Ubiquitylation of p53 by the APC/C inhibitor Trim39

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Tripartite motif 39 (Trim39) is a RING domain-containing E3 ubiquitin ligase able to inhibit the anaphase-promoting complex (APC/C) directly. Through analysis of Trim39 function in p53-positive and p53-negative cells, we have found, surprisingly, that p53-positive cells lacking Trim39 could not traverse the G1/S transition. This effect did not result from disinhibition of the APC/C. Moreover, although Trim39 loss inhibited etoposide-induced apoptosis in p53-negative cells, apoptosis was enhanced by Trim39 knockdown in p53-positive cells. Furthermore, we show here that the Trim39 can directly bind and ubiquitylate p53 in vitro and in vivo, leading to p53 degradation. Depletion of Trim39 significantly increased p53 protein levels and cell growth retardation in multiple cell lines. We found that the relative importance of Trim39 and the well-characterized p53-directed E3 ligase, murine double minute 2 (MDM2), varied between cell types. In cells that were relatively insensitive to the MDM2 inhibitor, nutlin-3a, apoptosis could be markedly enhanced by siRNA directed against Trim39. As such, Trim39 may serve as a potential therapeutic target in tumors with WT p53 when MDM2 inhibition is insufficient to elevate p53 levels and apoptosis.

E3 ubiquitin ligases are essential for cell cycle transitions, controlling the abundance of many key cell cycle regulators, such as cyclin proteins and their inhibitors (1). In addition, E3 ligases, such as murine double minute 2 (MDM2), a ligase that targets the p53 tumor suppressor, are known to control the cellular response to DNA damage, including cell cycle arrest and the induction of apoptosis (2–4).

Recently, we reported that involvement of the tripartite motif 39 (Trim39) E3 ubiquitin ligase in controlling DNA damage-induced apoptosis through inhibition of the anaphase promoting complex (APC/C), a multiprotein ubiquitin ligase that controls multiple cell cycle regulators, including cyclins, geminin, and others (5). Surprisingly, analysis of cell cycle progression in cells lacking Trim39 now suggests that it also might be important for G1/S progression/initiation of DNA replication in some cell types but that a target other than the APC/C was likely involved.

The sequence-specific transcription factor p53 controls a host of genes important for cell death and proliferation (6). The p53 protein is crucial for protecting cells in times of stress through a combination of cell cycle arrest, apoptosis, cellular senescence, DNA repair, and autophagy (7, 8). A variety of stressors can engage the p53 pathway, and p53 mutations, often lying within the p53 DNA-binding domain or within its protein interaction domains, are present in greater than half of human cancer cases (9, 10).

Given the centrality of p53 in the cellular stress response and the potential deleterious impact of alterations in p53 levels (e.g., cell cycle arrest, cell death) under nonstressed conditions, levels of cellular p53 are tightly controlled via ubiquitin-mediated proteasomal degradation (11, 12). The most intensively studied p53-directed E3 ligase is MDM2, proven in many settings to promote p53 proteasomal degradation (2–4). Indeed, MDM2 inhibitors, such as nutlin-3a, are potential cancer therapeutics aimed at increasing p53 abundance to reduce proliferation and enhance tumor cell death/senescence (13, 14). Despite the importance of MDM2 in regulating p53 levels, p53 is susceptible to degradation in *MDM2*-deficient mice (15), indicating the presence of other E3 ligases able to target p53. In this regard, several E3 ligases, including Pirh2, COP1, ARF-BP1, CHIP, CARP1, and CARP2, can promote p53 ubiquitylation and degradation under specific circumstances and/or in specific cell types (12).

Here, we report that the Trim39 E3 ubiquitin ligase, previously identified as a regulator of the APC/C, has a dual role as a direct E3 ubiquitin ligase for p53. Accordingly, the G1/S arrest observed following depletion of Trim39 was dependent on p53. The ability of Trim39 to induce p53 ubiquitylation and degradation did not depend on the APC/C and was also independent of MDM2. Accordingly, for several cell lines that were relatively insensitive to nutlin-3a, depletion of Trim39 increased apoptotic cell death. This identification of a previously unknown negative regulator of p53 suggests that there are likely to be as yet unexplored strategies for elevating p53 for therapeutic benefit.

