Ubiquitin-dependent Degradation of p73 Is Inhibited by PML

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Abstract

p73 has been identified recently as a structural and functional homologue of the tumor suppressor p53. Here, we report that p73 stability is directly regulated by the ubiquitin–proteasome pathway. Furthermore, we show that the promyelocytic leukemia (PML) protein modulates p73 half-life by inhibiting its degradation in a PML–nuclear body (NB)–dependent manner. p38 mitogen-activated protein kinase–mediated phosphorylation of p73 is required for p73 recruitment into the PML-NB and subsequent PML-dependent p73 stabilization. We find that p300-mediated acetylation of p73 protects it against ubiquitinylation and that PML regulates p73 stability by positively modulating its acetylation levels. As a result, PML potentiates p73 transcriptional and proapoptotic activities that are markedly impaired in $Pml^{-/-}$ primary cells. Our findings demonstrate that PML plays a crucial role in modulating p73 function, thus providing further insights on the molecular network for tumor suppression.

Key words: ubiquitinylation • acetylation • nuclear body • transcription • apoptosis

Introduction

p73 is a structural and functional homologue of p53 (1, 2), able to transactivate the promoters of several p53-responsive genes involved in apoptosis and cell cycle regulation (3–7). Consequently, p73 can regulate cell death and growth arrest as well as differentiation (3–7).

p73 displays a modular structure similar to p53, with extensive homology to p53, particularly in the central DNA binding domain. However, unlike p53, p73 exists as multiple isoforms arising from both the usage of a cryptic promoter in intron 3 (ΔNp73 isoforms) and differential splicing of the COOH-terminal exons (α,β,δ,γ,ε,ζ, and η isoforms), of which the two major forms are p73α and p73β (5, 8).

p53 stability is regulated by ubiquitinylation, a multi-step process in which substrate proteins are covalently modified by the formation of polyubiquitin chains, and hence targeted for proteasomal degradation. p53 ubiquitinylation is predominantly regulated by the oncoprotein MDM2, which functions as a ubiquitin E3 ligase (9, 10). Cellular oncoproteins, such as c-myc, E1A, Ras, and E2F1, lead to p53 accumulation through the induction of the alternative reading frame, which, in turn, inhibits the ubiquitin ligase activity of MDM2 (10). Cellular stress leads to stabilization and accumulation of p53, mainly via posttranslation modification of the p53 protein (11). In contrast, the molecular mechanisms regulating the steady-state levels of p73 as well as its stability upon DNA damage remain poorly understood. Unlike p53, binding of p73 to MDM2 does not target p73 for ubiquitin-mediated proteolysis through the ubiquitin

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Abbreviations used in this paper: APL, acute promyelocytic leukemia; CHX, cycloheximide; DAPI, 4′,6-diamidino-2-phenylindole; HAT, histone acetyltransferase; HA-Ub, HA-tagged ubiquitin; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; NB, nuclear body; PML, promyelocytic leukemia; RARα, retinoic acid receptor.

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ligase function of MDM2 (12). In response to cisplatin treatment, p73 is stabilized by c-Abl (13) as well as by the mismatch repair protein PMS2 (14). Recent evidence suggests that the p38 mitogen-activated protein kinase (MAPK) pathway is required for c-Abl to promote p73 stabilization through phosphorylation by p38 (15). In addition to cisplatin, other DNA-damaging agents (16–19), TCR stimulation (20), and oncogenic stimuli (21) have been reported to induce and activate p73.

In contrast with p53, no functionally significant mutations of p73 have been reported so far in cancer cells, and p73 knockout mice did not show an increased susceptibility to spontaneous tumorigenesis (22). Nevertheless, p73 silencing by hypermethylation has been reported to contribute to the pathogenesis and progression of both B- and T-lymphoblastic leukemias (23). Interestingly, p73 has been shown recently to play a role in determining cellular sensitivity to chemotherapeutic agents (19, 24). In addition, some p53 mutants and polymorphisms can inactivate p73 proapoptotic function, thus possibly contributing to tumor development and drug resistance (19).

The promyelocytic leukemia (PML) tumor suppressor gene, *Pml*, is implicated in the pathogenesis of acute promyelocytic leukemia (APL), where, as a result of a reciprocal chromosomal translocation, it fuses with the retinoic acid receptor α (RARα) gene, *RARα* (25, 26). The resulting PML–*RARα* fusion gene encodes a chimeric oncoprotein known to act as a dominant negative PML and RARα mutant (27). PML is a RING finger nuclear matrix-associated protein that typically concentrates within discrete speckled multiprotein subnuclear domains, termed the PML–nuclear bodies (NBs; reference 28). PML is essential for the proper formation and stability of the PML-NB, as its inactivation leads to the disruption of the PML-NB and the aberrant localization of PML-NB components (28, 29). In APL blasts, PML-RARα causes the delocalization of PML into aberrant microspeckled nuclear structures and the subsequent disruption of the PML-NBs (30). PML exerts crucial tumor-suppressive functions such as induction of cell cycle arrest, cellular senescence, and apoptosis (31). We and others have recently shown that upon DNA damage and oncogenic transformation, PML acts as a transcriptional coactivator of p53 by favoring p53 acetylation by CBP/p300 (32–34). Here, we report that p73 turnover is directly regulated through a ubiquitin-dependent pathway and that PML is a key regulator of p73 function through its ability to inhibit this process.

Materials and Methods

Plasmids, Cell Culture, and Transfection Conditions. We have used human HA-tagged p73 isoforms and p53 in pCDNA3 (5), Flag-tagged PML (PML isoform IV), PML ΔRING, PML RBCC, and PML ΔXcmI in pCMV-Tag 2B (32) expression vectors. The PML ΔXcmI mutant was generated by introducing a frameshift at amino acid 496. The following cell lines were used: Chinese hamster–derived lung ts20 (provided by R. Schneider, New York University School of Medicine, New York, NY); CHO ts41 (provided by M. Brandeis, The Hebrew University of Jerusalem, Jerusalem, Israel); monkey kidney Cos-1; human lung carcinoma H1299; colon carcinoma HCT-116(3); osteosarcoma Saos-2 cell lines inducible for the expression of p73α, p73β, and p73γ (provided by K.H. Vousden, Beatson Institute for Cancer Research, Glasgow, Scotland, UK; reference 35); $Pml^{-/-}$, and wild-type mouse embryo fibroblasts (MEFs). MEFs were transfected by using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

Immunoblot Analysis and Coimmunoprecipitation Assays. For immunoblot analysis, the following antibodies were used: anti-p73 (clone 5B429; Imgenex), a rabbit polyclonal anti-p73 directed against the carboxy terminus of simian p73 recognizing both the α and β isoforms, a rabbit polyclonal anti-p73β (35), two rabbit anti-PML polyclonal antibodies (a gift from P. Freemont [Imperial College of Science, Technology and Medicine, London, England, UK] and K.S. Chang [The University of Texas, Houston, TX]), anti-HA (Covance), anti-GFP (CLONTECH Laboratories, Inc.), anti-Flag (M2; Sigma-Aldrich), anti-p27 (BD Biosciences), anti–Cul-2 (Zymed Laboratories), anti–acetyl-lysine (Cell Signaling), anti-PML (PG-M3), anti-Lamin A/C, anti-Bax, anti-p21, anti-p300, anti-p38, and anti-RARα (Santa Cruz Biotechnology, Inc.). For coimmunoprecipitation experiments, cell extracts were prepared as described previously (32). After the lysates had been precleared, proteins were immunoprecipitated for 2 h at 4°C with 1 μg per sample of antibody and preadsorbed on protein G–Sepharose beads (Amersham Biosciences).

Half-Life Determination. H1299 cells were transfected with either the empty vector or Flag-PML for 24 h, split, and seeded in 100-mm dishes. Both H1299 and MEFs were exposed to 20 μg/ml cycloheximide (CHX). Cells were harvested at different time points after CHX treatment, and cell extracts were immunoblotted with anti-HA and anti-p73 antibodies, respectively.

