BRCA1 and Tip60 determine the cellular response to ultraviolet irradiation through distinct pathways

Dominique Kranz,[1] Christoph Dohmesen,[1] and Matthias Dobbelstein[1,2]

[1]Medical Biotechnology Center, Institute for Medical Biology, University of Southern Denmark, 5000 Odense C, Denmark
[2]Department of Molecular Oncology, Göttingen Center of Molecular Biosciences, University of Göttingen, D-37077 Göttingen, Germany

The histone acetyltransferase Tip60 regulates the apoptotic response to ultraviolet (UV) irradiation. A previously suggested mechanism for this regulation consists of the ability of Tip60 to coactivate transcription by the tumor suppressor p53. In this study, we show that Tip60 is required for the early DNA damage response (DDR) to UV, including the phosphorylation of histone 2AX, c-Jun N-terminal kinases (JNKs), and ataxia telangiectasia–related substrates. In contrast, p53 was not required for UV-induced DDR. Rather, p53 accumulation by either knockdown of Mdm2 or addition of an Mdm2 inhibitor, Nutlin-3, before irradiation strongly attenuated the UV-induced DDR and increased cell survival. This protective effect of preaccumulated p53 was mediated, at least in part, by the increased expression of CDKN1A/p21, subsequent down-regulation of BRCA1, and impaired JNK activation accompanied by decreased association of replication protein A with chromatin. We conclude that Tip60 enables UV-induced DDR signaling even in the absence of p53, whereas preaccumulated p53 suppresses UV-induced DDR by reducing the levels of BRCA1.

Introduction

UV irradiation represents a major challenge to genomic integrity throughout the evolution of terrestrial organisms, resulting in the development of specific mechanisms that govern the cellular response to UV-induced DNA damage. The specific changes on the DNA upon UV exposure are different from those induced by γ irradiation, mostly resulting in chemical modifications of single DNA strands such as cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (Balajee and Bohr, 2000). Single-strand breaks are mainly recognized by the heterotrimeric complex of replication protein A (RPA; RPA1, 2, and 3; Iftode et al., 1999), which plays important roles in DNA replication, damage repair, and recombination (Binz et al., 2004). Mammalian cells respond to UV by activating the kinases ataxia telangiectasia related (ATR), checkpoint kinase 1 (Chk1), JNK1/2, and others (Latonen and Laiho, 2005), resulting in the phosphorylation of numerous proteins (Matsuoka et al., 2007) that include the C-terminal part of histone H2AX (γH2AX) as well as the N-terminal and N-terminal portions of the tumor suppressor p53. The ultimate result of the UV response consists either of repair of the damaged DNA or the death of the cell, frequently displaying the hallmarks of apoptosis. JNKs are required for the UV-induced mitochrondrial death pathway (Tournier et al., 2000) and for H2AX phosphorylation (Lu et al., 2006).

It recently became clear that the DNA damage response (DDR) is not only a function of the frequency with which chemical modifications occur in cellular DNA. Rather, DDR can also be influenced by the general "vigilance" of the cell toward such damage. Most notably, tumor cells tend to respond more readily and more extensively to DNA damage than their normal counterparts, and they often display signs of DDR even in the absence of any exogenous DNA-damaging agent (Bartkova et al., 2005). However, the exact mechanisms and factors that influence the cellular sensitivity to DNA damage are largely unknown at present.

The tumor suppressor p53 is phosphorylated and activated in response to a variety of DNA-damaging mechanisms (Bode and Dong, 2004). As a result, the ability of p53 to activate a large set of promoters is enhanced unless p53 is mutated by tumor-associated alterations in the corresponding gene. p53 can be manipulated pharmacologically, even without inducing DNA damage. Nutlin-3 and similar compounds activate it by disrupting the interaction of p53 with Mdm2 (Vassilev et al., 2004). Some of the p53 target gene products induce cell cycle arrest and/or DNA repair, whereas others mediate programmed cell death.

Correspondence to Matthias Dobbelstein: mdobbel@uni-goettingen.de

Abbreviations used in this paper: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia related; CPD, cyclobutane pyrimidine dimer; DDR, DNA damage response; NHEK, normal human epidermal keratinocyte; PARP, poly-ADP ribose polymerase; RPA, replication protein A.

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This proapoptotic activity in response to DNA damage is probably the most widely acknowledged property of p53. However, in some situations, p53 can also act as a protector of cells. For instance, we have recently found that nongenotoxic activation of p53 through blocking the p53-antagonizing ubiquitin ligase Mdm2 can render cells resistant to nucleoside analogues such as gemcitabine or cytosine-arabinoside (Ara-C; Kranz and Dobbelstein, 2006). It is currently not known how prevalent such prosurvival functions of p53 are.

The histone acetyltransferase Tip60 has been described as a cofactor of p53, contributing to induction of the *CDKN1A/p21* gene by p53 (Berns et al., 2004) but also enhancing proapoptotic p53-responsive genes (Sykes et al., 2006; Tang et al., 2006). Interestingly, Tip60 also interacts with Mdm2, raising the possibility that p53 and Tip60 are coregulated (Legube et al., 2002). Tip60 contributes to the activity of various promoters (Sapountzi et al., 2006; Squatrito et al., 2006); however, transcription-independent functions of Tip60 have been described (e.g., its ability to acetylate the ataxia telangiectasia mutated [ATM] kinase [Sun et al., 2005] or its contribution to the exchange of phosphorylated histones in *Drosophila melanogaster* [Kusch et al., 2004]).

The exact role of Tip60 in response to double-stranded DNA breaks raised the question of whether it might also contribute to the UV response. Recently, it was shown that Tip60 is indeed required for efficient UV-induced apoptosis. Importantly, it was suggested in the same study that this contribution of Tip60 to cell death is caused by its activity as a cofactor of p53 (Tyteca et al., 2006).

Although our data corroborate the idea of Tip60 being necessary for UV-induced apoptosis, they suggest an unexpected role of p53 that contradicts the previously published concept. The function of Tip60 in UV DDR does not require p53 and consists of the activation of DDR-induced phosphorylation cascades. In contrast, when p53 is accumulated and activated by blocking Mdm2, this attenuates the early signaling response to UV irradiation. Further delineation of the underlying mechanisms implied the induction of *CDKN1A/p21* as well as the suppression of BRCA1 levels in the attenuation of UV DDR by p53. Moreover, BRCA1 is required for the recruitment of RPA to chromatin upon UV light exposure. Thus, preaccumulated p53 suppresses the early steps of UV DDR and protects rather than eliminates UV-exposed cells.

Results

Tip60 is essential for the UV-induced DDR, independent of p53

We first tested the impact of Tip60 knockdown on the UV response. U2OS cells (p53 wild type) were transfected with siRNA targeting Tip60, p53, and Mdm2 or with two different negative control siRNAs. All knockdowns were verified by immunoblot analysis and/or quantitative real-time PCR (Fig. 1 A and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). Subsequently, the cells were irradiated with UV-C light and harvested 6 h later. Immunoblot analysis revealed that, as expected (Tyteca et al., 2006), the removal of Tip60 reduced the amounts of cleaved caspase 3 and of caspase-cleaved poly-ADP ribose polymerase (PARP; Fig. 1, A and B), indicating that Tip60 is required for efficient UV-induced apoptosis. Surprisingly, however, the knockdown of p53 did not influence UV-induced caspase activity in either direction, arguing that Tip60 knockdown does not exert its antiapoptotic effect through the attenuation of p53 activity.

siRNA to Mdm2 activated p53, as verified by the increased levels of p53 and the p53 target gene product p21 as well as decreased levels of the p53-repressed genes (Lohr et al., 2003) *BRCA1* and *Chk1*. Strikingly, the knockdown of Mdm2 and concomitant increase in p53 activity were found to reduce rather than increase UV-induced caspase activity. This result was obtained with two independent siRNA species directed against Mdm2 (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). Again, this indicated that the role of Tip60 in UV-mediated apoptosis is not based on coactivating p53. Therefore, we investigated how Tip60 influences the upstream DDR after exposure of cells to UV light. The phosphorylation of histone H2AX (γH2AX), JNKs, and Chk1 (Fig. 1, A and B) was detected by phosphospecific antibodies. The phosphorylations of H2AX and JNKs but not Chk1 were found to depend on Tip60. Thus, Tip60 is required for triggering at least an important subset of the early signaling cascade that responds to UV rather than merely the downstream proapoptotic mechanisms.

Even more surprising, however, we observed that activation of p53 by Mdm2 knockdown resulted in attenuated phosphorylation of H2AX and JNKs as well. This argues that p53 is not only unnecessary for UV-induced apoptosis; rather, when preactivated, it can prevent both the upstream signaling and the downstream caspase activity in response to UV. This is in contrast to the previously proposed concept in which Tip60 was suggested to mediate UV-induced apoptosis by increasing DNA binding and transactivation by p53 (Tyteca et al., 2006). UV irradiation decreased the levels of Tip60 (Fig. 1 A), but knocking down Mdm2 prevented this reduction of Tip60 levels (see Discussion).

Next, we asked whether the impact of Tip60 or Mdm2 on the UV response results from its influence on gene expression after irradiation. Therefore, we analyzed UV DDR while blocking translational elongation using cycloheximide. Interestingly, the preceding knockdown of Tip60 or Mdm2 still attenuated the UV response, even when the cells were incubated with cycloheximide immediately after UV irradiation (Fig. 1 C). This indicates that the reductions of Tip60 or Mdm2 do not confer their protective effects by changes in gene expression and protein synthesis after UV exposure, further arguing against an essential role of postirradiation p53-mediated transactivation in apoptosis.

To corroborate the independence of UV-induced Tip60-mediated DDR from the presence of p53, we performed similar assays in Saos-2 (Fig. 2 A) cells that lack p53 entirely. These cells displayed similar H2AX phosphorylation and caspase cleavage in response to UV, which was still inhibited by Tip60 knockdown (Fig. 2 A). Furthermore, these cells also showed an inhibition of UV-induced JNK phosphorylation when Tip60 was removed (Fig. 2 A). As expected, siRNA to Mdm2 did not change the response to UV in the $p53^{-/-}$ Saos-2 cells (Fig. S2 B).

