ATR Is Not Required for p53 Activation but Synergizes with p53 in the Replication Checkpoint*

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ATR (ataxia telangiectasia and Rad-3-related) is a protein kinase required for survival after DNA damage. A critical role for ATR has been hypothesized to be the regulation of p53 and other cell cycle checkpoints. ATR has been shown to phosphorylate p53 at Ser[15], and this damage-induced phosphorylation is diminished by expression of a catalytically inactive (ATR-kd) mutant. p53 function could not be examined directly in prior studies of ATR, however, because p53 was mutant or because cells expressed the SV40 large T antigen that blocks p53 function. To test the interactions of ATR and p53 directly we generated human U2OS cell lines inducible for either wild-type or kinase-dead ATR that also have an intact p53 pathway. Indeed, ATR-kd expression sensitized these cells to DNA damage and caused a transient decrease in damage-induced serine 15 phosphorylation of p53. However, we found that the effects of ATR-kd expression do not result in blocking the response of p53 to DNA damage. Specifically, prior ATR-kd expression had no effect on DNA damage-induced p53 protein upregulation, p53-DNA binding, p21 mRNA up-regulation, or G_1 arrest. Instead of promoting survival via p53 regulation, we found that ATR protects cells by delaying the generation of mitotic phosphoproteins and inhibiting premature chromatin condensation after DNA damage or hydroxyurea. Although p53 inhibition (by E6 or MDM2 expression) had little effect on premature chromatin condensation, when combined with ATR-kd expression there was a marked loss of the replication checkpoint. We conclude that ATR and p53 can function independently but that loss of both leads to synergistic disruption of the replication checkpoint.

The response to DNA damage is a complex process that is crucial in maintaining the fidelity of the genome amid diverse stresses. A conserved and critical aspect of this response is cell cycle arrest immediately following damage, which allows for DNA repair prior to progression. The mechanisms of arrest following DNA damage are becoming increasingly well understood and in many cases involve a member of a conserved

family of very large protein kinases called the phosphatidyl inositol kinase-related kinases (1, 2). In yeast, the Rad-3 (*Schizosaccharomyces pombe*) or Mec1 (*Saccharomyces cerevisiae*) family members are essential protein kinases that have been shown to be required for the response to diverse stresses including ultraviolet light, ionizing radiation, and hydroxyurea (3, 4). In humans, deficiency in ATM function leads to a complex phenotype including extreme sensitivity to ionizing radiation (IR),[1] loss of p53 activation after IR, IR-resistant DNA synthesis, insulin resistance, chromosomal instability, loss of cerebellar neurons leading to ataxia, and development of lymphoid malignancies (5). The development of ATM-deficient mice and the human disease ataxia telangiectasia have led to a greater understanding of the functions of the ATM protein kinase.

The role of ATR (ataxia telangiectasia and Rad-3-related) has remained more mysterious than ATM because the ATR −/− mouse is early embryonic lethal (6, 7), and there is no specific small molecule inhibitor for ATR. In particular, the role of ATR in regulating p53 function has remained unclear. Several studies have demonstrated that *in vitro* ATR phosphorylates p53 on serine 15 (8–11), a site that is phosphorylated in response to DNA damage and plays a role in its transcriptional activation (12). In addition, Tibbetts *et al.* (13) demonstrated that in cells overexpressing ATR-kd, this serine 15 phosphorylation did not occur normally in response to damage by ultraviolet light or ionizing radiation, implicating ATR in the regulation of p53 in cells. It is also plausible that ATR may regulate p53 indirectly via Chk-1. Several studies have documented that ATR activates Chk-1 (14–16), and Chk-1 has been shown to phosphorylate p53 (17). Moreover, p53 levels are decreased when Chk-1 is inhibited, suggesting that p53 expression may be regulated by Chk-1 (17). Importantly, these prior studies have not been able to examine the role of ATR in regulating p53 function or the induction of a G_1 arrest because the p53 response was defective because of p53 mutations or expression of SV40 large T antigen, which binds p53 and blocks its function (18).

To address the role of ATR in DNA damage-induced p53 activation and G_1 arrest, we generated U2OS-derived stable cell lines that can inducibly overexpress either wild-type or kinase-dead ATR and that are functional in p53 and G_1 arrest pathways. Here we report our results using these cell lines that demonstrate that ATR is not required for p53 activation or G_1 arrest and that ATR works together with p53 in the replication checkpoint to block cells from prematurely condensing their chromatin.

[1] The abbreviations used are: IR, ionizing radiation; GFP, green fluorescent protein; PCC, premature chromatin condensation.

EXPERIMENTAL PROCEDURES

Generation of Cell Lines—U2OS (human osteosarcoma) cells stably expressing the tetracycline repressor (Invitrogen) and verified to have intact p53 and G_1 checkpoint function were used to generate doxycycline-inducible (19) stable cell lines. Amino-terminal FLAG epitope-tagged full-length constructs of ATR-wt or ATR-kd were inserted into the cytomegalovirus promoter-based plasmid pcDNA4/TO (Invitrogen), which contains two tetracycline operator-binding sites. These constructs were co-transfected with a 20-fold lower amount of pcDNA3.1, which contains the neomycin resistance gene. Beginning 2 days later, G418 (400 μg/ml)-resistant clones were selected. Hygromycin was always present at 200 μg/ml to maintain expression of the tetracycline repressor as described (19). Approximately 120 G418-resistant clones were screened by FLAG immunoprecipitation and FLAG Western blotting to obtain a pair of clones that were highly inducible for ATR-wt or ATR-kd and that had undetectable expression of the FLAG-tagged protein in the absence of doxycycline induction.

