Oxidative stress–induced assembly of PML nuclear bodies controls sumoylation of partner proteins

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The promyelocytic leukemia (PML) protein organizes PML nuclear bodies (NBs), which are stress-responsive domains where many partner proteins accumulate. Here, we clarify the basis for NB formation and identify stress-induced partner sumoylation as the primary NB function. NB nucleation does not rely primarily on intermolecular interactions between the PML SUMO-interacting motif (SIM) and SUMO, but instead results from oxidation-mediated PML multimerization. Oxidized PML spherical meshes recruit UBC9, which enhances PML sumoylation, allow partner recruitment through SIM interactions, and ultimately enhance partner sumoylation. Intermolecular SUMO–SIM interactions then enforce partner sequestration within the NB inner core. Accordingly, oxidative stress enhances NB formation and global sumoylation in vivo. Some NB-associated sumoylated partners also become polyubiquitinated by RNF4, precipitating their proteasomal degradation. As several partners are protein-modifying enzymes, NBs could act as sensors that facilitate and confer oxidative stress sensitivity not only to sumoylation but also to other post-translational modifications, thereby explaining alterations of stress response upon PML or NB loss.

Introduction

The eukaryotic nucleus contains domains organized by master proteins, such as promyelocytic leukemia (PML), which drives the formation of PML nuclear bodies (NBs; Lallemand-Breitenbach and de Thé, 2010). PML NBs are stress-regulated, dynamic structures that concentrate hundreds of proteins and finely tune multiple pathways including senescence, stemness, stress response, and defense against viruses (Koken et al., 1995; Dellaire and Bazett-Jones, 2004; Bernardi and Pandolfi, 2007; Ching et al., 2013). Functionally, NB disruption through expression of the PML/RARA oncogene has been implicated in acute promyelocytic leukemia (APL) pathogenesis. As₂O₃ (arsenic), an effective APL therapy, restores NBs through PML and PML/RARA oxidation, disulfide-mediated multimerization, or direct binding to PML, both followed by PML/RARA sumoylation and degradation (Jeanne et al., 2010; Zhang et al., 2010; de Thé et al., 2012). Arsenic similarly enhances NB biogenesis and nuclear matrix association in non-APL cells (Zhu et al., 1997). Yet, PML NB assembly and function remain imperfectly understood (Lallemand-Breitenbach and de Thé, 2010).

PML is sumoylated on three target lysines and displays a SUMO-interacting motif (SIM; Hecker et al., 2006; Kamitani et al., 1998). Accordingly, it has been proposed that NB nucleation depends on intermolecular interactions between a sumoylated
stress modulates global sumoylation through multiple mechanisms (Bossis and Melchior, 2006; Xu et al., 2009), and some key enzymes in the SUMO conjugation/deconjugation pathways are oxidative stress sensitive, including SUMO proteases and the SAE2-activating enzyme (Bossis and Melchior, 2006; Xu et al., 2009; Yeh, 2009). Among SUMO-regulated processes, poly- or multi-sumoylation may initiate polyubiquitination by the SUMO-targeted ubiquitin ligase (STUbL) Ring-finger protein 4 (RNF4) and proteasome-mediated degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Although this pathway was initially described for arsenic-induced PML or PML/RARA degradation, other NB-associated proteins may also be subjected to RNF4-mediated degradation.

The diversity of PML partner proteins recruited onto NBs has suggested a general function for NBs in their sequestration and/or activation (Eskiw et al., 2003; Lin et al., 2006; Bernardi and Pandolfi, 2007). In particular, overexpression of PML and/or of some specific partners modulates post-translational modifications of these partners (Bernardi and Pandolfi, 2007; Lallemand-Breitenbach and de Thé, 2010). Nevertheless, a global systematic analysis of PML NB function that establishes a role for NBs as global post-translational regulation sites is lacking.
Here, we dissect the mechanisms underlying NB biogenesis and show that NB formation regulates oxidative stress–responsive sumoylation through cooperation between PML, UBC9, and RNF4. Our results shed new light on how PML–dependent sumoylation could finely tune senescence or self-renewal.

