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The anaphase-promoting complex/cyclosome is an E3 ubiquitin ligase for Mdm2

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Abbreviations: APC/C, anaphase-promoting complex/cyclosome; Mdm2, mouse double minute 2; 4-OHT, 4-Hydroxytamoxifen; MEFs, mouse embryonic fibroblasts; UV, ultraviolet; TCGA, The Cancer Genome Atlas

The Mdm2 proto-oncoprotein is the primary negative regulator for p53. While it is believed that Mdm2 degradation is regulated via its own E3 ubiquitin ligase activity, recent development of knock-in mouse models demonstrates that Mdm2 E3 ligase function is dispensable for self-degradation in vivo. Here, we show that the anaphase-promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase for Mdm2 degradation. We demonstrate that APC2, a scaffold subunit of APC/C, binds to Mdm2 and is required for Mdm2 polyubiquitination and proteasomal degradation. Downregulation of APC2 by RNAi results in transcription-independent accumulation of Mdm2 and attenuation of stress-induced p53 stabilization, leading to decreased senescence and increased cell survival. Furthermore, APC2 expression is frequently downregulated in human cancers; in tumor cell lines, APC2 downregulation correlates with Mdm2 overexpression. Our study shows the regulation of Mdm2 by the E3 ubiquitin ligase APC/C and has important therapeutic implications for tumors with Mdm2 overexpression.

Introduction

Mdm2 is well characterized as the major negative regulator of the tumor suppressor p53.[1-3] The C terminus of Mdm2 confers intrinsic E3 ubiquitin ligase activity, which promotes the ubiquitination and degradation of p53.[4-7] *Mdm2* is a transcriptional target of p53, forming a regulatory feedback loop between Mdm2 and p53 to maintain cellular homeostasis.[4,8-11] The importance of Mdm2 in proper p53 regulation makes the understanding of its own regulation of critical concern. In order for the Mdm2-p53 feedback loop to function properly, tight regulation of Mdm2 degradation is essential. Previous in vitro and overexpression studies have demonstrated that Mdm2 regulates its own degradation by autoubiquitination, targeting itself for proteasome-mediated degradation.[12,13] However, recent studies of $Mdm2^{C462A/C462A}$ knock-in mice have challenged the Mdm2 autoubiquitination dogma. In $Mdm2^{C462A/C462A}$ mouse embryonic fibroblasts (MEFs), the mutant $Mdm2^{C462A}$ protein is degraded as rapidly as wild-type Mdm2 while p53 degradation is blocked, indicating that Mdm2 E3 ligase activity is not required for its own degradation when endogenously expressed,[14,15] and suggesting that other E3 ubiquitin ligases regulate Mdm2 stability.[14,15]

In light of this in vivo data, the potential for an outside E3 ubiquitin ligase to function in Mdm2 regulation has been more expressly studied. The Cullin1/β-TRCP E3 ubiquitin ligase complex was identified to interact with Mdm2, and this interaction was demonstrated to lead to the poly-ubiquitination and degradation of Mdm2.[16] However, the regulation of Mdm2:Cullin1/β-TRCP interaction suggests that this interaction only occurs following DNA damage. Furthermore, knockdown of Cullin1-βTRCP does not block p53 activation; instead, it affects the regulation of Mdm2 and p53 during the recovery of cells to basal conditions following exposure to stress.[16] More recently, NEDD4–1 was biochemically identified to contribute to the regulation of Mdm2 protein stability in cells by functioning as an E3 ligase; however, NEDD4–1 catalyzes the formation of K63-type polyubiquitin chains on Mdm2 that are distinct from the K48-type polyubiquitin chains typically required for proteasomal degradation.[17] Notably, K63-type polyubiquitination by NEDD4–1 competes with K48-type polyubiquitination of Mdm2 in cells, and, as a result, NEDD4–1-mediated ubiquitination stabilizes Mdm2. Our study was designed to identify E3 ubiquitin ligases responsible for the regulation of Mdm2 stability under physiological conditions.

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Results

Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 self-degradation under physiological conditions

To examine the role of Mdm2 E3 ligase function in Mdm2 degradation in vivo, we compared the degradation dynamics of Mdm2 in MEFs expressing wild-type (WT) Mdm2 or E3 ligase inactive mutant Mdm2. We generated 2 Mdm2 knock-in mouse models with inactivated Mdm2 E3 ubiquitin ligase activity toward p53: *Mdm2*^{Y487A} (corresponding to human Mdm2^{Y489A})[18] and *Mdm2*^{C462A} (corresponding to human Mdm2^{C464A}).[14] Both mutated *Mdm2* genes are under the control of the native promoter and hence result in physiological levels of *Mdm2* expression. While both the Mdm2^{Y487A} and Mdm2^{C462A} proteins display disrupted E3 ubiquitin ligase activity for p53, the mechanism of disruption in each of these mutants is unique. The Mdm2^{Y487A} mutation is C-terminal of the RING domain, in a region previously demonstrated to be critical for Mdm2 E3 ubiquitin ligase activity toward p53; however, this mutation retains an intact RING domain.[18] In contrast, the Mdm2^{C462A} mutation affects one of the 4 critical cysteine residues responsible for maintaining the RING domain structure; disruption of the Mdm2 RING domain structure results in the loss of E3 ubiquitin ligase activity.[18] Because the *Mdm2*^{C462A} mutation results in early embryonic lethality in homozygous mice, we crossed *Mdm2*$^{C462A/+}$ mice with mice expressing inducible p53ER^{TAM} (p53ER hereafter); the inducible nature of the p53ER fusion protein allows for generation of mice and MEFs with inactive p53.[19] The addition of 4-Hydroxytamoxifen (4-OHT) activates p53ER to allow for transactivation of Mdm2 and the study of Mdm2 dynamics in the *Mdm2*$^{C462A/C462A}$ background. In the presence of 4-OHT, the half-life of p53ER was approximately 30 min, and the half-life of Mdm2 approximately 15 min in *Mdm2*$^{+/+}$*;p53*$^{ER/-}$ MEFs (**Fig. 1A**). The disruption of Mdm2 E3 ligase activity greatly extended the half-life of p53ER, as observed in *Mdm2*$^{Y487A/Y487A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ MEFs (**Fig. 1B and C**). These results support previous findings and indicate that in vivo the Mdm2^{Y487A} and Mdm2^{C462A} mutant proteins have indeed lost E3 ubiquitin ligase activity for p53 degradation.