Semiquantitative RT-PCR. Total RNA was isolated using TRIzol reagent (Invitrogen). RNA was converted to cDNA by reverse transcription and a 376-bp DNA fragment was amplified by PCR using the following primers: TAp73 F1, 5′-GTGGGGAAGATGGCCCAGTC-3′ (+); and p73 R1, 5′-CGGGGTAGTCGGTGTTGGAG-3′ (−). Expression of β-actin was used as an internal standard for RNA integrity and loading. β-actin amplification (660 bp) has been performed using commercial primers (human no. 302010 and mouse no. 302110; Stratagene) according to the manufacturer's instructions.

In Vivo p73 Degradation Assay. Cells were transfected at 34°C. 24 h after transfection, cells were split, seeded in 100-mm dishes, and cultured at either 34 or 40°C for 36 (ts20) or 48 h (ts41). In some experiments, cells were treated with the p38 MAPK-specific inhibitor SB 202190 (30 μM; Calbiochem) for 20 h.

In Vivo Ubiquitinylation Assay. H1299 cells were transiently transfected with cDNAs encoding human GFP-p73α or p27 and HA-tagged ubiquitin (HA-Ub) with or without PML or p300bromo–histone acetyltransferase (HAT) expression vectors for 24 h. In some experiments, proteasome inhibitors MG132 and lactacystin (20 μM; Calbiochem) or their solvent DMSO (0.2%) were added for the last 6 h of transfection. GFP-p73– and p27-ubiquitin conjugates were immunoprecipitated and analyzed by SDS-PAGE.

siRNA studies. The sequence of small interfering (si)RNA was selected for the following target sequence of PML cDNA: 5′-AACGACAGCCCAGAAGAGGAA-3′. Control Lamin A/C siRNA oligonucleotides were purchased from Dharmacon. Cos-1 cells were transfected as described previously (36).

Immunofluorescence Microscopy. MEFs were transiently transfected with an expression vector for human GFP-p73α in the absence or in the presence of full-length PML or PML-RARα for 24 h. Cells were washed and fixed in 4% paraformaldehyde solution for 10 min at room temperature and permeabilized with 0.1% Triton X-100. For p73 and PML staining, cells were incubated with anti-p73 (clone 5B429; Imgenex) and a rabbit polyclonal anti-PML antibody in PBS and 10% goat serum.

Phosphorylation Assay. Cells were washed twice with DMEM without sodium phosphate and sodium pyruvate and incubated at 37°C in the same medium supplemented with 10% fetal bovine dialysed serum. After 1.5 h, medium was replaced with the same incubation medium containing 111 MBq/ml (0.3 μCi/ml) of [^{32}P]orthophosphate, and cells were incubated at 37°C for 3 h. After washing cells with PBS, 1 mg of cell lysate was used for immunoprecipitation with anti-HA antibody. p73 phosphorylation was visualized by autoradiography.

p38 MAPK Assay. In vitro p38 MAPK assay was performed at 30°C for 30 min in the presence of MAPK buffer (20 mM Hepes, pH 7.4, 20 mM $MgCl_2$, 2 mM DTT, and 0.5 mM Na_3VO_4 and NaF) with 200 μM of cold ATP, 5 μCi [^{32}P]ATP, recombinant active p38 MAPK (Upstate Biotechnology), and either 2 μg of ATF-2 (Santa Cruz Biotechnology, Inc.) or 6 μg of purified NH_2-terminally His-tagged full-length p73α.

Luciferase Reporter Assay. For luciferase assays, cells were seeded in duplicate into 12-well plates and cotransfected with a luciferase reporter plasmid driven by the *bax* (*bax*-Luc) or the *p21waf1* (*p21*-Luc) promoters and a pRL-TK encoding *Renilla* luciferase cDNA, together with a combination of different plasmids. Luciferase activity was assayed 24 (H1299 and Saos-2) or 48 h (MEFs) after transfection by a dual luciferase reporter assay system (Promega) and normalized against *Renilla* luciferase activity.

Determination of Apoptosis. Hypodiploid events were evaluated by FACS® analysis using a propidium iodide staining.

Retroviral Infection. Retroviruses were generated by transient transfection of Phoenix cells with pBabe or pBabe-p73α retroviral vectors, using the calcium phosphate method. Filtered supernatants were harvested every 12 h, and used to infect MEFs in the presence of 4 μg/ml of polybrene. 48 h after infection, cells were selected in 2.5 μg/ml puromycin for 3 d.

Online Supplemental Material. In Fig. S1, the effect of proteasome inhibitor treatment and inactivation of the NEDD8-activating E1 enzyme on p73 ubiquitinylation and degradation was analyzed. Fig. S2 shows that PML specifically stabilizes TA and DNp73 without generically affecting protein ubiquitinylation. In Fig. S3, we show that stabilization of p73 by PML is isoform specific because PML III is not able to inhibit p73 ubiquitinylation. In Fig. S4, the effect of histone deacetylase inhibitor treatment and p300-mediated acetylation of p73 on its protein expression levels is shown. Figs. S5 and S6 show that PML potentiates p73 transcriptional and proapoptotic functions, respectively. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20031943/DC1.

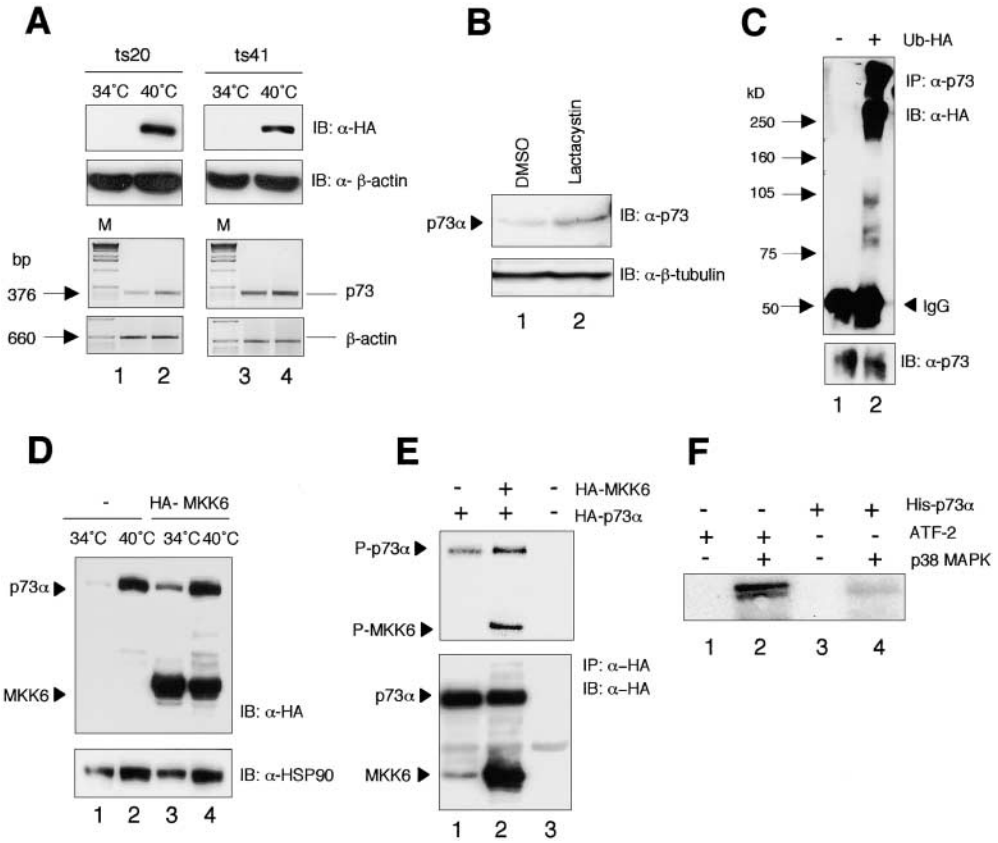

Figure 1. p73 is degraded through the ubiquitin–proteasome pathway. (A) ts20 and ts41 cells were transfected with HA-p73α and either left at 34°C (lanes 1 and 3) or shifted to 40°C (lanes 2 and 4). Cell lysates were analyzed by immunoblot (IB) with anti-HA and anti–β-actin (top) antibodies. Expression of mRNA for p73 and β-actin was assessed by semi-quantitative RT-PCR analysis (bottom). (B) HCT-116(3) cells were either left untreated (lane 1) or incubated with 10 μM or lactacystin (lane 2) for 12 h. Cellular extracts were analyzed with anti-p73 antibody (clone 5B429). (C) Cos-1 cells were either transfected with the empty vector (lane 1) or with HA-Ub (lane 2) and treated with MG132 for 6 h. Cell lysates were immunoprecipitated with a rabbit polyclonal anti-p73 antibody followed by IB with anti-HA antibody. (D) ts20 cells grown at 34°C were transiently transfected with HA-p73α alone or in combination with a constitutively active form of MKK6. 8 h after transfection, some cultures were shifted to 40°C. (E) Phosphorylation of p73 in H1299 cells transfected with HA-p73α alone (lane 1) or in combination with HA-MKK6 (lane 2). Cells were cultured in [^{32}P]orthophosphate-containing medium for 3 h, and phosphorylation was assessed by autoradiography. (F) In vitro phosphorylation of human purified His-p73α by p38 MAPK. 6 μg His-p73α was incubated with 1.2 U of recombinant purified p38 MAPK. ATF-2 was included as a control.

