DNA Damage and Cellular Stress Responses

The Atr Protein Kinase Controls UV-Dependent Upregulation of p16\(^{\text{INK4A}}\) Through Inhibition of Skp2-Related Polyubiquitination/Degradation

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Abstract

The tumor suppressor p16\(^{\text{INK4A}}\), a phosphoprotein that exists in human cells under both phosphorylated and nonphosphorylated forms, plays crucial roles during the cellular response to UV light. However, it is still unclear how this protein is activated in response to this carcinogenic agent. We have shown here that UVC upregulates p16\(^{\text{INK4A}}\) and the phosphorylated form of the protein at the 4 serine sites; Ser-7, Ser-8, Ser-140, and Ser-152. This accumulation of p16\(^{\text{INK4A}}\) occurred through increasing the stability of both forms of the protein. Importantly, phospho-p16\(^{\text{INK4A}}\) showed much higher stability, and UV treatment strongly increased its level in absence of \textit{de novo} protein synthesis. Furthermore, we have shown that the UV-dependent upregulation of both forms of p16\(^{\text{INK4A}}\) is under the control of the protein kinase Atr, which suppresses their UVC-dependent proteasomal degradation. Interestingly, although this degradation is ubiquitin-related for p16\(^{\text{INK4A}}\) through the Skp2 ubiquitin ligase protein, it is ubiquitin-independent for the phosphorylated form. In addition, we present clear evidence that Skp2 is upregulated in ATR-deficient cells, leading to the downregulation of the p27\(^{\text{Kip1}}\) protein in response to UV light. Moreover, we have shown a preferential association of endogenous phospho-p16\(^{\text{INK4A}}\) with Cdk4. This association increased following UV-treatment mainly for p16\(^{\text{INK4A}}\) phosphorylated at Ser-140 and Ser-152. Besides, we have shown that Atr regulates UV-related p16/Cdk4-dependent and -independent phosphorylation of pRB and G1 cell cycle delay. Together, these results indicate that p16\(^{\text{INK4A}}\) and p27\(^{\text{Kip1}}\) are key targets in the Atr-dependent signaling pathway in response to UV damage.

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Introduction

Exposure to ultraviolet (UV) radiation induces the formation of various cellular damages and triggers the activation of various pathways in order to promptly cope with the damage, and therefore eliminates the potentially carcinogenic genetic instability. Posttranslational modifications are of great importance during the cellular DNA damage response (DDR). Indeed, different protein kinases responsible for phosphorylating a plethora of proteins are involved (1, 2). Furthermore, tremendous work during the last 2 decades showed the crucial role of protein ubiquitination in the cellular DDR (3). The E3 ubiquitin ligase Skp2 plays a pivotal role in the cellular response to DNA damage by targeting a number of cell cycle and tumor suppressor proteins for proteolysis (4, 5). Interestingly, several proteins are posttranslationally modified by different molecules and the cross-talk between protein phosphorylation and ubiquitination seems to be a major regulator of the cellular response to genotoxic stresses (6). During DNA damage signaling cascade, the protein kinase Atr acts as a sensor of DNA damage (3, 7). In response to UV light, Atr phosphorylates different proteins and is implicated in the arrest of the cell cycle (8, 9). Furthermore, it has been recently shown that members of the ubiquitin-proteasome system are substrates of the Atr protein kinase during the DDR (10).

\textit{p16}\(^{\text{INK4A}}\) (p16) is also involved in blocking the progression of the cell cycle by binding and inhibiting the action of cyclin-dependent kinases (CDKs) that are responsible for the phosphorylation of the retinoblastoma tumor suppressor protein, pRB (11). p16 has been found mutated or silenced in various types of cancer and is linked to familial melanoma (12). Compelling epidemiological and basic science data support a critical causal role of UV sunlight...
exposure in the development of melanoma (13), suggesting an important role of p16 in the cellular response to UV-induced DNA damage. However, the effect of UV radiation on p16 protein and its upstream regulators are still not defined.