To evaluate the importance of Mdm2 E3 ligase function in the degradation of Mdm2 itself, we assessed the half-life of Mdm2 in *Mdm2*$^{+/+}$*;p53*$^{ER/-}$, *Mdm2*$^{Y487A/Y487A}$*;p53*$^{ER/-}$ and *Mdm2*$^{C462A/C462A}$*;p53*$^{ER/-}$ MEFs. Despite disrupting p53 degradation, Mdm2^{Y487A} and Mdm2^{C462A} protein itself was degraded normally when compared with WT Mdm2 (**Fig. 1A–C**). Furthermore, treatment with the proteasome inhibitor MG-132 led to similar stabilization of WT Mdm2, Mdm2^{Y487A}, and Mdm2^{C462A} (**Fig. 1D**), suggesting that each of these proteins is degraded at a similar rate in a proteasome-dependent manner. To eliminate the possible aberrant effect of p53ER on transcriptional regulation of Mdm2, *Mdm2*$^{+/+}$*;p53*$^{-/-}$, *Mdm2*$^{Y487A/Y487A}$*;p53*$^{-/-}$, and *Mdm2*$^{C462A/C462A}$*;p53*$^{-/-}$ mice were generated, and MEFs were isolated and similarly subjected to MG-132 treatment. In the absence of p53, MG-132 treatment resulted in a similar level of Mdm2 stabilization, regardless of the E3 ligase status of Mdm2 and consistent with observations made in the *p53*$^{ER/-}$ genetic background (**Fig. 1E**). These results suggest that while Mdm2 E3 ligase function is necessary for the degradation of p53, under physiological conditions, this function is dispensable for proteasome-dependent degradation of Mdm2.

In light of the normal, proteasome-dependent Mdm2 degradation dynamics observed in Mdm2 E3 inactive MEFs, we postulated that an alternative E3 ubiquitin ligase(s) is responsible for Mdm2 degradation under physiological conditions. To test this hypothesis, we used an in vitro ubiquitin ligase assay to assess the E3 ubiquitin ligase activity of HeLa cell lysate toward bacterial purified Mdm2 protein. Consistent with previous studies, and similar to most other RING finger domain-containing proteins that confer E3 ligase activity, purified WT Mdm2 protein demonstrated intrinsic autoubiquitination activity in the in vitro ubiquitin ligase assay; in contrast, Mdm2^{C464A} mutant protein does not demonstrate intrinsic autoubiquitination activity (**Fig. 1F**).[12,20] Interestingly, following the addition of HeLa cell lysate to the Mdm2^{C464A} protein reaction samples, strong polyubiquitin chain formation was observed (**Fig. 1F**). The ability for Mdm2^{C464A} to be polyubiquitinated in the presence of HeLa cell lysate suggests that an E3 ubiquitin ligase(s) capable of polyubiquitinating Mdm2 is present in the lysate. To rule out the possibility that WT Mdm2 present in the HeLa lysate is responsible for the observed polyubiquitination of Mdm2^{C464A}, we assessed polyubiquitin chain formation in cell lysate isolated from *WT, Mdm2*$^{+/+}$*;p53*$^{-/-}$ and *Mdm2*$^{-/-}$*;p53*$^{-/-}$ MEFs. Polyubiquitin chain formation in *WT, Mdm2*$^{+/+}$*;p53*$^{-/-}$ and *Mdm2*$^{-/-}$*;p53*$^{-/-}$ MEF cell lysate occurred at similar levels (**Fig. 1G**), indicating that a component in the MEF cell lysates, independent of both Mdm2 and p53, is capable of promoting Mdm2 polyubiquitination.

We then tested if this polyubiquitin chain formation is dependent on E1 and E2 supplied in vitro by individually removing each of these components. Polyubiquitin chain formation was dependent on E2 supplied in vitro, but HeLa cell lysates contained sufficient E1 ubiquitin activating enzyme to support the in vitro ubiquitination reaction (**Fig. S1A**). Furthermore, we found that the E3 ubiquitin ligase activity from HeLa cell lysate was diminished in the presence of SDS, providing further evidence that an enzymatic activity in the HeLa lysate is essential for the observed polyubiquitin chain formation (**Fig. S1B**).

Mdm2 interacts with APC2

Previous studies have indicated that Mdm2 protein levels oscillate with the cell cycle.[16,21] Accordingly, we also found that in *WT* and *p53*$^{-/-}$ MEFs synchronized by serum starvation, Mdm2 protein levels oscillated in a manner correlated with cyclin B1 oscillations (**Fig. 2A and B**). Similar results were also observed in U2OS cells synchronized with serum starvation (**Fig. S2A**), and HeLa and HCT116/p53$^{-/-}$ cells synchronized by double thymidine block (**Fig. S2B and C**). Together, these results suggest that Mdm2 levels may be regulated throughout the cell cycle, presumably by a member of the Cullin family E3 ubiquitin ligases, in a manner similar to cyclin B1. Furthermore, the observed oscillation of Mdm2 in p53-null cells suggests that this manner of Mdm2 regulation is p53-independent.

To test the possibility that a Cullin family E3 ubiquitin ligase is responsible for the ubiquitination and degradation of Mdm2, we first determined the binding affinity of Cullin family E3 ubiquitin ligases with Mdm2 via immunoprecipitation. As shown in **Figure 2C**, Mdm2 interacted with Cullin1 when co-overexpressed, consistent with previous studies characterizing Cullin1:Mdm2 binding.[16] Additionally, we observed Mdm2 interaction with APC2, a scaffold subunit of APC/C, which is a multi-subunit E3 ubiquitin ligase complex that controls the degradation of many proteins involved in cell cycle regulation (**Fig. 2C**). Most APC/C substrates oscillate their protein levels throughout the cell cycle, including the metaphase–anaphase transition and mitotic exit.[22-24] We confirmed APC2:Mdm2 interaction with reciprocal immunoprecipitation of ectopically expressed proteins (**Fig. 2D and E**), as well as with immunoprecipitation of endogenous Mdm2 in 293T and H1299 cells. 293T and H1299 cells express different levels of Mdm2, which allowed for correlation of the levels of Mdm2 with the levels of APC2 being co-immunoprecipitated (**Fig. 2F**).

