Promyelocytic Leukemia Protein is Required for Gain of Function by Mutant p53

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Abstract

Mutations in the p53 tumor suppressor are the most common genetic events in human cancer. These mutations not only result in a loss of wild-type p53 activity, but can also lead to a gain of new oncogenic properties. Understanding how these gained functions are regulated is in its infancy. In this study, we show that the promyelocytic leukemia (PML) protein is an important regulator of mutant p53. We show that PML interacts with mutant p53. Importantly, PML enhances the transcriptional activity of mutant p53. Unexpectedly, PML is required for the proliferation and colony formation of cancer cells bearing mutant p53. Down-regulation of PML expression inhibits the growth of mutant p53-expressing cancer cells, predominantly by promoting cell cycle arrest. Our results suggest that the tumor suppression function of PML depends on the status of p53. In the context of mutant p53, PML enhances its cancer-promoting activities. [Cancer Res 2009;69(11):4818–26]

Introduction

P53 mutation can corrupt the tumor-suppressive functions of the wild-type (wt) protein. In response to specific cellular stresses, wt p53 either initiates a temporary interruption of the cell cycle to enable DNA repair; or triggers cellular senescence or apoptosis when damage is excessive (reviewed in ref. 1). In contrast, cells bearing common p53 mutations are released from these constraints. Furthermore, mutant p53 may acquire distinct properties from its wild-type counterpart, referred to as a "gain of function" (GOF) phenomena (reviewed in ref. 2). The contribution of certain p53 mutations to chemotherapeutic drug resistance was shown in human cancer cells cultured *in vitro* (reviewed in refs. 3, 4). A role for mutant p53 in the development and spread of the tumors was also corroborated by these studies. The most compelling evidence for the GOF of mutant p53 was shown in knock-in mutant p53 mice, which developed tumors with a distinct spectrum from p53+/− or p53−/− mice. Furthermore, tumors in these knock-in mice exhibited an enhanced metastatic potential (5, 6).

Wt p53 is subject to tight regulation that is affected through protein-protein interactions and by extensive posttranslational modifications (7, 8). In contrast, less is known about the regulation of mutant p53, although mutant p53 is subject to at least certain modifications (8). Strikingly, higher mutant p53 levels have been identified in (most) tumor cells than in the surrounding healthy tissues of mutant p53 knock-in mice (9). Recent work by Terzian and colleagues (10) showed that, as in the case of wt p53, the stabilization of mutant p53 is regulated by Mdm2. Mice lacking *mdm2* express higher levels of mutant p53, succumb earlier to cancer onset and develop metastatic tumors (reviewed in ref. 11).

Certain modifications of wt p53 are regulated by the promyelocytic leukemia (PML) protein (12). PML is a key factor in the formation of PML nuclear bodies, which are distinct nuclear multiprotein complexes that have been associated with critical cellular processes, including tumor suppression, gene regulation, posttranslational modifications, and protein catabolism (reviewed in refs. 12, 13). PML KO mice develop normally, but are resistant to lethal doses of γ-ionizing irradiation (γ-IR; ref. 14). In addition, they are prone to tumorigenesis in response to carcinogens (15), or an additional oncogenic event, such as the loss of PTEN (16). In humans, a complete or partial loss of PML has been observed in multiple types of cancers, including breast colon and prostate (17). PML is considered as a bona fide tumor suppressor.

PML regulates some of the key modifications of wt p53 (reviewed in ref. 12). We have previously shown that NH_2-terminal phosphorylations of p53 are facilitated by PML. These include serine 20 (Ser^{20}) by checkpoint kinase 2 (18), and more recently, threonine 18 (Thr^{18}) by CK1 (19). Because mutant p53 is also subjected to posttranslational modifications, it is pertinent to question whether PML also regulates mutant forms of p53. Here, we addressed this question and found that PML interacts and colocalizes with mutant p53. Surprisingly, we found that PML activates mutant p53 transcriptional activity and is important for its gain of function in cultured human cancer cells. Our results support the notion that as is the case for wt p53, PML is a key regulator of mutant p53. The implications to anticancer treatment are discussed.

Materials and Methods

All materials were purchased from Sigma Chemical, Co., unless otherwise stated. Solvents were analytical grade and water was double distilled. All experiments were performed in triplicate and repeated at least thrice.

Cell culture. Human colon adenocarcinoma cell lines HT29 [mutant p53(R273H); $p53^{273}$], SW480 mutant p53(R273H/P309S; $p53^{273/309}$), human colon carcinoma cell line HCT116 p53+/+ and its engineered counterpart HCT116 p53−/− (B. Vogelstein) and breast cancer cell lines SKBR3 [containing mutant p53(R175H); $p53^{175}$] and MCF7 (wt p53) and their derivatives, were cultured in DMEM containing 10% FCS (Biological Industries). Promyelocytic leukemia cells NB4 [mutant p53(R248Q/R273H), $p53^{248/273}$; ref. 20], human lung adenocarcinoma cell line H1299 lacking p53 expression, and its derivatives stably expressing either mutant

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-08-4010

$p53^{273}$ or $p53^{175}$ (ref. 21) were grown in RPMI medium supplemented with 10% FCS.

Knockdown of p53 and PML. Cell lines with stably knocked-down p53 were generated by transduction using p53 short hairpin RNA (shRNA; ref. 22; shp53) inserted into a retroviral vector (pRetro-SUPER) and polyclonal populations were selected with puromycin; or p53 was transiently by transfection with short interfering RNA (siRNA) to p53 (sip53; ref. 23). Cell lines in which PML was inducibly knocked-down were generated by coinfection with a lentivirus (pLVTHM) expressing the shRNA PML target sequence GACCAACAACATCTTCTGC ($shPML_1$) or AGATGCAGCTGTATCCAAG (ref. 24, $shPML_2$) and a lentivirus expressing a tetracycline repressor (pLV-Ttrkrab-Red). Control cell lines with shLacZ and relevant wobble shPML control sequences GACCAACAATATATTCTGC (w-$shPML_1$) and AGATGCAGCTGTATCCAAG (w-$shPML_2$) were also generated under the control of a tetracycline repressor. PMLi to target sequence GAGTCGGCCGACTTCTGGT (25), referred to subsequently as PML_3i was transfected into SKBR3 cells using LipofectAMINE 2000 (Invitrogen Corporation), according to the instructions of the manufacturer.