Materials and Methods

Cells, cell culture, and chemicals

HFSN1 (primary normal human skin fibroblast) cells were routinely cultured in DMEM/F12 (50:50) medium supplemented with 10% CFS. The HNEC1 cells (primary normal human breast epithelial) were cultured in DMEM/F12 (50:50) supplemented with 1% antibiotic antimycotic, 10 ng/mL insulin-like growth factor (IGF), 0.5% insulin transferin selenium (ITS), 5 μg/mL insulin, 250 ng/mL hydrocortisone, and 0.1 μmol/L of each of phosphoethanolamine and ethanolamine.

MG-132 was purchased from Sigma (USA) and the protein phosphatase PP1 from Biolabs.

Irradiation

The medium was removed and the monolayers in dishes were covered with PBS and exposed to a germicidal UV lamp (254 nm) at a fixed distance. The UV dosimetry was conducted using an ultraviolet meter (Spectronics Corporation).

Cellular lysate preparation

This has been carried out as previously described (14).

Immunoblotting

SDS-PAGE was conducted using 12% separating mini gels as previously described (14). The antibodies directed against p53 (DO-1), PCNA (PC-10), Ubiquitin (P4D1), Atr (N-19), α-tubulin (B-5–1-2), and β-actin (C-11) were purchased from Santa Cruz (USA); the specific p16 Phospho-Ser7, 8, 140 and 152 antibodies were purchased from ABGENT; p16 from BD Biosciences, pPRB Ser-612 (4E4) from ABNOVA, pPRB Ser-780 (9307), p27 and Skp2 from Cell Signaling.

Analysis of protein half-life

This was carried out as previously described (14).

shRNA Transfection

Specific shRNAs against ATR (Cat # KH01318N) and SKP2 (Cat # KH00232N) were purchased from SA Biosciences. Stable transfection was carried out using human dermal fibroblast Nucleofector Kit (Amaxa Biosystems) following the protocol recommended by the manufacturer as previously described (14).

Immunoprecipitation

Cell lysates were prepared using RIPA buffer containing protease inhibitors, and then centrifuged at 14,000 rpm at 4°C. About 300 μg of protein extracts were precleaned with 20 μL protein A/G agarose for 2 hours at 4°C and then incubated for 2 hours with 2 μg of specific antibody at 4°C. Subsequently, 50 μL of A/G agarose was added for 2 hours at 4°C. After centrifugation the pellet was washed with the RIPA buffer and the proteins were recovered by boiling in Laemmli buffer.

Cell cycle analysis by flow cytometry

PI stained cells were analyzed for DNA content and the percentage of cells in various cell cycle phases was determined by using Cell Quest software (Becton Dickinson).

Quantification of protein expression level

The expression levels of the immunoblotted proteins were measured using the densitometer (BIO-RAD GS-800 Calibrated Densitometer) as previously described (14).

Results

UV light upregulates p16 protein level in human cells

Sub-confluent normal human skin fibroblast HFSN1 cells were challenged with a UV fluence of 5 Jm⁻² and then re-incubated for different periods of time (0–24 hrs). Figure 1A shows that 75% of cells were at the prereplicative stage before UV-treatment and remained in G0/G1 phase.

Figure 1. UVC upregulates p16 protein in human fibroblast and epithelial cells. Cells were challenged with UVC, re-incubated and harvested after the indicated time periods for protein purification or FACScan analysis. About 30 μg of proteins was used for western blot analysis using the indicated antibodies. β-actin was used as internal control A. FACScan analysis following UV irradiation (5 Jm⁻²) of HFSN1 cells. B, Western blots using HFSF1 cells. C, Western blots using HNEC1 cells.
during the whole period of reincubation that followed the irradiation. Subsequently, cells were harvested and protein extracts were prepared and used in immunoblotting assay to assess the levels of p16 and p53 using specific antibodies. Anti β-actin antibody has been used as internal control. Figure 1B shows that p16 protein level increased 4.2 fold only 1 hour following UV-treatment, and reached its maximum level (4.9-fold higher) after 24 hours. The level of p53 started to increase 2 hours postirradiation and reached its maximum level (6.1 fold higher) 24 hours after the treatment (Fig. 1B). This indicates that, like p53, p16 protein level was upregulated in response to UV damage. Subsequently, p16 level was assessed in UV-challenged primary human epithelial cells. For comparison, the level of the p53 protein was also evaluated. HNEC1 cells were treated as described above and cell lysates were used for Western blot analysis. The exposure of these cells to 5 J/m² increased the expression of p16 in a time-dependent manner reaching the maximum level (4.8-fold higher) 24 hours post-UV treatment. Similarly, p53 level also increased in these cells with the maximum level of induction (3.4 fold) reached 24 hours after UV-treatment (Fig. 1C). This indicates that low UV dose triggers p16 accumulation in both fibroblast and epithelial cells.

