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DNA polymerase eta is targeted by Mdm2 for polyubiquitination and proteasomal degradation in response to ultraviolet irradiation

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Abstract

DNA polymerase eta (PolH), the product of the xeroderma pigmentosum variant (XPV) gene and a Y-family DNA polymerase, plays a pivotal role in translesion DNA synthesis. Loss of PolH leads to early onset of malignant skin cancer in XPV patients and increases UV-induced carcinogenesis. Thus, the pathways by which PolH expression and activity are controlled may be explored as a strategy to prevent UV-induced cancer. In this study, we found that Mdm2, a RING finger E3 ligase, promotes PolH degradation. Specifically, we showed that knockdown of Mdm2 increases PolH expression in both p53-proficient and -deficient cells. In addition, we showed that UV-induced PolH degradation is attenuated by Mdm2 knockdown. In contrast, ectopically expression of Mdm2 decreases PolH expression, which can be abrogated by the proteasome inhibitor MG132. Moreover, we showed that Mdm2 physically associates with PolH and promotes PolH polyubiquitination *in vivo* and *in vitro*. Finally, we showed that knockdown of Mdm2 increases the formation of PolH replication foci and decreases the sensitivity of cells to UV-induced lesions in a PolH-dependent manner. Taken together, we uncovered that Mdm2 serves as an E3 ligase for PolH polyubiquitination and proteasomal degradation in cells under the basal condition and in response to UV irradiation.

Keywords

PolH; Mdm2; E3 ligase; Ubiquitination; UV

1. Introduction

DNA polymerase eta (PolH), a member of the Y-family DNA polymerases, is originally identified as an enzyme with the ability to bypass translesion DNA synthesis (TLS) across ultraviolet (UV)-induced pyrimidine dimers [1]. The UV light is the major carcinogen involved in the etiology of skin cancer [2]. In humans, the defect in the *PolH* gene results in xeroderma pigmentosum variant syndrome (XPV) [1, 3]. XPV patients are highly sensitive to UV and have a much greater risk of developing sun-induced skin cancer than that of normal population [4–7]. Due to the role of PolH in repairing UV-induced DNA damage, current studies have been focused on identifying various pathways that regulate PolH

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expression and activity. Recently, we demonstrated that Pirh2 E3 ubiquitin ligase promotes PolH degradation in a ubiquitin-independent manner [8]. In addition, Kim *et al.* showed that GEI-17 SUMO E3 ligase protects PolH protein from CRL4^{Cdt2}-mediated degradation [9]. Other studies showed that the activity of PolH can be regulated by posttranslational modifications, such as monoubiquitination and sumoylation [9–11].

The murine double minute (Mdm2) oncogene is an important negative regulator of p53 [12, 13]. In unstressed cells, the level of p53 is tightly controlled by Mdm2, which associates with p53 and promotes p53 degradation via polyubiquitination. However, Mdm2 is dissociated from phosphorylated p53 induced by stress signals, leading to p53 activation. In addition, Mdm2 is also a target of p53. Given the importance of p53 in maintaining tissue homeostasis, the feedback loop between Mdm2 and p53 has been intensively explored. Interestingly, accumulating evidence showed that Mdm2 also acts as an E3 ubiquitin ligase for other targets, including itself, Mdmx, RPL26, Tip60, and other substrates [12, 14–19].

Here, we found that Mdm2 associates with PolH and targets PolH for ubiquitination, leading to proteasomal degradation. Importantly, we showed that depletion of Mdm2 protects cells from UV-induced cell death in a PolH-dependent manner. Thus, we showed that through regulating PolH stability, Mdm2 is linked to the PolH-mediated pro-survival pathway in response to UV irradiation.

2. Materials and Methods

2.1. Cell culture

RKO, H1299, MCF7, and HCT116 p53^{-/-} were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). MCF7-Mdm2-KD (clone #25) cell line is a derivative of MCF7 cell line in which Mdm2 is inducibly knocked down as previously described [20]. GM00024 primary human fibroblasts were cultured in MEM medium containing 10% FBS.