Western blot analysis and immunoprecipitation. Western blot analysis and immunoprecipitation assays were carried out as previously described (18). For mapping the interaction site in PML, the following plasmids were used: expression plasmids for mutant $p53^{273}$, $p53^{175}$, and Flag-tagged PML deletion mutants (PMLRBB, PML RBCC, PMLδRINGB1; ref. 14). The antibodies used in this study were anti-human p53 monoclonal antibodies PAb1801 and DO1, anti–phospho-p53 Thr^{18} polyclonal antibody (26), anti–phospho-p53 Ser^{20} polyclonal antibody (Cell Signaling Technology); anti-PML monoclonal antibody (clone PML-97; Sigma Chemicals), anti-Flag (Sigma Chemicals), anti-GFP (Roche Applied Science), HRP-conjugated goat anti-mouse IgG, Envision peroxidase anti-mouse, or anti-rabbit (Dako, Corp.). The antibodies anti-p53 goat polyclonal antibody (FL-393, AC), anti-p53 rabbit polyclonal antibody (FL-393, FC), and anti-PML polyclonal rabbit antibody [(H-238)sc-5621] were from Santa Cruz Biotechnology.

Transactivation assays. The dual luciferase assay was performed in triplicate according to the instructions of the manufacturer (Promega Corporation). Briefly, for H1299 and its derivatives (200,000/3.5 cm dish) were transiently cotransfected using PEI with reporter constructs pCCAAT-B2LUC (27) or pCCAAT-cdc25CLUC (28) 1 μg, 6 μg of plasmid expressing PML IV, or an equal amount of empty vector and 0.25 ng of Renilla luciferase SV40 reporter (Promega Corporation). After 48 h, the fold change in relative firefly to Renilla luciferase activity was compared between cell lines without p53 to those bearing either exogenously expressed $p53^{273}$ or $p53^{175}$ mutants.

For LacZi-SKBR3 and PML_3i-SKBR3, cells (1.5×10^5) were transiently transfected with expression plasmids, reporter constructs, and 0.5 μg of CMV-β-galactosidase plasmid (pCDNA3-β-gal vector) as an internal control for transfection efficiency. Precipitates were removed and cells were treated with 0.5 μg/mL of ADR for 48 h. Luciferase activity was assayed on whole-cell extract, as described (28). The luciferase values were normalized to β-galactosidase activity and protein content.

Flow cytometry analyses: analysis of live cells. Cells were cultured in the absence or presence of doxycycline (0.2 μg/mL added every second day) for 3 days; then, 25,000 cells were plated (with doxycycline maintenance in culture for continued shRNA induction) and harvested at selected time points. Cells adherent to the plate were released with trypsin-EDTA and combined with those suspended in the culture supernatant prior to flow cytometric analysis of live cell numbers, as discriminated by propidium iodide exclusion using a cell sorter (FACSCalibur) harnessed to CellQuest software (BD Biosciences). Cell cycle analysis of fixed cells was done as previously described (18).

BrdUrd. Cells were plated 2×10^6/10 cm dish and allowed to recover for 24 h. BrdUrd was incorporated (20 μmol/L, 4 h), prior to cell harvest with trypsin-EDTA and fixation in 70% methanol at −20°C overnight. BrdUrd detection was undertaken subsequent to sequential rehydration in HCl(2N)/Triton X (0.5%) then incubation in boric acid (0.1 mol/L), and anti-BrdUrd FITC antibody (BD Biosciences) in PBS/BSA (1%)/Triton X (0.5%). DNA was labeled using propidium iodide (0.5 mg/mL) and incorporation was analyzed using the FACSCalibur (BD Biosciences).

Colony formation assay. Cancer cell lines were plated (700/well) in 5 cm diameter dishes in the absence or presence of tetracycline (0.2 μg/mL added every second day) for 21 days. At harvest, the cells were fixed in ethanol and stained in 0.1% crystal violet.

MTT metabolic assay. Cells either untreated or treated with doxycycline (0.2 μg/mL) to knock down PML were plated 4,000 per well in a 96-well plate and allowed to recover for 24 h. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT; 0.5 μg/mL final concentration) was added to the wells and the experiment was terminated after an additional 4 h incubation, by aspiration of the liquid and addition of 200 μL of DMSO. The absorbance was read at 540 nm, relative to a reference wavelength of 630 nm (29).

Reverse transcription-PCR (RT-PCR) analysis. Cellular RNA was isolated by Tri-Reagent (MRC, Inc.) using the manufacturer's instructions, DNA was removed from the samples using DNase treatment (DNA-free kit; Ambion Applied Biosystems), cDNA was synthesized from the purified RNA using Moloney murine leukemia virus reverse transcription kit (Promega). Primers for ribosomal 18S were forward, ctaccacatccaaggaaggc, and reverse, aagaatttcacctctagcggc. Primers for PML were forward, cgccctggataacgtcttt, and reverse, actgtggctgctgtcaagg. Primers for cyclin B2, for cdc252c and for adolase were as previously reported (23).

Immunofluorescent staining analysis. For immunofluorescent staining, cells were plated on coverslips. Twenty-four hours later, cells were washed and fixed with 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. p53 was visualized with the simultaneous exposure of DO1 and 1801 antibodies in conjunction with goat anti-mouse FITC-conjugated secondary antibody or Cy2-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). DNA was stained with DAPI (4′,6′-diamidino-2-phenylindole). PML was detected using anti-PML polyclonal antibody rhodamine conjugate (PG-M3 TRITC; sc-966; Santa Cruz Biotechnology) or Cy5-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Proteins were visualized by confocal fluorescence microscopy using a Zeiss 410 microscope (PlanApochromat ×40), and Olympus FV1000 microscope (×60), using FluView 1000 v.1.5 software.

Results

Mutant p53 and PML interact and colocalize. We have previously shown that PML facilitates the phosphorylation of wt p53 at Ser^{20} by checkpoint kinase 2 (18) and Thr^{18} by CK1 (19). These observations raised the possibility that PML may also facilitate the phosphorylation of mutant p53. To investigate this suggestion, NB4 acute promyelocytic leukemia cells were exposed to ionizing irradiation in order to induce p53 phosphorylation in the absence of active PML, or in the presence of arsenic trioxide, which activates PML. Consolidating previous findings (19), activation of PML by arsenic trioxide facilitated IR-induced phosphorylation of p53 on Thr^{18} (Supplementary Fig. S1), suggestive of PML involvement.

