The function of PML in p53-dependent apoptosis

Ailan Guo*, Paolo Salomoni*, Jianyuan Luo†, Alan Shih*, Sue Zhong*, Wei Gu† and Pier Paolo Pandolfi*‡

**Department of Human Genetics and Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA*
†Institute of Cancer Genetics and Department of Pathology, College of Physicians & Surgeons, Columbia University, New York, New York 10032, USA
‡e-mail: p-pandolfi@ski.mskcc.org

The *PML* gene of acute promyelocytic leukaemia (APL) encodes a growth- and tumour-suppresor protein that is essential for several apoptotic signals. The mechanisms by which PML exerts its pro-apoptotic function are still unknown. Here we show that PML acts as a transcriptional co-activator with p53. PML physically interacts with p53 both *in vitro* and *in vivo* and co-localizes with p53 in the PML nuclear body (PML-NB). The co-activatory role of PML depends on its ability to localize in the PML-NB. p53-dependent, DNA-damage-induced apoptosis, transcriptional activation by p53, the DNA-binding ability of p53, and the induction of p53 target genes such as *Bax* and *p21* upon γ-irradiation are all impaired in *PML*$^{-/-}$ primary cells. These results define a new PML-dependent, p53-regulatory pathway for apoptosis and shed new light on the function of PML in tumour suppression.

The *PML* gene is involved in reciprocal chromosomal translocations with the retinoic acid receptor-α (*RARα*) locus which are specifically observed in almost 100% of cases of APL, a distinct subtype of myeloid leukaemia[1–6]. This translocation leads to the production of a PML–RARα chimaeric oncoprotein, which is thought to interfere with both the PML and RAR/RXR pathways[5,6]. PML belongs to a family of proteins that are characterized by the presence of the RBCC (RING B-box coiled-coil) motif. The RBCC consists of a C_3HC_4 zinc-finger motif (RING-finger) and one or two additional cysteine-rich, zinc-binding regions (B-boxes), followed by a predicted coiled-coil region. The RBCC domain mediates homodimerization of PML, protein–protein interactions and the subnuclear localization of PML[7–9]. PML localizes in discrete, speckled subnuclear structures termed PML-NBs, where it co-localizes with several other proteins[10]. PML is essential for formation of the PML-NB; in *PML*$^{-/-}$ cells other PML-NB components exhibit aberrant nuclear localization[11]. In APL cells, PML–RARα delocalizes PML from the PML-NB through physical interactions, thus leading to the disruption of the PML-NB.

PML antagonizes the initiation, promotion and progression of tumours of various histological origins, acting *in vivo* as a cell-growth and tumour suppressor[12]. In addition, *PML*$^{-/-}$ mice and cells are protected from several apoptotic pathways including those of Fas, tumor-necrosis factor-α (TNFα), ceramide, and IFN types I and II, as well as DNA damage-induced apoptosis[13]. However, the molecular pathways through which PML can modulate response to pro-apoptotic stimuli are currently unknown.

Recent evidence has linked PML and the PML-NB to transcriptional regulation[10]. Although PML does not bind directly to DNA, when tethered to DNA its RBCC domain exhibits a cryptic transactivating activity, which is dependent on the presence of the RING-finger motif[14]. PML acts as a co-activator in the RARα–RXRα transcriptional complex, possibly through its interaction with the transcriptional co-activators CBP and/or TIF1α[15,16], or through its participation in the transcriptionally active DRIP complex[15,17] (also known as ARC[18] or TRAP[19]). The ability of PML to interact with co-activators such as CBP indicates that PML could modulate transcription through its ability to stabilize complexes of cofactors and transcription factors.

The tumour-suppressor protein p53 has a key function in regulating cell growth and cell death in the response of cells to cellular stress such as DNA damage or hypoxia[20,21]. The *p53* gene is mutated in the majority of human cancers, indicating that loss of p53 function may have an important role in tumorigenesis[22]. *p53*$^{-/-}$ mice are developmentally normal, but are highly prone to developing spontaneous tumours[23]. Furthermore, *p53*$^{-/-}$ cells such as primary thymocytes are protected from DNA-damage-induced apoptosis[24,25]. The ability of p53 protein to induce cell-cycle arrest and apoptosis

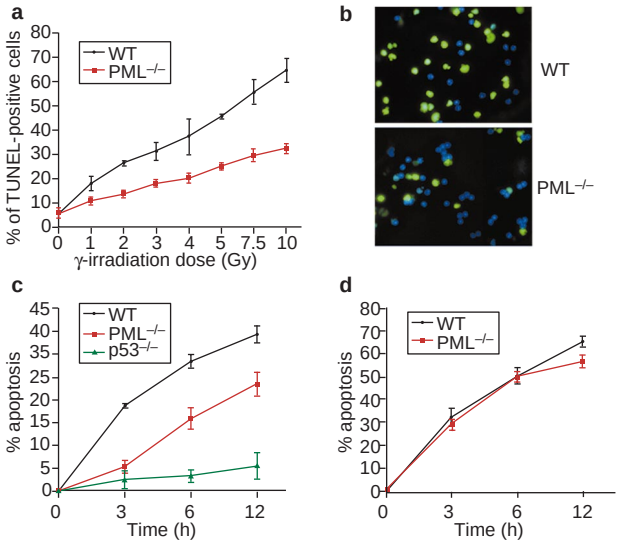

Figure 1 **DNA-damage-induced apoptosis is reduced in *PML*$^{-/-}$ thymocytes. a**, Dose–response curve of γ-irradiation-induced apoptosis in wild-type (WT, black line) and *PML*$^{-/-}$ (red line) thymocytes. Thymocytes from wild-type and *PML*$^{-/-}$ mice were subjected to different doses of γ-irradiation; cells were collected after 8 h and apoptosis was analysed by TUNEL staining. Values are means ± s.d. from triplicate experiments scoring at least 300 cells for each sample. **b**, Representative TUNEL staining of wild-type and *PML*$^{-/-}$ thymocytes after irradiation. Cells shown were subjected to a 7.5-Gy dose of irradiation; apoptosis was scored after 8 h. Apoptotic cells are shown in green (TUNEL-positive); nuclei are shown in blue (DAPI staining). **c**, Time course of apoptosis induced by γ-irradiation (5 Gy) in wild-type (black line), *PML*$^{-/-}$ (red line), and *p53*$^{-/-}$ (green line) thymocytes. **d**, Time course of apoptosis induced by treatment with dexamethasone (1 μM) in wild-type (black line) and *PML*$^{-/-}$ (red line) thymocytes. Values in **c** and **d** are means from three independent experiments. Percentages of apoptotic cells were normalized for apoptosis in untreated cells.