2.2. Antibodies

Rabbit polyclonal anti-PolH and mouse anti-ubiquitin were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-Mdm2 and anti-PCNA were obtained from Santa Cruz Biotechnology and Calbiochem. Other antibodies were anti-HA (HA11, Covance), anti-FLAG (Sigma), anti-p53 (DO-1, PAb1801, PAb240, and PAb421), and anti-actin (Sigma).

2.3. Immunoprecipitation-Western blot analysis

The experiment was carried out as described [8]. Briefly, RKO cells were transfected with indicated plasmids and harvested 24 h post transfection. Cleared cell lysates (300–500 µg of total proteins) were immunoprecipitated with 1 µg of indicated antibodies and then subject to Western blot analysis.

2.4. Small interference RNA (siRNA)

Scramble and Mdm2 siRNA (sense 5'-AAG CAG UAG CAG UGA AUC dTdT-3' and antisense 5'-GAU UCA CUG CUA CUG CUU dTdT-3') were purchased from Dharmacon. The siRNA duplex was introduced into cells using SilentFect (Bio-Rad) according to the manufacturer's protocol. Cells were harvested 72 h after transfection for Western blot, immunoprecipitation, and FACS analysis.

2.5. Plasmids

pcDNA3 vectors expressing HA-PolH and FLAG-Ubiquitin were described [8]. To generate 2× FLAG-tagged Mdm2, the cDNA fragment was amplified with Mdm2-BF (5'-GAGCGGATCCATGTGCAATACCAACATGTCTGTACCTAC -3') and Mdm2-XR (5'-GACACTCGAGCTAGGGGAAATAAGTTAGCACAATCATTG -3'). Mdm2 (1–441) fragment lacking RING domain was amplified with Mdm2-BF (5'-GAGCGGATCCATGTGCAATACCAACATGTCTGTACCTAC -3') and Mdm2-441-XR (5'-TAGGCTCGAGAATCACACAAGGTTCAATGGCATTG -3'). These products were cloned into pcDNA3-2× FLAG vector via BamHI and XhoI sites.

To generate a construct that expresses shRNA against Mdm2 under the control of the tetracycline-regulated H1 promoter, one pair of oligonucleotides were synthesized, annealed and then cloned into pBabe-H1 at HindIII and BglII sites [20]. The resulting construct was designed pBabe-H1-siMdm2. The sense oligonucleotide is 5'-gatcccc GAAGCAGTAGCAGTGAATC ttcaagaga GGAGAGCAATTAGTGAGAC ttttta-3', and the antisense oligonucleotide is 5'-agcttaaaaa GAAGCAGTAGCAGTGAATC tctcttgaa GGAGAGCAATTAGTGAGAC ggg-3' (the shRNA targeting region is shown in capital).

2.6. Ubiquitination assay

In vivo ubiquitination assay was performed as described [21]. RKO cells were transfected with indicated plasmids and treated with 5 μM MG132 for 6 h prior to harvest. Cleared cell lysates were immunoprecipitated with anti-HA antibody followed by Western blot with anti-HA and anti-Ubiquitin antibodies to detect PolH ubiquitination.

In vitro ubiquitination assay using immunopurified proteins was performed as described [21]. ³⁵S-labeled PolH proteins were mixed with immunopurified FLAG-Mdm2 or Mdm2 (1–441) and then incubated on ice for 1 h to form Mdm2:PolH complexes. The complexes were added to ubiquitination buffer containing E1, E2, and ubiquitin, and incubated at 30°C for 2 h. Finally, the reaction mixtures were separated on SDS-PAGE and analyzed by autoradiography. E1, E2, and ubiquitin were purchased from Boston Biochem. ³⁵S-labeled PolH was produced by the TNT T7-coupled reticulocyte lysates system (Promega). To purify Mdm2 from RKO cells, whole cell lysates from RKO cells transfected with FLAG-Mdm2 or FLAG-Mdm2(1–441) expression vector were then immunoprecipitated by anti-FLAG (Sigma) antibody.