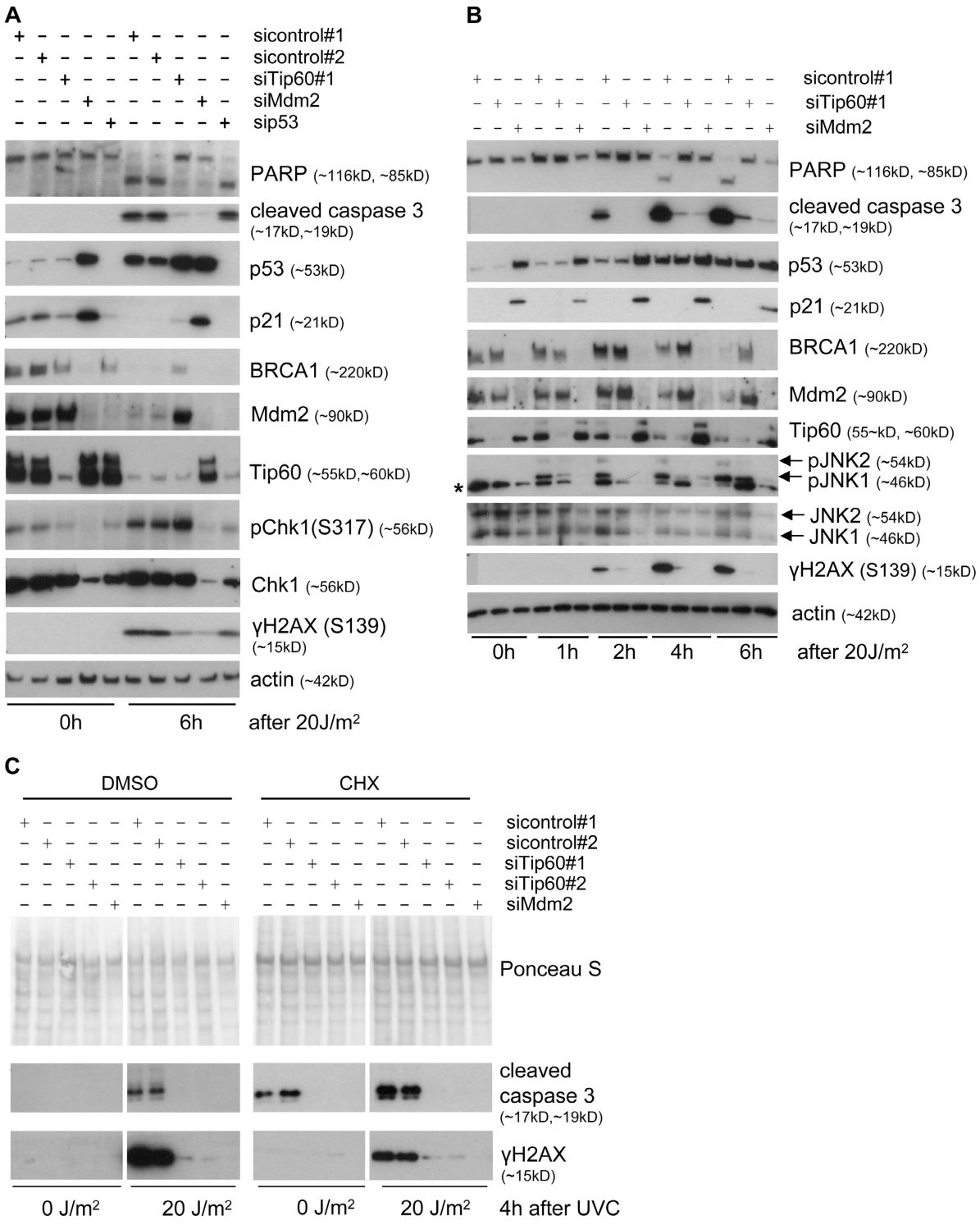

Figure 1. **Knockdown of Tip60 or Mdm2 but not p53 impairs the UV-induced DDR.** (A) U2OS cells were transfected with siRNAs as indicated followed by UV irradiation with 20 J/m^2 48 h later. The cells were harvested and subjected to immunoblot analysis 6 h after irradiation. (B) U2OS cells were transfected with siRNAs and irradiated as in A. After further incubation for the indicated periods of time, the cells were harvested and subjected to immunoblot analysis. The asterisk indicates a nonspecific band (compare with Fig. 7 B). (C) U2OS cells were treated as in A, but here, cycloheximide (CHX) or the DMSO solvent alone was added immediately after irradiation.

To investigate the effect of Tip60 knockdown after a longer time after irradiation, flow cytometry was performed to measure the percentage of cells with DNA content <2N. Cells depleted of Tip60 displayed a reduction of sub-G1 cells even 48 h after UV irradiation (Figs. 2 B and S1 B). Together, these results indicate that Tip60 is a critical component of the UV response but acts on a p53-independent signaling cascade rather than merely on the proapoptotic activity of p53. In contrast, preaccumulated

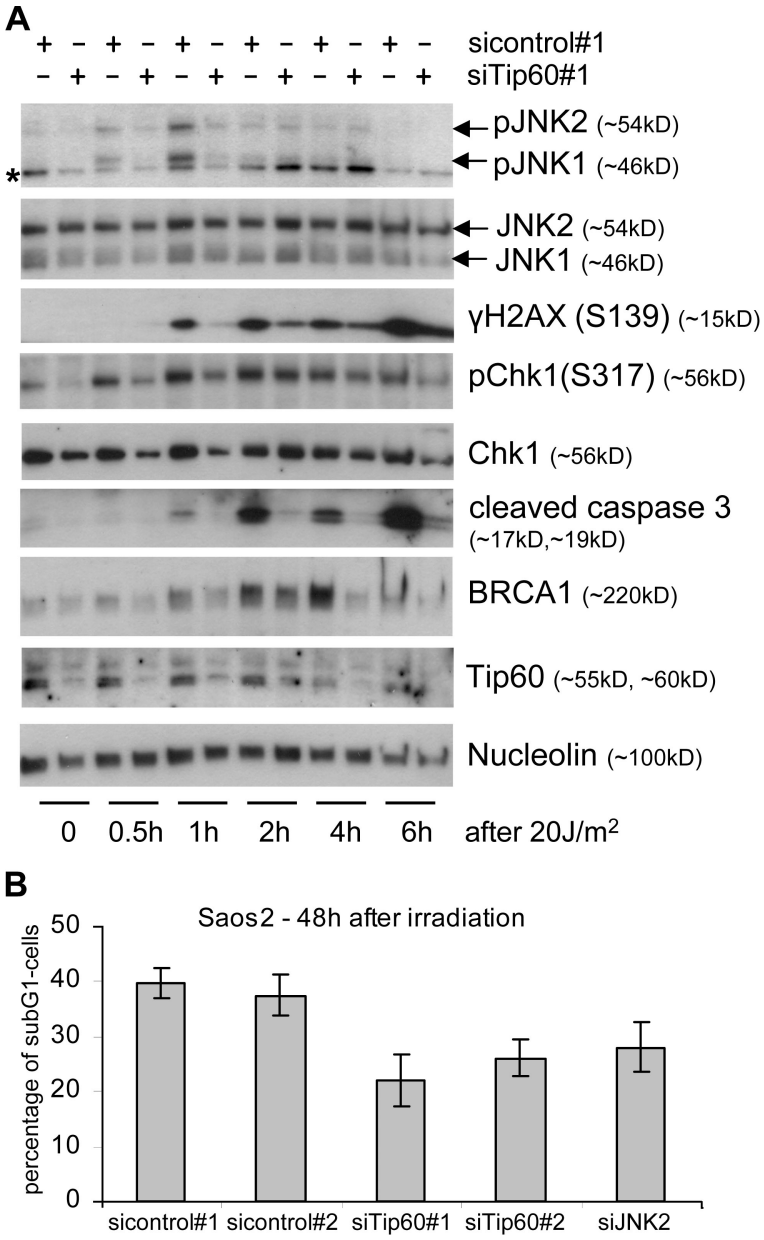

Figure 2. **Impairment of the response to UV irradiation by Tip60 knockdown is independent of p53.** (A) Saos-2 cells were transfected with 30 nM siRNA (siTip60#1 or sicontrol#1). 48 h later, cells were exposed to 20 J/m^2 UV and harvested after the indicated time points for Western blotting. The asterisk indicates a nonspecific band. (B) Saos-2 cells were transfected with 30 nM of the indicated siRNAs and UV irradiated (20 J/m^2) 48 h later. 48 h after UV irradiation, cells were harvested for FACS analysis, and the percentage of sub-G1 cells was determined. The results shown represent the mean percentages of sub-G1 cells from four independent experiments with their corresponding SD (error bars).

p53 appears to be capable of attenuating the signals in response to UV exposure.

Nongenotoxic pharmacological accumulation of p53 confers resistance to UV irradiation

To further investigate the apparent protective role of p53 upon UV irradiation, we pretreated U2OS cells with the drug candidate Nutlin-3, a compound that specifically binds to the hydrophobic pocket of Mdm2, thereby disrupting the interaction of p53 and Mdm2 (Vassilev et al., 2004). Nutlin-3 prevents the Mdm2-mediated ligation of ubiquitin to p53, thereby increasing p53-mediated transcription. Initially, the idea behind the development of Nutlin-3 was mainly to induce p53-dependent apoptosis in tumor cells, and this turned out to be achievable in selected tumor species (e.g., certain leukemias and lymphomas; Kojima et al., 2005; Stuhmer et al., 2005; Petre et al., 2007; Sarek et al., 2007). However, our previous experiments revealed that Nutlin-3 induces cell cycle arrest but does not induce detectable apoptosis in U2OS cells and several other cell species (Kranz and Dobbelstein, 2006).