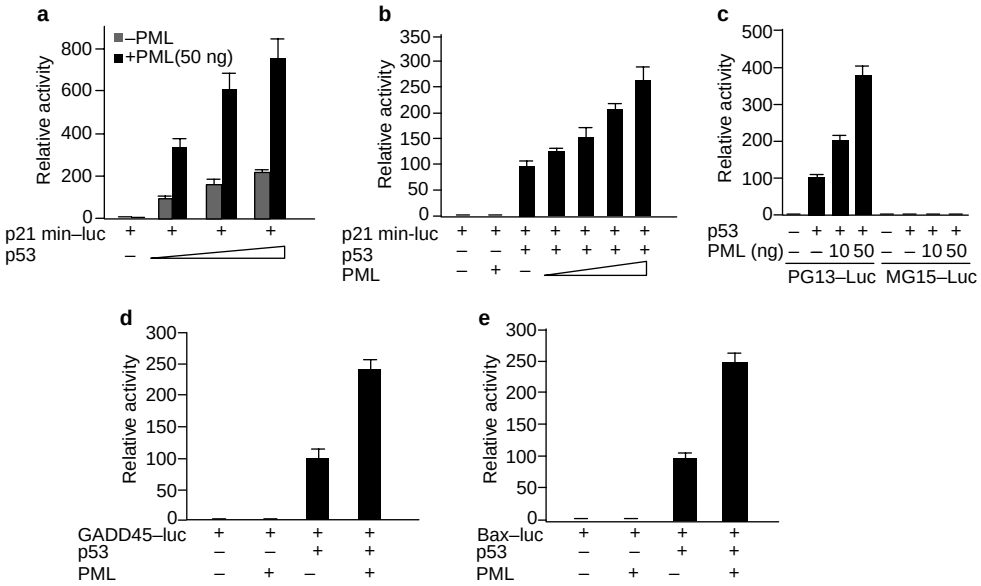

Figure 2 **PML enhances p53 transcriptional activation. a**, SaoS2 cells were transfected with 160 ng p21min–luc, 50 ng pSG5–PML and various amounts of pCMV–p53 (0, 5, 10 or 20 ng). Relative activities were derived from arbitrary light units of luciferase activity normalized for β-galactosidase activity. The value obtained by transfecting 5 ng p53 was arbitrarily designated as 100. Values are means ± s.d. from triplicate platings. Data shown are from one out of three independent experiments with comparable results. **b**, PML enhances p53 transactivation of a p21min–luc reporter in a dose-dependent manner. SaoS2 cells were transfected with 160 ng p21min–luc, 10 ng pCMV–p53, and various amounts of pSG5–PML (0, 10, 20, 50 or 90 ng). The value obtained by transfecting 10 ng of p53 was arbitrarily designated as 100. Data are expressed as in **a** and are from one out of two independent experiments with comparable results. **c**, Mutations in p53-responsive elements in the p21 promoter abolish transactivation by PML and p53. SaoS2 cells were transfected with 200 ng PG13 (wild-type responsive elements) or MG15 (mutant responsive elements), 5 ng pCMV–p53 and the indicated doses of pSG5–PML. Data are expressed as in **a**. **d**, **e**, PML enhances p53 transactivation of the *GADD45* (**d**) and *Bax* (**e**) promoters. SaoS2 cells were transfected with160ng GADD45–luc or Bax–luc together with 5 ng pCMV–p53 and/or 10 ng pSG5-PML where indicated. The value obtained by transfecting 5 ng of p53 was arbitrarily designated as 100. Values are means ± s.d. from triplicate platings. Data shown are from one out of two independent experiments with comparable results for each promoter. In **a–d**, 50 ng of pSG5–PML were used when PML was transfected alone with the reporter.

is due, at least in part, to its function as a sequence-specific transcriptional activator. In particular, p53 can transactivate target genes such as that encoding the pro-apoptotic factor Bax, which is involved in the mitochondrial checkpoint of apoptosis, and neutralizes the anti-apoptotic functions of BCL-X_L and BCL-2 (ref. 26). In response to stress signals, levels of p53 protein are rapidly upregulated, and its activity is enhanced upon post-translational modification[20,21]. Each of these modifications or their combinations have been proposed to be important for transcriptional activation of p53. Here we define a function of PML in p53-dependent transcription and apoptosis.

Results

DNA-damage-induced apoptosis is reduced in *PML*$^{-/-}$ thymocytes. We tested the responses of *PML*$^{-/-}$ and wild-type thymocytes to γ-irradiation. In these cells, DNA-damage-induced apoptosis depends entirely on the presence of normal p53 function[24,25]. Thymocytes express PML, which is found in its classical speckled distribution in PML-NBs[27] at the steady state and is induced upon treatment with IFN (data not shown). Thymuses from wild-type and *PML*$^{-/-}$ mice contained comparable numbers of $CD4^+/CD8^+$ immature cells (data not shown), which are susceptible to apoptosis[24,25]. As expected, wild-type thymocytes rapidly underwent radiation-induced apoptosis. In contrast, apoptosis was markedly impaired in *PML*$^{-/-}$ thymocytes, although not to the same extent as in *p53*$^{-/-}$ cells (Fig. 1a–c). At later time points (24 h), and at any radiation dose between 1 to 10 Gy, inactivation of PML resulted in a consistent reduction in thymocyte death relative to wild-type samples, to a level that was comparable to that observed in *p53*$^{+/-}$