Results

Knockdown of Trim39 Induces G1/S Arrest and Inhibition of Proliferation. Based on its ability to inhibit the APC/C, we had postulated that Trim39 protein might be a component of the spindle assembly checkpoint that prevents APC/C activation when chromosomes are not properly aligned on the mitotic spindle. To assess this, we transfected cells with Trim39-directed siRNA able to deplete greater than 90% of Trim39 from human telomerase reverse transcriptase-immortalized retinal pigment epithelial cell line (hTERT-RPE) cells (where we had seen a marked effect on APC/C-mediated protein degradation), synchronized these cells using a double thymidine block, released them into nocodazole to disrupt the spindle, and monitored them by time-lapse microscopy or analyzed their DNA content by flow cytometry (Fig. S1 *A* and *B*). We were surprised to find that the great majority of cells lacking Trim39 (93%) never entered mitosis (Fig. S1 *A* and *B*). These data suggested that there might be another critical role for Trim39 in promoting cell cycle progression. Indeed, proliferation of the RPE cells was markedly dampened by loss of Trim39, as shown by both cell growth curves and colony-forming assays (Fig. 1 *A* and *B*). Moreover, EdU (5-ethynyl-2′-deoxyuridine) incorporation was significantly decreased after knockdown of Trim39 (1.3% vs. 25.9% in control; $P < 0.05$), demonstrating that the lack of Trim39 prevented DNA replication (Fig. 1*C* and Fig. S1*C*). Together, these data suggested that Trim39 has a role in the G1/S transition (under normal, nonstressed conditions), in addition to its role in controlling apoptosis in response to DNA damage. Indeed, cell death did not contribute to failure of Trim39-deficient cells to proliferate, because depletion of Trim39 had little effect on basal rates of cell death in hTERT-RPE cells as determined by annexin V/propidium iodide (PI) staining (Fig. S1*D*).

G1 Arrest Induced by Depletion of Trim39 Is Dependent on p53. The APC/C inhibitor Emi1 enables DNA synthesis by allowing accumulation of APC/C substrates, such as cyclin A, and other

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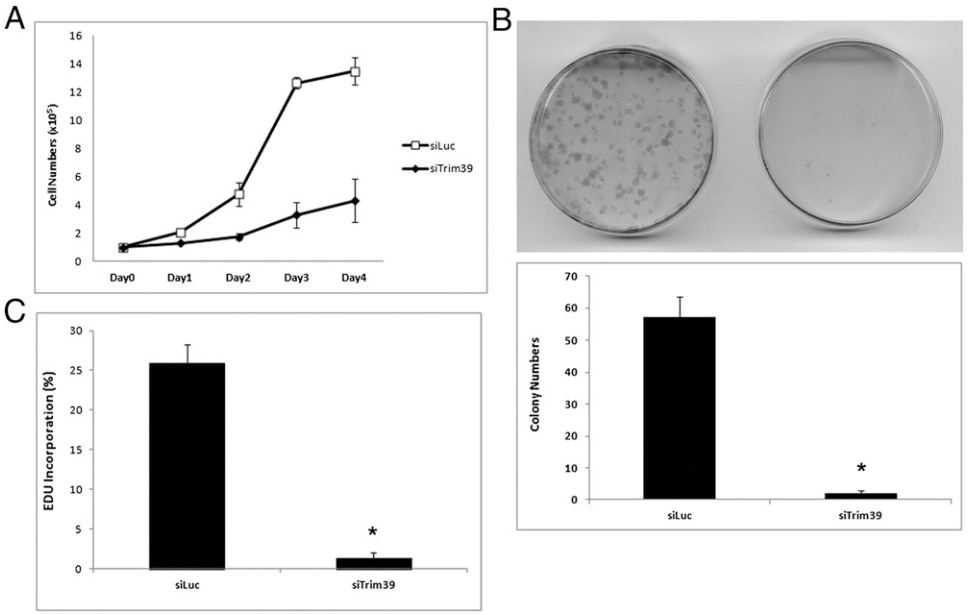

Fig. 1. Trim39 depletion induces cell growth retardation. (*A*) hTERT-RPE cells were transfected with control or Trim39 siRNA, and 1×10^5 cells were seeded in six-well plates 2 d after siRNA transfection. Cells were counted each day for 4 d. Luc, luciferase (*B*) Colony assay. Two days after siRNA transfection, 500 cells were seeded in 60-mm dishes and cultured for 12 d, and the number of colonies was counted. $*P < 0.05$ vs. control. (*C*) hTERT-RPE cells were transfected with control or Trim39 siRNA, and DNA replication was monitored by EdU incorporation. $*P < 0.05$ vs. control.

factors required for the G1/S transition (16–19). Thus, it remained possible that Trim39 also controlled G1/S through its ability to modulate the APC/C. The hTERT-RPE cells were serum-starved to arrest at G0/G1 phase and then released into serum-containing medium. As shown in Fig. 2*A*, the levels of the APC/C substrates geminin and cyclin A rapidly increased in a time-dependent manner in control cells, whereas these increases were not observed in cells treated with Trim39 siRNA. A similar decrease in cyclin A and geminin accumulation could be seen on loss of Emi1 (Fig. 2*B*). However, additional analysis revealed a fundamental difference between loss of Emi1 and Trim39; proteasome inhibition by MG132 was able to restore cyclin A and geminin levels following knockdown of Emi1, whereas proteasome inhibition had a minimal effect on the levels of these proteins following Trim39 knockdown (Fig. 2*B*). These data suggested that the effect of Trim39 on cyclin A and geminin expression was unlikely to be a result of Trim39-mediated APC/C inhibition.

