Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion

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p19ARF has been implicated as a key regulator of p53 stability and activation. While numerous stresses activate the p53 growth arrest pathway, those requiring p19ARF remain to be elucidated. We used p19ARF knockout mouse embryo fibroblasts to show that DNA damage and microtubule disruption require p19ARF to induce p53 responses, whereas ribonucleotide depletion and inhibition of RNA synthesis by low doses of actinomycin D do not. The data provide evidence that the arrest pathway activated by ribonucleotide depletion involves some different signal transducers than those activated by DNA damage or microtubule disruption. We also present biochemical analyses that provide insights into the mechanism by which p53 and p19ARF cooperate in normal cells to induce cell cycle arrest.

The tumor suppressors p53 and pRb participate in signal transduction pathways that respond to various environmental and intracellular challenges, such as hypoxia, ionizing and ultraviolet radiation, ribonucleotide depletion, microtubule disruption, and oncogene activation (1–8). The responses to these stresses range from induction of a reversible cell cycle arrest when DNA damage is not incurred to damage-dependent activation of pathways that lead to premature senescence or apoptosis. The net result of activation of the pathway, therefore, is to remove cells exposed to stressing conditions from the replicative cycle. During neoplastic progression, there is a strong selection for mutations that inactivate the p53–pRb pathways, thus enabling cells to avoid apoptosis or senescence and to continue dividing under conditions that can increase genetic instability (9, 10).

Recent experiments have focused attention on the *Ink4a/ARF* locus, which has the potential to regulate both p53 and pRb function. The p16Ink4a protein (inhibitor of cdk4) ensures that pRb remains active by preventing the inhibitory phosphorylations by cyclin D/CDK4,6 (11). p19ARF (mouse; p14ARF, human), partly encoded by exons 2 and 3 of the p16Ink4a gene in an *a*lternative *r*eading *f*rame, regulates the stability of p53 by binding to mdm2 (12–15). mdm2 and p53 participate in an autoregulatory feedback loop in which p53 induces mdm2 expression (16). mdm2, in turn, mediates p53 degradation and prevents p53 association with the basal transcriptional machinery (17–19). The binding of mdm2 to p53 is affected by phosphorylation of p53 at the N terminus, which compromises mdm2 binding (20, 21). More recently, mdm2 was shown to be regulated by interaction with p19ARF, which binds to and alters the subcellular localization of mdm2 so that it has reduced access to p53 (13, 14, 22, 23).

The available data predict that disruption of the *Ink4a/ARF* locus alone should disable the two tumor suppressors most commonly mutated in human cancers. Indeed, mice lacking functional p16Ink4a and/or p19ARF develop normally but are highly susceptible to tumor formation (11, 24). However, the frequency of mutations at the *Ink4a* and/or *ARF* locus is not as high as the frequency of p53 mutations in human cancers, suggesting that loss of p53 confers a greater advantage during tumor progression.

The data discussed above raise the possibility that p19ARF functions in some, but not all, of the p53-responsive pathways such that loss of p19ARF may not be functionally equivalent to loss of p53. It has been suggested that oncogene-mediated activation of p53 requires p19ARF, and occurs in the absence of DNA damage (25, 26). By contrast, damage induced by ionizing radiation (IR) involves p53 activation through N-terminal phosphorylations and other modifications, but is reported to be independent of p19ARF (20, 24, 27). However, E1A-expressing $p19ARF^{-/-}$ mouse embryo fibroblasts (MEFs) were nearly as radioresistant after exposure to IR as E1A-expressing $p53^{-/-}$ MEFs (25), and p19ARF deficiency prevents induction of premature senescence in $ATM^{-/-}$ MEFs (28). One interpretation of the data is that loss of p53 or p19ARF reduces the E1A-induced radiosensitivity because of the requirement for these proteins in arrest responses triggered by E1A. Alternatively, the data raise the possibility that the observed radioresistance and rescue of premature senescence is due to the requirement for p53, and possibly p19ARF, in the arrest induced by DNA damage. To distinguish between these possibilities, we assessed the role of p19ARF in the p53-mediated arrest responses induced by IR as well as other stresses that do not induce DNA damage.

Materials and Methods

Cell Culture. Pools of wild-type, $p53^{+/-}$, $p19ARF^{-/-}$, $p53^{-/-}$, and $p16^{-/-}p19ARF^{-/-}$ double-knockout MEFs were maintained in DMEM (Cellgro, Waukesha, WI) containing 10% dialyzed fetal bovine serum, kanamycin sulfate (GIBCO), L-glutamine (GIBCO), penicillin/streptomycin (GIBCO), and nonessential amino acids (GIBCO). $p19ARF^{-/-}$ MEFs (passage 6) were generously provided by Charles Sherr and Martine Roussel; $p16^{-/-}p19ARF^{-/-}$ double-knockout MEFs (passage 3) were kindly provided by Manuel Serrano. Cells were maintained at 37°C in an atmosphere containing 7% CO_2.

Cell Cycle Analysis. Cell cultures were split 1:3 or 1:4 into media containing 0.05 μg/ml nocodazole, 5 nM actinomycin D, or 100 μM *N*-phosphonacetyl-L-aspartate (PALA), or were exposed to γ radiation by using a Gammabeam 150-C irradiator with a ^{60}Co source at a dose rate of 2 Gy/min. At the indicated time points, the fibroblasts were pulse labeled with 10 μM BrdUrd for 1 h before fixation in 70% ethanol. Analysis of BrdUrd-labeled cells was performed as described previously (3). For DNA content analysis, cells were stained with propidium iodide only. The samples were analyzed on a Becton Dickinson FACScan and

Abbreviations: IR, ionizing radiation; MEFs, mouse embryo fibroblasts; PALA, *N*-phosphonacetyl-L-aspartate.

