PML IV/ARF interaction enhances p53 SUMO-1 conjugation, activation, and senescence

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Promyelocytic leukemia protein (PML) nuclear bodies (NBs) recruit multiple partners, including p53 and many of its regulators. NBs are believed to facilitate several posttranslational modifications and are key regulators of senescence. PML, the organizer of NBs, is expressed as a number of splice variants that all efficiently recruit p53 partners. However, overexpression of only one of them, PML IV, triggers p53-driven senescence. Here, we show that PML IV specifically binds ARF, a key p53 regulator. Similar to ARF, PML IV enhances global SUMO-1 conjugation, particularly that of p53, resulting in p53 stabilization and activation. ARF interacts with and stabilizes the NB-associated UBC9 SUMO-conjugating enzyme, possibly explaining PML IV-enhanced SUMOylation. These results unexpectedly link two key tumor suppressors, highlighting their convergence for global control of SUMO conjugation, p53 activation, and senescence induction.

UBC9 | posttranslational modification | nuclear matrix | splicing | tumor suppression

Promyelocytic leukemia protein (PML) is the key organizer of PML nuclear bodies (NBs), organelles that recruit an ever-growing number of unrelated partner proteins (1). Initially discovered through its targeting by an oncogenic translocation (2), PML has drawn considerable attention through its complex link with SUMOylation, a posttranslational modification (PTM) by ubiquitin-like peptides (SUMO). Indeed, PML must be SUMOylated to recruit its partners within PML NBs, together with the universal SUMO-conjugating enzyme UBC9 then facilitating the partner's modification (1, 3–5). Alternative splicing of human PML yields at least seven isoforms, and previous work has implicated these isoforms in specific interactions or functions (6–8). In particular, overexpression of a single isoform, PML IV, initiates p53 activation and drives senescence (8). Conversely, the requirement for PML in senescence induction was demonstrated by Ras's inability to induce senescence in *pml*^{−/−} cells (9, 10). In many other experimental systems, p53 activation and/or senescence induction can be antagonized by PML extinction (11, 12).

p53 is a multifunctional transcription factor, which is a key regulator of survival and proliferation (13). p53 activity is tightly regulated through multiple PTMs that control interactions with partner proteins or DNA. How exactly PML controls p53 is still debated. Numerous studies have pointed out the ability of PML to recruit p53 and many of its specific regulators (HIPK2, HAUSP, DAXX, CBP, HDM2, SIRT1, Chk2, MageA2, MOZ, etc.) into NBs (10, 14–20). Partner recruitment/retention within NBs was proposed to enhance various p53 PTMs, particularly acetylation of lysine K382. However, neither partner nor p53 recruitment is specific for PML IV (8), so the basis for the exquisite specificity of PML IV to control p53 signaling remains ill-understood. A study proposed that PML IV represses *TBX2*, a gene amplified in some cancers that controls the *CDKN2A* promoter (21). The *CDKN2A* locus encodes two related products, ARF and p16INK4A, both involved in senescence activation

through p53 and pRB, respectively. Besides its control of p53 abundance, ARF also exerts p53-independent functions, notably on the regulation of cell growth (22). These functions were proposed to rely, at least in part, on ARF's ability to enhance global SUMOylation (23–27).

Here, we demonstrate that PML IV specifically binds ARF and enhances p53 SUMO-1 conjugation, resulting in p53 stabilization and senescence. Our results unexpectedly bridge PML and ARF, two key senescence players, and highlight their roles in SUMOylation and control of p53-induced senescence.

Results

PML IV C Terminus Is Required to Trigger Senescence. PML IV exon 8a is shared with PML I (Fig. 1A) and is thus unlikely to account on its own for senescence induction. Exon 8b, specific for PML IV, encodes a 13-amino-acid sequence conserved among species (Fig. 1A and Fig. S1). Remarkably, this short amino acid stretch contains seven cyclic amino acids with high steric hindrance. In particular, three bulky aromatic residues (human residues F623, Y627, and F631) are perfectly conserved between man and mouse (Fig. 1A) and in most mammals (Fig. S1).