Wt p53 and PML (isoform IV, formerly PML3) interact and colocalize (30) upon exposure to stress (18, 31). The observed PML-mediated phosphorylation of mutant p53 provoked the notion that mutant p53 and PML may also interact and colocalize. To examine for PML and mutant p53 interaction, the following mutant p53-expressing cell lines were studied: HT29 ($p53^{273}$), SW480 ($p53^{273/309}$), and SKBR3 ($p53^{175}$). As controls, the wt p53-expressing cell lines MCF7 and HCT116 p53+/+ were studied. Genotoxic stress was induced either by exposure to UV light (40 μJ ×100), or by treatment with doxorubicin (2 μg/mL, 1 hour) as indicated. Extracts from mutant p53-bearing cells were sequentially subjected to coimmunoprecipitation using anti-p53 antibodies (FL393 goat), followed by PML blotting (Fig. 1*Ai–iii*). Conversely, due to low endogenous p53 levels, extracts of wt p53-expressing cells were immunoprecipitated with anti-PML antibodies prior to p53

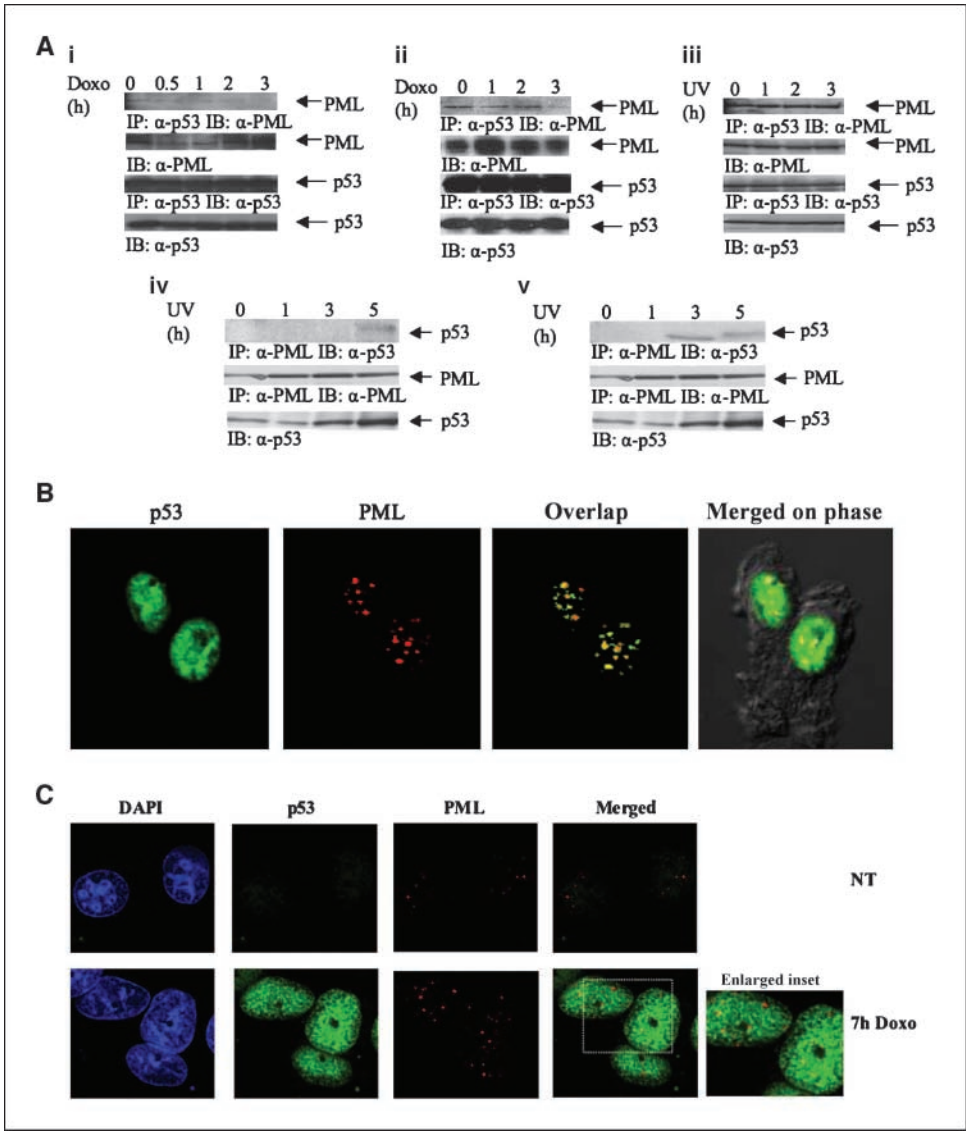

Figure 1. Mutant p53 and PML interact without extracellular stress. *A,* cells bearing either mutant (*i,* HT29; *ii,* SW480; and *iii,* SKBR3) or wt p53 (*iv,* HCT116 p53+/+; and *v,* MCF7) were subjected to UV (40 μJ ×100) or doxorubicin (2 μg/mL, 1 h) as indicated, harvested at designated times, and subjected to immunoprecipitation and blotting with either a consecutive combination of polyclonal antibodies to p53 (FL393 goat) then PML (Sigma Chemicals; *i–iii*), or PML (Sigma Chemicals) then polyclonal antibodies to p53 (FL393 rabbit; *iv* and *v*). *B,* SW480 cells were plated on coverslips and 24 h later were fixed and subjected to immunofluorescent staining. p53 was stained with the simultaneous exposure of DO1 and 1801 antibodies in conjunction with goat anti-mouse FITC-conjugated secondary antibody (*green*). PML was detected using anti-PML polyclonal antibody rhodamine conjugate (*red,* PG-M3 TRITC). Cells were visualized using an Olympus FV1000 confocal microscope (×60 objective, zoom 2.5). *Right,* merged images (*yellow,* colocalization). *C*, Mcf7 cells bearing wt p53 were plated on coverslips and 24 h subsequently were alternatively either left untreated or exposed to doxorubicin (2 μg/mL for 1 h), washed out and harvested after an additional 6 h. Fixed cells were either DNA-stained with DAPI or with anti-PML polyclonal antibodies in conjunction with Cy5-conjugated secondary antibody (*red*) and p53 monoclonal antibodies followed by Cy2-conjugated secondary antibody (*green*).

blotting (Fig. 1*Aiv–v*). This analysis revealed a temporally distinct interaction between PML and mutant or wt p53, respectively. Mutant p53 was identified to be associated with PML in the absence of additional external genotoxic stress. In contrast, wt p53 association with PML was enhanced in a time-dependent manner subsequent to UV exposure. This increase correlates well with the increase in wt p53 levels.

Given the interaction between PML and mutant p53, the possible colocalization of the two proteins in SW480 was examined using immunofluorescence staining. Critically, localization of endogenous mutant p53 and PML was detected in these cells. The choice of studying these proteins at endogenous levels was made to avoid the criticism that transfected proteins often colocalize with PML in the PML-NBs, due to their overexpression. Stained cells were analyzed by confocal microscopy (Fig. 1*B*). Although mutant p53 seems to be expressed throughout the nucleus, PML was localized to the PML-NBs. We therefore analyzed the extent of colocalization (using the FluView 1000 v.1.5 software) from the perspective of PML, and identified a significant extent of colocalization of mutant p53 (~10%). This observation that without additional exogenous stress, mutant p53 interacts with PML and colocalizes with it in the PML-NBs (Fig. 1*B*), is reminiscent of the interaction of genotoxically stressed wt p53 with PML, as observed with MCF7 cells stressed with doxorubicin (Fig. 1*C*) and RKO (Supplementary Fig. S2).