Accordingly, treatment with Nutlin-3 led to the accumulation of p21 in U2OS cells, which is in analogy to Mdm2 knockdown. However, in contrast to Mdm2 siRNA, Nutlin-3 induced the accumulation of Mdm2 (Fig. 3 A) as observed previously (Vassilev et al., 2004; Kranz and Dobbelstein, 2006), perhaps as a result of increased p53-mediated transcription of the *Mdm2* gene. Next, the cells were UV irradiated, and their response was assessed by immunoblot analysis at various time points after irradiation. p21 decreased upon UV irradiation, presumably as a result of proteasomal degradation, as described previously (Bendjennat et al., 2003). Strikingly, blocking Mdm2 by Nutlin-3 or siRNA attenuated caspase 3 and PARP cleavage as well as the accumulation of phospho-H2AX, phospho-JNKs, and Chk1

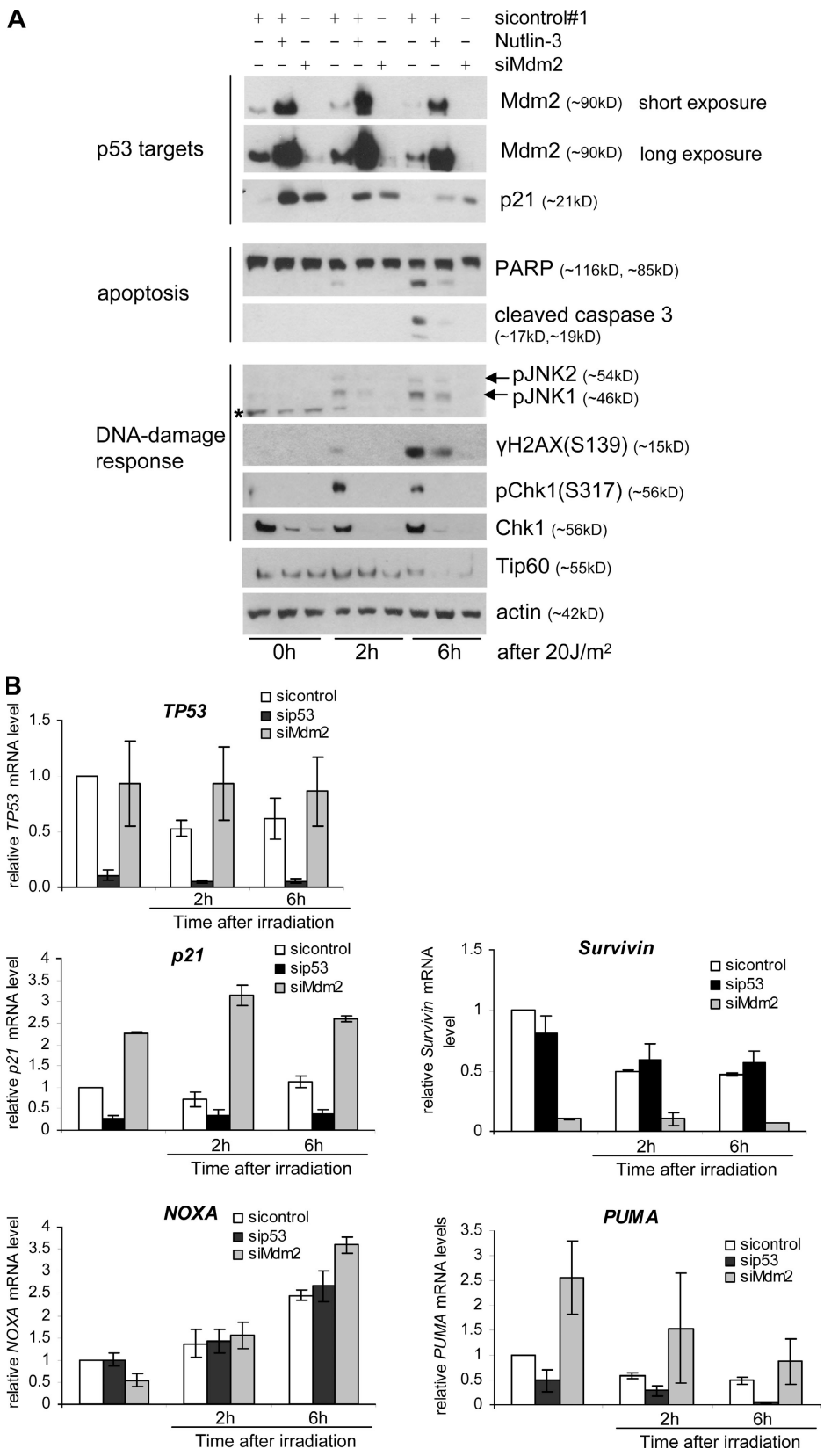

Figure 3. **Pharmacological as well as siRNA-mediated preactivation of p53 inhibits the UV response.** (A) U2OS cells were transfected with 30 nM siRNA (sicontrol#1 or siMdm2). After 24 h, cells were treated with 8 μM Nutlin-3 or the solvent DMSO. After an additional incubation for 24 h, the cells were UV irradiated (20 J/m^2) and harvested 0, 2, and 6 h after irradiation for Western blotting. The asterisk indicates a nonspecific band. (B) Quantitative real-time PCR analysis of *TP53*, *p21*, *Survivin*, *NOXA*, and *PUMA* mRNA in U2OS cells was performed to determine the corresponding relative mRNA levels after transfection with 30 nM of the indicated siRNAs 0, 2, and 6 h after UV irradiation (20 J/m^2). *Glyceraldehyde 3-phosphate dehydrogenase* mRNA was used as an internal control. Error bars represent SD.

(Fig. 3 A). Thus, Nutlin-3 confers protection to the UV response, as did Mdm2 knockdown.

We also determined whether the removal of photoproducts in UV-irradiated U2OS cells was affected by Nutlin-3. However, pretreatment of cells with Nutlin-3 did not result in a difference in the immunostaining intensity of CPDs or 6-4 photoproducts at various time points after irradiation (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). Thus, the antiapoptotic effect of p53 activation by Nutlin-3 before UV irradiation does not appear to be based on accelerated DNA repair. Instead, preaccumulated p53 blocks signaling in response to UV exposure despite the unchanged rate of removing DNA lesions.

To clarify the involvement of p53 target genes in the UV response, we determined the mRNA levels of *TP53* and some of its target genes upon UV irradiation in cells depleted of p53 or Mdm2 (Fig. 3 B). The transfection of siMdm2 alone increased *p21* mRNA and *PUMA* mRNA levels, whereas mRNA levels of the p53-repressed gene *Survivin* decreased as expected. *NOXA* mRNA levels increased upon irradiation, but this was observed regardless of p53 or Mdm2 knockdown, arguing that this activation is p53 independent. *PUMA* mRNA levels decreased upon irradiation, again independently of the transfected siRNA. Thus, none of the genes that responded to UV irradiation did so in a p53-dependent manner, which is in line with our observation that UV-induced apoptosis does not require p53 (Fig. 2).

Long-term cytotoxicity of UV irradiation is attenuated by Nutlin-3

Next, we assessed whether the short-term reduction of caspase activity conferred by Nutlin-3 on UV-irradiated cells is reflected by increased long-term survival. Again, U2OS cells were treated with Nutlin-3 or only the DMSO solvent (control) 24 h before irradiation. Subsequently, the cells were irradiated (or not) with UV light. After an additional incubation for 24 h, the cellular DNA content was quantified by flow cytometry. As expected, the proportion of cells with a sub-G1 DNA content was high (~18%) upon UV irradiation in DMSO-pretreated cells. However, pretreatment with Nutlin-3 reduced this to <6%. Costaining of living cells with propidium iodide and annexin V confirmed the proapoptotic effect of UV light and its inhibition by Nutlin-3, as revealed by a reduced proportion of annexin V–positive cells (Fig. 4 A). Caspase cleavage was also found to be reduced in the Nutlin-treated cells at this time point (Fig. S2 C). This strongly suggests that the cell death response to UV is still attenuated by Nutlin-3 at 24 h after irradiation. To test the long-term outcome, clonogenic assays were performed. After pretreatment with Nutlin-3 and/or UV light, U2OS cells were seeded for 7–10 d to form colonies, which were then stained and counted. Again, pretreatment with Nutlin-3 strongly increased the proportion of colonies that formed from UV-exposed cells as compared with nonirradiated cells (Fig. 4 B). We conclude that pretreatment of cells with the nongenotoxic p53 activator Nutlin-3 increases the long-term survival of cells upon UV irradiation.

Preactivation of p53 attenuates the UV response in keratinocytes

Because U2OS cells, despite their convenient handling and transfection properties, are not derived from naturally UV-exposed tissue, we assessed whether keratinocytes would behave similarly in response to Nutlin-3 and/or UV irradiation. Immortalized keratinocytes (EPC2hTert; Fig. 5 A; Harada et al., 2003) as well as primary normal human epidermal keratinocytes (NHEKs; Fig. 5 B) were protected by Nutlin-3 from UV-induced phosphorylation of H2AX and caspase activity, similar to U2OS cells. Phospho-JNK accumulation, although occurring with different kinetics, was also largely abolished by pretreatment with Nutlin-3 (Fig. 5 C). Furthermore, flow cytometry revealed that the same cells accumulated in the sub-G1 fraction (indicative of cell death) upon UV irradiation but did so to a much lesser extent when the cells were pretreated with Nutlin-3 (Fig. 5 D). Thus, preactivation of p53 by Nutlin-3 protects keratinocytes against UV-induced DDR.

p21 is required for UV protection and for suppression of BRCA1 levels by p53

p21, the cyclin-dependent kinase inhibitor and product of the *CDKN1A* gene, has been reported to confer protection against apoptosis in various settings (Roninson, 2002). On the other hand, the expression of *CDKN1A/p21* is strongly enhanced by p53. Therefore, we tested whether the increased amounts of p21 in cells that were pretreated with Nutlin-3 might be required for UV protection. In parallel to treatment with Nutlin-3 and/or UV irradiation, U2OS cells were transfected with siRNA to knock down p21 or with control siRNA. As shown in Fig. 6 A, the knockdown of p21 largely abolished the preventive effect of Nutlin-3 on UV-induced PARP and caspase cleavage, indicating that the induction of p21 is essential for the prevention of UV-induced apoptosis by p53 activation. However, loss of p21 somewhat impaired caspase activation in response to UV treatment when Nutlin-3 was absent, arguing that a minimum amount of p21 is required for apoptosis despite the protective effect of larger amounts. Such dose-dependent opposite roles of CDK inhibitors have been described previously (Di Cunto et al., 1998; Denicourt and Dowdy, 2004). In any case, long-term assays did not show a significant difference in UV-surviving colonies when p21 was knocked down, as detailed at the end of this section.

In addition to caspase cleavage, we observed that knockdown of p21 to some degree permitted JNK and H2AX phosphorylation even in the presence of Nutlin-3, at least at 6 h after irradiation (Fig. 6 A). Thus, p21 appears to be not only responsible for attenuating the apoptotic response but also for reduction in the primary DNA damage–induced signaling cascade.

To understand how p21 suppresses UV DDR, we took into account that the repression of E2F/NF-Y–responsive genes (such as *Stathmin*, *Survivin*, *Cdc25c*, *Chk1*, and *BRCA1*) by p53 depends on p21 (Lohr et al., 2003). Indeed, we observed that the DDR mediator BRCA1, but not a functionally related gene product, 53BP1, was suppressed in its levels by Nutlin-3. However, BRCA1 suppression was fully abolished by siRNA targeting p21 (Fig. 6 A). siRNA to Mdm2 also reduced the levels of BRCA1 (Fig. 1, A and B). These observations raised the possibility that p53 attenuates the response to UV irradiation through the suppression of *BRCA1* by p21.