DNA Damage—IR was delivered by Cesium-137 irradiation at a rate of 2.5 Grays/min. UV was delivered at a rate of 4 J/m²/s from a panel of four UV bulbs (8 watts/bulb, RPR-3000; Southern New England Ultraviolet Co., Branford, CT) that had peak emission at 312 nm. For UV irradiation, phenol red-containing medium was removed for the brief irradiation period and then immediately replaced. A 5 mil Kodacel filter (K6808, Eastman Kodak Co., Rochester, NY) was used to filter UV <295 nm, which is not encountered in the environment.

Long Term Survival Assay—The cells were plated in duplicate and incubated in the presence or absence of doxycycline for 6 weeks. Every 3–4 days the cells were split, taking care to replate each set of uninduced and induced cultures at the exact same dilution. At each passage the cells were counted by hemocytometer and trypan blue exclusion. The percentages denote the number of induced cells relative to uninduced at that point in time, and each data point represents the average of two separate cultures, with a completely independent experiment yielding essentially the same results.

Clonogenic Survival Assays—The cells were plated in triplicate for each of two plating densities (one 10-fold lower than the other, designed to yield between 50 and 500 colonies) on 6-cm dishes in the presence or absence of doxycycline (1 μg/ml). After 2 days, the cells were irradiated or treated with the indicated agents and incubated until colonies developed (roughly 11 days). The colonies were visualized by fixation with 3.7% formaldehyde and staining with 0.1% crystal violet. To be counted, a colony had to contain more than 50 cells. The percentages were calculated relative to the appropriate doxycycline-treated or untreated cells that had not received radiation or chemical treatment. Each data point represents the average of six independent samples, and the error bars indicate standard errors of the mean.

Antibodies—For detection of ATR, a rabbit polyclonal antibody was generated against amino acids 1–20. M-5 monoclonal anti-FLAG antibody (Sigma) was used to detect FLAG-ATR, and cross-immunoprecipitations validated the specificity of these antibodies. Other antibodies used were ATM antibody-1 (Oncogene) and p53 Ab-6 (Oncogene), and the rabbit polyclonal phosphoserine 15 p53 antibody was a gift from Y. Taya.

Ribonuclease Protection Assay—The cells used in this assay were transiently transfected using Lipofectin (Invitrogen) according to the manufacturer's instructions with a GFP expression construct (SRα) for all cells and an equal amount of empty expression vector (Vector), ATR-wt, or ATR-kd construct as indicated. Two days after transfection, the cells were trypsinized and sorted by flow cytometry, and cells with GFP expression greater than one log above background were harvested. Control immunofluorescence studies looking for FLAG-tagged ATR showed nearly 100% co-expression of FLAG-ATR in cells that were GFP-positive. GFP-positive cells were replated in duplicate and treated with UV as indicated and harvested 16 h later. RNeasy (Qiagen) was used to isolate total RNA, and 1 μg of RNA was used in the RiboQuant assay system (PharMingen) with the h-STRESS-1 probe template set. Bands corresponding to p21 and L32 (internal control ribosomal RNA) were quantitated on a PhosphorImager. Fig. 5*B* indicates the normalized level of the indicated mRNA relative to the L32 control from two independent experiments.

Electrophoretic Mobility Shift Assay—DNA binding of p53 was analyzed by electrophoretic mobility shift assay using p53 consensus sequence double-stranded oligonucleotides: top, 5′-AGC TTA GAC ATR CCT AGA CAT GCC AAG CT-3′; bottom, 5′-AG CTT GGC ATG TCT AGG CAT GTC TAA GCT-3′. The oligonucleotides were labeled with ³²P using polynucleotide kinase (New England Biolabs) and separated from free ATP by QiaQuick column (Qiagen). The cells were treated

with doxycycline (1 μg/ml) for 48 h and exposed to UV as indicated. The cells were harvested with trypsin, washed, lysed in two packed cell volumes of hypotonic buffer (10 mM Hepes, pH 7.6, 10 mM KCl, 1 mM MgAc, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride), triturated with a pipitter, and placed on ice for 10 min. After centrifugation at $12,000 \times g$ for 5 min at 4 °C, the cytosolic supernatant was saved on ice, and the pellet was extracted with two packed cell volumes of hypertonic buffer (hypotonic plus 500 mM KCl) for 30 min on ice after trituration. Nuclei were then spun at $12,000 \times g$ for 5 min, and the supernatant was combined with the prior cytosolic extract. The DNA binding reaction was carried out at 23 °C for 30 min by adding 2.5 μl of this combined extract to 7.5 μl of a master mix containing 1 μl of dithiothreitol (50 mM stock), 1 μl of ZnCl₂ (500 μM stock), 1 μl of nonspecific DNA (0.1 μg/ul, Bluescript plasmid DNA, digested with *Hha*I), 1 μl of probe (1 ng/μl of labeled, annealed, double-stranded DNA), 1.2 μl of glycerol, 0.5 μl of polyclonal antibody 421 (p53 Ab-1, 2 mg/ml, Oncogene), and 1.8 μl of Tris-buffered saline (25 mM Tris, pH 7.5, 130 mM NaCl, 3 mM KCl). The mixes were then loaded and run at 4 °C on a 4% nondenaturing polyacrylamide gel with 1× Tris-borate EDTA (with final EDTA of 1 mM to avoid chelating the zinc), and the gel was dried and exposed to film. Antibody 421 was included because (*a*) signal in the absence of this antibody was poor in these cells, (*b*) no activation occurred merely by adding this antibody in the absence of damage, and (*c*) it is often included in gel shift studies of p53 function (20).

