PML3 Orchestrates the Nuclear Dynamics and Function of TIP60*

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The promyelocytic leukemia (PML) protein is a major component to govern the PML nuclear body (NB) assembly and function. Although it is well defined that PML NB is a site recruiting sumoylated proteins, the mechanism by which PML protein regulates the process remains unclear. Here we show that PML3, a specific PML isoform, interacts with and recruits TIP60 to PML NBs. Our biochemical characterization demonstrates that PML3 physically interacts with TIP60 via its N-terminal 364 amino acids. Importantly, this portion of PML60 is sufficient to target to the PML NBs, suggesting that PML3-TIP60 interaction is sufficient for targeting TIP60 to the NBs. The PML3-TIP60 interaction is specific, since the region of TIP60 binding is not conserved in other PML isoforms. The physical interaction between PML3 and TIP60 protects TIP60 from Mdm2-mediated degradation, suggesting that PML3 competes with MDM2 for binding to TIP60. Fluorescence recovery after photobleaching analysis indicates that the PML3-TIP60 interaction modulates the nuclear body distribution and mobility of TIP60. Conversely, the distribution and mobility of TIP60 are perturbed in PML3-deficient cells, accompanied by aberrations in DNA damage-repairing response. Thus, PML3 orchestrates the distribution, dynamics, and function of TIP60. Our findings suggest a novel regulatory mechanism by which the PML3 and TIP60 tumor suppressors cooperate to ensure genomic stability.

The histone acetyltransferase TIP60 regulates the DNA damage response following genotoxic stress by acetylating histone and remodeling chromatin (1–5). Besides histone acetylation activity on chromatin remodeling, recent data indicate that TIP60 has more divergent functions in many processes. In transcriptional regulation, TIP60 can acetylate various transcriptional factors besides histones. For instance, androgen receptor (AR) is acetylated by TIP60, which enhances its trans-activation in a ligand-dependent manner (6, 7). Interaction of TIP60 with BLC-3 regulates the transcriptional activity of NFkB (8); The ability of c-MYC to activate transcription relies in part on the recruitment of cofactor complexes containing TIP60, which directly acetylates c-MYC (9). Moreover, the kinase ataxia telangiectasia-mutated (ATM) activation is dependent on its acetylation by TIP60 in response to DNA double break repair (10), indicating that TIP60 plays crucial roles in DNA damage response. Indeed, mounting evidence implies that TIP60 functions at multiple levels in DNA damage repair, and its histone acetyltransferase activity acts as an upstream component in DNA damage checkpoint activation (11).

So far, regulation of TIP60 by post-translational modification has been documented. Similar to p53, TIP60 is subjected to proteasome-dependent proteolysis by interacting physically with Mdm2, a specific E3 ligase in ubiquitination cascade and accumulates following UV irradiation, indicating that ubiquitination of TIP60 could be part of the mechanism in tumorigenesis induced by Mdm2 and p53 following cellular stress (12). Combining biochemical assay with mass spectrometric analysis, Lemercier et al. (13) revealed that TIP60 can be phosphorylated at Ser286 and Ser90, whereas TIP60 histone acetyltransferase activity is controlled by its phosphorylation. Specifically, the phosphorylation of Ser90 is modulated by cyclin B/Cdc2; accordingly, phosphorylated TIP60 is accumulated after drug-induced arrest of cells in G2/M or at the G2/M transition, which strongly suggests a cell cycle-dependent control of TIP60 activity. As a histone acetyltransferase, TIP60 can also be acetylated by p300/CREB-binding protein in the zinc finger domain at Lys268 and Lys282, although the impact of acetylation on TIP60 function has yet to be elucidated (14). Recently, we identified that TIP60 is a novel substrate of sumoylation, and SUMO

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attachment remarkably translocates TIP60 to PML nuclear bodies (PML NBs), which is a well defined pool of sumoylatable substrates. Significantly, p53 is recruited by sumoylated TIP60 to PML NBs in response to UV irradiation (15), suggesting that PML NBs are an essential location to monitor TIP60 function.

PML NBs are subnuclear protein structures from 0.3 to 1.0 μm in diameter and were first identified as nuclear domain 10 (ND10) by human autoantibodies recognizing Sp100 protein (16, 17). Numerous dynamic components of PML NBs, including ATM, Chk2, p53, TIP60, Mdm2, Daxx, SUMO-1, and Ubc9, prominently implicate a role for PML NBs in DNA repair, apoptosis, gene regulation, and post-translational modification (18, 19). Despite the fact that the PML NB is a highly dynamic molecular machine that undergoes dramatic structural and numerical changes during cell cycle and in response to various cellular conditions, the plasticity of PML NB is essential to sustain normal cell growth and development, since perturbation of PML NB plasticity and structure has been observed in human diseases such as in acute promyelocytic leukemia (20). In acute promyelocytic leukemia cells, during treatment with therapeutic agents, such as As$_2$O$_3$, the disrupted PML NBs reorganize to a normal NB pattern (21), suggesting that PML NBs are potential targets for acute promyelocytic leukemia therapy.

Among the factors essential for the integrity of PML NBs, sumoylation plays a key role in the formation of mature PML NBs. Not only is sumoylation of PML protein an indispensable prerequisite to sustain PML NB integrity (22), but also multiple sumoylated substrates reside in PML NBs, including p53 and TIP60 (15, 23), indicating that SUMO modification may mediate the translocalization or that PML NBs are locations where sumoylation takes place. Once nonsumoylatable PML protein in which conservative SUMO attachment sites are mutated is overexpressed, the integrity of PML NBs is disrupted (20). In acute promyelocytic leukemia cells, the disrupted PML NBs derive from the expression of PML-RARα fusion protein, which is found fused to the retinoic acid receptor-α as a consequence of the t(15,17) chromosomal translocation (24–26). Under As$_2$O$_3$ treatment, the reorganized integrity of PML NBs is due to the induced sumoylated PML-RARα and relocation of PML protein to NBs (27).

