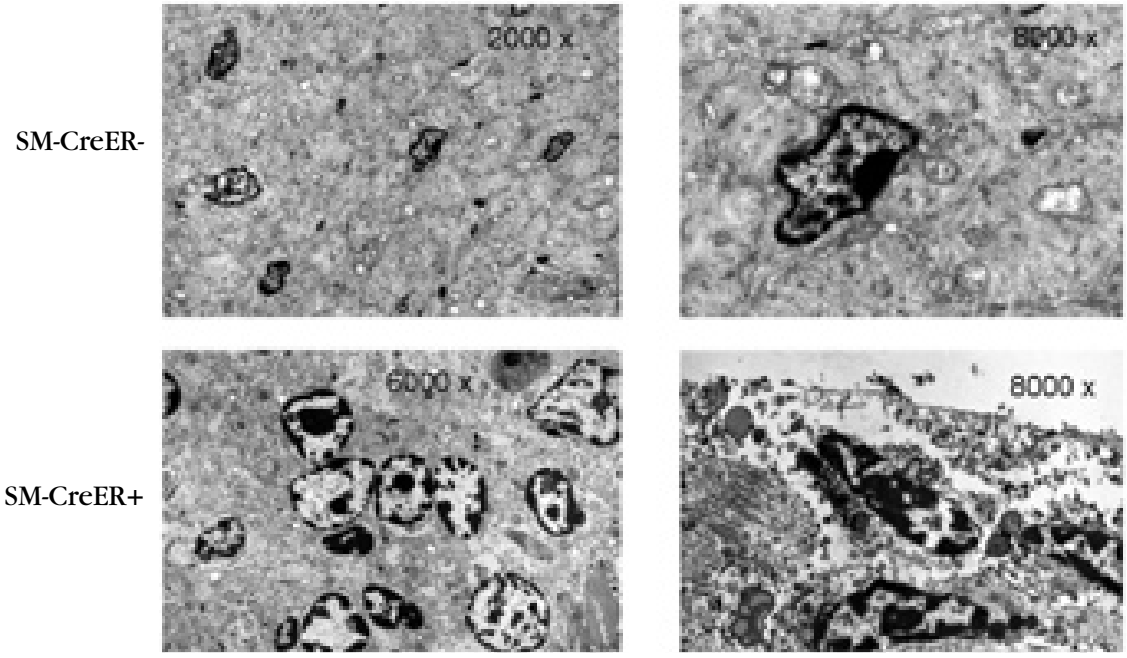


Figure 5. Electron microscopy of the small intestine of control (creER-negative; top panels) and CreER^{T2}(ki);*mdm2*$^{FM/FM}$ (low panels) TMX-treated mice. Nuclei in control tissues are sparse, equally distributed and surrounded by a plasma membrane. Nuclei in tissues from CreER^{T2}(ki);*mdm2*$^{FM/FM}$ mice are clustered and not surrounded by a plasma membrane. Evidence of plasma membrane ruptures is shown in the bottom right panel. Magnifications are indicated.

whereas apoptosis includes phagocytosis, even heavily altered cells appeared not to be within phagocytes. Surprisingly, absence of phagocytosis did not lead in this case to local signs of inflammation, but this absence might be related to local anatomical constraints.

DISCUSSION

It is well documented that the p53 protein is maintained at low levels in embryonic and adult tissues; however, it remained to be established whether p53 degradation occurs in a strict Mdm2-dependent manner. In addition, while downregulation of Mdm2 appears to be sufficient to activate a p53 response in homeostatic tissues,[12] its relevance in non-proliferating, terminally differentiated cells remained unexplored. Here, we provide the first genetic evidence that Mdm2 plays a key role in the regulation of p53 levels and activity in quiescent, terminally differentiated, SMCs.

Notably, we have recently confirmed this finding in other cellular contexts. Indeed, conditional inactivation of mdm2 was achieved in mouse embryonic fibroblasts (MEFs), in neuronal stem/progenitor cells and in post-mitotic neurons (Francoz et al., submitted 2005), in erythroid progenitor cells (Maetens et al., in preparation), in thymocytes and cardiac muscle cells (G. Lozano, Personal communication). In all these settings, an increase in p53 levels and activity was observed and this activation of function resulted in caspase 3-dependent cell death *in vivo*. Thus, even if other E3 protein ligases have been reported to induce p53 ubiquitination and

degradation,[19-22] none of these proteins can fully compensate for loss of Mdm2 function *in vivo*. Therefore, even if these data do not exclude the possibility that these proteins might aid in p53 degradation, they strongly suggest that p53 degradation *in vivo* occurs through Mdm2-dependent pathways.

