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PML Is a Direct p53 Target that Modulates p53 Effector Functions

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Summary

The p53 tumor suppressor promotes cell cycle arrest or apoptosis in response to stress. Previous work suggests that the promyelocytic leukemia gene (*PML*) can act upstream of p53 to enhance transcription of p53 targets by recruiting p53 to nuclear bodies (NBs). We show that *PML* is itself a p53 target gene that also acts downstream of p53 to potentiate its antiproliferative effects. Hence, p53 is required for PML induction in response to oncogenes and DNA damaging chemotherapeutics. Furthermore, the *PML* gene contains p53 binding sites that confer p53 responsiveness to a heterologous reporter and can bind p53 in vitro and in vivo. Finally, cells lacking PML show a reduced propensity to undergo senescence or apoptosis in response to p53 activation, despite the induction of several p53 target genes. These results identify an additional element of PML regulation and establish PML as a mediator of p53 tumor suppressor functions.

Introduction

The p53 tumor suppressor is activated in response to many stimuli, including hyperproliferative signals, DNA damaging agents, and viral infection (Prives, 1998, and references therein). Once activated, p53 functions as a transcription factor to promote several antiproliferative responses, including cell cycle checkpoints, cellular senescence, and apoptosis (Oren, 2003). Tumor-derived *p53* mutations disable its transcription functions, thereby compromising the arrest or death programs that would otherwise limit the proliferation of damaged cells. Although the precise nature of these p53 transcriptional programs remains to be elucidated, p53 acts to coordinate each process through a combination of effectors (Fridman and Lowe, 2003; Vogelstein et al., 2000). Given the importance of the p53 network in human cancer, it seems likely that key p53 target genes will also be linked to cancer development.

The promyelocytic leukemia (*PML*) gene also plays an important role in human cancer, although its functions and regulation are less well understood than p53's. *PML* initially was identified in patients with acute promyelocytic leukemia (APL), where it is fused to the retinoic acid receptor α (*RAR*α) gene as a result of the reciprocal t(15;17) chromosomal translocation (Piazza et al., 2001, and references therein). The resulting two fusion proteins (PML-RARα and RARα-PML) are each expressed in leukemia cells from APL patients (Melnick and Licht, 1999), and expression of the PML-RARα fusion protein in transgenic mice is sufficient to produce leukemia with features of APL (Piazza et al., 2001). PML loss also contributes to the pathogenesis of APL, since dominant-negative *RAR*α mutants fail to produce APL in vivo, whereas *PML* gene deletions enhance PML-RARα-induced leukemogenesis (Rego et al., 2001). The potential importance of PML loss to the development of non-hematologic malignancies is only now emerging, but PML-deficient mice are more susceptible to chemically induced skin carcinogenesis, and PML inactivation has been noted in cancer patients, where it correlates with poor prognosis (Gurrieri et al., 2003; Wang et al., 1998a).

Like p53, PML participates in several biological processes, including differentiation, senescence, and apoptosis (Salomoni and Pandolfi, 2002). PML is an essential component of highly dynamic nuclear structures known as PML oncogenic domains (PODs) or nuclear bodies (NBs). In addition to PML, NBs contain the autoimmune antigen Sp100 and a variable number of accessory proteins known to regulate processes, such as transcription or repair (Borden, 2002). Accordingly, studies suggest that NBs may act as transient storage sites for regulatory proteins and/or organizing centers where nuclear processes are regulated or executed (Borden, 2002; Zhong et al., 2000). The potential importance of NBs for PML function is underscored by the fact that the PML-RARα fusion protein disrupts NBs in a manner that is restored by the differentiating agent retinoic acid (Dyck et al., 1994) and that several viruses encode essential proteins that disrupt NB formation (Everett, 2001; Regad and Chelbi-Alix, 2001).

Little is known about how PML is regulated or how it might be activated to suppress tumor growth. PML expression and NB formation can increase in response to certain forms of stress, including heat shock, arsenic treatment, DNA damage, and aberrant oncogene expression (Pearson and Pelicci, 2001), although the mechanisms underlying this regulation remain unexplored. The best-characterized PML inducer is interferon (IFN), which induces *PML* mRNA expression through Jak/Stat signaling and through IFN-stimulated response elements (ISREs) and IFNγ-activated sites (GAS) in the PML promoter (Chelbi-Alix et al., 1995). PML-deficient cells are unable to induce NBs and do not respond appropriately to IFN, suggesting a role for PML in antiviral responses (Everett, 2001).

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Given that p53 and PML have overlapping biological activities, it is interesting that these proteins functionally and physically interact. For example, both *p53*$^{-/-}$ and *PML*$^{-/-}$ thymocytes are resistant to radiation-induced apoptosis (Lowe et al., 1993; Wang et al., 1998b). Similarly, *p53*$^{-/-}$- and *PML*$^{-/-}$-deficient mouse embryo fibroblasts (MEFs) show defects in a premature senescence program induced by oncogenic *ras* (Pearson et al., 2000; Serrano et al., 1997). Here, PML is required for the appropriate formation of certain p53 posttranscriptional modifications that potentiate its activity (Ferbeyre et al., 2000; Guo et al., 2000; Pearson et al., 2000). Moreover, overexpression of PML can recruit p53 to NBs, enhance the transcription of p53 target genes, and promote cell cycle arrest in a p53-dependent manner (Bischof et al., 2002; Ferbeyre et al., 2000; Fogal et al., 2000; Guo et al., 2000; Pearson et al., 2000). Finally, PML can bind and recruit the negative p53 regulator Mdm2 into NBs and protect p53 from Mdm2-mediated degradation (Kurki et al., 2003; Louria-Hayon et al., 2003; Wei et al., 2003). Collectively, these studies suggest that PML acts as an upstream regulator of p53.

The current study was initiated to understand the regulation of PML during cellular senescence, an apparently permanent form of cell cycle arrest that can act as a potent barrier to oncogenic transformation (Campisi, 2003). Although senescence was initially linked to the replicative exhaustion of human fibroblasts in culture, this program can also be induced acutely in response to DNA damage and hyperproliferative signals. For example, in MEFs, oncogenic *ras* can induce premature senescence in a manner that depends on the ARF-p53 tumor suppressor pathway, such that loss of either ARF or p53 prevents *ras*-induced senescence and allows oncogenic transformation (Lin et al., 1998; Palmero et al., 1998; Serrano et al., 1997). We have previously shown that PML and NBs accumulate in senescent cells (Ferbeyre et al., 2000). Surprisingly, we now see that ARF and p53 are required for PML upregulation in response to oncogenic *ras* and that *PML* is a direct p53 target gene. Moreover, we find that PML participates in additional p53-mediated programs, including reversible cell cycle arrest and apoptosis. These data imply that PML is not solely an upstream regulator of p53 but also acts as a general p53 effector.

Results

Oncogenic Ras Induces PML through the MAPK Cascade

Previous studies have shown that PML accumulates during cellular senescence following replicative exhaustion and in response to oncogenic *ras* (Ferbeyre et al., 2000; Pearson et al., 2000). In the case of Ras-induced arrest, activation of the MAPK cascade is both necessary and sufficient for the establishment of the senescence program (Lin et al., 1998; Zhu et al., 1998). To determine whether the MAPK cascade was also involved in Ras signaling to PML, we examined the impact of activating or inhibiting MAPK signaling on PML induction and NB formation in mouse embryo fibroblasts (MEFs). A control vector, oncogenic Ras, or an activated form of MEK (MEK^{Q56P}) were introduced into wild-type MEFs, and the resulting cell populations were examined for PML expression and NB formation using a newly generated monoclonal antibody that specifically recognizes several murine PML isoforms (Figure 1A, compare lanes 1–3 to lane 4). Of note, *MEK* acts downstream of *ras* to activate MAPK independently of other Ras effector functions and is sufficient to induce premature senescence (Lin et al., 1998).