Although the nuclear antigen Sp100 was the first characterized protein to localize in PML NBs, the PML protein is the major structural component of the PML NBs. According to C-terminal sequence variation, seven PML isoforms have been identified (28). However, most studies on these isoforms have concentrated on PML3, which has been revealed to interact with p53, increasing p53 transcriptional activity and recruiting it to PML NBs following cellular stress (29). Given our novel discovery that sumoylated TIP60 targets to PML NBs and the functional similarity of p53 and TIP60, we are intrigued to explore the impact of PML3 on TIP60 functions in PML NBs.

In this study, we present evidence that PML3 recruits TIP60 into PML NBs by specifically mutual binding, and we also observe that the interaction of the two proteins is independent of their sumoylation. In addition, we demonstrate that PML3 protects TIP60 from degradation by sequestering Mdm2 binding through a ubiquitination cascade reaction. Significantly, we confirm that physical interaction allows PML3 to control the dynamics of TIP60 in PML NBs. Collectively, this study provides a novel mechanism to further understand the interactive network among TIP60, PML3, and Mdm2 in transcriptional regulation and tumorigenesis.

MATERIALS AND METHODS

Cell Culture and Transfection and Small Interfering RNA Experiment—HeLa cells, H1299 cells, U2OS cells, and 293T cells were obtained from ATCC (Manassas, VA) and cultured in media containing 10% fetal bovine serum at 37 °C with 10% CO$_2$. Antibiotics (penicillin/streptomycin, 100 mg/ml) and L-glutamine (30 mg/ml) were obtained from Cellgro. Fetal bovine serum was from Hyclone. For cycloheximide (CHX; Sigma) treatment, cells were treated with CHX (30 μg/ml) to inhibit protein translation at a desirable time after 24 h of transfection. For MG132 and INF-α treatments, cells were cultured in media containing MG132 (20 μM) and INF-α (2 kilounits/ml) for a desirable time. For UV-irradiation treatment, cells were cultured in media and irradiated with UV (100 J/m$^2$) using a cross-linker (Hoefer).

Lipofectamine 2000 for transfection was obtained from Invitrogen. For cell transfection, cells were plated at the appropriate dilution and then transfected with Lipofectamine 2000 according to the manufacturer.

Antibodies, Plasmids, and Small Interfering RNA—The monoclonal anti-GFP antibody was obtained from BD Biosciences; anti-FLAG and tubulin were from Sigma; anti-GST, anti-HA, and anti-maltose-binding protein (MBP) antibodies were all purchased from Cell Signaling. The polyclonal anti-PML and anti-TIP60 antibodies were purchased from Upstate Biotechnology. The affinity-purified anti-PML3 rabbit antibody was characterized as described previously (30). Secondary antibodies were from Jackson ImmunoResearch Laboratories, including rhodamine-conjugated goat anti-rabbit IgG and goat anti-mouse IgG.

Details of constructs will be provided upon request. Anti-PML3 small interfering RNA was described by Xu et al. (30) and purchased from Dharmaco. The small interfering RNA sequence is as follows: PML3, 5’-AATGAAAGTGGGTTCTCCTGG-3.

Immunoprecipitation, in Vivo and in Vitro Binding Assay, and Western Blotting Analysis—Whole cell protein extracts were obtained by lysing cells in SDS-containing lysis buffer. For Western blotting, aliquots of total cell extracts were resolved on a 5–15% SDS-polyacrylamide gel, following transfer onto nitrocellulose membranes and blocking with 5% nonfat dried milk diluted in Tris-buffered saline, 0.05% Tween 20 (Sigma). Proteins of interest in membrane were detected by incubating with primary antibodies overnight at 4 °C. Then the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at room temperature and developed with the standard ECL detection method under conditions recommended by the manufacturer (Amersham Biosciences).

For the co-immunoprecipitation assay, transfected cells were rinsed with phosphate-buffered saline and scraped into 500 μl of lysis buffer (150 mmo NaCl, 50 mmo Tris–HCl, pH 7.5, and 0.2% Nonident P-40), supplemented with a complete protease-inhibitor mixture. The scraped cells were lysed on ice for 30 min with occasional light vortexing, followed by 15 min of centrifugation to remove cellular debris. The supernatant was incubated with
FIGURE 1. PML3 recruits TIP60 to PML NBs. A, TIP60 and nonsumoylatable TIP60dm diffuse throughout the nucleus. U2OS cells were stained using TIP60 antibody (a), or HeLa cells were transfected to express GFP-TIP60 (b) or GFP-TIP60dm (c). Thirty-six hours post-transfection, cells were fixed, permeabilized, and stained with PML antibody to indicate PML NBs and FLAG-PML3. Bar, 10 μm. B, PML3 facilitates TIP60 and nonsumoylatable TIP60dm to PML NBs. U2OS cells treated with UV (100 J/m^2) were stained using TIP60 antibody (a), or HeLa cells were transfected to co-express FLAG-PML3 and GFP-TIP60 (b) or GFP-TIP60dm (c). Thirty-six hours post-transfection, cells were fixed, permeabilized, and stained with PML3 antibody or FLAG antibody to indicate FLAG-PML3. Bar, 10 μm. DAPI, 4',6-diamidino-2-phenylindole. C, PML3 recruits TIP60 into PML NBs. U2OS cells grown on coverslips were transfected with Flag-PML3. Thirty-six hours post-transfection, cells were in normal condition (a) or exposed to UV (100 J/m^2) (b), then cells were fixed, permeabilized and stained with TIP60 antibody and Flag antibody to indicate TIP60 and Flag-PML3. Bar: 10 μm. D, specific knockdown expression of PML3 by siRNA. U2OS cells were transfected with the PML3 siRNA oligonucleotide (150 nM) for different intervals (0, 24, 36 and 48 hours) and subjected to SDS-PAGE and immunoblotting. Top panel: PML3; middle panel: PML4; lower panel: tubulin. E, depletion of PML3 abrogates TIP60 targeting to PML NBs. U2OS cells were transfected with/without PML3 siRNA oligonucleotide (150 nM) (b, d and a, c). Forty-eight hours after transfection, cells were in normal condition or treated with UV-irradiation (100 J/m^2) (a, b and c, d), and then fixed, permeabilized and stained with TIP60 and PML3 antibody to indicate TIP60 and PML3. Bar: 10 μm.
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FLAG antibody-conjugated FLAG M2 beads at 4 °C for 1 h or more. Immunoprecipitates were washed five times with ice-cold immunoprecipitation buffer and resolved by SDS-PAGE. For in vivo binding assays, transfected 293T cells with FLAG-PML3, FLAG-PML4, and FLAG-PML-RARα were lysed in 300 mM NaCl-containing lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 10% glycerol). The lysates were then diluted twice and incubated with 4 µg of MBP-TIP60 or MBP alone. For the in vitro binding assay, GST fusion protein-bound Sepharose beads were used as an affinity matrix to iso-
late proteins interacting with TIP60 by purified MBP-tagged full-length and mutant of TIP60 that were expressed in bacteria. Briefly, the GST-PML3 fusion protein-bound Sepharose beads were incubated with purified proteins for 4 h at 4 °C. After the incubation, the beads were extensively washed with PBS and boiled in SDS-PAGE sample buffer, followed by fractionation of bound proteins on 10% SDS-polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane for Western blotting using an appropriate antibody. Western blotting was performed according to standard procedures.