APC2 is important for Mdm2 degradation

To test if APC/C is a physiological E3 ubiquitin ligase for Mdm2, we knocked down APC2 in human tumor cell lines and examined the levels of Mdm2. Knockdown of APC2 by siRNA in both U2OS (p53-positive) and HCT116/p53$^{-/-}$ (p53-negative) cells led to accumulation of Mdm2 (**Figs. 3A and B**), as well as extension of Mdm2 protein half-life (**Figs. S3A and B**). The limited increase of Mdm2 level upon APC2 knockdown suggested that there are other E3 ligase(s), in addition to

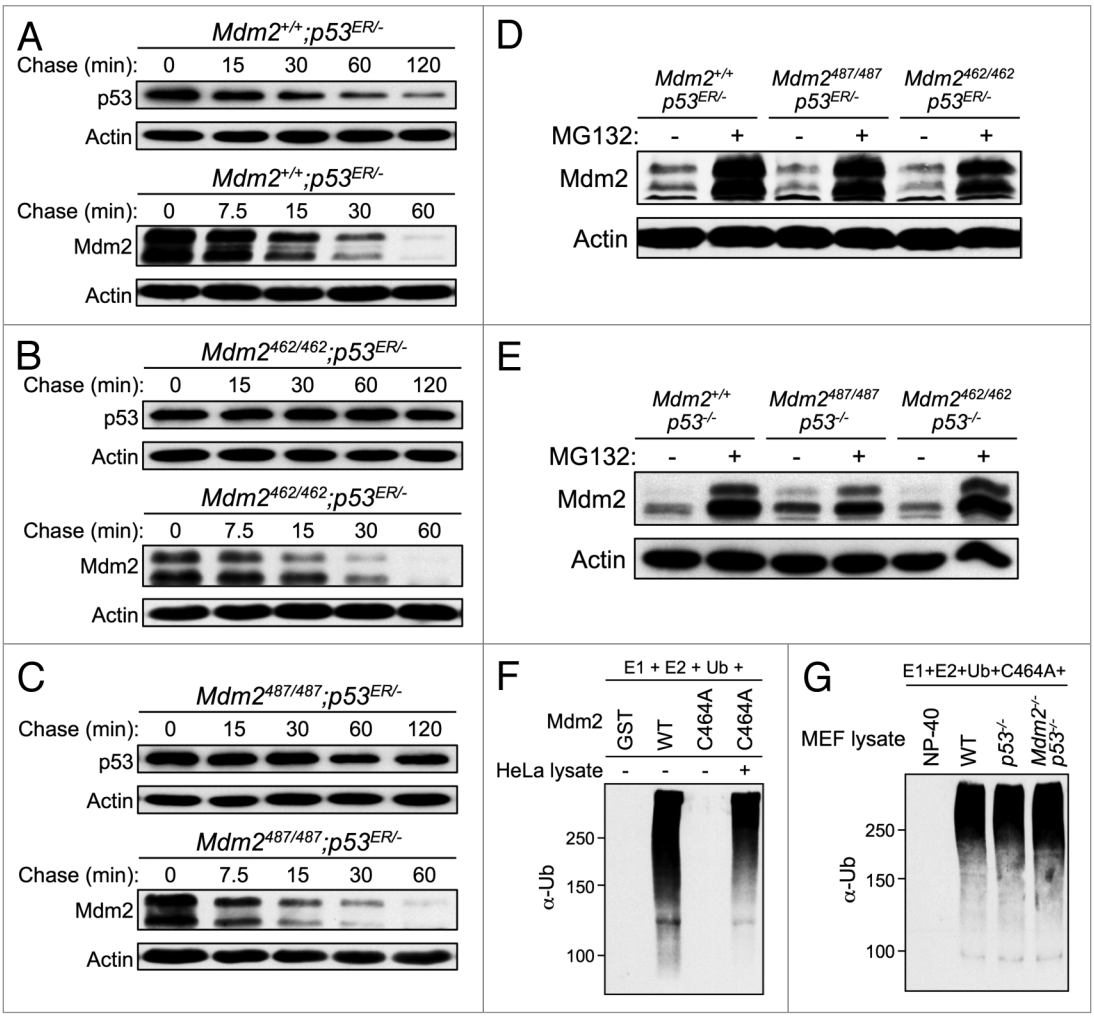

Figure 1. Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 self-degradation under physiological conditions. (**A**) *Mdm2*$^{+/+}$;*p53*$^{ER/-}$ MEFs were treated with 100 nM 4-OHT for 24 h before treatment with 150 μg/mL cycloheximide. Cell lysates were collected at the indicated times following cycloheximide treatment and p53ER and Mdm2 levels were analyzed by western blot. Actin was used as a loading control. (**B**) *Mdm2*$^{C462A/C462A}$;*p53*$^{ER/-}$ MEFs were treated and analyzed as described in (**A**). (**C**) *Mdm2*$^{Y487A/Y487A}$;*p53*$^{ER/-}$ MEFs were treated and analyzed as described in (**A**). (**D**) *Mdm2*$^{+/+}$;*p53*$^{ER/-}$, *Mdm2*$^{Y487A/Y487A}$;*p53*$^{ER/-}$, *and Mdm2*$^{C462A/C462A}$;*p53*$^{ER/-}$ MEFs were treated with 25 uM MG-132 for 8 h, as indicated. Mdm2 levels were analyzed by western blot. Actin was used as a loading control. (**E**) *Mdm2*$^{+/+}$;*p53*$^{-/-}$, *Mdm2*$^{Y487A/Y487A}$;*p53*$^{-/-}$, *and Mdm2*$^{C462A/C462A}$;*p53*$^{-/-}$ MEFs were treated and analyzed as in (**D**). (**F**) An equal amount of bacterial purified GST, GST-tagged wild-type Mdm2, and GST-tagged Mdm2^{C464A} protein bound to glutathione beads was incubated with E1, E2, and ubiquitin (Ub) with or without addition of HeLa cell lysate for 1 h at 37° C as indicated. Ubiquitin bound to the glutathione bead associated materials was analyzed by western blot. (**G**) Experiment is done as in (**F**), except cell lysate from wild-type (*WT*), *Mdm2*$^{+/+}$;*p53*$^{-/-}$ (*p53*$^{-/-}$), or *Mdm2*$^{-/-}$;*p53*$^{-/-}$ MEFs is used as indicated.