Mdm2 ubiquitination activity and the physical interaction between Mdm2 and p53 have become the targets of adjuvant chemotherapies designed to sensitize human tumors to cancer therapies.[32-35] Indeed, there is evidence that cancer cells are more sensitive to activation of p53 apoptotic function than the resting host cells. However, the data presented herein suggest that Mdm2 is critical for maintaining p53 activity at low levels also in quiescent, terminally differentiated cells. Therefore, this study raises concerns about the benefit of such approaches for the patients. Indeed, our data would predict that Mdm2 inhibition of function *in vivo* would be detrimental not only to cancer cells but also to most of the resting host cells.

Interestingly, the data presented herein also provide the first compiling evidence that p53 is able to activate a caspase-3-independent cell death program with a necrotic morphotype *in vivo*. All studies converge to the crucial role of both the mitochondrial pathway (cytochrome c release,[36] ROS production and/or $\Delta\psi$m drop)[37] and activation of caspase-9 and its downstream caspases[38] in p53-induced apoptosis. In agreement with this view, Apaf-1[39] and several genes encoding for the BH3-containing proapoptotic proteins such as Bax, Noxa and Puma are among the many reported p53-regulated genes and key mediators of p53-induced apoptosis *in vivo*.[40,41] Caspase-3, the main effector caspase acting dowstream of caspase-9, is also of fundamental importance for many forms of apoptosis[42] and required for p53-induced apoptosis. Since we show here that caspase-3 protein is either absent or expressed at very low levels (below detectable levels) in the SMCs, these data provide a simple explanation for our failure to detect apoptosis, despite the fact that p53 was stabilized and transcriptionally active in these cells. One possibility is that p53 once activated provokes, through some of its mediators, mitochondrial damages that are sufficient to induce cell death in a caspase-independent manner. We could indeed detect in *mdm2*-deficient SMCs high levels of expression of bax, a protein particularly important for the triggering of mitochondrial outer membrane permeabilization (MOMP) and cytochrome c release. MOMP frequently marks the "point of no return" of the lethal process. It is clear today that artificial or genetic inhibition of caspases is often not sufficient to avoid physiological cell death, and in this case this often leads to a shift in the morphology of cell death, from classical apoptosis to "apoptosis-like cell death", autophagic cell death or necrosis.[43] For instance, the loss of interdigital cells in the mouse embryo, a prototype of mitochondrial apoptotic cell death, still occurs by necrosis in mice deficient for Apaf-1.[30] Similarly, because they do not express caspase-3, the *mdm2*-deficient SMCs could undergo necrotic cell death following activation of MOMP by p53. In order to test whether necrotic cell death observed in these cells occurs through a MOMP-dependent mechanism, one could check whether overexpression of Bcl-2, a BH3-containing protein that can inhibit mitochondrial channel opening, is able to rescue the phenotype. Alternatively, eventhough less likely, p53 might be able to activate directly a genetic program leading to necrotic cell death *in vivo* in a tissue-specific manner. Interestingly, p53 was recently shown to be able to activate a MOMP-independent (and Bcl-2

insensitive) cell death program in cultured cells.[44] The existence of such a pathway *in vivo* remains, however, to be demonstrated. A careful analysis of the transcription program activated by p53 in the *mdm2*-deficient SMCs might help to identify key mediator(s) of this putative pathway.

Regardless the molecular mechanism, this study opens new perspectives for cancer therapies. For instance, inactivation of Apaf-1, which is essential for p53-induced caspase-dependent apoptosis, may contribute to the low frequency of p53 mutations observed in therapy-resistant melanomas.[38] The ability of p53 to induce a caspase-independent and MOMP-independent type of cell death may be the basis for new therapies killing cells in which p53 is wild-type but have acquired defects in the signaling pathways that are downstream p53.