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quantitated by gating events on dot plots by using CELLQUEST software. All experiments were repeated a minimum of three times, 10,000 events were analyzed per sample, and data from representative experiments are shown.

Western Blot Analysis. Treated cells were lysed in RIPA buffer (150 mM NaCl/1% Nonidet P-40/0.5% sodium deoxycholate/0.1% SDS/50 mM Tris·HCl, pH 8.0) supplemented with fresh 1 mM PMSF, 1 mM sodium vanadate, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 1 mg/ml pepstatin, 1 mg/ml 1,10-phenanthroline·HCl, and 160 μg/ml benzamidine·HCl. Lysates were sonicated and protein concentrations were quantitated with a Bio-Rad protein assay (modified Lowry assay). Fifty or 100 μg of protein was resolved by electrophoresis on a 10% (p21 and p53) or 12% (p19ARF) polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher and Schuell). p53 was detected by using a mixture of monoclonal antibodies 421 at 1:50 (Ab-1, Oncogene Science) and 240 at 1:100 (Ab-3, Oncogene Science). p21 was detected by using the polyclonal antibody C-19 (Santa Cruz Biotechnology) diluted 1:100; p19ARF was detected by using a polyclonal antibody (200-106; Novus Biologicals, Littleton, CO) diluted 1:200; and protein loading was measured with an anti-actin polyclonal antibody (A2066, Sigma) diluted 1:50. Membranes were incubated with secondary antibodies conjugated to horseradish peroxidase, and proteins were detected by using ECL chemiluminescence reagents according to the manufacturer's protocol (New England Nuclear). Bands were quantitated by computer scan densitometry (NIH IMAGE), experiments were repeated at least three times, and representative blots are shown. In all cases, actin was used as an internal control to enable quantification of relative band intensities.

Results

p19ARF$^{-/-}$ MEFs Become Arrested After Extended Exposure to PALA. p19ARF has been proposed to mediate p53 activation in response to oncogene overexpression in a manner that is independent of DNA damage (25). Depletion of ribonucleotide pools in cells also activates p53 without producing DNA damage in human and mouse fibroblasts (3, 29). Therefore, we determined whether p19ARF is involved in the activation of p53 after exposure to the UMP synthesis inhibitor PALA (30). As previous reports showed no significant differences in the results obtained after ribonucleotide depletion of asynchronous or G_0/G_1 synchronized MEFs, the PALA studies reported here were performed on exponentially growing populations of MEFs (31).

Fig. 1 shows that PALA induces a cell cycle arrest in MEFs regardless of their p19ARF status. Approximately 20–25% of untreated wild-type, p53$^{+/-}$, p53$^{-/-}$, or p19ARF$^{-/-}$ MEFs were in S phase (BrdUrd positive) in exponentially growing populations (Fig. 1 *A–D*). The fraction of BrdUrd-positive p53$^{+/-}$, p53$^{-/-}$, and p19ARF$^{-/-}$ MEFs increased after 24 h in PALA, presumably because of depletion of ribonucleotide and deoxyribonucleotide pools, which slows the progression of replication forks (32, 33). After 48 h of PALA treatment, the fraction of S-phase cells decreased in wild-type, p53$^{+/-}$, and p19ARF$^{-/-}$ populations (Fig. 1 *A, B,* and *D*), whereas the percentage of BrdUrd-positive p53$^{-/-}$ MEFs remained elevated, as observed previously (Fig. 1*C*) (31). By 72 h, significantly fewer wild-type and p19ARF$^{-/-}$ MEFs were in S phase compared with untreated populations or PALA-treated p53$^{-/-}$ MEFs. Although the percentage of p53$^{-/-}$ S-phase cells decreased between 48 and 72 h of PALA treatment, the fraction of cycling cells was as high as in the untreated population, and significantly higher than PALA-treated wild-type, p53$^{+/-}$, or p19ARF$^{-/-}$ populations. There appears to be a slight increase in the population of cells in S phase that are BrdUrd negative (below the diagonal line) in wild-type, p53$^{+/-}$, and p19ARF$^{-/-}$ MEFs; however, this population accounts for only 5% of the total population. As reported

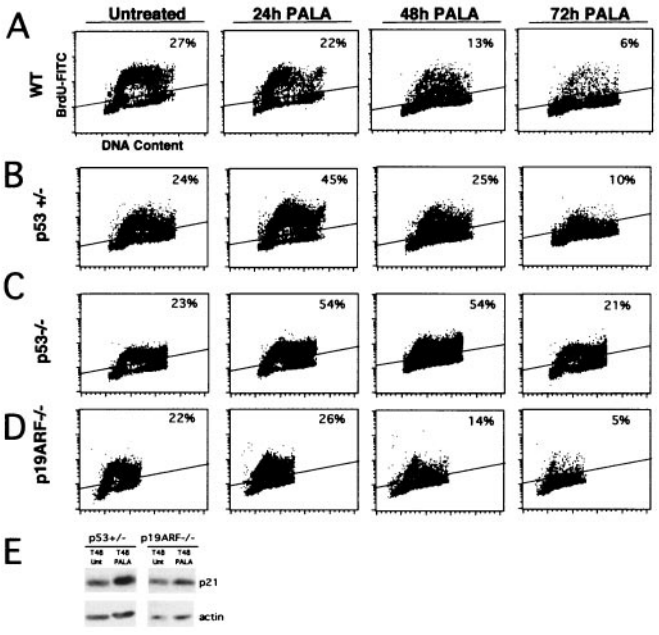

Fig. 1. p19ARF-null MEFs become arrested during ribonucleotide depletion. Asynchronous populations of wild-type (WT), p53$^{+/-}$, p53$^{-/-}$, and p19ARF$^{-/-}$ MEFs were untreated or treated with 100 μM PALA for 24, 48, or 72 h. Cells were pulse labeled with BrdUrd and analyzed by flow cytometry. Cells that incorporated BrdUrd are shown above the diagonal line. The percentages of BrdUrd-positive cells are shown in the upper right corner of each plot. Representative dot plots for untreated or PALA-treated MEFs are shown in *A* (WT), *B* (p53$^{+/-}$), *C* (p53$^{-/-}$), and *D* (p19ARF$^{-/-}$). (*E*) Western blot analysis of p21 and actin after 48-h PALA treatment of p53$^{+/-}$ and p19ARF$^{-/-}$ MEFs. Relative band intensities were quantified in all Western analyses with actin as an internal control.