Cytometry/Cell Cycle Analysis—Unsynchronized ATR-kd-inducible U2OS cells were treated with doxycycline (1 μg/ml) for 48 h prior to UV treatment (200 J/m²) and nocodazole (100 ng/ml) as indicated. 16 h later cells were harvested with trypsin and resuspended in 700 μl of phosphate-buffered saline, and 300 μl of ice-cold ethanol was added and mixed well. After at least 15 min on ice, the cells were spun at $300 \times g$, resuspended in 1 mg/ml RNase A in phosphate-buffered saline, and incubated at 37 °C for 30 min. The cells were spun at $300 \times g$ and resuspended in 50 μg/ml propidium iodide in 0.1% sodium citrate. Flow cytometry was carried out on a FACScan (Becton Dickenson) apparatus as per the manufacturer's recommendations.

Double Thymidine Block—The cells were plated into normal medium, and after adherence, 2.5 mM thymidine was added for 17 h. The cells were washed twice with phosphate-buffered saline and placed in normal thymidine-free medium for 12 h. Thymidine (2.5 mM) was added for a second 17-h period, at which time cells were washed with phosphate-buffered saline; this point was designated $t = 0$, at which 98% of cells were found to be at the G_1/S border by propidium iodide flow cytometry analysis.

Adenovirus-mediated Expression—Adenoviral constructs and virus preparations were performed as described (21). Purified virus was added to the cell medium to a final titer of 10^{10} viral particles/ml 24 h before UV treatment. Expression constructs were driven by the cytomegalovirus promoter, and protein expression was verified by Western blotting. Function of each protein was verified by flow cytometry for its expected cell cycle effects. Based on GFP expression performed in parallel, this concentration of 10^{10} viral particles/ml yielded 100% infection and expression in these cells.

Mitotic Spreads—Doxycycline was added on the day of plating. 24 h later, adenovirus was added as indicated to 10^{10} plaque-forming units/ml. 24 h later, the cells were treated with ionizing radiation or hydroxyurea, and all cells received nocodazole (100 ng/ml). 24 h later the cells were harvested with trypsin and spun ($300 \times g$ for 10 min). All but about 50 μl of supernatant was discarded, and the cells were resuspended with a pipetter. 1 ml of 75 mM KCl was added for 10 min at room temperature. The cells were spun, the supernatant was discarded, and the cells were resuspended in 300 μl of freshly prepared Carnoy's fixative (three parts methanol and one part glacial acetic acid) for 10 min at room temperature. The cells were spun, the supernatant was discarded, and the cells were resuspended in 100 μl of Carnoy's fixative. 10 μl of this cell suspension was dropped from a height of 10 cm onto a glass slide and allowed to dry. 12 μl of 4,6-diamidino-2-phenylindole solution (Vectashield with 4,6-diamidino-2-phenylindole, Vector Laboratories, Burlingame, CA) was spotted onto the slide, a coverslip was placed above it, and the edges were sealed with clear nail polish. A fluorescence microscope was used to count mitotic cells that had characteristic features of either a normal mitosis or premature chromatin condensation (PCC). Interphase cells and cells that were intermediate in morphology between normal and PCC were not counted. The following criteria were used to identify mitoses as PCC or normal. PCC characteristics include well defined particles of DAPI-staining material that were round, not oblong; space between the particles with no hazy chromatin material between particles; no chromatid-like pairs present;
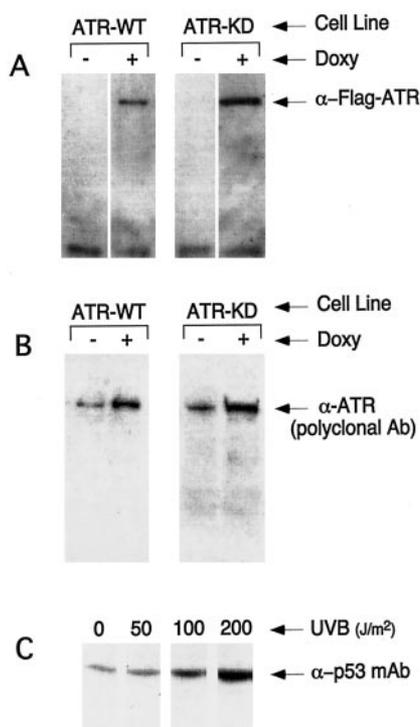
FIG. 1. **Characterization of p53 wild-type, ATR-inducible cell lines.** *A*, Western blots using anti-FLAG antibody in the two cell lines expressing wild-type and kinase-dead ATR. Doxycycline (*Doxy*, 1 μg/ml) was added 48 h prior to harvest. 10 μg of total protein was loaded per lane. A nonspecific band at the *bottom* of each *lane* serves as a loading control. *B*, a polyclonal anti-ATR antibody was used to detect endogenous and recombinant ATR, demonstrating that induced recombinant expression is roughly 3–5-fold endogenous ATR expression. *C*, p53 expression was examined 16 h after the indicated UV dose in ATR-kd inducible cells, to which no doxycycline was added (uninduced) to demonstrate p53 up-regulation by UV.

and borders of the chromatin of the cell are jagged and composed of speckles, not smooth or with a creamy hazy appearance (all characteristics must be met to be counted). Normal mitosis characteristics include well formed oblong chromatids present in pairs and at least 20 such chromosome pairs found in a cluster.