To test whether these amino acids are involved in the specific function(s) of PML IV, we generated two mutants, either deleted for the PML IV-specific sequence (PML IVΔC) or with substitutions of aromatic residues for alanine (PML IV3A: F623A, Y627A, and F631A) (Fig. 1A). Overexpression of PML IVΔC, PML IV3A, or PML I in WI38 human primary fibroblasts failed to induce growth arrest, whereas PML IV or oncogenic Ras did (Fig. 1B) (8, 10). Moreover, Ras- and PML IV-transduced cells

Significance

Promyelocytic leukemia protein (PML) nuclear bodies (NBs) are subnuclear domains proposed to facilitate posttranslational modifications. Among NB partners, proteins are p53 and are most of the regulators. Overexpression of a single PML splice variant, PML IV, triggers p53-driven senescence. We demonstrate an interaction between PML IV C terminus and the ARF tumor suppressor. This interaction is required to promote p53 SUMOylation and subsequent stabilization. These results unexpectedly bridge three key tumor suppressors and stress the key role of PML NBs as SUMOylation factories and regulators of senescence.

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Fig. 2. PML IV increases p53 SUMOylation by SUMO-1. (A) H1299 cells stably expressing His-SUMO-1 or His-SUMO-2 were transfected with the indicated expression vectors 2 d before analysis. Purification of His-SUMO-1 (Top) and His-SUMO-2 (Right) conjugates followed by p53 Western blot. Samples were normalized for p53 expression to compare SUMOylation efficiency. (B) Conjugation of endogenous p53 by SUMO-1 was assessed by PLA in WI38 cells. (Left) Quantification of p53-S1 amplified dots (mean $\pm$ SD from three independent experiments). * P < 0.05. (Right) p53-SUMO-1 red dots; PML was detected by immunofluorescence (green). (Scale bar, 5 μ m.) (C) H1299-HisS1 cells were transfected with p53, p53K386R, or p53E388A, in combination with empty vector, PML IV, or PML IVΔC. Protein extracts were immunoblotted with anti-PML, p53, acetyl K382 p53, and actin antibodies. Asterisk, nonspecific band. (D) H1299-HisS1 cells were transfected with p53K382R acetylation mutant, in combination with empty vector, PML IV, or PML IVΔC. Purification of His-SUMO-1 conjugates was followed by p53 Western blot. (E) Cotransduction of PML with SUMO paralogs in WI38 cells. Protein extracts were normalized for GFP level and immunoblotted with PML, p53, p21, and GFP antibodies. (F) His-SUMO-1 or His-SUMO-2 purification was performed in stably SUMO-expressing HeLa cells, transiently transfected by HDMX and PML or ARF constructs. Shown is the immunoblotting with HDMX or PML antibodies. (G) WI38 primary cells were transduced with PML expression vectors. After 3 d, global conjugation of endogenous SUMO-1 (Left) or SUMO-2 (Right) was analyzed by Western blot. Levels of GFP and PML are shown as controls.

ARF to PML NBs (Fig. 3B). Interestingly, PML IV K160R, a SUMOylation mutant that fails to recruit most NBs partners (4), still recruits ARF (Fig. 3A and B). In stable PML transfectants

derived from primary *pml*^{-/-} mouse embryo fibroblasts (MEFs), only PML IV, but not other isoforms (PML I or V), was able to recruit ARF from the nucleolus into NBs (Fig. 3C). Localization

Fig. 3. PML IV specifically interacts with ARF through its C terminus. (A) ARF or PML immunoprecipitations followed by PML or ARF Western blot in SaOs-2 cells. Inputs are shown. (B) PML (green) and ARF (red) immunostaining in H1299 cells transfected as indicated. Arrows point to NBs where the two proteins colocalize. (Scale bar, 5 μ m.) (C) Primary *pml*^{-/-} MEFs were stably transduced by expression vectors encoding PML I, IV, or V isoforms and ARF. Cells were labeled with anti-PML antibody (red) and anti-ARF antibody (green). (Scale bar, 5 μ m.) Arrows denote PML/ARF colocalization. (D) Interaction of endogenous PML IV and ARF was tested by coimmunoprecipitation in 293T cells. Control serum (Ctrl) or anti-ARF antibody was used for immunoprecipitation. PML was revealed either with pan-PML antibody or isoform-specific antibodies (6). (E) Empty vector (Ctrl), PML I, or PML IV was transduced in WI38 cells. PLA was assessed between PML and endogenous ARF (red dots), and PML was immunostained (green). (Scale bar, 5 μ m.)

of other nucleolar proteins like B23/NPM was unaffected by PML IV (Fig. S5). Using isoform-specific PML antibodies (6), we demonstrated endogenous PML IV/ARF interaction by coimmunoprecipitation in 293T cells (Fig. 3D). Existence of this PML IV/ARF interaction was also substantiated by a significantly increased PLA in PML IV-transduced WI38 cells (Fig. 3E), PML IV/ARF-cotransduced WI38 cells (Fig. S6B), or H1299-transfected cells (Fig. S6C). Collectively, these results demonstrate that PML IV specifically interacts with ARF through its C terminus.