previously, a majority of the PALA-treated MEFs that were in S phase at the beginning of the experiment appear to accumulate in G_2/M, as this population shows a 10% increase relative to the G_2/M population in untreated cells (31). p16$^{-/-}$p19ARF$^{-/-}$ double-knockout MEFs also became arrested after 72 h of PALA treatment, suggesting that the loss of pRb regulation does not abrogate the arrest response (data not shown). These data clearly show that the p53-mediated arrest induced by PALA is intact in MEFs lacking p19ARF function.

The p53-dependent cell cycle arrest induced by PALA requires activation of p53 target genes such as p21 (3, 31). An analysis of the p21 protein levels in p19ARF$^{-/-}$ MEFs revealed that p21 was up-regulated 48 h after exposure to PALA compared with untreated cells (Fig. 1*E*). The fold induction of p21 (normalized with actin protein loading) is approximately 2-fold in p19ARF$^{-/-}$ and p53$^{+/-}$ MEFs after 48 h of PALA treatment. This finding is consistent with the previous observation that p19ARF function is not required for the arrest induced by ribonucleotide depletion. Induction of p21 was not observed in p53$^{-/-}$ MEFs treated with PALA for 48 h, which supports previous reports that p21 expression is activated by p53 in response to ribonucleotide depletion (data not shown) (3).

To assess whether ribonucleotide depletion was unique in eliciting a p53-dependent arrest that is independent of p19ARF, we determined whether the arrest induced by the intercalating agent actinomycin D required p19ARF function. Recent data indicate that low doses (20 nM) of actinomycin D did not induce DNA double-strand breaks but were sufficient to inhibit transcription and activate p53 in normal human fibroblasts, whereas doses of 200 nM induced DNA damage (34, 35). We found that p53$^{+/-}$ and p19ARF$^{-/-}$ MEFs become arrested after 24 h of

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Table 1. ARF dependence of p53-mediated stress responses

MEFs	% S γ/% S untreated				% BrdUrd-positive			Polyploidy
	5 Gy	6 Gy	7 Gy	20 Gy	Untreated	T72 PALA	T24 Act-D	T48 Noc
$p53^{+/-}$	39.6 ± 0.5	36.9 ± 1.8	34.4 ± 5.2	25.9 ± 4.5	22.8 ± 2.4	12.0 ± 3.1	5.1 ± 0.1	6.5 ± 3.4
$p53^{-/-}$	101.0 ± 1.4	100.8 ± 3.2	105.2 ± 8.3	83.5 ± 0.7	24.4 ± 3.8	27.7 ± 3.3	20.8 ± 5.4	60.1 ± 2.2
$p19ARF^{-/-}$	75.0 ± 9.9	85.0 ± 2.8	81.5 ± 0.7	64.5 ± 0.7	19.7 ± 1.2	6.9 ± 2.6	4.6 ± 0.5	26.1 ± 4.9

Asynchronous populations of $p53^{+/-}$, $p53^{-/-}$, and $p19ARF^{-/-}$ MEFs were irradiated with 5, 6, 7, or 20 Gy, pulse-labeled with BrdUrd, and fixed 24 h later. The values represent the percentage of BrdUrd-positive cells in γ-irradiated cultures (% S γ) relative to untreated. Cells were also untreated or treated with 100 μM PALA for 72 h or 5 nM actinomycin D for 24 h, and the percentage of BrdUrd-positive cells is shown. The polyploid fraction was assessed by treating cells with 0.05 μg/ml nocodazole for 48 h and quantitating the percentage of cells with $> 4N$ DNA content. The values represent the mean of at least two independent experiments and standard deviations are shown.

exposure to 5 nM actinomycin D, whereas most of the $p53^{-/-}$ MEFs continue cycling (Table 1). Moreover, 5 nM actinomycin D treatment induced a reversible arrest in wild-type and $p19ARF^{-/-}$ MEFs; that is, once the drug was removed, the cells resumed cycling as efficiently as untreated populations (data not shown). This result provides further evidence that the arrest induced by 5 nM actinomycin D is unlikely to result from residual DNA damage. Therefore, two treatments that reduce RNA synthesis in the absence of detectable DNA damage activate a cell cycle arrest requiring p53, but not p19ARF.

$p19ARF^{-/-}$ MEFs Show an Increase in Polyploidy After Nocodazole Treatment. We and others showed previously that the microtubule-depolymerizing agents nocodazole and Colcemid provoke an arrest in a $4N$ G_1-like state in cells with an intact p53–pRb pathway (5, 36–39). By contrast, mutants with defects in this pathway undergo rereplication during continuous exposure to these drugs. As with PALA, the arrest induced by these agents is largely reversible and is not associated with detectable DNA damage (39). It was shown previously that $p16^{-/-}$ MEFs also undergo rereplication after exposure to such drugs (39). However, these MEFs have nonfunctional p16Ink4a and p19ARF because of targeted disruption of exons shared by the two proteins (11). The dual inactivation of the proteins in this mutant raised the important question of whether this damage-independent stress required p19ARF to induce a cell cycle arrest, or whether p16Ink4a deficiency alone allowed rereplication to occur.