**Figure 2.** UV-related p16 phosphorylation at four different serine sites. A, 60 μg of proteins from HFSN1 cells were either treated or not with the protein phosphatase1 (PP1) (7.5 U) for 1 hour and then were used for immunoblotting analysis using the indicated antibodies. Samples were loaded on the SDS-PAGE gel and the membrane was then cut vertically and probed with the indicated antibodies separately. α-tubulin antibody served as loading control. B, Protein lysates were prepared from HFSN1 cells expressing either control-siRNA or CDKN2A-siRNA and were used for immunoblotting using the indicated antibodies. C, D, HFSN1 cells were either mock-treated or UV-irradiated (5 J/m²), and then reincubated and harvested at the indicated periods of time. Fifty μg of proteins were used for Western blot analysis using the indicated antibodies. The numbers below the bands indicate the folds of protein induction.

UV light upregulates phosphorylated p16 at Ser-7, Ser-8, Ser-140, and Ser-152

To further investigate the effect of UV light on p16, we used anti-p16 antibodies that recognize the phosphorylated form of the protein. To confirm the specificity of these antibodies, we treated HFSN1 cell lysate with the protein phosphatase1 (PP1) and anti-p16 as well as anti-phospho-p16 antibodies were used for detection. Figures 2A and 2D show that the phosphorylated form of p16 migrated at a MW of 25 kDa, and that after the treatment with PP1 the level of the phosphorylated forms of p16 decreased, whereas no effect was observed on the non-phosphorylated form of the protein. This shows that the used antibodies recognize specifically the phosphorylated form of the protein. Furthermore, the 2 forms of p16 were not detected in the p16-defective U2OS cells (data not shown) and their levels decreased significantly in p16-siRNA expressing HFSN1 cells (14) (Fig. 2B), confirming the specificity of the utilized antibodies. Next, HFSN1 cells were UV-treated as described above and the phosphorylated form of p16 was assessed using specific antibodies. Figure 2C shows that the 4 antibodies detected the phosphorylated form of the protein before UV treatment, showing the existence of phosphorylated p16 in the absence of the genotoxic stress. Following UV irradiation, significant increase in the level of phospho-p16 occurred at the 4 serine sites. This increase was more pronounced at Ser-8, which was phosphorylated to a level 17-fold higher 14 hours postirradiation. The UV-dependent phosphorylation of p16 at Ser-140, Ser-152 and Ser-7 reached levels 3.6-, 3-, and 2.1-fold higher, respectively (Fig. 2C).
2D also shows the simultaneous upregulation of both phosphorylated and nonphosphorylated forms of p16. Similar results were obtained when the other anti-phospho-p16 antibodies were used (data not shown).