Results

p73 Degradation Occurs through Ubiquitinylation and Is Negatively Regulated by the p38 MAPK Pathway. p73 protein levels have been shown to increase upon proteasome inhibitor treatment (12, 36, 38). However, whether p73 turnover is directly regulated by ubiquitin-mediated proteolysis remains to be proven. The mutant ts20 cell line has a thermolabile ubiquitin-activating E1 enzyme that is inactivated at the nonpermissive temperature of 40°C, which leads to disruption of the ubiquitinylation process. Complete degradation of p73α occurred at 34°C (Fig. 1 A, lane 1), whereas incubation of ts20 cells at 40°C resulted in marked accumulation of p73 protein (Fig. 1 A, lane 2), thus indicating that an intact ubiquitin system is required for p73 degradation. On the contrary, p73 mRNA levels were considerably unaltered upon temperature switch (Fig. 1 A, bottom left). Proteasome inhibitor treatment of ts20 cells resulted in p73 accumulation in a polyubiquitinylated state (Fig. S1 A, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1) and in the appearance of higher molecular weight immunoreactive bands of p73 that contained ubiquitin (Fig. S1 B).

Next, we used a different temperature-sensitive cell line, ts41, expressing a thermolabile NEDD8-activating E1 enzyme mutant (39, 40). NEDD8 is an ubiquitin-like protein whose covalent attachment to cullins, its only known substrates, appears to increase the activity of cullin-containing ubiquitin E3 ligase complexes (39). As shown in Fig. 1 A (top right), p73 degradation was abrogated at 40°C (lane 2), thus suggesting that ubiquitinylation of p73 requires an in-

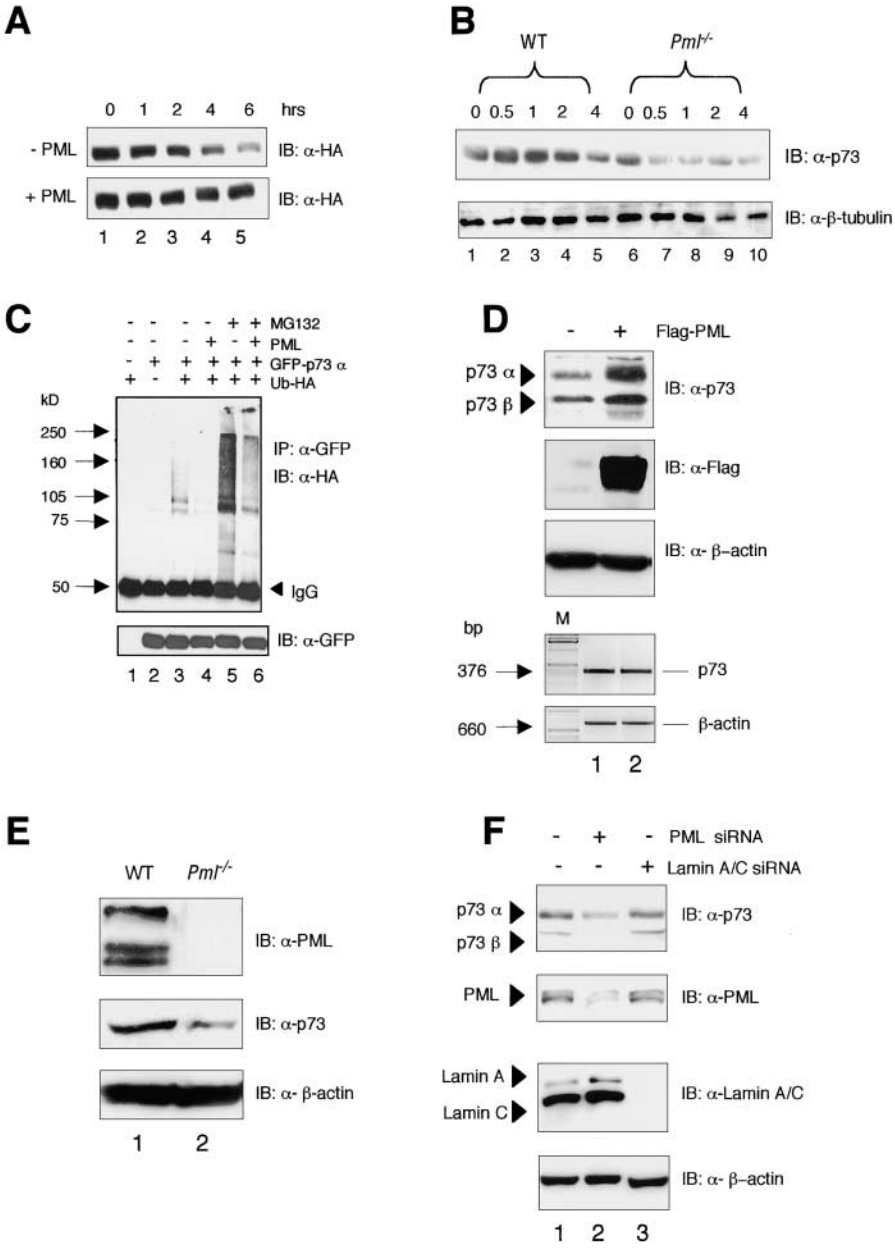

Figure 2. PML protects p73 from ubiquitinylation and proteasome-mediated degradation. (A and B) PML increases the half-life of both exogenous and endogenous p73. p73 half-life was measured upon CHX (20 μg/ml) addition in H1299 cells transfected with either HA-p73α alone or with PML (A) and in wild type and *Pml*$^{-/-}$ MEFs (B). Cells lysates were examined by IB using anti-HA (A) or anti-p73 (B, clone 5B429) antibody. (C) H1299 cells were cotransfected with the indicated plasmids for 24 h. Some cultures were incubated with MG132 (lanes 5 and 6). p73-Ub immunocomplexes were analyzed by IB analysis with anti-HA (top) and anti-GFP (bottom) antibodies. (D) Cos-1 cells were either transfected with the empty vector (lane 1) or with Flag-PML (lane 2) for 24 h. Cell lysates were analyzed with rabbit polyclonal anti-p73, anti-Flag, and anti–β-actin antibody. Semi-quantitative RT-PCR analysis on p73 and β-actin mRNAs in Cos-1 cells was treated as aforementioned (bottom). (E) Cell lysates from wild type and *Pml*$^{-/-}$ MEFs were examined by IB using anti-p73 (clone 5B429) antibody. (F) Cos-1 cells were either left untreated (lane 1) or transfected with 200 pmol PML (lane 2) and Lamin A/C (lane 3) siRNA. After 48 h, cell lysates were prepared and analyzed for p73, PML, Lamin A/C, and β-actin expression by IB.

tact NEDD8 pathway and presumably a cullin-dependent ubiquitin protein ligase. Under these experimental conditions, stabilization of p73 mRNA did not occur at 40°C because p73 expression levels were substantially unaffected.