**Immunofluorescence Staining**—For immunofluorescence staining, cells were plated on glass coverslips. Thirty-six hours after transfection, cells were fixed in PBS supplemented with 3.7% paraformaldehyde for 5 min and permeabilized in PBS supplemented with 0.5% Triton for 5 min. After a 30-min incubation in blocking buffer (1% bovine serum albumin in PBS), cells were stained with primary antibodies overnight at 4 °C and then incubated with the secondary fluorochrome-conjugated antibodies for 30 min at room temperature. After several washes, coverslips were mounted in Dabco 4',6-diamidino-2-phenylindole and analyzed with a microscope.

**Fluorescence Recovery after Photobleaching (FRAP) Analysis**—For FRAP analysis, HeLa cells were cultured in a glass-bottom culture dish (MatTek) with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37 °C. Cells were visualized by an inverted microscope (Leica SP5) with a 63 × 1.3 numerical aperture PlanApo objective and observed by using an argon laser at 543-nm excitation and 560–700-nm band pass emission filters for RFP or argon laser at 488-nm excitation and 500–530-nm band pass emission filters for GFP. An ~2-μm² bleach spot was generated by using the 70% power setting of the appropriate excitation laser. The times of 50% fluorescence intensity recovery (t₁/₂) at the bleached area from 10 independent experiments were quantified.

**RESULTS**

**PML3 Recruits TIP60 to the Nuclear Bodies**—It is well defined that TIP60 co-localizes with p53 in PML NBs to share similar biological significance, and PML3 recruits p53 to the NBs (12, 31). Taking into account our latest observation that UV radiation promotes the translocation of TIP60 to the PML NBs (15), we are intrigued to investigate whether PML3 assist the redistribution of TIP60 to PML NBs. To this end, we initially examined the localization of TIP60 under various conditions. U2OS cells stained with TIP60 antibody to detect endogenous TIP60 or HeLa cells transfected with GFP-TIP60 presented no PML NB localization, indicated by PML or PML3 polyanitibody staining (Fig. 1A, a and b), whereas overexpression of PML3 or UV treatment distinctly strengthened TIP60 localization in PML NBs in size and quantity (Fig. 1B, a and b).

Expressing PML3 alone also recruited TIP60 into PML NBs with or without UV irradiation (Fig. S1). Since TIP60 has been identified as a novel site-specific sumoylatable substrate (15), we next examined whether PML3 impacts on the distribution of nonsumoylatable TIP60 mutant (TIP60dm), in which two SUMO-conjugating sites (Lys⁴₃₀ and Lys⁴₅₁) were replaced by arginines. GFP-TIP60dm is diffused in whole nucleus (Fig. 1A, c). However, co-expression with FLAG-PML3 resulted in an apparent enrichment of TIP60dm in the PML body (Fig. 1B, c), suggesting that targeting of TIP60 to the PML body is independent of its sumylation.

Moreover, we sought to repress PML3 protein level using siRNA and examine if the localization of TIP60 depends on PML3. As shown in Fig. 1D, siRNA oligonucleotide-targeted PML3 selectively suppressed PML3 protein level without altering PML4 and tubulin levels. As reported previously, UV irradiation induced concentration of TIP60 in NBs, where it co-localized with PML3 (Fig. 1E, a). As predicted, repression of PML3 abolished the UV-induced NB localization of TIP60 (Fig. 1E, b), indicating that PML3 is required for NB localization of TIP60.

**PML3 Interacts with and Specifies TIP60 Localization to the PML Body**—To examine further whether PML3 recruits TIP60 to NB due to their physical interaction, we used PML3 antibody to carry out immunoprecipitation experiments with cell lysates from U2OS cells irradiated in vivo. As expected, UV irradiation of U2OS cells resulted in up-regulation of TIP60 expression and a concomitant increase in the amount of TIP60 co-immuno-