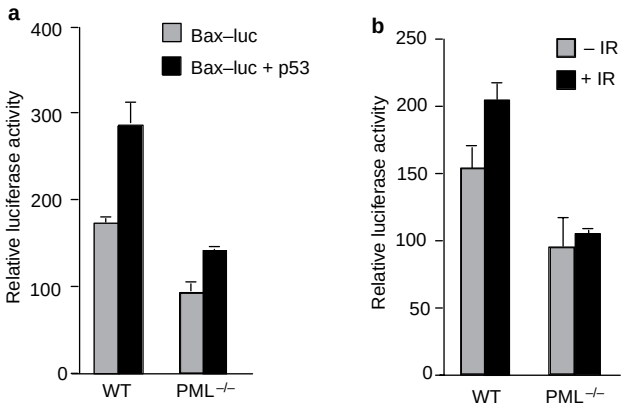

Figure 3 **Transcriptional activity of p53 is impaired in *PML*$^{-/-}$ cells. a**, Bax–luc reporter alone (0.6 μg, gray bar) or Bax–luc reporter (0.6 μg) and pCMV–p53 (50 ng, black bar) were transfected into wild-type (WT) or *PML*$^{-/-}$ primary embryonic fibroblasts. Luciferase activity was assessed 48 h after transfection. Relative luciferase activity is expressed as light units normalized for β-galactosidase activity. Values are means ± s.d. from triplicate samples and are representative of two independent experiments. **b**, γ-irradiation-induced p53 transcriptional activity is impaired in *PML*$^{-/-}$ MEFs. Wild-type and *PML*$^{-/-}$ MEFs were transfected with Bax–luc reporter (0.6 μg). Forty hours after transfection, one set of samples (triplicate) was subjected to 10 Gy γ-irradiation. Cells were then incubated for 8 h before measurement of luciferase activity. Data are expressed as in **a** and are representative of two independent experiments.

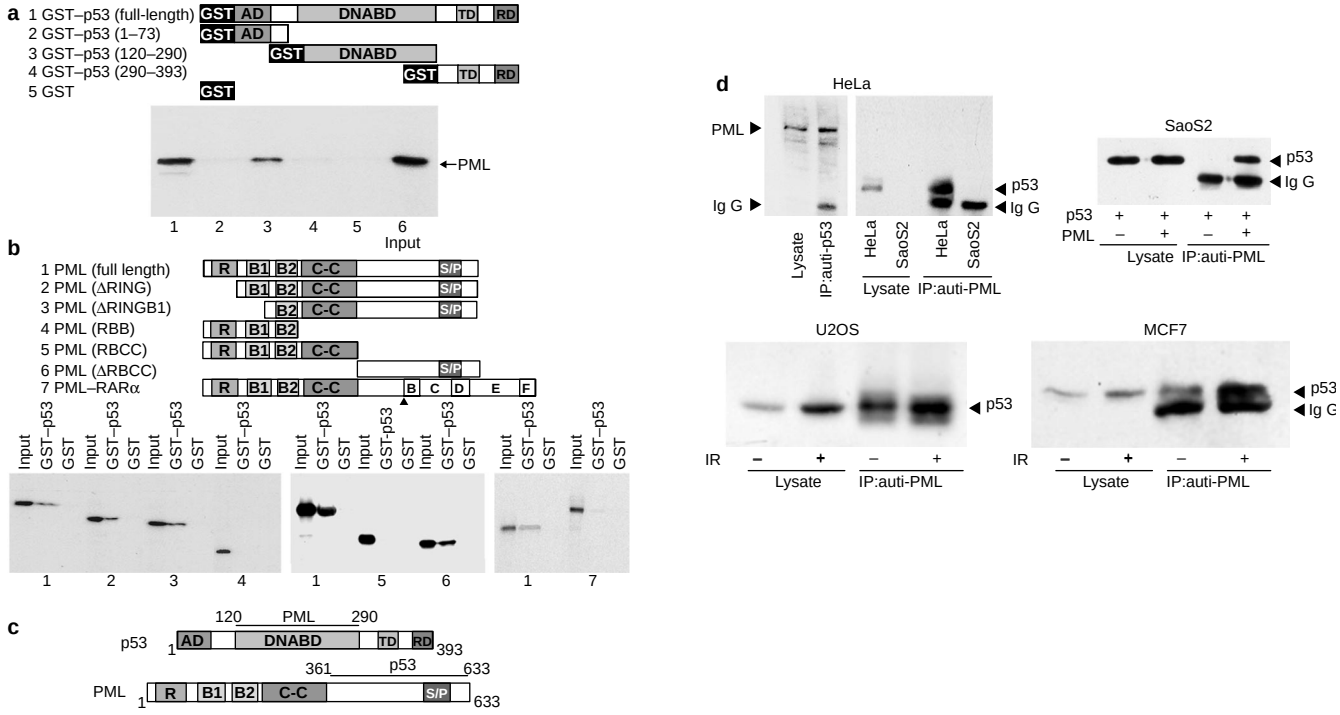

Figure 4 **PML interacts with p53 *in vitro* and *in vivo*. a**, Mapping of the PML-interacting domain of p53. The various p53 constructs used in GST pull-down assays are shown in the upper panel. AD, activation domain; DNABD, DNA binding domain; TD, tetramerization domain; RD, regulatory domain. These proteins were incubated with ^{35}S-labelled, *in vitro*-translated PML and subjected to the pull-down assay (lower panel). Input PML (10 %) is shown in lane 6. **b**, Mapping of the p53-interacting domain of PML. The PML deletion mutants used in pull-down assays are shown in upper panel. R, RING-finger motif; B1, B2, B boxes; C-C, coiled-coil domain; S/P, serine/proline-rich region. These ^{35}S-labelled, *in vitro*-translated proteins (input shown in the lane 1 of each group) were incubated with GST–p53 or GST alone (lanes 2 and 3, respectively) in pull-down assays (lower panel). **c**, Schematic representation of the p53 and PML interaction moieties. PML interacts with amino acids 120–290 of p53, which also contains the DNA-binding domain. p53 binds to the C-terminal portion of PML (amino acids 361–633). **d**, PML and p53 interact *in vivo*. Upper-left panel, co-immunoprecipitation of PML and p53 in HeLa cells. Extracts from 10^7 HeLa and SaoS2 cells were used for immunoprecipitation (IP). As controls, the gel was loaded with 5% of the lysate used for the p53 IP, and 2% of the lysate used for the PML IP. Western blotting was carriedout using anti-PML and anti-p53 (DO-1, see Methods) antibodies. Upper-right panel, SaoS2 cells were transfected with p53 or co-transfected with p53 and PML (see Methods); and whole-cell extracts were subjected to co-immunoprecipitation and western blotting with anti-p53 antibody as above. The lysate loaded onto the gel was 5% of that used in the IP. Lower panels, whole-cell extracts from U2OS and MCF7 cells before and 5 h after γ-irradiation (IR, 8 Gy) were subjected to co-immunoprecipitation and western blotting with anti-p53 antibody as above. Lysates loaded onto the gel were 10% and 2% of those used for co-immunoprecipitation in U2OS and MCF7 cells, respectively. Cell extracts were run on a 10% polyacrylamide gel. For U2OS cells, a longer run allowed better separation of the immunoglobulin G (Ig G) band from the p53-specific band.

thymocytes (specific cell death at 7.5 Gy at 24 h: wild-type, 73 ± 2.3%; *PML*$^{-/-}$, 63.2 ± 2.4%; *p53*$^{+/-}$, 62.4 ± 2.7%). We next tested whether PML inactivation could protect thymocytes from other p53-independent apoptotic stimuli, such as dexamethasone. As in *p53*$^{-/-}$ thymocytes[24,25], the ability of dexamethasone to induce apoptosis was unaffected in *PML*$^{-/-}$ thymocytes (Fig. 1d). Thus, inactivation of *PML* impairs both the rate and overall extent of p53-dependent cell death upon ionizing irradiation in mouse thymocytes.