ACKNOWLEDGMENTS

We thank the technical staffs of TNO-Biomedical Research, Dieter Defever, Ines Bonk and Riet De Rycke for their excellent technical assistance. We thank Guillermina Lozano for the *mdm2* conditional knockout mice. This work was supported by Public Health Service grant 2000B051 from the Netherlands Heart Foundation and by grants 902-26-242 and 912-02-03 from NWO/ ZonMw. The research of B.J.M. van Vlijmen has been made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. S. Francoz was supported by grants from "Télévie (FNRS)". This work was supported in part by grants from Association for International Cancer Research (J-C Marine), Belgium Federation against Cancer (non profit organization; J-C Marine) and by EC FP6 funding (AG Jochemsen and J-C Marine). This publication reflects only author's views. The commission is not liable for any use that may be made of the information herein.

REFERENCES

1. Lane DP. Cancer. p53, guardian of the genome. Nature 1992;358:15-16.
2. el Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.
3. Doumont G, Martoriati A, Beekman C, Bogaerts S, Mee PJ, Bureau F, Colombo E, Alcalay M, Bellefroid E, Marchesi F, Scanziani E, Pelicci PG, Marine JC. G1 checkpoint failure and increased tumor susceptibility in mice lacking the novel p53 target Ptprv. EMBO J 2005;24:3093-3103.
4. Chipuk JE, Green DR. p53's believe it or not: lessons on transcription-independent death. J Clin Immunol 2003;23:355-361.
5. Momand J, Jung D, Wilczynski S, Niland J. The MDM2 gene amplification database. Nucleic Acids Res 1998;26:3453-3459.
6. Riemenschneider MJ, Buschges R, Wolter M, Reifenberger J, Bostrom J, Kraus JA, Schlegel U, Reifenberger G. Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. Cancer Res 1999;59:6091-6096.
7. Fakharzadeh SS, Trusko SP, George DL. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J 1991;10:1565-1569.
8. Cahilly-Snyder L, Yang-Feng T, Francke U, George DL. Molecular analysis and

chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somat Cell Mol Genet 1987;13:235-244.
9. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237-1245.
10. Jones SN, Roe AE, Donehower LA, Bradley A. Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature 1995;378:206-208.
11. Montes de Oca LR, Wagner DS, Lozano G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature 1995;378:203-206.
12. Mendrysa SM, McElwee MK, Michalowski J, O'Leary KA, Young KM, Perry ME. mdm2 Is critical for inhibition of p53 during lymphopoiesis and the response to ionizing irradiation. Mol Cell Biol 2003;23:462-472.
13. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes the rapid degradation of p53. Nature 1997;387:296-299.
14. Honda R, Tanaka H, Yasuda H. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett 1997;420:25-27.
15. Honda R, Yasuda H. Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. Oncogene 2000;19:1473-1476.
16. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. Nature 1997;387:299-303.
17. Lai Z, Ferry KV, Diamond MA, Wee KE, Kim YB, Ma J, Yang T, Benfield PA, Copeland RA, Auger KR. Human mdm2 mediates multiple mono-ubiquitination of p53 by a mechanism requiring enzyme isomerization. J Biol Chem 2001;276:31357-31367.
18. Li M, Brooks CL, Wu-Baer F, Chen D, Baer R, Gu W. Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science 2003;302:1972-1975.
19. Chen D, Kon N, Li M, Zhang W, Qin J, Gu W. ARF-BP1/Mule is a critical mediator of the ARF tumor suppressor. Cell 2005;121:1071-1083.
20. Dornan D, Wertz I, Shimizu H, Arnott D, Frantz GD, Dowd P, O'Rourke K, Koeppen H, Dixit VM. The ubiquitin ligase COP1 is a critical negative regulator of p53. Nature 2004;429:86-92.
21. Leng RP, Lin Y, Ma W, Wu H, Lemmers B, Chung S, Parant JM, Lozano G, Hakem R, Benchimol S. Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. Cell 2003;112:779-791.
22. Sui G, Affar eB, Shi Y, Brignone C, Wall NR, Yin P, Donohoe M, Luke MP, Calvo D, Grossman SR, Shi Y. Yin Yang 1 is a negative regulator of p53. Cell 2004;117:859-872.
23. Kuhbandner S, Brummer S, Metzger D, Chambon P, Hofmann F, Feil R. Temporally controlled somatic mutagenesis in smooth muscle. Genesis 2000;28:15-22.
24. Grier JD, Yan W, Lozano G. Conditional allele of mdm2 which encodes a p53 inhibitor. Genesis 2002;32:145-147.
25. Jacks T, Remington L, Williams BO, Schmitt EM, Halachmi S, Bronson RT, Weinberg RA. Tumor spectrum analysis in p53-mutant mice. Curr Biol 1994;4:1-7.
26. Mao X, Fujiwara Y, Orkin SH. Improved reporter strain for monitoring Cre recombinase-mediated DNA excisions in mice. Proc Natl Acad Sci U S A 1999;96:5037-5042.
27. Iwakuma T, Lozano G. MDM2, an introduction. Mol Cancer Res 2003;1:993-1000.
28. Ruest LB, Khalyfa A, Wang E. Development-dependent disappearance of caspase-3 in skeletal muscle is post-transcriptionally regulated. J Cell Biochem 2002;86:21-28.
29. Gavrieli Y, Sherman Y, Ben Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119:493-501.
30. Chautan M, Chazal G, Cecconi F, Gruss P, Golstein P. Interdigital cell death can occur through a necrotic and caspase-independent pathway. Curr Biol 1999;9:967-970.
31. Searle J, Kerr JF, Bishop CJ. Necrosis and apoptosis: distinct modes of cell death with fundamentally different significance. Pathol Annu 1982;17 Pt 2:229-259.
32. Issaeva N, Bozko P, Enge M, Protopopova M, Verhoef LG, Masucci M, Pramanik A, Selivanova G. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. Nat Med 2004;10:1321-1328.