Upon temperature switch to 40°C, we found both p27 (a target of the SKP1-cullin1–F box-Roc1 E3 ubiquitin ligase complex) accumulation and disappearance of a slower migrating NEDD8-modified form of cul-2, hence proving that neddylation is effectively inhibited under these experimental conditions (Fig. S1 C).

To assess whether this mechanism is of physiological relevance, we studied the effect of proteasome inhibitors on endogenous p73 protein levels. Indeed, proteasome inhibitor treatment led to the accumulation of both p73α and p73β (Fig. 1 B, lane 2, and Fig. S1 D, lanes 2 and 3). Importantly, endogenous p73 was polyubiquitinylated in vivo (Fig. 1 C, lane 2). Therefore, we conclude that the regulation p73 degradation involves the ubiquitin-dependent proteasome pathway.

p38 MAPK pathway plays a role in p73 stabilization and activation (18). In agreement with these findings, we found that the overexpression of a constitutively active form of MKK6, the immediate upstream regulator of p38 MAPK, led to reduced degradation of p73 (Fig. 1 D) and increased p73 total phosphorylation levels (Fig. 1 E). In vitro kinase assays showed that p73 is directly phosphorylated by p38 MAPK (Fig. 1 F). Thus, p38 MAPK signaling contributes to both p73 phosphorylation and stabilization.

PML Protects p73 from Proteasome-mediated Degradation. PML isoform IV regulates p53 induction and transcriptional activation (32–34, 41) and protects p53 from MDM2-mediated degradation (42, 43). Therefore, we set out to test whether PML isoform IV might regulate p73 stability. PML overexpression indeed led to a dose-dependent p73 accumulation (Fig. S2, A and B, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1). To test whether PML regulates p73 stability, we followed the half-life of ectopically expressed p73 and found that it was greatly increased by PML overexpression (Fig. 2 A), thus demonstrating that PML stabilizes p73 in vivo. Importantly, and in agreement with these results, we observed that the half-life of endogenous p73α was prolonged in cells overexpressing PML (not depicted) and resulted markedly shortened in *Pml*$^{-/-}$ compared with wild-type MEFs (Fig. 2 B).

Next, we investigated whether PML might interfere with the proteasome-dependent degradation of p73 and observed that it was dose-dependently reduced by increasing amounts of exogenous PML in ts20 cells (Fig. S2 C). Similar results were obtained by using the ts41 cell line (unpublished data).

The ubiquitinylation levels of p73 were significantly reduced in PML-overexpressing cells (Fig. 2 C). PML also prevented p73 ubiquitinylation in MG132-treated cells (Fig. 2 C, lane 6), indicating that PML acts upstream of the proteasome degradation process. Importantly, PML did not

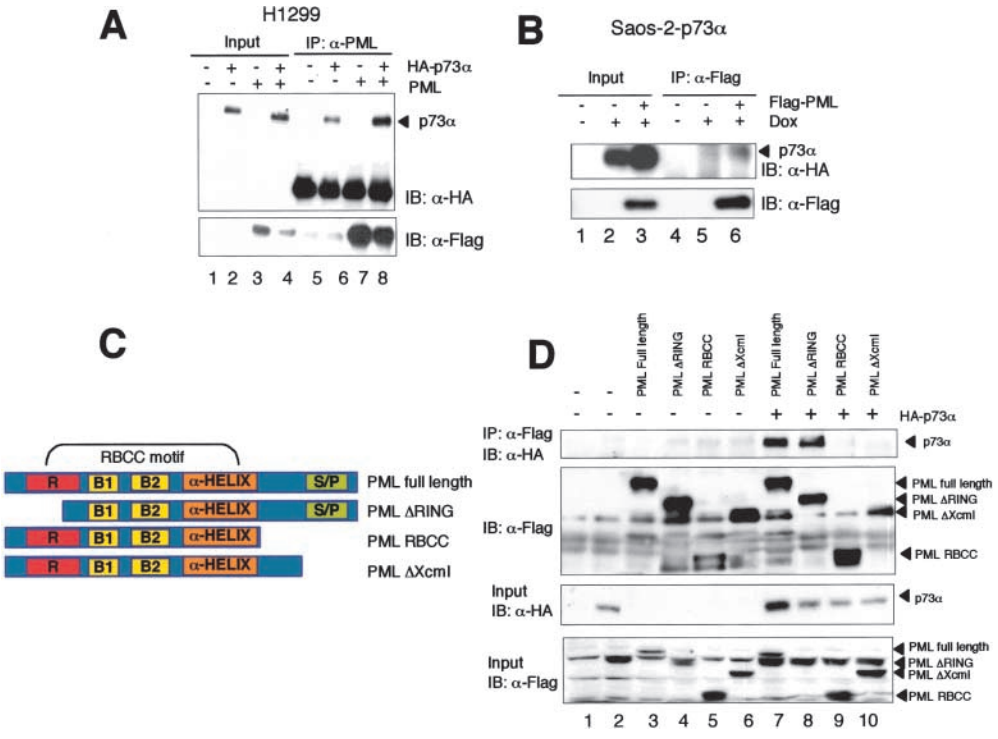

Figure 3. p73 and PML physically interact in vivo. (A) H1299 cells were transiently transfected with expression vectors encoding HA-p73α along with Flag-PML. p73–PML complexes were analyzed by IP using a monoclonal anti-PML (PG-M3) followed by IB with anti-HA and anti-Flag antibodies. Unspecific bands were recognized by the anti-Flag antibody in extracts from cells that did not overexpress PML (lanes 5 and 6). (B) Saos-2 cells inducible for p73α expression were transfected with Flag-PML. 4 h after transfection, cells were either left untreated or stimulated with 2 μg/ml doxycycline (Dox) for 24 h. Cell lysates were immunoprecipitated with anti-Flag antibody, and the immunoprecipitate was analyzed by IB using anti-HA antibody. (C) Schematic presentation of the structure of PML and its deletion mutants. (D) p73 interacts with the carboxy-terminal domain of PML in vivo (amino acids 436–633). Transformed 3T3 *Pml*$^{-/-}$ MEFs were cotransfected with GFP-p73α and full-length Flag-PML (lane 7), Flag-PML ΔRING (lane 8), Flag-PML RBCC (lane 9), or Flag-PML ΔXcmI. Cell lysates were immunoprecipitated with anti-Flag antibody and the immunoprecipitate analyzed by IB using anti-HA antibody. Inputs for both p73 and PML expression are shown. The ΔRING mutant comigrated with unspecific bands recognized by the anti-Flag antibody in the extracts (lanes 4 and 8).

exert a global inhibitory effect on the ubiquitin-dependent degradation machinery because, for instance, it did not affect p27 ubiquitinylation levels (Fig. S2 D).

Next, we examined the effect of PML on the induction of endogenous p73 protein. Furthermore, PML overexpression led to a significant accumulation of endogenous p73α and p73β isoforms (Fig. 2 D, top). PML-mediated accumulation of p73 was not caused by any increase in p73 gene transcription or mRNA stabilization because p73 expression levels were unaffected (Fig. 2 D, bottom). p73α protein levels were significantly reduced in *Pml*$^{-/-}$ versus wild-type MEFs (Fig. 2, B and E). We down-regulated PML expression by using siRNA oligonucleotides and found that reduction of steady-state levels of PML resulted in a marked decrease of both p73α and p73β protein levels (Fig. 2 F, lanes 1 and 2). In contrast, the control Lamin A/C siRNA did not affect either PML or p73 protein levels (Fig. 2 F, lane 3). Together, these results demonstrate that PML protects p73 against ubiquitinylation and its subsequent proteasome-dependent degradation.