ARF Binds to and Stabilizes UBC9. PML IV-induced p53 SUMOylation was dampened upon ARF extinction, suggesting that ARF could be implicated in PML IV-enhanced SUMOylation of p53 (Fig. 4A). Global ARF-enhanced SUMOylation was suggested to rely on its ability to interact with UBC9 (26). Indeed, UBC9 protein and ARF coimmunoprecipitated (Fig. 4B). The association between endogenous ARF and UBC9 was further demonstrated by PLA in WI38 cells (Fig. 4C) and was significantly enhanced by PML IV, but not PML IV Δ C, expression (Fig. 4C and Fig. S6). Moreover, we observed a sharp stabilization of transfected UBC9 upon PML IV or ARF expression, with a synergistic impact for PML IV–ARF combination, which is suggestive for complex formation (Fig. 4D). PML binds UBC9 through its RING domain and ARF through its C terminus, which could favor formation of a tripartite complex that could explain their mutual stabilization in an environment with active proteolysis (37, 38).

NB Recruitment of Partners Is Essential for PML IV-Driven Senescence. Our studies suggest that ARF recruitment within NBs enhances activity of UBC9, notably on p53 and its modulators, and drives PML IV-initiated senescence. However, a previous study argued that PML IV does not need to form NBs and/or recruit a partner to initiate senescence. A single SUMOylation site in PML, K160, is required for recruitment of partner proteins through their own SUMO-interacting motif (SIM) (4, 5). We therefore assessed whether PML IV K160R would induce senescence. Remarkably, PML IV K160R did not affect the growth of WI38 cells (Fig. 5A) or p53 activation, as shown by p21 stabilization 48 h postinfection (Fig. 5B). Absence of p53 stabilization upon expression of PML IV K160R was also observed upon transfection of HeLa-S1 (Fig. S7).

We also cotransduced PML IV with CMV IE1, a protein disrupting PML NBs or its variant IE1core, which spares NB structure

and only impedes partner recruitment (39). Critically, coexpression of either IE1 constructs with PML IV completely reversed growth arrest, whereas these viral proteins alone did not significantly alter cell proliferation (Fig. 5C). Therefore, PML IV-driven senescence relies on its ability to recruit partners, such as ARF and p53, into NBs (Fig. 5D).

Discussion

We unravel a specific interaction between two key senescence drivers, PML IV and ARF. This interaction likely accounts for PML IV control over p53 stability, activation, and subsequent

Fig. 4. PML IV/ARF interaction promotes UBC9 stabilization and p53 SUMOylation. (A) H1299-HisS1 cells were transfected with p53 and PML IV-expressing constructs in combination with control or ARF shRNA. His purification was followed by p53 Western blot. Samples were normalized for p53 expression to compare SUMOylation efficiency. (B) H1299-HisS1 cells were transfected as indicated. ARF or HA immunoprecipitation is analyzed by Western blot. IP Ctrl, immunoprecipitation with nonrelevant serum. (C) Empty vector (Ctrl), PML IV, or PML IV Δ C expression vectors were transduced in WI38 cells. PLA was assessed between both endogenous UBC9 and ARF (Left), and dots per cell were quantified (means from 50 cells) (Right). * P < 0.05; ** P < 0.01. (D) UBC9 protein is stabilized by ARF or PML IV in H1299-HisS1 cells. The remaining UBC9 signal is indicated (*).

Fig. 5. NB recruitment of partners is essential for PML IV-driven senescence. (A) Proliferation of primary human WI38 fibroblasts infected by lentiviruses expressing the indicated constructs. (B) Western blot analysis of PML, p53, p21, and actin in WI38 cell extracts 2 d after transduction. (C) Proliferation of primary human WI38 fibroblasts transduced by PML IV-expressing lentiviruses in combination with the IE1 core (39). (D) PML IV/ARF-controlled senescence model. Although UBC9 is recruited through the PML RBCC domain (5), PML IV specifically interacts with ARF, enhancing formation of an ARF/UBC9 complex within PML NBs. This leads to an increase of SUMO-1 conjugation of p53 and some of its partners. ARF enrichment into PML NBs also enhances ARF-mediated HDM2 inhibition, leading to p53 stabilization. Finally, enhanced global SUMOylation could also promote senescence. RING, RING-finger domain; S1, SUMO-1.

senescence induction. These unexpected results shed a new light on the relationship between PML, ARF, or p53 and further link PML NBs to regulation of global SUMOylation.

