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Inactivation of the MDM2 RING domain enhances p53 transcriptional activity in mice

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Running Title: *MDM2^{C462A} mutation activates p53*
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ABSTRACT

The MDM2 RING domain harbors E3 ubiquitin ligase activity critical for regulating the degradation of tumor suppressor p53, which controls many cellular pathways. The MDM2 RING domain also is required for an interaction with MDMX. Mice containing a substitution in the MDM2 RING domain, MDM2^{C462A}, disrupting MDM2 E3 function and the MDMX interaction, die during early embryogenesis that can be rescued by p53 deletion. To investigate whether MDM2^{C462A}, which retains p53 binding, has p53-suppressing activity, we generated *Mdm2$^{C462A/C462A}$;p53$^{ER/-}$* mice, in which we replaced the endogenous *p53* alleles with an inducible *p53$^{ER/-}$* allele, and compared survival with that of similarly generated *Mdm2$^{-/-}$;p53$^{ER/-}$* mice. Adult *Mdm2*-null mice died ~7 days after tamoxifen-induced p53 activation, indicating that in the absence of MDM2, MDMX cannot suppress p53. Surprisingly, *Mdm2$^{C462A/C462A}$;p53$^{ER/-}$* mice died ~5 days after tamoxifen injection, suggesting that p53 activity is higher in the presence of MDM2^{C462A} than in the absence of MDM2. Indeed, in MDM2^{C462A}-expressing mouse tissues and embryonic fibroblasts, p53 exhibited higher transcriptional activity than in those expressing no MDM2 or no MDM2 and MDMX. This observation indicated that MDM2^{C462A} not only is unable to suppress p53 but may have gained the ability to enhance p53 activity. We also found that p53 acetylation, a measure of p53 transcriptional activity, was higher in the presence of MDM2^{C462A} than in the absence of MDM2. These results reveal an unexpected role of MDM2^{C462A} in enhancing p53 activity and suggest the possibility that compounds targeting MDM2 RING domain function could produce even more robust p53 activation.

INTRODUCTION

As a transcription factor, p53 induces the transcription of several downstream targets in the presence of both intrinsic and extrinsic cellular stress signals; many of these targets regulate the cell cycle, apoptosis, DNA repair, senescence, and angiogenesis, and other cellular pathways (*1-4*). The activation of p53-dependent pathways is thought to prevent and resist tumor development and growth by preventing the proliferation of damaged cells with oncogenic potential. For that reason, p53 has been called "the guardian of genome" due to its indispensable contribution to genome integrity (*5-7*). However, because of its global and heterogeneous functions, endogenous p53 activity must also be carefully regulated.

Murine double minute 2 (MDM2) and 4 (MDM4, also known as MDMX) are widely considered to be the primary negative regulators of p53 (*8, 9*). Considering that *Mdm2* and *MdmX* knockout mice exhibit p53-dependent early embryonic lethality, MDM2 and MDMX play unique and nonredundant roles in the control of p53 activation (*10-12*). Though MDM2 and MDMX are structurally similar, they exhibit differing activities towards p53 regulation.

Through its RING domain, MDM2 not only functions as an E3 ubiquitin ligase for p53 (*13-15*) but also binds to MDMX in a heterodimer that is required for p53 regulation during embryogenesis (*16, 17*). Recent evidence has demonstrated that the RING finger motif of MDM2 is indispensable for the suppression of p53 activity (*18, 19*), and we have previously demonstrated that the MDM2 RING domain plays a crucial role in p53 regulation during embryonic development (*14*). Interestingly, two functions of the MDM2 RING domain, E3 ubiquitin ligase activity and MDMX binding, have been found to be independently dispensable in unstressed adult mice (*15, 17*). Thus, the function of RING domain towards the regulation of physiological p53 transcriptional activity in the adult mouse still remains inadequately understood. In this study we sought to address the following questions: (1) Does the MDM2 RING domain function to regulate p53 transcriptional activity in adult mice? (2) Is MDM2-p53 binding sufficient for p53 regulation and adult organismal survival? (3) In the absence of MDM2 or MDM2 RING domain function, does MDMX play a role in regulating p53 transcriptional activity in adult mice?

To study the role of the MDM2 RING domain *in vivo*, we crossed mice bearing a single residue substitution in the MDM2 RING domain (*Mdm2*$^{C462A/C462A}$) that abolishes several key RING domain functions, including MDM2 E3 ligase activity and MDM2-MDMX binding. Since *Mdm2*$^{C462A/C462A}$ single-residue substitution results in the embryonic lethality, we substituted endogenous *p53* with *p53*$^{ER/-}$, an inactive p53 estrogen receptor fusion allele, which allows for tamoxifen mediated p53 functional restoration (*20*). At the same time, we generated *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ as control groups in order to define the function of the MDM2 RING domain on regulation of physiological p53. Strikingly, following p53^{ER} functional restoration, mice bearing the MDM2^{C462A} mutation die earlier than mice bearing MDM2 deletion, and this early lethality correlates with increased p53 activity. These results provide evidence to suggest that loss of MDM2 RING domain function through MDM2^{C462A} mutation stimulates spontaneous p53 activation beyond what occurs when MDM2 is deleted.