Both oncogenic Ras and activated MEK induced PML expression as assessed by immunoblotting (Figure 1A, compare lane 1 to lanes 2 and 3). This induction was observed as early as 2 days postselection, a time when Ras- and MEK-expressing cell populations begin to senesce (data not shown). Conversely, a single treatment of the Ras-expressing MEFs with 10 μM U0126 (a highly specific MEK inhibitor) prevented PML upregulation and the establishment of senescence (Figure 1B; data not shown). Analysis of PML-containing NBs by indirect immunofluorescence produced a similar result: NBs increased dramatically in size and number in response to oncogenic Ras or activated MEK and were inhibited by treatment with U0126 (Figure 1C). Therefore, MAPK signaling is necessary and sufficient for PML induction in response to oncogenic Ras.

The ARF-p53 Pathway Is Required for PML Induction in Response to Oncogenic Ras

Like PML, the ARF and p53 tumor suppressors are also induced in response to MAPK signaling and contribute to the establishment of senescence (Lin et al., 1998; Palmero et al., 1998; Serrano et al., 1997). To determine whether ARF, p53, and PML influence each other's expression, we introduced oncogenic Ras or a control vector into early passage MEFs deficient for each gene (*p53*$^{-/-}$, *ARF*$^{-/-}$, and *PML*$^{-/-}$, respectively) and analyzed PML expression by immunoblotting (Figure 2A) and immunofluorescence (Figure 2B). Surprisingly, neither PML nor PML-containing NBs were induced in *ARF*$^{-/-}$ or *p53*$^{-/-}$ MEFs in response to Ras (Figure 2A, compare lane 2 with lanes 4 and 6; Figure 2B, "Ras" panels), implying that ARF and p53 are required for PML induction. Conversely, ARF, p53, and two well-characterized p53 targets (p21 and Mdm2) were induced in response to Ras in *PML*$^{-/-}$ cells (Figure 2A, compare lanes 2 and 8). These data indicate that PML is dispensable for the Ras-induced activation of the ARF-p53 pathway and identify PML as a potential p53 effector.

PML Is Induced by p53

To directly test whether *PML* is a p53-inducible gene, we introduced p53 into *p53*$^{-/-}$ MEFs expressing oncogenic Ras or the p53-deficient H1299 human lung carcinoma line using adenovirus-mediated gene transfer. Forty-eight hours later, PML expression was assessed by immunoblotting (Figures 3A and 3C) and immunofluorescence (Figures 3B and 3D) and compared to that of control cells infected with a GFP-expressing adenovirus. PML protein increased in response to p53 in both cell types, as did the number and size of PML-containing NBs. Interestingly, β interferon (IFN), the best-characterized PML regulator, also induced PML, implying that IFN signaling to PML is p53-independent (Figure 3A,

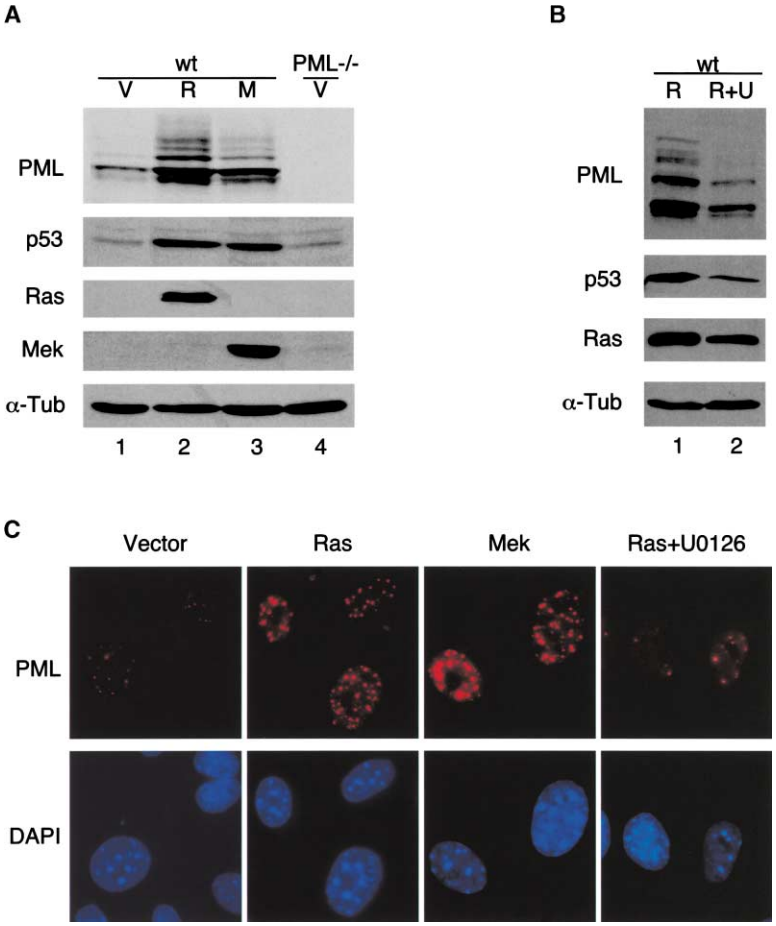

Figure 1. Ras Induces PML through the MAPK Pathway

(A) The expression of PML, p53, Ras, and Mek was measured by immunoblotting of lysates from wild-type (wt) or PML null (*PML*$^{-/-}$) MEFs containing a control vector (V), oncogenic Ras (R), or activated Mek1 (M). α-tubulin (α-Tub) was used as loading control.

(B) PML, p53, and Ras expression in Ras-expressing cells following treatment with the Mek inhibitor U0126 (10 μM).

(C) PML expression in the same cells described in (A) and (B) was visualized by indirect immunofluorescence using an anti-PML antibody. 4′6-diamidino-2-phenylindole (DAPI) was used as counterstain to highlight the nucleus.

data not shown). We conclude that p53 can regulate PML expression through a conserved mechanism.

p53 Controls PML mRNA Expression

We previously showed that PML protein accumulation in senescent cells is accompanied by increases in PML mRNA (Ferbeyre et al., 2000). To determine whether p53 can also influence PML mRNA expression, we examined PML transcripts in the cell types described above using Northern blotting or a real-time quantitative RT-PCR assay (Figure 4). In MEFs, PML transcripts increased in response to Ras in a manner that was p53-dependent and analogous to PAI-1 and p21, two senescence-associated genes also controlled by p53 (el-Deiry et al., 1993; Kunz et al., 1995) (Figure 4A, compare lane 2 and 4; see also Figure 4B). Similarly, in H1299 cells, PML mRNA was substantially induced, following reintroduction of p53, to levels comparable to those produced by IFN treatment (Figure 4C). Therefore, p53 can control PML mRNA expression in a manner comparable to an established PML regulator.

PML Is a Direct Transcriptional Target of p53

The results described above suggest that PML may be a direct transcriptional target of p53. Consistent with this view, PML mRNA expression increases following activation of a temperature-sensitive p53 in the presence of the protein synthesis inhibitor cycloheximide (Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/13/4/523/DC1). Bona fide p53 targets contain defined p53 responsive elements (REs) in their promoters that physically associate with p53 and activate transcription (see Supplemental Table S1 on *Molecular Cell*'s website). To assess whether *PML* falls into this category, we assembled the human and mouse PML promoters from sequence information available in private and public databases (Figure 5A). We then analyzed them using the p53Scan program (http://bioinformatics.med.ohio-state.edu/P53), which can identify putative p53 REs on the basis of consensus sequences and additional contextual motifs (see Experimental Procedures). Interestingly, both the mouse and human *PML* genes contain putative p53 binding sites in their promoters and first introns, some of which were given a high statistical score (black boxes in Figure 5A; see Supplemental Table S1 on *Molecular Cell*'s website). Similarly, a genome-wide scan for p53 consensus sites using a different algorithm also identified putative p53 REs in the human *PML* gene (Hoh et al., 2002). The identification of p53 binding sites in the first intron of target genes is not unusual and has been confirmed for established p53 targets, such as *mdm2*, *bax*, and *puma* (Juven et al., 1993; Nakano and Vousden, 2001; Thornborrow et al., 2002; Yu et al., 2001).