In vitro ubiquitination assay using bacterial purified proteins was carried out as described above except that recombinant GST-Mdm2 and GST-Mdm2(1–441) were used as E3 ligase.

2.7. GST fusion protein preparation

DNA sequences corresponding to the ORFs of human Mdm2 and Mdm2(1–441) were subcloned into the bacterial expression vector pGEX-4T-3 (Amersham Pharmacia Biotech) to produce GST-tagged fusion protein. The GST fusions of Mdm2 and Mdm2 (1–441) were expressed in *E. coli* BL21 (DE3) (Novagene) upon induction with 0.5 mM IPTG for 4 h at 37°C. Bacterial cells were harvested, sonicated, and clarified by centrifugation. The recombinant GST-tagged proteins were purified by glutathione-Sepharose beads (Amersham Pharmacia Biotech) as described previously [22].

2.8. DNA histogram analysis

DNA histogram analysis was performed as previously described [20]. RKO, H1299 and GM00024 cells were transfected with scramble or Mdm2 siRNA for 2 days. Both floating cells in the medium and live cells on the plates were collected 24 h post 15 J/m² UV

irradiation and fixed in 70% prechilled ethanol and followed by PI staining. Stained samples were analyzed by BD FACS Calibur™ flow cytometer.

2.9. Cell proliferation assay

H1299 cells were transfected with scramble or Mdm2 siRNA for 48 h and then an appropriate number of cells were inoculated in 6 well plates in triplicate. 24 h after seeding, cells were washed with PBS and exposed to 15 J/m² UV irradiation and cultured over a 9-day period. Cells were harvested and counted at the indicated times.

2.10. Immunofluorescence assay

Immunofluorescence assay was performed as previously described [22]. Briefly, H1299 cells were transfected with scramble or Mdm2 siRNA for 72 h and then an appropriate number of cells were seeded on cover slide in triplicate. 6 h after UV irradiation (15 J/m²), cells were fixed with formaldehyde and then incubated with a mixture of anti-PolH and anti-PNCA antibodies. Nuclei were visualized using 4'-6-Diamidino-2-phenylindole (DAPI).

3. Results

3.1. Mdm2 regulates the steady-state level of PolH

In our previous study, we showed that PolH protein stability can be regulated by Pirh2 [8]. However, Pirh2-mediated PolH degradation is accomplished in a ubiquitin-independent manner. To investigate whether PolH stability is regulated by other E3 ubiquitin ligases, we showed that upon transient knockdown of Mdm2, the level of PolH was increased (Fig. 1A, left panel). To confirm this, Mdm2 was inducibly knocked down in MCF7 cells (Mdm2-KD). Similarly, PolH was increased upon knockdown of Mdm2 (Fig. 1A, middle panel). To confirm the effect of Mdm2 on PolH expression in a p53-independent manner, we showed that PolH was increased upon Mdm2-KD in p53^{-/-} HCT116 cells (Fig. 1A, right panel). To further validate this, Mdm2 was knocked down in MCF7 cells over a 3-day period. We showed that as the level of Mdm2 was progressively decreased (Fig. 1B, Mdm2 panel), the level of endogenous PolH protein was gradually increased with concomitant induction of p53 (Fig. 1B, PolH and p53 panels). To rule out a potential effect of p53 induction of PolH [20], we showed that the level of PolH was increased upon Mdm2-KD in p53-null H1299 cells (Fig. 1C).