APC2, that degrade Mdm2. To further rule out the possibility that the increase in Mdm2 is due to increased transcription of *Mdm2*, rather than decreased degradation, we generated stable cell lines expressing GFP-tagged WT Mdm2 (GFP-Mdm2) or GFP-tagged Mdm2^{C464A} mutant under the control of the CMV promoter. As shown in **Figure 3C**, knockdown of APC2 by siRNA led to a strong accumulation of ectopic GFP-tagged Mdm2, indicating that the accumulation of Mdm2 is independent of transcriptional regulation. More importantly, knockdown of APC2 by siRNA also led to a strong accumulation of ectopic GFP-tagged Mdm2^{C464A}, which suggests that the Mdm2 accumulation observed following downregulation of APC2 is independent of Mdm2 E3 ligase activity. Disruption of proteasome-mediated degradation by MG-132 treatment resulted in an accumulation of Mdm2 in U2OS cells transfected with a control knockdown siRNA (siCtrl), but did not further stabilize Mdm2 in cells transfected with APC2 knockdown siRNA (siAPC2), which had higher levels of Mdm2 prior to MG-132 treatment (**Fig. 3E**). Together, these results indicate that APC2 is important for Mdm2 degradation in a manner independent of p53 status and *Mdm2* transcriptional regulation.

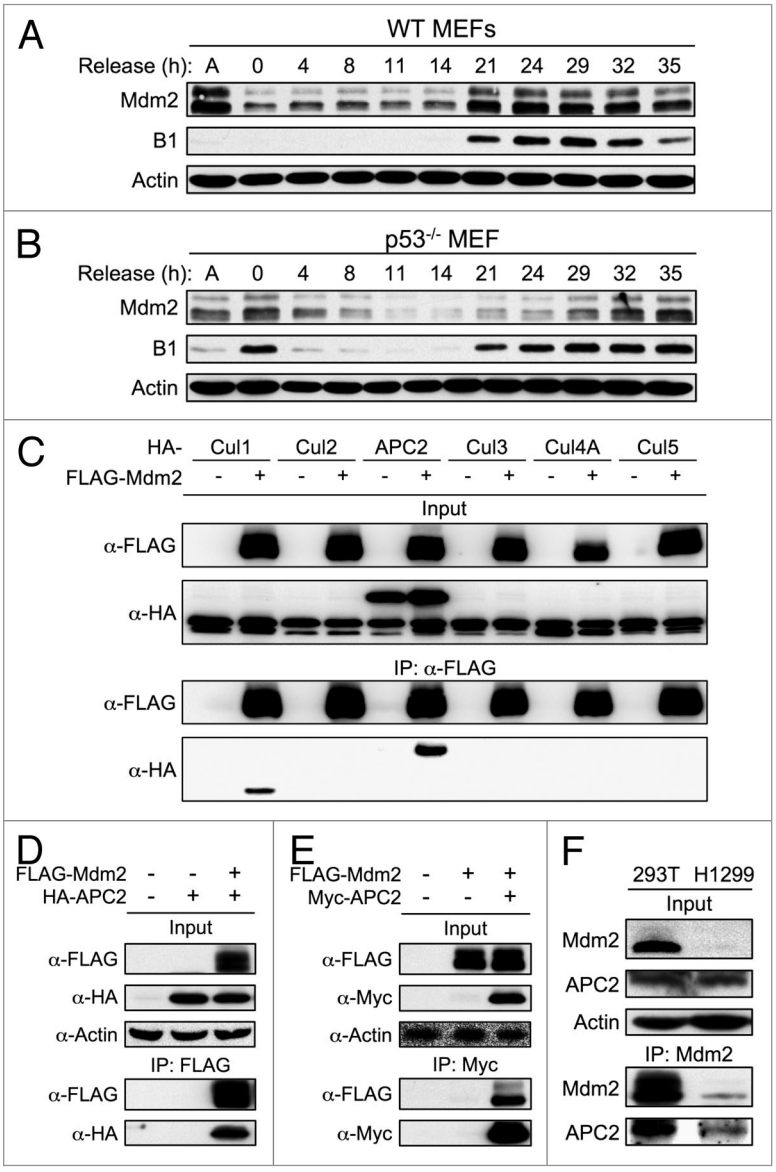

Figure 2. Mdm2 interacts with APC2. (**A**) *WT* MEFs were incubated with DMEM with 0.1% FBS for 72 h for serum starvation; media was then replaced with fresh DMEM with 10% FBS. Cells were harvested at the indicated time points after addition of FBS and analyzed by western blot. Actin was used as a loading control. (**B**) *p53*$^{-/-}$ MEFs were treated, harvested, and analyzed as in (**A**). (**C**) 293T cells were transfected with FLAG-tagged Mdm2 together with HA-tagged Cullin1 (Cul1), Cullin2 (Cul2), APC2, Cullin3 (Cul3), Cullin4A (Cul4A), or Cullin5 (Cul5) as indicated. Cell lysate was immunoprecipitated and immunoblotted with the indicated antibodies. Input represents 4% of total cell lysate utilized for IP. (**D**) 293T cells were transfected as indicated. Cell lysate was immunoprecipitated with anti-FLAG and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP. Actin was used as a loading control for the input. (**E**) 293T cells were transfected as indicated. Cell lysate was immunoprecipitated with anti-Myc antibody and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP. Actin was used as a loading control for the input. (**F**) 293T and H1299 cell lysates were immunoprecipitated with anti-Mdm2 antibody and immunoblotted as indicated. Input represents 2% of total cell lysate utilized for IP. H1299 cells, with a very low level of Mdm2, were used as a negative control. Actin was used as a loading control for the input.