We investigated this possibility by determining whether nocodazole induced rereplication in MEFs solely lacking p19ARF. Asynchronous populations of $p53^{+/-}$, $p53^{-/-}$, $p19ARF^{-/-}$, and $p16^{-/-}p19ARF^{-/-}$ MEFs were treated with 0.05 μg/ml nocodazole for 48 h, after which polyploidy was measured by DNA content analysis (see *Materials and Methods*). After 48 h in nocodazole, 8.7% of $p53^{+/-}$ MEFs were polyploid compared with 60.3% in $p53^{-/-}$ MEFs, 18.3% in $p19ARF^{-/-}$ MEFs, and 37.9% in $p16^{-/-}p19ARF^{-/-}$ double-knockout MEFs (Fig. 2 and Table 1). In three independent experiments, the percentage of polyploid cells in nocodazole-treated $p19ARF^{-/-}$ populations consistently doubled compared with the untreated control (Fig. 2*C* and Table 1). Furthermore, the percentage of cells with greater than $4N$ DNA content was consistently higher in the $p16^{-/-}p19ARF^{-/-}$ double-knockout MEFs (Fig. 2*C*), suggesting that the loss of both tumor suppressors is additive in this response. While it remains to be determined whether the loss of p16Ink4a alone induces polyploidy after microtubule disruption, we predict this is likely, as pRb deficiency produces substantial polyploidy (≈50%) under the same conditions (36).

The polyploid population was significantly higher in $p53^{-/-}$ MEFs compared with either $p19ARF^{-/-}$ or $p16^{-/-}p19ARF^{-/-}$ MEFs, indicating that the loss of p19ARF is not equivalent to loss of p53 in the nocodazole-induced arrest response. Similarly, a reduced level of polyploidy was observed in $p21^{-/-}$ MEFs compared with MEFs lacking p53 (39). This observation may be due to a p53-dependent activation of additional pathways that may compensate for the loss of p16, p19ARF, or p21, such that pRb is eventually restored to the antiproliferative form to promote cell cycle arrest.

$p19ARF^{-/-}$ MEFs Exhibit a Defective DNA-Damage Response. The p53-dependent cell cycle arrest induced by ionizing radiation requires prolonged induction of p21 (7, 40). Previous reports indicated that p53 and p21 induction occurs in $p19ARF^{-/-}$

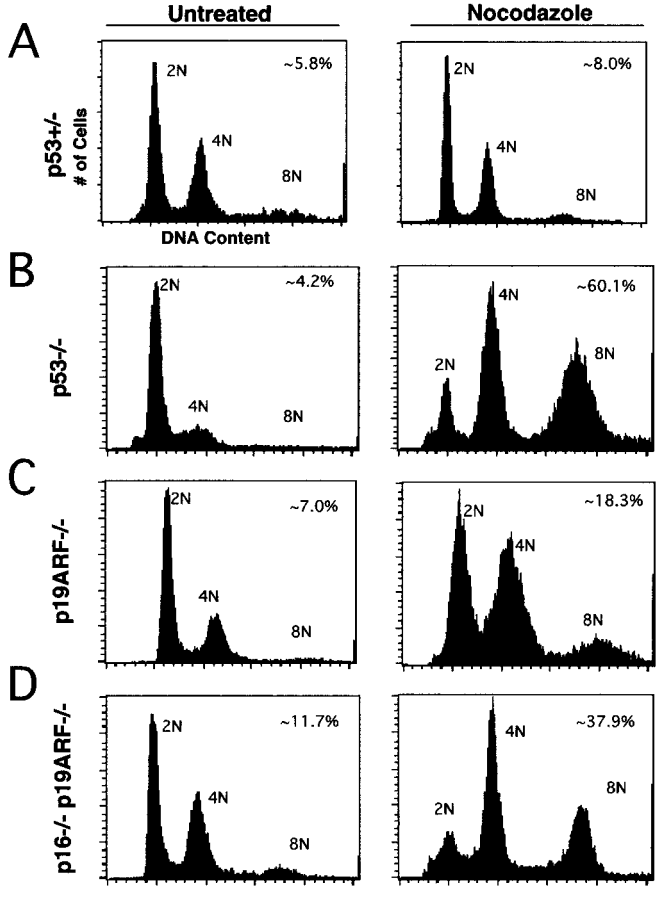

Fig. 2. Increased polyploidy in p19ARF-null fibroblasts after nocodazole treatment. Asynchronous cultures of $p53^{+/-}$, $p53^{-/-}$, $p19ARF^{-/-}$, and $p16^{-/-}p19ARF^{-/-}$ MEFs were treated with or without 0.05 μg/ml nocodazole for 48 h, fixed, and stained with propidium iodide for FACS analysis. Values in the upper right corner of each plot represent the percentage of cells with $>4N$ DNA content. Histogram plots of untreated or nocodazole-treated MEFs are shown in *A* ($p53^{+/-}$), *B* ($p53^{-/-}$), *C* ($p19ARF^{-/-}$), and *D* ($p16^{-/-}p19ARF^{-/-}$).