UV damage stabilizes the p16 protein

Next, HFSN1 cells were treated with 20 μg/mL of cycloheximide and either mock-treated or challenged with a UV fluence of 5 J/m², and then reincubated for different periods of time. Subsequently, whole cell extracts were prepared and used to assess the levels of the p16 and p53 proteins. Figures 3A and 3B show that in the absence of UV light the half-lives of p16 and p53 were 4 hours 20 minutes and 40 minutes, respectively. As expected, the p53 half-life increased and reached 5 hours 20 minutes following UV treatment (Fig. 3B). Interestingly, UV radiation increased the level of p16 during the first 2 hours reaching a level 1.8-fold higher, and then the level decreased slightly without going below the basal level, which is considered as 100% (Fig. 3B). This clearly shows that like p53 (15), UV light stabilizes p16 by reducing its turnover. Figure 3A shows also that the phosphorylated form of p16 is much more stable than the nonphosphorylated form. Interestingly, the irradiation of the HFSN1 cells increased the level of phospho-p16, despite the presence of cycloheximide (Fig. 3A). These results might suggest posttranslational UV-dependent phosphorylation of the phosphorylated form of p16 on other Serines of the protein.

Atr is required for UV-related p16 accumulation

To investigate whether the protein kinase Atr is involved in UV-related p16 upregulation, ATR was down-regulated using specific shRNA. Figure 4A shows a significant decrease in the expression level of the Atr protein in ATR-shRNA-expressing HFSN1 cells as compared to their control counterparts. Next, these cells were treated with UV light (5 J/m²) and re-incubated for different periods of time. Figure 4B shows that while p16 level increased in the control cells, it rather decreased following UV-treatment in the ATR-deficient cells. Indeed, p16 was almost undetectable only 2 hours post-UV irradiation (Fig. 4B). Similar results were obtained when antibodies specific for the phosphorylated form of p16 at Ser-7,
Ser-8, Ser-140, and Ser-152 were used (Fig. 4B). These results clearly show that ATR plays a critical role in the UV-dependent upregulation/stabilization of both p16 and phospho-p16.

**Atr controls ubiquitin-dependent proteasomal degradation of p16 in response to UV light**

To investigate the possible role of proteasome in UV-dependent degradation of p16 in ATR-shRNA expressing cells, we have challenged these cells and their corresponding control with a UV fluence of 5 Jm^{-2}, and then we reincubated them in a culture medium containing MG-132, an inhibitor of the proteasome activity, for different periods of time. Cell lysates were prepared and used for immunoblotting analysis using specific antibodies. Figure 5A shows that p16 was upregulated in these conditions in both ATR-deficient and ATR-proficient cells. However, the upregulation was delayed in ATR-shRNA expressing cells wherein p16 reached a level 4.5-fold higher but only 6 hours post-UV treatment. On the other hand, 4-fold increase was reached after only 2 hours in the control cells (Fig. 5A). Similarly, in ATR-shRNA expressing cells the levels of phospho-p16 at Ser-8 and Ser-152 did not decrease in the presence of MG-132, and rather strongly increase in control cells reaching levels 25-fold and 4-fold higher, respectively (Fig. 5A). Together, these results show that UV-related decrease in p16 and phospho-p16 levels in ATR-shRNA cells is mainly due to proteasomal degradation.

Since proteasomal-dependent degradation is usually linked to protein ubiquitination, which plays important roles in the regulation of UV damage response (16), we investigated the possible UV-dependent ubiquitination of p16 in the control and ATR-shRNA expressing cells. These cells were either mock-treated or challenged with UV light (5 Jm^{-2}) with or without the presence of MG-132 and then reincubated for 3 hours. Whole cell extracts were prepared and specific antiubiquitin antibody was used for immunoprecipitation and specific anti-p16 and phospho-p16 antibodies were utilized for immunoblotting. Figure 5B shows high molecular weight p16 bands corresponding to the polyubiquitinated form of the protein that were almost undetectable in nontreated cells and appeared in the UV-treated ones. The level of the polyubiquitinated form of p16 was much higher in ATR-shRNA cells than in the control cells (Fig. 5B). Interestingly, the presence of MG-132 further increased the level of polyubiquitinated p16.
especially in the ATR-shRNA expressing cells. The presence of little polyubiquitination in UV-treated HFSN1 control cells reflects the decrease in p16 levels that followed the UV-dependent upregulation (Fig. 1B). On the other hand, the presence of MG-132 did not lead to an increase in the level of the high molecular weight bands detected with the anti-phospho-p16 (Ser-8) antibody in both the control and ATR-shRNA cells (Fig. 5B). This shows UV-dependent poly-ubiquitination of p16 but not phospho-p16 in ATR-deficient cells, suggesting ubiquitin-dependent proteasomal degradation of p16, but ubiquitin-independent proteasomal degradation of phospho-p16 in ATR-deficient cells.