![Figure 2. Direct interaction between PML3 and TIP60 is independent of their sumoylation. A, PML3 interacts with TIP60 in vivo. Whole cell extracts from normal U2OS cells and irradiated U2OS cells (UV, 100 J/m²) were subjected to co-immunoprecipitation (IP) and Western blotting with anti-TIP60 and PML3 antibody (Ab) as above. Lysates loaded onto the gel were 5% of those used for co-immunoprecipitation in U2OS cells. Cell extracts were run on a 10% SDS-polyacrylamide gel. Lanes 1, normal cells; lanes 2, cells treated with UV (100 J/m²) incubated with PML3 antibody; lanes 3, cells treated with UV (100 J/m²) incubated with control IgG. B, TIP60 interacts with PML3 in vivo. Whole-cell extracts from normal U2OS cells and U2OS cells irradiated with 100 J/m² UV were subjected to co-immunoprecipitation (IP) and western blotting with anti-TIP60 and PML3 antibodies (Ab) as above. Lysates loaded onto the gel were 5% of those used for co-immunoprecipitation in U2OS cells. Cell extracts were run on a 10% SDS-PAGE gel. Lane 1, normal cell; lane 2, cells treated with UV (100 J/m²) were incubated with TIP60 antibody; lane 3, cells treated with UV (100 J/m²) were incubated with control IgG. C, PML3 interacts with TIP60 in vivo. 293T cells were transfected to co-express FLAG-PML3 and the indicated GFP-tagged TIP60 and GFP-TIP60dm constructs. Thirty-six hours after transfection, cells were collected for a co-immunoprecipitation assay with FLAG M2 beads. Immunoprecipitates were identified by Western blotting with GFP and FLAG antibodies. Lane 1, GFP + FLAG-PML3; lane 2, GFP-TIP60 + FLAG-PML3; lane 3, GFP-TIP60dm + FLAG-PML3. D, TIP60 interacts with PML3 in vivo. 293T cells were transfected to co-express FLAG-TIP60 or TIP60dm and the indicated GFP-tagged PML3 construct. Thirty-six hours after transfection, cells were collected for a co-immunoprecipitation assay with FLAG M2 beads. Immunoprecipitates were identified by Western blotting with GFP and FLAG antibodies. Lane 1, GFP + FLAG-TIP60; lane 2, GFP + FLAG-TIP60dm; lane 3, GFP-PML3 + FLAG-TIP60; lane 4, GFP-PML3 + FLAG-TIP60dm. E, schematic diagram of PML3 SUMO modification sites. F, nonsumoylatable PML3Δ₇₈ disrupts PML NB localization of TIP60. HeLa cells were transfected to co-express the indicated constructs. Thirty-six hours after transfection, cells were fixed, permeabilized, and stained with FLAG antibody to the indicated FLAG-PML3 and FLAG-PML3Δ₇₈. Bar, 10 μm. G, PML3 interacts with TIP60 in vivo. 293T cells were transfected to co-express FLAG-PML3 or FLAG-PML3Δ₇₈ and the indicated GFP-tagged TIP60 construct. Thirty-six hours after transfection, cells were collected for a co-immunoprecipitation assay with FLAG M2 beads. Immunoprecipitates were identified by Western blotting with GFP and FLAG antibodies. Lane 1, GFP + FLAG-PML3; lane 2, GFP + FLAG-PML3Δ₇₈; lane 3, GFP-PML3 + FLAG-TIP60; lane 4, GFP-PML3 + FLAG-TIP60dm. H, PML3 interacts with TIP60 in vitro. GST-tagged recombinant PML3 protein purified on glutathione-agarose beads was used as an affinity matrix to absorb purified MBP-TIP60 and MBP-TIP60dm proteins from bacteria. GST-PML3 and MBP fusion proteins were used as loading controls. Pull-downs were identified by Western blotting with MBP antibody. Lane 1, MBP; lane 2, MBP + GST-PML3; lane 3, MBP + TIP60; lane 4, MBP + TIP60 + GST; lane 5, MBP + TIP60 + GST + PML3; lane 6, MBP + TIP60dm; lane 4, MBP + TIP60dm + GST; lane 8, MBP + TIP60 + GST + PML3. DAPI, 4',6-diamidino-2-phenylindole.
precipitated with PML3 (Fig. 2A, lanes 1 and 2). Conversely, we use TIP60 antibody to perform immunoprecipitation. As shown in Fig. 2B, TIP60 also co-immunoprecipitated with PML3, which demonstrates that PML3 can interact with TIP60. Previous studies showed that sumoylation plays an important role in PML NBs assembly and relocalization of other components into PML NBs (32, 33). We then examined whether sumoylation of PML3 or TIP60 is essential for their interaction. We used co-immunoprecipitation experiments from transfected 293T cells in vivo and found that co-expression of FLAG-PML3 with GFP-TIP60 or GFP-TIP60dm and immunoprecipitation of PML3 led to co-immunoprecipitation of TIP60 or TIP60dm (Fig. 2C, lanes 2 and 3), and immunoprecipitation of TIP60 and TIP60dm also led to co-immunoprecipitation of PML3 (Fig. 2D, lanes 2 and 3). These observations therefore demonstrate that SUMO modification of TIP60 does not affect its interaction with PML3.

To confirm that recruitment of TIP60 into PML NBs depends on SUMO modification of PML3, we initially generated a nonsumoylatable form of PML3 in which all three known SUMO conjugation sites (Lys<sup>65</sup>, Lys<sup>160</sup>, and Lys<sup>490</sup>) were mutated to arginine (Fig. 2E). As expected, wild type GFP-PML3 exhibited a typical sumoylated pattern, whereas nonsumoylatable GFP-PML3<sup>3R</sup> mutant contained no detectable SUMO conjugation as judged by PML3 Western blotting analysis (Fig. S1A).

We next examined whether sumoylation of PML3 is essential for the NB distribution of TIP60. As shown in Fig. S1B, FLAG-tagged PML3<sup>3R</sup> diffused in whole nucleus in HeLa cells, whereas wild type PML3 was readily apparent in the PML NBs, consistent with previous observations (e.g. see Ref. 34). Overexpression of FLAG-PML3 efficiently recruited TIP60 into PML NBs (Fig. 2F, a), whereas PML3<sup>3R</sup> sequestered the PML NBs localization of TIP60 (Fig. 2F, b).

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![Diagram](https://via.placeholder.com/150)

**Table:**

| PML NBs Localization | PML3 Binding |
|----------------------|--------------|
| WT                   | +            |
| 1-211                | +            |
| 212-364              | +            |
| 212-511              | +            |
| 235-511              | +            |

**Figure Legends:**

- **A:** Schematic representation of PML domain structures and their localization.
- **B:** Immunofluorescence images showing the interaction between TIP60 and PML3.
- **C:** Western blot analysis of TIP60 and PML3 interaction.