Knockdown of PML reduces the number of mutant p53-bearing cancer cells. To assess the influence of PML in human cancer cell lines bearing endogenous mutant p53, PML expression was knocked down using RNA interference. HT29 and SW480 were transduced with lentivirus encoding shRNA to PML. The efficacy of PML down-regulation was evaluated by Western blotting using an antibody that detects multiple PML isoforms (clone PML-97; Sigma Chemicals). Under the influence of $shPML_1$, expression of major PML isoforms was reduced in HT29 and SW480 (Fig. 2*A* and *B*, respectively). Control shRNA to LacZ (shLacZ) and wobble shRNA to PML (w-$shPML_1$), failed to reduce PML levels, indicating the specificity of PML knockdown. A reduction in PML levels in response to $shPML_1$ was also confirmed at the mRNA level. RT-PCR directed to a region common to multiple isoforms of PML revealed a reduction in PML transcript, consistent with the results observed

at the protein level (Fig. 2*C* and *D*). Of note, PML knockdown did not influence the p53 RNA levels in these cells (data not shown).

The influence of PML knockdown on the growth of HT29 and SW480 was examined. $ShPML_1$ was induced by growing the cells in the presence of doxycycline. Live cell numbers were measured at selected time intervals by flow cytometry. Dead cells were excluded using propidium iodide staining. Down-regulation of PML (induced by 7 or 8 days of doxycycline exposure) significantly reduced the number of live cells in HT29 and SW480 cultures (Fig. 3*A* and *B*, respectively). Further confirmation of the influence of PML down-regulation was provided with $shPML_2$ in HT29 and SW480, where similar results to those using $shPML_1$ in Fig. 3 were recorded (data not shown). Depletion of endogenous mutant p53 has been shown to reduce colony formation competence (32), whereas over-expression of mutant p53 proteins in p53-null cells was shown to enhance plating efficiency (21, 33–35). We therefore measured the effect of PML depletion on colony formation of the mutant p53 cell lines HT29 and SW480 (graphed in Fig. 3*Bi* and *Bii*, respectively; with representative plates in Fig. 3*C*). PML knockdown in HT29 almost completely blocked colony formation (>90% inhibition), and in SW480, >70% inhibition was observed. This result is consistent with PML knockdown reducing cancer cell numbers. Importantly, down-regulation of PML (for 8 days) in cells lacking p53, HCT116 p53−/− (Supplementary Fig. S3), had no significant effect on their proliferation (Fig. 3*D*), demonstrating that the observed effect on growth was dependent on mutant p53.

Two possible explanations were envisaged for these reductions in viable cell numbers: these included a greater susceptibility to cell death, or a reduced rate of proliferation. To test the first possibility, the numbers of dead HT29 and SW480 cells were measured in the presence of shPML or controls. Flow cytometric analysis using propidium iodide exclusion revealed a modest increase in cell death after 7 or 8 days of PML down-regulation for HT29 and SW480 (Fig. 4*Ai* and *ii*, respectively).

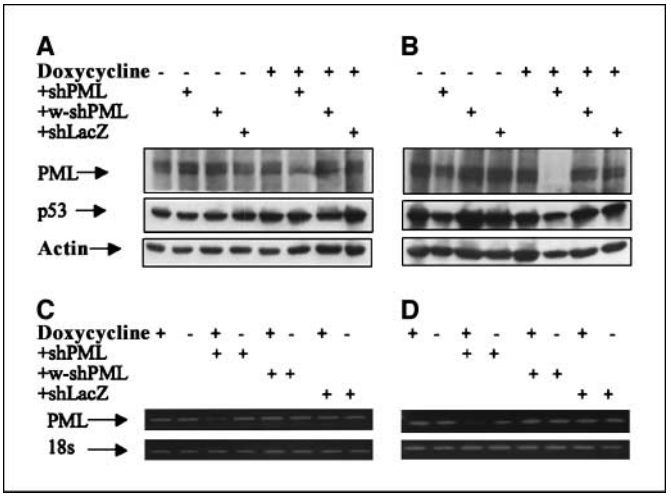

Figure 2. Knockdown of major PML isoforms was achieved with shRNA technology. Cell lines HT29 (*A*) and SW480 (*B*) either nontransduced or transduced with $shPML_1$, w-$shPML_1$, or shLacZ were exposed to doxycycline for 72 h for PML knockdown as indicated. Immunoblotting was performed with anti-PML (Sigma Chemicals), anti-p53 (DO1 and 1801), and anti-actin monoclonal antibodies. RT-PCR examination of these cell lines corroborated the reduction in PML in these HT29 and SW480 cells (*C* and *D,* respectively), in which PML levels were normalized to 18s.

To ascertain the influence of PML levels on the proliferation of HT29 human cancer cells, they were subjected initially to metabolic evaluation using an MTT assay and cell cycle analysis using BrdUrd incorporation. MTT analysis identified a slightly lower rate of metabolism in HT29 cells expressing $shPML_1$ as compared with control w-shPML (Fig. 4*B*), after 3, 4, and 11 days of doxycycline exposure. This reduction was consistent with the reduced growth rate, but cannot explain the marked difference in cell numbers. It is important to clarify that because the MTT assay involved plating cells of equal number 24 hours in advance of each assay time point, there was a continual selection for live cells, which is likely to have masked the accrued effects detected with the longer-term plating for the flow cytometric analysis and the colony assay. After 3 days of doxycycline induction, the relative number of cells cycling through the S phase was comparable for the parental line and those expressing w-shPML and $shPML_1$, as assessed by BrdUrd incorporation, with a slightly higher level of G_1 and a lower level of G_2 for cells expressing $shPML_1$ (data not shown). Significantly, after 8 days of continuous PML knockdown, the level of BrdUrd incorporation into the S phase was reduced markedly, with corresponding increases in G_1 and G_2 (Fig. 4*C*). To further substantiate the mutant p53 dependence of cell cycle perturbation induced through PML knockdown, H1299 cells either without, or bearing exogenous mutant $p53^{175}$, were subjected to cell cycle analysis and flow cytometry (Supplementary Fig. S4). Although the cell cycle profile of p53-null H1299 cells exposed to either a control siRNA vector, PML_3i, or siRNA to p53 were not disrupted; a striking increase in G_2 was shown in response to either siPML or shp53 in the mutant p53 context (Fig. 4*D*). This data further substantiates the observed PML dependence of mutant p53-bearing cancer cells for cell cycle progression (as seen for HT29 in Fig. 4*C*).