To address these issues further, we performed a quantitative real-time PCR assay on control cells or cells treated with Trim39 siRNA. As shown in Fig. 2*C*, there was a significant decrease in the levels of cyclin A, geminin, and Emi1 mRNAs in the absence of Trim39. However, we observed a marked increase in the expression of mRNAs encoding p21 (but not p27 and p16), MDM2, and Bax after Trim39 depletion. These genes are well-known targets of the tumor suppressor p53, raising the possibility that p53 was involved in some way in the G1/S transition defect observed in cells lacking Trim39. Therefore, we examined the effects of codepletion of p53 and Trim39. Although the protein levels of p53 and p21 were increased coordinately with decreased cyclin A and geminin proteins after depletion of Trim39, control levels of these proteins were restored after codepletion of Trim39 and p53 (Fig. 2*D*). These data strongly suggested that the G1/S arrest resulting from Trim39 depletion in RPE cells was exerted through p53. Indeed, knockdown of p53 largely (albeit not completely) restored EdU incorporation and DNA replication following knockdown of Trim39 (Fig. 2*E* and Fig. S2 *A–C*). Extending these results to other cell types, and consistent with this idea, we found that depletion of Trim39 decreased EdU incorporation in cells with WT p53 (A549, U-87 MG, 22Rv1, MCF7, HepG2, and HCT116 $P53^{+/+}$) but had either a minor or no effect on EdU incorporation in p53 mutant or null cells (H1299, T98G, PC3, and HCT116 $P53^{-/-}$) (Fig. 2*F*). Collectively, these data suggest that Trim39 depletion can induce accumulation of p53 and its target, p21, resulting in a failure to traverse the G1/S transition.

Trim39 Interacts with p53 and Regulates Its Stability. Given the effects of Trim39 on p53 levels, we tested whether Trim39 and p53 could interact. As shown in Fig. 3*A*, Trim39 and p53 proteins were both predominantly nuclear and the nuclear fraction of p53 was dramatically increased in abundance following Trim39 depletion (Fig. 3*A*; also shown in micrographs in Fig. S2*D*). In addition, we were able to coimmunoprecipitate p53 and Trim39 with antisera directed against either protein (Fig. 3 *B–D* and Fig. S3 *A* and *B*). Moreover, we were able to demonstrate a direct interaction between recombinant Trim39 and p53 proteins in vitro (Fig. S3 *C* and *D*).

Because Trim39 depletion increased p53 protein levels and we had previously characterized Trim39 as a RING E3 ligase, we monitored the effect of Trim39 on p53 protein stability and found, after depletion of Trim39, that the half-life of p53 was significantly increased (Fig. 4*A* and Fig. S4 *A* and *B*). Importantly, although protein levels of p21 were also dramatically increased after depletion of Trim39, the half-life of p21 was not changed, indicating that Trim39 does not regulate protein levels of p21 directly (Fig. 4*A* and Fig. S4 *A* and *B*). Rather, it is likely that changes in p21 levels reflect changes in its transcriptional activator, p53. Trim39 overexpression decreased levels of p53; this decrease was dependent on Trim39 E3 ligase activity, because overexpression of ligase-deficient RING domain mutants could not promote p53 degradation, despite their ability to bind p53 (Figs. 3*D* and 4*B*). The proteasome inhibitor MG132 allowed preservation of p53 protein in the face of WT Trim39 overexpression, consistent with a role for Trim39 in p53 degradation (Fig. S5*A*). In addition, the half-life of exogenously expressed p53 was significantly decreased on coexpression of WT Trim39 and was actually increased with coexpression of the ligase-deficient RING domain mutant, likely acting in a dominant-negative fashion to impede endogenous Trim 39 (Fig. S4*C*).

Furthermore, ectopic expression of WT Trim39, but not the RING mutant protein, increased p53 ubiquitylation, consistent with the possibility that Trim39 acts as a p53-directed E3 ubiquitin ligase (Fig. S5*B*). In support of this idea, Trim39 was able to ubiquitylate p53 directly in vitro (Fig. 4*C* and Fig. S5*C*). Given that we had previously reported that Trim39 could regulate APC/C