p16 ubiquitination in ATR-deficient cells is Skp2-dependent

To test whether the ubiquitin protein ligase Skp2 is responsible for UV-dependent poly-ubiquitination of p16 in ATR-defective cells, we first assessed Skp2 levels following UV-treatment in cells expressing either ATR-shRNA or control-shRNA. Figure 6A shows that while UV light upregulated Skp2 expression (3.4-fold) in ATR-deficient cells, it has no effect on Skp2 level in ATR-proficient cells.

Skp2 is known to regulate p27Kip1 (thereafter p27) ubiquitination and consequently proteasomal degradation (5). Therefore, to further proof UV-dependent increase of Skp2 protein level and activity in ATR-shRNA expressing cells, we assessed the level of p27. Figure 6A shows that following UV treatment the level of p27 decreased in ATR-deficient cells, while it increased 3.2-fold 14 hours post-UV treatment in the control cells. This indicates that ATR suppresses Skp2 upregulation and therefore positively controls the upregulation of p27 in response to UV light. As Skp2 induction was concomitant with p16 and p27 degradation, Skp2 could be also responsible for p16 ubiquitination upon UV treatment in ATR-deficient cells. To test this hypothesis, we investigated the possible interaction between Skp2 and p16. HFSN1 cells expressing either ATR-shRNA or control-shRNA were challenged with 5 Jm⁻² and either harvested immediately or reincubated for 1 hour. Figure 6B shows that in ATR-deficient cells p16 was co-precipitated with anti-Skp2 and its level strongly increased following UV-treatment. However, there was a marked decrease in the level of co-precipitated p16 in the UV-irradiated control counterparts. Similar results were obtained for Skp2 when the complex was immunoprecipitated with anti-p16 antibody (Fig. 6B). This may indicate that, upon UV-treatment, Skp2 binds to p16 and target it for proteasomal degradation in an ATR-dependent manner.

Next, we tested whether downregulation of SKP2 in ATR-deficient cells could inhibit the UV-dependent degradation of p16. Therefore, ATR-deficient cells expressing either control-shRNA or SKP2-shRNA were treated with a UV fluence of 5 Jm⁻² and the levels of p16 and p27 were assessed by immunoblotting. Figure 6C shows that while
p16 and p27 levels decreased in the control cells, they were upregulated in SKP2-shRNA-expressing cells. This indicates that the observed decrease in the levels of p16 and p27 in the ATR-deficient cells after UV-treatment is Skp2-dependent, probably through ubiquitination. To test the possible Skp2-dependent ubiquitination of p16, ATR-deficient cells expressing either control-shRNA or SKP2-shRNA were either mock-treated or challenged with UV light (5 J m^{-2}) and then reincubated for 6 hours. Whole cell extracts were prepared and specific antibiubiquitin antibody was used for immunoprecipitation and specific anti-p16 antibody was utilized for immunoblotting. Figure 6D shows high molecular weight p16 bands corresponding to the polyubiquitinated form of the protein. As expected, the level of polyubiquitinated p16 increased upon UV-treatment in ATR-deficient SKP2-proficient cells (control). However, no increase was observed in SKP2-shRNA expressing ATR-deficient cells (Fig. 6D).

This suggests that UV-dependent polyubiquitination of p16 in ATR-deficient cells is indeed Skp2-related. Together, these results indicate that Atr protein kinase controls UV-dependent upregulation of p16 through inhibition of Skp2-related ubiquitination and proteasomal degradation.