APC2 is important for stress-induced p53 stabilization and activation

Because Mdm2 is the primary regulator of p53, mechanisms functioning to regulate Mdm2 will inevitably affect p53 function as well. To investigate the functional connections of APC2 with p53 activity, we examined the effect of downregulation of APC2 on p53-induced growth inhibition. We generated MEF cells with stable knockdown of APC2 in both *WT* and *p53*$^{-/-}$ genetic backgrounds. Stable knockdown of APC2 in the *WT,* but not *p53*$^{-/-}$ background, corresponded to a higher number of cells displaying flattened and enlarged morphology, indicative of senescence. Assessment of β-galactosidase activity was utilized to determine whether the flattened and enlarged MEF cells were indeed senescent. MEFs with APC2 knocked down exhibited significantly weaker activity of senescence-associated β-galactosidase than control cells (**Fig. 4A**). Additionally, quantitative analysis indicated that APC2 knockdown decreased the percentage of senescent cells by more than half (**Fig. 4A**). We then analyzed the effect of APC2 knockdown on p53 activity by examining p53-dependent transcription using a luciferase assay. As shown in **Figure 4B**, p53-dependent activation of the p21 promoter was greatly reduced by knockdown of APC2.

To investigate the role of APC2 in the p53 stress response, we knocked down APC2 by shRNA in *WT* MEFs and examined cell survival following exposure to UV radiation. Under unstressed conditions, APC2 knockdown did not affect cell survival and resulted in a slight increase in p53 levels; however, following UV-induced DNA damage, knockdown of APC2 increased cell survival (**Fig. 4C and E–G**). This increase in cell survival following irradiation was attenuated

when Mdm2–p53 interaction was disrupted by Nutlin-3 (**Fig. 4D**), suggesting that the effect of APC2 on cell survival following UV irradiation is p53-dependent.

To determine whether APC2 regulates stress-induced p53 expression, siRNA was used to knockdown APC2 in U2OS cells, followed by treatment with genotoxic agents to challenge the cells. Indeed, downregulation of APC2 substantially reduced the level of p53 induced by the DNA damaging agents, as observed following treatment with UV (**Fig. 4E**) and doxorubicin (**Fig. 4F**), as well as in response to ribosomal stress, as induced by actinomycin D (**Fig. 4G**). These results indicate that APC2 plays an important role in p53 stabilization in response to genotoxic stress.

APC2 downregulation correlates with cancer development

To further investigate the physiological relevance of APC2 regulation of Mdm2, we assessed the expression levels of APC2 and Mdm2 in various cancer cell lines. Interestingly, JAR and SJSA cells expressed very low levels of APC2 and, conversely, very high levels of Mdm2 compared with HepG2, MCF7, HeLa, A375, U2OS, and H1299 cells, which expressed higher levels of APC2 and correspondingly lower levels of Mdm2 (**Fig. 5A**). Of note, in addition to expressing low levels of APC2, JAR and SJSA cells contain Mdm2 gene amplifications; we speculate that the high levels of Mdm2 observed are the combined effect of low levels of APC2 and gene amplification of Mdm2.[25,26] To investigate the clinical relevance of variation in APC2 expression, we interrogated available tumor data sets for APC2. mRNA levels of APC2 were significantly decreased in breast, liver, and lymphocyte cancer tissues compared with that observed in corresponding normal tissue, based on an analysis of the Oncomine database (http://www.oncomine.com) (**Fig. 5B**). Investigation of breast, ovarian, lung, thyroid, liver, and renal cancer tissues in The Cancer Genome Atlas (TCGA) gene copy data set also revealed that *APC2* gene copy number was decreased compared with the gene copy number observed in normal tissues (**Fig. 5C**). The decreased levels of *APC2* mRNA and gene copy number in a variety of cancer types suggest that APC2 may be clinically relevant in the development or progression of cancer.

Discussion

Here, we report that Mdm2 protein stability is regulated by the APC/C E3 ubiquitin ligase complex through APC2:Mdm2 interaction. Various biochemical analyses, including protein binding and degradation assays, as well as functional assays, identify Mdm2 as a substrate of APC2. Depletion of APC2 results in increased levels of Mdm2, which, in turn, leads to attenuation of stress-induced p53 stabilization and increased cell survival. Mitotic inhibitors that activate the spindle assembly checkpoint by perturbing microtubule function are commonly used in the treatment of breast, ovarian, and lung cancers; however, as activation of the spindle assembly checkpoint does not suppress APC/C activity completely, it is believed that inhibitors directly targeting APC/C could provide a better therapeutic method for inducing mitotic arrest.[27,28] In contrast, our study suggests that inhibition of APC/C activity by downregulation of APC2 can result in oncogenic consequences due to accumulation of Mdm2, and the resulting inactivation of p53. In light of this finding, the prospect of pharmacologic inhibition of APC/C as an anti-cancer therapeutic needs to be carefully reevaluated. The observation that APC2 depletion effectively stabilizes Mdm2 and leads to inhibition of p53 activation indicates that cautious consideration of p53 status should be taken prior to the use of cancer treatments targeting APC/C-related pathways.