Most studies assessing the role of PML on p53 function used PML IV, the only isoform that stabilizes p53 and induces senescence. However, to date, the molecular basis for this striking selectivity remained poorly understood. Here, we demonstrate that the C terminus of PML IV is required for ARF binding, p53 SUMOylation, stabilization, and activation. Although ARF notoriously engages in nonspecific binding when overexpressed, the specific interaction between endogenous ARF and PML IV (Fig. 3 C–E) is supportive for a physiologic relevance. When expressed in *pml*^{−/−} MEFs, PML IV may be observed surrounding the nucleolus, where ARF is normally located and fusion to GFP of an exon 8b-containing peptide enforced its nucleolar localization (40). Recruitment of ARF in the p53-enriched PML NB environment can activate p53 signaling by multiple mechanisms. First, PML IV/ARF-dependent p53 SUMO-1 conjugation is critical for p53 stabilization (Fig. 2C). Note that P53 SUMO-1 conjugation was implicated in apoptotic control or senescence (11, 41) and that the unexpected SUMO-1 paralog specificity for p53 stabilization and p21 activation deserves further investigation. Second, ARF directly interacts with several NB-associated p53 regulators, such as HDM2 (Fig. 5D). Third, NB-associated p53 regulators (HIPK2, HAUSP, DAXX, CBP, HDM2, SIRT1, Chk2, MageA2, or MOZ) may all be SUMOylated. PML and/or ARF-enhanced SUMOylation could alter their functions, as proposed for HDMX (34). Note that NB-enhanced p53 signaling is not always associated with p53 stabilization (Fig. 5B) (12), suggesting that

PML-enhanced PTMs may actually suffice for p53 activation and senescence induction.

As proposed for DNA damage domains, recruitment of the SUMOylation machinery into PML NBs targets a functionally defined group, rather than an individual protein (42). NBs appear as scaffolds that facilitate interactions of ARF, UBC9, and multiple p53 regulators to favor their reciprocal SUMOylation and interactions, ultimately activating p53 signaling. However, that PML IV, physiologically a minor isoform (6), fails to trigger senescence in *pml*^{−/−} cells (8) stresses the importance of the other, more abundant, isoforms. These recruit p53 and HDM2 (16, 32, 33) and could enforce efficient NB recruitment of other p53 modifiers by SUMO/SIM interactions (5). A previous study had argued that PML IV-initiated senescence does not require NBs (8). Technical differences in the lentiviral expression vector used and culture protocols that result in higher PML expression levels may explain these discrepancies. NBs facilitate a number of PTMs by a scaffolding effect, concentrating enzymes and protein substrates within a restricted domain (1). With high levels of PML expression, NB formation may no longer be required for p53 activation.

ARF tumor-suppressive activities are only partially p53-dependent and were proposed to also rely on enhanced global SUMO-1 conjugation. ARF interacts with recombinant UBC9 in vitro and favors global SUMO-1 conjugation in cell lines (26). Our results suggest that PML IV favors endogenous ARF/UBC9 interaction, at least in part by recruiting them within NBs (Fig. 4C). The ARF/UBC9 interaction stabilizes the proteins (Fig. 4B and D) and could also enhance the intrinsic activity of UBC9. In that respect, PML IV sharply enhanced SUMO-1 conjugation of a p53–UBC9 fusion (Fig. S8). These enhanced ARF/UBC9 interactions could explain global PML IV-mediated hyper-SUMO-1 conjugation (Fig. 2G), which might contribute to PML IV-driven senescence independently of p53 activation (Fig. 5D).

Our findings have important implications for cancer biology, as they directly link two major senescence drivers and upstream activators of p53. These results highlight the importance of PML or ARF loss in tumors (1, 43) and that of SUMOylation in cancer progression (44, 45).