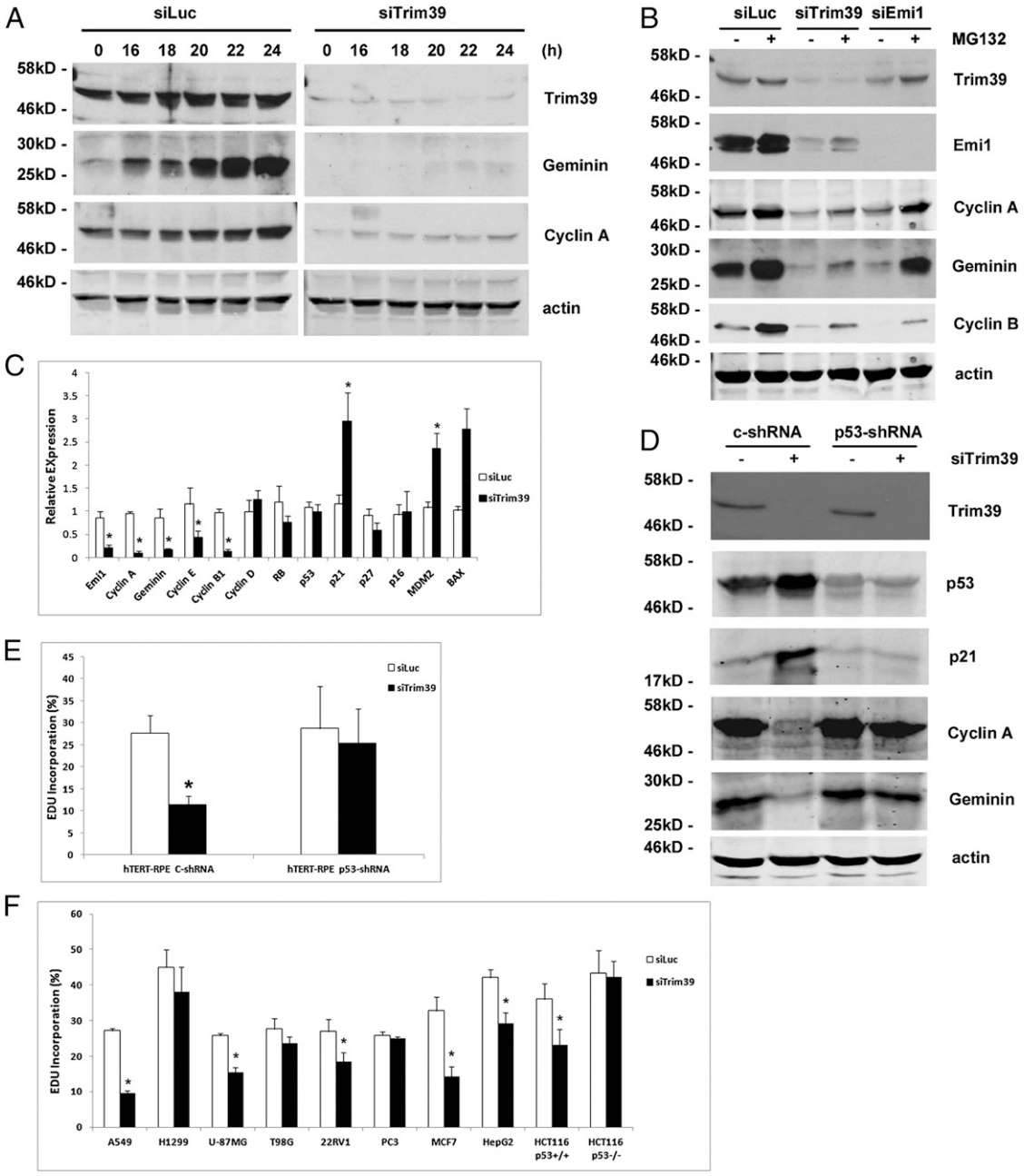

Fig. 2. Cell growth inhibition induced by Trim39 depletion is p53-dependent. (*A*) hTERT-RPE cells were arrested at G1 phase by serum starvation, and cells were harvested at different time points after adding back media with 10% FBS. The protein levels were detected by Western blotting with the indicated antibodies. Luc, luciferase. (*B*) Cells were transfected with control, Trim39, or Emi1 siRNA; 48 h later, cells were treated with MG132 for a further 8 h. Western blotting was carried out to detect the protein level with the indicated antibodies. (*C*) Real-time PCR was carried out to detect the gene expression in control or Trim39 knockdown cells. *$P < 0.01$ vs. control. (*D*) Control-shRNA or p53-shRNA cells were transfected with control or Trim39 siRNA, and the protein levels were detected by Western blotting with the indicated antibodies. (*E*) Control-shRNA (c-shRNA) or p53-shRNA cells were transfected with control or Trim39 siRNA, and cell proliferation was measured by EdU incorporation. *$P < 0.05$ vs. control. (*F*) Panel of cell lines with different p53 background was transfected with control or Trim39 siRNA, and cell proliferation was detected by EdU incorporation. *$P < 0.05$ vs. control.

activity, we speculated that there might be an unknown connection between the APC/C and p53 stability. However, depletion of CDH1, the activator of APC/C in interphase, or APC11, a core subunit of the APC/C, had no effect on the ability of Trim39 loss to elevate p53 levels (Fig. S6 *A* and *B*), strongly suggesting that Trim39 did not affect p53 levels by modulating the APC/C.