PML regulates p53 transcriptional activity. To define the function of PML in the p53 pathway and the mechanisms that underlie the unresponsiveness of *PML*$^{-/-}$ cells to γ-irradiation, we investigated whether PML might regulate the transcriptional activity of p53, which is required, at least in part, to mediate the pro-apoptotic activity of p53 (refs 20,21). To this end, we co-transfected SaoS2 cells (which are p53-null in view of a deletion of the *p53* gene[28]) with vectors expressing p53 and/or PML and tested whether PML could affect the ability of p53 to transactivate through the p53-responsive element found in the p21 promoter, which was fused to a luciferase reporter (p21min–luc). Transiently expressed p53 activated this reporter construct, and co-transfection of PML enhanced p53 transactivation (Fig. 2a, b). Notably, transcriptional activation of p21min–luc by PML was completely p53-dependent (Fig. 2a, b). Furthermore, neither p53 nor PML could activate a p21mini–luc construct in which the p53-responsive elements have been mutagenized and do not bind to p53 (ref. 29; Fig. 2c). We next investigated whether PML could enhance the p53-dependent transactivation of the promoters for *Bax* or *GADD45*. The *Bax* and *GADD45* promoters contain p53-responsive elements that mediate the induction of expression of these genes by p53 upon ionizing irradiation[30,31]. PML markedly potentiated p53 transactivation of both promoters (Fig. 2d, e). Once again, transcriptional activation by PML was totally p53-dependent (Fig. 2d, e). Levels of p53 co-activation by PML were comparable to those observed when p53 was co-transfected with the CBP transcriptional co-activator (data not shown)[32]. Furthermore, PML co-activated the *Bax* and *GADD45* promoters in a dose-dependent manner, whereas it repressed p53 transactivation of these promoters at very high doses (data not shown). This may represent a 'squelching' effect that is due to the fact that PML acts as a transcriptional co-activator, but does not directly bind to the p53-responsive element of these promoters (data not shown).

We next tested if p53 transcriptional activity is affected in *PML*$^{-/-}$ cells. Western-blot analysis revealed that wild-type and *PML*$^{-/-}$ mouse embryonic fibroblasts (MEFs) expressed comparable levels of p53 protein, which was similarly induced by γ-irradiation (data not shown). However, when transfected into MEFs, a Bax–luc reporter