Next, we examined whether increased expression of Mdm2 leads to decreased expression of PolH. To test this, FLAG-tagged Mdm2 was transiently overexpressed in RKO, MCF7, and p53^{-/-} HCT116 cells. We showed that upon ectopic expression of Mdm2, the level of PolH protein was decreased in RKO, MCF7, and p53^{-/-} HCT116 cells (Fig. 1D). To further examine the effect of Mdm2 on PolH degradation, HA-PolH was transiently expressed in MCF7 cells along with an increased dose of FLAG-Mdm2 (Fig. 1E, Mdm2 panel). We showed that the level of exogenous PolH protein was gradually decreased by Mdm2 in a dose-dependent manner (Fig. 1E, PolH panel). The level of p53 was also measured and found to be decreased by Mdm2 in a dose-dependent manner (Fig. 1E, p53 panel), consistent with previous reports [23–25]. Moreover, Mdm2-mediated PolH degradation was abolished by MG132 treatment, indicating that PolH was degraded by a proteasomal degradation pathway (Fig. 1E, compare lane 5 and 6). Similarly, we showed that the level of PolH was decreased by ectopically expressed Mdm2 in a dose-dependent manner in p53-null H1299 cells (Fig. 1F). These results suggest that PolH stability is regulated by the proteasomal degradation pathway in a Mdm2-dependent manner.

3.2. Mdm2 plays a role in UV-induced PolH degradation

Proteasomal degradation of PolH is known to be induced by UV irradiation [8, 9]. Thus, we examined whether Mdm2 E3 ubiquitin ligase, which is known to be increased by UV, plays a role in UV-induced degradation of PolH. Indeed, we found that upon UV irradiation, the level of Mdm2 protein was increased whereas the level of PolH protein was decreased (Fig. 2A, left panel). In contrast, upon knockdown of Mdm2, PolH degradation was attenuated, indicating that Mdm2 is involved in PolH destabilization (Fig. 2A, left panel). Similar observation was detected in p53-null H1299 cells (Fig. 2A, right panel). Next, the relative level of PolH protein was quantified in RKO cells with or without knockdown of Mdm2. As shown in Fig. 2B, the half life of PolH protein in RKO cells was markedly increased by Mdm2 knockdown. We would like to mention that PolH is a target of p53 [20]. Thus, we would like to emphasize that upon UV irradiation, the level of PolH protein was still decreased although the level of p53 protein was increased (Fig. 2A). This suggests that Mdm2-mediated degradation of PolH cannot be compensated by increased transcriptional upregulation of PolH by stabilized p53. We would also like to mention that Pirh2, a target of p53, is also known to degrade PolH in a ubiquitin-independent but proteasome-dependent manner [8]. These results suggest that in addition to Pirh2, Mdm2 regulates PolH protein stability in a manner independent of Pirh2 function.

3.3. Mdm2 binds to PolH and promotes PolH degradation via polyubiquitination

Since Mdm2 targets are known to interact with Mdm2, we wanted to examine whether Mdm2 associates with PolH. To address this, potential PolH and Mdm2 complexes were immunoprecipitated with anti-PolH and anti-Mdm2, respectively. We showed that PolH and Mdm2 were detected in the same immunocomplexes in RKO and MCF7 cells (Fig. 3A-B). p53, a well-defined binding partner of Mdm2, was also detected in the Mdm2 immunocomplex (Fig. 3B). To confirm the association between Mdm2 and PolH, FLAG-tagged Mdm2 (FLAG-Mdm2) and HA-tagged PolH (HA-PolH) were cotransfected in RKO cells, which were then immunoprecipitated by anti-HA and anti-FLAG, respectively. We showed that ectopically expressed HA-PolH was coimmunoprecipitated with FLAG-Mdm2 (Fig. 3C). Conversely, ectopically expressed FLAG-Mdm2 was detected in the anti-HA (PolH) immunocomplex (Fig. 3D). Thus, we conclude that PolH physically interacts with Mdm2.