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To examine whether sumoylation of PML3 determines the PML3-TIP60 interaction, we performed co-immunoprecipitation experiments from transfected 293T cells in vitro. As shown in Fig. 2G, the level of TIP60 co-immunoprecipitated by PML3[3R] or PML3 is virtually the same (Fig. 2G, lanes 2 and 3). These findings therefore implicate that SUMO modification of PML3 is indispensable for TIP60 translocalization to PML NBs but is dispensable for interaction between PML3 and TIP60. To confirm their directly physical interaction, we carried out a pull-down assay in which GST-tagged recombinant PML3 was purified on glutathione-agarose beads and used as an affinity matrix to absorb purified MBP-TIP60 and MBP-TIP60dm proteins in solution. We found that GST-PML3 efficiently bound to MBP-TIP60 and MBP-TIP60dm proteins (Fig. 2H, lanes 5 and 8), indicating a specific physical interaction between PML3 and TIP60 and TIP60dm in vitro.

These findings therefore verify that PML3 physically interacts with TIP60 independent of their sumoylation status. However, SUMO modification of PML3 is dispensable for TIP60 localization to the nuclear bodies.

Mapping the TIP60 Region Responsible for PML3 Binding and NB Localization—As our data show, PML3 physically interacts with TIP60 and recruits TIP60 into PML NBs. To map the precise region of TIP60 required for PML binding, we generated various TIP60 deletion mutants according to its functional domains depicted in Fig. 3A and transiently transfected 293T cells to express GFP-tagged TIP60 proteins and FLAG-tagged PML3 proteins. The immunoprecipitates were then washed and resolved in SDS-PAGE, followed by Western blotting analysis. As shown in Fig. 3C, FLAG-PML3 immunoprecipitation pulls down GFP-tagged TIP60 deletion mutants containing amino acids 1–211, 1–364, 212–364, and 212–511 but not 365–511 (indicated with asterisks), demonstrating that the N-terminal region of TIP60-(1–364) is responsible for the PML3 binding. Consistent with the co-immunoprecipitation results, GFP-tagged TIP60 deletion mutants containing amino acids 1–211, 1–364, 212–364, and 212–511 are co-localized with FLAG-PML3 in the NBs (Fig. 3B, a–d), whereas GFP-TIP60-(361–511) failed to localize to PML NBs despite overexpression of PML3 (Fig. 3B, e). Thus, we conclude that the N terminus (amino acids 1–364) of TIP60 containing the chromodomain and zinc finger is required for PML3 association and responsible for its targeting to NBs.

TIP60 Specifies PML3 Association by Binding to C-terminal PML3—It has been reported that overexpression of PML-RARα fusion protein leads to the disruption of PML NBs due mainly to an inhibition of SUMO-binding activity located to the C terminus of PML (20, 33). We thus also introduced PML-RARα (Fig. 4A) to examine whether TIP60 recruitment to PML NBs was specific to PML3 but not the PML-RARα product.

To begin with, FLAG-PML-RARα and GFP-TIP60 were co-transfected into HeLa cells to investigate the localization of the two exogenously expressed products by immunofluorescence. As expected, FLAG-PML-RARα appeared to diffuse throughout the nucleus rather than concentrate at the NBs, confirming that PML-RARα disrupts the integrity of PML NBs (Fig. 4B, c). Although exogenous expression of wild type FLAG-PML3 promotes the localization of TIP60 to the NBs (Fig. 4B, a, b), FLAG-PML-RARα abrogates the TIP60 localization into PML NBs. In the PML-RARα-expressing cells, GFP-TIP60 is diffused throughout the nucleus (Fig. 4B, c).

Since the PML-RARα fusion protein lacks the PML C-terminal region (Fig. 4A), we hypothesized that this domain is required to recruit TIP60 into NBs. Among seven isoforms of the PML protein, PML4 shares the highest similarity with PML3, which only presents a slight variation from the C terminus of PML4 (Fig. 4A) (28). To test the specificity of the PML3-TIP60 interaction for localizing TIP60 to the NBs, HeLa cells were transiently transfected to co-express FLAG-PML4 and GFP-TIP60. The transfected cells were then immunostained and analyzed as above. Although PML4 is also one of the important PML proteins to form NBs, we found that the TIP60 remained diffusing in the nucleus of PML4-overexpressing HeLa cells (Fig. 4B, b), confirming the specificity of the PML3-TIP60 interaction.

To further demonstrate that TIP60 recruitment to the NBs is specifically mediated by its PML3 C-terminal binding but not PML-RARα or PML4 products, we conducted in vitro binding experiments with MBP-TIP60 on lysates from 293T cells expressing FLAG-PML3, FLAG-PML4, or FLAG-PML-RARα and verified that TIP60 bound strongly only to PML3 (Fig. 4C, lane 3), whereas the interaction with PML4 and with PML-RARα was severely impaired (Fig. 4C, lanes 6 and 9). We also performed pull-down assay to test whether we would obtain the same result as in Fig. 3C in vitro. As shown in Fig. 4D, only the full-length PML3, and not PML4 or PML3-(1–394), binds to TIP60. Next, we examined whether TIP60 only forms a cognate complex with PML3 and not PML4. As shown in Fig. 4E, our immunoprecipitation experiment demonstrated that FLAG-TIP60 forms a complex GFP-tagged C-terminal region of PML3-(480–633) and full-length PML3 (asterisks) but not other PML3 deletion mutants and the C-terminal region of PML4-(480–641), validating that TIP60 specifies PML3 binding via the C-terminal fraction of PML3 (amino acids 480–633; Fig. 4E). Thus, we conclude that localization of TIP60 to the NBs depends on its physical interaction with a specific isoform of PML protein, PML3.