Results

**NB nucleation does not rely on PML SIM-SUMO interactions**

The current model proposes that interaction of PML-conjugated SUMO with PML SIM nucleates NB biogenesis (Fig. 1 A). To test this, we stably expressed, in immortalized pml−/− mouse embryonic fibroblasts (MEFs), a PML mutant the SIM of which was excised (PMLΔSIM). However, this did not impede the formation of PML bodies (Fig. 1 B and Fig. S1 A). Similarly, a PML mutant devoid of its three sumoylation sites (PML3KR), and even PML3KRΔSIM, allowed the formation of the spherical PML lattice when stably expressed in pml−/− cells (Fig. 1, B and C). Interestingly, the latter did not exhibit a diffuse nuclear staining, possibly suggesting that SUMO and SIM allow interaction of PML with components of the chromatin. Although there were some changes in the mean number of bodies (Fig. S1 A), those directly reflected expression levels of the different PML mutants that have different stabilities or toxicities (Fig. S1, B and C; unpublished data). Importantly, super-resolution structured illumination microscopy (Gustafsson, 2000) revealed that these mutants form spherical structures exactly like wild-type PML (PML WT; Fig. 1 C). Finally, siRNA extinction of three mutants that have different stabilities or toxicities (Fig. S1, D and E), although it decreased their number and completely abrogated NB association of partners such as SP100 or RNF4. Our findings imply that SIM and SUMOs are NB-targeting/retention signals, explaining the NB association of SIM–SUMO-containing partners.

**Partner recruitment/sequestration onto PML NBs are controlled by sequential and polarized SUMO-SIM interactions**

Using FRAP analysis, we measured the exchange rate of the GFP fusions between NBs and the nucleoplasm. S3ΔGG-GFP was retained in NBs, and adding SIM to the S3ΔGG-GFP fusion enhanced its retention in NBs (Fig. 2, D). Similarly, a photo-switchable fusion between Dendra and DAXX (a well-known SUMO–SIM-containing NB partner) stably expressed in CHO-PML cells, diffused much faster in the nucleoplasm than in NBs, again evidencing a PML role in partner protein sequestration (Fig. 2 E). Our findings imply that SIM and SUMOs are NB-targeting/retention signals, explaining the NB association of SIM–SUMO-containing partners.

We then questioned how partner SIM or SUMO could interact with PML. Unexpectedly, NBs generated in CHO cells or pml−/− MEFs by PMLΔSIM stable expression efficiently recruited DAXX, RNF4, or SP100, whereas this was not observed with PMLK160R (Fig. 2 F; unpublished data; Ishov et al., 1999; Lallemand-Breitenbach et al., 2001). Note that PMLΔSIM was sumoylated and degraded upon arsenic exposure, corroborating the efficient recruitment of RNF4 (Fig. S1 C). Thus, PML SIM is unessential for partner recruitment.

Examining the partner side, multiple studies have demonstrated the requirement of SIM for the recruitment of some specific proteins in NBs and for their sumoylation (Lin et al., 2006). Indeed, deletion of DAXX C-terminal SIM abrogated its association to NBs (Fig. 2 G and Fig. S2; Lin et al., 2006). Although irreversible fusion of SUMO1 or -2 clearly enhanced DAXX association to NBs, this was insufficient to rescue DAXXΔSIM NB localization (Fig. 2 G). These data demonstrate that SIM and SUMO are not equivalent in promoting partner NB recruitment. Collectively, these data argue against the previously proposed role of PML SIM in either NB morphogenesis or partner recruitment. They strongly suggest that SUMO–SIM interactions are polarized and sequential. PML sumoylation on K160 recruits partners through their SIM. Partners undergo sumoylation and are then sequestered through interactions with partner, or PML, SIMs.
are unlikely to secure partner association to the NM. To test this, in situ NM preparations were labeled with antibodies against PML or partners. Intense PML and SUMO2/3 staining was observed in NM preparations, whereas SUMO1, RNF4, and DAXX staining was dramatically diminished when compared with whole-cell preparations (Fig. 3 A).