Trim39 Can Synergize with MDM2 to Affect Cell Growth and Apoptosis. There are a number of E3 ubiquitin ligases that have been reported to act on p53, most prominently MDM2 (2–4). We wished to determine whether the regulation of p53 stability by Trim39 was dependent on MDM2; thus, we transfected H1299 cells lacking p53 with control or MDM2 siRNA and examined the effects of Trim39 on the stability of cotransfected p53. As shown in Fig. 5*A*, Trim39 expression was able to induce p53 degradation even in the absence of MDM2. We also did not observe any direct effects of Trim 39 on MDM2 (or vice versa). Although Trim 39 knockdown was able to elevate MDM2 levels

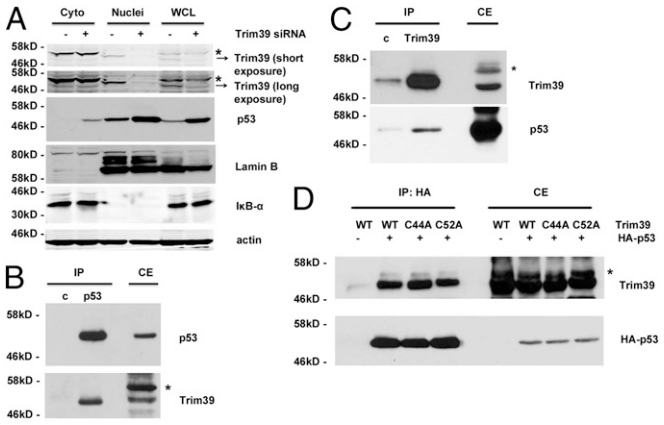

Fig. 3. Trim39 localizes to the nucleus and interacts with p53. (*A*) hTERT-RPE cells were transfected with control or Trim39 siRNA, and cytoplasmic (Cyto) and nuclear extracts (Nuclei) were fractionated and analyzed by Western blotting with the indicated antibodies. The asterisk indicates nonspecific signal. (*B* and *C*) hTERT-RPE cells were cross-linked with DSP (Dithiobis[succinimidyl propionate]), and immunoprecipitation was carried out to detect the interaction between Trim39 and p53. The asterisk indicates nonspecific signal. (*D*) 293T cells were transfected with HA-p53, Trim39 WT, C44A, or C52A, respectively, as indicated. HA-p53 was immunoprecipitated with anti-HA antibody, and Western blotting was carried out to detect the interaction between Trim39 and HA-p53. The asterisk indicates nonspecific signal. c, control; CE, cell extract; IP, immunoprecipitation; WCL, whole cell lysate.

(Fig. S7*A*), this was an indirect effect resulting from stabilization of p53 (and thus enhanced MDM2 transcription) because knockdown of Trim 39 in H1299 cells lacking p53 had no effect on MDM2 levels (Fig. S7*B*). Moreover, it appears that Trim39 and MDM2 bind to different domains of p53, because p53 mutants (e.g., L14Q/F19S, L22Q/W23S) that were unable to bind MDM2 bound as well to Trim39 as the WT p53 (20) (Fig. S8*A*).

To assess the relative contributions of Trim39 and MDM2 in controlling cell cycle progression across a range of p53 WT cell types, we knocked down Trim39, MDM2, or both and measured EdU incorporation as well as protein levels of p53 and p21. As shown in Fig. 5*B* and Fig. S7*A*, MDM2 and Trim39 knockdown had a variable impact on cell cycle progression in a variety of different cell types, with both ligases contributing to the overall p53 and p21 levels as well as possessing the ability to undergo DNA replication. That said, in cells in which another p53-directed negative regulator (e.g., murine double minute 4 (MDMX) in JEG-3 cells) is known to play a dominant role, knockdown of neither Trim39 nor MDM2 (or both) had a significant impact on EdU incorporation (Fig. S8*B*).

MDM2 has been considered a promising target for cancer chemotherapy in that elevating levels of p53 in p53-positive tumors has the potential to induce cell cycle arrest and/or apoptosis. A number of MDM2 inhibitors have been developed, the most well known of which is nutlin-3a, a compound that interferes with the binding of p53 to MDM2 (13, 14). In contrast, we found that nutlin-3a was unable to disrupt the interaction of Trim39 with p53 (Fig. S8*C*, compare lanes 2 and 5). Given the variable relative importance of MDM2 in different cell types, these data suggest that nutlin-3a might have only partial effects in cells expressing both MDM2 and Trim39. Therefore, the utility of nutlin-3a as a therapeutic agent is likely to be restricted by the population of E3 ligases other than MDM2 (in this case, Trim39) that contribute to p53 stability in a given cell type, particularly if those ligases, like Trim39, are refractory to inhibition by nutlin-3a. Given this, we hypothesized that coinhibition of Trim39 would increase the efficacy of nutlin-3a in killing p53-positive cells. Consistent with this idea, knockdown of Trim39 enhanced cell killing by nutlin-3a in p53 WT cells expressing both ligases, even in cells that were relatively refractory to nutlin-3a, such as MCF-7 breast cancer cells and A549 lung cancer cells (Fig. 5 *C* and *D*). Importantly, nutlin-3a, either alone or in combination with Trim39 knockdown, did not kill cancer cells in the absence of p53 (Fig. 5*D*; H1299, p53 null cancer cell).