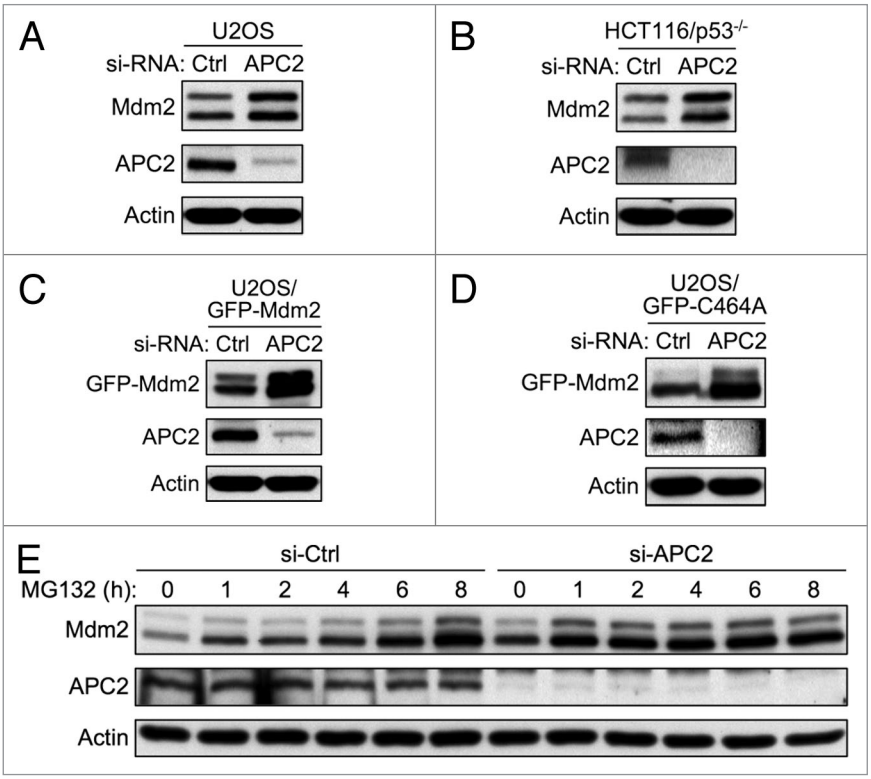

Figure 3. APC2 is important for Mdm2 degradation. (**A**) U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 72 h. Cell lysates were analyzed by western blotting for Mdm2 and APC2. Actin was used as a loading control. (**B**) HCT116/p53$^{-/-}$ cells were transfected, harvested, and analyzed as in (**A**). (**C**) Clonal stable cell lines stably expressing a GFP-Mdm2 fusion protein under the control of CMV promoter in U2OS cells were transfected, harvested, and analyzed as in (**A**). (**D**) Clonal stable cell lines stably expressing a GFP-Mdm2^{C464A} fusion protein under the control of CMV promoter in U2OS cells were transfected, harvested, and analyzed as in (**A**). (**E**) U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 h, cells were then split, and 25 uM MG-132 was added 48 h later. Cells were collected at indicated time after MG-132 treatment and analyzed by western blot. Actin was used as a loading control.

Materials and Methods

Cell culture, transfection

All cells were cultured at 37° C, and 5% CO_2 in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 ug/mL streptomycin. DNA transfections were performed using the Calcium phosphate method[29] and Fugene-6 (Promega #E2691, http://www.promega.com/products/reporter-assays-and-transfection/transfection-reagents/fugene-6-transfection-reagent/).

In vitro ubiquitination assay

Bacterial purified GST (Glutathione S-transferase) or GST-tagged $Mdm2^{C464A}$ bound to glutathione beads (GE Healthcare, #17–0756–01 http://www.gelifesciences.com/webapp/wcs/stores/servlet/productById/en/GELifeSciences/17075601) were incubated with 50 ng E1 (UBE1, Millipore, #23–021 https://www.millipore.com/catalogue/item/23-021-K), 50 ng E2 (bacterial purified UBCH5C), E3 (cell lysate or APC2 immunocomplex), 5 ug ubiquitin, and reaction buffer (50 mM TRIS-HCl pH 8.0, 0.1 M NaCl, 2 mM DTT, 5 mM MgCl2, 2 mM