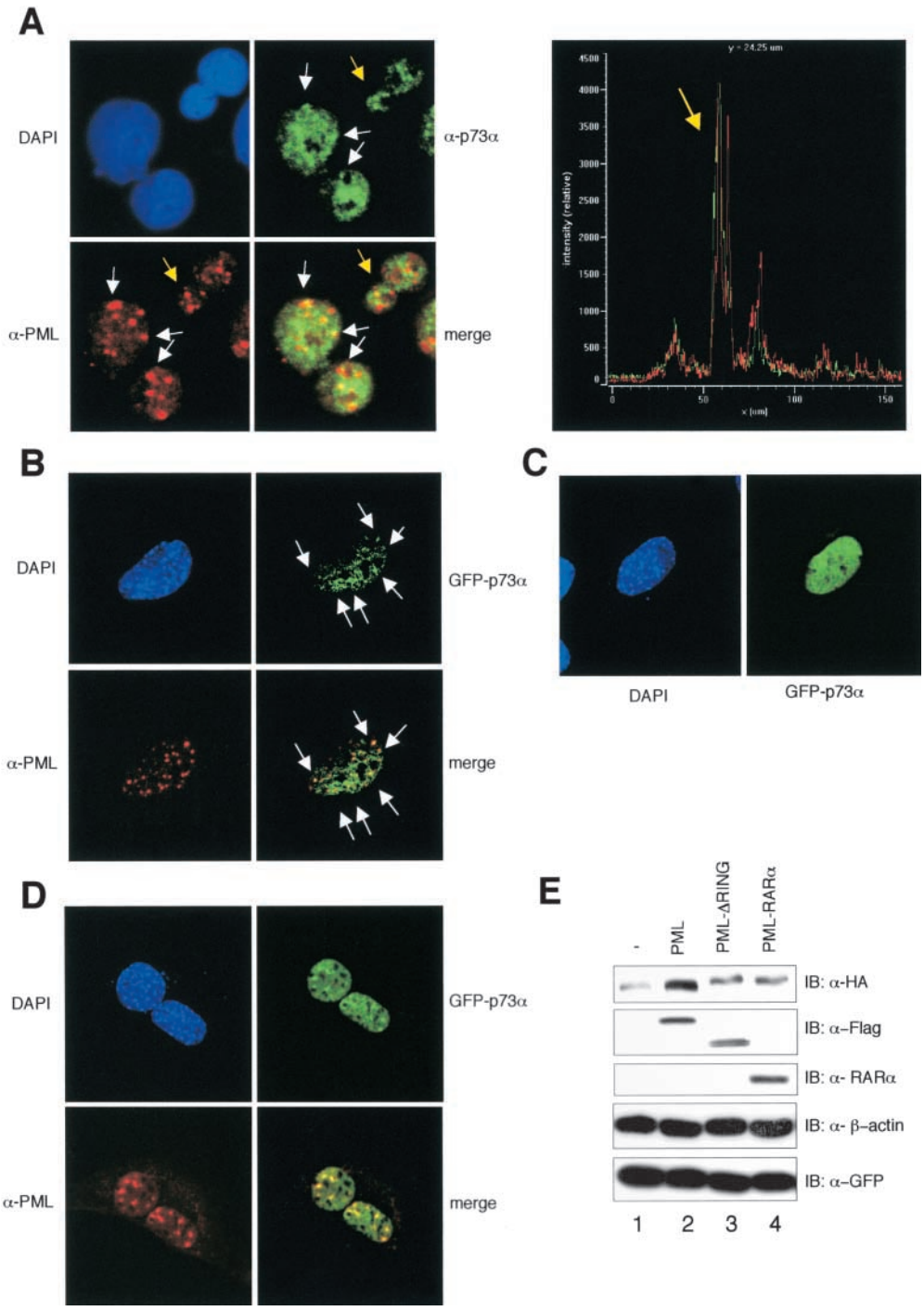

Figure 4. p73 and PML colocalization within the PML-NBs is required for p73 stabilization (A) Representative image of Cos-1 cells costained with anti-p73 (green, clone 5B429) and anti-PML (red) antibodies and analyzed by confocal microscopy (left). Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. The arrows indicate p73-containing speckles. (right) A quantitative analysis of the green and red fluorescence intensity at distinct nuclear speckles as indicated by the yellow arrow. (B–D) GFP-p73α was overexpressed into wild type (B) and *Pml*$^{-/-}$ (C) MEFs. Full-length PML was cotransfected with GFP-p73α into *Pml*$^{-/-}$ MEFs (D). Nuclei were visualized by DAPI staining. The arrows indicate p73-containing PML-NBs. (E) *Pml*$^{-/-}$ MEFs were transiently transfected with HA-p73α alone (lane 1) or in combination with full-length Flag-PML (lane 2), Flag-PML ΔRING (lane 3), or PML-RARα (lane 4). Cell extracts were subjected to IB with anti-HA, anti-Flag, anti-RARα, and anti–β-actin antibodies. (bottom) Normalization of transfection efficiency by quantitation of GFP expression.

PML Inhibits p73 Degradation in a PML-NB–dependent Manner. Because PML interacts with p53 through the DNA binding domain (32), which is highly homologous to that of p73 (1), next we examined whether PML and p73 also physically interact in vivo. Complexes immunoprecipitated with anti-PML antibody were found to contain p73 (Fig. 3 A). Similarly, doxycycline-induced p73α coimmunoprecipitated with PML (Fig. 3 B). To identify the PML domain responsible for the interaction with p73, we expressed HA-p73α along with the PML ΔRING (32), PML RBCC (32), and PML ΔXcm1 deletion mutants (Fig. 3 C) in $Pml^{-/-}$ MEFs to avoid heterodimerization of the various mutants with the endogenous PML isoforms. As shown in Fig. 3 D, the two PML mutants lacking the carboxy-terminal region lost the ability to interact with p73, whereas the absence of the PML amino-terminal domain did not influence its ability to associate with p73.

Because PML interacts with p73, next we investigated whether PML could affect p73 sub-cellular localization. Endogenous p73 showed a diffuse as well as a nuclear punctuate pattern partially colocalizing with PML (Fig. 4 A). Similar results were obtained in HCT116(3) cells (unpublished data). Ectopically expressed GFP-p73α partly accumulated in speckles in wild-type MEFs (in ~70% of cells), which colocalized with PML (~40% of PML-NBs per cell were found to contain p73) (Fig. 4 B and see Fig. 5 D). In sharp contrast, GFP-p73α did not accumulate in speckles, and its staining pattern remained entirely diffuse in $Pml^{-/-}$ MEFs (Fig. 4 C).

Add-back of PML into $Pml^{-/-}$ MEF cells resulted in restoration of p73 PML-NB localization (Fig. 4 D). On the contrary, transfection of $Pml^{-/-}$ MEFs with the PML ΔRING mutant, which lacks the RING domain and, therefore, does not accumulate in the PML-NBs (32), but retains the ability to physically interact with p73 (Fig. 3 D), did not alter the diffuse distribution pattern of p73 (not depicted). Similarly, the PML-RARα oncogenic fusion protein, which exhibits an aberrant microspeckled nuclear distribution pattern, did not colocalize with p73 in these microspeckles (unpublished data).

To examine whether colocalization of p73 and PML into the PML-NBs is required for p73 stabilization, we compared the effect of the aforementioned PML mutants on p73 accumulation. We expressed HA-p73α in $Pml^{-/-}$ MEFs along with full-length PML, PML-ΔRING, or PML-RARα. Strikingly, both PML-ΔRING and PML-RARα were significantly less effective (two- vs. sixfold induction) than wild-type PML in stabilizing p73 (Fig. 4 E and Fig. 3 D). Similar results were obtained by using PML mutants that had lost the ability to interact with p73 (Fig. 3 D). Furthermore, the PML III isoform, which showed a shorter carboxy-terminal tail and, therefore, was unable to