RESULTS

Mice expressing a functionally inactive Mdm2 RING domain exhibit early lethality following p53 activation—In vitro, MDM2 has been shown to modulate p53 transcriptional activity by direct inhibitory binding to the p53 transactivation domain (*21, 22*). The RING domain of MDM2, which is important for MDM2-mediated p53 ubiquitination and degradation and MDM2-MDMX heterodimer formation, is also critical for p53 regulation *in vivo*. Several studies using mouse models have suggested that each of these RING domain functions are important to p53 regulation during embryogenesis; however, MDM2-MDMX binding and MDM2 E3 ligase activity have been shown to be independently dispensable in the adult mouse (*14, 15, 17*). To confirm whether MDM2 retains p53 inhibitory capabilities in the absence of either of the canonical MDM2 RING domain functions in adult mice, we utilized mice bearing the MDM2^{C462A} mutation (*Mdm2*$^{C462A/C462A}$), which disrupts both MDM2-MDMX binding and MDM2 E3 ligase function. Because *Mdm2*$^{C462A/C462A}$ mice exhibit p53-dependent embryonic lethality (*14*), we also utilized mice bearing the inactive p53 estrogen receptor fusion allele (hereafter referred to as *p53*^{ER}) which allows for tamoxifen mediated p53 functional restoration (*20*). We crossed *Mdm2*$^{C462A/C462A}$ mice with *p53*$^{ER/-}$ mice to generate *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice. We also generated the mice with the following genotypes as positive and negative controls for MDM2 function, respectively: *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;p53*$^{ER/-}$. Previous studies have shown that *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice tolerate daily injections of tamoxifen (*20*). Our study replicated these results. Following daily injection of tamoxifen, *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice survived the duration of the study (up to 40 days) with no apparent abnormalities. Surprisingly, under the same conditions the maximum survival time of *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice was approximately 4-5 days (Figure 1A). This dramatic difference between *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice suggests that a functional MDM2 RING domain is integral for maintaining homeostasis in adult mice. Further, mice lacking MDM2 (*Mdm2*$^{-/-}$*;p53*$^{ER/-}$) have been shown to perish approximately 6 days after a single tamoxifen injection (*23*). We also compared the survival of *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$

mice with that of *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ mice following tamoxifen injection. Surprisingly, we found that *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice lived an evidently shorter lifespan than *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ mice (Figure 1B), which suggests that loss of MDM2 RING domain function through the MDM2^{C462A} mutation may alter MDM2 functions towards p53 regulation and enhance p53 function. Since the MDM2^{C462A} mutation disrupts MDM2-MDMX interaction and MDMX has been shown to be important for MDM2-mediated p53 regulation (*24-26*), we sought to determine whether the differences in survival between *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice and either *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice or *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ mice could be dependent on differential MDMX protein stability or p53 regulation. To this end, we generated each of these mice in the background of *MdmX* deletion. We found that following tamoxifen injection, the median survival time of *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mice was significantly shorter than that of *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*; p53*$^{ER/-}$ mice (Figure 1C) and was equivalent to *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice (Figure 1D), indicating that changes in MDMX-mediated p53 regulation or protein stability are unlikely to contribute to survival differences present in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice. Further, the loss of MDMX did not contribute to changes in survival upon *Mdm2* deletion, as *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mice and *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ mice display similar survival times following tamoxifen injection (Figure 1E), suggesting that in the absence of MDM2, MDMX plays a minimal role in contributing to organismal survival during basal levels of p53 activity. This notion is in line with previous studies in *MdmX*$^{-/-}$*; p53*$^{ER/-}$ mice, which tolerate daily injections of tamoxifen for a median of ~29 days (*27*), which showed the significantly longer than *Mdm2*$^{-/-}$*; p53*$^{ER/-}$ surviving 7 days.

Loss of Mdm2 RING domain function exacerbates intestinal atrophy and cell apoptosis—Because loss of MDM2 in *p53*$^{ER/-}$ mice leads to p53-induced apoptosis and organismal lethality, we sought to determine whether *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mouse death is correlated with changes in tissue homeostasis, including increased apoptosis. We injected tamoxifen into *Mdm2*$^{+/+}$*;p53*$^{ER/-}$, *Mdm2*$^{-/-}$*;p53*$^{ER/-}$, *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$, *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mice once daily for three days and then collected tissues sensitive to p53-induced apoptosis, including colon and small intestine. We first performed H&E staining in order to observe any changes in tissue architecture. The overall integrity of both small intestine and colon tissues harvested from *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice was maintained, and these tissues appeared relatively normal (Figure 2A-B). In contrast, the small intestine and colon from *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ or *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ exhibited severe intestinal atrophy characterized by shortening of villi and marked hypocellularity of crypts compared with the corresponding MDM2 WT and MDM2-null control groups. In order to detect apoptosis in these tissues, we performed a TUNEL assay. Colon tissue from *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice exhibited more extensive apoptosis compared to either *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ or *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ colon tissue (Figure 2C). Small intestine from *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice exhibited light TUNEL staining compared to *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ mice (Figure 2D), but we suspect that may be because the majority of cells from *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ small intestine have already completed the apoptotic process by this time, as indicated by a reduction in nuclear staining (blue). Notably, *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ tissues exhibited similar morphological changes to *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ tissues, which suggests that MDMX does not play a role in the morphological changes.

Mdm2 RING mutation enhances p53 target gene transcription—The accelerated organismal lethality and increased apoptosis in tissues of *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice is consistent with increased p53 activity. In order to assess the effect of the MDM2^{C462A} mutation on p53 transcriptional activity, we isolated mouse embryonic fibroblasts (MEFs) from each of the five genotypes and treated these cells with 4-hydroxytamoxifen (4-OHT). We then utilized qRT-PCR to detect changes in the transcription of several p53 target genes including *p21*, *puma* and *bax* (Figure 3A) (*28-30*). Consistent with previous studies, *p21* transcription was elevated in *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ MEFs and was increased to a greater degree in *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ MEFs (*23*). While *puma* and *bax* transcription were nearly unchanged following 4-OHT treatment in *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ MEFs, an

increase in their transcription was apparent in *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ MEFs. Interestingly, following 4-OHT treatment, *p21*, *bax*, and *puma* transcription were significantly increased in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ MEFs to an even greater extent than in *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ or *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ MEFs, suggesting that p53 activity is greatest in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ MEFs. Further, the deletion of MDMX did not significantly alter p53 activity in the presence of the MDM2^{C462A} mutation. In order to confirm these observations *in vivo*, we harvested small intestine and lung from the p53^{ER} mice after the daily administration of tamoxifen for 3 days. The trend that we observed in MEFs was also reflected in mouse tissue, including small intestine and lung, with *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mice exhibiting the highest expression of each target gene (Figure 3B-C). Intriguingly, since mice bearing the MDM2^{C462A} mutation exhibit greater p53 activity than mice lacking MDM2 altogether, these data suggest the possibility that MDM2-mediated p53 activity suppression is dependent on MDM2 RING function, and in the absence of this function MDM2 may have alternate p53 regulating capabilities *in vivo*.