RESULTS

ATR-inducible Cell Lines—Fig. 1 shows that these cell lines have several important characteristics desirable for studying the role of ATR in cell function. Using an epitope (FLAG) tag to detect expression of the recombinant ATR, Fig. 1*A* shows there is no detectable expression of the recombinant ATR protein in the absence of doxycycline. The addition of doxycycline (1 μg/ml) caused a marked induction of recombinant ATR expression. For both wild-type and kinase-dead inducible cells lines, the addition of doxycycline caused roughly a 5-fold increase in total cellular ATR relative to the endogenous ATR level as revealed by a rabbit polyclonal antibody that detects both endogenous and recombinant ATR. UV treatment of cells caused an increase in p53 levels that was proportional to the dose of UV administered, demonstrating that the p53 pathway is intact in these cells (Fig. 1*C*).

ATR-kd Expression, DNA Damage, and Survival—The effect of prolonged induction of ATR-wt or ATR-kd constructs was investigated in cells that were not treated with DNA damage, and cell number in the doxycycline-induced case was compared with paired cultures of the same cell line (either ATR-wt or ATR-kd) to which no doxycycline was added. Ongoing induction of ATR-wt had no effect on growth of undamaged cells over 11 passages and 40 days in culture (Fig. 2*A*). In contrast, after a 2-week lag, ongoing expression of ATR-kd decreased the num-
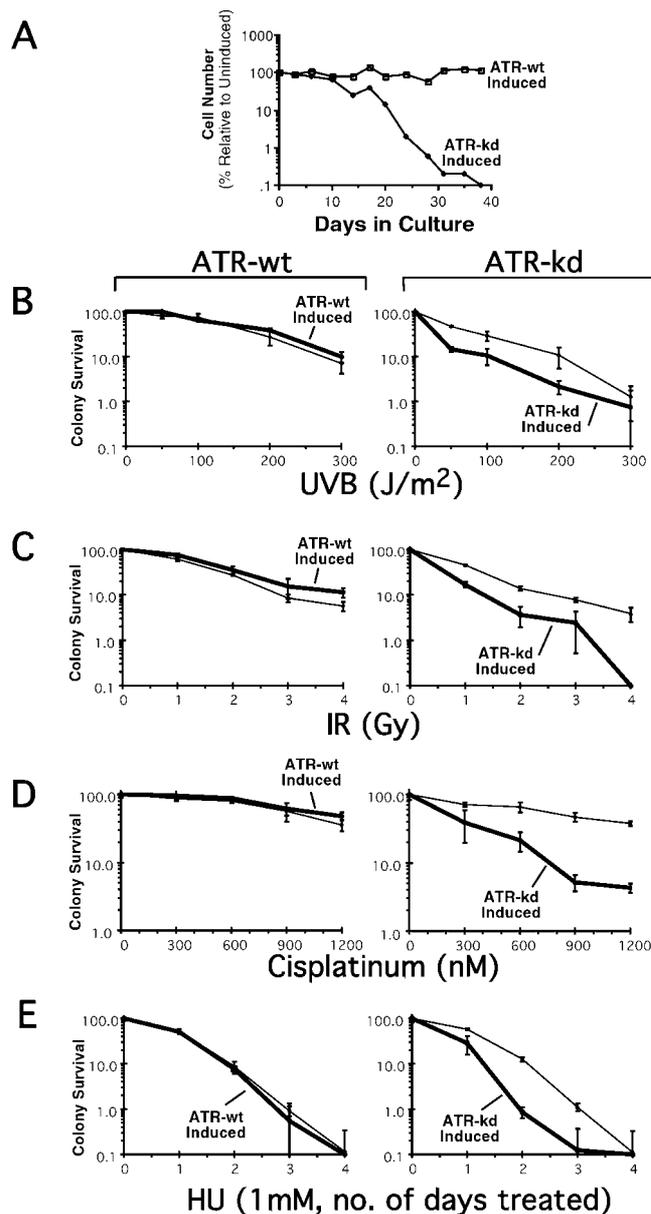
FIG. 2. **Effect of long and short term ATR induction and DNA damaging agents.** *A*, relative number of cells present after ongoing exposure to doxycycline (1 μg/ml) for the ATR-kd or -wt inducible cell lines. ATR-wt expression had no effect, but ATR-kd expression suppressed cell viability/growth starting about 2 weeks after induction. The results are the means of two independent cultures for each condition and each cell line. Colony survival assays after UV (*B*), IR (*C*), *cis*-platinum (*D*), and hydroxyurea (*E*) reveal no effect for ATR-wt expression but a marked sensitization to ATR-kd induction. The *bold lines* represent doxycycline-induced cells (ATR-wt in *left column*; ATR-kd in *right column*). The results are the means of two independent experiments each carried out in triplicate ± S.E. *HU*, hydroxyurea.

ber of viable cells to the point that none could be found after 40 days (Fig. 2*A*). In an identical experiment, inducible ATR-wt and ATR-kd cells generated by Cliby *et al.* (22) yielded the same results despite the expression of SV40 large T antigen in these other cell lines.