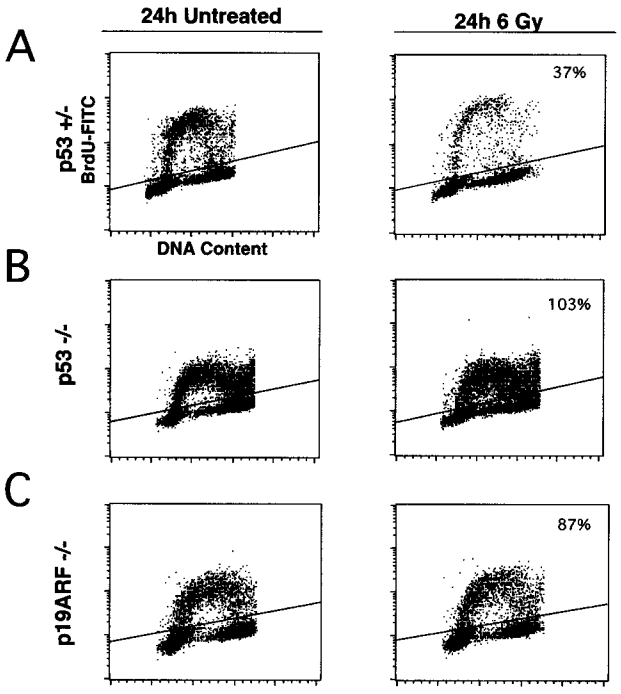

Fig. 3. Defective DNA-damage response in p19ARF-null MEFs. Asynchronous cultures of $p53^{+/-}$, $p53^{-/-}$, and $p19ARF^{-/-}$ MEFs were exposed to 6 Gy of γ-radiation. After 24 h, cells were pulse labeled with BrdUrd and analyzed by FACS. Cells above the diagonal line are BrdUrd positive. The values shown in the upper right represent the percentage of irradiated cells in S phase relative to untreated (% S irradiated/% S untreated). Representative plots are shown for $p53^{+/-}$ (*A*), $p53^{-/-}$ (*B*), and $p19ARF^{-/-}$ (*C*).

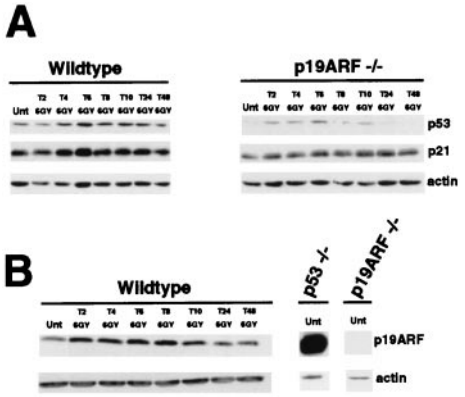

Fig. 4. Involvement of p19ARF in the sustained induction of p21 after IR. Asynchronous populations of wild-type (WT) and $p19ARF^{-/-}$ MEFs were treated with 6 Gy of γ-radiation and harvested after 2, 4, 6, 8, 10, 24, or 48 h. For p53 and p21 analysis, 50 μg of protein was resolved on an SDS/10% polyacrylamide gel. For analysis of p19ARF, 100 μg of protein was resolved on a 12% polyacrylamide gel. (*A*) p53, p21, and actin protein levels in WT and $p19ARF^{-/-}$ MEFs 2 h (T2), 4 h (T4), 6 h (T6), 8 h (T8), 10 h (T10), 24 h (T24), and 48 h (T48) after IR compared with untreated (Unt). (*B*) p19ARF and actin protein levels in WT MEFs at the indicated times after IR compared with untreated (Unt). $p53^{-/-}$ and $p19ARF^{-/-}$ MEFs are also shown as positive and negative controls for p19ARF protein detection.

MEFs 4 h after IR, but protein analyses at later time points were not carried out and the stringency of the cell cycle arrest was not shown (24). The increased viability of E1A-expressing $p19ARF^{-/-}$ MEFs after IR invites the speculation that these cells are compromised in their ability to undergo cell cycle arrest or apoptosis in response to IR (25). Hence, we assessed whether the $p19ARF^{-/-}$ MEFs exhibit an altered cell cycle response after IR.

The cell cycle responses of asynchronous $p53^{+/-}$, $p53^{-/-}$, and $p19ARF^{-/-}$ MEFs are shown in Fig. 3 and Table 1. In the experiment shown, the percentage of BrdUrd-positive cells in irradiated populations relative to untreated cells was approximately 37% for $p53^{+/-}$ MEFs, 103% in $p53^{-/-}$ MEFs, and 87% in $p19ARF^{-/-}$ MEFs 24 h after exposure to 6 Gy of γ-radiation (Fig. 3 *A, B,* and *C,* respectively). This observation suggests that loss of p19ARF results in a defective DNA-damage arrest pathway even though these cells express wild-type p53 and p21. The experiments were repeated a minimum of five times at passages 8–10 with various radiation dosages (Table 1). Similar results were observed in irradiated populations of $p19ARF^{-/-}$ MEFs that were synchronized in G_0/G_1 by serum deprivation (data not shown). We also found that the percentage of irradiated cells in S phase relative to untreated was $\approx$70% in $p16^{-/-}p19ARF^{-/-}$ MEFs after 24 h (data not shown), suggesting that the loss of pRb regulation does not augment the defective DNA-damage arrest created by loss of p19ARF alone.

The data show that $p19ARF^{-/-}$ MEFs continue cycling after DNA damage, although not to the same extent as $p53^{-/-}$ MEFs. Interestingly, the lack of cell cycle arrest in $p19ARF^{-/-}$ MEFs is similar to that reported for $p21^{-/-}$ MEFs, in which $\approx$80% of the population continued to cycle 24 h after DNA damage (31). This observation implies that factors in addition to p19ARF and p21 contribute to the p53-dependent DNA-damage-induced arrest in wild-type cells.