PML IV enhances the transcriptional activity of mutant p53 and interacts through its COOH terminus with mutant p53. The next obvious question was, at what level does PML benefit the survival of these mutant p53-bearing cancer cell lines? PML has been shown to enhance the transcriptional activity of wt p53 (18). Because mutant p53 has transcriptional activity (ref. 23 and reviewed in ref. 4), it was of interest to examine whether PML IV (the isoform identified to interact with wt p53; ref. 31) influences this activity of mutant p53. For this purpose, we used a luciferase reporter assay in p53-deficient H1299 lung carcinoma cells to measure the effect of PML-IV (referred to as PML hereafter) on the activation of a number of well characterized transcriptional targets of mutant p53 (23). Parental and H1299 cells stably expressing exogenous p53 mutants (R273H and R175H) were transfected with luciferase reporter plasmid under the control of cyclin B2 or Cdc25C promoters. In contrast to the known repression of cyclin B2 by wt p53 (ref. 28; data not shown), mutant $p53^{175}$ induced the cyclin B2 promoter, consistent with previous studies (ref. 23; Fig. 5*A*). In this assay, mutant $p53^{273}$ had no major effect on this promoter, as previously reported (23). Strikingly however, inclusion of PML enhanced the reporter activity of this promoter under the influence of both these p53 mutants. Similarly, expression of PML enhanced the induction of the Cdc25C promoter (Fig. 5*B*). It should be noted that expression of PML alone also enhanced the activity of this promoter in a mutant p53-independent manner by an unknown mechanism. Nevertheless, these results show that expression of PML enhances the transcriptional activity of at least two forms of mutant p53.

To define the nature of the interaction between mutant p53 and PML, PML IV domain mutants bearing a Flag-tag were transfected

together with either mutant $p53^{273}$ or $p53^{175}$ into H1299 and coimmunoprecipitations were performed by immunoprecipitation using anti-p53 and immunoblotting using anti-Flag antibody. The PML IV carboxyl-terminus was identified as the region interacting with the p53 mutants (Fig. 5*Ci* and *ii*, respectively).

Down-regulation of PML reduces the growth of cells expressing mutant p53 in response to genotoxic stress. Expression of certain p53 mutants confers cells with some resistance to genotoxic stress (2). It was therefore important to define whether PML contributes to this biological effect of mutant p53. To this end, PML expression was down-regulated in SKBR3 cells and cells were exposed to 0.5 μg/mL of Adriamycin for 24 hours. The effect on cell survival was determined by trypan blue dye exclusion. Down-regulation of PML reduced the numbers of live cells (Fig. 6*Ai*), whereas no significant increase in cell death was identified (Fig. 6*Aii*). Importantly, this reduction in cell numbers did not correspond to a significant increase in cell death (consistent with short-term PML down-regulation in HT29 and SW480; Fig. 4*Ai* and *ii*; respectively).

In addition, the effect of PML down-regulation on the transcription of the mutant p53 targets cyclin B2 and Cdc25C, in response to genotoxic stress, was examined in the endogenous mutant $p53^{175}$ background of SKBR3 cells. SKBR3 cells were transfected with either PML_3i or sip53, and luciferase reporter

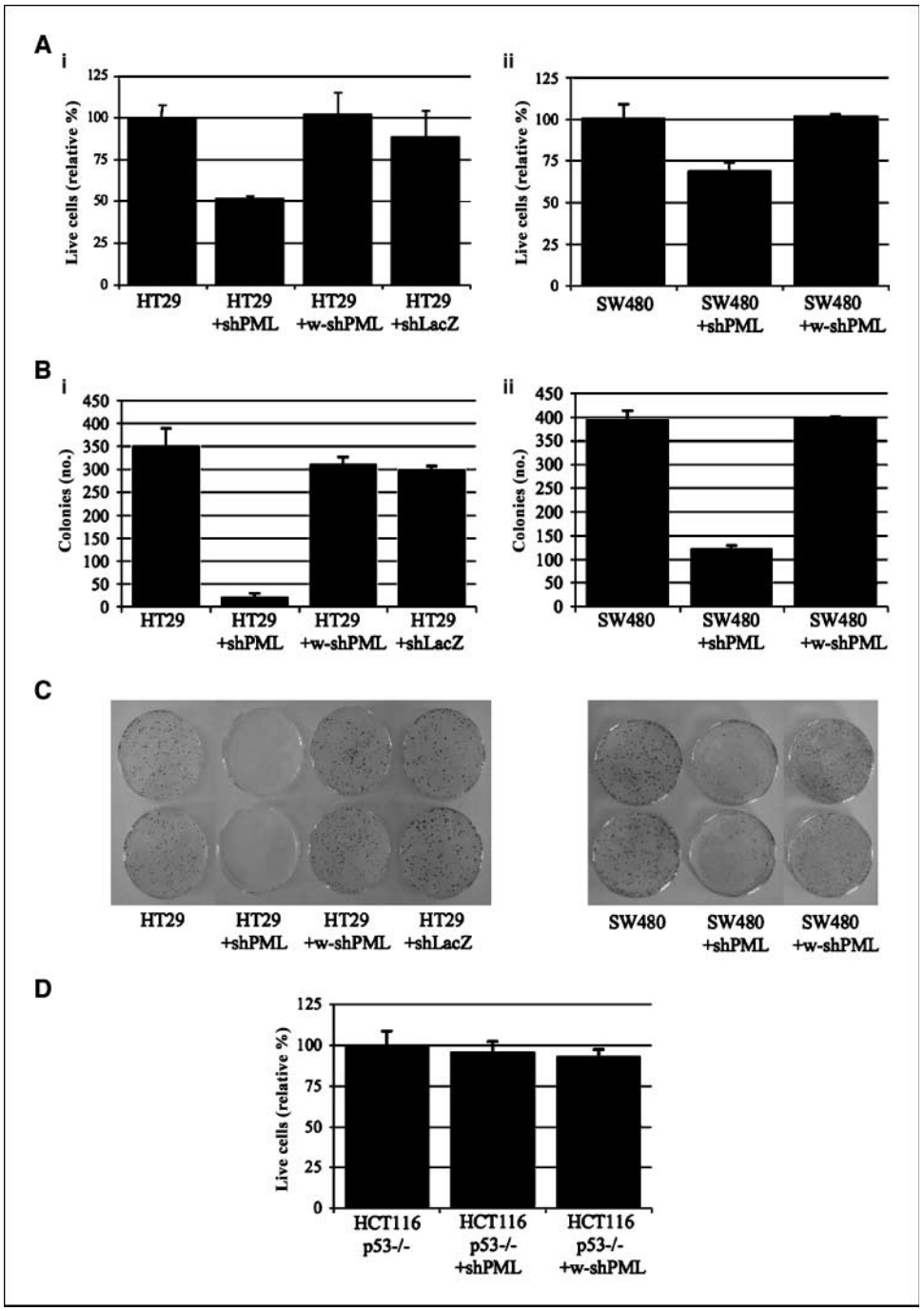

Figure 3. Knockdown of PML inhibited cell proliferation in mutant p53 cell lines. Cell proliferation decreased in HT29 (*A*) and SW480 (*B*) cells when subjected to PML knockdown (induced with doxycycline exposure) for 7 or 8 d, as identified using flow cytometry to distinguish propidium-negative live cells. Colony formation was significantly impaired in HT29 (graphed in *Bi*, with representative plates in *C*) and SW480 (graphed in *Bii,* with representative plates in *C*) cells subjected to doxycycline-induced PML knockdown for 21 d. In contrast, HCT116 p53−/− cells were not growth-inhibited by PML knockdown as assessed by flow cytometry of the propidium iodide–excluded live cell population, 8 d after transduction: HCT116 p53−/− not transduced (*D, column 1*), transduced for $shPML_2$ without repressor (*column 2*), or w-$shPML_2$ without repressor (*column 3*).