To determine whether any of the putative p53 REs in the *PML* gene are functional, we tested whether they

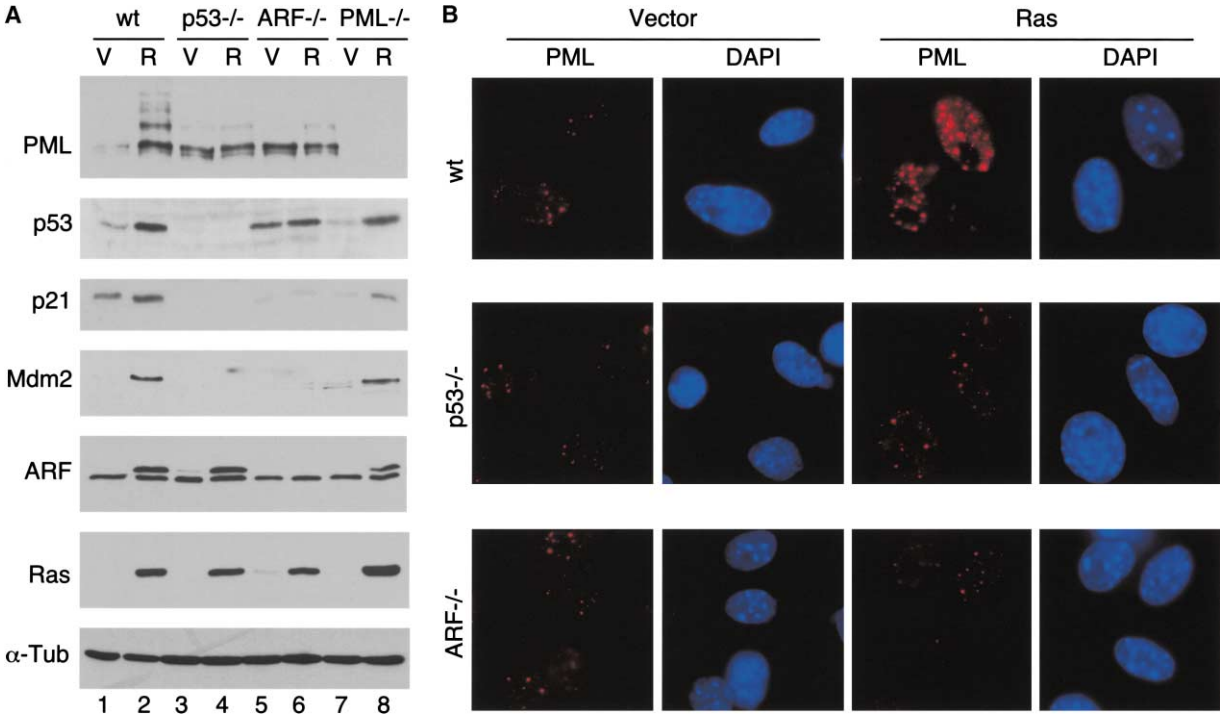

Figure 2. ARF and p53 Are Required for PML Induction during Premature Senescence

(A) Wild-type (wt), p53 null (*p53*$^{-/-}$), ARF null (*ARF*$^{-/-}$), or PML null (*PML*$^{-/-}$) MEFs containing a control vector (V) or oncogenic Ras (R) were collected and analyzed for the expression of PML, p53, p21, Mdm2, ARF, and Ras by immunoblotting. α-tubulin (α-Tub) was used as loading control.

(B) Immunofluorescence staining of the cells described in (A) using an anti-PML antibody and DAPI counterstain.

could confer p53 responsiveness to a heterologous reporter. DNA fragments containing either the murine PML promoter (sequences 4410–5189) or intron 1 (sequences 5246–6191) were isolated from genomic DNA and subcloned into a luciferase reporter plasmid. Next, these constructs were transfected into *p53*$^{-/-}$ MEFs along with increasing concentrations of a p53 expression plasmid and a normalization control (Figures 5B and 5C). Of note, the murine PML promoter contains both GAS and ISRE elements that confer IFN responsiveness to the human counterpart (Stadler et al., 1995). In fact, IFN induced a 3-fold increase in reporter activity over basal levels in cells transfected with the promoter construct but had no effect on the intron 1 construct (Figure 5B). Conversely, p53 triggered a 5-fold increase in luciferase activity from the intron 1 reporter but had no effect on the promoter construct. The ability of p53 to transactivate the intron 1 reporter was more pronounced in the presence of oncogenic Ras, perhaps because Ras signaling enhances p53 activity (Figure 5B). Similar results were also obtained in H1299 cells using the corresponding human promoter and intron 1 reporters (data not shown). These data imply that sequences in the intron 1 region are largely responsible for p53 regulation of the *PML* gene.

We next engineered point mutations in p53 REs of the murine intron 1 reporter construct at sites known to abolish p53 binding and tested their impact on p53's ability to transactivate the reporter construct (Figure 5C and Supplemental Table S1 on *Molecular Cell*'s website). Constructs that contained point mutations in all three REs were unable to respond to p53 (Figure 5C, compare intron 1 to RE 1,2,3 mut). Double mutant constructs retaining an intact RE3 were also nonresponsive to p53, whereas those retaining either RE1 or RE2 responded in a manner comparable to the intron 1 control (Figure 5C, compare RE 1,3 mut and RE 2,3 mut with intron 1). These data imply that RE3 does not play a major role in p53-mediated transactivation of the *PML* gene and that RE1 and RE2 have redundant activities.

We also asked whether p53 could physically associate with PML intronic sequences in vitro and in vivo. First, we tested several p53 REs (murine RE1-3 and human RE4) in an electrophoretic mobility shift assay (EMSA). Increasing amounts of recombinant p53 protein were added to each ^{32}P-labeled p53 RE, and the resulting products were resolved on polyacrylamide gels. In all instances, we observed a shift in the migration of the probes in the presence of p53 (Figure 5D; see lanes 3 and 7 for RE1 and RE4, respectively; data not shown for RE2 and RE3) but not to BSA (data not shown). Moreover, addition of the p53-specific antibody pAb421 to the reaction resulted in a supershift of the probe, confirming that the complexes in fact contained p53 (Figure 5D, see lanes 4 and 8).

To determine whether endogenous p53 occupies the PML promoter in vivo, we used chromatin immunoprecipitation (ChIP) to detect p53 bound to specific regions of DNA in live cells. MEFs harboring a control vector or oncogenic Ras were fixed in formaldehyde to crosslink