Mdm2 RING functional inactivation potentiates p53 acetylation and downstream target gene expression—To confirm whether the shortened survival time of *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice is associated with p53 transcriptional activation at the protein level, we cultured early-passage MEFs in either the absence or presence of 4-OHT for different time points. Then, we assayed the expression of p53 target genes p21 and MDM2 (Figure 4A). In *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ MEFs, MDM2 and p21 levels were increased after 4-OHT exposure, which is consistent with the idea that that the activation of p53^{ER} by 4-OHT results in increased *mdm2* and *p21* transcription and translation. Consistent with *in vivo* survival trends, *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ MEFs exhibited high p21 expression when compared to either *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ or *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ MEFs, and the deletion of *MdmX* did not significantly impact this difference in p21 expression (Figure 4B). Unsurprisingly, p53^{ER} levels were also significantly increased in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ MEFs compared with *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ MEFs (Figure 4A), which is consistent with the role of the MDM2 RING domain in p53 degradation (*25*). Similarly, MDMX was induced by the MDM2 deletion or the RING domain mutation due to the loss of E3 ligase function, which is also explained by the previous studies (*31-33*). We also noticed that following 4-OHT treatment p53^{ER} levels continued to increase in cells *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ MEFs (Figure 4A). This effect is likely due to the regulation of p53 by other ubiquitinating and deubiquitinating enzymes, such as HAUSP (*34*).

Previous studies have shown that p53 activation requires its own acetylation (*35*). The acetylation of p53 through interaction with cofactors p300/CBP is required for p53 to bind to DNA and promote the transcription of p21 and other downstream targets (*36, 37*). Consequently, we sought to ascertain whether the MDM2^{C462A} mutation affects p53 acetylation, thereby elevating p53 transcriptional activity. In order to normalize for the variations in p53^{ER} protein levels in each of the different MEFs, we calculated the ratio of acetylated p53^{ER} to total p53^{ER} protein levels (Figure 4C). Though p53^{ER} protein levels were consistently highest in cells lacking MDM2 (Figure 4A), relative p53^{ER} acetylation levels were highest in cells harboring the MDM2^{C462A} mutation (Figure 4C).

To confirm these results *in vivo*, we injected tamoxifen into *Mdm2*$^{+/+}$*;p53*$^{ER/-}$, *Mdm2*$^{-/-}$*;p53*$^{ER/-}$, *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$, *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mice once daily for three days and then analyzed the protein levels of total and acetylated p53^{ER}, as well as p53 target gene protein levels in spleen, small intestine, colon and lung tissues (Figure 5A-D). In all tissues, MDMX protein was accumulated in *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mouse tissues, however it barely controlled p53 transcriptional activity in the absence of MDM2 or MDM2 RING domain function. On the other hand, p21 and MDM2 protein levels were highest in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mouse tissues. We further quantified p21 levels in three independent experiments and found that p21 was significantly elevated in all *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mouse tissues

compared to their respective *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ and *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ counterparts (Figure 5E). Further, differences in p53^{ER} acetylation were far more apparent in mice than in MEFs, and relative p53^{ER} acetylation levels were significantly increased in *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ mouse tissues compared to either *Mdm2*$^{-/-}$*;p53*$^{ER/-}$ or *Mdm2*$^{-/-}$*;MdmX*$^{-/-}$*;p53*$^{ER/-}$ tissues (Figure 5F). These increases in relative acetylation correlate with both the increased p53 activity and early lethality observed in mice bearing the MDM2^{C462A} mutation and suggest that MDM2 RING function may be required for MDM2-mediated p53 transcriptional regulation *in vivo*.

DISCUSSION

The results of this study have provided some clarification of the role of the MDM2 RING domain in the regulation of p53 post-embryogenesis. First, while the two key functions of this domain (E3 ligase activity and MDMX binding) can be individually lost with little consequence in adult, unstressed mice (*15, 17*), at least one of these functions is required to maintain tissue homeostasis (Figure 2). When both E3 ligase activity and MDMX binding capabilities are lost, MDM2-p53 binding is not sufficient to control basal p53 transcriptional activity and prevent p53-induced apoptosis and organismal lethality (Figure 1). This is principally demonstrated by the early lethality of *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ mice compared to *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ mice (Figure 1A).

Second, the presence of the MDM2^{C462A} mutation correlated with both increased p53 transcriptional activation and acetylation compared to the complete absence of MDM2 (Figure 5). There are several possibilities that could explain this observation. First, studies in our lab have revealed that the MDM2^{C462A} mutation facilitates greater interaction of p53 with histone acetyl transferase CBP/p300 (*15, 38*), an important co-activator of p53 that potentiates p53 acetylation and transcriptional activity (*37*). Previous *in vitro* studies have also shown that disruption of the MDM2 RING domain disrupts MDM2-MDMX binding, but not MDM2 homodimer formation (*39*). Although the MDM2^{C462A} mutation clearly disrupts MDM2-MDMX binding, whether or not it affects MDM2 homodimerization *in vivo* remains unknown. It is possible that MDM2-MDMX binding serves to inhibit MDM2 p300-mediated acetylation of p53, but that monomeric or homodimeric MDM2 could enhance p300-p53 interaction and functional activation. As we have recently shown that the MDM2 RING domain is required for MDM2-MDMX heterodimerization but not for MDM2-MDM2 homodimerization (*39*), it is possible that the MDM2^{C462A} mutant forms homodimer, which may have a stronger binding affinity to actyltransferase. This would suggest that MDM2-mediated inhibition of p53 acetylation by p300 requires MDM2-MDMX interaction, which is supported by the correlation of the MDM2^{C462A} mutation with increased p53 acetylation in mouse tissues (Figure 5F). Although overexpressed MDMX has been shown to inhibit p300-mediated p53 acetylation (*40*), our data could suggest that MDMX alone cannot inhibit p300-p53 interaction *in vivo*, as the loss of MDMX does not appear to increase p53 acetylation or activity beyond that which is observed in the presence of the MDM2^{C462A} mutation (Figure 5), though this hypothesis remains to be tested *in vivo*.