As the response to ionizing radiation involves rapid p53-dependent induction of p21, it is conceivable that p53 activation and p21 induction could be compromised in p19ARF-null cells. We investigated this possibility by measuring p53 and p21 protein levels as a function of time after IR. As reported previously, p53 induction is biphasic in wild-type cells, consisting of a rapid increase, followed by a decrease, and then a second increase to a level that remained above the uninduced control up to 96 h after IR (7, 8). The p53 levels in $p19ARF^{-/-}$ MEFs showed a similar initial increase in abundance 2–10 h after irradiation, but then diminished to undetectable levels by 24 h and remained undetectable 48 h after IR (Fig. 4*A*). In contrast, the p53 levels were elevated between 1.9- and 3.5-fold at each of the time points analyzed after IR in wild-type MEFs (Fig. 4*A*). A possible explanation for the apparent p53 instability is that the absence of p19ARF allows a higher proportion of p53 to be bound by mdm2, which increases p53 turnover.

Consistent with the p53 expression levels, p21 protein levels started to increase 4 h after irradiation in wild-type MEFs and remained elevated up to 48 h (Fig. 4*A*). While the p21 levels are detectable in $p19ARF^{-/-}$ MEFs at all times tested, the induced levels are lower than observed in wild-type MEFs. Wild-type MEFs show a 1.9- to 5.0-fold increase in p21 between 2 and 48 h after exposure to IR, whereas the maximum induction of p21 in $p19ARF^{-/-}$ MEFs during the same time course is 2-fold. Furthermore, 24–48 h after IR the levels of p21 are not increased relative to the untreated control in $p19ARF^{-/-}$ MEFs (Fig. 4*A*). $p53^{-/-}$ MEFs showed no detectable levels of p21 at several time points after exposure to IR; hence the induction of p21 in this stress response is largely, if not entirely, dependent on transactivation by p53 (data not shown) (40).

We next determined whether p19ARF is induced by DNA damage by measuring the p19ARF protein levels at several intervals after IR. As expected, p19ARF was not present in the p19ARF-null MEFs, and it is present at high levels in $p53^{-/-}$ MEFs (Fig. 4*B*) (25, 26). p19ARF levels increased significantly (2- to 8-fold induction) 2–10 h after γ-irradiation in wild-type MEFs (Fig. 4*B*). Although the level of p19ARF appears to

decrease 24–48 h after IR, the protein levels were still 1.5- to 1.7-fold elevated relative to untreated controls at the latest time points measured (Fig. 4*B*). These results show that p19ARF protein levels increase after exposure to γ-radiation.

Discussion

The studies reported here reveal that p19ARF contributes to the ability of MEFs to undergo p53-dependent cell cycle arrest induced by IR and by microtubule disruption. The lack of a damage-induced arrest in p19ARF$^{-/-}$ MEFs correlates with decreased basal p53 levels and an inability to sustain the induction of p53. This, in turn, appears to affect the induction of the CDK inhibitor p21, a key downstream target gene in the damage response. By contrast, the arrest induced by ribonucleotide depletion and actinomycin D treatment is intact in p19ARF-deficient fibroblasts. The data are consistent with the model that mdm2 is a key negative regulator of p53 function and that p19ARF contributes to a subset of p53-dependent arrest pathways to counteract the inhibitory effects of mdm2.

The requirement for p19ARF in the p53-dependent arrest induced by DNA damage is consistent with current models that p19ARF is a regulator of mdm2–p53 interactions (12–15, 22). p19ARF appears to reduce the ability of mdm2 to interact with p53 by changing mdm2 subcellular localization (22, 23). Our observation that p53 levels are reduced in p19ARF-null MEFs is consistent with the increased p53–mdm2 associations that should occur in cells lacking p19ARF. It is conceivable that the reduced p53 levels may contribute to the defective arrest induced by IR in the p19ARF-null MEFs. However, the arrest induced by IR appears to be normal in cell lines expressing low amounts of a transfected wild-type p53 gene and high quantities of dominant-negative p53 encoded by the mutated endogenous alleles (e.g., see ref. 41). This observation leads us to favor the interpretation that it is the altered p53 and p21 induction kinetics in the p19ARF-null MEFs that prevents them from mounting a durable arrest response. This view is compatible with the observation of long-term induction of p53 and p21 in irradiated fibroblasts that undergo a senescent-like arrest (7, 42). The difference in the response kinetics at later times suggests that p19ARF and/or its induction by DNA damage may contribute to the sustained activation of p53 and its target genes. While the mechanism for p19ARF induction remains to be elucidated, it is tempting to speculate that the transcriptional regulator E2F-1 may be involved, as p19ARF expression can be transactivated by E2F-1 (43), and recent studies show that IR increases E2F-1 abundance by a posttranslational mechanism (44). We also note that the rapid induction of p53 and p21 in p19ARF-null MEFs appears to be equivalent to that of wild-type MEFs. We suggest that kinases such as ATM, ATR, and others that modify the N terminus of p53 to limit interactions with mdm2 may be involved in establishing the early stages of the damage response (20, 21, 45–47).

The involvement of p19ARF in the DNA-damage-inducible arrest is compatible with studies analyzing the cell cycle responses and radiosensitivity of MEFs with various genetic deficiencies in the p53 pathway. p21-null MEFs continue to cycle to approximately the same extent as p19ARF-null MEFs after IR, and both are more radioresistant than are wild-type MEFs (31, 48). By contrast, ATM-deficient MEFs are more radiosensitive than wild-type MEFs, and they senesce prematurely (49, 50). These phenotypes are consistent with the generation and accumulation of DNA damage when cells cycle in the absence of the ATM kinase (51). Importantly, premature senescence is rescued in MEFs doubly deficient in ATM and p19ARF, p53, or p21 (28, 52, 53). Thus, p19ARF deficiency results in defects similar to those observed in cells lacking two key members of the damage-response pathway, p53 and p21. However, it is important to note that neither p19ARF, p21, nor p53 deficiency rescues the radiosensitivity of ATM-deficient mice (28, 53, 54). This observation is most likely explained by the fact that intestinal epithelia and other cell types in ATM-deficient mice appear to be exquisitely sensitive to damage-induced apoptosis that occurs independent of p53 function (54). Hence, the survival of ATM-deficient mice after radiation is influenced by factors outside of the p53 signaling pathway, whereas the isolated cells of specific tissues appear to be profoundly dependent on p53, p21, and p19ARF.