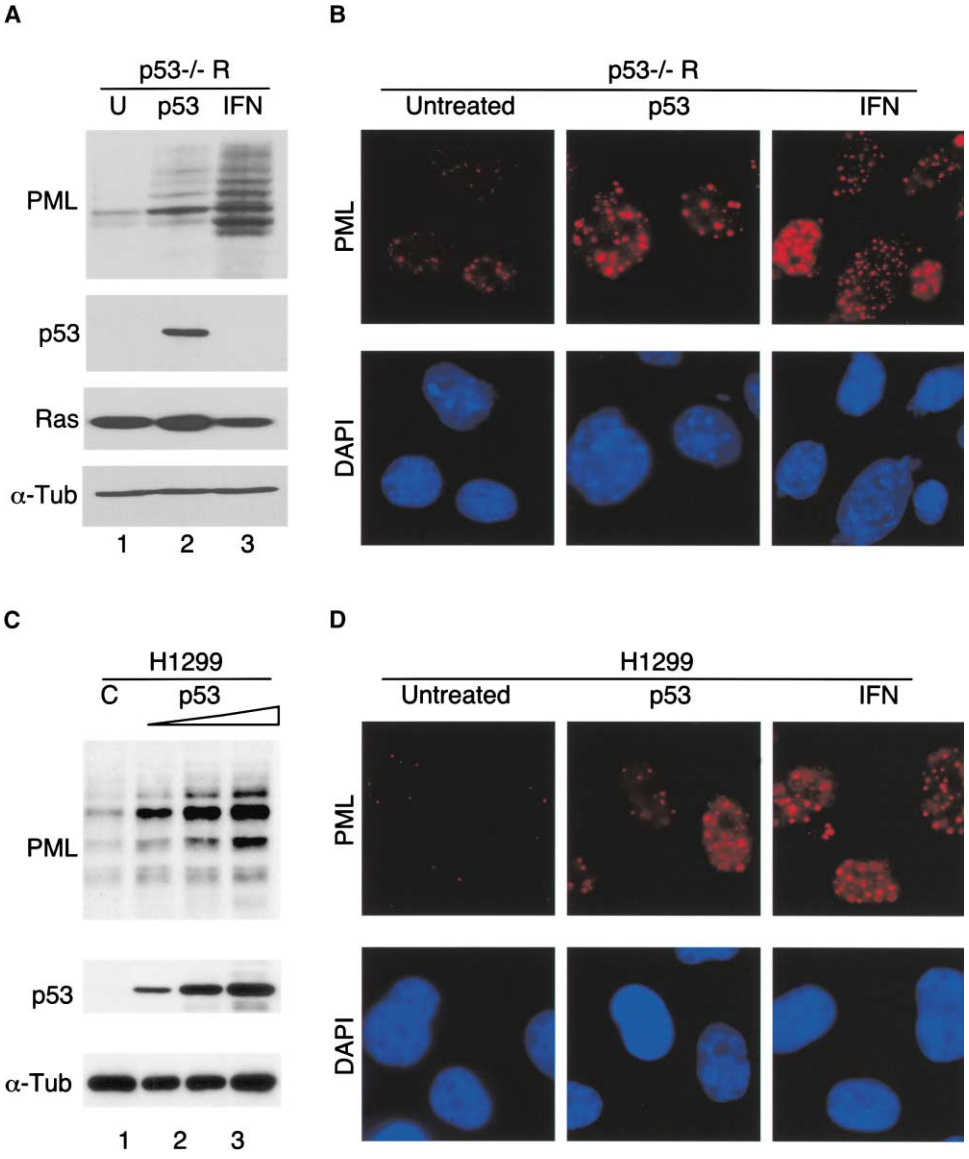

Figure 3. p53 Induces PML

(A) p53 null MEFs expressing Ras ($p53^{-/-}$ R) were left untreated (U), infected with an adenovirus expressing murine p53 (p53), or treated with 1000 U of mouse β interferon (IFN) and analyzed for the expression of PML, p53, and Ras by immunoblotting. α-tubulin (α-Tub) was used as loading control.

(B) PML localization of the cells described in (A) was visualized by indirect immunofluorescence using an anti-PML antibody and DAPI counterstaining.

(C) H1299 cells infected with a control adenovirus (C) or increasing amounts of an adenovirus expressing human p53 (p53) were analyzed by immunoblotting for the expression of PML and p53. α-tubulin (α-Tub) was used as loading control.

(D) H1299 cells either untreated, infected with an adenovirus expressing human p53 (p53), or treated with 1000 U of human β interferon (IFN) were fixed in 4% paraformaldehyde, stained with a polyclonal antibody against human PML, and counterstained with DAPI.

proteins to DNA, and the p53:DNA complexes were precipitated using a cocktail of p53 monoclonal antibodies (Flores et al., 2002). Following purification, the released DNA was analyzed by PCR using primers that flank the putative p53 REs. PML intron 1 sequences were detected in immunoprecipitates from Ras-expressing cells, indicating that p53 was bound to the *PML* gene during senescence (Figure 5E, lane 6). As controls, the same procedure amplified sequences corresponding to the bona fide p53 RE in the p21 promoter but not sequences in the actin promoter. Taken together, our data show that *PML* expression is directly regulated by p53.

PML Contributes to p53-Mediated Senescence and Cell Cycle Arrest

Depending on context, p53 can induce reversible cell cycle checkpoints, senescence, or apoptosis. Having established that PML is a direct p53 target, we then asked whether disruption of PML could interfere with any of these p53 activities. First, we examined the contri-

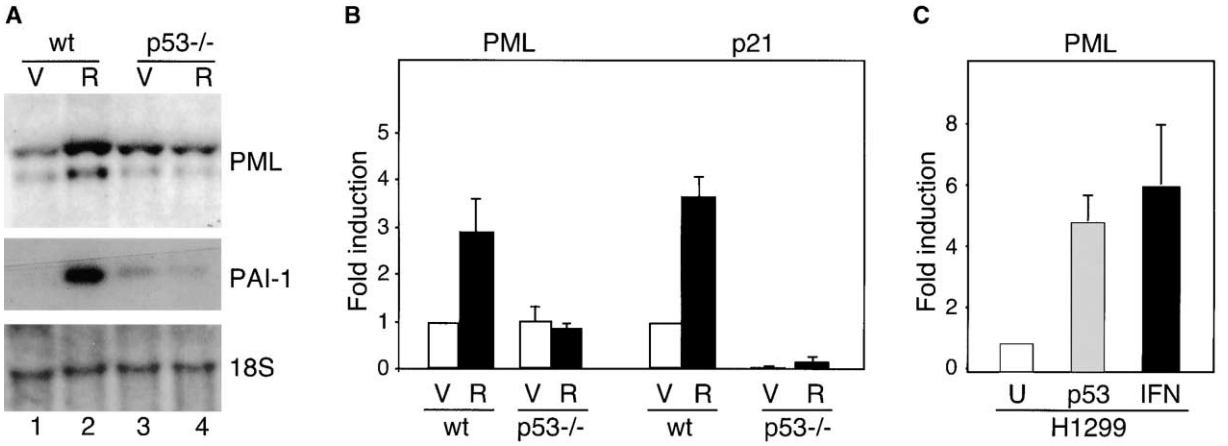

Figure 4. PML Is a p53 Responsive Gene

(A) Northern blot for PML of total RNA purified from wild-type (wt) and p53 null (*p53*$^{-/-}$) MEFs infected with an empty vector (V) or a vector expressing oncogenic Ras (R). PAI-1 was used as positive control, since it is known to be induced during premature senescence. 18S RNA was used as loading control.

(B) Total RNA from the same cells described in (A) was analyzed by quantitative RT-PCR analysis using primers for murine PML and p21 cDNAs. The data represent the mean (± the standard deviation) of three independent experiments.

(C) Total RNA was extracted from H1299 control cells (U), H1299 cells infected with an adenovirus expressing human p53 (p53), or treated with 1000 U of β interferon (IFN) and was analyzed by quantitative RT-PCR analysis using primers specific for the human PML cDNA. The data represent the mean (± the standard deviation) of three separate experiments.

bution of PML to p53-mediated cell cycle arrest. To suppress PML, we designed a PML short-hairpin RNA (shPML) that can effectively suppress PML levels (see Figure 6D) and introduced this by retroviral-mediated gene transfer into wild-type or *p53*$^{-/-}$ MEFs. These cells were then superinfected with a control or Ras-expressing retrovirus. Four days after drug selection to eliminate uninfected cells, the cell populations were plated for BrdU incorporation assays. As expected (Serrano et al., 1997), wild-type cells expressing oncogenic Ras showed a marked decrease in BrdU incorporation and accumulated senescence features, such a flat cell morphology and an increase in senescence-associated β-gal (SA-β-gal) activity, while their *p53*$^{-/-}$ counterparts continued to proliferate and did not display senescence markers (Figures 6A and 6B). Consistent with a previous report (Pearson et al., 2000), wild-type MEFs expressing both shPML and oncogenic Ras initially did not arrest or accumulate senescence markers, although we noted that these cells (as well as *PML*$^{-/-}$ MEFs) efficiently induced some p53 targets (see Figure 2A) and eventually arrested (data not shown). *p53*$^{-/-}$ MEFs expressing shPML showed no added defect in Ras-induced arrest compared to the parental *p53*$^{-/-}$ MEFs (Figure 6A).

To test more directly whether PML loss can attenuate p53 arrest functions through a downstream defect, we overexpressed p53 using a p53-deficient MEF line expressing a temperature-sensitive p53 mutant (TSP). These cells, which also lack *p21*, undergo a reversible cell cycle arrest following p53 activation (Ferbeyre et al., 2002). TSP cells expressing either shPML or a control vector were plated either at 39°C (the restrictive temperature) or at 32°C (the permissive temperature), treated for 3 hr with BrdU, and fixed for BrdU incorporation assays. At 39°C, both cell types grew at similar rates (Figure 6C, time 0) despite a near-complete ablation of PML protein expression by the short hairpin RNA (Figure 6D, see lanes 1 and 2). However, upon transfer to 32°C, the shPML-expressing cells showed a substantial delay in the establishment of the p53-induced cell cycle arrest (Figure 6C, see 24 and 36 hr). Importantly, at time points when shPML-expressing cells showed the greatest defect in p53-induced arrest, another p53 target (Mdm2) was efficiently induced (Figure 6D). Hence, in this setting, loss of PML does not affect p53 activity per se but instead impairs the downstream response.