It is also possible that the MDM2 RING domain is required for the inhibitory functions of MDM2-p53 binding. For example, *in vitro* assays using an overexpressed human equivalent HDM2^{C464A} mutation have demonstrated that a functional RING domain is necessary for MDM2-mediated p53 export (*41*). In this context, MDM2^{C462A} could tether p53 to the nucleus, allowing for increased interaction with DNA and greater transcriptional activity. The combination of increased p53 acetylation and activity with nuclear sequestration could explain why mice bearing the MDM2^{C462A} mutation demonstrate increased p53 activity than mice lacking MDM2 (Figure 3).

This study suggests the following about *in vivo* MDM2-mediated p53 regulation: (1) The MDM2 RING domain contributes to the regulation of p53 transcriptional activity in adult mice (2) MDM2-p53 binding is not sufficient for p53 regulation and adult organismal survival (3) In the absence of MDM2 or MDM2 RING domain function, MDMX does not appear to play a role in regulating p53 transcriptional activity in adult mice. However, it remains possible that the observations made in this study could be specific

to the MDM2^{C462A} mutation or p53^{ER}. In future studies it would be useful to confirm these results using another model system, such as mice lacking the MDM2 RING domain, containing an alternate RING-disrupting mutation, or an alternate inducible p53 model. The results of this study also have implications for p53-inducing therapies. Small molecule compounds that inhibit MDM2-p53 interaction, like nutlin-3a, have been widely used to facilitate p53 activation (*42*). Our study suggests the possibility that compounds targeting MDM2 RING domain function could produce even more robust p53 activation.

EXPERIMENTAL PROCEDURES

Genetically engineered mice and in vivo studies—To generate mice bearing the genotypes used in this study, we bred *p53$^{ER/-}$* mice with mice from several different backgrounds, including *Mdm2$^{-/-}$*, *Mdm2$^{C462A/C462A}$*, and *MdmX$^{-/-}$* (*10, 14, 20*). All experimental procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. To restore p53^{ER} function, mice were intraperitoneally injected with 1mg/kg tamoxifen (Sigma, T5648) according to body weight.

Cell culture—MEF cells were isolated from E13.5-E16.5 embryos and cultured in a 37^{o}C incubator with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (Gibco) and 100 IU/ml penicillin (Gibco). To restore p53ER function *in vitro*, cultured MEFs were exposed to 100 nM 4-OHT (Sigma, H7904) for the indicated amounts of time.

Immunoblot analysis—Protein was extracted from organs and cells as described previously (*14*). Protein lysates were fractionated on SDS polyacrylamide gels and transferred onto nitrocellulose membranes. Procedures and conditions for immunoblotting were described previously. The following antibodies were purchased commercially: monoclonal mouse anti MDMX (MDMX-82, Sigma), monoclonal mouse anti actin (MAB1501, EMD Millipore), polyclonal rabbit anti acetyl-p53 (K379, Cell Signaling Technology Inc) and polyclonal goat anti p21 (C-19, Santa Cruz Biotechnology). The monoclonal mouse anti p53 (pAB122) and monoclonal mouse anti MDM2 (2A10) were homemade by our laboratory. The intensity of the bands in the linear range of exposure was quantified using ImageJ 1.46r.

qRT-PCR analysis of mRNA—Total RNA was prepared from mouse tissues using Trizol® Reagent (Invitrogen, #15596-026). RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Scientific, NanoDrop™ 2000c) and quality was assessed by agarose gel electrophoresis. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen, 18080-051). qRT-PCR was performed with SYBR Green probes using the Applied Biosystems 7900HT Fast Real-Time PCR system. Thermal cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Target gene transcript levels were normalized to beta-actin transcript levels obtained in each sample via the subtraction of the Ct value of beta-actin from the Ct value for each target gene. Results were expressed as the fold-change in transcript levels. Primers used for qRT-PCR were as follows:
p21: 5'TCCACAGCGATATCCAGACA3' and 5'AGACAACGGCACACTTTGCT3'.
Puma: 5'TGTGGAGGAGGAGGAGTGG3' and 5'TGCTGCTCTTCTTGTCTCCG3'.
Bax:
5'GGACAGCAATATGGAGCTGCAGAGG3'
and 5'GGAGGAAGTCCAGTGTCCAGCC3'.
β-Actin: 5'GGCTGTATTCCCCTCCATCG3' and 5'CCAGTTGGTAACAATGCCATGT-3'.

Hematoxylin and eosin staining and TUNEL assay—Tissues were dissected, flushed gently with cold PBS, fixed in 10% formalin overnight, dehydrated in 50% ethanol, and stored in 70% ethanol until they were transferred to the Histology Research Core Facility at UNC for paraffin embedding. The tissues were then cut into 4 µm sections and then processed for Hematoxylin and eosin staining (H&E). TUNEL staining for apoptosis was performed using the Apoptag Peroxidase In Situ Apoptosis Detection Kit according to manufacturer's instructions. TUNEL stained tissue was analyzed using an Olympus IX-81 microscope fitted with a SPOT camera and software.

Statistical analysis—Results are represented as mean ± standard error of the mean. Quantitative

PCR data and immunoblotting quantifications were evaluated for significance using the unpaired t test. A p value <0.05 was considered significant for all analyses. Significant differences between experimental groups were: $^{*}P < 0.05$ or $^{**}P < 0.01$. Calculations were performed using GraphPad Prism 5 software.