To determine whether Mdm2 can promote ubiquitination of PolH *in vivo*, PolH and ubiquitin were cotransfected into RKO cells along with FLAG-tagged Mdm2 or Mdm2 (1–441), which lacks the RING domain (Fig. 4A). We showed that PolH was polyubiquitinated in the presence of wild-type Mdm2 but not mutant Mdm2 (1–441) (Fig. 4B, PolH panel, compare lanes 4–5). We also showed that the slow migrating species were recognized by anti-ubiquitin (Fig. 4B, Ub panel).

To validate whether Mdm2 acts as an E3 ubiquitin ligase for PolH, *in vitro* ubiquitination assay was performed with ³⁵S-labeled PolH in the presence of GST-Mdm2 or GST-Mdm2 (1–441). We showed that PolH was polyubiquitinated in the presence of GST-Mdm2 but not GST-Mdm2 (1–441) (Fig. 4C). To confirm this, *in vitro* ubiquitination assay was performed with ³⁵S-labeled PolH in the presence of immunopurified FLAG-Mdm2 or FLAG-Mdm2 (1–441). Similarly, we showed that PolH was polyubiquitinated by FLAG-Mdm2 but not FLAG-Mdm2 (1–441) (Fig. 4D, compare lanes 3–4). These observations suggest that PolH is a substrate for Mdm2-mediated polyubiquitination *in vivo* and *in vitro*.

3.4. Mdm2-KD makes cells resistant to UV-induced cell death in a PolH-dependent manner

To investigate the functional consequences of Mdm2-mediated PolH polyubiquitination, we analyzed the effect of Mdm2 knockdown on PolH-mediated UV resistance. In this

experiment, p53-null H1299 cell line was used to avoid any effect of p53 on UV-induced cell death. We showed that cell proliferation was significantly increased when Mdm2 was knocked down (Fig. 5A).

Upon UV irradiation, the level of PolH protein was decreased whereas the level of Mdm2 protein was increased (Fig. 2). Thus, we wanted to examine whether Mdm2 plays a role in UV-induced apoptosis via regulating PolH. To address this, DNA histogram analysis was performed to measure the sub-G₁ population of cells, which is believed to represent apoptotic cells. We showed that UV-induced apoptosis was markedly decreased by Mdm2-KD in RKO cells (Fig. 5B). To avoid the effect of p53 induction of PolH [20], DNA histogram analysis was performed in p53-null H1299 cells. Similarly, we observed that knockdown of Mdm2 desensitized H1299 cells to UV-induced apoptosis (Fig. 5C, compare left panel with middle panel).

Next, we examined whether the decreased sensitivity to UV irradiation upon knockdown of Mdm2 is PolH-dependent. To test this, Mdm2 and PolH were knocked down together in H1299 cells and normal human fibroblasts (GM00024). We showed that upon knockdown of Mdm2, the decreased sensitivity to UV irradiation was detected in GM00024 cells (Fig. 5D, compare left panel with middle panel). In contrast, upon knockdown of both Mdm2 and PolH, the decreased sensitivity to UV irradiation was abolished in H1299 and GM00024 cells (Fig. 5C-D, compare left panel with right panel).

Finally, we examined whether Mdm2 has an effect on PolH-dependent lesion bypass in response to UV irradiation. To test this, H1299 cells were transfected with scramble or Mdm2 siRNA. We found that upon UV irradiation, PolH and PCNA replication foci were accumulated (Fig. 5E, two left panels), which were further increased in the intensity and the number by Mdm2-knockdown (Fig. 5E, two right panels). Together, these data indicate that Mdm2 plays a crucial role in UV-induced DNA damage tolerance via controlling PolH stability in a p53-independent manner.