PML3 Modulates TIP60 Stability by Competing with Mdm2 for TIP60 Binding—Previous studies showed that PML3 protects Mdm2-mediated p53 degradation (35, 36). Given the facts
FIGURE 4. TIP60 specifies PML3 association by binding to PML3 C terminus. A, schematic diagram of PML3, PML4, PML-RARα, and various deletions. B, overexpression of PML3 isoform recruits PML NBs targeting of TIP60. HeLa cells were transfected to co-express FLAG-PML3, FLAG-PML4, FLAG-PML-RARα and the indicated GFP-tagged TIP60. Thirty-six hours after transfection, cells were fixed, permeabilized, and stained with FLAG antibody to indicate exogenous PML3, PML4, and PML-RARα. Bar, 10 μm. C, TIP60 specifically associates with PML3. 293T cells were transfected to express FLAG-PML3, FLAG-PML4, and FLAG-RARα constructs. Thirty-six hours after transfection, cells were collected and lysed in 300 mM NaCl-containing lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 10% glycerol) for an in vitro binding assay. The lysates were then diluted twice and incubated with 4 μg of MBP-TIP60 or MBP alone. Pull-downs were identified by Western blotting with FLAG antibody. D, TIP60 specifically associates with PML3 in vitro. MBP fusion protein-bound amylose resin (Biolab) was used as an affinity matrix to isolate proteins interacting with TIP60 by purified MBP-tagged full-length that was expressed in bacteria. Briefly, the MBP-TIP60 fusion protein-bound amylose resin was incubated with purified proteins (GST-PML3, GST-PML4, and GST-PML-(1–394)) for 4 h at 4 °C. After the incubation, the resin was extensively washed with 0.25% Triton X-100 PBS and boiled in SDS-PAGE sample buffer, followed by fractionation of bound proteins on a 10% SDS-polyacrylamide gel. Proteins were then transferred onto a nitrocellulose membrane for Western blotting using GST antibody. E, TIP60 specifically interacts with PML3 C terminus. 293T cells were transfected to co-express FLAG-TIP60 and indicated GFP-tagged PML constructs. Thirty-six hours after transfection, cells were collected for a co-immunoprecipitation assay with FLAG antibody. Immunoprecipitates were identified by Western blotting with GFP antibody. Asterisks indicate TIP60 specifically associated with the C terminus of PML3 and PML3 full-length in vivo. Lanes 1, GFP; lanes 2, GFP-PML3-(1–394); lanes 3, GFP-PML3-(228–394); lanes 4, GFP-PML3-(1–570); lanes 5, GFP-PML4-(480–641); lanes 6, GFP-PML3-(480–633); lanes 7, GFP-PML3. DAPI, 4',6-diamidino-2-phenylindole.
that Mdm2 acts as E3 ligase to regulate both TIP60 and p53 stability and TIP60 shares similar biological significance with p53, we hypothesize that PML3 may also modulate Mdm2-mediated TIP60 stabilization. To this end, we first tested whether PML3 stabilizes TIP60 by blocking Mdm2-mediated degradation. As shown in Fig. 5A, in comparison with TIP60 expression alone (Fig. 5A, lane 1), co-expression of Mdm2 markedly reduced the protein level of TIP60 (Fig. 5A, lane 2), consistent with the role of Mdm2 in promoting TIP60 turnover. If PML3 competes Mdm2 for TIP60 binding, overexpression of PML3 would stabilize the TIP60 protein level. To test this hypothesis, U2OS cells were transiently transfected to express increased amounts of FLAG-PML3 and assessed for the protein levels of GFP-TIP60 level in transfected cells. As shown in Fig. 5A (lanes 3–6), increased levels of exogenous expression of FLAG-PML3 gradually elevated TIP60 protein level, whereas the Mdm2 protein level was relative constant, supporting the notion that PML3 probably antagonizes Mdm2-mediated TIP60 ubiquituous degradation. To test further whether PML3 modulates the stability of the TIP60 protein, H1299 cells were co-transfected with GFP-TIP60 and GFP expression vectors either in the presence or in the absence of PML3 and treated with CHX, which inhibits protein synthesis. We used Western blotting analyses to quantify the relative amounts of TIP60 at different time intervals after the CHX treatment. As shown in Fig. 5B, quantification of band density of TIP60 indicated that expression of PML3 protects TIP60 from decay, indicating that PML3 stabilizes the TIP60 protein. Importantly, we observed that TIP60 protein levels decreased following PML3 depression in U2OS cells (Fig. S2).

To further understand the molecular mechanism underlying this PML3-mediated protection, three PML constructs, PML(1–394) (PML-RBCC domain), PML-RARα, and PML4, as well as nonsumoylatable PML3 mutant PML3CR were expressed to assess their ability to protect TIP60 from degradation. In contrast to Mdm2-mediated TIP60 degradation (Fig. 5C, top, lane 2), only PML3 and PML3CR effectively stabilized TIP60 under Mdm2 expression (Fig. 5C, top, lanes 5 and 7), whereas PML(1–394) (PML-RBCC domain), PML-RARα, and PML4 could not rescue TIP60 from Mdm2-mediated degradation (Fig. 5C, top, lanes 3, 4, and 6), which further implies that PML3 is specific for TIP60 stabilization.