Cdk4 binds preferentially to phospho-p16, and Atr controls both Cdk4-dependent and -independent phosphorylation of pRB in response to UV light.

p16 blocks the progression of the cell cycle by binding to either Cdk4 or Cdk6 and inhibiting the action of cyclin D (17–20). In order to investigate the binding of p16 to Cdk4 in the absence and presence of UV damage we performed immunoprecipitation experiments using anti-Cdk4 antibody. HFSN1 cells expressing either ATR-specific shRNA or control shRNA were challenged with 5 J m^{-2} and either harvested immediately or re-incubated for 6 hours. The presence of p16 and phospho-p16 in the pulled down material was assessed by immunoblotting using specific antibodies. Figure 7A shows that in the control cells at time 0 only phospho-p16 (at the 4 serine sites) was co-precipitated with anti-Cdk4 antibody but not the nonphosphorylated form, indicating that the phosphorylated form of p16 was selectively associated with Cdk4. Interestingly, 6 hours post UV-treatment, the level of coprecipitated phosphorylated p16 at Ser-140 and Ser-152 increased significantly in the control cells. On the other hand, the binding to Cdk4 of UV-dependent phosphorylated p16 molecules at Ser-7 and Ser-8 did not increase.
epithelial cells were exposed to a UVC fluence as low as 5 J m\(^{-2}\). This indicates that UV-mediated increase in the phosphorylated form of p16 at Ser-140 and Ser-152 preferentially associate with Cdk4. Interestingly, no increase, but rather slight decrease, in the Cdk4-associated phospho-p16 occurred in UV-treated ATR-shRNA expressing cells (Fig. 7A). This shows that the Atr-dependent accumulation of phospho-p16 in response to UV damage increases the binding of p16 to Cdk4. This binding is known to inhibit the kinase activity and therefore leads to a decrease in the phospho-pRB. To test this, we assessed the level of the phosphorylation of pRB at the Ser-780 site, which is Cdk4-dependent, using the cell lysates prepared above (Fig. 7A). Figure 7B shows that the level of phospho-pRB at Ser-780 decreased about 2-fold in UV-treated control cells, but rather increased 2.3-fold in ATR-shRNA expressing cells. This indicates that Atr is involved in UV-dependent inhibition of Cdk4 by p16. On the other hand, in ATR proficient cells a significant increase in the phosphorylated form of pRB was observed at Ser-612, which is Cdk4-independent. Interestingly, this increase was abolished in the ATR-deficient cells (Fig. 7B). This shows that ATR is also required for the UV-related Cdk4-independent phosphorylation of pRB, which plays a key role in the arrest of the cell cycle at G1 phase. Accordingly, we have shown that ATR is indeed required for UV-related cell cycle delay at the G1 phase of the cell cycle (Fig. 7C).

**Discussion**

The tumor suppressor p16 exists in human cells under phosphorylated and nonphosphorylated forms in absence of stress (21, 22). In this report, we have used specific antibodies for each form of the protein and have shown that both accumulate in response to UVC. Indeed, p16 protein level increased several times when human skin fibroblast and epithelial cells were exposed to a UVC fluence as low as 5 J m\(^{-2}\). This upregulation occurred as early as 1 hour after UV treatment and cells remained at the G0/G1 phase of the cell cycle throughout the 24 hours that followed the irradiation (Fig. 1), indicating that p16 accumulation is UV-dependent and not due to the accumulation of cells in the G2/M phase of the cell cycle as has been previously reported (23–25).

Interestingly, we have shown here that UV damage stabilizes p16 protein by increasing its half-life. Furthermore, using specific anti-phospho-serine antibodies we have confirmed the existence of phosphorylated form of p16 at 4 sites, Ser-7, Ser-8, Ser-140 and Ser-152 (21), and UV light triggered great accumulation of phospho-p16 at the 4 sites, but to different extents. Importantly, the phosphorylated form of p16 showed significantly lower turnover-over than the nonphosphorylated one, and following UV-treatment the level of the phosphorylated form of the protein increased despite the presence of cycloheximide, indicating the presence of UV-dependent phosphorylation of phospho-p16 (Fig. 3).