NBs consist of a matrix PML shell and a nonmatrix SUMO–SIM core

Our findings suggest that NBs could consist of two compartments: one, formed by a mesh of covalent PML multimers and associated to the NM; the other, formed by partner proteins, relying on multiple weak SUMO–SIM interactions. Yet, the latter are unlikely to secure partner association to the NM. To test this, in situ NM preparations were labeled with antibodies against PML or partners. Intense PML and SUMO2/3 staining was observed in NM preparations, whereas SUMO1, RNF4, and DAXX staining was dramatically diminished when compared with whole-cell preparations (Fig. 3 A).

Figure 2. SIM and SUMOs are NB-targeting signals. (A) Prototypical PML partner with both a SIM and a SUMO conjugation site may interact with PML SUMO (1) and SIM (2) in an ordered manner. (B) Colocalization of GFP-SIM or SUMO1/2/3ΔGG-GFP fusions with NBs. (Top) As2O3 (arsenic)-enhanced NB recruitment of GFP-SIM fusions stably expressed in CHO-PML (arsenic: 10⁻⁶ M, 1 h). (Bottom) Recruitment to NBs of SUMO1, 2, or 3ΔGG-GFP fusions in CHO-PML but not in CHO cells. Insets show GFP labeling alone. (C) Quantification of GFP fusions’ recruitment on NBs: ratios of GFP intensities (in NBs versus in the nucleoplasm) were calculated cell by cell, and averaged from 20 cells. P-values are indicated. (D) FRAP analysis of NB-associated SUMO3ΔGG-GFPs or SIM-GFPs in CHO-PML cells (the graph represents means of five experiments); standard deviations are shown for GFP and 3ΔGG-GFP SIM. t₁/₂ recoveries after photo-bleaching are shown below (table). (E) Real-time diffusion analysis after Dendra-DAXX photoconversion performed on NBs or in the nucleoplasm of CHO-PML cells (means of five experiments are represented below). Error bars represent standard deviation. Half times of Dendra-DAXX diffusion after photobleach are indicated below (t₁/₂). Insets show a representative green to red photoconverted ROI surrounding NB or in the nucleoplasm at t = 2.5 s, t = 9.5 s, and t = 45.3 s after switch. Bar, 0.5 µm. (F) Immunolocalization of PMLΔSIM or PMLK160R stably expressed in pml⁻/⁻ MEFs or CHO cells, and of endogenous DAXX or transfected SP100, as indicated. (G) Immunolocalization of DAXX mutants stably expressed in CHO-PML cells. Bars, 5 µm.
To illustrate this biochemical difference at the level of localization, PML was stably expressed in CHO cells stably expressing SP100 (CHO-SP100). This led to the recruitment of diffuse SP100 into typical NBs (Fig. 3 B, top). A distinct PML-labeled shell was observed while SP100 staining concentrated within the central micro-granular core in electron microscopy (Fig. 3 B, bottom). Similarly, in transformed (SaOS) and primary (MRC5) cells, endogenous SP100, DAXX, SUMO1, and SUMO2/3, as well as ectopically expressed RNF4, were all found in this central core, and only SUMO2/3 was present together with PML on the outer shell (Fig. 3 C; unpublished data). Absence of SUMO1 on the external shell contrasts with a previous report and may be due to different cell types used (Lang et al., 2010). Collectively, in keeping with the super-resolution images, these data demonstrate that NBs consist of a matrix-associated peripheral shell, containing oxidized and SUMO-conjugated PML, and an inner nonmatrix core that accumulates partners through SUMO–SIM interactions.