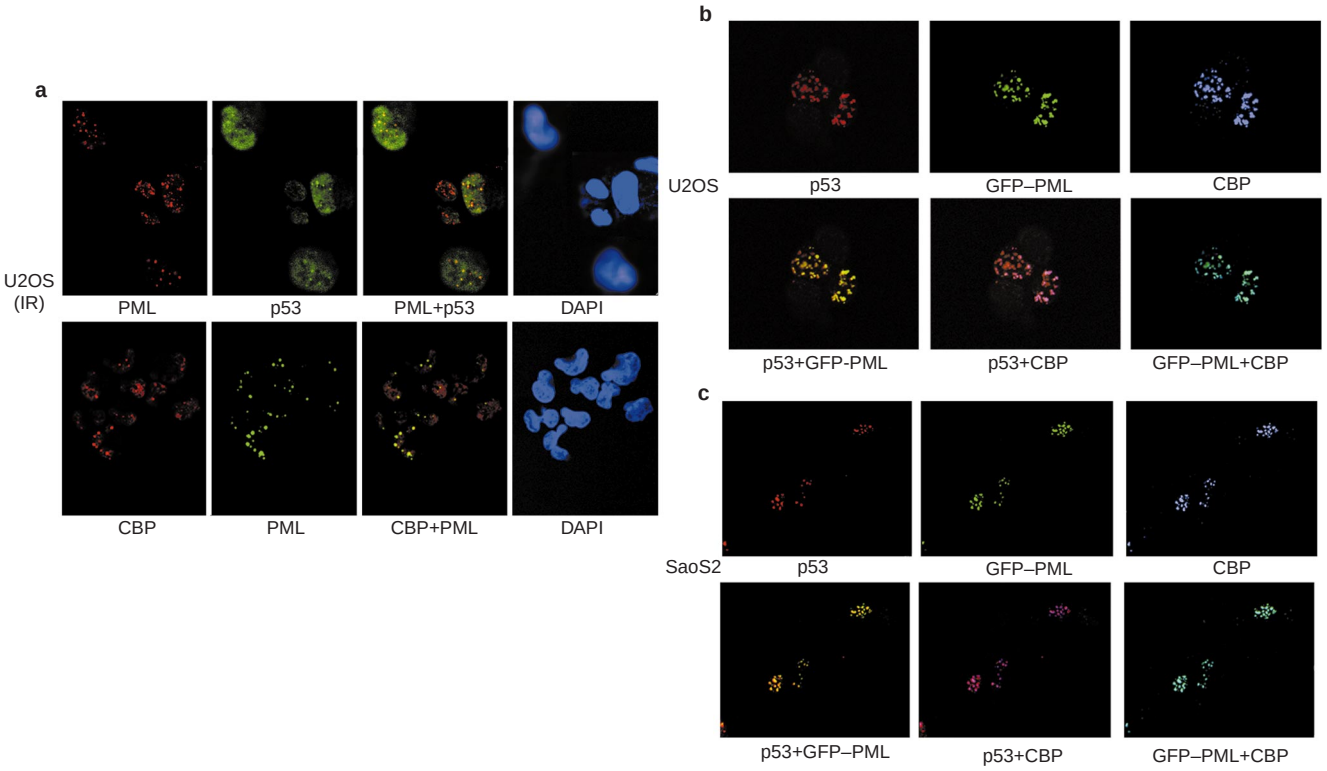

Figure 5 **p53, CBP and PML co-localize in the PML-NB. a**, Upper panels, p53 and PML co-localize in discrete nuclear regions in irradiated (IR) U2OS cells. U2OS cells were stained with anti-p53 (FL-393, green) and monoclonal anti-PML (red) antibodies and analysed by confocal microscopy. Yellow indicates co-localization. Nuclei are shown in blue (DAPI staining). Lower panels, CBP and PML co-localize in PML-NBs in irradiated U2OS cells. Cells were stained with anti-CBP (red) and anti-PML (green) antibodies. Yellow indicates co-localization. Nuclei are shown in blue (DAPI staining). **b**, **c**, Triple immunofluorescence staining showing co-localization of p53, PML and CBP in U2OS and SaoS2 cells. **b**, U2OS cells were transfected with GFP–PML (green); endogenous p53 and CBP were stained with anti-p53 (DO-1, red) and anti-CBP (A22, blue) antibodies, respectively. Lower panels show co-localization. Red and green overlap results in yellow, red and blue in violet, and green and blue in aquamarine. **c**, SaoS2 cells were co-transfected with p53 and GFP–PML (green); p53 was stained with anti-p53 antibody (red); endogenous CBP was stained with anti-CBP antibody (blue). Lower panel shows co-localization as in **b**.

had higher relative luciferase activity in wild-type cells than in $PML^{-/-}$ cells, indicating that transcriptional activation by p53 may be impaired in $PML^{-/-}$ cells (Fig. 3a). The transcriptional impairment of p53 observed in $PML^{-/-}$ cells was completely rescued by addition of exogenous PML, whereas addition of mutated PML proteins that do not localize in the PML-NB and do not bind to p53 did not rescue the impaired p53 transactivation (see below). Moreover, γ-irradiation induced further detectable activation of the *Bax* promoter, but failed to induce activation of Bax–luc in $PML^{-/-}$ cells (Fig. 3b).

PML physically interacts with p53. To investigate whether PML can modulate the transcriptional function of p53 through physical interactions, we carried out pull-down experiments using glutathione-*S*-transferase (GST). We cloned various deletion mutants of p53 in fusion with the GST moiety and purified the recombinant proteins (Fig. 4a). We incubated ^{35}S-labelled, *in vitro*-translated PML with the various immobilized GST–p53 fusion proteins, eluted the bound proteins, and analysed them by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) for autoradiography. PML interacted with both full-length p53 as well as with a p53 mutant comprising the DNA-binding domain only, indicating that the PML–p53 interaction occurs through this domain (Fig. 4a). To identify the PML domain that is required for the interaction with p53, we incubated various ^{35}S-labelled, *in vitro*-translated PML mutants with immobilized GST–p53 and analysed them as described above (Fig. 4b). Mutants lacking the three amino-terminal zinc-finger motifs of PML still interacted with p53, whereas deletion of the carboxy-terminal region abrogated the ability of PML to interact with p53 (Fig. 4b). The PML–RARα fusion protein interacted very poorly with p53 (Fig. 4b). Thus, the C terminus of PML interacts with the DNA-binding domain of p53 (Fig. 4c).

To determine whether PML and p53 can interact *in vivo*, we firstly co-transfected COS-1 and SaoS2 cells with vectors expressing PML and p53. Overexpressed PML and p53 proteins could be co-immunoprecipitated (Fig. 4d and data not shown). We then investigated whether endogenous PML and p53 can interact *in vivo*, using HeLa, MCF7 and U2OS cells. Endogenous PML and p53 also co-immunoprecipitated in these cells (Fig. 4d). As expected, irradiation of these cells led to upregulation of p53 expression (two- and threefold in MCF7 and U2OS cells, respectively) and to a concomitant increase in the amount of p53 that co-immunoprecipitated with PML (Fig. 4d). Quantification of the relative amounts of p53 in complex with PML, by comparing the amounts of p53 that immunoprecipitated with the protein present in the lysate and that were left in the supernatant, revealed that ~5–10% of the total p53 was complexed with PML, depending on the cell line analysed (Fig. 4d and data not shown). Together, these data show that PML physically interacts with p53 and that, through this interaction, PML can potentiate transcriptional activation by p53.

p53 and PML co-localize in the PML-NB. Recent evidence has linked PML and the PML-NB to transcriptional regulation[10]. We therefore used co-immunofluorescence staining to study the localization of endogenous p53, PML and CBP. The transcription co-activator CBP can localize in the PML-NB[11,15,16,33], and is also known to act as a co-activator of p53 through its ability to interact