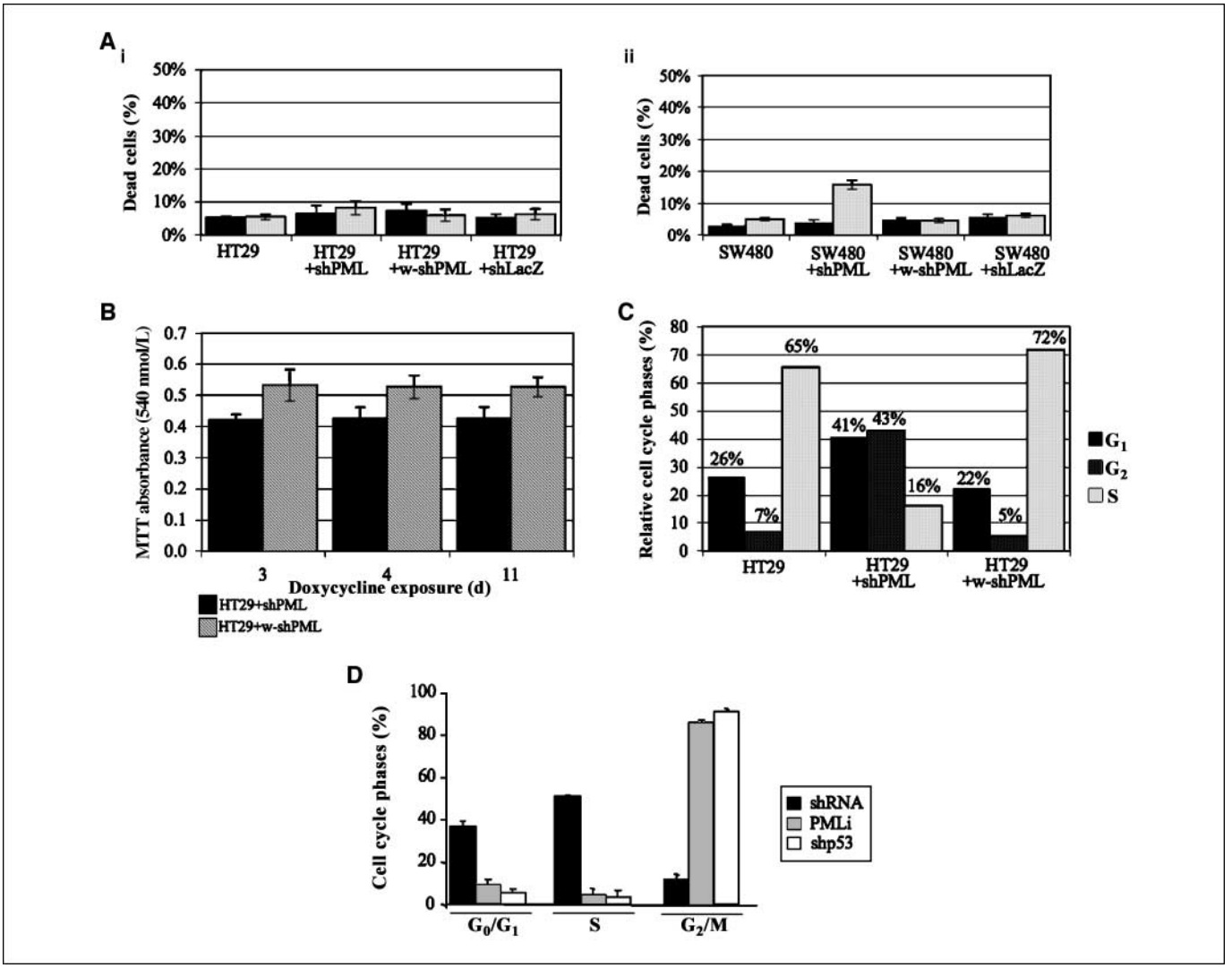

Figure 4. Knockdown of PML affects cell cycle progression in a mutant p53-dependent manner, with only a modest effect on cell death or metabolism. *A,* knockdown of PML induced little additional cell death in the mutant p53-bearing lines HT29 and SW480, as assessed with propidium iodide exclusion using flow cytometric analysis. shPML expression was induced by doxycycline exposure in HT29 (for 7 d; *i*) and SW480 (for 8 d; *ii*). *B,* MTT metabolic assay of HT29 cells transformed with either $shPML_1$ or $w\text{-}shPML_1$ and exposed to doxycycline (0.2 μg/mL) over a period of 3, 4, or 11 d, exhibited only a minor reduction in metabolic activity in response to PML knockdown when cells were plated (4,000 cells per 96 wells) 24 h prior to each assay time point. *C,* sustained PML knockdown in HT29 cells (involving 8 d of doxycycline induction of $shPML_1$) selectively reduced the cell cycle S phase and perturbed G_1 and G_2 in HT29 cells, as compared with either nontransduced or $w\text{-}shPML_1$ controls, identified through flow cytometric analysis of BrdUrd incorporation. *D*, knockdown of PML and p53 significantly increased G_2 in H1299-expressing exogenous mutant $p53^{175}$ cells.

constructs for either cyclin B2 or Cdc25c. After 24 h, the cells were exposed to Adriamycin (0.5 μg/mL). Down-regulation of p53 suppressed the induction of both promoters (Fig. 6*Bi* and *ii*, respectively). Strikingly, down-regulation of PML also reduced cyclin B2 and Cdc25C promoter activity (Fig. 6*Bi* and *ii*, respectively). The suppression of endogenous cyclin B2 and Cdc25C in response to either p53 or PML down-regulation, under the influence of Adriamycin, was also confirmed at the RNA level by RT-PCR (Fig. 6*C*). Together, these results strongly support a role for PML in the transcriptional activity of mutant p53.

Discussion

Wt p53 can lose its tumor-suppressive reflex to stress exposure through substitutions of specific single amino acids, some of which can confer GOF properties (reviewed by Strano and colleagues 4). Intriguingly, despite differences between wild-type and mutant p53 protein conformation, stability and diametrically distinct effects on cell growth and survival, they seem to share some aspects of their regulation. Here, we studied the role of PML in the regulation of mutant p53. We found a physical and functional link between PML and mutant p53. PML interacts and colocalizes with mutant p53 (Fig. 1). Although the same region of PML interacts with wild-type and mutant p53 (Fig. 5*C*), the pattern of interaction differs markedly. Whereas the interaction between PML and wt p53 occurs in response to DNA damage (in correlation with increased p53 expression; Fig. 1*Aiv* and *v*), PML interaction with mutant p53 in tumor cells occurs in the absence of genotoxic stress (Fig. 1*Ai-iii*). This suggests that oncogenic and oxidative stress in the tumor cell may suffice to trigger PML-mutant p53 interactions.