Colony survival assays were used to evaluate whether DNA damage selectively sensitized cells in which ATR function was inhibited by ATR-kd expression. Although induction of wild-type ATR expression had no significant effect on colony survival after any of the four treatments, ATR-kd expression decreased colony survival by roughly 10-fold in each case (Fig. 2, *B* and *C*). In the case of UV (Fig. 2*B*) a small difference in the
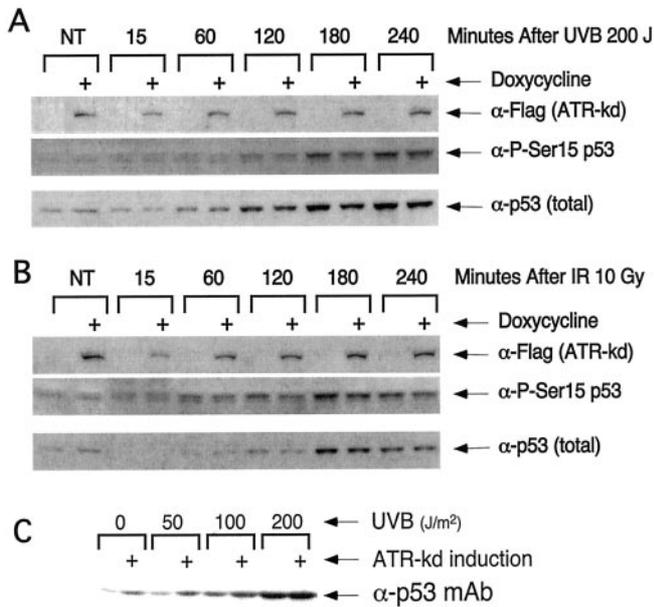
FIG. 3. **Effect of ATR-kd expression on p53 Ser[15] phosphorylation and p53 up-regulation in response to UV and IR.** *A*, Western blots using the indicated antibodies show a subtle decrease in phosphoserine 15 on p53 in the ATR-kd expressing cells, most prominent at 180 min. *B*, IR-induced serine 15 phosphorylation is also subtly inhibited at 120 and 180 min. *C*, UV-induced p53 protein up-regulation is unaffected at 16 h after damage. *NT*, not treated.

FIG. 4. **ATR-kd has no effect on p53-DNA binding following UV.** An electrophoretic mobility shift assay was used to assess UV-induced p53-DNA binding at the indicated times after 200 J/m² of UV. There was no effect of ATR-kd expression on p53-DNA binding induced by UV. *NT*, not treated.

FIG. 5. **UV-induced p21 mRNA transcription is not affected by ATR-kd expression.** *A*, ribonuclease protection was carried out on 10 μg of total RNA isolated from cells transiently expressing a control (empty) expression vector or ATR-wt or -kd. The cells had been treated 16 h before with UVB 200 J/m² as indicated. There was no significant inhibition of p21 mRNA induction. *B*, a PhosphorImager was used to quantitate the radioactivity in each *band* and normalize it to the riboprotein L32 (control). The results of two independent experiments are shown.

sensitivity of the uninduced cell lines is present with uninduced ATR-kd cells showing 10% survival at 200 J, whereas uninduced ATR-wt cells had 10% survival at 300 J. This effect may be due to low level leakiness not visible by Western blotting but detectable functionally. We have observed a similar phenomenon with caffeine sensitivity of these cells (16).[2] These data suggest that ATR plays an essential role in rapidly dividing cells in an ongoing manner and that its role is especially critical immediately following DNA damage or an S phase arresting agent such as hydroxyurea.

p53 Serine 15 Phosphorylation and ATR—We used a phospho-specific antibody to assess the extent of serine 15 phosphorylation on p53 at various times after UV or IR as indicated in Fig. 3 (*A* and *B*). Similar to a prior study in SV40-transformed cells (13), our p53 wild-type cells showed a small but reproducible decrease in UV- or IR-induced serine 15 phosphorylation at roughly 120 or 180 min after radiation. In contrast, the level of p53 protein (as opposed to its phosphorylation) was essentially unaffected by ATR-kd at these time points up to 240 min after damage (Fig. 3, *A* and *B*). At 16 h after UV damage, there was no inhibitory effect of ATR-kd on p53 protein induction at any dose of UV treatment. At this later time point there was also no effect on UV or IR-induced serine 15 phosphorylation of p53 (data not shown).