We and others have reported that Trim39 helps to induce apoptosis following DNA damage, due to its ability to elevate levels of the Bax activator modulator of apoptosis 1 (MOAP-1; by means of MOAP-1 degradation by the APC/C). However, based on the data shown here, it seems that Trim39 can also be considered antiapoptotic, because its loss can promote stabilization of p53 and cell death. To evaluate this issue further, we knocked down Trim39 in a p53 WT cell (A549) or a p53 null cell (H1299) and treated with etoposide. As shown in Fig. S8*D*, knockdown of Trim39 in the presence of functional p53 enhanced

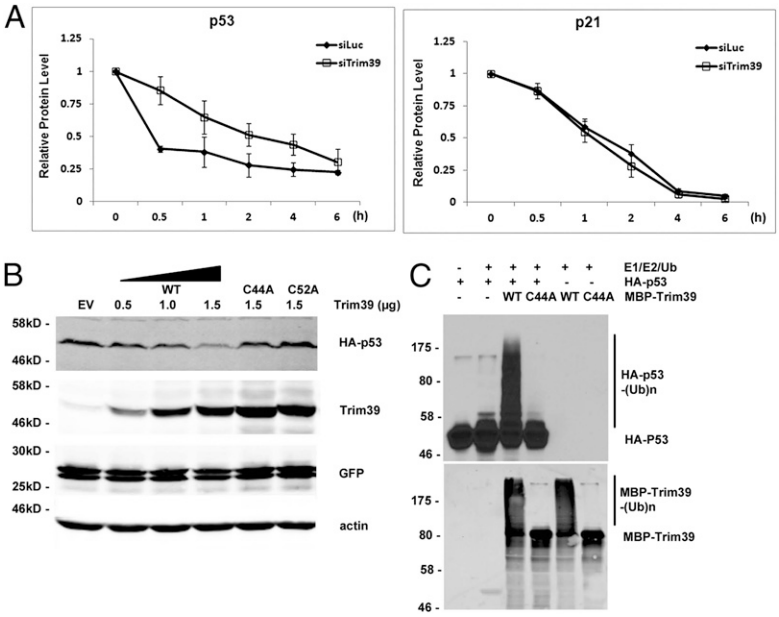

Fig. 4. Trim39 negatively regulates p53 levels. (*A*) hTERT-RPE cells were transfected with control or Trim39 siRNA, and cells were treated with cycloheximide for the time courses indicated. The protein levels of p53 and p21 were detected by Western blotting, quantified using Odyssey software, and plotted against time to determine protein half-lives. Luc, luciferase. (*B*) H1299 cells were transfected with 0.2 μg of HA-p53, 0.1 μg of pEGFP-C1, and different amounts of Trim39 WT or RING mutant-encoding plasmids as indicated. Cells were harvested 48 h after transfection, and protein levels were detected by Western blotting with the indicated antibodies. (*C*) In vitro ubiquitylation of p53 by Trim39. 293T cells were transfected with HA-p53, and HA-p53 was immunoprecipitated and mixed with myelin basic protein (MBP)-Trim39 and E1/E2/ubiquitin as indicated for 1 h at 30 °C. Reactions were stopped by adding sample buffer, and Western blotting was carried out to detect the ubiquitylation of p53.