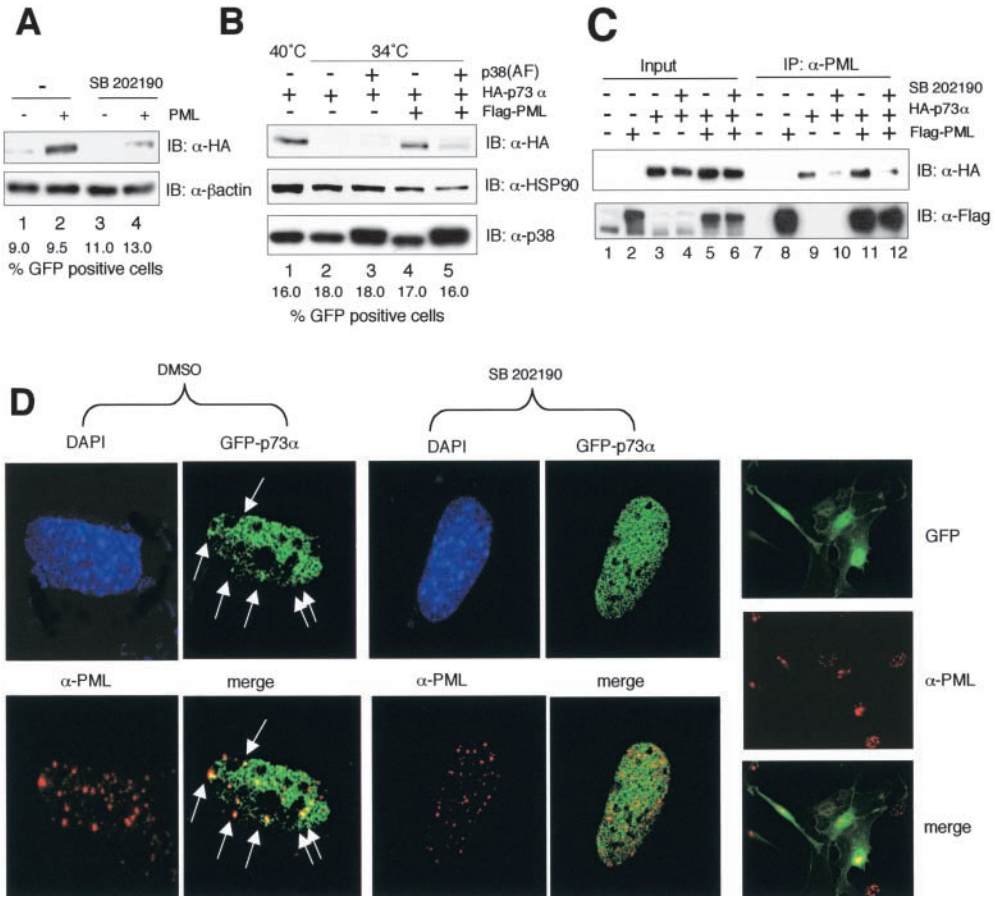

Figure 5. p38 MAPK is required for PML-mediated inhibition of p73 degradation. (A) ts20 cells were cotransfected with HA-p73α, GFP-spectrin with (lanes 2 and 4) or without (lanes 1 and 3) Flag-PML for 48 h at 34°C. Some cultures were treated with the p38 MAPK inhibitor SB202190 (lanes 3 and 4). (B) ts20 cells were cotransfected with GFP-spectrin, HA-p73α alone (lanes 1 and 2) or in combination with PML (lanes 4 and 5) in the absence (lane 4) or in the presence (lane 5) of p38(A/F). Whole cell extracts were immunoblotted with anti-HA, anti-p38, anti–β-actin, or anti-HSP90 antibodies. (C) Transfection and IP experiments were performed as described in Fig. 3 A. 8 h after transfection, cells were incubated with SB 202190 or DMSO for 20 h. (D) Wild-type MEFs were transfected with GFP-p73α and either left untreated or incubated with SB 202190 for 20 h. Cells were analyzed by confocal microscopy. Nuclei were visualized by DAPI staining. The arrows indicate p73-containing speckles. As a control, wild-type MEFs were also transfected with a GFP expression vector and stained with anti-PML (red) antibody (right).

bind p53, did not affect p73 ubiquitinylation (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1). In agreement with these results, treatment of Cos-1 cells with arsenic trioxide, which promotes PML modification by SUMO-1 and its subsequent recruitment to the PML-NB (44), enhanced endogenous p73 protein levels (unpublished data), further substantiating the notion that PML localization to the PML-NB is crucial for regulation of p73 stability. Together, these results indicate that PML influences p73 subcellular localization and that this is crucial for regulation of p73 stability.

Furthermore, because in APL blasts, PML activity is compromised due to both the dominant negative action of PML-RARα and the reduction of *Pml* gene to hemizygosity (45), the fact that PML-RARα is defective in stabilizing p73 strongly suggests that p73 function may be impaired in APL.

The p38 MAPK Pathway Is Required for PML-dependent p73 Stabilization. Because p38 MAPK plays a role in p73 stabilization (Fig. 1 E and reference 15), next we asked whether the inhibition of p38 MAPK signaling might affect the protective effect exerted by PML on p73 degradation. Treatment of ts20 and H1299 cells with SB 202190, a selective inhibitor of p38 MAPK (46), reduced the steady-state levels of p73 and dramatically attenuated the ability of PML to prevent its degradation (Fig. 5 A and not depicted). Overexpression of a dominant negative mutant of p38 (47), in which the TGY dual phosphorylation site is mutated to AGF (p38[A/F]), also led to a significant reduction of PML-induced p73 accumulation (Fig. 5 B, compare lanes 4 and 5).

To elucidate the mechanism underlying the cross-talk between the p38 MAPK pathway and PML, we measured p38 MAPK activation levels in wild type and $Pml^{-/-}$ MEFs at steady-state and upon UV irradiation, and found that they were comparable between the two genotypes (unpublished data). In addition, in vivo p38 MAPK phosphorylation assays performed by using immunoprecipitates of both endogenous and exogenous p38 MAPK did not show increased kinase activity upon PML overexpression, and total phosphorylation levels of p73 were not altered by PML overexpression (unpublished data).

Therefore, we sought to examine whether p38 MAPK activation is required for the ability of p73 to interact with PML and localize in the PML-NB. Strikingly, the inhibition of p38 MAPK strongly diminished the amount of p73 associated with PML protein (~73% reduction; Fig. 5 C). In addition, treatment of wild-type MEFs with SB 202190 led to a decrease in the percentage of cells showing colocalization of p73 and PML into the PML-NBs (71% ± 3 and 56% ± 7 in untreated vs. SB 202190–treated MEFs, re-