p53 DNA Binding and Transcriptional Activity—We performed electrophoresis mobility shift assays to investigate a possible role of ATR in the induction of p53 DNA binding activity. p53 DNA binding could be observed initially about 2 h after UV treatment and peaked after 4 h (Fig. 4). In each case, there was no effect of ATR-kd on the ability of p53 to bind the labeled double-stranded oligonucleotide. An important transcriptional target of p53 in the DNA damage response pathway is p21. We used ribonuclease protection to detect p21 mRNA levels in U2OS cells overexpressing ATR-wt or ATR-kd. p21 mRNA levels increased roughly 3-fold following UV treatment
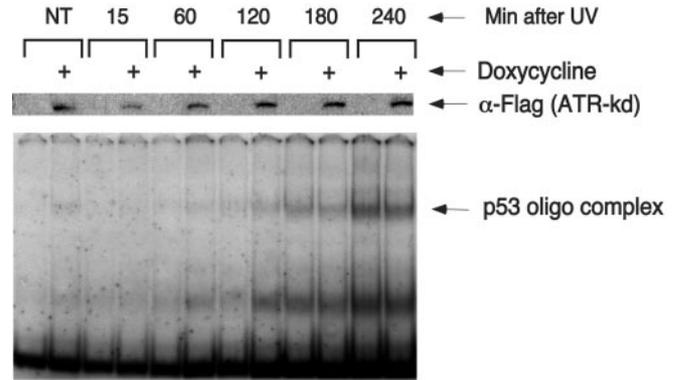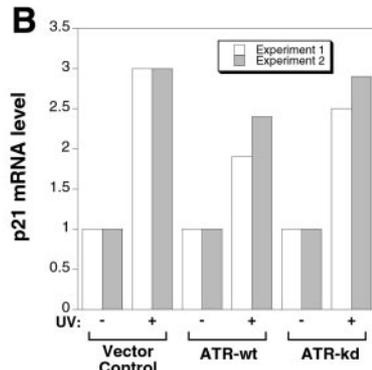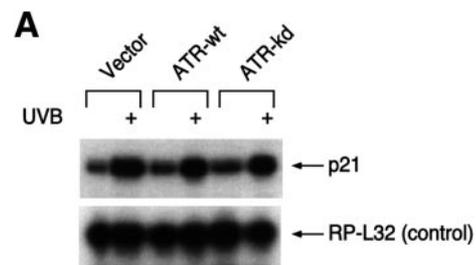
in control cells, and this induction was not affected by ATR-kd (Fig. 5). These data suggest that ATR is not required for damage-induced p53 DNA binding or transcriptional activity.

G_1 Arrest—We studied the effect of ATR-kd expression on G_1 cell cycle arrest after UV-induced DNA damage by using flow cytometry of unsynchronized propidium iodide-stained cells. Nocodazole was used as a mitotic inhibitor to show that during the 18-h treatment period undamaged cells would proceed from 2 N DNA content into arrest at 4 N DNA content (Fig. 6). UV treatment caused a significant G_1 arrest as evidenced by a persistent peak at 2 N DNA content in the presence of nocodazole, which prevents mitotic cells from cycling back to G_1 content. Indeed, this G_1 arrest was unaffected by ATR-KD overexpression. These data demonstrate that G_1 arrest occurs

ATR and p53 in the G$_1$ and Replication Checkpoints
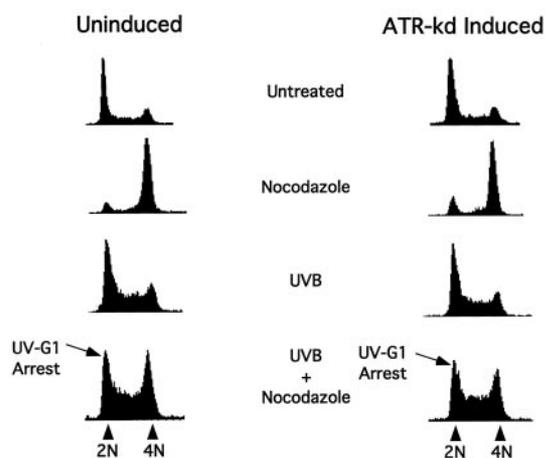
Uninduced   **ATR-kd Induced**

FIG. 6. **UV-induced G$_1$ arrest is not affected by ATR-kd expression.** Asynchronous ATR-kd-inducible cells were treated with doxycycline for 48 h (*Induced*) or not (*Uninduced*) and then given nocodazole (100 ng/ml) or UVB (200 J/m^2) as indicated. The population of G$_1$-arrested cells is indicated by the *arrow*.

FIG. 7. **ATR-kd promotes premature phosphorylation of nucleolin following UV.** ATR-kd-inducible cells were treated with doxycycline as indicated for 48 h during which time they were synchronized to the G$_1$/S border with a double thymidine block. The cells were UV-irradiated (200 J/m^2) at the time of release and harvested at the times indicated for Western blot analysis with an anti-phosphonucleolin antibody. Nucleolin is phosphorylated only in mitosis and serves as an indicator of the progression of cells into mitosis. A nonspecific band at the *bottom* of each *lane* acts as a loading control.

FIG. 8. **ATR and p53 cooperate in preventing premature chromatin condensation.** The cells were treated with doxycycline as indicated, and 24 h later the indicated adenovirus was added. 24 later the cells were treated with IR (10 Grays, *A*) or hydroxyurea (*HU*, 1 mM, *B*). Premature chromatin condensation occurs after IR or UV in ATR-kd-expressing cells. PCC is rescued by p21 expression and markedly augmented by overexpression of proteins that destroy p53 function, MDM2, and E6.

normally after DNA damage in cells that have been made sensitive to UV by ATR-kd overexpression. Taken together, we conclude that the major effect of ATR-kd is not on p53 or the G$_1$ arrest.

The Replication Checkpoint and ATR—To investigate whether ATR regulates entry into mitosis after DNA damage, we used a phospho-specific antibody to assess phosphorylation of nucleolin (an event that indicates entry into mitosis (23)) in cells that had been synchronized to the G$_1$/S boundary using a double thymidine block protocol. By 8 h after release from this G$_1$/S block, a significant proportion of untreated or ATR-kd-expressing cells progressed into mitosis as indicated by phosphorylation of nucleolin (Fig. 7). UV-treated cells, however, were fully arrested prior to mitosis at 8 h as evidenced by the absence of phosphonucleolin. This effect of UV was significantly blocked by ATR-kd expression (Fig. 7), suggesting that ATR is involved in delaying mitotic entry after DNA damage.