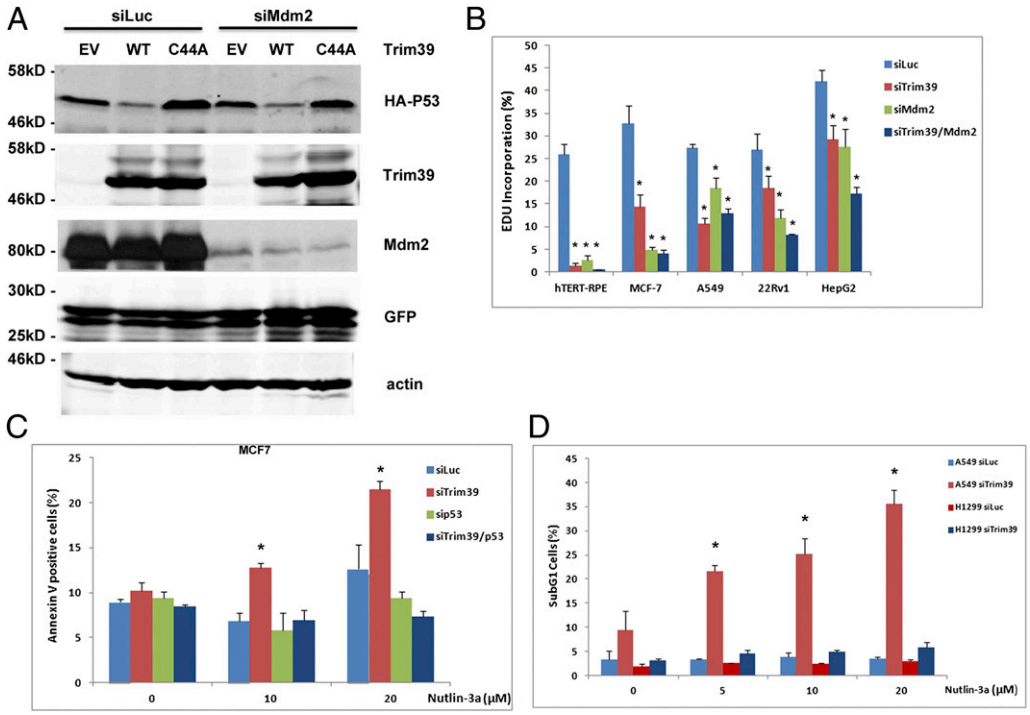

Fig. 5. Regulation of p53 stability by Trim39 is independent of MDM2. (*A*) H1299 cells were transfected with control or MDM2 siRNA first, and cells were then transfected with HA-p53, Trim39 WT or RING mutants, and pEGFP-C1 as indicated 8 h after siRNA transfection. Cells were harvested 48 h later, and protein levels were detected by Western blotting with the indicated antibodies. Luc, luciferase; EV, empty vector. (*B*) hTERT-RPE, MCF7, A549, 22Rv1, or HepG2 cells were transfected with control, Trim39, or MDM2 siRNA as indicated. Forty-eight hours later, cell proliferation was monitored by EdU incorporation. $*P < 0.05$ vs. control. (*C*) MCF7 cells were transfected with control, Trim39, or p53 siRNA as indicated; 48 h later, cells were treated with nutlin-3a for a further 48 h. Cells were harvested and stained with annexin V and PI, and apoptosis was detected by flow cytometry. $*P < 0.05$ vs. control. (*D*) A549 or H1299 cells were transfected with control or Trim39 siRNA, and 48 h later, cells were treated with nutlin-3a for a further 48 h. Apoptosis (as indicated by the presence of a sub-G1 population) was detected by flow cytometry. $*P < 0.05$ vs. control.

etoposide-induced cell death. In contrast, depletion of Trim39 in cells lacking p53 dampened etoposide-induced apoptosis, which is likely due, at least in part, to the effects of Trim39 on the APC/C and its substrate, MOAP-1, as discussed further below.

Discussion

p53 is a central regulator of cell death, proliferation, and senescence and enables the cell to withstand a wide variety of stressors (1, 11, 21). For this reason, p53 mutation or deficiency contributes to a significant fraction of human tumors. Although p53 functions are required in the face of cellular stress or damage, normal proliferation of the unperturbed cell requires that intracellular levels of p53 be restrained. Accordingly, a complex network of posttranslational modifications (e.g., acetylation, methylation, phosphorylation) fine-tunes p53's half-life, rendering it more or less susceptible to the action of E3 ubiquitin ligases (10, 11).

Although the E3 ubiquitin ligase MDM2 is thought to be the primary determinant of p53 ubiquitylation and stability in a variety of cell types, it is clearly not the only relevant p53-directed ligase (15, 22). Aside from the observation that p53 can be degraded in MDM2-deficient mice, mutation of the known MDM2-targeted lysines on p53 does not prevent p53 ubiquitylation and degradation in cells (22). Moreover, several other E3 ligases, including another Trim family member, Trim24, are capable of promoting p53 degradation (23). An additional level of complexity stems from the ability of several of the p53-directed ligases to control each other's stability, potentially through direct interaction (24).

In this study, we identified Trim39 as a unique E3 ligase directly regulating p53 stability and compared the differential effects of Trim39 and MDM2 knockdown on cell proliferation, p53 stability, and p21 induction in multiple cell lines. Interestingly, we found that the effect of these ligases varied in different cell types. For example, it seems that Trim39 contributes more significantly than MDM2 to cell growth and p53 stability in hTERT-RPE cells. This may be of potential significance for diseases of the retina, for example, macular degeneration, where p53 is important for controlling cell death in response to oxidative stress (25).