**PML recruits UBC9 into NBs favoring ROS-enhanced sumoylation**

We found that UBC9 was concentrated into NBs (Fig. 4 A), consistent with reports showing that UBC9 may bind PML in vitro (Duprez et al., 1999). UBC9 NB association was observed in H1299 cells for endogenous proteins (Fig. 4 A, left), as well as in CHO cells stably expressing both UBC9-GFP and PML (Fig. 4 A, right). UBC9, which harbors a SIM and may be sumoylated (Knipscheer et al., 2008), was associated with the soluble inner fraction of the bodies (Fig. S3, A and B).
UBC9 to NBs and may thus specifically favor sumoylation of PML partner proteins in situ, enhancing their sequestration by SIM–SUMO interactions.

Arsenic, which induces oxidative stress, increased UBC9 recruitment into NBs (Fig. 4 A and Fig. S3 C), and actually depleted UBC9 from the nucleoplasm in both H1299 and CHO-PML cells (Fig. 4 A). Interferon (IFNα), used to boost endogenous PML expression and NB formation (Stadler et al., 1995), enhanced UBC9 targeting to NBs in the same manner as arsenic (Fig. 4 A, bottom). These findings imply that PML recruits UBC9 to NBs and may thus specifically favor sumoylation of PML partner proteins in situ, enhancing their sequestration by SIM–SUMO interactions.

SP100 is one of the most studied PML partners, whose basal sumoylation was proposed to be influenced by PML (Cuchet et al., 2011; Fig. S4 A, left). Arsenic elicited hyper-sumoylation of endogenous SP100 in HeLa and H1299 cells, which was...
be sumoylated, whereas GFP itself, which is not targeted to NBs, did not undergo significant modifications (Fig. 4 D).

Collectively, we formally establish that PML NBs enhance in situ the sumoylation of partner proteins. Most importantly, it confers oxidative stress and interferon sensitivity to partner sumoylation through concentration of UBC9 and its substrates within NB cores.

RNF4 recruitment into NBs induces loss of conjugated partners

Upon exposure to arsenic, RNF4 is recruited into NBs together with proteasome subunits (Lallemand-Breitenbach et al., 2008). Proximity ligation assays, performed to better assess PML–RNF4 interactions, revealed that the interaction signals predominantly took place in NBs and were sharply increased upon exposure to arsenic, implying that RNF4 primarily interacts with NB-associated PML (Fig. 5 A).

When examining arsenic-induced SP100, TDG, or HIPK2 hyper-sumoylation, we noted loss of sumoylated species after 24 h exposure (Fig. 5, B and C; and Fig. 4 C). This loss was reversed by the proteasome inhibitor MG132, and also by RNF4 siRNA silencing (Fig. 5 B, arrows; and Fig. 5 C; unpublished data), suggesting that with time some sumoylated species were ubiquitinated sharply delayed and reduced by PML silencing (Fig. 4 B, left; and Fig. S4, A and B). Proximity ligation assays, which allow in situ detection of closely interacting individual proteins, confirmed that endogenous interactions between SP100 and SUMO1 occurred mainly in NBs and increased with exposure to arsenic or IFNα (Fig. S4 C). This strongly suggests that SP100 sumoylation occurs in NBs. Similarly, exposure to IFNα increased SP100 sumoylation in a PML-dependent manner, as assessed in pml−/− or pml+/− MEFs stably expressing SP100A isoform (MEF-SP100A; Fig. 4 B, right) or in HeLa cells expressing endogenous SP100 (Fig. S4 D).

We finally assessed modifications of SIM- and SUMOΔGG-GFP fusions that are artificially recruited into NBs by performing His-purification from cells coexpressing (His)x6-SUMO2 together with SUMO3ΔGG-GFP, GFP-SIM, or the TDG sumoylation site fused to either GFP or GFP-SIM (site-GFP and site-GFP-SIM, respectively). All the GFP fusions were found to be sumoylated, whereas GFP itself, which is not targeted to NBs, did not undergo significant modifications (Fig. 4 D).