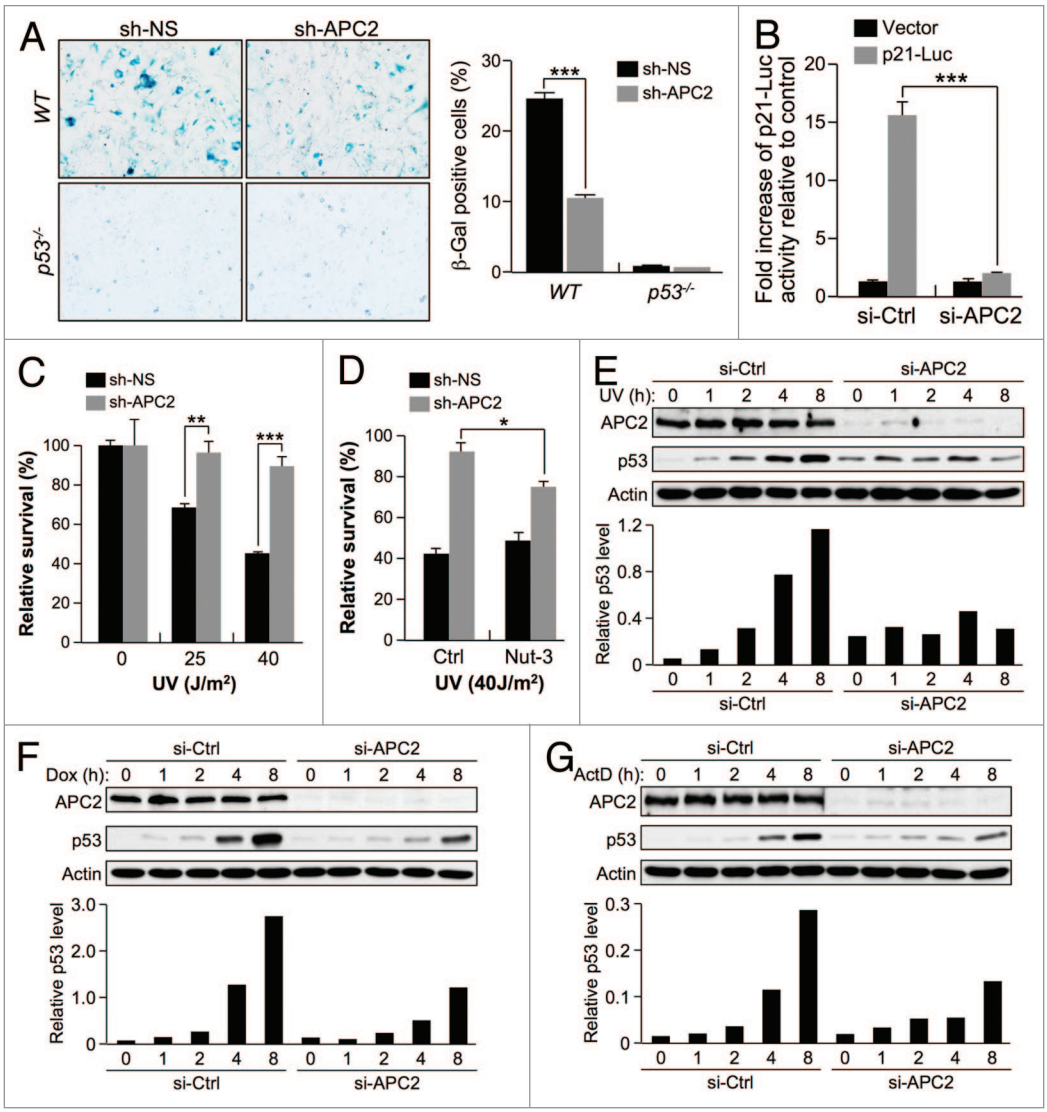

Figure 4. APC2 is important for stress-induced p53 stabilization and activation. (**A**) *WT* MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2) Lentiviral based shRNA were cultured for 12 passages. Cells were stained with β-gal and the percentage of β-gal-positive cells was quantified and plotted. (**B**) U2OS cells were transiently transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 h. Cells were split, followed by transfection with a pGL3-Basic luciferase reporter plasmid containing the wild-type p21 promoter (p21-Luc), or an empty vector (Vector). Cells were collected 24 h later and assayed for luciferase activity. (**C**) *WT* MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2). Lentiviral-based shRNA were treated with different dosages of UV as indicated. Cells were collected 24 h after UV treatment, stained with trypan blue, and counted to determine cell survival. (**D**) *WT* MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2) Lentiviral-based shRNA were treated with 40 J/m^2 UV and 8 μM Nutlin-3. Cells were collected 24 h after treatment, stained with trypan blue, and counted to determine cell survival. (**E**) U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 h, cells were split, and 15 J/m^2 UV was added 48 h later. Cells were collected at the indicated time points after UV treatment, and analyzed for Mdm2 and p53 by western blot. Actin was used as a loading control. Bottom: p53 levels were measured by densitometry, normalized to actin, and plotted. (**F**) U2OS cells were treated and analyzed as described in (**E**) except treatment is 0.5 μg/mL doxorubicin. (**G**) U2OS cells were treated and analyzed as described in (**E**), except treatment is 5 nM actinomycin D.

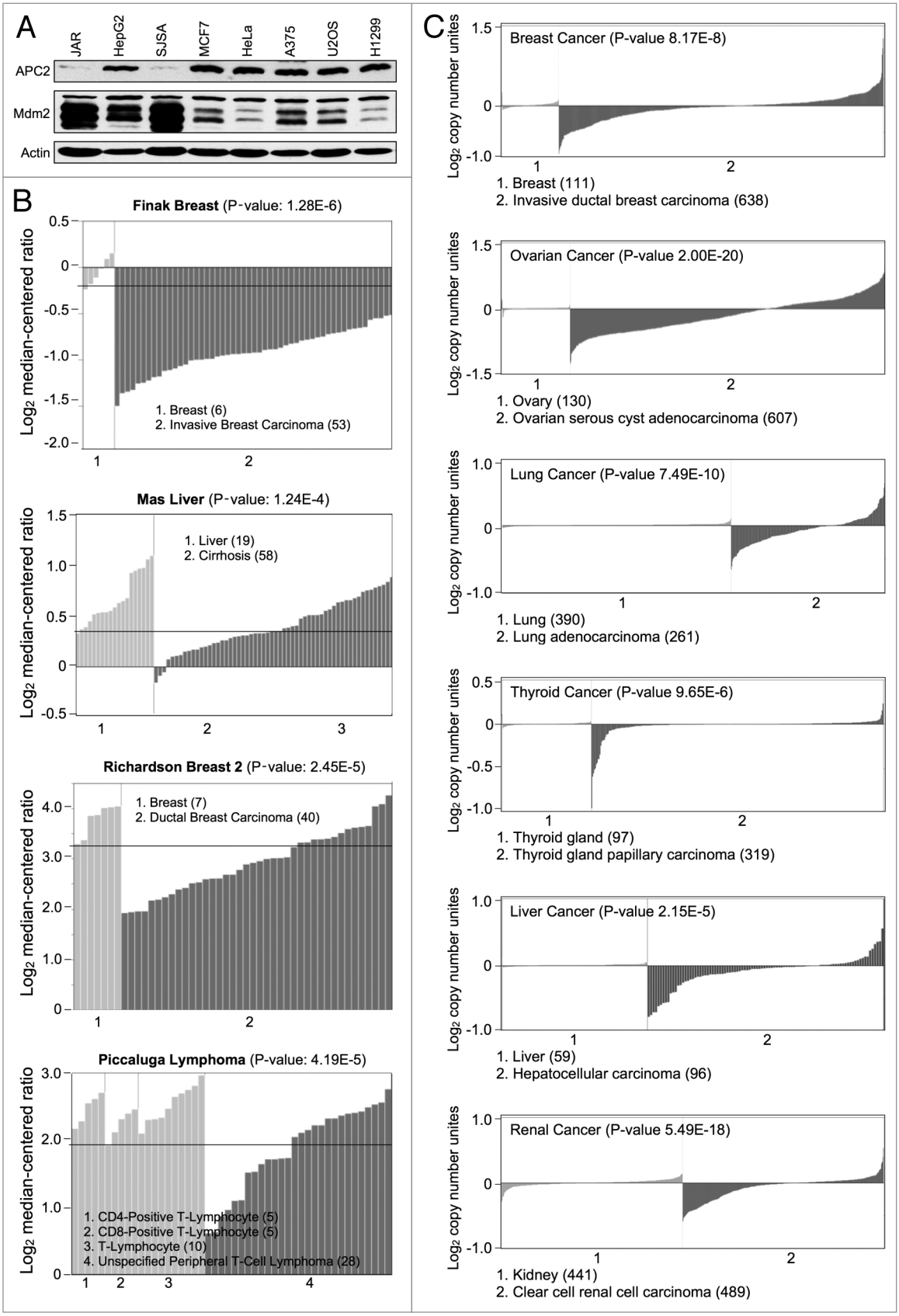

Figure 5. For figure legend, see page 2108.