For tumors with WT p53, numerous pharmacological approaches have been taken to upregulate or stabilize p53 protein (26, 27). Small molecules, such as nutlin-3a and MI-219, are able to disrupt MDM2–p53 interactions, thereby preventing MDM2-mediated p53 ubiquitylation (13, 14, 27). Although this approach to induce p53-mediated cell cycle arrest/apoptosis has been successful in many cancer cell lines and xenograft models (and is under investigation for treating human cancers), significant expression of other E3 ligases, such as Trim39, would be expected to diminish the effects of nutlin-3a and similar molecules, unless the interaction of p53 with these other ligases was also disrupted. For example, nutlin-3a is already known to be ineffective in cells that express high levels of MDMX, another known p53-directed ligase (28–30). Given this, effective elevation of p53 in tumors may well require a mixture of E3 ligase inhibitors tailored to the population of p53-directed ligases present in an individual tumor. Indeed, unique inhibitors that target both MDM2 and MDMX can impede growth and induce apoptosis in cells with high levels of both MDM2 and MDMX (26, 31, 32).

In this study, we found that combining nutlin-3a with knockdown of Trim39 was effective in triggering apoptosis in cells that were otherwise insensitive to nutlin-3a (e.g., MCF7, A549). However, this combination was not effective in JEG-3 cells,

consistent with previous reports documenting high levels of MDMX expression in these cells.

Many of the E3 ligases that target p53 are also transcriptional targets of p53 (12). Thus, although E3 ligase inhibition may elevate p53, p53, in turn, can promote transcriptional up-regulation of the ligases, potentially overcoming the effect of the inhibitors. Trim39 does not appear to be regulated by p53, because depletion of MDM2 significantly increases the levels of p53 without affecting the levels of Trim39. This might make Trim39 a promising therapeutic target, but we would note that depletion of Trim39 significantly increases protein levels of both p53 and MDM2; induced MDM2 inhibition might therefore counter the effects of Trim39 inhibition. These observations again support the notion of an inhibitor mixture as perhaps the most effective approach to elevating p53 levels.

The identification of Trim39 as a p53-directed ligase raises the question of how this activity may be related to its previously described role as an APC/C inhibitor. It does not appear that the effects of Trim39 on p53 are exerted through the APC/C, nor do effects on the APC/C require p53 (because Trim39 can inhibit the APC/C in p53-null cells). Although Trim39 E3 ligase activity is required to inhibit the APC/C and Trim39 can inhibit the purified APC/C in vitro, the direct target of Trim39 in the APC/C has not yet been identified. It is possible that identification of determinants of p53 recognition/binding by Trim39 may shed light on the direct interactors of Trim39 within the APC/C. DNA damage-induced activation of Trim 39 appears to have different consequences depending on the p53 status of the cell. In the absence of p53, the Trim 39-mediated inhibition of the APC/C results in stabilization of MOAP-1, promoting Bax activation and apoptosis. Additionally, although the ability of Trim39 to inhibit the APC/C seems to be activated by DNA damage, Trim39 knockdown can affect p53 levels even in the unperturbed cell cycle. Taken together, these data place Trim39 as a regulator of several key processes in the proliferative cycle. Additional experiments will determine whether these functions are entirely independent and whether they can be manipulated to therapeutic advantage in the setting of cancer, eye disease, or other pathologies involving misregulation of p53.

Materials and Methods

Flow Cytometry Analysis. For the proliferation assay, the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) was used to measure cell proliferation following the manufacturer's instructions. For cell cycle analysis, cells were fixed in 70% ethanol (vol/vol) (prechilled at −20 °C) for at least 30 min and incubated in 15 μg/mL RNase A (Roche) for 15 min, followed by incubation in 50 μg/mL PI (Sigma) for 30 min. For the annexin V/PI apoptosis assay, cells (~1×10^5) were collected and washed once with PBS, followed by incubation in annexin V (A13201; Invitrogen) solution [2.5 μL of annexin V in 50 μL of annexin V binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl2)] for 15 min and 300 μL of 2 μg/mL PI in annexin V binding buffer. Flow cytometry analysis was carried out with the use of a Becton–Dickinson FACScan apparatus and CellQuest software.

Quantitative Real-Time PCR Analysis. Quantitative real-time PCR analysis was carried out as described (33). All samples were amplified in triplicate, and every experiment was repeated at least two times. Relative gene expression was converted using the $2^{-\Delta\Delta Ct}$ method against the internal control GAPDH housekeeping gene. The primers are listed in *SI Materials and Methods*.

Measurement of p53 and p21 Half-Life. For protein half-life assays, 100 μg/mL cycloheximide was added to cell cultures to block protein synthesis. Cells were collected at the indicated time points, and protein levels were measured and quantified by Western blotting using LI-COR Biosciences Odyssey software. The half-lives of proteins were calculated from three independent experiments. Also shown are immunoblots developed using chemiluminescent reagents, but these were not used for quantitation.

In Vitro Ubiquitylation. In vitro ubiquitylation was performed as described (34). More information is provided in *SI Materials and Methods*. Additional information on materials and methods can also be found in *SI Materials and Methods*.

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