PML Contributes to p53-Mediated Apoptosis

In addition to its role in cell cycle control, PML also promotes apoptosis (Guo et al., 2000; Quignon et al., 1998; Wang et al., 1998b; Yang et al., 2002). Interestingly, PML-deficient hematopoietic cells are resistant to radiation-induced cell death (Wang et al., 1998b), a process also mediated by p53 (Lowe et al., 1993). We previously showed that p53 promotes apoptosis in response to anticancer agents in *Eμ-myc* lymphomas, which arise in transgenic mice that constitutively express c-Myc in the B cell lineage (Adams et al., 1985). Thus, in lymphomas containing functional p53, chemotherapy induces p53 protein leading to massive apoptosis and a sustained response to therapy. In contrast, p53 null lymphomas show little apoptosis and a poor therapeutic response. Therefore, we envisioned that these lymphomas would provide an ideal setting to determine whether PML might act downstream of p53 during apoptosis in vivo.

Mice harboring p53-expressing (control) and p53 null (*p53*$^{-/-}$) lymphomas were treated with either cyclophosphamide (CTX) or γ-radiation (IR), and tumor material was isolated for immunoblotting 4 hr later (Figure 7A). In control lymphomas, anticancer therapy produced a massive induction of p53 and several PML isoforms. Similarly, *INK4a/ARF*$^{-/-}$ lymphomas, which do not disable DNA damage signaling to p53 (Schmitt et al., 1999),

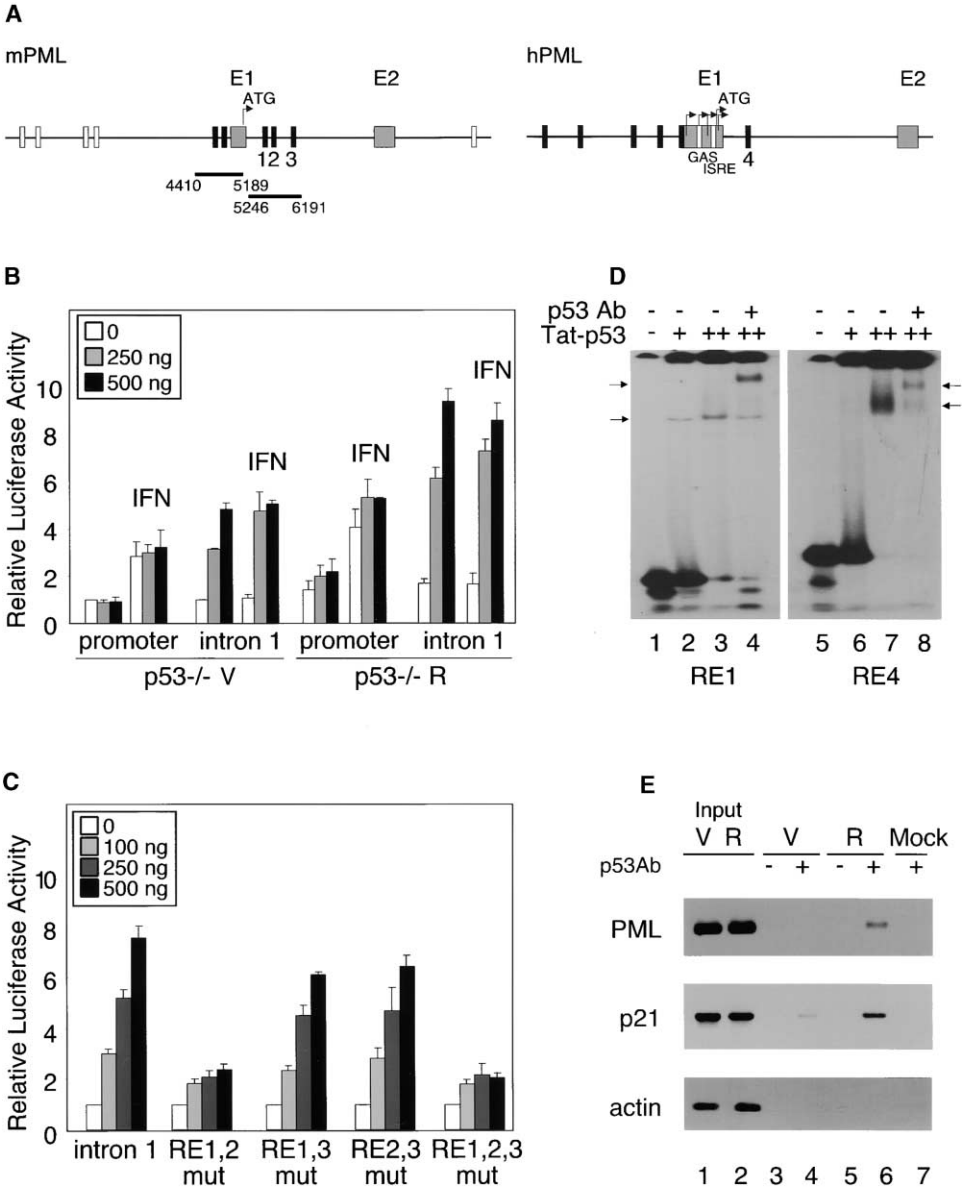

Figure 5. PML Is a Direct p53 Target

(A) Schematic diagram depicting the genomic regions spanning 5000 bp upstream of the predicted PML transcriptional start site and 5000 bp into the first (E1) and second (E2) exons of mouse (mPML) and human *PML* (hPML). Predicted p53 responsive elements (REs), identified by the p53Scan program, are represented by black (high-score sites) and white boxes (low-score sites) (see Supplemental Information Table S1 available on *Molecular Cell*'s website). RE 1, 2, 3, and 4 represent the REs subsequently tested by EMSA (see [D]). The GAS and ISRE boxes represent sequences identified previously. Sequences corresponding to the PML promoter (4410–5189) or intron 1 region (5246–6191) studied in (B) and (C) are highlighted by black bars.

(B) p53 null MEFs expressing either a control vector (*p53*$^{-/-}$ V) or *Ras* (*p53*$^{-/-}$ R) were cotransfected with increasing concentrations of a p53 expression plasmid and a luciferase reporter plasmid containing either the PML promoter or the PML intron 1 region. Some of the cells were treated with β interferon (IFN). Luciferase activity was plotted as the induction relative to basal luciferase activity. The data represent the mean (± the standard deviation) of three separate experiments.

(C) Luciferase reporter assay using p53 null Ras MEFs cotransfected with increasing concentrations of a p53 expression plasmid and a luciferase reporter plasmid containing either the PML intron 1 (intron 1) or intron 1 point mutants in the p53 REs (RE mut). The data represent the mean (± the standard deviation) of at least three independent experiments.

(D) In vitro binding of p53 to p53 REs in the mouse (RE1) and human (RE4) PML intron 1 region using EMSA. Increasing amounts of Tat-p53 protein were added to ^{32}P-labeled p53 RE1 and RE4 probes (lanes 2, 3, 4, 6, 7, and 8). Mobility shift of the probes was visualized by autoradiography (see arrows). A p53-specific antibody (p53Ab) was added to some of the reactions (lanes 4 and 8).

(E) In vivo detection of promoter occupancy by p53. Chromatin from wt MEFs infected with an empty vector (V) or Ras (R) was immunoprecipitated with a cocktail of p53-specific antibodies (lanes 4, 6, and 7). PCR amplification was performed on corresponding templates using p21, PML, and actin primers. Equal loading of templates in V and R samples is shown in the input lanes (lane 1 and 2), which correspond to 0.2% of total chromatin used in the immunoprecipitation reaction. Parallel immunoprecipitations without antibody (lanes 3 and 5) or without chromatin (mock, lane 7) failed to yield a detectable signal in any of the samples.