33. Midgley CA, Desterro JM, Saville MK, Howard S, Sparks A, Hay RT, Lane DP. An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene 2000;19:2312-2323.
34. Vassilev LT. Small-molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. Cell Cycle 2004;3:419-421.
35. Yang Y, Ludwig RL, Jensen JP, Pierre SA, Medaglia MV, Davydov IV, Safiran YJ, Oberoi P, Kenten JH, Phillips AC, Weissman AM, Vousden KH. Small molecule inhibitors of HDM2 ubiquitin ligase activity stabilize and activate p53 in cells. Cancer Cell 2005;7:547-559.
36. Gao CF, Ren S, Zhang L, Nakajima T, Ichinose S, Hara T, Koike K, Tsuchida N. Caspase-dependent cytosolic release of cytochrome c and membrane translocation of Bax in p53-induced apoptosis. Exp Cell Res 2001;265:145-151.
37. Li PF, Dietz R, von Harsdorf R. p53 regulates mitochondrial membrane potential through reactive oxygen species and induces cytochrome c-independent apoptosis blocked by Bcl-2. EMBO J 1999;18:6027-6036.
38. Soengas MS, Alarcon RM, Yoshida H, Giaccia AJ, Hakem R, Mak TW, Lowe SW. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science 1999;284:156-159.
39. Moroni MC, Hickman ES, Lazzerini DE, Caprara G, Colli E, Cecconi F, Muller H, Helin K. Apaf-1 is a transcriptional target for E2F and p53. Nat Cell Biol 2001;3:552-558.
40. Jeffers JR, Parganas E, Lee Y, Yang C, Wang J, Brennan J, MacLean KH, Han J, Chittenden T, Ihle JN, McKinnon PJ, Cleveland JL, Zambetti GP. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 2003;4:321-328.
41. Michalak E, Villunger A, Erlacher M, Strasser A. Death squads enlisted by the tumour suppressor p53. Biochem Biophys Res Commun 2005;331:786-798.
42. Woo M, Hakem R, Soengas MS, Duncan GS, Shahinian A, Kagi D, Hakem A, McCurrach M, Khoo W, Kaufman SA, Senaldi G, Howard T, Lowe SW, Mak TW. Essential contribution of caspase 3/CPP32 to apoptosis and its associated nuclear changes. Genes Dev 1998;12:806-819.
43. Kroemer G, Martin SJ. Caspase-independent cell death. Nat Med 2005;11:725-730.
44. Godefroy N, Lemaire C, Renaud F, Rincheval V, Perez S, Parvu-Ferecatu I, Mignotte B, Vayssiere JL. p53 can promote mitochondria- and caspase-independent apoptosis. Cell Death Differ 2004;11:785-787.