Collectively, we formally establish that PML NBs enhance in situ the sumoylation of partner proteins. Most importantly, it confers oxidative stress and interferon sensitivity to partner sumoylation through concentration of UBC9 and its substrates within NB cores.

Figure 5. RNF4 recruitment into NBs results in sumoylation decay through partner ubiquitination. (A, top) PML–RNF4 interactions detected by PLA Duolink assay (red dots) and PML NB immunolocalization (green); Z-stack projections are shown. (Bottom) Quantification of the Duolink dots per cell and percentages of colocalization with NBs (means from 20 cells). (B) Western blot analysis of endogenous SP100 hyper-sumoylation (bracket) upon exposure to arsenic in H1299 transfected with the indicated siRNAs. (C) Western blot analysis of transduced hISP100A in MEFs treated as indicated, demonstrating arsenic-induced proteasomal degradation after 24 h. (D) SUMO and ubiquitin conjugation of SP100 upon exposure to arsenic. (Left) Endogenous SP100 immunoprecipitates from arsenic-treated Hela cells probed with anti-SUMO1 and anti-SP100 antibodies. (Right) Nickel-purified His-ubiquitin conjugates from His-ubiquitin-overexpressing Hela cells, probed with antibodies to SP100 and ubiquitin. (E) Polyubiquitination of indicated GFP fusions in CHO-PML cells overexpressing (His)x6-Ubiquitin, purified over nickel column and analyzed by Western blot using anti-GFP antibodies. Left, inputs; right, denaturing purification of ubiquitinated proteins.
and degraded. Endogenous SP100 was therefore immunoprecipitated and this demonstrated that arsenic enhanced both endogenous SUMO1 and polyubiquitin conjugation (Fig. 5 D). A fraction of SP100 species was dually conjugated by both SUMO and ubiquitin, as demonstrated by two-step purification (Fig. S4 E).

We similarly assessed the ubiquitination status of site-GFP-SIM, GFP-SIM, and SUMOΔGG-GFP fusions that are recruited in NBs and sumoylated. His-purifications from cells coexpressing (His)6-Ubiquitin and SUMO3ΔGG-GFP, GFP-SIM, or site-GFP-SIM (see previous section) indicated that these GFP fusions were also ubiquitinated, but not GFP itself (Fig. 5 E).

**PML-dependent biphasic response of global sumoylation to oxidative stress**

We then analyzed global sumoylation in response to ROS induced by arsenic (Fig. S3 C; Kawata et al., 2007). Arsenic elicited transient formation of high molecular weight (HMW) SUMO conjugates, while protein species in the 50–100 kD range were marginally affected (Fig. 6 A). After more than 12 h exposure to arsenic, both HMW SUMO1 and SUMO2/3 conjugates declined (Fig. 6 A). MG132 proteasome inhibitor dampened this late arsenic-triggered loss of sumoylated proteins, showing that sumoylation decrease reflects the degradation of some hyper-sumoylated proteins rather than de-sumoylation (Fig. 6 A). In that respect, SENP3 and SENP5, which were proposed to traffic between nucleoli and NBs (Gong and Yeh, 2006), were not recruited to NBs upon arsenic exposure (unpublished data).

Arsenic’s biphasic effect on global sumoylation again depended on both PML and RNF4 (Fig. 6 B). Indeed, PML silencing abrogated the initial arsenic-enhanced conjugation, whereas RNF4 extinction affected only the late degradation phase. HMW SUMO conjugates did not co-migrate with SUMO-modified PML and there was a delay between PML degradation and sumoylated protein decrease (Fig. 6 B; and not depicted), implying that arsenic-induced HMW species do not correspond solely to PML conjugates. Moreover, arsenic-induced degradation of sumoylated proteins was confirmed to be PML dependent in MEFs isolated from pml+/− and pml−/− animals (Fig. S5 A).