4. Discussion

Our findings provide evidence that Mdm2 serves as an E3 ubiquitin ligase in the polyubiquitination of PolH. First, we demonstrate a physical interaction between Mdm2 and PolH; second, overexpression of Mdm2 led to polyubiquitination of PolH in an *in vivo* ubiquitination assay; third, in the presence of GST-Mdm2 together with PolH in an *in vitro* ubiquitination assay, PolH was polyubiquitinated. Previous work from our laboratory showed that Pirh2 promotes PolH degradation via 20S proteasome in a ubiquitin-independent manner [8]. Here, we showed that Mdm2 promotes PolH degradation via the ubiquitin-dependent proteasomal pathway. These observations suggest that PolH degradation occurs via both 20S and 26S proteasomes.

While Pirh2 can recruit 20S proteasome to destruct PolH, the expression level of Pirh2 was not significantly altered in response to UV irradiation [8]. This suggests that Pirh2 may regulate the proteasomal degradation of PolH to sustain an appropriate level of PolH expression at an unstressed condition. Here, we showed that the expression level of PolH was decreased concomitant with an increase in the expression level of Mdm2 (Fig. 2A) upon UV irradiation. In addition, we showed that Mdm2 is able to promote the PolH ubiquitination and degradation. Therefore, our study indicated that Mdm2 may be responsible for UV-induced and potentially other stress-induced degradation of PolH.

Here, we showed that Mdm2 knockdown attenuates UV-induced cell death in both p53-proficient (RKO) and -deficient (H1299) cells (Fig. 5A-D). However, when PolH is knocked down, Mdm2 knockdown would have no effect. This implies that PolH-proficient

cells are less sensitive to UV-induced cell death due to the bypass of UV-induced DNA lesions by PolH. Since the level of PolH is decreased upon UV irradiation, low amounts of PolH may not be sufficient to bypass the UV-induced DNA lesions. This would activate other DNA repair pathways and checkpoint to take care of the UV-induced DNA lesions. In some cases, this would lead to DNA mutations and subsequently genomic instability and cancer formation. Therefore, whether the Mdm2-PolH complex is involved in other DNA repair pathways is worth further investigation.

So far, two E3 ubiquitin ligases are found to promote PolH degradation [8, 9]. Here, we identified another E3 ubiquitin ligase (Mdm2) for PolH ubiquitination. Thus, like p53, PolH stability is regulated by multiple E3 ubiquitin ligases. Due to the unique role of PolH in TLS activity, these PolH regulators might be explored as a strategy for cancer prevention and therapy.

Acknowledgments

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Highlights

- Mdm2 interacts with Polymerase eta (PolH)
- Mdm2 functions as an E3 ubiquitin ligase for PolH polyubiquitination
- PolH-dependent translesion synthesis is modulated by Mdm2

Fig. 1. Knockdown of Mdm2 increases, whereas overexpression of Mdm2 decreases, PolH expression. (A) Cell lysates were prepared from RKO (left panel), MCF7 (middle panel), and p53^{-/-} HCT116 (right panel) cells transfected with scramble siRNA or Mdm2 siRNA to knock down Mdm2 expression for 72h. PolH, Mdm2, and actin were measured with their respective antibodies. Densitometry values for PolH protein expression levels were shown below the blots. The nonspecific band is marked by *. (B) Cell lysates were prepared from MCF7 cells transfected with Mdm2 siRNA for 0, 24, 48, and 72 h. PolH, Mdm2, p53, and actin were measured with their respective antibodies. (C) The experiment was performed as described for panel (B) except that H1299 cells were used for transfection. (D) Western blots were prepared with extracts from RKO (left panel), MCF7 (middle panel), and p53^{-/-} HCT116 (right panel) cells that were mock-transfected or transfected with FLAG-Mdm2 for 36 h. The blots were analyzed as in (A) except anti-FLAG antibody was used to detect FLAG-Mdm2. (E) Western blots were prepared with extracts from MCF7 cells transfected with HA-PolH along with an increasing dose of FLAG-Mdm2 for 36 h. The blots were analyzed as in (B) except anti-FLAG antibody was used to detect FLAG-Mdm2. (F) Western blots were prepared with extracts from H1299 cells transfected with FLAG-Mdm2 for 36 h. The blots were analyzed as in (E).