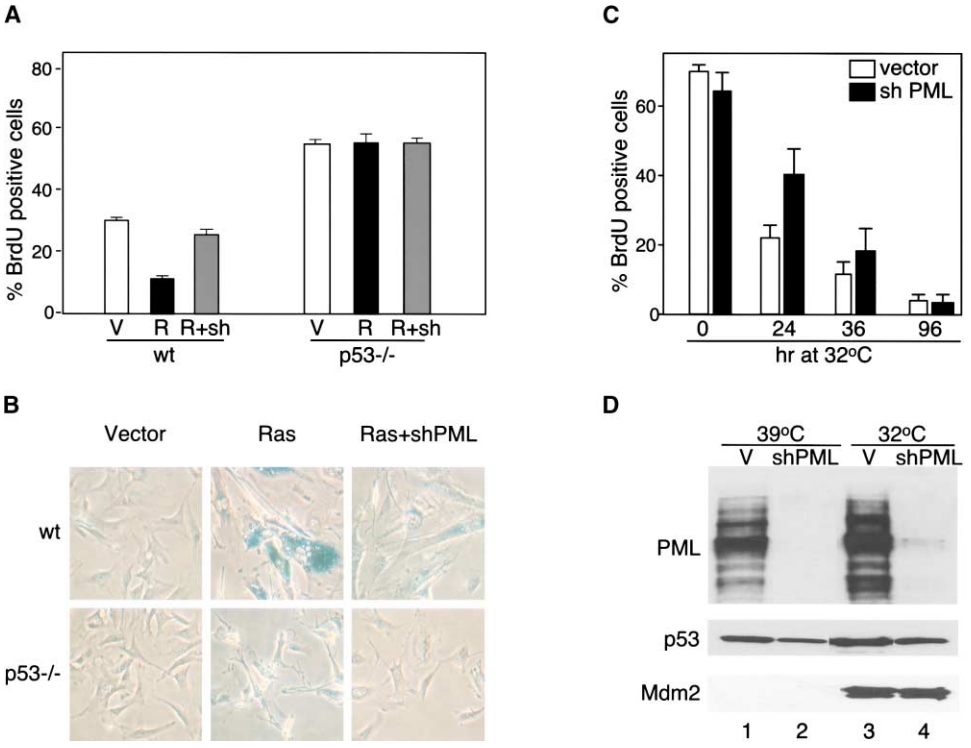

Figure 6. PML Potentiates p53-Mediated Premature Senescence and Cell Cycle Arrest

(A) Wild-type (wt) or p53 null (*p53*$^{-/-}$) MEFs infected with either an empty vector (V), Ras (R), or Ras and a PML short hairpin RNA (R + sh) were pulsed with 10 μM BrdU for 3 hr and stained with an anti-BrdU antibody to identify cycling cells. At least 200 nuclei per sample were counted. Results are presented as the percentage of BrdU positive nuclei and represent the mean of two independent experiments.

(B) SA-β-gal staining of wild-type (wt) or p53 null (p53$^{-/-}$) MEFs infected with either an empty vector, Ras, or Ras and a PML short hairpin RNA (Ras + shPML). Cells were plated 4 days after selection and fixed 12 hr later for staining. Representative images are shown.

(C) In situ BrdU incorporation of TSP cells containing an empty vector (white bars) or a PML short hairpin RNA (black bars). Cells were pulsed with 10 μM BrdU for 3 hr at the indicated time following transfer of the cells at 32°C to activate p53. At least 200 nuclei per each sample were counted. Results are presented as the percentage of BrdU positive nuclei and represent the mean (± standard deviation) of three separate experiments.

(D) TSP cells infected with a control vector (V) or a PML short hairpin (shPML) were collected following 24 hr incubation at either the restrictive (39°C) or permissive (32°C) temperature, and PML, p53, and Mdm2 protein levels were visualized by immunoblotting.

also induced p53 and PML in response to therapy. In contrast, *p53*$^{-/-}$ lymphomas were unable to induce PML in response to chemotherapy, implying that p53 controls PML expression in response to anticancer therapy in vivo.

c-Myc sensitizes both hematopoietic cells and MEFs to apoptosis in a p53-dependent manner (Eischen et al., 1999; Hermeking and Eick, 1994). To determine whether PML contributes to p53-mediated cell death, we introduced c-Myc into wild-type, *p53*$^{-/-}$, and *PML*$^{-/-}$ MEFs by retroviral gene transfer and examined the resulting populations for PML expression and apoptosis in response to several stimuli. Consistent with our in vivo results, PML was induced in wild-type MEFs following adriamycin treatment and, once again, this effect was p53 dependent (Figure 7C, compare lanes 2 and 4). Importantly, this increase is functionally relevant, as *PML*$^{-/-}$ cells expressing Myc showed substantial apoptotic defects following treatment with adriamycin, IR, or 0.1% serum, albeit not to the same extent as p53$^{-/-}$ cells (Figure 7B). Apoptosis was largely restored by reintroduction of PML into the *PML*$^{-/-}$ but not *p53*$^{-/-}$ cells (Figure 7B). Thus, p53 loss decreases both PML accumulation and activity following stress.

To determine whether PML-deficient cells were capable of efficiently activating p53, we also examined the expression of p53 and several well-characterized p53 transcriptional targets by immunoblotting and/or quantitative real-time RT-PCR. Of note, two of these targets—*puma* and *bax*—functionally contribute to p53-dependent apoptosis in oncogene-expressing MEFs (McCurrach et al., 1997; Villunger et al., 2003). Despite their apoptotic defect, *PML*$^{-/-}$ cells induced p53, p21, Mdm2, Puma, and, to a lesser extent, Bax following adriamycin treatment (Figure 7C, compare lanes 2 and 6, and Figure 7D), implying that their resistance to apoptosis is not due to a global defect in p53-mediated transactivation but, at least in part, to a defect downstream of p53. Therefore our data demonstrate that PML can contribute to p53-mediated cell cycle arrest, premature senescence, and apoptosis and, together with the expression data, establish PML as a mediator of p53 tumor suppressor functions.

Discussion

In this study, we identify *PML* as a p53 target gene that contributes to p53 tumor suppressor functions. Thus,

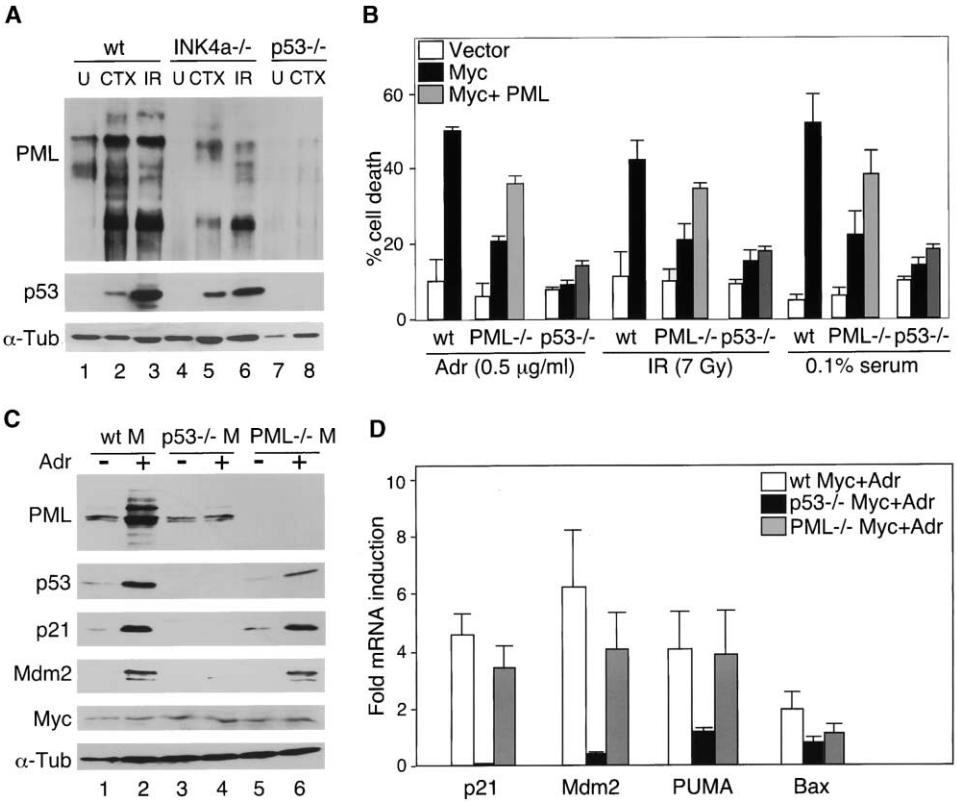

Figure 7. PML Potentiates p53-Mediated Apoptosis

(A) Control (wt), INK4a/ARF null (*INK4a/ARF*$^{-/-}$), and p53 null (*p53*$^{-/-}$) *Eμ-myc* lymphoma cells were isolated from lymph nodes of untreated animals (U) or 4 hr after treatment with cyclophosphamide (CTX) or ionizing radiation (IR), and PML and p53 expression was assessed by immunoblotting. α-tubulin (α-Tub) was used to verify protein loading.