MEFs were then treated with both arsenic and IFNα to enhance NB formation. Polyubiquitinated conjugates were immunoprecipitated and probed with antibodies to SUMO1, SUMO2/3, or PML. Formation of mixed ubiquitin–SUMO2/3 conjugates, a read-out for RNF4 activity, was strongly induced in pml+/− but not in pml−/− MEFs (Fig. 6 C), suggesting that RNF4 required PML to efficiently ubiquitinate sumoylated proteins, at least in this setting of oxidative stress.

We then investigated whether overexpressing RNF4 could lead to the clearance of SUMO conjugates in a pml-dependent manner. RNF4 or a dominant-negative mutant (RNF4-DN) thereof was transduced in pml+/− or pml−/− MEFs treated with IFNα to increase the differences in PML content. RNF4 overexpression markedly decreased the amounts of HMW SUMO1 or SUMO2/3 conjugates in pml+/− MEFs only (Fig. 6 D). In contrast, these conjugates accumulated in RNF4-DN–transduced cells, also in a pml-dependent manner (Fig. 6 D). SUMO1 conjugation to Ran-GAP1 was unaffected (Fig. 6 D, star; also see star in Fig. 6 B). Similar effects, albeit of smaller magnitude, were noted in the absence of IFNα (unpublished data). Interestingly, in the absence of treatment, pml−/− MEFs reproducibly exhibited higher levels of HMW SUMO1 and 2/3 conjugates than pml+/− MEFs (Fig. 6 E, also see untransfected cell lanes in Fig. 6 D). Accumulation of SUMO conjugates in the absence of PML was also noted in primary tissues such as bone marrow (Fig. S5 B). Collectively, these data demonstrate that upon oxidative stress PML NB formation enhances first the sumoylation of partner proteins and subsequently the degradation of some of these, revealing NBs as global sumoylation control machineries.

**Oxidation triggers NB formation and sumoylation in vivo and in APL cells**

To address the role of oxidation in a physiological setting, we analyzed NB formation in vivo in conditions of oxidative stress. For this we examined livers from mice after a short (1–2 h) exposure to acetaminophen, doxorubicin, gamma irradiation, or paraquat (Kawata et al., 2007). Remarkably, the size and number of NBs were dramatically increased (Fig. 7 A and Fig. S5 C). Whereas doxorubicin and paraquat led to formation of much larger NBs, acetaminophen and irradiation led to a dramatic increase in the number of NBs (Fig. S5 C and not depicted). This was not associated with increased amounts of PML (unpublished data), except a twofold increase for doxorubicin, which activates pml expression through P53 (de Stanchina et al., 2004). Collectively, these data support the direct role of PML oxidation in NB nucleation and are consistent with previous pathology studies showing an increased amount of NBs in inflammatory tissues (Koken et al., 1995; Terris et al., 1995). Importantly, in mouse liver or bone marrow, in vivo treatments with arsenic or paraquat also led to a transient boost of sumoylation (Fig. 7 B).

In APL, PML NBs are disrupted in a treatment-reversible manner (de Thé and Chen, 2010; Lallemand-Breitenbach et al., 2012). We thus examined whether NB reformation by arsenic or paraquat would change the global sumoylation profile of APL cells (Fig. 7, C–E). Leukemic cells from APL mice were transduced or not with His-SUMO1 and transplanted into healthy mice, which were then treated after leukemia development. After short in vivo exposure to arsenic, APL cells displayed a transient increase in HMW SUMO1 and mixed SUMO1/2/3 conjugates (Fig. 7 D), concomitant with NB reformation (Fig. 7 C). Similarly, paraquat treatment in vivo induced both NB reformation and SUMO1 conjugation (Fig. 7, E and C). Rapid arsenic-induced degradation of sumoylated PML/RARA confirmed that the fusion did not account for the increase in global SUMO conjugation (Fig. 7 D). Thus, in vivo NB reformation is accompanied by enhanced global sumoylation, raising the issue that this may contribute to the efficacy of arsenic or retinoic acid therapy to cure APL.