To understand the importance of p21 and p53 for long-term survival, we performed clonogenic assays after knocking down

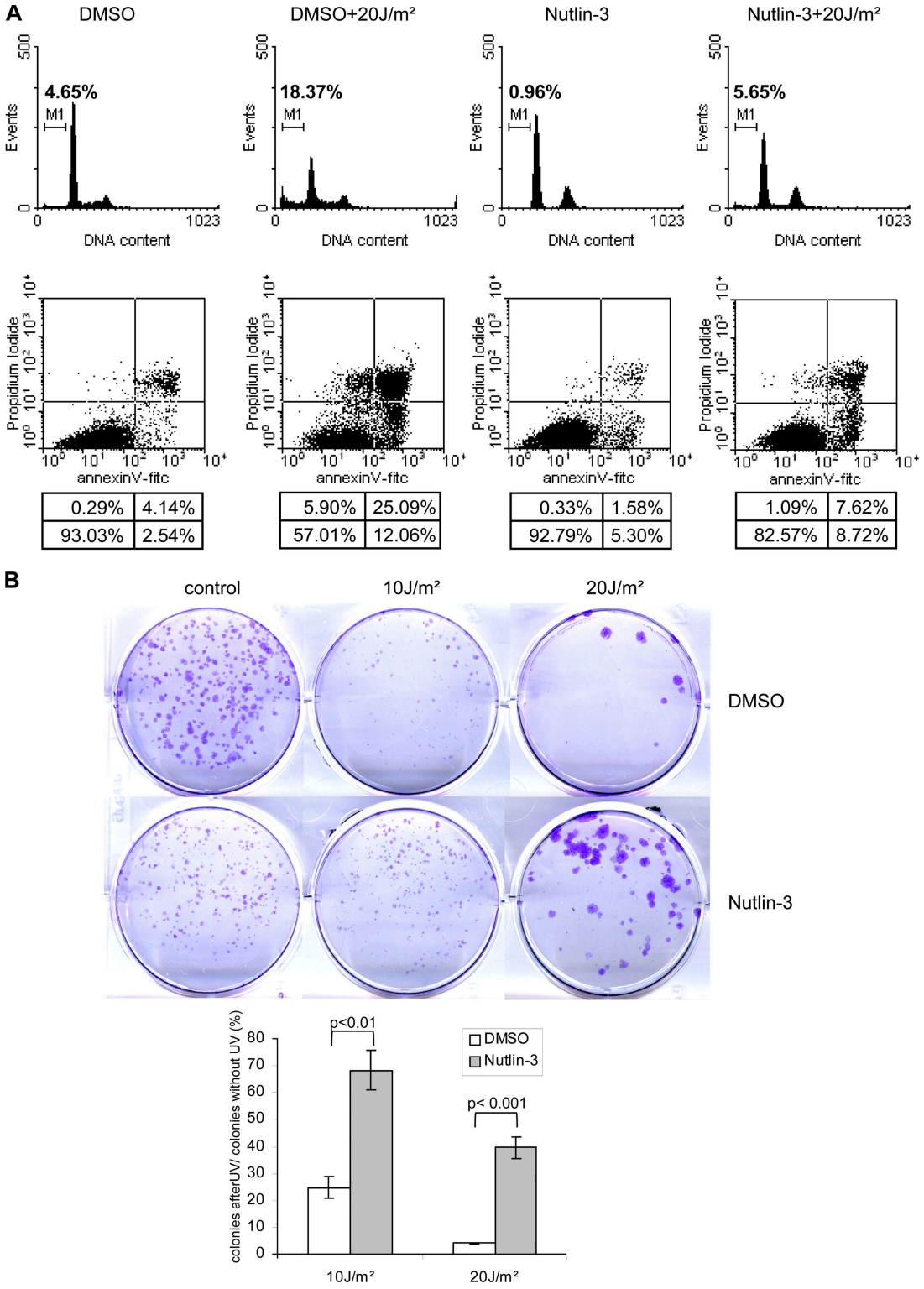

Figure 4. **Nutlin-3 enhances long-term survival upon UV irradiation.** (A) U2OS cells were treated with 8 μM Nutlin-3 or the solvent DMSO. After 24 h, they were UV irradiated. The cell cycle profile was determined by FACS analysis 24 h after irradiation. The percentage of cells in the sub-G1 fraction (M1) is indicated (top). For the bottom panel, the cells were first treated the same way. After harvesting, they were incubated with annexin V–FITC, counterstained with propidium iodide, and analyzed by flow cytometry. Percentages of cells in each quadrant were determined. (B) U2OS cells were seeded in triplicate at 500 cells per 3.5-cm dish. Thereafter, they were treated with 8 μM Nutlin-3 or DMSO. After 24 h, the cells were UV irradiated or not irradiated followed by further incubation with Nutlin-3 or DMSO for another period of 24 h. Thereafter, the drug was removed, and the medium was replaced. After 7 (no UV; 10 J/m^2) or 10 d (20 J/m^2), emerging colonies were stained with crystal violet. The results represent the percentage of surviving colonies and are shown as the means from three independent experiments with their corresponding SEM (error bars).

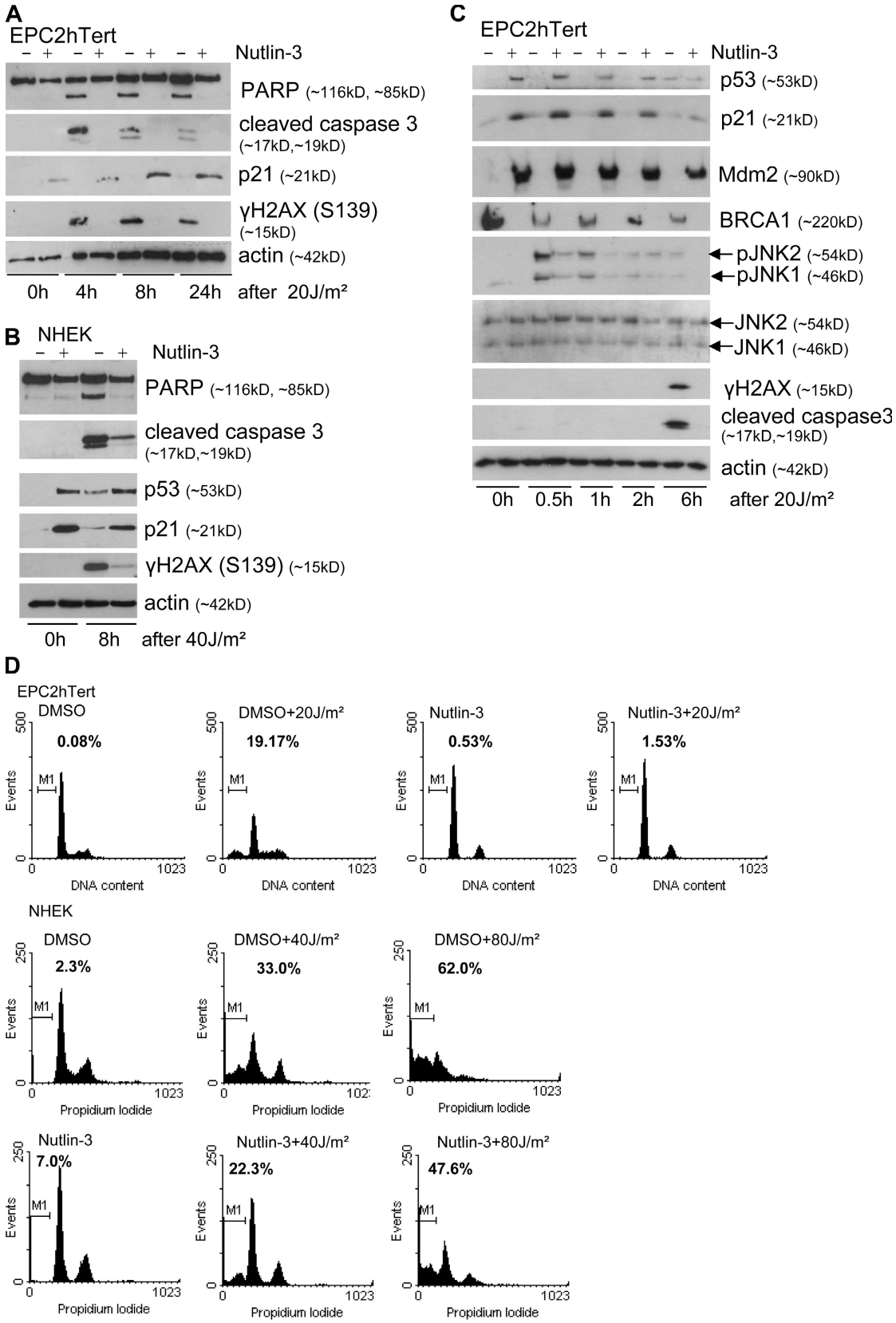

Figure 5. **Nutlin-3 treatment inhibits the UV response in keratinocytes.** (A–C) Immortalized keratinocytes (EPC2hTERT; A and C) and NHEKs (B) were pretreated with Nutlin-3, UV irradiated after 24 h as indicated, harvested at the indicated time points, and subjected to immunoblot analysis as described in Fig. 2 A. (D) EPC2hTERT cells or NHEKs were treated with 8 μM Nutlin-3 or DMSO for 24 h and UV irradiated. The cell cycle profile was determined by flow cytometry 24 h after irradiation. The percentage of cells in the sub-G1 fraction is indicated in each panel.