PML3 protecting TIP60 degradation raises the possibility that PML3 may interfere with the Mdm2-dependent TIP60 ubiquitination. To address this hypothesis, transfected cells were treated with MG132 to minimize the proteosome activity (Fig. 5D). In addition, aliquots of cells were treated with INF-α to increase the synthesis of PML protein (37) as well as PML3 protein (Fig. 5D). Thereafter, cells were harvested, and TIP60-ubiquitin conjugates were detected by a Western blotting assay with HA antibody. As shown in Fig. 5D, following MG132 treatment, co-expression of Mdm2 facilitated the ubiquitination of exogenous HA-TIP60 judged by the high molecular weight shift bands (Fig. 5D, lane 2). Interestingly, expression of PML3 alone or in combination with INF-α treatment prevents TIP60 from ubiquitination (Fig. 5D, lanes 3 and 4). As a control, PML4 expression did not block the TIP60 ubiquitination under Mdm2 expression (Fig. 5D, lane 5), whereas PML4 expression with additional INF-α treatment visibly reduced the extent of TIP60 ubiquitination (Fig. 5D, lane 6). Therefore, we propose that PML3 protects TIP60 degradation by interfering with Mdm2-mediated ubiquitination. Since TIP60 fraction 258–364 involved in binding to Mdm2 is required for TIP60 degradation (12) and 1–364 is responsible for PML3 association (Fig. 3B), we speculate that the mechanism by which PML3 contributes to TIP60 stability may result from PML3 competing with Mdm2 to bind to the overlap region 258–364 of TIP60. To this end, we carried out a competitive experiment to investigate whether PML3 competes with Mdm2 to associate with TIP60 in vivo. As shown in Fig. 5E, with the increase of GFP-PML3 expression, gradual increments of GFP-PML3 were precipitated by FLAG-TIP60, whereas the immunoprecipitates of GFP-Mdm2 gradually decreased (Fig. 5E), suggesting that PML3 competes with Mdm2 for the association with TIP60. Collectively, we conclude that PML3 modulates TIP60 stability by impairing Mdm2-mediated ubiquitination with competitive binding to TIP60.

**PML3 Controls the Dynamics of TIP60 in PML NBs**—PML nuclear body formation is determined by PML protein structural integrity and its SUMO modification and SUMO binding (33). In addition, sumoylated PML protein regulates the molecular dynamics of nuclear body proteins and protein translocation between nucleoplasm and nuclear body. For example, SUMO modification of PML protein is necessary for p53 and Daxx accumulation within PML NBs (17, 29, 38, 39). To validate the functional importance of PML3 in recruiting and stabilizing TIP60 in the PML nuclear body, we performed FRAP experiments to evaluate the molecular kinetics of TIP60 in the PML NBs. To this end, HeLa cells were transiently transfected to express GFP-TIP60 wild type and GFP-TIP60 deletions alone or together with FLAG-PML3. We chose individual nuclear dot areas and areas in the nucleoplasm for photo-bleaching using a pulse of 2 s, and the dynamics of the recovery of fluorescence intensity at the bleached spots were measured. Recovery of full-length GFP-TIP60 and its NB-binding domain GFP-TIP60(1–364) in the presence of PML3 is relatively slow in the NB compared with that in the nucleoplasm. The time for reaching 50% the prebleach GFP-TIP60 and GFP-TIP60(1–364) intensity in NB where contains PML3 is 90–100 s (Fig. 6, A and B, d and f). On the other hand, the time for reaching 50% the prebleach GFP-TIP60 intensity in nucleoplasm where PML3 is absent is 12 s (Fig. 6, A and B, c), which is significantly faster than that in NB, suggesting that PML3 stabilizes the localization of TIP60 in the NB. Consistent with this notion, the time for reaching 50% of the prebleach GFP-TIP60 intensity in both the nucleus is 11.5 s on average (Fig. 6, A and B, c), which is significantly faster than that in NB, suggesting that PML3 stabilizes the localization of TIP60 in the NB. Consistent with this notion, the time for reaching 50% of the prebleach GFP-TIP60 intensity in the nucleus is 11.5 s on average (Fig. 6, A and B, c), which is significantly faster than that in NB, suggesting that PML3 stabilizes the localization of TIP60 in the NB. Consequently, PML3 and TIP60 stabilize the localization of TIP60 in the NB to ensure its function.

**DISCUSSION**

Our recent study revealed the critical role for TIP60 in the UV irradiation response and showed its involvement in cell
FIGURE 5. PML3 modulates TIP60 stability by impairing Mdm2 binding to TIP60. A, PML3 protects TIP60 stability in the presence of Mdm2. U2OS cells were transfected to express GFP-tagged TIP60 construct alone (0.5 μg; lane 1), together with HA-Mdm2 (1 μg; lane 2) or together with HA-Mdm2 and increasing amounts of PML3 (0.25 μg lane 3), 1 μg lane 4), 2 μg lane 5), and 4 μg (lane 6). Thirty-six hours after transfection, cells were harvested and subjected to SDS-PAGE for Western blotting detection. Transfection efficiency was monitored by expressing a constant amount of GFP expression plasmid (0.25 μg) in each sample.

B, PML3 delays the degradation of TIP60. H1299 cells were transfected with GFP-TIP60 (1 μg) and GFP vector (0.25 μg) in the presence or absence of FLAG-PML3 (3 μg) and treated with CHX for the indicated durations. Whole cell extracts were then subjected to Western blotting using anti-tubulin, anti-FLAG, and anti-GFP antibodies. TIP60 densitometric signals were normalized to tubulin as a loading control. A 100% value was arbitrarily assigned to the signal obtained at zero time of cycloheximide treatment. Results are the means ± S.D. of three independent experiments.