**Discussion**

This study presents an integrated view of the sequential steps of NB biogenesis and is the first proposal for an implication of NBs in global oxidative stress–induced partner sumoylation and degradation.
tissues (Daniel et al., 1993; Koken et al., 1995; Gambacorta et al., 1996). UBC9 binds PML (Duprez et al., 1999), accumulates in NBs (Fig. 4), and enhances PML sumoylation in trans on the PML mesh (Jeanne et al., 2010). This sumoylated PML lattice then becomes a docking site for the dynamic association of partners through multiple labile SUMO–SIM interactions (Fig. 8), in line with the seeding model proposed for other nuclear domains (Rajendra et al., 2010). The initial SIM–SUMO interaction is polarized as the SIM anchors partners to the SUMO of PML K160, and in situ sumoylation then enforces partner retention within the NB core (Fig. 2). PML SIM is dispensable for partner recruitment and the fusion of SUMO to DAXX.SIM fails to rescue NB association. This rules out the alternative model whereby partner SIM would recruit UBC9, triggering partner sumoylation in the nucleoplasm and subsequent recruitment onto NBs, partner SUMO binding PML SIM. Collectively, distribution of partner proteins

Whereas previous studies had noted the requirement of the SIM for sumoylation (Takahashi et al., 2005; Knipscheer et al., 2008; Meulmeester et al., 2008) or for NB association of specific proteins (DAXX, HIKP2, Topors) (Weger et al., 2003; Lin et al., 2006; Sung et al., 2011), we provide evidence for a mechanism of NB biogenesis involving two distinct steps: an initial oxidation-sensitive PML multimerization and subsequently, a polarized SIM–SUMO-dependent recruitment of partner proteins. Indeed, analysis of PML3KRΔSIM demonstrates that SUMO–SIM interactions are not responsible, as previously thought (Müller et al., 1998; Shen et al., 2006), for the initial nucleation of the PML spherical mesh (Fig. 1). The latter is triggered by a complex polymerization scheme involving PML coiled-coil and disulfide bonds (Jeanne et al., 2010). Our in vivo experiments demonstrate that NB formation primarily mirrors cellular oxidative stress (Fig. 7), explaining NB prevalence in diseased, but not normal,
within the nucleus will primarily depend on PML level as well as on its self-assembly and sumoylation, both reflecting redox status.

Consistent with the recruitment of UBC9 within NBs, we demonstrate that PML aggregation upon oxidative stress enhances global sumoylation (Figs. 6 and 7), most likely of NB partners. Overexpression of TRIM proteins was suggested to modulate sumoylation, notably in yeast (Quimby et al., 2006; Chu and Yang, 2011). Concentration of targets and enzymes or different components of protein–RNA complexes constitute a common feature of nuclear bodies (Cajal bodies, nuclear speckles…), which enables chemical reactions or complex formation between low-abundance nuclear species (Rajendra et al., 2010). That NBs constitute stress-responsive sumoylation factories may define a new class of E3 ligase acting through physical concentration, as suggested for the nuclear pore (Fig. 8; Zhang et al., 2002; Melchior et al., 2003; Nagai et al., 2011). It is possible that SUMO E3 ligases are also recruited into NBs (Rabellino et al., 2012), and that NB inner core provides a favorable redox environment for thiol enzymes involved in the sumoylation cascade (Bosiss and Melchior, 2006). Recent studies have similarly demonstrated that concentration of viral genomes and transactivators into PML NBs contributes to viral fitness (Teng et al., 2012). The wave of sumoylation induced by PML NBs upon oxidative stress resembles the one occurring after DNA damage, in which SIMs orchestrate the coordinated sumoylation of DNA repair proteins (Psakhye and Jentsch, 2012). Interestingly, many of these proteins may be observed in PML NBs (Lallemand-Breitenbach and de Thé, 2010), and PML or RNF4 absence is associated with persistent DNA damage (Zhong et al., 1999; Galanty et al., 2012; Yin et al., 2012), both supporting a role of PML in sumoylation/degradation of DNA repair proteins.