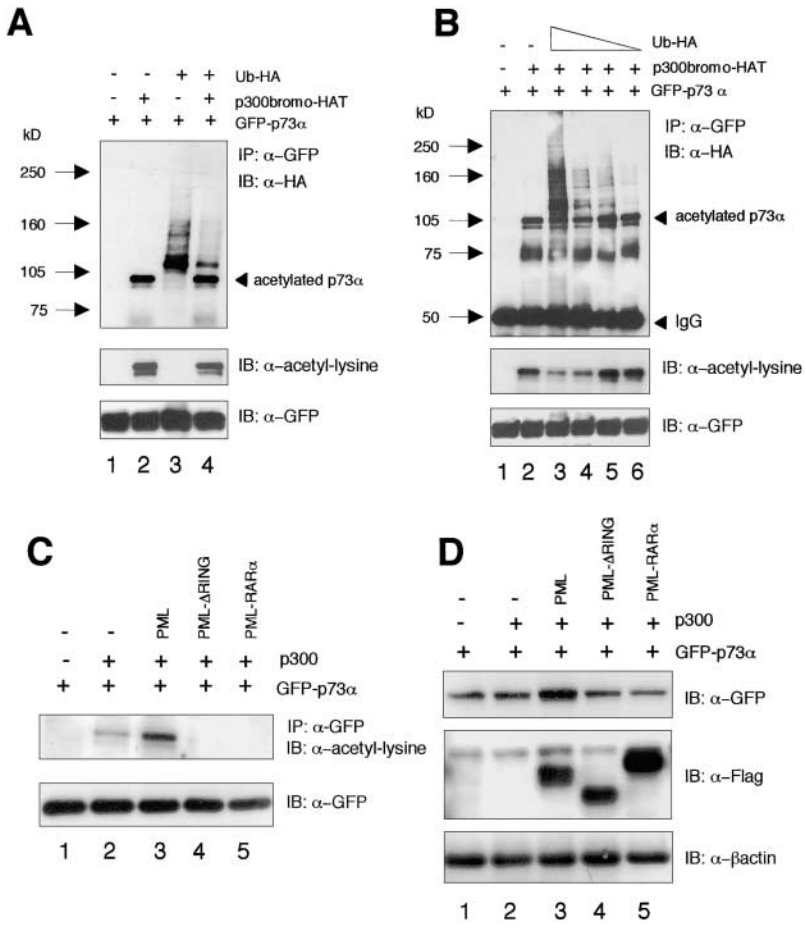

Figure 6. PML-regulated acetylation of p73 by p300 protects p73 against degradation. (A) GFP-p73α and HA-Ub were expressed in 293T cells in the absence (lane 3) or in the presence (lane 4) of p300bromo-HAT for 24 h. Cells were incubated with 1 μM trichostatin A (TSA) and 1 mM niacinamide for the last 12 h of transfection. After IP of GFP-p73, ubiquitinylation levels of p73 were detected with anti-HA antibody. Acetylated and unacetylated p73 in the immunoprecipitates were determined with anti–acetyl-lysine and anti-GFP antibody, respectively. (B) 293T cells were transfected with a plasmid encoding GFP-p73α alone (lane 1) or in combination with p300bromo-HAT (lanes 2–6), in the absence (lane 2) or in the presence (lanes 3–6) of decreasing doses of HA-Ub for 24 h. Treatment with deacetylase inhibitors and IP experiments were performed as described in A. (C and D) Transformed 3T3 $Pml^{-/-}$ MEFs were transiently transfected with GFP-p73α alone (lane 1) or along with p300 in the absence (lane 2) or in the presence of full-length Flag-PML (lane 3), Flag-PML-ΔRING (lane 4), or Flag-PML-RARα (lane 5) for 24 h. Cells were incubated with deacetylase inhibitors for 8 h. p73 was immunoprecipitated using anti-GFP antibody followed by IB with anti–acetyl-lysine antibody (C). Whole cell extracts were immunoblotted with anti-GFP, anti-Flag, and anti–β-actin antibodies (D).

spectively) as well as a decrease of PML-NBs found to contain p73 per cell (36% ± 6 vs. 20% ± 1) and resulted in a more diffuse p73 distribution pattern (Fig. 5 D, middle). These effects were specific to p73 as GFP did not unspecifically accumulate in the PML-NBs (Fig. 5 D, right). Altogether, these findings demonstrate that p38 MAPK plays a key role in modulating the ability of PML to stabilize p73 and further support the notion that the PML-NB–dependent interaction of p73 and PML is critical for PML to inhibit p73 degradation.

PML Promotes p300-mediated Acetylation and Stabilization of p73. p73 is a substrate for the histone acetyltransferase (HAT) activity of p300 (18). In addition, reports have correlated p53 acetylation and stability (11, 48). We found that p300-mediated acetylation of p73 as well as treatment with histone deacetylase inhibitors increased p73 protein levels (Fig. S4, A and B, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1). Importantly, the effect of p300 on p73 accumulation was dependent on the intrinsic HAT activity of p300 because expression levels of p73 were also increased by simply overexpressing the p300bromo-HAT domain (Fig. S4 C).

To provide direct evidence that p300-mediated enhanced expression of p73 reflects increased stability of the protein, we tested the effect of p73 acetylation on its ubiquitinylation levels in vivo. As shown in Fig. 6 A, acetylated p73 (~90 kD band as indicated by the arrow) was significantly less ubiquitinylated than the unacetylated protein (compare lanes 3 and 4), thus demonstrating that p300-mediated modification of p73 inhibits its ubiquitinylation. In accordance with the previous results, we found that p73 acetylation levels were strongly reduced by high levels of protein ubiquitinylation (Fig. 6 B). On the contrary, p73 acetylation could be restored by decreasing its ubiquitinylation levels (Fig. 6 B). Thus, competition between acetylation and ubiquitinylation may result in regulation of p73 protein stability.

Because CBP and p300 have both been shown to colocalize with PML to the PML-NBs and PML is a crucial regulator of CBP-mediated acetylation of p53 (32, 34, 49), we investigated whether p300-mediated acetylation of p73 is modulated by PML. Restoration of PML expression in $Pml^{-/-}$ cells significantly increased p73 acetylation levels (Fig. 6 C), demonstrating that PML also promotes the acetylation of p73 in vivo. On the contrary, both the PML-ΔRING PML-NB–defective mutant and PML-RARα were unable to affect p73 acetylation levels (Fig. 6 C). Furthermore, the overexpression of PML and p300 in $Pml^{-/-}$ cells resulted in a cooperative stabilization of p73, whereas the add-back of PML-ΔRING or PML-RARα did not affect the ability of p300 to accumulate p73 (Fig. 6 D). Together, these findings demonstrate that acetylation leads to p73 stabilization and PML positively modulates this process in a PML-NB–dependent manner.

PML Regulates p73 Transcriptional and Biological Activities. Overexpression of PML resulted in enhancement of the transactivation potential of various p73 isoforms on both the *bax*-Luc and the *p21*-Luc promoters (Fig. S5, A and B, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1, and not depicted). Accordingly, Bax and p21 protein levels were enhanced by coexpression of p73 and PML as compared with cells expressing p73 alone (Fig. S5 C). *Pml* inactivation resulted in a marked impairment of p73-dependent transcription. Indeed, p73α transcriptional activity was diminished up to 65% in $Pml^{-/-}$ cells (Fig. 7 A and not depicted). In agreement with the inability of the PML-ΔRING mutant to efficiently stabilize

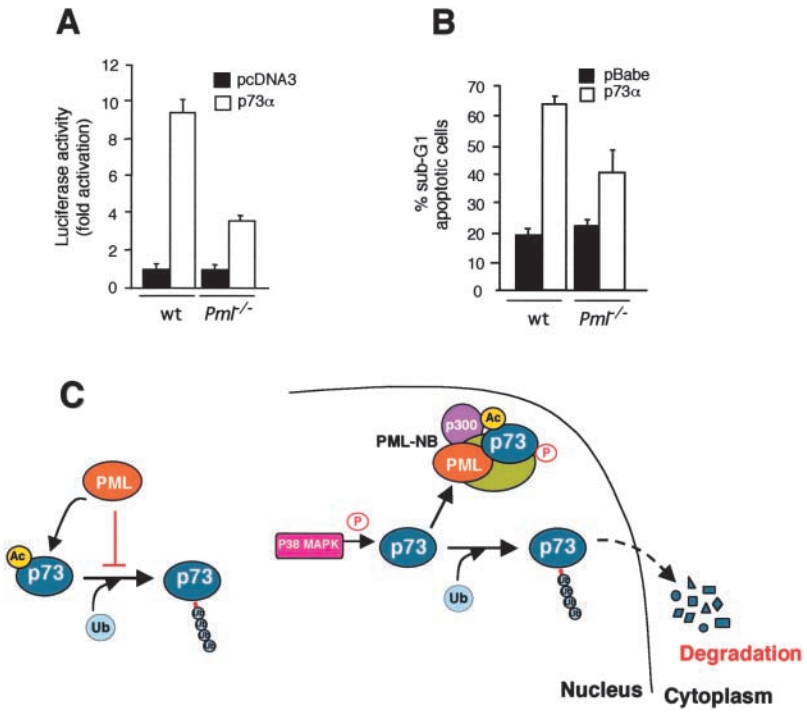

Figure 7. PML is required for p73 transcriptional and proapoptotic activity. (A) A bax promoter-driven luciferase reporter plasmid (bax-Luc) alone or in combination with p73α was transfected into wild type and $Pml^{-/-}$ MEFs, and luciferase activity was assayed 48 h after transfection. pRL-TK vector was included to normalize transfection efficiency, and reporter basal luciferase activity was normalized as 1. Values are mean ± SEM of two separate experiments each performed in duplicate. (B) Wild type and $Pml^{-/-}$ MEFs were retrovirally transduced with a pBabe-p73α construct for 48 h, selected in puromycin-containing medium for 3 d, and scored for apoptosis by FACS® analysis using propidium iodide staining. Values are mean ± SEM of two separate experiments each performed in duplicate. (C) A model for PML-mediated protection against p73 degradation. PML inhibits p73 ubiquitin-dependent degradation (left). PML controls p73 stability by regulating p73 acetylation levels in the PML-NB, thereby preventing its ubiquitinylation (right).

p73, the transcriptional defect observed in the *Pml*$^{-/-}$ MEFs could be rescued by PML, but not by PML-ΔRING add-back (Fig. S5 D). Furthermore, SB 202190 treatment reduced the ability of p73 to transactivate the *bax*-Luc promoter (up to 44% inhibition; Fig. S5 E). Remarkably, PML coactivation of p73-dependent transcription was additionally impaired upon SB 202190 treatment (up to 56% inhibition; Fig. S5 E).