Mutant p53, in cooperation with NF-Y, transcriptionally activates a number of cell cycle genes, such as cyclin B2 and cdc25c, which

have been proposed to contribute to a GOF by mutant p53 (23). These genes are repressed by wt p53 (28, 36, 37). Intriguingly, the induction of these target promoters was augmented by exogenous expression of PML, and their expression was reduced by down-regulating PML expression (Figs. 5 and 6, respectively). The mechanism by which PML affects the transcriptional activity of mutant p53 is yet to be defined. Nevertheless, these findings raised the surprising possibility that PML may promote mutant p53 gain of function.

Indeed, we found that PML is critical for the proliferation of tumor cells expressing mutant p53 (Fig. 3). Temporal down-regulation of PML expression reduced cell numbers by depleting the number of cells in S phase and increasing the proportion of cells in G_1 and G_2 cell cycle arrest, and was associated with a modest induction of cell death after extended shPML induction (Fig. 4). Strikingly, down-regulation of PML blocked the ability of HT29 and SW480 cells to form colonies (Fig. 3). In marked contrast, down-regulation of PML had no effect on the proliferation of cells lacking p53 (HCT116 p53−/−; Fig. 3), or of cultured human foreskin fibroblasts expressing wt p53 (38). Furthermore, PML-deficient mice are viable and develop normally. Embryo fibroblasts derived from these mice exhibit no growth impairment, and seem to grow even faster than control cells (17). Therefore, our results suggest that the growth-inhibitory effects induced by down-regulation of PML expression are mutant p53 context–dependent.

In stark distinction, overexpression of PML is lethal in normal cells (39). Specifically, PML isoform IV, which interacts with p53, induces apoptosis (40). Critically, PML overexpression is growth-inhibitory to cancer cells with wild-type, mutant (41), and without functional p53 (42). Elevated PML expression perturbs the cell cycle (41, 42). Thus, there is a differential response to PML manipulation, in which normal cells with wt p53 can tolerate its reduction, but not elevation, whereas mutant p53 cancer cells are disrupted by both PML overexpression and knockdown.

Normal PML expression is critical for the growth of tumor cells bearing mutant p53. This conclusion is supported by several clinical observations. Patients with acute promyelocytic leukemias, expressing PML-RARα oncogenic fusion (13), rarely contain p53 mutations (41, 43). Furthermore, PML is commonly down-regulated

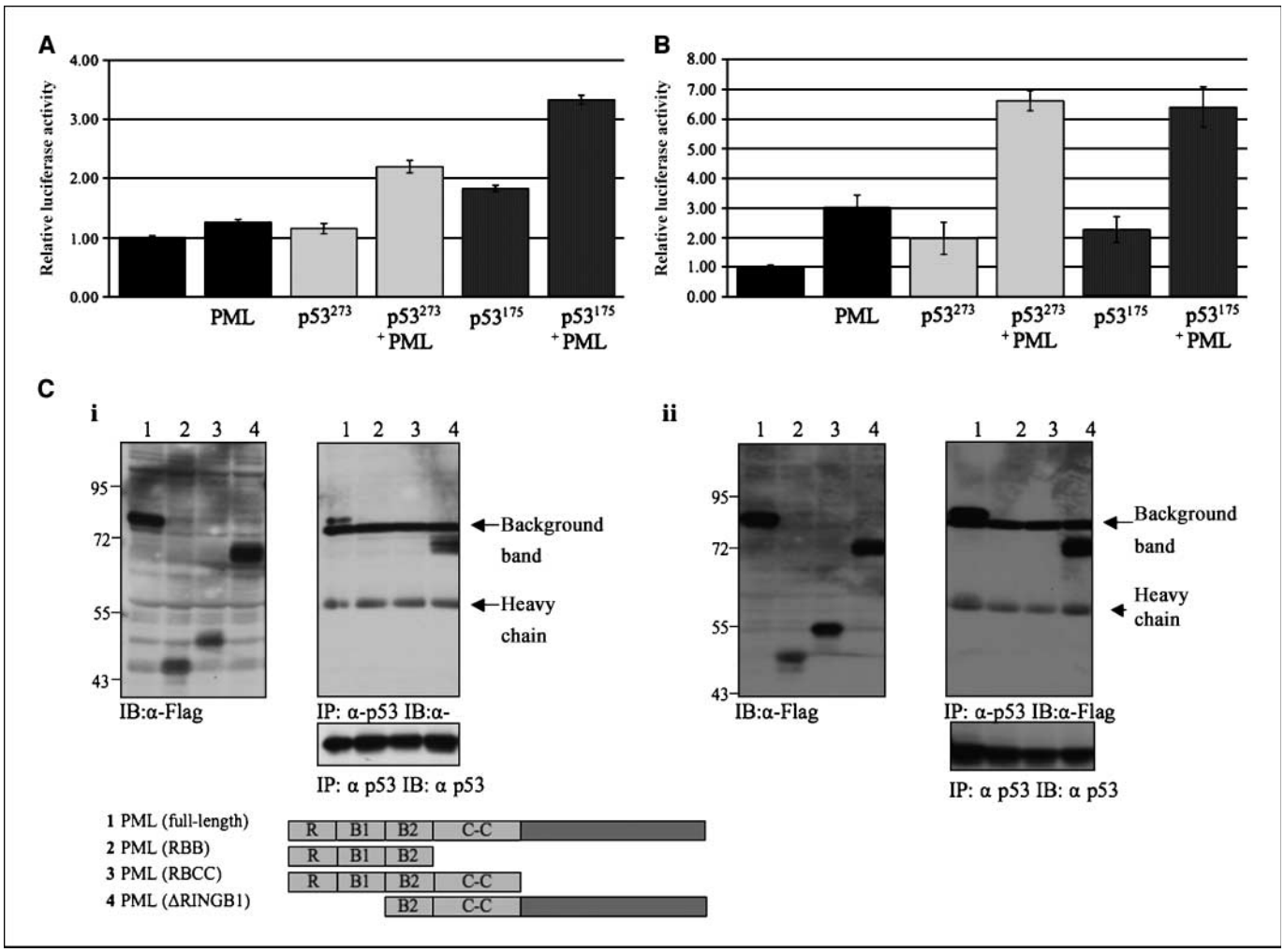

Figure 5. PML IV promoted mutant p53 transcriptional activation in H1299 cells exogenously expressing either mutant $p53^{273}$ or $p53^{175}$, and was identified to interact with mutant p53 through its COOH terminus. H1299, null for p53 (*columns 1* and *2*), or exogenously expressing either mutant $p53^{273}$ (*columns 3* and *4*), or $p53^{175}$ (*columns 5* and *6*) were compared for their effect on pCCAAT-B2LUC (*A*) or pCCAAT-cdc25CLUC (*B*). Renilla was cotransfected either in the absence or presence of PML (*columns 2, 4*, and *6*) and after 48 h, expression was assessed. *Columns,* mean of triplicate determinations representative of at least three separate experiments; *bars,* SD. *C,* H1299 cells were transfected with PML deletion mutants (*1–4* as schematically presented) together with mutant $p53^{273}$ (*i*) or $p53^{175}$ (*ii*). Twenty-four hours after transfection, p53 was immunoprecipitated from the cell extracts using goat polyclonal anti-p53 antibody followed by immunoblotting with anti-Flag antibody to detect PML Flag-tagged proteins.