Previous studies showed that p19ARF cooperates with p53 to mediate cell cycle arrest or programmed cell death in response to activated oncogenes such as *E1A, Myc, Ras,* and *E2F-1* (25, 26, 43, 55). E1A overexpression did not lead to phosphorylation of p53 at serine-15, which is one residue that is modified after DNA damage (20, 25, 27). This finding led to the proposal that overexpression of E1A activates p53 independent of DNA damage. However, phosphorylation at serine-15 is just one of many p53 modifications that occur in response to DNA damage; recent data indicate the importance of serine-20 phosphorylation in the DNA-damage response (21, 47). Our data linking p19ARF to the damage-induced arrest raise the possibility that activated oncogenes may induce DNA damage, which could then lead to senescence in fibroblasts with an intact p53 pathway. Indeed, this model would be compatible with numerous reports showing that activated c-myc, ras, or mos can induce chromosome breakage in cycling cells (refs. 56–58; O. Vafa and G.M.W., unpublished results).

Our data demonstrate that stresses such as ribonucleotide depletion or RNA synthesis inhibition clearly function independently of p19ARF. PALA treatment is associated with increased p53 abundance and activation of p21 transcription, but it remains to be determined whether induction of mdm2 is involved (3). It is possible that p19ARF is not required for the arrest induced by stresses that reduce transcription because such conditions may preferentially reduce the levels of the unstable mdm2 transcript. Consistent with this proposal, previous studies showed that low doses of actinomycin D and UV doses that reduce transcription also reduce the abundance of mdm2 mRNA (44, 59). Similarly, we found reduced levels of mdm2 protein in PALA-treated cells, but not in those treated with nocodazole (S.H.K. and G.M.W., unpublished data). Hence, inhibition of mdm2 expression by agents such as actinomycin D, and possibly PALA, serves as a p19ARF-independent mechanism to promote the stability of p53.

These and other data also emphasize the complexity of the biochemical and cell cycle responses to diverse stresses that activate the p53 pathway. The different responses of cells with defects in this pathway indicate that additional genes remain to be elucidated. Thus, as p21$^{-/-}$ and p53$^{-/-}$ MEFs exhibit comparable defects in PALA-induced arrest, we surmise that p21 is a key downstream modulator of this response. By contrast, since p21$^{-/-}$ and p19ARF$^{-/-}$ MEFs retain a partial ability to undergo arrest after IR and show less rereplication after nocodazole treatment than do p53$^{-/-}$ MEFs (Figs. 1, 2, and 3; refs. 31 and 39), we infer that additional upstream effectors and downstream targets remain to be defined. Additional studies to elucidate the mechanisms by which p19ARF is activated and the kinetics of p53, mdm2, and p19ARF associations will enhance our understanding of the cellular response to DNA damage and damage-independent stresses.