Figure 5 (See previous page). APC2 downregulation correlates with cancer development. (**A**) Western blot was used to assess APC2 and Mdm2 in JAR, HepG2, SJSA, MCF7, HeLa, A375, U2OS, and H1299 cancer cell lines. Actin was used as a loading control. (**B**) mRNA microarray data for APC2 in Invasive breast carcinoma (Finak Breast), liver cancer with cirrhosis (Mas Liver), ductal breast carcinoma (Richardson Breast 2), and unspecified peripheral T-cell lymphoma (Piccaluga Lymphoma). Data was obtained from Oncomine. (**C**) TCGA Gene copy number data for APC2 in breast, ovarian, lung, thyroid, liver, and renal cancer. Data was obtained from Oncomine.

ATP). Reaction mixes were incubated at 37° C for 1 h, the beads were washed with 3 times with washing buffer (50 mM TRIS-HCl pH 8.0, 0.1 M NaCl, 1 mM DTT, 5 mM MgCl2, 1% TritonX-100, 1 mM PMSF), and analyzed by western blotting.

Double thymidine block synchronization

Cells were cultured with 2 mM thymidine (Sigma #T9250, http://www.sigmaaldrich.com/catalog/product/sigma/t9250?lang=en&region=US) for 19 h, washed once with 1× PBS, incubated with fresh media without addition of thymidine, and then 2 mM thymidine was added back 8 h later. After another 16 h, cells were washed once with 1× PBS, fresh media without thymidine addition was added back. Cells were collected every 2 h and analyzed by western blotting.

DNA plasmids, siRNA, and lentiviral-based shRNA

All cloned constructs were confirmed by DNA sequencing. siRNA duplexes targeting APC2 (5′-AACGATCTGC AGGCCAACAT C-3′) and nonspecific control siRNA (5′-CAGUCGCGUU UGCGACUGG-3′) were manufactured by Dharmacon. Stealth siRNAs targeting APC2 (HSS121004, HSS179082, HSS179083, http://www.lifetechnologies.com/order/genome-database/browse/sirna/keyword/HSS121004) were manufactured by Invitrogen. Lenti-viral based shRNA clones were purchased commercially from Open Biosystems (pLKO.1 shRNA sets RMM4534-NM_175300, http://www.thermoscientificbio.com/shrna/trc-lentiviral-shrna/?productId=1961A1D5-D4C1-4A23-A7D0-81D8B30525CB). Transfections of siRNA were performed with RNAiMax (Invitrogen # 13778, https://www.lifetechnologies.com/order/catalog/product/13778075?ICID=search-product) according to the manufacturer's instructions 48 h before additional treatments

SDS-page and western blotting

Cells were lysed in 0.5% NP-40 lysis buffer (50 mM TRIS-HCl pH7.5, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM NaVO3, 1 mM DTT, 1 mM PMSF, 1× protease inhibitor cocktail), lysates were resolved on a 12.5% polyacrylamide gel and transferred onto a 0.45 μM nitrocellulose membrane. Membranes were blocked for at least 30 min in blocking buffer (PBS with 0.1% Tween-20 and 5% nonfat dried milk). Membranes were incubated for overnight in primary antibody diluted in blocking buffer, incubated for 1–2 h in secondary HRP-conjugated antibody diluted in block buffer, and exposed after incubate in Supersignal West Pico (Pierce #34080, http://www.piercenet.com/product/supersignal-west-pico-chemiluminescent-substrate) or Dura (Pierce #37075, http://www.piercenet.com/product/supersignal-west-dura-chemiluminescent-substrate) for 5 min.

IP-western blotting

MEF cells were lysed in 0.5% NP-40 lysis buffer. The lysates were pre-cleared with Sepharose CL4B beads (Sigma #CL4B200, http://www.sigmaaldrich.com/catalog/product/sigma/cl4b200?lang=en&region=US) for 30 min and then immunoprecipitated with the specified antibody overnight at 4 °C, followed by incubation with protein A/G beads (Pierce #20333, http://www.piercenet.com/product/protein-a-agarose), (Pierce #20399, http://www.piercenet.com/product/protein-g-agarose-kits) for 2 h at 4° C. The beads were washed 4 times with cold 0.5% NP-40 lysis buffer, and analyzed by SDS-PAGE and western blotting.

Half-life assay

Cells were treated with cycloheximide (100–150 μg/mL) (Sigma #C7698, http://www.sigmaaldrich.com/catalog/product/sigma/c7698?lang=en&region=US) for the indicated length of time and then harvested using 1× SDS lysis buffer (50 mM TRIS-HCl, pH 7.5, 0.5 mM EDTA, 1% SDS, and 1 mM DTT). The Mdm2 level was analyzed by western blotting, and the intensity of the bands in the linear range of exposure was quantified by densitometry and normalized to Actin using ImageJ.

Beta-gal staining

Beta-Gal staining was done with Senescence β-Galactosidase Staining Kit (Cell Signaling #9860, http://www.cellsignal.com/product/productDetail.jsp?productId=9860) according to the manufacturer's instructions.

Luciferase assay

Beta-Gal staining was done with Dual-Light® Luciferase and β-Galactosidase Reporter Gene Assay System (Life technologies #T1004, http://www.lifetechnologies.com/order/catalog/product/T1004?CID=bnk) according to the manufacturer's instructions.

Antibodies

Rabbit anti-APC2 antibody was kindly provided by Dr Yue Xiong (UNC at Chapel Hill). HA (12CA5), myc (9E10), Mdm2 (2A10), and Mdm2 (4B11) antibodies were made from the culture medium of the hybridoma cell line. The following antibodies were purchased commercially: Actin (Chemicon #MAB1501, http://www.millipore.com/catalogue/item/mab1501), hp53 (DO-1 Labvision AB-6 #MS-187 – P, http://www.fishersci.com/ecomm/servlet/fsproductdetail_10652_1672983__-1_0), mp53 (Leica # NCL-p53–505, http://www.leicabiosystems.com/ihc-ish/novocastra-reagents/primary-antibodies/details/product/p53-protein/), FLAG (M2 Sigma #F3165, http://www.sigmaaldrich.com/catalog/product/sigma/f3165?lang=en&region=US). Anti-UB (Covance #PRB-268C, https://store.crpinc.com/datasheet.aspx?catalogno=prb-268C), Cyclin B1 (GNS1 Santa Cruz #sc-245, http://www.scbt.com/datasheet-245-cyclin-b1-gns1-antibody.html)

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/29106

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