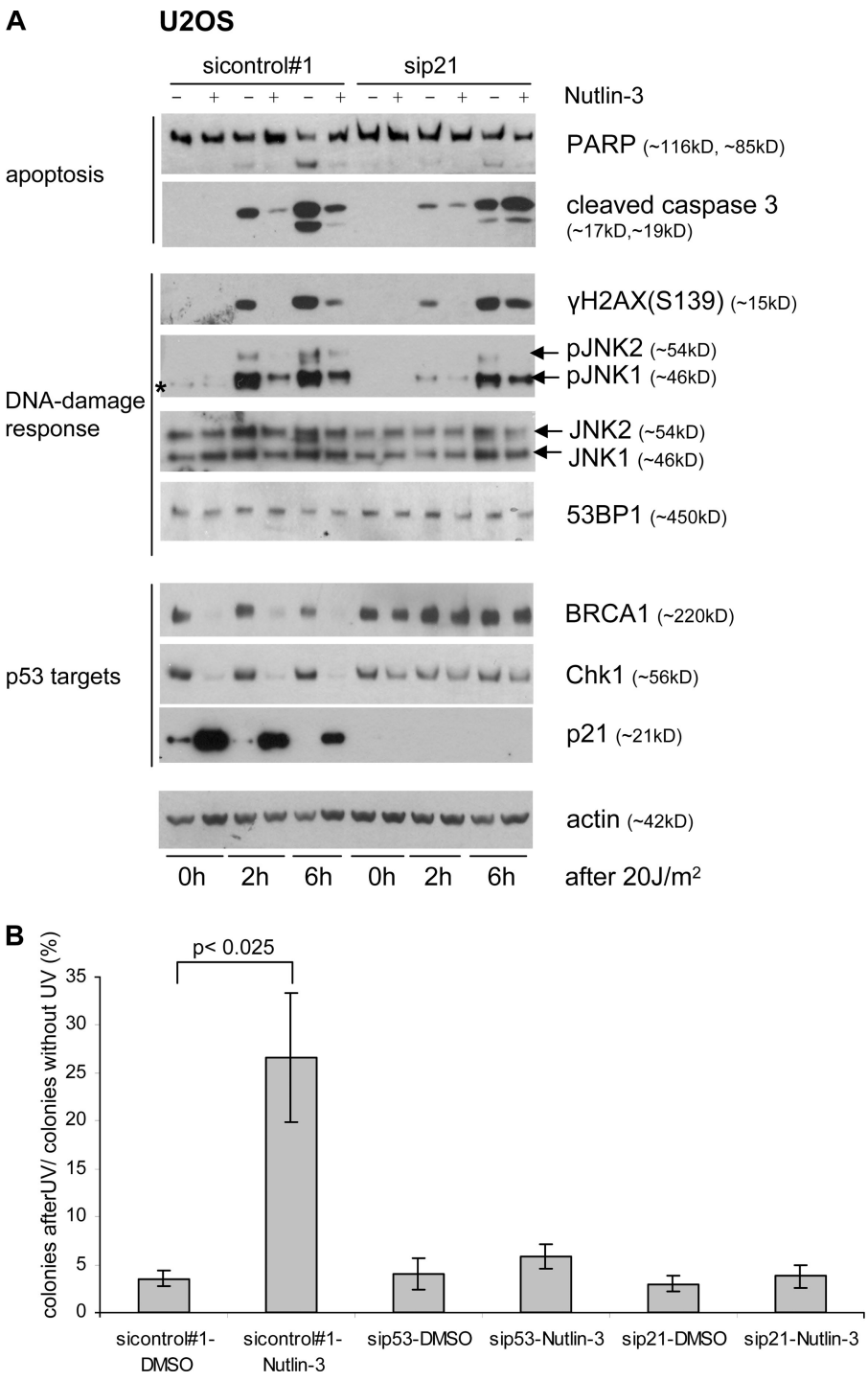

Figure 6. **p21 is required for Nutlin-mediated protection against UV irradiation.** (A) U2OS cells were transfected with siRNA control#1 or siRNA to p21. After 24 h, cells were treated with 8 μM Nutlin-3 or DMSO and incubated for another period of 24 h. After 48 h in total, the cells were UV irradiated (20 J/m^2) and harvested after the indicated time points. Cell extracts were analyzed by Western blotting. The asterisk indicates a nonspecific band (compare with Fig. 7 B). (B) U2OS cells were transfected with 100 nM siRNAs and treated as described in A. After UV irradiation (20 J/m^2), the cells were trypsinized, and a constant fraction of them was seeded in 3.5-cm dishes followed by incubation for 7–10 d and colony staining. The results represent the percentage of surviving colonies and are shown as the means from three independent experiments with their corresponding SEM (error bars).

p21 or p53 followed by treatment with Nutlin and/or UV irradiation (Fig. 6 B). Only control-transfected cells pretreated with Nutlin-3 but not Nutlin-treated cells transfected with siRNA to p21 or p53 showed a significant increase in the proportion of surviving colonies compared with their DMSO-treated counterparts. Thus, p53 and p21 are necessary for UV protection by Nutlin-3.

BRCA1 is required for the cellular response to UV-induced DNA damage

To assess the role of BRCA1 in the response to UV, we tested three different siRNAs to BRCA1 concerning their efficiency in U2OS cells (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). We achieved a reduction to 20–30% of the original mRNA levels, which was comparable with Nutlin-3–treated cells. The knockdown of BRCA1 largely abolished the phosphorylation of JNK and H2AX upon UV irradiation as well as the cleavage of PARP and caspase 3 (Figs. 7 A and S4 B). When BRCA1 was eliminated, the addition of Nutlin-3 had little additional effect on phospho-JNK and phospho-H2AX levels (Fig. 7 A). This suggests that BRCA1 elimination is part of the pathway (epistatic) induced by p53 to attenuate UV DDR. Of note, BRCA1 levels were reduced upon UV irradiation, even without

previous knockdown, perhaps as part of a mechanism that provides a negative feedback on the UV response (Andres et al., 1998).

To identify downstream transmitters of BRCA1, we also eliminated the JNKs. As expected from a previous study (Lu et al., 2006), the knockdown of JNK1 or JNK2 each attenuated the UV response in a manner analogous to the knockdown of BRCA1 (Fig. 7 B), suggesting that the requirement of BRCA1 in UV DDR is at least partially based on its contribution to JNK activation. In contrast, the phosphorylation of Chk1 was neither affected by BRCA1 nor by JNK levels (Fig. 7, A and B), and knocking down Chk1 did not attenuate the apoptotic response to UV (Fig. S4 C), which is in agreement with an earlier study (Niida et al., 2005). Thus, it appears that Chk1 is not a positive contributor to UV DDR in the system under study despite its known role in p53 phosphorylation (Shieh et al., 2000) and despite the ability of preactivated p53 to repress the levels of phosphorylated and total Chk1 in a p21-dependent fashion, as shown in Figs. 6 A and 7 A as well as in our previous study (Lohr et al., 2003).

To analyze the protective effect of removing BRCA1 or JNK1/2 at a later time point, we harvested the transfected cells 24 h after UV irradiation for flow cytometry (Fig. 7 C). Upon transfection with siRNAs targeting BRCA1 as well as JNK2 and Mdm2, a reduced proportion of cells was found in the sub-G1 fraction compared with two different control siRNAs. Unlike the knockdown of Mdm2, neither the removal of BRCA1 nor that of Tip60 or JNK1/2 induced a detectable shift in cell cycle distribution, as determined by DNA content analysis and by BrdU incorporation (Fig. S5, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). Also, eliminating BRCA1 did not detectably alter the levels of p21 (Fig. 7 A), in contrast to a recent study that described an inhibitory effect of BRCA1 on p21 expression (Moisan and Gaudreau, 2006). This suggests that the inhibitory effects of the siRNAs to BRCA1 and Tip60 on DDR do not represent a result of cell cycle regulation (see Discussion). To assess long-term survival, we again performed clonogenic assays, revealing that reduction of JNK1 or JNK2, like that of Mdm2, also resulted in an increased proportion of colonies upon UV treatment (Fig. 7 D). Curiously, BRCA1 knockdown largely abolished the formation of colonies both in unirradiated and UV-treated cells (unpublished data), perhaps reflecting the role of BRCA1 in the maintenance of genomic stability. In agreement, BRCA1-deficient cells were reported to contain lagging chromosomes, indicating defects in DNA decatenation and chromosome segregation (Lou et al., 2005). In conclusion, the removal of JNKs and, at least on the short term, that of BRCA1 is sufficient to confer a similar protection against UV irradiation as the preactivation of p53 by Nutlin-3. Thus, the suppression of BRCA1 and phospho-JNK1/2 represents at least one, and possibly the major, contribution of p53 to attenuation of the cellular response to UV-induced damage.

Reductions in Mdm2 or BRCA1 impair RPA loading and the phosphorylation of ATR substrates, whereas knockdown of Tip60 only inhibits the latter

Association of the RPA complex with single-stranded DNA is required for the recruitment of ATR/ATR-interacting protein to the sites of DNA damage, leading to activation of the DDR (Zou and Elledge, 2003). Upon DNA damage by UV irradiation, RPA2 becomes highly phosphorylated (Binz et al., 2004). To investigate the early UV response, we analyzed the levels and phosphorylation of RPAs upon UV irradiation (Fig. 8 A), and we isolated chromatin from U2OS cells after the same treatment (Fig. 8 B). RPA levels were not detectably affected by UV light. However, as reported before, RPA1 and RPA2 were recruited onto the chromatin, and RPA2 was phosphorylated, as determined by staining with a phospho-specific antibody. Some Tip60 was also present in the chromatin-associated unsoluble fraction, perhaps reflecting its association with histones (Fig. 8 B). To investigate the influence of Tip60, Mdm2, and BRCA1 on RPA loading and phosphorylation, cells were transfected with the corresponding siRNAs, and chromatin was extracted 4 h after irradiation. Depleting cells of Tip60 did not detectably affect RPA loading (Fig. 8 B) or RPA phosphorylation (Fig. 8, A and B). Analogous results were obtained in the p53-deficient cell line Saos-2 (unpublished data). In contrast, cells transfected with siRNA to Mdm2 or BRCA1 showed decreased RPA loading and RPA2 phosphorylation upon UV irradiation. We conclude that a reduction in Mdm2 and/or BRCA1 levels prevents one of the earliest known steps in the UV response (i.e., loading of RPA onto chromatin). Mdm2 knockdown might also elicit this effect by cell cycle arrest, but in the case of BRCA1, it appears that its elimination interferes with RPA phosphorylation and chromatin loading despite ongoing DNA replication (Fig. S5 B).

Tip60 levels were reduced upon UV irradiation but were retained in whole cell extracts of siBRCA1- and siMdm2-transfected cells upon UV exposure (Figs. 1 A and 8 A). This reduction appears to occur through a combination of enhanced degradation and reduced RNA levels (unpublished data).

The ATR kinase is generally considered as the primary mediator of the UV-induced DDR. Therefore, we investigated the impact of Tip60, Mdm2, and BRCA1 on the phosphorylation of ATR substrates. U2OS cells were transfected with control siRNAs or siRNAs targeting Tip60, Mdm2, BRCA1, and ATR. Cells were harvested 4 h after UV irradiation (Fig. 8 C). In sicontrol-transfected cells, UV irradiation induced the appearance of several epitopes corresponding to ATR/ATM substrates that are phosphorylated at specific S*/T*Q sites (Stokes et al., 2007). As expected, siRNA to ATR abrogated the phosphorylation of a substantial portion of proteins, confirming the specificity of the antibody for ATR substrates. Strikingly, Tip60 knockdown also abolished the occurrence of the majority of ATR/ATM substrate epitopes in U2OS cells (Fig. 8 C) and Saos-2 cells that lack p53 (Fig. 8 D), strongly suggesting a crucial and p53-independent role of Tip60 in the activation of ATR. However, Tip60 knockdown did not alter total ATR protein levels. Similar to Tip60 knockdown, Mdm2 and BRCA1 knockdown also decreased the phosphorylation of ATR/ATM substrates, albeit to different extents for different protein substrates (Fig. 8 C). Thus, reductions in Mdm2, BRCA1, and Tip60 each appear to interfere with the phosphorylation of ATR substrates, although only the Mdm2–p53–p21/BRCA1 pathway affects the loading of RPA onto chromatin.