(B) Apoptosis assays in MEFs. Wild-type (wt), p53 null (*p53*$^{-/-}$), or PML null (*PML*$^{-/-}$) MEFs infected with retroviruses expressing Myc (black bars) or Myc + PML (gray bars) were monitored for cell death 24 hr after treatment with adriamycin (Adr, 0.5 μM), ionizing radiation (IR, 7 Gy), or serum depletion (0.1% serum). Cell viability was assessed by trypan blue exclusion. Each point represents the mean from two separate experiments.

(C) Lysates from wild-type (wt), p53 null (*p53*$^{-/-}$), or PML null (*PML*$^{-/-}$) MEFs expressing Myc (M) were collected either prior to or 12 hr after treatment with 0.2 μM adriamycin (Adr), and PML levels, along with levels of p53, p21, Mdm2, Myc, and α-tubulin (α-Tub, as loading control), were determined by immunoblotting.

(D) Total RNA from the same cells described in (C) was analyzed by quantitative RT-PCR analysis using primers specific for murine p21, Mdm2, Puma, and Bax cDNAs. The data represent the mean (± the standard deviation) of three independent experiments.

p53 is required for oncogene and drug-induced increases in PML and NB levels and is capable of activating *PML* transcription through p53 response elements in the *PML* gene. Remarkably, cells lacking *PML* show a reduced propensity to undergo cell cycle arrest, cellular senescence, and apoptosis in response to p53 activation, despite the efficient induction of some other p53 target genes. Although p53 may also affect PML and NB formation through nontranscriptional mechanisms (see, for example, Seker et al., 2003), our results establish *PML* as an important p53 target gene. They also reveal an additional mode of PML regulation and suggest a prominent role for PML in tumor suppression.

How does PML mediate p53 functions? Despite the importance of PML in cancer biology, its biochemical activities are poorly understood. Most, but not all, studies correlate PML activity with its ability to nucleate NBs (Bischof et al., 2002; Ferbeyre et al., 2000; Guo et al., 2000; Pearson et al., 2000; Zhu et al., 1997). Although the precise functions of NBs are controversial, they may act as transient storage sites or organizing centers where nuclear processes are carried out (Borden, 2002). Interestingly, NBs can recruit proteins involved in both transcription and repair, and perhaps the ability of p53 to increase the number and size of PML-containing NBs modifies parallel transcriptional programs and/or modifies repair processes (Borden, 2002; Seker et al., 2003). Irrespective of its precise mechanism, PML is distinguished among p53 effectors by its ability to influence multiple p53 activities. However, as is true for other p53 effectors, PML is not sufficient to mediate each effect but acts in concert with other p53 effectors to execute specific p53 responses. Future studies elucidating the biochemical activities of PML and NBs should provide new insights into p53 action as well.

Our data are intriguing in light of previous reports suggesting that PML acts genetically and biochemically upstream of p53. Specifically, p53-deficient cells are resistant to PML-induced arrest and, as shown here, apoptosis, and PML can recruit p53 to the NBs and

enhance its transcriptional activity (Ferbeyre et al., 2000; Fogal et al., 2000; Guo et al., 2000; Pearson et al., 2000; Zhong et al., 2000). Interestingly, we also see that ARF-deficient cells are resistant to PML-induced arrest (our unpublished data). At face value, these observations place PML genetically upstream of the entire ARF-p53 pathway. Nevertheless, our data clearly demonstrate that PML is also a p53 effector.

How can we reconcile these observations? One possibility is that PML does not act upstream of p53 but instead requires other p53 effectors to exert its biological activities. *ARF*- and *p53*-deficient cells, which do not express these p53 effectors, would therefore be resistant to PML-mediated effects. The p21 cyclin-dependent kinase inhibitor represents a strong candidate for such an effector, since *p53*-deficient cells do not express p21 and *p21*-deficient cells are resistant to PML-induced arrest (Pearson et al., 2000). However, p21 cannot be the only p53 target that cooperates with PML, since p21 does not contribute to apoptosis (Attardi et al., 1996) and cells lacking both PML and p21 (as well as the related p27 protein) retain the ability to undergo a delayed p53-mediated arrest (our unpublished data).

Alternatively, PML might act in a positive feedback loop that, once initiated by p53, further potentiates p53 activity to reinforce or redirect the downstream response. In this scenario, the crucial p53 effector function mediated by PML is to act on p53 itself. Indeed, enforced PML expression can increase p53 levels and activity (Bischof et al., 2002; Ferbeyre et al., 2002; Pearson et al., 2000), and disruption of PML can prevent oncogene-induced modifications on p53 (Pearson et al., 2000). Such a model predicts that PML should be required for an optimal induction of at least some p53 effectors. Although PML loss had little impact on the ability of p53 to induce *p21*, *Mdm2*, and *puma* following stress, *bax* expression was impaired (see Figure 7D). Accordingly, *PML*$^{-/-}$ thymocytes fail to induce *bax* in response to radiation and PML can differentially activate certain p53 targets in transient reporter assays (Fogal et al., 2000; Guo et al., 2000). Irrespective of whether PML acts on p53 effectors or p53 itself, p53 loss short circuits the network and prevents increases in both PML levels and activity following stress.

Our demonstration that PML is a p53 target provides additional insights into the regulation and roles of PML. Prior to this work, the best-characterized PML regulator was interferon (Regad and Chelbi-Alix, 2001). Interestingly, p53 mRNA expression can increase in response to α and β IFN (Takaoka et al., 2003). However, it is unlikely that IFN-induced increases in *PML* expression are mediated by p53, since IFN controls *PML* expression primarily through IRES and GAS elements in the *PML* promoter, whereas p53 acts via consensus p53 response elements in the *PML* first intron (Stadler et al., 1995; Figure 5). Moreover, IFN efficiently induces *PML* in *p53* null cells (see Figures 3A, 3B, and 3D). Therefore, IFN and p53 regulate PML through distinct mechanisms.

The regulation of PML by interferon suggests that PML contributes to cellular antiviral responses. Accordingly, several viral proteins disrupt NBs, and *PML*-deficient mice are infection prone (Regad and Chelbi-Alix, 2001; Wang et al., 1998a). By analogy, the regulation of PML by p53 implies that PML also acts as an integral component of cellular stress responses that limit the proliferation of damaged cells. Indeed, previous studies implicate PML in cellular senescence, apoptosis, and DNA repair, all processes controlled by p53 (Pearson and Pelicci, 2001; Seker et al., 2003). Our results provide a mechanistic explanation for these overlapping activities and establish PML as a component of a bona fide tumor suppressor network.

In conclusion, p53 participates in a complex tumor-suppressor network that integrates stress signals leading to one of several antiproliferative responses. These responses are, in turn, carried out by a series of p53 effectors that act in concert to elicit changes required for the tumor-suppressive effect. In principle, the identification of additional network components that are altered in cancer cells reveals key "nodes" in the network (Vogelstein et al., 2000). Although the extent to which PML mediates p53 responses during the course of tumorigenesis or therapy remains to be determined, the fact that *PML* is an established cancer gene with an emerging role in tumor suppression suggests that it will play an important role. If so, then PML and PML-containing NBs should serve as useful surrogate markers for the p53 pathway in cancer specimens and may act as determinants of therapeutic responses.