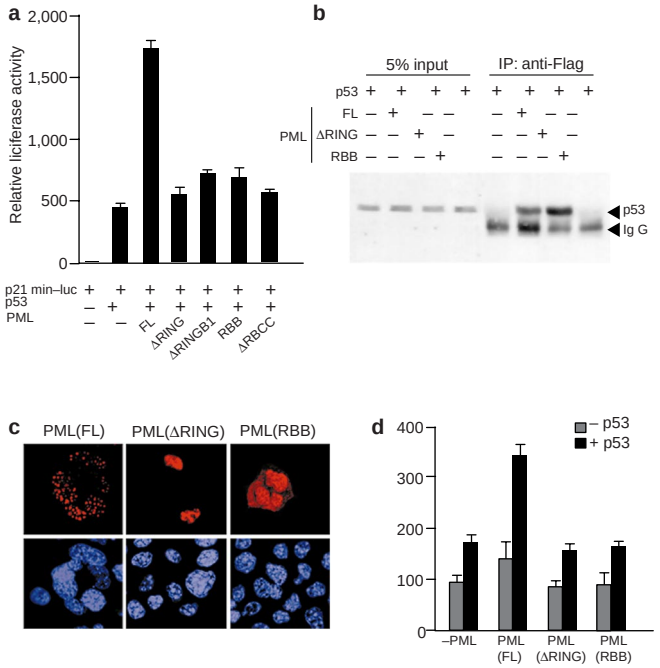

Figure 6 **Function of PML-NB localization in transcriptional co-activation by PML. a**, PML mutants are transcriptionally inactive. SaoS2 cells were transfected with 200 ng p21min–luc, 50 ng of full-length (FL) PML or of the indicated PML mutants, and 5 ng pCMV–p53 where indicated. Relative activity was derived from arbitrary light units of luciferase activity normalized for β-galactosidase activity. Values are means ± s.d. from triplicate platings. Data shown are from one out of four independent experiments with comparable results. **b**, PML(ΔRING) interacts with p53 *in vivo*, whereas PML(RBB) does not. SaoS2 cells were transfected with p53, PML(FL) and PML(ΔRING) and PML(RBB) where indicated; whole-cell extracts were subjected to co-immunoprecipitation (IP) with anti-Flag antibody and western blotting with anti-p53 antibody as described above. **c**, Subnuclear localization of PML(FL), PML(ΔRING) and PML(RBB) in *PML*$^{-/-}$ MEFs. Cells were transiently transfected with expression vectors, stained with monoclonal anti-Flag antibody (red) and analysed by confocal microscopy. Nuclei are shown in blue (DAPI staining). **d**, Addition of full-length PML but not of PML(ΔRING) or PML(RBB) rescues impaired p53 transactivation in *PML*$^{-/-}$ MEFs. Cells were transfected with a Bax–luc reporter alone (gray bars) or with Bax–luc reporter and pCMV–p53 (black bars) as in Fig. 3a. The indicated PML constructs (150 ng) were co-trasfected with the above plasmids. Relative luciferase activity was assessed 48 h after transfection, and is expressed as light units normalized for β-galactosidase activity. Values are means ± s.d. from triplicate samples and are representative of two independent experiments.

with and acetylate p53 (refs 32, 34). Confocal microscopic analysis of irradiated U2OS cells ($n = 200$) revealed that 100% of cells analysed contained speckles of PML staining. In a subset of these cells (10%), p53 accumulated in nuclear dots, which co-localized exactly with PML speckles (Fig. 5a, upper panel). Similarly, in U2OS cells, >95% of cells contained speckles of CBP staining. 100% of these CBP speckles co-localized with PML speckles (Fig. 5a, lower panel). In nonirradiated cells, a similar localization pattern was observed, however, the percentage of cells that contained p53 speckles was only 6%. To demonstrate that p53, CBP and PML co-localize in the same subnuclear compartment, we carried out triple staining using PML proteins tagged with green fluorescent protein (GFP, see Methods). In U2OS cells transfected with GFP–PML, endogenous CBP and p53 were recruited to PML-NBs (Fig. 5b), whereas in untransfected cells, p53 and CBP exhibited both speckled and diffuse nuclear staining. When p53 and GFP–PML were co-transfected into SaoS2 cells, they also co-localized in PML-NBs (Fig. 5c). These findings indicate that p53, CBP and PML can interact with each other in PML-NBs.

Localization of PML in PML-NBs is necessary to potentiate p53 transcriptional activation. The co-localization data led us to investigate whether PML can exert its co-transcriptional function on p53 in the PML-NB and whether this would require a direct PML–p53 interaction. We therefore transfected the various PML mutants shown in Fig. 4b into SaoS2 cells (under the control of the CMV promoter, see Methods) and tested their ability to potentiate p53 transactivation. Strikingly, the co-activation capacity of PML mutants such as PML(ΔRING) and PML(ΔRINGB1) was almost completely abrogated (Fig. 6a). These PML mutants could still physically interact with both p53 and CBP (Fig. 6b and ref. 16), but no longer accumulated in the PML-NB (Fig. 6c and data not shown). This is consistent with the observation that, whereas PML rescued the impaired p53 transactivation in *PML*$^{-/-}$ fibroblasts, PML(ΔRING) did not (Fig. 6d). The interaction of PML with p53 was also critical for co-activation, as PML(RBB) mutants, which do not interact with p53, were also transcriptionally inactive (Fig. 6a–d). Together, these data support the idea that the influence of PML on transcriptional activation by p53 is mediated by the physical interaction of these two proteins in the PML-NB.

p53–DNA binding and induction of p53 target genes are impaired in *PML*$^{-/-}$ thymocytes. p53 acts as a sequence-specific transcription factor, and acetylation and phosphorylation of p53 enhance its DNA-binding capacity[20,34,40]. We therefore investigated whether inactivation of *PML* would affect the ability of p53 to bind to DNA, by carrying out an electrophoretic mobility-shift analysis (EMSA). In the absence of PML, the DNA-binding ability of p53 upon γ-irradiation was impaired in *PML*$^{-/-}$ thymocytes (Fig. 7a). However, the mobility of the p53–DNA complex was not altered by the absence of PML, nor could an anti-PML antibody supershift the p53–DNA complex, indicating that PML may activate p53 before the p53–DNA interaction occurs (Fig. 7a and data not shown). This is supported by the fact that *in vitro*-translated PML did not enhance the DNA-binding activity of baculovirus-purified p53 (data not shown).

We next investigated whether p53-dependent induction of target genes such as *Bax* and *p21* would be affected by γ-irradiation in *PML*$^{-/-}$ thymocytes[30,35,36]. As recently reported, *Bax* and *p21* were not induced in *p53*$^{-/-}$ thymocytes upon ionizing irradiation (ref. 37 and data not shown). After 2 h of γ-irradiation, *Bax* was induced up to threefold in irradiated wild-type thymocytes at both the mRNA and protein levels (Fig. 7b and data not shown). Conversely, the induction of *Bax* upon γ-irradiation was profoundly impaired in *PML*$^{-/-}$ thymocytes, as assessed by northern and western blotting (1.3-fold; Fig. 7b and data not shown). At later time points (6–12 h), *Bax* was also induced in *PML*$^{-/-}$ cells (data not shown). Similarly, upon γ-irradiation, expression of *p21* mRNA was markedly induced in irradiated wild-type thymocytes, whereas *p21* induction was greatly impaired in *PML*$^{-/-}$ thymocytes (Fig. 7b). However, the induction of *Fas* and *Gadd45*, also putative p53 target genes, was unaffected in *PML*$^{-/-}$ thymocytes upon irradiation (Fig. 7b and data not shown). Once again, under these experimental conditions, the induction of p53 in response to DNA damage was comparable in wild-type and *PML*$^{-/-}$ cells (data not shown). Thus, the inactivation of PML impairs the ability of p53 to induce expression of target genes, such as *p21* and *Bax*, that are important mediator of the growth-inhibiting and pro-apoptotic functions of p53 upon DNA damage.