Furthermore, we have shown that UV-dependent accumulation of p16 and phospho-p16 is under the control of the Atr protein kinase (Fig. 4B). Indeed, the levels of p16 and phospho-p16 decreased after UV-treatment in the ATR-deficient cells owing to proteasomal degradation. Surprisingly, although p16 degradation occurred following ubiquitination, phospho-p16 was not ubiquitinated in response to the same UV fluence. This shows that the proteasomal degradation of p16 and its phosphorylated form is carried out through 2 different pathways that are both repressed by the Atr protein kinase.

For the majority of proteins, conjugation of ubiquitin to an internal lysine is the initial event in their degradation by the ubiquitin-proteasome system (26). However, some studies have shown that the internal lysine is not necessary for ubiquitination that can take place at the N-terminal residue of the protein (27). This is the case for p16 and p14, 2 lysine-less proteins that can be degraded through N-terminal ubiquitination (28, 29).

Here we provide evidence that the UV-dependent ubiquitination/degradation of p16 in ATR-deficient cells is Skp2-dependent (Fig. 6). This ubiquitin ligase is involved in the turnover of many cell cycle regulatory substrates, including p27 and E2F1 (5). It is therefore tempting to speculate that in ATR-deficient cells Skp2 targets p16 for ubiquitination on its N-terminal residue in response to UV damage. Importantly, similar results were obtained for p27. Indeed, we provide here the first indication that p27 expression is also upregulated following UV damage. Like p16, p27 accumulation is mediated through Atr-dependent inhibition of Skp2-related ubiquitination and degradation. Here we have shown that Atr is an inhibitor of Skp2 upregulation upon UV-treatment. The molecular basis of this inhibition is still undefined and based on the important functions of these proteins during the cellular DDR, it will be of great importance to elucidate how Atr inhibits Skp2 upregulation in response to DNA damage; especially that the increase in the expression of the Skp2 and its subsequent degradation of p27 promotes the growth of cancer cells. Indeed, deregulation in the expression of p27 has been reported in several types of human cancer, and was correlated with poor prognosis (5).

The induction of p16 in response to UV irradiation could lead to the inhibition of both, cell proliferation and/or cell death to allow prompt and efficient removal of the UV-induced DNA damage. Indeed, it has been previously shown that p16 delays the cell cycle after exposure to low UV fluences (30). Here we have shown that Cdk4 preferentially associates with phosphorylated p16 at the 4 serine sites. This corroborates what has been previously shown by Gump et al. who have also shown an association of Cdk4 with endogenous p16 phosphorylated at Ser-152 (21). However, although the phosphorylation at the 4 sites increased following UV-treatment, the association with the Cdk4 kinase increased only for p16 phosphorylated at the C-terminal part of the protein at Ser-140 and Ser-152. Interestingly, this UV-mediated association between Cdk4 and phospho-p16 decreased in ATR-shRNA expressing cells. As expected, the UV-dependent upregulation of p16 and its binding to Cdk4 correlated with a decrease in phospho-pRB at Ser-780. On the other hand, the phosphorylation of this site rather
increased in UV-treated ATR-defective cells, wherein the level of phospho-p16 decreased. Furthermore, we have shown that Atr controls the UV-related phosphorylation of pRB at the Scr612 site. This phosphorylation is Chk1/2-dependent but Cdk4-independent and it enhances the formation of the pRB-E2F1 complex required for cell cycle arrest in response to DNA damage (31). Together, these results show that following UV light, the Atr kinase controls both the p16/Cdk4-dependent and -independent phosphorylation of pRB, which plays a key role in the arrest of the cell cycle at the G1 phase. Accordingly, we have shown that ATR is indeed required for UV-related cell cycle delay at the G1 phase of the cell cycle (Fig. 7C). However, the significance of p16 phosphorylation in the G1 checkpoint remains to be determined.

In conclusion, we present here clear evidence that the cyclin-dependent kinase inhibitors p16 and p27 accumulate in response to the carcinogenic UV damage through suppression of their Skp2-dependent ubiquitination/degradation by the Atr protein kinase.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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The Atr Protein Kinase Controls UV-Dependent Upregulation of p16INK4A Through Inhibition of Skp2-Related Polyubiquitination/Degradation

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