Experimental Procedures

Cell Culture

Primary MEFs from wild-type, *ARF*, *p53*, and *PML* knockout mice were derived from day 13.5 embryos (Serrano et al., 1997). TSP cells, expressing p53^{val135}, were generated by retroviral-mediated gene transfer of *p53*^{val135} into *p53*$^{-/-}$ *p21*$^{-/-}$ MEFs, followed by clonal selection. H1299 cells were obtained from ATCC (CRL-5803). Cells were cultured in Dulbecco's modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin G/streptomycin sulfate (Sigma). For some experiments, cells were treated with U0126 (10 μM, Calbiochem) for 2 days, with β interferon (1000 U/ml, Biosource or a gift of D.P. Backer [Biogen]) for 24 hr or with cycloheximide (40 μg/ml) for 4–6 hr.

Viruses and Gene Transfer

Retroviral-mediated gene transfer was performed using Phoenix packaging cells (G. Nolan, Stanford University) as previously described (Serrano et al., 1997). Retroviral vectors were as follows: pBabe vector and its derivatives with oncogenic *ras* (H-Ras^{V12}) and *mek1*^{Q56P}, pMARXHygro and its derivative with *c-myc*, pLPC and its derivative with murine *p53*, and a murine PML hairpin was cloned into MSCVpuro (Clontech) as previously described (Hemann et al., 2003) (see also Supplemental Table S2 on *Molecular Cell*'s website). Infected cell populations were selected in puromycin (2 μg/ml, 2 days) or hygromycin (100 μg/ml, 3 days). The Ad GFP control (a gift of J.R. Nevins), Ad hp53 (a gift of F.L. Graham), and Ad mp53 vectors were produced according to standard procedures (Bett et al., 1994). In general, exponentially growing cells were infected at a moi (multiplicity of infection) of 100, as determined on 293 cells.

Promoter Analysis

The sequences of the murine and human PML promoter regions were assembled using both public and private databases. The first nucleotide of exon 1 of PML was assigned position 5000, as shown in Figure 5A. Genomic DNA isolated from wt MEFs was amplified by PCR in the presence of 5% DMSO. To clone the murine PML promoter and intron 1 region, PCR reactions were performed with oligos annealing at position 4410 in the promoter and at the PML translation start site (PML promoter) or oligos annealing at position 5246 and 6400 (PML intron 1); the resulting fragments were cloned into the pGL3-Basic, pGL3-Enhancer, or pGL3-Promoter vectors (Promega). Point mutations to inactivate each of the three potential

p53 binding sites present in the mPML intron 1 sequence were introduced by single site-directed mutagenesis. The sequences of the original and mutated p53 REs are listed in Supplemental Table S1 (available on *Molecular Cell*'s website). To identify putative p53-response elements in the PML promoter and intron 1 regions, we used the p53Scan program (http://bioinformatics.med.ohio-state.edu/P53) (see Supplemental Table S1 on *Molecular Cell*'s website).

Luciferase Reporter Assays

Cells were transfected using Fugene 6 (Roche) according to the manufacturer's instructions, then harvested and processed for dual-Luciferase assay (Promega). To test the murine PML promoter region, *p53*$^{-/-}$ MEFs were plated at 5×10^5 cells per plate and harvested 48 hr after transfection. All cells were transfected with 2.5 μg of the firefly luciferase reporter plasmid (pGL3, Promega), varying quantity of a plasmid expressing murine p53 (pLPCmp53), and an empty vector (pLPC) to keep the amount of total plasmid constant. A small amount (0.5 μg) of plasmid expressing Renilla Luciferase under the β-globin promoter was included to equalize for transfection efficiency in the dual-Luciferase assay.

Northern Blots and Real-Time RT-PCR

Northern blots were performed as previously described (Ferbeyre et al. 2000). Total RNA (30 μg), extracted from cells at day 4 postselection, were loaded in formaldehyde/agarose gels and transferred to Hybond membranes (Amersham). Probes for murine PML and PAI-1 consisting of overlapping 50-mer oligonucleotides were labeled with Klenow polymerase. 18S rRNA was used to confirm equal loading. For quantitative RT-PCR, total RNA was converted into cDNA using the TaqMan Reverse Transctiption Kit (Applied Biosystems). Gene-specific primer sets are listed in Supplemental Table S2, available on *Molecular Cell*'s website. Real-time PCR was carried out in triplicate with the SYBR Green PCR Master Mix (Applied Biosystems) with β actin primer sets used to produce a normalization control. Data analysis utilized the Sequence Detector Software (version 1.7) to determine the threshold cycle for each amplified product.

p53 Binding Assays

For electromobility shift assays (EMSA), single-stranded oligonucleotides corresponding to the RE1 and RE4 sequences (see Table S2 in the Supplemental Data on *Molecular Cell*'s website) were end labeled with T4 polynucleotide kinase and annealed to obtain the double-stranded probe used in the EMSA. An aliquot of Tat-p53 protein (a generous gift of S. Mayilvahanan) was incubated with 0.2–0.5 ng of probe (2×10^4 cpm) in a 50 μl reaction containing 1 μg poly(dI-dC), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10% glycerol, 1 mM DTT, and 0.1 mM EDTA. After a 15 min incubation at RT, DNA-protein complexes were resolved on a 6% polyacrylamide gel. For some reactions, the p53 protein was preincubated with 0.5 μg of p53 antibody (Ab1, Oncogene) for 10 min at RT in the same reaction before adding the probe.

ChIP was performed as previously described (Flores et al., 2002) using the p53 antibodies Ab1, Ab3 (Oncogene), and FL-393G (Santa Cruz). DNA released from precipitated complexes was amplified using primers for the p21 and actin promoters and for the PML intron 1 region. Primer sequences are provided in Supplemental Table S2 on *Molecular Cell*'s website.

Protein Expression

Immunoblots were performed from whole-cell lysates obtained by boiling cell pellets solubilized in Laemmli sample buffer (de Stanchina et al., 1998). Samples of 30 μg of protein (Bio-Rad protein assay) were separated on SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore). The antibodies used were anti-p53 (CM5, Novocastra, 1:1000 dilution), anti-Mdm2 (2A10, provided by A. Levine, 1:250), anti-p21 (C-19, Santacruz, 1: 500), anti-Mek (αMEK1, Calbiochem, 1:500), anti-Ras (Op23, Oncogene, 1:200), anti-p19^{ARF} (Novus Biologicals, 1:500), anti-Tubulin (B-5-1-2, Sigma, 1:2000), anti-Myc (Ab1, Oncogene, 1:200), and anti-human PML (1:500, a kind gift of K.S. Chang, and 1:10, a kind gift of K. van der Kraan). A monoclonal anti-mouse PML (36-1-104, 1:1000) antibody was produced in our laboratory using His-tagged full-length mouse PML (a gift of T. Ley) as antigen. Proteins were visualized using ECL (Amersham) or SuperSignal West Femtomaximum (Pierce).

For immunofluorescence assays, cells were plated on coverslips and fixed using 4% paraformaldehyde in PBS for 15 min at room temperature. After washing with PBS, cells were permeabilized for 5 min with 0.2% Triton X-100 in PBS with 0.5% normal goat serum (PBS/NGS). Next, cells were washed with PBS/NGS, incubated for 1 hr with antibodies against mouse PML (36-104, 1:100) or human PML (PGM3, Santacruz 1:100), washed in PBS/NGS, and stained with Alexa 595 secondary antibodies (1:1000) for 1 hr at room temperature in a humidified chamber. Finally, cells were washed in PBS, stained with 0.1 μg/ml DAPI, and mounted on microscope slides for fluorescence detection.

Cell Viability and Cell Proliferation Assays

Cells were distributed into 12 well plates (10^5 cells/22 mm well) 12 hr prior to treatment. Adherent and nonadherent cells were pooled 24 hr after treatment with γ-radiation (7 Gy), adriamycin (0.5 μg/ml), or 0.1% FBS and analyzed for viability by trypan blue exclusion; at least 200 cells were scored for each point. To measure BrdU incorporation, cells were plated on coverslips (8×10^4 cells/well in 6 well plates). After 12 hr, the cells were incubated for 3 hr in the presence of 10 μM BrdU, fixed, and nuclei incorporating BrdU were visualized by immunostaining using a monoclonal anti-BrdU antibody (Becton Dickinson, 1:200). At least 200 nuclei were counted for each sample. In the case of TSP cells, before adding BrdU the cells were grown at 32°C for 0, 24, 36, and 96 hr. SA-β-gal assays were performed as described (Ferbeyre et al., 2002).

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