Discussion

We have drawn two principal conclusions. First, we have defined a new PML-dependent regulatory pathway for p53-dependent apoptosis. Inactivation of this pathway results in impaired DNA-damage-induced apoptosis in thymocytes as well as in other cell types, including bone marrow haemopoietic cells[13], thus protecting *PML*$^{-/-}$ mice from the lethal effects of ionizing radiation[13]. However, PML is also critical for DNA-damage-induced apoptosis of mitogen-activated

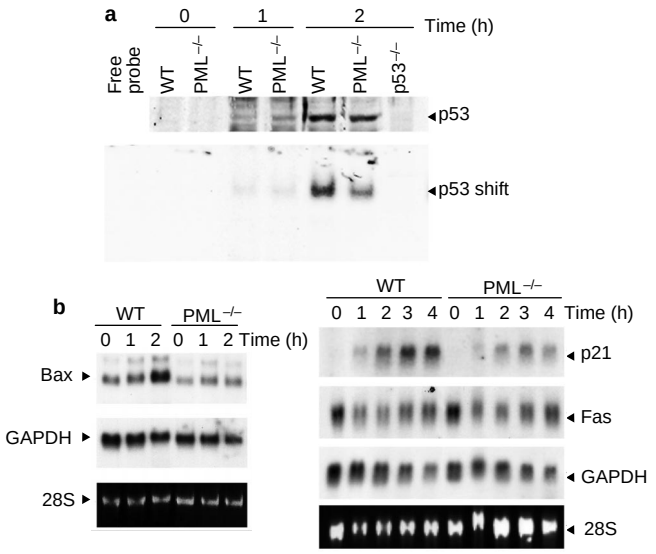

Figure 7 **Impaired p53–DNA binding and induction of p53 target genes in PML$^{-/-}$ thymocytes. a.** Impaired DNA-binding activity of p53 in *PML*$^{-/-}$ thymocytes upon γ-irradiation. Lower panel, EMSA using radiolabelled oligonucleotides spanning a p53 DNA-binding site. The specificity of the shifted bands was confirmed using the nuclear extract from irradiated *p53*$^{-/-}$ thymocytes (right lane). The specificity of the shifted bands was also confirmed by the fact that a 100-fold excess of a cold oligonucleotide containing the wild-type p53 binding site competed the shift, but a cold oligonucleotide containing a mutated p53 binding site did not (data not shown; see Methods). Upper panel, levels of p53 in the nuclear extracts used in the EMSA. Data shown are from one out of two experiments with similar results. **b**, Northern-blot analysis of expression of *Bax*, *p21* and *Fas* after γ-irradiation (5 Gy) in wild-type and *PML*$^{-/-}$ thymocytes. Each lane was loaded with 15 μg of total RNA. Expression levels of *GAPDH* mRNA and 28S rRNA were monitored as a loading control. Data shown are from one out of three experiments with comparable results.

mature splenic lymphocytes, which seems not to depend on p53 function[13]. In this respect, we have recently described a pathway for apoptosis that is dependent on Daxx and PML-NBs, and could mediate programmed cell death and the Fas response in B and T splenocytes[38]. A possible relationship between the p53 and the Daxx pathways remains to be explored, although splenocytes do not depend on p53 to undergo programmed cell death in response to Fas or to large doses of ionizing radiation[39]. Thus, PML can participate in both p53-dependent and p53-independent programmes for apoptosis. Together, these findings provide an explanation for the increased incidence of lymphoma observed in *PML*$^{-/-}$ mice when challenged with carcinogens[12].

Second, we have specified a distinct function of PML in this pathway as a p53 transcriptional co-activator that is required for proper transactivation of p53 target genes upon γ-irradiation. Our findings demonstrate that this transcriptional function depends on the ability of PML to localize into PML-NBs. Acetylation is one of the post-translational modifications that p53 undergoes in order to become transcriptionally active[34]. This occurs through interaction with acetyltransferases such as CBP and p300 (ref. 34). Although PML does not possess intrinsic acetyltransferase activity[15], it can directly interact with CBP (refs 15, 16). The reciprocal interactions between PML, CBP and p53 may be essential for the stability of the p53–CBP acetylation complex. This is supported by the fact that p53, PML and CBP co-localize in PML-NBs (Fig. 5b), and that the dimerization interfaces of p53, CBP and PML do not overlap (p53 interacts with CBP through amino acids 1–73 and with PML through amino acids 120–290; PML interacts with CBP through amino acids 216–331 and with p53 through amino acids 361–633; CBP interacts with PML through amino acids 357–452 and with p53 through amino acids 1,990–2,441; refs 15, 16, 32 and this present study). Thus, p53 and CBP could be recruited to PML-NBs for acetylation. Acetylation of p53 would in turn enhance its DNA-binding ability[34]. This idea is supprted by the finding that in *PML*$^{-/-}$ thymocytes, upon γ-irradiation-dependent induction, p53 can no longer accumulate in PML-NBs, and acetylation of p53 is reduced relative to that in wild-type thymocytes (A.G. Guo *et al*, unpublished observations). However, in *p53*$^{-/-}$ MEFs and SaoS2 cells, PML was still able to co-activate a p53(K382R) mutant (in which the lysine that is preferentially acetylated by CBP/p300 (lysine 382, refs 40, 41) was mutated to arginine) to a similar extent to its co-activation of wild-type p53, indicating that PML-NB-dependent transcriptional activation by p53 does not entirely dependent on acetylation by CBP.

PML is required for proper induction of p53 target genes such as *Bax* and *p21* in primary thymocytes in response to DNA damage, whereas the regulation of other genes such as *Fas* and *Gadd45* is not perturbed in the absence of *PML*. However, the regulation of these two genes upon damage to DNA is not solely dependent on p53 (refs 39, 42, 43).

PML interacts with the DNA-binding domain of p53, where the vast majority of p53-inactivating mutations are found[22]. Some of these mutations could affect the ability of p53 to bind to PML, resulting in impaired transcriptional activation by p53. Conversely, mutation or functional inactivation of PML could affect the transcriptional function of p53. This, combined with the ability of PML to control cell-cycle progression[12,15], genomic stability[44] and cell survival through p53-independent mechanisms[13,38], provides a strong basis for the tumour-suppressing function of PML, and indicates that functional inactivation of PML may underlie the pathogenesis of cancers other than APL. In APL, the PML–RARα oncoprotein, although it does not seem to efficiently interact with p53, may disrupt the PML/p53 pathway as a result of its ability to physically interact with and sequester PML, which in turn disrupts the PML-NB[5,6]. Furthermore, as PML modulates p53 activity in a dose-dependent manner, the reduction of PML to heterozygosity in APL, in view of the chromosomal translocation, could further impair the transcriptional function of p53. This is consistent with the observed protection from several pro-apoptotic stimuli that is conferred by the PML–RARα oncoprotein[13,45], as well as with the surprising observation that in APL, unlike in other acute myeloid leukaemia subtypes, p53-inactivating mutations are extremely rare[46]. □

Methods

Cell culture.