Premature chromatin condensation is a hallmark of cells that have an inadequate replication checkpoint and have begun chromatin condensation, an early stage of mitosis, before completing DNA synthesis (24). In a separate set of studies we have found that ATR-kd expression markedly promotes the occurrence of premature chromatin condensation after DNA damaging treatments or replication inhibitors such as hydroxyurea (16). Fig. 8 shows that ATR-kd expression sensitizes IR- and hydroxyurea-treated cells to premature chromatin condensation as revealed by mitotic spread analysis. Expression of the G$_1$-arresting cyclin-dependent kinase inhibitors p21 or p27 rescued ATR-kd expressing cells from PCC. In contrast, we investigated whether PCC would be promoted by inhibiting p53 function via MDM2 or E6 expression. If ATR function was necessary for p53 activation, one would predict that ATR-kd expression would inactivate p53 and that addition of MDM2 or E6 would have no effect. In fact, MDM2 or E6 expression each sharply increased the extent of PCC observed (Fig. 8), suggesting that p53 had been activated in the presence of ATR-kd expression. Taylor and Stark (25) recently reviewed several mechanisms by which p53 may promote a G$_2$/M arrest (discussed below). Regardless of the mechanism of p53 function in preventing PCC, it is clear that ATR-kd does not significantly inhibit p53 function because of the profound additional effect of MDM2- or E6-mediated p53 inhibition. These data also indicate that ATR and p53 represent two converging pathways that cooperate to maintain genomic integrity via the replication checkpoint.
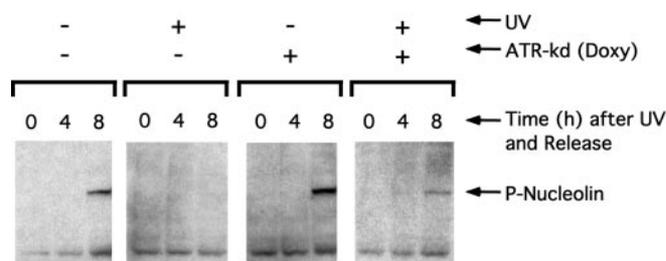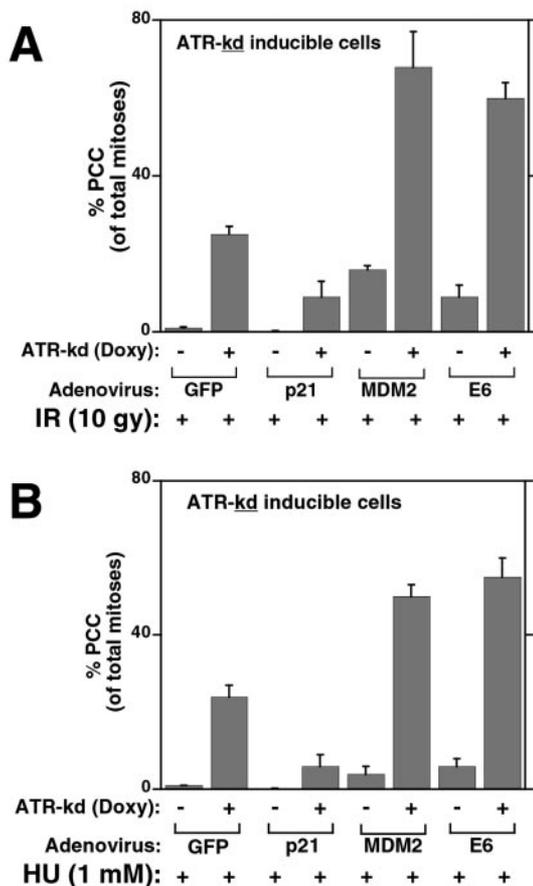
DISCUSSION

Several studies have shown that ATR can phosphorylate p53 on Ser[15] *in vitro* (8–11) and *in vivo* (13), raising the possibility that ATR is involved in p53 regulation after DNA damage. Because of a variety of technical issues, prior studies could not examine the role of ATR in the functional regulation of p53. The data presented here are thus the first to examine the functional interactions of ATR and p53. Surprisingly, although expression of a catalytically inactive ATR caused marked sen-

sitivity to DNA damage and a transient decrease in Ser[15] phosphorylation, ATR-kd had no effect on p53 activation by DNA damage. The simplest explanation for this finding is that ATR does not play a significant role in p53 functional activation. A related possibility is that there is functional overlap in p53 activation by multiple kinases such as ATM (26, 27), p38 (28, 29), ATR, and possibly DNA-PK (10). In such a case, inhibition of one of these could be compensated by others. In either case, the data suggest that the major role of ATR in the response to DNA damage does not involve p53 regulation.

Our data help in defining the relative roles of ATR and ATM in the response to DNA damage. There are many reasons to suspect that in some cases there may be functional overlap in the roles of ATR and ATM; both kinases phosphorylate p53 at Ser[15] (11), they share overlapping substrate specificities (10), and ATR has been shown to rescue the radio-resistant DNA synthesis defect of ATM cells (22). Indeed, Zhou and Elledge (2) have raised the concern that overexpression of ATR-kd may in fact be mediating some of its effects by inhibition of ATM function. There are several lines of evidence that suggest this is not the case: (*a*) ATM is well established as important in the activation of p53 and G_1 arrest (especially following ionizing radiation) (26, 27). In contrast, here we show that ATR-kd overexpression does not affect p53 activation by ultraviolet (Figs. 3–6) or ionizing radiation (Fig. 3*B* and data not shown). (*b*) In a separate study (16), we show that ATM does not play a role in the replication checkpoint that is likely a major function of ATR. In summary, it appears that in the p53/G_1 checkpoint ATM plays a dominant role over ATR and that in the replication checkpoint, ATR is the relevant mediator rather than ATM. In the S phase (radio-resistant DNA synthesis) checkpoint, ATM has been well established to be involved through several mechanisms (30, 31), and ATR likely plays a role (22), perhaps through Chk-1 activation and cdc25A degradation (32).