Fig. 2. PolH turnover in response to UV irradiation is regulated by Mdm2. (A) RKO (left panel) and H1299 (right panel) cells were transfected with scramble or Mdm2 siRNA for 48 h, followed by UV irradiation (15 J/m^2). After UV irradiation, cells were harvested at the indicated times. PolH, Mdm2, p53, and actin were measured with their respective antibodies. A nonspecific band is marked by *. (B) The relative intensity of PolH in RKO cells was determined by densitometric analysis.

Fig. 3.

PolH interacts with Mdm2. (A) Cell extracts from RKO and MCF7 cells were immunoprecipitated with anti-PolH or control IgG. The immunocomplexes were then used to detect PolH and Mdm2 along with whole-cell lysates as input control. (B) The experiment was performed as described for panel (A) except that immunoprecipitation was performed with anti-Mdm2. (C) The experiment was performed as in panel (A) except anti-HA used to immunoprecipitate HA-PolH in RKO cells transiently transfected with HA-PolH and FLAG-Mdm2. (D) The experiment was performed as in panel (A) except anti-FLAG used to immunoprecipitate FLAG-Mdm2 in RKO cells transiently transfected with HA-PolH and FLAG-Mdm2.

Fig. 4. Mdm2 promotes PolH polyubiquitination. (A) Schematic presentation of Mdm2 domains (p53-BD, p53 binding domain; acidic, acidic domain; Zn, Zn finger domain; RING, RING finger E3 ubiquitin ligase domain) along with location of deletion. (B) Cell lysates were prepared 6 h following MG132 (5 μ M) treatment from RKO cells transfected with HA-PolH along with ubiquitin and wild-type Mdm2 or Mdm2 (1-441). Whole cell lysates (WCLs) were immunoprecipitated with anti-HA antibody followed by Western blot with anti-HA or anti-ubiquitin to detect PolH ubiquitination (upper panels). The levels of ectopically expressed proteins were detected with anti-HA and anti-FLAG, respectively (lower panels). (C) *In vitro* ubiquitination of PolH was performed with GST-Mdm2 and GST-Mdm2 (1-441) along with E1, E2, and ubiquitin. The reaction mixtures were separated on SDS-PAGE gel and analyzed by autoradiography. (D) The experiment was carried out as in (C) except that immunopurified FLAG-Mdm2 and Mdm2 (1-441) from RKO cells was used.

Fig. 5.

Mdm2-knockdown cells are resistance to UV-induced cell death. (A) H1299 cells were transfected with scramble or Mdm2 siRNA. 24 h after transfection, appropriate cells were seeded in 6 well plates in triplicate, followed by mock-treatment or treatment with UV (15 J/m²), and then cultured for a 9-day period. The cell number was measured at the indicated times. (B) RKO cells were transfected with scramble or Mdm2 siRNA for 2 days, followed by mock-treatment or treatment with UV (15 J/m²). 24 h post-UV irradiation, DNA content was measured using FACS Calibur™ flow cytometer as described in “Materials and Methods”. (C-D) H1299 cells and normal human fibroblasts (GM00024) were transfected with scramble or Mdm2 siRNA along with PolH siRNA for 2 days, followed by mock-treatment or treatment with UV (15 J/m²). 24 h post-UV irradiation, DNA content was measured as in (B). (E) H1299 cells were transfected with scramble or Mdm2 siRNA. After 72 h, cells were exposed to UV (15 J/m²) and then incubated for 6 h. PolH (green) image was captured by anti-PolH and FITC conjugated secondary antibody. PCNA (red) image

was obtained by anti-PCNA and Texas-Red conjugated secondary antibody. Nuclei were visualized using 4'-6-Diamidino-2-phenylindole