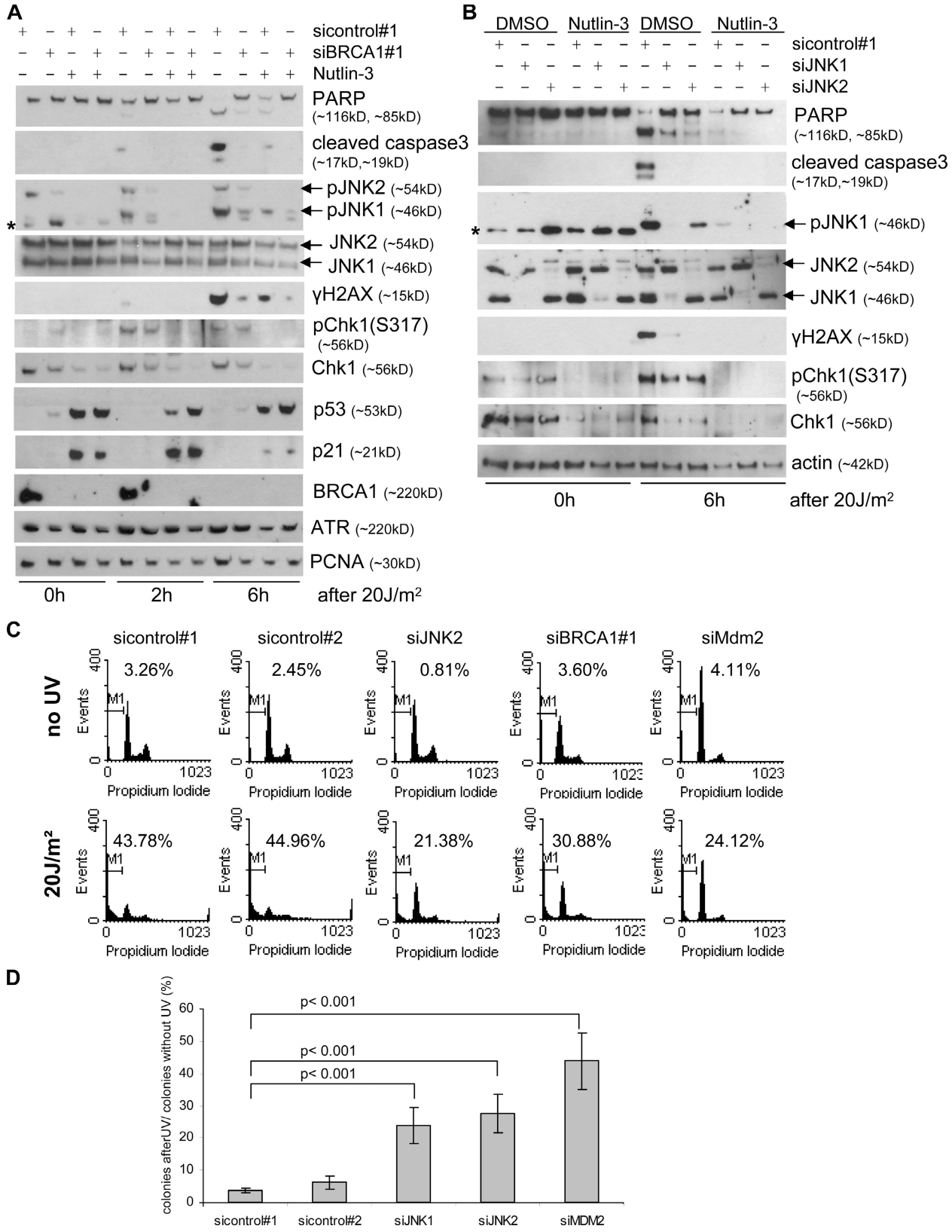

Figure 7. **Knockdown of BRCA1, JNK1, or JNK2 impairs the DDR upon UV irradiation.** (A and B) U2OS cells were transfected with 30 nM siRNAs targeting BRCA1 (A) or JNKs (B) and were processed as described in Fig. 5 A. Asterisks indicate nonspecific bands that were not eliminated by JNK siRNAs. Note that Nutlin-3 strongly reduced BRCA1 levels, apparently as a function of p21 induction (compare with Fig. 6 A). (C) U2OS cells were transfected with 30 nM of the indicated siRNAs. After 48 h, the cells were UV irradiated (20 J/m^2) or not irradiated, and the proportion of the sub-G1 fraction (M1) was determined by flow cytometry 24 h later. (D) U2OS cells were transfected with 30 nM siRNAs and treated as described in Fig. 5 A. Emerging colonies were counted, and statistical analysis was performed as described in Fig. 6 B. The results represent the percentage of surviving colonies and are shown as the means from three independent experiments with their corresponding SEM (error bars).

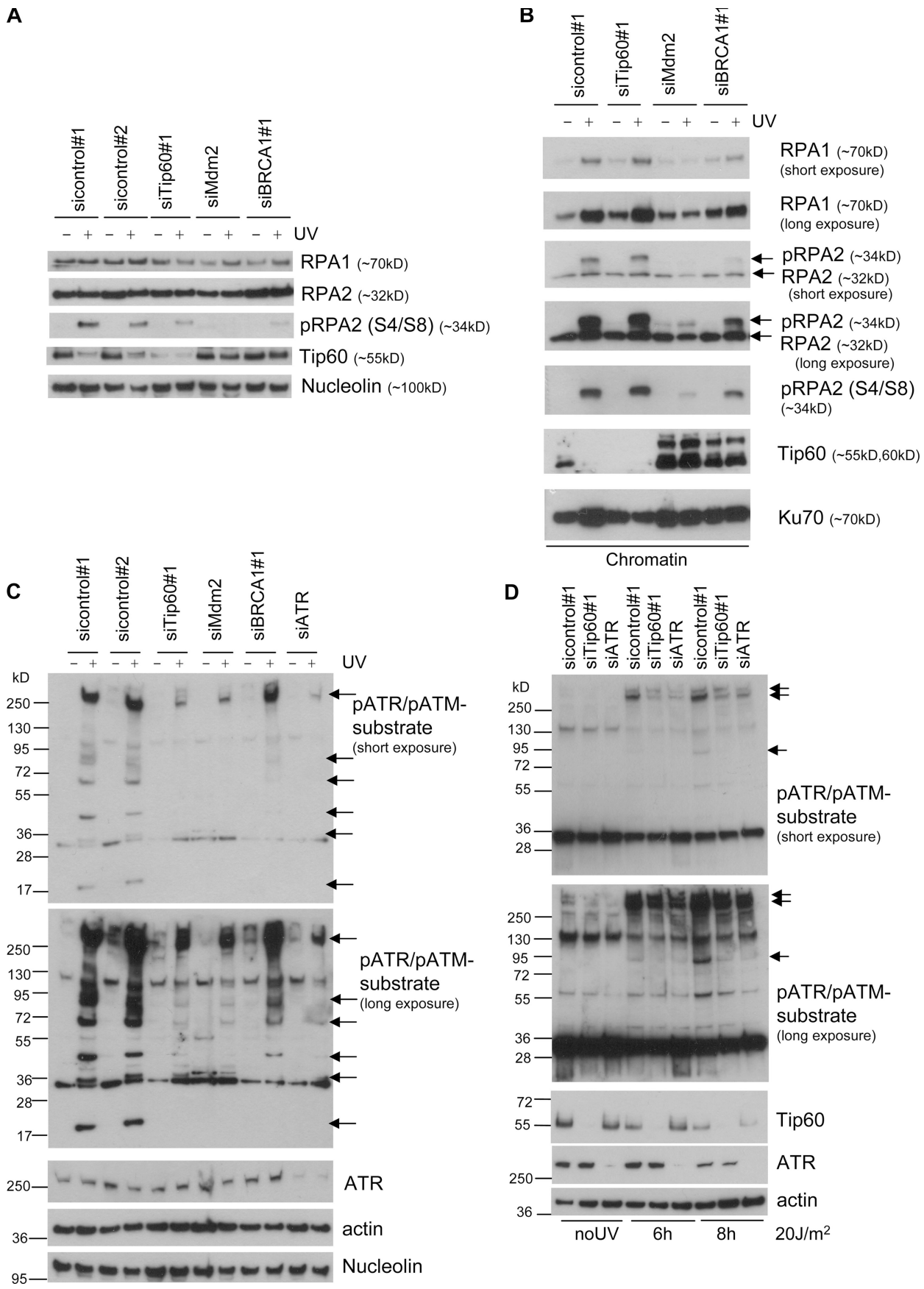

Figure 8. **Knockdown of Mdm2 or BRCA1 but not Tip60 impairs RPA2 phosphorylation and RPA1/RPA2 recruitment to chromatin, whereas all three siRNA species diminish the phosphorylation of ATR substrates.** (A) U2OS cells were transfected with 30 nM siRNA and UV irradiated (20 J/m^2) followed by

Discussion

In an attempt to identify the mechanisms by which Tip60 contributes to UV-induced apoptosis, we were surprised to find p53 completely dispensible for this response. On the contrary, preaccumulated p53 efficiently prevented the signaling and apoptotic response to UV irradiation. At least one of the underlying mechanisms appears to consist of the induction of p21, with subsequent suppression of the DDR mediator BRCA1. BRCA1 enables the loading of RPA onto chromatin and the phosphorylation of some ATR substrates upon UV irradiation. Tip60, on the other hand, is not required for RPA loading, but it does mediate ATR substrate phosphorylation. Both Tip60 and BRCA1 function as mediators of JNK activation. This model is summarized in Fig. 9.

Tip60 as an upstream component of UV DDR

In a previous study, the role of Tip60 in response to UV irradiation was attributed to its role as a cofactor of p53-induced transcription (Tyteca et al., 2006). In contrast to such a model, our results show that the knockdown of p53 does not affect the UV response under circumstances in which a reduction of Tip60 largely eliminates UV-induced apoptosis. Moreover, knocking down Tip60 diminishes the UV response even in cells that lack p53 entirely. These findings make it clear that the role of Tip60 in the UV response is independent of p53. Instead, we observed that Tip60 is required for the phosphorylation of factors involved in the DNA damage–induced signaling cascade, such as H2AX, JNK1/2, and ATR substrates. Thus, Tip60 is required for the upstream UV-induced DDR mechanisms.