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1. Huang, L. C., Clarkin, K. C. & Wahl, G. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4827–4832.
2. Hermeking, H. & Eick, D. (1994) *Science* **265,** 2091–2093.
3. Linke, S. P., Clarkin, K. C., Di Leonardo, A., Tsou, A. & Wahl, G. M. (1996) *Genes Dev.* **10,** 934–947.
4. Maltzman, W. & Czyzyk, L. (1984) *Mol. Cell. Biol.* **4,** 1689–1694.
5. Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., Raskind, W. H. & Reid, B. J. (1995) *Science* **267,** 1353–1356.
6. Graeber, T. G., Peterson, J. F., Tsai, M., Monica, K., Fornace, A. J., Jr., & Giaccia, A. J. (1994) *Mol. Cell. Biol.* **14,** 6264–6277.
7. Di Leonardo, A., Linke, S. P., Clarkin, K. & Wahl, G. M. (1994) *Genes Dev.* **8,** 2540–2551.
8. Kastan, M. B., Onyekwere, O., Sidransky, D., Vogelstein, B. & Craig, R. W. (1991) *Cancer Res.* **51,** 6304–6311.
9. Paulson, T. G., Almasan, A., Brody, L. L. & Wahl, G. M. (1998) *Mol. Cell. Biol.* **18,** 3089–3100.
10. Chernova, O. B., Chernov, M. V., Agarwal, M. L., Taylor, W. R. & Stark, G. R. (1995) *Trends Biochem. Sci.* **20,** 431–434.
11. Serrano, M., Lee, H., Chin, L., Cordon-Cardo, C., Beach, D. & DePinho, R. A. (1996) *Cell* **85,** 27–37.
12. Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H. & Peters, G. (1998) *EMBO J.* **17,** 5001–5014.
13. Zhang, Y., Xiong, Y. & Yarbrough, W. G. (1998) *Cell* **92,** 725–734.
14. Pomerantz, J., Schreiber-Agus, N., Liegeois, N. J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H. W., *et al*. (1998) *Cell* **92,** 713–723.
15. Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F. & Sherr, C. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 8292–8297.
16. Barak, Y., Juven, T., Haffner, R. & Oren, M. (1993) *EMBO J.* **12,** 461–468.
17. Kubbutat, M. H., Jones, S. N. & Vousden, K. H. (1997) *Nature (London)* **387,** 299–303.
18. Haupt, Y., Maya, R., Kazaz, A. & Oren, M. (1997) *Nature (London)* **387,** 296–299.
19. Thut, C. J., Goodrich, J. A. & Tjian, R. (1997) *Genes Dev.* **11,** 1974–1986.
20. Shieh, S. Y., Ikeda, M., Taya, Y. & Prives, C. (1997) *Cell* **91,** 325–334.
21. Unger, T., Juven-Gershon, T., Moallem, E., Berger, M., Vogt Sionov, R., Lozano, G., Oren, M. & Haupt, Y. (1999) *EMBO J.* **18,** 1805–1814.
22. Tao, W. & Levine, A. J. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 6937–6941.
23. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J. & Bar-Sagi, D. (1999) *Nat. Cell Biol.* **1,** 20–26.
24. Kamijo, T., Zindy, F., Roussel, M. F., Quelle, D. E., Downing, J. R., Ashmun, R. A., Grosveld, G. & Sherr, C. J. (1997) *Cell* **91,** 649–659.
25. de Stanchina, E., McCurrach, M. E., Zindy, F., Shieh, S. Y., Ferbeyre, G., Samuelson, A. V., Prives, C., Roussel, M. F., Sherr, C. J. & Lowe, S. W. (1998) *Genes Dev.* **12,** 2434–2442.
26. Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J. & Roussel, M. F. (1998) *Genes Dev.* **12,** 2424–2433.
27. Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E. & Kastan, M. B. (1997) *Genes Dev.* **11,** 3471–3481.
28. Kamijo, T., van de Kamp, E., Chong, M. J., Zindy, F., Diehl, J. A., Sherr, C. J. & McKinnon, P. J. (1999) *Cancer Res.* **59,** 2464–2469.
29. Agarwal, M. L., Agarwal, A., Taylor, W. R., Chernova, O., Sharma, Y. & Stark, G. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 14775–14780.
30. Collins, K. D. & Stark, G. R. (1971) *J. Biol. Chem.* **246,** 6599–6605.
31. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J. & Leder, P. (1995) *Cell* **82,** 675–684.
32. Chernova, O. B., Chernov, M. V., Ishizaka, Y., Agarwal, M. L. & Stark, G. R. (1998) *Mol. Cell. Biol.* **18,** 536–545.
33. Moyer, J. D., Smith, P. A., Levy, E. J. & Handschumacher, R. E. (1982) *Cancer Res.* **42,** 4525–4531.
34. Chang, D., Chen, F., Zhang, F., McKay, B. C. & Ljungman, M. (1999) *Cell Growth Differ.* **10,** 155–162.
35. Ljungman, M., Zhang, F., Chen, F., Rainbow, A. J. & McKay, B. C. (1999) *Oncogene* **18,** 583–592.
36. Di Leonardo, A., Khan, S. H., Linke, S. P., Greco, V., Seidita, G. & Wahl, G. M. (1997) *Cancer Res.* **57,** 1013–1019.
37. Minn, A. J., Boise, L. H. & Thompson, C. B. (1996) *Genes Dev.* **10,** 2621–2631.
38. Lanni, J. S. & Jacks, T. (1998) *Mol. Cell Biol.* **18,** 1055–1064.
39. Khan, S. H. & Wahl, G. M. (1998) *Cancer Res.* **58,** 396–401.
40. el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., *et al*. (1994) *Cancer Res.* **54,** 1169–1174.
41. Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C. & Wahl, G. M. (1992) *Cell* **70,** 937–948.
42. Robles, S. J. & Adami, G. R. (1998) *Oncogene* **16,** 1113–1123.
43. Bates, S., Phillips, A. C., Clark, P. A., Stott, F., Peters, G., Ludwig, R. L. & Vousden, K. H. (1998) *Nature (London)* **395,** 124–125.
44. Blattner, C., Sparks, A. & Lane, D. (1999) *Mol. Cell. Biol.* **19,** 3704–3713.
45. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B. & Siliciano, J. D. (1998) *Science* **281,** 1677–1679.
46. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y. & Ziv, Y. (1998) *Science* **281,** 1674–1677.
47. Shieh, S. Y., Taya, Y. & Prives, C. (1999) *EMBO J.* **18,** 1815–1823.
48. Brugarolas, J., Chandrasekaran, C., Gordon, J. I., Beach, D., Jacks, T. & Hannon, G. J. (1995) *Nature (London)* **377,** 552–557.
49. Barlow, C., Hirotsune, S., Paylor, R., Liyanage, M., Eckhaus, M., Collins, F., Shiloh, Y., Crawley, J. N., Ried, T., Tagle, D. & Wynshaw-Boris, A. (1996) *Cell* **86,** 159–171.
50. Xu, Y., Ashley, T., Brainerd, E. E., Bronson, R. T., Meyn, M. S. & Baltimore, D. (1996) *Genes Dev.* **10,** 2411–2422.
51. Elson, A., Wang, Y., Daugherty, C. J., Morton, C. C., Zhou, F., Campos-Torres, J. & Leder, P. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 13084–13089.
52. Westphal, C. H., Schmaltz, C., Rowan, S., Elson, A., Fisher, D. E. & Leder, P. (1997) *Cancer Res.* **57,** 1664–1667.
53. Wang, Y. A., Elson, A. & Leder, P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 14590–14595.
54. Westphal, C. H., Hoyes, K. P., Canman, C. E., Huang, X., Kastan, M. B., Hendry, J. H. & Leder, P. (1998) *Cancer Res.* **58,** 5637–5639.
55. Palmero, I., Pantoja, C. & Serrano, M. (1998) *Nature (London)* **395,** 125–126.
56. Felsher, D. W. & Bishop, J. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 3940–3944.
57. Denko, N., Stringer, J., Wani, M. & Stambrook, P. (1995) *Somatic Cell Mol. Genet.* **21,** 241–253.
58. Fukasawa, K. & Vande Woude, G. F. (1997) *Mol. Cell. Biol.* **17,** 506–518.
59. Wu, L. & Levine, A. J. (1997) *Mol. Med.* **3,** 441–451.