Ethics statement—This investigation has been conducted in accordance with ethical standards, the Declaration of Helsinki, national and international guidelines, and has been approved by the authors' institutional review board.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

H.T., N.R.T., and A.J. researched data. H.T., and N.R.T. wrote the manuscript. Y.Z. reviewed and edited the manuscript. J.Z. contributed to discussion and reviewed and edited the manuscript.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Mice bearing Mdm2^{C462A} exhibit early lethality following p53^{ER} activation by 4-OHT.
(A) Kaplan-Meier curve depicting the survival of *Mdm2$^{+/+}$;p53$^{ER/-}$* (n=5) and *Mdm2$^{C462A/C462A}$;p53$^{ER/-}$* (n=5) mice treated with daily injection of tamoxifen. (B) Kaplan-Meier curve depicting the survival of *Mdm2$^{-/-}$;p53$^{ER/-}$* (n=5) and *Mdm2$^{C462A/C462A}$;p53$^{ER/-}$* (n=5) mice treated with daily injection of tamoxifen. (C) Kaplan-Meier curve depicting the survival of *Mdm2$^{-/-}$;MdmX$^{-/-}$;p53$^{ER/-}$* (n=5) and *Mdm2$^{C462A/C462A}$;MdmX$^{-/-}$;p53$^{ER/-}$* (n=5) mice treated with daily injection of tamoxifen. (D) Kaplan-Meier curve depicting the survival of *Mdm2$^{C462A/C462A}$;p53$^{ER/-}$* (n=5) and *Mdm2$^{C462A/C462A}$;MdmX$^{-/-}$;p53$^{ER/-}$* (n=5) mice treated with daily injection of tamoxifen. (E) Kaplan-Meier curve depicting the survival of *Mdm2$^{-/-}$;p53$^{ER/-}$* (n=5) and *Mdm2$^{-/-}$;MdmX$^{-/-}$;p53$^{ER/-}$* (n=5) mice treated with daily injection of tamoxifen.

Figure 2. Mdm2 RING mutation Mdm2$^{C462A/C462A}$ exacerbated p53-induced mouse intestine atrophy and cell apoptosis.
(A-B) Mice of the indicated genotypes in a *p53$^{ER/-}$* background were treated with daily tamoxifen injection for 3 days and harvested. Hematoxylin and eosin (H&E) staining was used to detect the tissue structure of colon (A) and small intestine (B) from the different genotypes. Scale bar 50μm. (C-D) Peroxidase TUNEL staining was perform as a cellular marker of apoptosis in colon (C) and small intestine (D) from the above mice. The TUNEL stain is brown and hematoxylin nuclear counterstain is blue. Scale bar 50μm.

Figure 3. Functional Mdm2 RING inactivation correlates with enhanced p53 target gene transcription *in vivo*.
(A) MEFs of the indicated genotypes in a *p53$^{ER/-}$* background were treated with 4-OHT for the indicated times and harvested. Then, qRT-PCR was used to measure the mRNA levels of *p21*, *puma*, and *bax* relative to those present in untreated *Mdm2$^{+/+}$;p53$^{ER/-}$* MEFs. (B-C) Mice of each indicated genotypes (n=3) in a *p53$^{ER/-}$* background were treated with daily tamoxifen injection for 3 days and harvested. Then, qRT-PCR was used to measure the mRNA levels of *p21*, *puma*, and *bax* present in small intestine (B) and lung (C) relative to those present in tissues from *Mdm2$^{+/+}$;p53$^{ER/-}$* mice. Data are means ± SD calculated from three independent experiments and normalized to *Actin*. **p*<0.05, ***p*<0.01.

Figure 4. Mdm2 RING mutation potentiates p53 acetylation and downstream target protein expression in MEF cells.
(A) MEFs of the indicated genotypes in a *p53$^{ER/-}$* background were treated with 4-OHT for the indicated times and harvested for Western blot analysis with the indicated antibodies. (B) The levels of p21 from three independent experiments as in (A) were quantified using densitometry and normalized to untreated *Mdm2$^{+/+}$;p53$^{ER/-}$* MEFs. (C) The ratio of acetyl-p53^{ER} to total p53^{ER} protein from three independent experiments as in (A) were quantified using densitometry and normalized to untreated *Mdm2$^{+/+}$;p53$^{ER/-}$* MEFs. Data are represented as means ± SD calculated and were normalized to *Actin*. **p*<0.05, ***p*<0.01.

Figure 5. Mdm2 RING mutation potentiates p53 acetylation and downstream target protein expression *in vivo*.
(A-D) Mice of the indicated genotypes in a *p53$^{ER/-}$* background were treated with daily tamoxifen injection for 3 days. Spleen (A), small intestine (B), colon (C), and lung (D) tissues were harvested for Western blot analysis with the indicated antibodies. (E) The levels of p21 from three independent experiments as in (A-D) were quantified using densitometry and normalized to *Mdm2$^{+/+}$;p53$^{ER/-}$* mouse tissues. (F) The acetyl-p53^{ER} protein level from three independent experiments as in (A-D) were quantified using densitometry and normalized to *Mdm2$^{+/+}$;p53$^{ER/-}$* mouse tissues. Data are means ± SD and were normalized to Actin. **p*<0.05, ***p*<0.01.