Tip60 was previously implicated in the activation of transcription by p53. Initially, its major role was seen in the activation of *CDKN1A/p21* (Berns et al., 2004; Legube et al., 2004). However, in more recent studies, Tip60 was found to be mainly required for the activation of proapoptotic genes through the acetylation of p53 (Sykes et al., 2006; Tang et al., 2006). Our siRNA studies revealed that the impact of different Tip60-targeted siRNAs on p21 levels varied (unpublished data), whereas their impact on Tip60 levels and on the UV response remained constant, suggesting some caution about the previously suggested role of Tip60 in *CDKN1A/p21* expression. In any case, our results strongly suggest that the role of Tip60 in the UV response does not depend on its putative impact on p53.

We found that Tip60 plays an important role in early phosphorylation of the JNKs after UV irradiation, as siRNA to Tip60 consistently decreased the levels of phospho-JNK1 and -JNK2 independently of p53 status (Figs. 1 B and 2 A). We speculate that the interaction of Tip60 with MEKK1, one of the upstream kinases of JNK in the UV response, might enable JNK phosphorylation (Zhu et al., 2006; Song and Lee, 2007).

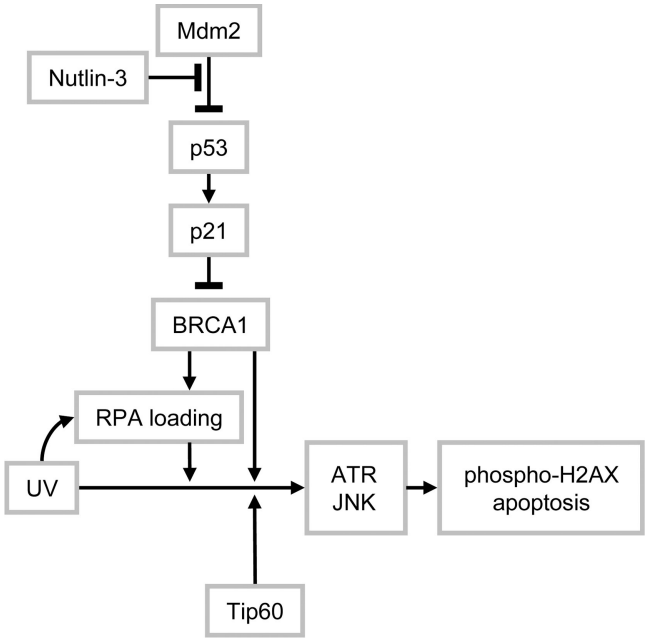

Figure 9. **p53 and Tip60 each modulate the response to UV irradiation.** A schematic model is provided to summarize the pathways that enhance or inhibit the UV-induced DDR. Arrows indicate activation, and bars represent inhibition. p53 activity leads to a reduction in BRCA1 levels. BRCA1 but not Tip60 triggers the recruitment of RPA on chromatin, contributing to ATR and JNK activation. Tip60 also mediates the activation of ATR and JNK, but without affecting RPA recruitment. ATR and JNK activity trigger histone 2AX phosphorylation and apoptosis.

A role of Tip60 in the regulation of phospho-H2AX was previously described in the *Drosophila* system (Kusch et al., 2004). There, however, Tip60 was found to promote the removal of phospho-H2AX from DNA lesions, not its accumulation. Possibly, Tip60 is required for the dynamics of phospho-H2AX in mammalian cells as well, but with an opposite net effect. Along this line, it has been previously found that Tip60 binds, acetylates, and activates the ATM kinase (Sun et al., 2005; Jiang et al., 2006). ATM is induced by DNA double-strand breaks, typically resulting from γ irradiation but not primarily from UV irradiation. Furthermore, Tip60 is involved in the repair of DNA double-strand breaks (Murr et al., 2006) and is required to activate ATM by p14ARF (Eymin et al., 2006). On the other hand, Tip60 was found to be capable of interacting with the FATC domain of ATR (Jiang et al., 2006) and enhancing ATR phosphorylation (Cheng et al., 2008), which is compatible with our observations that suggest ATR regulation by Tip60 (Fig. 8). In addition, Tip60 may modulate the DDR through its impact on gene expression. Tip60 represents a cofactor for numerous transcription factors (Sapountzi et al., 2006; Squatrito et al., 2006), and its knockdown is therefore likely to affect the transcription of many genes. Although the impact of Tip60 on postirradiation protein synthesis does not appear to be required for its effect on

immunoblot analysis of the indicated proteins. (B) U2OS cells were transfected and UV irradiated as in A. Subsequently, the chromatin-associated insoluble fraction was extracted 4 h after irradiation (see Materials and methods), and proteins were analyzed by Western blotting. (C) U2OS cells were transfected with 30 nM siRNAs as indicated. After 48 h, they were UV irradiated (20 J/m^2) and harvested 4 h later for Western blot analysis of phosphorylated ATR/ATM substrates. (D) Saos-2 cells were treated as in C and harvested at the indicated time points after irradiation. Arrows indicate ATR/ATM substrates that were phosphorylated by UV irradiation.

DDR (Fig. 1 C), the removal of Tip60 may still affect DDR through altered gene product composition that already occurs before the onset of DNA damage.

Mdm2 has been reported to mediate the ubiquitination of Tip60 (Legube et al., 2002). Indeed, we observed a reduction of Tip60 in response to UV, and this was actually prevented by knocking down Mdm2. However, siRNA to BRCA1 abolished Tip60 reduction with similar efficiency (Fig. 8 A). Moreover, Nutlin-3 strongly augmented Mdm2 but did not affect Tip60 levels in nonirradiated cells (Fig. 3 A), although the binding site on Mdm2 for Nutlin-3 (and p53) does not appear to overlap with the Tip60-binding site (Legube et al., 2004). Therefore, we propose that Mdm2 contributes to Tip60 degradation indirectly, preventing BRCA1 repression through p53 and thereby enabling the UV response.

Suppression of BRCA1 levels by p53 and p21

We propose that enhanced p21 levels inhibit UV DDR, at least in part, by repressing *BRCA1* expression. In agreement with this, a previous study suggested that p400 is required for UV DDR and that p400 knockdown leads to an inhibition of UVC-induced apoptosis while increasing p21 (Tyteca et al., 2006). On the other hand, it is of interest to note that in response to UV irradiation, the amounts of p21 are reduced by several mechanisms including proteasomal degradation (Bendjennat et al., 2003). Moreover, the transcription of *CDKN1A/p21* is only poorly induced by p53 under such circumstances, presumably as a result of the recruitment of a transcriptionally inactive mediator complex to its promotor (Donner et al., 2007). The repression of p21 levels upon UV exposure may ensure a death response when the DNA damage is too severe to be repaired.

It was suggested that p53 might interfere with JNK by direct physical interaction (Lo et al., 2004). In contrast, we found the protective effect of Nutlin-3 to strictly depend on p21 induction (Fig. 6 A), strongly suggesting that the activity of p53 as a transcription factor is required for its ability to attenuate the UV DDR. The antiapoptotic effect of p21 may partially be caused by reduced DNA replication, possibly preventing cells that carry single-strand breaks from entering S phase and undergoing replication catastrophe-induced apoptosis. Importantly, however, reduction of the BRCA1 levels by siRNA did not alter the cell cycle distribution but nevertheless conferred protection against UV-induced apoptosis. Thus, the reduction in BRCA1 levels alone is sufficient for UV protection, but additional mechanisms such as cell cycle arrest or interaction with apoptosis signal-regulating kinase 1 (ASK1; Huang et al., 2003) may further contribute to the ability of p21 to attenuate the UV response.

Several genes, including *BRCA1*, can be negatively regulated rather than induced by p53. We have previously shown that this negative regulation requires the induction of *CDKN1A/p21*, possibly through the inhibition of cyclin-dependent kinases, the hypophosphorylation of Rb pocket proteins, and the negative regulation of E2F-induced transcription (Lohr et al., 2003). In agreement with this, *BRCA1* was found to be induced by E2F-1 (Wang et al., 2000) and repressed by E2F-6 (Yang et al., 2007). On top of this, p53-repressed genes were found to accumulate a complex of NF-Y and p53 on their promoters (Imbriano et al., 2005), and the same may also be true for BRCA1 (Ceribelli et al., 2006).

The role of BRCA1 in response to UV exposure was partially explored in previous studies, with controversial results. BRCA1 is capable of interacting with ATR, resulting in BRCA1 phosphorylation in response to UV irradiation (Tibbetts et al., 2000). This phosphorylation event was found to be required for the activation of caspase-3 under similar conditions (Martin and Ouchi, 2005). Indeed, BRCA1 may activate caspases by binding the X-linked inhibitor of apoptosis protein (XIAP) in a phosphorylation-dependent manner (Martin and Ouchi, 2005). Vice versa, it was reported that caspases activate BRCA1 by specific cleavage (Zhan et al., 2002). The results shown here imply that BRCA1 may not only act as a proapoptotic transmitter of ATR signaling but also as an upstream regulator of RPA recruitment and ATR substrate phosphorylation (Fig. 8). Moreover, our data suggest that the knockdown of BRCA1 impairs activation of the JNK pathway in response to UV irradiation. In support of this, BRCA1 may promote JNK activation and apoptosis through GADD45 expression (Harkin et al., 1999). More directly, however, it has been shown that BRCA1 interacts with MEKK3, an upstream regulator of the JNK pathway, and this interaction was found to be required for JNK activation (Gilmore et al., 2004).

We observed that depletion of Mdm2 or BRCA1 decreased RPA loading on the chromatin and RPA2 phosphorylation upon UV exposure (Fig. 8, A and B). In the case of siRNA to Mdm2, cell cycle arrest may contribute to this effect because RPA is known to be active during DNA replication (Szuts et al., 2003). In the case of BRCA1, however, this explanation does not apply (Fig. S5 B, available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). Rather, it seems that BRCA1 largely contributes to recruitment of the RPA complex to damaged DNA, possibly allowing efficient ATR activation.