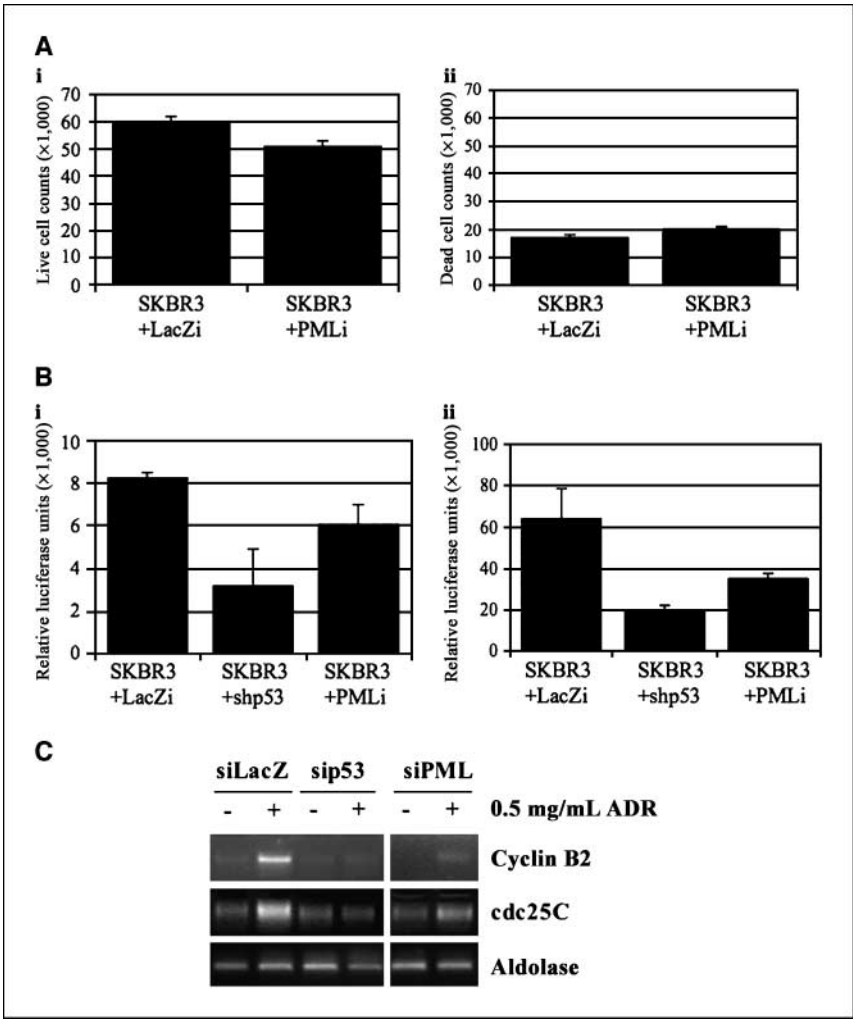

Figure 6. PML knockdown in SKBR3 under the influence of genotoxic stress, had only a modest effect on cell proliferation and viability, but suppressed mutant p53 transcriptional target activation. Knockdown of PML using siRNA in SKBR3, in conjunction with Adriamycin exposure (0.5 μg/mL; 24 h), slightly reduced SKBR3 live cell numbers, without significant cell death. *A,* live cells under the influence of either LacZi or PMLi (*i*), were differentiated from dead cells (*ii*), using trypan dye exclusion. *B,* knockdown of endogenous PML or mutant p53 in genotoxically stressed SKBR3 cells suppressed the activation of mutant p53 transcriptional targets in luciferase reporter constructs pCCAAT-B2LUC (*i*) or pCCAAT-cdc25CLUC (*ii*). SKBR3 were transfected with either LAcZi, sip53, or PML_3i simultaneously with β-galactosidase vector and luciferase reporter constructs, subsequently after 16 h, cells were then exposed to Adriamycin (0.5 mg/mL for 24 h). Luciferase activity was normalized to β-galactosidase activity and protein content. Knockdown of endogenous PML or mutant p53 in genotoxically stressed SKBR3 cells reduced RNA expression of endogenous cyclin B and Cdc25C (*C*). SKBR3 cells were transfected with either LAcZi, sip53, or PML_3i prior to Adriamycin (0.5 mg/mL; 24 h) treatment and subsequent RNA extraction and RT-PCR analysis.

in breast and prostate cancer, in which p53 mutations are less frequent (19). Additional analyses are required to substantiate this apparent correlation. Our findings, together with these studies, suggest that deregulation of PML and p53 mutations are incompatible with tumor development. The differential effect of PML reduction on the growth of cancer cells expressing wild-type or mutant p53 suggests a selective therapeutic opportunity that could be exploited by targeting mutant p53-bearing tumors.

Our study suggests that PML not only acts as an activator of wt p53 but also of mutant p53. This raises the possibility that in response to certain stress signals, such as genotoxic stress, PML enhances the gain of function of mutant p53. This intriguing finding is paralleled by a recent report of the regulation of mutant p53 by the PTEN tumor suppressor. PTEN, which normally cooperates with wt p53 in tumor suppression, stabilizes mutant p53 and promotes tumor cell growth in the context of mutant p53 (42). Thus, a pattern is emerging suggesting that in an insidious manner, mutation of p53 allows it to harness the normal regulatory machinery of wt p53 for its cancer-promoting activities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 10/16/08; revised 3/5/09; accepted 4/6/09.

Grant support: Association for International Cancer Research, the Israel Science Foundation (grant no. 1341/05), NHMRC project (grant no. 509197), and by the EC FP6 funding of the European Commission (contract 503576). This publication reflects only the author's views. The European Commission is not liable for any use that may be made of the information herein.

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We thank M. Oren and P.P. Pandolfi for their generous gifts of expression plasmids and cells, R. Agami for shRNA to p53, to D. Trono for lentiviral vectors, and B. Vogelstein for HCT116 cells.

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Cancer Res 2009;69:4818-4826.