Figure 1

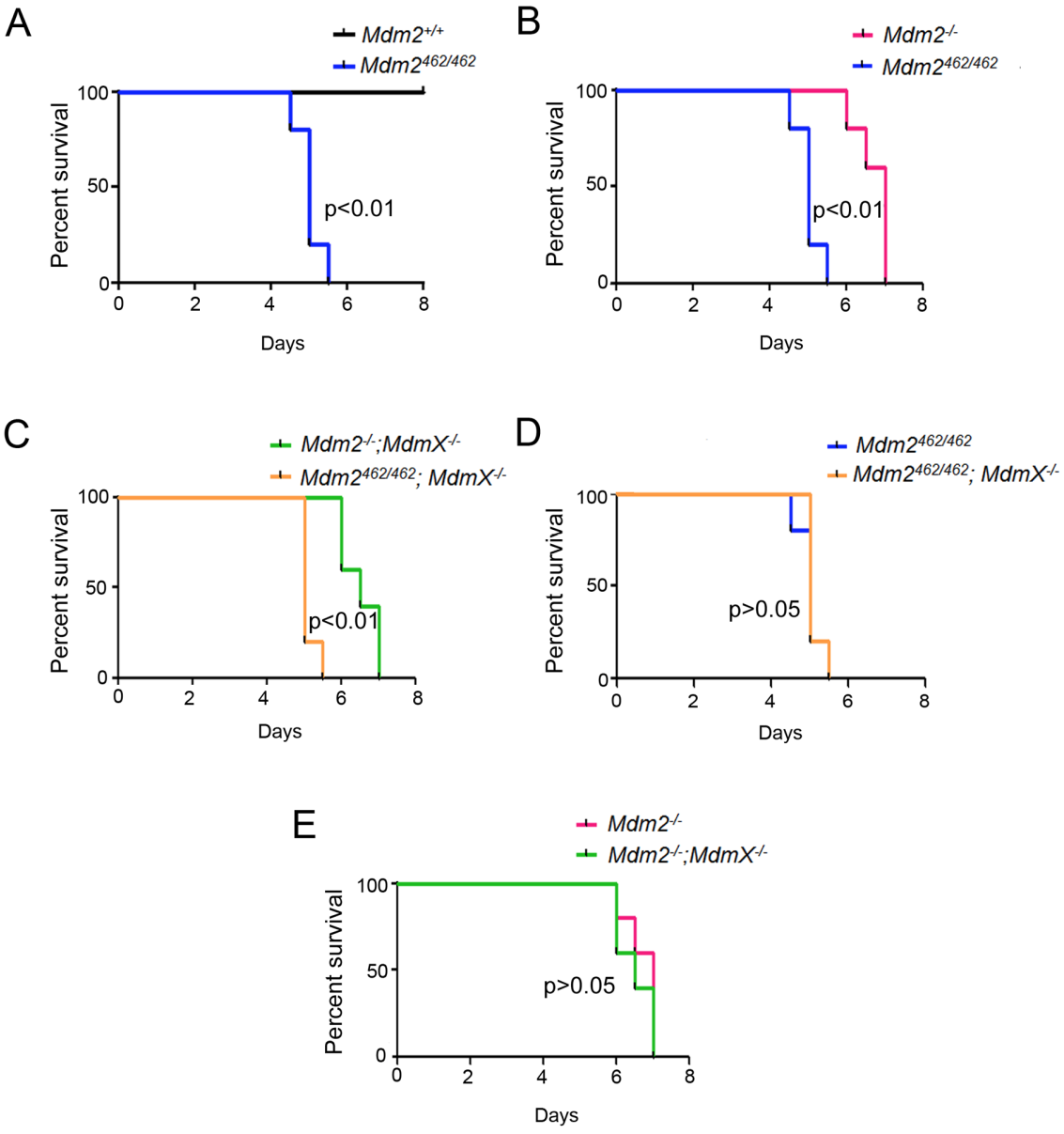


Figure 2

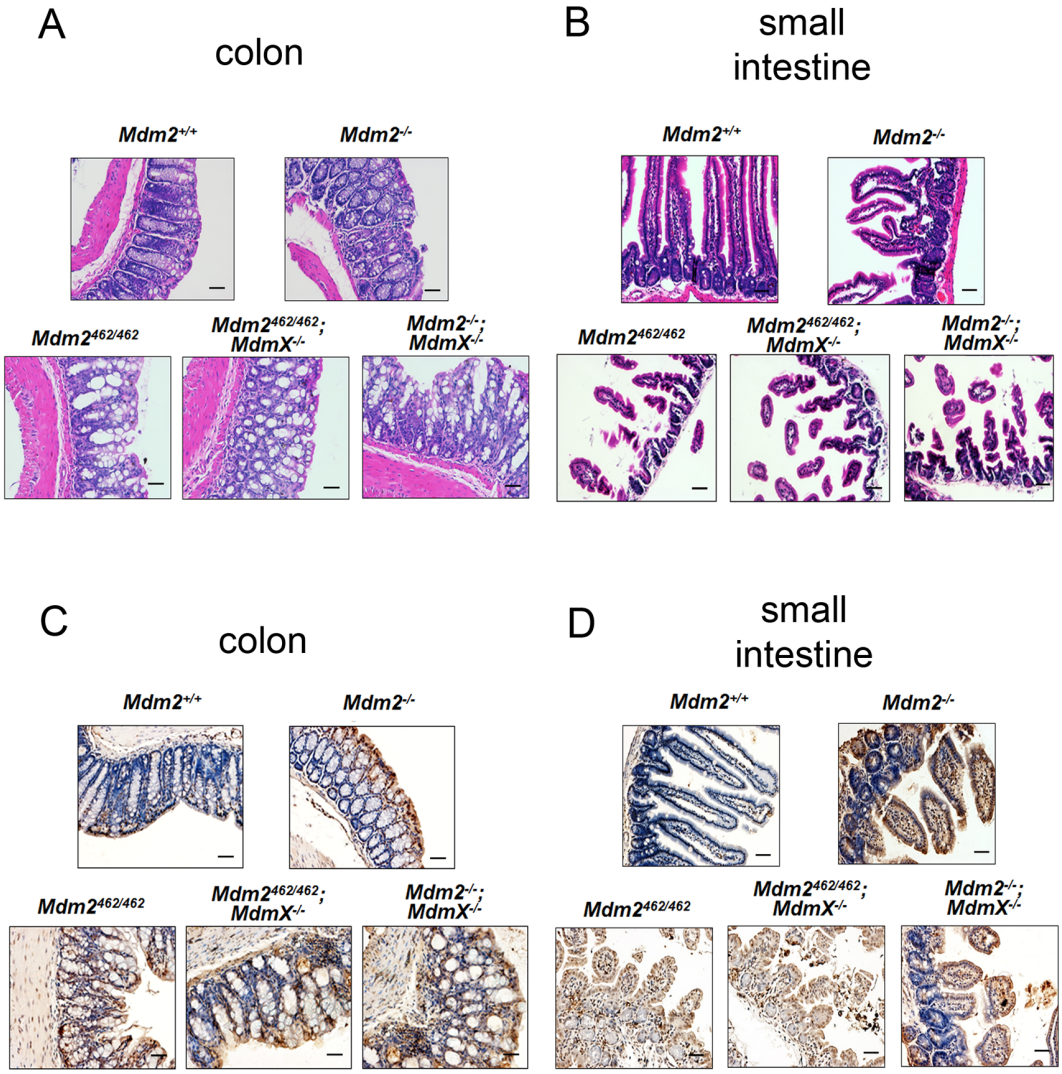


Figure 3