Next, we evaluated the ability of PML to affect p73 biological functions, such as induction of apoptosis, and found that PML can potentiate it (Fig. S6, A and B, available at http://www.jem.org/cgi/content/full/jem.20031943/DC1, and not depicted). Furthermore, *Pml* inactivation markedly impaired p73-induced apoptosis in MEFs retrovirally transduced with p73α or p73β constructs (Fig. 7 B and not depicted). Thus, PML is a critical regulator of p73 transcriptional and biological functions through its ability to regulate p73 acetylation in a NB-dependent manner.

Discussion

Our findings lead to three major conclusions. First, degradation of p53 family members is regulated through distinct molecular mechanisms. Although MDM2 has a critical function in the negative regulation of p53 activity in vivo (50), it does not lead to p73 degradation (12), but rather to its stabilization (51). We show that endogenous p73 is polyubiquitinylated in vivo and degraded through the ubiquitin–proteasome pathway. Cullin-dependent ubiquitinylation is positively regulated by the NEDD8 pathway (39). We also found that an intact NEDD8 system is required for p73 degradation, suggesting that p73 ubiquitinylation might require the NEDD8-mediated modification and consequent activation of a cullin-dependent ubiquitin ligase. Although NEDD8-mediated modification of cullin-containing complexes has been recently implicated in adenovirus-induced p53 degradation (52), p53 turnover at the physiological level is mainly controlled by MDM2. This is intriguing and surprising in view of the high degree of structural and functional homology among p53 family members and may suggest that the evolutionary divergence of degradation mechanisms has evolved to ensure that the possible deregulation of this process would not concomitantly affect the entire p53 family. Further studies will be required to identify the E3 ubiquitin ligase involved in p73 ubiquitinylation and to prove that it contains a NEDD8-modified cullin subunit. Our preliminary results indicate that either the SKP1-cullin1–F box-Roc1 and the VEC E3 ubiquitin ligase complexes are not responsible for p73 degradation.

Second, we demonstrate that PML increases p73 acetylation, thus preventing its ubiquitinylation and subsequent degradation (Fig. 7 C, left). Our findings identify a chain of molecular events by which p38 MAPK activation is a prerequisite for p73 binding to PML and recruitment into the PML-NB, PML-dependent p300-mediated acetylation and stabilization of p73 and its consequent transcriptional/functional activation (Fig. 7 C, right). In agreement with these findings, it has been reported that the activation of the p38 MAPK pathway enhances p73 stability by promoting its phosphorylation on threonine and threonine residues adjacent to prolines (15). Also in accordance with these results, here we demonstrate that p73 is a direct target for p38 MAPK phosphorylation.

PML mutants that exhibit aberrant nuclear distribution patterns and do not colocalize with p73 in the PML-NB are defective in stabilizing p73. Similarly, a recent work has demonstrated that PML isoform IV plays a role in regulating the stability of the DNA damage response protein topoisomerase IIβ–binding protein (TopBP1) through its recruitment into the PML-NB in response to γ-irradiation (36).

It is becoming apparent that the PML-NB can be regarded as a factory for posttranslational modifications (53). Key regulators of p53 function, such as HAUSP, a p53-specific deubiquitinylase (54); HIPK2, a novel p53 kinase (55); CBP; and p300 (32, 34, 49), colocalize with PML within the PML-NB. Therefore, as with p53, p73 recruitment to the PML-NB could result in multiple posttranslational modifications of the protein. Here, we show that PML indeed modulates p73 acetylation; in contrast, it does not affect p73 sumoylation levels (unpublished data).

Competition between ubiquitinylation and acetylation of overlapping lysine residues constitutes a novel mechanism to regulate protein stability (11, 48, 56). In particular, p300/CBP-mediated acetylation of p53 attenuates its degradation by blocking the ε-amino group of the lysine residues for ubiquitinylation (11, 48). Acetylation of p73 by p300 plays a key role in potentiating its apoptotic function in response to DNA damage by stimulating the ability of p73 to selectively transactivate proapoptotic target genes, such as the p53AIP1 (18). Here, we show that acetylation of p73 can also abrogate its ubiquitinylation and stabilize the protein, possibly by competition for the same overlapping lysine residues. Further studies will be needed to identify specific lysine residues, which are targeted for acetylation/ubiquitinylation modification.

PML positively regulates the ability of p300 to acetylate and stabilize p73, thus indicating that PML inhibits p73 ubiquitin-dependent degradation by affecting its acetylation levels. Importantly, the inability of PML mutants exhibiting an aberrant nuclear distribution pattern to potentiate p300-mediated acetylation and stabilization of p73 further supports a role for the PML-NB in regulation of p73 stability. The presence of p300 in the PML-NB suggests that p73 is recruited in these structures to facilitate and regulate its acetylation, which in turn promotes stabilization of the protein.

A recent paper (42) demonstrated that PML neutralizes the inhibitory effects of MDM2 and, therefore, inhibits p53 degradation by prolonging the stress-induced checkpoint kinase 2–mediated phosphorylation of p53 (42). In addition, we have found that PML is essential for MDM2 sequestration in the nucleolus upon DNA damage, which in turn leads to p53 accumulation (43). It remains to be established whether the ability of PML to modulate p53 acetyla-

tion also results in its stabilization. Thus, although the mechanisms that control p53 and p73 are distinct, our data identify PML as an important regulator of the stability of both proteins.

Our third major conclusion suggests a role for p73 in the pathogenesis of APL. Although the p73 gene is rarely mutated, interaction of the p73 protein with dominant negative forms of p53 and p73 (ΔNp73, a mutant lacking the NH_2-terminal transactivating domain) or hypermethylation of 5′ untranslated region of the p73 mRNA (23) has been observed in tumors of various histological origins. It has been reported recently that ΔNp73 may provide a proliferative advantage to cancer cells by counteracting the transactivation activities of p53 as well as TAp73 (the full-length p73 isoform) and, hence, their ability to induce apoptosis (1, 8). Indeed, several NH_2-terminally deleted variants of p73 are overexpressed in cancer cells (4) and represent a strong adverse prognostic marker in certain tumors such as neuroblastoma (57). Therefore, it is emerging that the balance between TAp73 and ΔNp73 isoforms and loss of expression, rather than gene mutation, could contribute to tumorigenesis. Although when overexpressed, PML appears to stabilize both TAp73 and ΔNp73 isoforms (Fig. S1, A and B), it remains to be determined whether or not in vivo PML would stabilize preferentially the TAp73 isoforms, an issue that cannot be presently addressed in view of the lack of antibodies that recognize endogenous ΔNp73 proteins.

Among hemopoietic malignancies, loss of p73 expression is observed in a subset of lymphomas and leukemias (23). In addition, analysis of samples from acute myeloid leukemia patients has revealed increased expression of low molecular weight p73 splicing variants (γ, δ, and ζ), which are weaker transcriptional activators than the larger isoforms (58). In APL, p73 function may be impaired for at least two reasons. First, the PML dose is reduced, due to the fact that one PML allele is involved in the chromosomal translocation, and PML-RARα is defective in p73 stabilization. Second, disruption of the PML-NBs by PML-RARα can further exacerbate this defect. Our observation that PML-RARα acts as a dominant negative regulator of p73 acetylation (Fig. 6 C) strongly supports the hypothesis that stability and activity of p73 may be compromised in APL blasts. In this respect, PML-RARα has been reported recently to destabilize p53 by promoting its deacetylation and its subsequent MDM2-dependent degradation (59). Also, in complete agreement with these observations, p73 protein levels are markedly increased upon retinoic acid treatment of the human APL cell line NB4 (58). As retinoic acid treatment of NB4 cells causes the degradation of the PML-RARα fusion protein and the reorganization of the PML-NB (28), this may restore the ability of PML to stabilize and functionally activate p73.

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