A further intriguing distinction between ATR and ATM lies in their mechanisms of activation; ATM kinase activity increases following DNA damage (11), whereas the kinase activity of ATR measured *in vitro* following *in vivo* DNA damage is unchanged (33). The most plausible model for ATR activation is that ATR co-localizes with its relevant substrates, such as BRCA-1, following DNA damage (33) and that this damage-induced co-localization involves DNA binding (14), which has been shown to increase the kinase activity of ATR (8, 9). Given these observations of how ATR is activated in cells, we believe that expression of ATR-kd likely blocks endogenous ATR function by binding its activation partners into inactive complexes rather than by directly blocking the catalytic activity of endogenous ATR.

Our studies of premature chromatin condensation are the first to investigate the functional interactions of ATR and p53 in the G_1 and replication checkpoints, which we summarize in Fig. 9. If ATR were required for p53 activation, one would expect that there would be little or no additional effect of p53 inhibition in cells expressing ATR-kd. In contrast we have found that inhibition of ATR and p53 function each independently sensitize cells to premature chromatin condensation and that the combination is more than additive (synergistic). Of note, our data suggest that ATR is more important (likely essential) in the replication checkpoint than p53 (see relative PCC rates for ATR-kd expression *versus* MDM2 or E6 expression). Portions of the pathway by which ATR likely functions in this checkpoint, via Chk-1 activation, have been relatively well characterized in *Xenopus* (14, 15) and mammalian studies (16, 34). In addition to the role of ATR in halting chromatin condensation before replication is complete, recent studies of Mec1 (the *S. cerevisiae* homolog of ATR) suggest ATR may also be
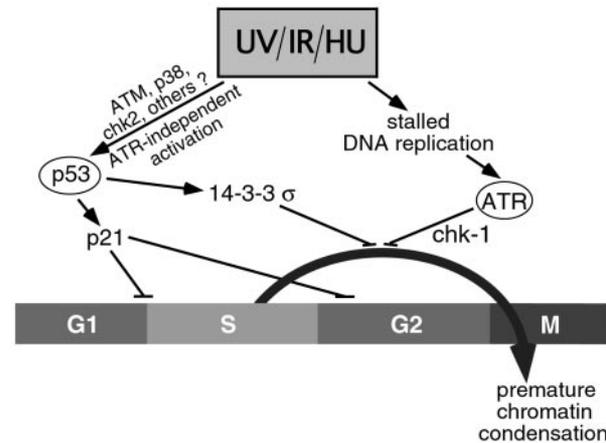

FIG. 9. **A model of ATR and p53 interactions in cell cycle checkpoints.** p53 is activated by DNA damaging agents in a pathway that does not require ATR function. Both ATR and p53 are involved in the replication checkpoint and loss of the functions of both proteins strongly promotes PCC by DNA replication stresses.

required for progression of DNA replication (35), for stability of the replication fork (36), and for blocking late origins of replication (35).

Regarding the role of p53 in the replication checkpoint (25), there are a number of mechanisms by which this tumor suppressor can prevent cells from entering mitosis before DNA replication is complete: (*a*) p53 up-regulates 14-3-3-σ, which sequesters cdc25 outside the nucleus, blocking activation of mitosis promoting factor (37). (*b*) p53 is well established as a regulator of p21, which arrests the cell cycle in G_1 and maintains a G_2 arrest (38). (*c*) p53 induces Gadd45, which has been implicated in inducing the G_2/M checkpoint (39). (*d*) p53 diminishes expression of Cdc2 and cyclin B1 (25). All of these p53 effects slow the cell cycle, allow time for repair, and decrease the immediate need for an ATR-mediated arrest during DNA synthesis. In contrast, p53-mediated apoptosis is not playing a role in reducing the rate of PCC observed after ATR-kd expression; p53-null status and caspase inhibition have each been shown not to prevent PCC in ATR-deficient mouse embryos (6).

Interestingly, we found that the cellular response to hydroxyurea (as well as to the more classical DNA damaging agent IR) was p53-dependent, as suggested by greater sensitivity to premature chromatin condensation upon HPV-E6 or MDM2 overexpression (Fig. 8). Others have reported that the response to hydroxyurea is dependent on p53 (40), but this has not been universally observed and may depend on the cell line.

An important implication of this work is that this level of ATR inhibition leaves p53 function fully intact, whereas the ATR/replication checkpoint is disabled. We have shown that defects in the G_1 checkpoint (universally present in cancer cells) sensitize cells to lethal premature chromatin condensation by ATR inhibition (16). This observation suggests that if a more potent and specific small molecule inhibitor of ATR can be discovered it may have cancer-selective properties by sensitizing these G_1 checkpoint-deficient cells to PCC. Our current results indicate that ATR inhibition does not disrupt p53 function and suggest that it may be important for an ATR inhibitor to be selective for ATR over ATM. This is because a small molecule that inhibited ATM function would cause the p53/G_1 checkpoint of normal cells to be impaired, diminishing the selectivity of the inhibitor for cancer cells over normal cells.

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