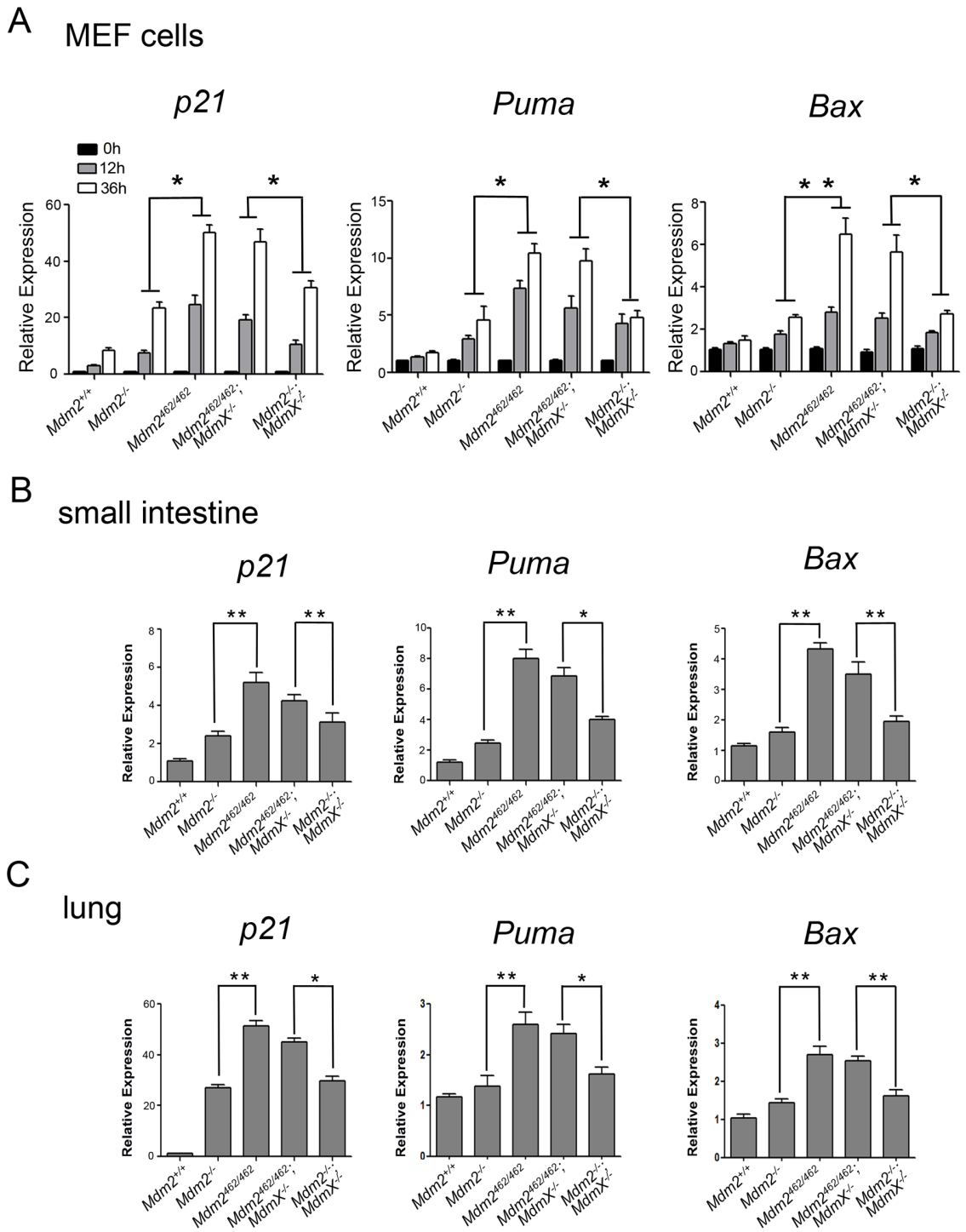


Figure 4

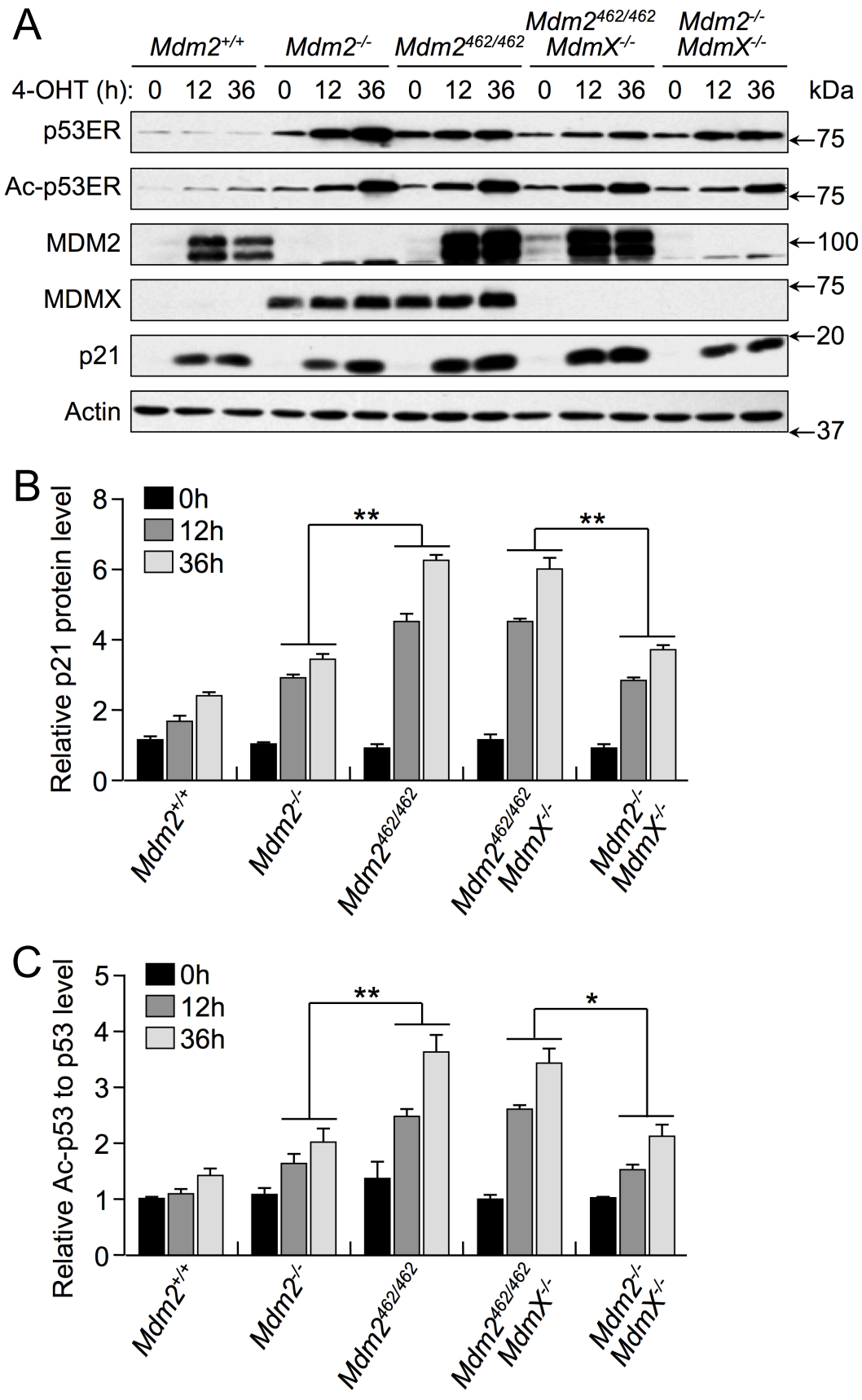
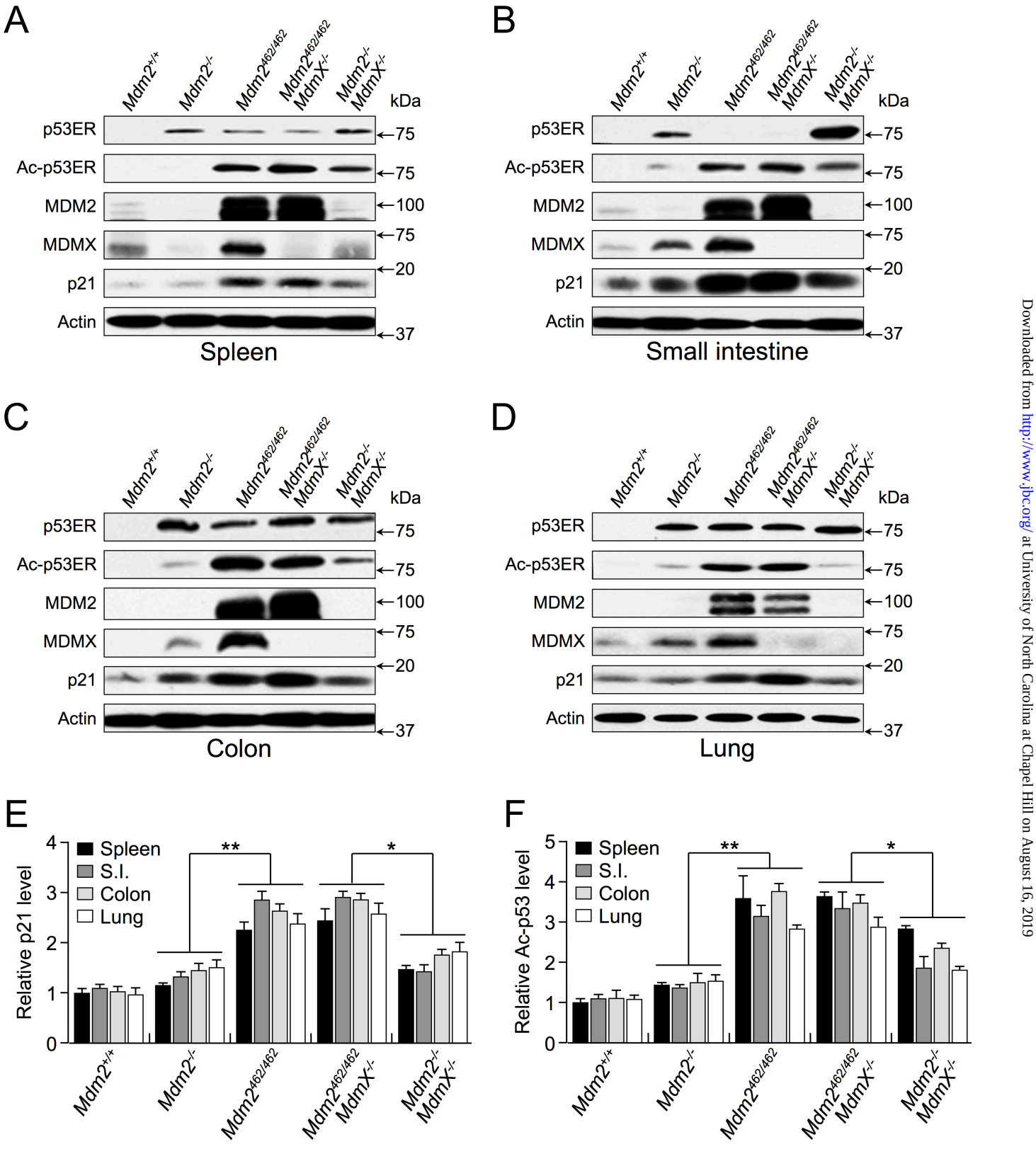


Figure 5



Inactivation of the MDM2 RING domain enhances p53 transcriptional activity in mice

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