Thymocytes were isolated from 5–8-week-old littermates matched for age and sex. After irradiation with a Cs^{137} irradiator (0.78 Gy min^{-1}), cells were cultured for the indicated times in RPMI medium containing 10% FBS and 5μM β-mercaptoethanol. Mouse primary embryonic fibroblasts were prepared from embryos at day 13.5 of development (E13.5), and cultured in DMEM with 10% FBS. HeLa, SaoS2, COS-1 and U2OS cells were cultured in DMEM with 10% FBS. MCF7 cells were cultured in RPMI medium with 10% FBS.

Plasmids and cell transfections.

p21min–luc, GADD45–luc and Bax–luc reporter plasmids were from C. Prives[47]; PG13–luc and MG15–luc[29] were from K. Scotto. The PML expression vector pSG5–PML was previously described[15]. PML deletion mutants were created by the polymerase chain reaction (PCR), re-sequenced and cloned into the pCMV-Tag 2B vector (Flag-Tag, Stratagene). MEFs were used at early passage (3–5) for transfection in 6-well plates. All transfection experiments were carried out using Effectene (Qiagen) according to the manufacturer's instructions. A TK–β-gal plasmid was co-transfected to normalize the transfection efficiency; the total amount of transfected plasmid was made equal by addition of empty pSG5 or pCMV-Tag2B vector. Luciferase activity was assayed 40 h (SaoS2 cells) or 48 h (MEFs) after transfection, using a luminometer.

GST pull-down.

Various domains of p53 were cloned into the relevant pGEX vector using PCR or engineered restriction sites. Recombinant proteins were expressed in and purified from BL21 cells as described[34]. PML deletion mutants were generated as described above. *In vitro*-translated products were generated using the TNT-coupled system (Promega). The ^{35}S-labelled wild-type PML and PML mutants were incubated with various immobilized GST-fusion proteins at 4 °C for 60 min. Beads were then washed 5 times in 1 ml BC200 buffer (20 mM Tris–HCl pH 8.0, 0.5 mM EDTA, 20% glycerol, 1mM dithiothreitol

(DTT) and 0.5 mM phenylmethyl sulphonyl fluoride (PMSF)) containing 200 mM KCl and 0.2% NP40. Bound proteins were eluted with SDS sample buffer and resolved by SDS–PAGE for autoradiography.

Western blotting and immunoprecipitation.

The following antibodies were used: anti-p53: DO-1 (Santa Cruz), pAb421 (Calbiochem) and pAb1801 (Santa Cruz); anti-PML: pG-M3 (Santa Cruz) and polyclonal anti-PML[13,38]; anti-Flag: M2 (Sigma). For immunoprecipitation, cells were lysed in E1A buffer (250 mM NaCl, 50 mM HEPES pH 7.0, 0.1% NP40 and 5 mM EDTA) supplemented with a complete protease-inhibitor cocktail (Boehringer), and precleared by incubating with 0.5% goat serum and protein A–sepharose beads (Pharmacia) for 1 h. The precleared lysate was then incubated with anti-p53 (pAb421 and pAb 1801) or anti-PML (pG-M3) antibody overnight at 4 °C. Protein A–sepharose was added and the immunoprecipitate was incubated for 1 h at 4 °C. After washing beads 5 times with E1A buffer, an equal volume of 2 × SDS sample buffer was added and the sample was boiled for 4 min and then loaded onto SDS–polyacrylamide gel. Western blotting was carried out according to standard procedures using anti-p53 DO-1 or anti-PML polyclonal antibody.

EMSA.

Oligonucleotides containing wild-type (wtp53) or mutated (mtp53) canonical p53-binding sites (wtp53: forward, 5′-GGAACATGTCTTGACATGTTC, reverse, 5′-GAACATGTCAAGACATGTTCC; mtp53: forward, 5′-GGAATATATCTTGAATTCTTC; reverse, 5′-GAAGAATTCAAGATATATTCC) were annealed and end-labelled by T4 polynucleotide kinase. Binding reactions for gel-retardation assays were set up in a 20-µl volume by incubating probes (50,000 c.p.m.) and 1 µg of poly[d(I-C)], 0.1 µg anti-p53 antibody pAb421 with 6 µg of nuclear extract from mouse thymocytes in binding buffer (40 mM KCl, 20mM HEPES pH 7.5, 1 mM$MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT and 0.4% Ficoll). The reaction was incubated 20 min at room temperature and then loaded onto 5% polyacrylamide gel. The gel was then dried and autoradiographed. Reactions were also carried out in the absence of anti-p53 antibody. This analysis revealed a comparable difference in the DNA-binding ability of p53 between wild-type and *PML*$^{-/-}$ samples, but, as previously reported[34], the signal obtained in the EMSA experiments was much weaker (data not shown).

Immunofluorsescence and confocal microscopy.

For immunostaining of U2OS cells, irradiated (6 Gy, 4 h) or non-irradiated U2OS cells were cytospun on slides. Cells were fixed in 4% paraformaldehyde for 10 min at room temperature, permeabilized for 1 h in PBS containing 10% goat serum and 0.3% Triton-100, and stained with monoclonal anti-PML (pG-M3), polyclonal anti-p53 (FL-393, Santa Cruz), and polyclonal anti-CBP (A22, Santa Cruz) antibodies. For triple staining, U2OS cells were transfected with GFP–PML; SaoS2 cells were co-transfected with p53 and GFP–PML. Forty hours after transfection, cells were collected and cytospun on glass slides. Cells were fixed and permeabilized as above, and stained with monoclonal anti-p53 (DO-1) and polyclonal anti-CBP (A22) antibodies for 3 h at room temperature. For detection, cells were incubated with a secondary-antibody mix containing rhodamine-conjugated goat anti-mouse and cyanine5-conjugated goat anti-rabbit immunoglobulin G antibodies (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Slides were then mounted in antifade mounting medium and analysed by confocal microscopy in the institute core facility.

Northern blotting.

Northern blotting was carried out as described[13]. Total cellular RNA was extracted from wild-type and *PML*$^{-/-}$ thymocytes at the indicated times after γ-irradiation (5 Gy) using Trizol (Life Technologies), and hybridized with complementary DNA probes for mouse *p21*, *Bax*, *Gadd45* and *Fas*. Levels of gene expression were quantified with a phosphorimager (Bio-Rad).

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Correspondence and requests for materials should be addressed to P.P.P.