Experimental Procedures

Plasmids, Transfection, and Transduction. ARF, PML isoforms I and IV, and PML IV mutants were expressed from the pSG5 vector. Oncogenic Ras was cloned in the pLPC vector, p53 in pCMV, HDM2 and HDMX in pcDNA3.1(+), and ARF shRNA in pSUPER (kindly provided by S. Lain, Karolinska Institutet, Stockholm). PML IV mutants (Δ C, 3A, K160, Δ CC, and Δ 8a) or p53 K386R and E388A mutants were obtained by site-directed mutagenesis (Quick Change Kit, Stratagene). HA-tagged UBC9 expression vector was obtained from Addgene (p3258, hCMV huUBC9-HA). PML RING-finger, B-boxes, coiled-coil domain (RBCC) fused to exon 8b (RBCC-8b) was obtained by assembly PCR. For lentiviral transduction, the pWPI expression vector containing GFP cDNA and either PML isoforms ARF or His-SUMO cDNAs was used, and the pTRIP vector was used for p53 extinction. For retroviral transduction, all of the constructs were expressed from MSCV-based retroviral vectors. Stable H1299 and HeLa cells expressing His-SUMO-1 or -2 were obtained by lentiviral transduction and GFP sorting. Transient transfections were performed with CaCl₂ or Effectene Transfection Reagent (Qiagen) and WI38 nucleofection with Amaxa technology (Lonza).

Cell Culture, β -Galactosidase Staining, and BrdU Labeling. H1299, SaOs-2, HEK293T, HeLa, and MEF cell lines were grown in 10% FCS-supplemented DMEM (GIBCO). WI38 primary cells were cultured in Eagle's minimal essential medium (EMEM) (ATCC) supplemented with 10% FCS and essential amino acids (GIBCO). WI38 population doublings were analyzed as previously described (8). SA- β -Gal activity was detected according to the manufacturer's instructions (Senescence β -Galactosidase Staining Kit, Cell Signaling).

Protein Purification, Immunoprecipitation, and Western Blot. His-tagged protein purification was performed after lysis into buffer (6 M guanidium-HCl, 0.1 M $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 10 mM imidazole pH 8). NiNTA resin from Invitrogen was incubated for 2 h, washes were performed with decreasing amounts of guanidium-HCl, and elutions were performed in Laemmli buffer with 200 mM imidazole. For PML or ARF immunoprecipitation, cells were lysed (lysis buffer, 10 mM Tris-HCl pH 7.5, 120 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 0.1% SDS, 1 mM DTT, protease inhibitor Complete Mini Roche). Immunoprecipitation was performed using our own designed PML rabbit polyclonal antibody or ARF monoclonal antibody (Calbiochem, clone 14P02) and protein A agarose beads (Roche). Western blots were performed using rabbit polyclonal PML isoform antibodies (6), chicken polyclonal PML antibody, p53 monoclonal DO-1 antibody (Santa Cruz, sc-126), Acetyl-p53 K382 polyclonal antibody (Cell Signaling, 25255), ARF polyclonal antibody (Biosource, clone AHZ0472), actin monoclonal antibody (Sigma, 20-33), HDM2 monoclonal antibody (Santa Cruz, SMP14), HDMX monoclonal antibody (Bethyl, A300-287A), SUMO-1 monoclonal antibody kindly provided by M. J. Matunis, Johns Hopkins Bloomberg School of Public Health, Baltimore, SUMO-2 polyclonal antibody (Invitrogen, 51-9100), GFP polyclonal antibody (Santa Cruz, sc-8334), UBC9 polyclonal antibody (Santa Cruz, clone H-81), and anti-His Tag monoclonal antibody (Novagen, 71941). Secondary antibodies were coupled to peroxidase (Jackson ImmunoResearch).

Immunostaining, PLA, and Microscopy Image Acquisition. Prepermeabilized (0.1% Triton-X100) cells were fixed in 4% paraformaldehyde and were immunolabeled with the indicated antibodies in 0.1% Triton-X100 and 1%

BSA. Primary antibodies using p53 (DO-1, Santa-Cruz, sc-126), chicken PML, ARF (Biosource, AHZ0472), and SUMO-1 polyclonal antibody (Cell Signaling, 4930) were detected by secondary antibodies (from Jackson Immuno-Research) coupled to fluorochromes [Alexa Fluor 488 or 594 or aminomethylcoumarin acetate (AMCA)] with Mowiol mounting medium. In situ PLAs were performed using the Duolink technology (Olink Bioscience). Confocal analyses were performed with a LSM510 meta-laser microscope (Carl Zeiss Micro-Imaging, Inc.) with a Plan-Apochromat 63 $\times$ N.A. 1.4 oil immersion objective at 20 °C. Images were acquired with LSM510 software (Carl Zeiss).

Statistical Analyses. Bilateral Student's *t* tests were used to assess the statistical significance of observed differences. All error bars are SD.

Note Added in Proof. ARF was similarly shown to cooperate with TRIM28, a PML family member, to promote NPM1 SUMOylation (46).

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