The question remains why the knockdown of BRCA1 resulted in reduced rather than increased colony formation, whereas Mdm2 knockdown or Nutlin-3 also reduced BRCA1 levels but still had the opposite effect on colony numbers (Fig. 7 D). Perhaps the elimination of BRCA1 is only tolerated when the cells are arrested in the G1 or G2 phases of the cell cycle. This model is supported by the notion that BRCA1 is required for chromosomal decatenation during DNA replication and cell division (Lou et al., 2005). Furthermore, cells transfected with siRNA targeting the BRCA1/BARD–heterodimer complex displayed defective chromosome segregation and malformed nuclei, supporting its requirement for accurate assembly of the mitotic spindle (Joukov et al., 2006). Other prosurvival activities of BRCA1 include its contribution to recombination repair while being recruited to nuclear foci upon UV exposure (Dunn et al., 2006; Wang, 2007). Furthermore, BRCA1 has been demonstrated to ubiquitinate RPB8, an RNA polymerase subunit, contributing to cell survival after UV irradiation (Wu et al., 2007).

p53 as a survival factor

A protective role of p53 for DNA-damaged cells is somewhat counterintuitive, at least at first glance, because p53 has long been known for its ability to induce apoptosis in response to

genotoxic stress. However, previous data suggest that p53 can actually act as a survival factor, at least under specific circumstances. For instance, the presence of p53 enhances the survival of retinal cells upon UV irradiation during *Drosophila* development (Jassim et al., 2003). Moreover, in mammals, the protective tan response to UV was recently found to depend on p53 in a paracrine fashion through the induction of proopiomelanocortin (Cui et al., 2007). Our findings point out an even more direct role of p53 in the protection of cells in that it attenuates the upstream signaling cascades in response to UV irradiation. We speculate that this might be of particular advantage during the adaptation to repeated sunlight exposure. In such a scenario, the activation of p53 by a nonlethal first exposure might render cells resistant to subsequent, more extensive irradiation.

These findings and considerations suggest that p53 is promoting cell survival in some instances, especially in UV-exposed tissues. Thus, even in tumor cells, mutating or inactivating p53 might not necessarily prevent cell death. In accordance with this, p53 mutations are rarely found in primary malignant melanoma but appear to be more prevalent in metastases of this tumor species (i.e., in cells that are no longer exposed to UV light; Hussein et al., 2003). Along the same line, not all human papillomaviruses encode E6 proteins that efficiently mediate the ubiquitination and degradation of p53, such as the types 16 and 18 associated with cervical carcinomas. In contrast, the E6 proteins from skin wart–associated papillomaviruses leave p53 intact (Storey, 2002), possibly because UV-exposed warts survive more easily when p53 remains functional.

Cellular vigilance to DNA damage subject to pharmacological manipulation

Irradiation with UV light is frequently used to treat dermatological disorders (e.g., psoriasis). Depending on the desired outcome (destruction or proliferation of cells), it might be beneficial to manipulate p53 activity before irradiation. Both activation and inhibition of p53 have been achieved through drug candidates such as Nutlin-3 (Vassilev et al., 2004) and pifithrin (Komarov et al., 1999), respectively. According to our study, it might become a useful therapeutic strategy to combine UV irradiation with the manipulation of p53. Earlier work from our group already suggested that Nutlin-3 or similar activators of p53 might help to protect normal cells from the cytotoxic effects of nucleoside analogues (Kranz and Dobbelstein, 2006). It is becoming increasingly clear that the DDR in general not only depends on the extent of DNA damage itself and is not only determined by the direct executors of the DDR-induced signaling cascades; rather, DDR can be tuned to different levels, depending on the vigilance toward genotoxicity required by individual cells. Manipulating this level of vigilance may enhance the efficacy and/or reduce the side effects of genotoxic cancer therapy.

Materials and methods

Cell culture, transfection, and irradiation

U2OS and Saos-2 cells were maintained in DME. Media were supplemented with 10% FBS. Immortalized keratinocytes (EPC2-hTERT; a gift from O.G. Opitz, Department of Medicine, University of Freiburg, Freiburg, Germany; Harada et al., 2003) were maintained in serum-free media supplemented with 0.2 ng/ml EGF and 25 µg/ml bovine pituitary extract (all from Invitrogen). NHEKs were obtained from PromoCell and were grown in keratinocyte growth medium 2 with supplements. Nutlin-3 (Qbiogene) was dissolved in DMSO and stored at −20°C as a stock solution of 25 mg/ml (43 mM). Cells were treated with 8 µM Nutlin-3 or its solvent DMSO alone. All siRNA duplexes, including siRNA control #1 and siRNA control #2, were purchased from Ambion (with the exception of the siRNA to p21, which was purchased from Santa Cruz Biotechnology, Inc.) and are described in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1). For siRNA transfection, cells were incubated with 30 or 100 nM siRNA and Lipofectamine 2000 (Invitrogen). UV-C irradiation was administered using a UV cross-linker (Hoefer).

Immunoblots and chromatin isolation

Whole cell extracts were prepared by lysing the cells in urea buffer (8 M urea, 0.1 M NaH_2PO_4, and 10 mM Tris-HCl, pH 7.5). Chromatin isolation was performed as described previously (Liu et al., 2007). Proteins were analyzed by Western blotting. The antibodies to p21/cip1/waf1 (Ab-1), BRCA1 (Ab-1), PARP (Ab-2), p53 (Ab-6 and Ab-2), RPA1 (Ab-1), and RPA2 (Ab-2) were purchased from EMD; those to β-actin (AC-15), Nucleolin, Chk1, and PCNA (PC-10) were purchased from Abcam; the antibody to γ-H2AX was obtained from Millipore; the antibody to S4/8P RPA2 was purchased from Bethyl Laboratories; the antibody to Ku-70 was obtained from Santa Cruz Biotechnology, Inc.; and antibodies to cleaved caspase 3 (5A1), JNK, 53BP1, S317P Chk1, T183P/Y185P JNK, and S/T-ATM/ATR substrates were purchased from Cell Signaling Technology. The monoclonal mouse antibody 2A9 against Mdm2 and the polyclonal rabbit antibody to Tip60 were gifts from A.J. Levine (The Cancer Institute of New Jersey, New Brunswick, NJ) and B. Amati (Department of Experimental Oncology, European Institute of Oncology, Milan, Italy), respectively. Primary antibodies were detected by chemiluminescence (Thermo Fisher Scientific) using a peroxidase-coupled secondary antibody (Jackson ImmunoResearch Laboratories).

Flow cytometric analysis

After the treatment, adherent and floating cells were pooled and processed for flow cytometry as described previously (Kranz and Dobbelstein, 2006). The intensity of staining was determined for 10,000 cells in each assay using the FACScan Flow Cytometry system (Becton Dickinson). The subdiploid population (M1) was calculated to reflect the proportion of apoptotic cells. For annexin V staining, the cells were treated and harvested by trypsination, incubated with FITC-conjugated annexin V (Abcam), and counterstained with propidium iodide. Thereafter, the samples were analyzed by flow cytometry for the presence of viable (double negative for annexin V and propidium iodide), early apoptotic (only annexin V positive), and late apoptotic cells (double positive for annexin V and propidium iodide).

Clonogenic survival assays

500 cells per plate were seeded in triplicates and treated with Nutlin-3/DMSO after 24 h for an additional period of 24 h. Then, the cells were UV irradiated and further incubated with Nutlin-3/DMSO. 24 h after irradiation, the medium was replaced by drug-free medium. 7–10 d later, the emerging colonies were fixed, stained with crystal violet, and counted. For other clonogenic assays, cells were transfected with the indicated siRNAs. After 48 h, the cells were UV irradiated and trypsinized, and a constant percentage of the cells in each sample was seeded in 3.5-cm wells in triplicate. After 7–10 d, the emerging colonies were fixed, stained with crystal violet, and counted. The ratio of surviving colonies was calculated as the number of colonies after UV versus the number of colonies without UV. Results presented indicate the means from three different experiments with their corresponding SEMs.

Statistics

For statistical analysis, the *t* test for two samples, assuming equal variances, was used.

RT-PCR analysis

Total RNA was extracted using TRIZOL (Invitrogen) followed by reverse transcription with the First Strand cDNA Synthesis kit (Roche). Quantitative real-time PCR was performed with a real-time PCR system (7300; Applied Biosystems) using the 2× Sybr green PCR Master Mix with AmpliTaq-gold-polymerase (Applied Biosystems). All samples were analyzed in triplicate. The data were analyzed with the 7300 system software (Applied Biosystems) using glyceraldehyde 3-phosphate dehydrogenase as an internal control. All primers are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1).

Immunofluorescence

Cells were grown on chamber slides (Thermo Fisher Scientific), treated with Nutlin-3, and UV irradiated. After different time points, the cells were fixed with 4% PFA in PBS and permeabilized with 0.2% Triton X-100 in PBS, and the DNA was denatured by incubation with 2 M HCl for 30 min at room temperature. After extensive washing with PBS, unspecific binding was blocked by incubation with 10% FBS in PBS. The cells were incubated with primary antibodies staining CPDs and 6-4 photoproducts (both were obtained from Medical and Biological Laboratories) followed by an incubation with AlexaFluor594-conjugated anti–mouse IgG antibody (Invitrogen). DNA was counterstained with DAPI followed by mounting with Fluoprep (bioMérieux). The samples were viewed with a fluorescence microscope (Bx51; Olympus) with a 20× UPlanFI NA 0.5 objective lens (Olympus) equipped with standard fluorescence filters. Images were obtained with a camera (DP-70; Olympus) using DP controller software (Olympus) and the same exposure times for each sample. Thereafter, the images were processed using Photoshop 8.0 (version 8.0; Adobe), applying the same settings for each sample.

BrdU staining

Cells were treated with 50 µM BrdU for 1 h, trypsinized, and fixed with 70% ethanol. The DNA was denatured by incubation with 2 M HCl for 30 min at 37°C. Thereafter, the cells were extensively washed with PBS and incubated for 1 h with anti-BrdU antibody (Abcam) and for 30 min with AlexaFluor488-conjugated anti–mouse IgG antibody (Invitrogen). The cells were stained with propidium iodide and analyzed by flow cytometry.

Online supplemental material

Fig. S1 shows that the efficient knockdown of Tip60 inhibits UV-induced apoptosis. Fig. S2 shows that Mdm2 knockdown inhibits the DDR upon UV irradiation by preactivating p53, whereas the depletion of Tip60 inhibits the UV response independently of p53. Fig. S3 shows that Nutlin has no detectable impact on the induction and removal of UV-induced photoproducts. Fig. S4 shows that efficient knockdown of BRCA1 using different siRNAs impairs the UV response, whereas Chk1 knockdown does not influence the DDR upon UV irradiation. Fig. S5 shows that knockdown of Mdm2 but not BRCA1 or other gene products induces cell cycle arrest. Table S1 and Table S2 present the siRNA sequences and RT-PCR primer sequences used in this study, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200712014/DC1.

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