C, PML3 specifies TIP60 stability protection in the presence of Mdm2. U2OS cells were transfected to express GFP-TIP60 (lane 1), together with HA-Mdm2 (lane 2) or together with HA-Mdm2 and the indicated FLAG-tagged constructs (lanes 3–7). Thirty-six hours after transfection, cells were harvested and subjected to SDS-PAGE for Western blotting detection. D, PML3 specifies TIP60 stability protection by blocking Mdm2-mediated ubiquitination degradation. U2OS cells were transfected to express HA-TIP60 (lane 1), together with GFP-Mdm2 (lane 2) or together with GFP-Mdm2 and the indicated FLAG-tagged constructs plus INF-α (2 kilounits/ml) treatment (lanes 3–7). Thirty-six hours after transfection, cells were treated with MG132 (20 μM) for 6 h to block Mdm2-mediated TIP60 degradation and, as indicated, treated with INF-α (2 kilounits/ml) for 24 h. After MG132 treatment, cells were harvested and subjected to SDS-PAGE for Western blotting with HA antibody to detect TIP60 and ubiquitination TIP60 shift bands. Long exposure indicated ubiquitination TIP60 shift bands, and short exposure indicated TIP60 stability. E, PML3 competes with Mdm2 to bind to TIP60 in vivo. 293T cells were transfected to express the indicated FLAG-TIP60, together with GFP-Mdm2, or to express GFP-PML3 alone. Thirty-six hours after transfection, cells were harvested for a co-immunoprecipitation assay with FLAG M2 beads. Then using co-immunoprecipitation, immunoprecipitated beads were incubated with increasing amounts of cell lysates of GFP-PML3. Immunoprecipitates were identified by Western blotting with GFP antibody.
FIGURE 6. PML3 governs the dynamics of TIP60 in PML NBs. A, dynamic association of TIP60 with PML-NBs. HeLa cells were transiently transfected to express RFP-PML3, GFP-TIP60, GFP-TIP60 plus RFP-PML3, GFP-TIP60(1–364), GFP-TIP60(1–364) plus RFP-PML3, GFP-TIP60(1–364), and GFP-TIP60(1–364) plus RFP-PML3. RFP and GFP fluorescence was monitored in the aforementioned experimentation live cells. After a region of interest was selected, corresponding to nuclear dots (a, d, and f) or areas in nucleoplasm (b, c, e, g, and h), photobleaching was initiated, and the fluorescence recovery was quantified. The images were collected every 4 s. Representative images were selected and presented from given time points. B, quantification fluorescence recovery $T_{1/2}$ for the bleached areas. The results are presented as mean values and S.E. of the percentages of the postbleach fluorescence intensities at the bleached areas relative to the prebleach intensity from 10 independent experiments.
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cycle checkpoint signaling via sumoylation in PML NBs (15). To delineate the molecular mechanisms underlying TIP60 localization to the NB, our present study discovered and characterized a novel interaction between PML3 and TIP60. Significantly, we mapped the binding interface between TIP60 and PML3 and revealed the role of such binding in linking TIP60 molecular stability to its function in the nuclear body.

Sumoylation plays a crucial role on PML NBs assembly and recruitment of other NB components into NBs. However, our data show that physical interaction between TIP60 and PML3 is independent of their sumoylation. Using deletion analysis, our study demonstrates that the interaction of TIP60 with PML3 is mediated by its chromodomain and zinc finger domain. The region containing the histone acetyltransferase domain from amino acid 365 to 511 of TIP60 is diffused in nucleoplasm and cytoplasm, since it lacks PML3 binding activity. Our previous study demonstrated that this region contains two sites for sumoylation, and the sumoylation increases TIP60 histone acetyltransferase activity in NB. These results show that PML3 recruits TIP60 into NBs to allow TIP60 for post-translational modification, such as sumoylation or acetylation (15, 40) in response to genomic stress, such as UV irradiation.

In human cells, the PML gene transcribes numerous alternatively spliced mRNAs, each of which encodes a distinct protein (28, 41, 42). All of them share an identical N-terminal region from amino acid 1 to 570 containing ring finger, B-box, and coiled-coil motifs (RBCC) known to be crucial for PML NB formation (43–45) and a nuclear localization signal (NLS) that localizes both to nucleoplasm and to a nuclear oncogenic body (PML-NB). PML genes encode similar isoforms, which may have largely distinct functions. It is known that the N-terminal region is important for PML homomultimerization, and a variable C-terminal region appears to determine its interaction, which supports the idea that different isoforms have distinct roles. According to this pattern, here we provide the first evidence that the recruitment of TIP60 into NBs is specific for the PML3 isoform, since there is no significant interaction of TIP60 with RBCC (PML3-(1–394)), PML4 (PML-L), and PML-RARα (Fig. 4, C and D). Significantly, as shown in Fig. 4E, TIP60 only binds to the C-terminal region of PML3. These data demonstrate that different PML isoforms specifically interact with variable cellular partners, which appear to have highly different functions, and these distinct interaction patterns may be essential for NB composition and carrying out of its functions.

A previous study reported that MDM2 binds to TIP60 via its amino acid 258–364 region and induces degradation of TIP60. As our data above demonstrate, the MDM2 binding region of TIP60 from amino acid 1 to 364 is also essential for PML3-TIP60 interaction. Thus, we hypothesize that PML3 may play an important role in stability of TIP60. Here we found a novel stability regulation that PML3 protects TIP60 from degradation by abrogating the ubiquitination of TIP60 (Fig. 5D), the mechanism of which is that PML3 binds to TIP60, disrupting MDM2-TIP60 interaction (Fig. 5E). These findings strongly indicate that direct interaction between PML3 and TIP60 is critical for TIP60 stability, which may be essential for sustaining TIP60 basic level and its functions.

The PML protein and PML NBs have been implicated in various functions. In addition to PML and PML-NBs playing a critical role in gene transcription, viral pathogenicity, tumor suppression, and DNA repair, accumulating evidence shows that PML-NBs may act as storage sites for inactive cargo proteins that could be specifically modified and form transcription complexes (46). In this study, we carry out a FRAP experiment to demonstrate the dynamics of TIP60. Fluorescence recovery of a TIP60 and TIP60 deletion located in the nucleoplasm shows high mobility compared with that targeted to NBs by PML. In the nucleoplasm, GFP-TIP60 and its deletion are highly mobile, and $t_{1/2}$ is no more than 12 s. In contrast, GFP-TIP60 and its deletion localized to PML bodies have restricted mobility, and $t_{1/2}$ is greater than 90 s. These observations provide evidence consistent with the proposal of speculative models in which PML-NB may provide a microenvironment that fosters specific activities in the proteins with which it associates (47).

Overall, our present study has revealed and characterized that a specific isoform, PML3, directly interacts with TIP60 and recruits TIP60 into PML-NB. PML3 plays a critical role in sustaining the stability of TIP60 and modulating the dynamics of TIP60. Thus, this study sheds new light on the function and regulation of TIP60 activity in the nuclear body.

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