Several NB partners are protein-modifying enzymes (RNF4, HIPK2, TDG, SIRT1, CBP, MDM2, HAUSP…), suggesting that partner NB association, through transient SIM–SUMO-mediated partner sequestration, could confer oxidative stress sensitivity to other post-translational modifications (Langley et al., 2002; Seet et al., 2006; Trotman et al., 2006; Kirkin and Dikic, 2007; Song et al., 2008; Regad et al., 2009; de la Vega et al., 2012; de Thé et al., 2012). In that respect, P53 is a downstream effector of PML-regulated senescence (Bischof et al., 2002; Chiantore et al., 2012), which together with many of its modifying enzymes, may be NB associated. This suggests that PML NB aggregation may confer redox sensitivity to P53 signaling. Supporting the hypothesis that NB aggregation facilitates other post-translational modifications through partner sumoylation, we demonstrate that PML is required for ubiquitination of many sumoylated proteins by the RNF4 ubiquitin ligase. Interestingly, UBC9 and RNF4 do not have the same requirements for NB association, as UBC9 directly binds to PML (Duprez et al., 1999), whereas RNF4 requires PML poly-sumoylation (Lallemand-Breitenbach et al., 2008). This likely contributes to slow kinetics of the degradation phase in arsenic-treated cells (Fig. 6 A), as opposed to immediate hyper-sumoylation upon NB formation. Degradation of PML upon longer arsenic exposure likely

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**Figure 7.** Induction of NB formation and sumoylation in oxidant-treated mice. (A) In vivo oxidative stress effects on NB formation: Immunofluorescence analysis of PML NBs on liver sections from mice exposed to APAP (N-acetyl-p-aminophenol), As2O3, doxorubicin (Doxo), or paraquat for 2 h. Quantifications are shown in Fig. S5 C. n ≥ 50 cells examined. (B) In vivo sumoylation after exposure to oxidative stress. (Left) Western blot analysis of liver cells from mice treated with paraquat. (Right) Western blot analysis of bone marrow (BM) cells from mice treated with arsenic; two mice are shown for each from two independent experiments. (C) Immunofluorescence analysis of APL cells obtained after in vivo administration of arsenic or paraquat for the indicated period of time. Bar, 5 µm. (D) His-SUMO1–transduced mouse APL bone marrow (BM) cells were isolated after in vivo arsenic administration. His-conjugates were purified and probed with antibodies to SUMO1, SUMO2/3, and RARA. PML/RARA-S, poly-sumoylated PML/RARA. Representative data from six mice, three independent experiments. (E) Western blot analysis of bone marrow cells of APL mice after in vivo treatment with arsenic or paraquat.
Underscore the relevance of this response pathway in physiological or pathological conditions. Remarkably, two key PML-regulated pathways, senescence and defense against microbes, are also activated by hyper-sumoylation (Yates et al., 2008; Ribet et al., 2010; Everett et al., 2013), fully in line with our model. Future studies should thus assess the contribution of deregulated sumoylation to other PML-dependent phenotypes.

**Materials and methods**

**Constructs and siRNAs**

PMLΔSIM, PMLΔKRΔSIM mutants and GFP-SIM<sub>N</sub> were constructed by deletion or insertion of the PML SIM coding sequence (aa 556–566) with the QuickChange II site-directed mutagenesis kit (QIAGEN) into MSCV-PML, MSCV-PMLΔKR, or pEGFP-N1. MSCV retroviral vectors for [His]<sub>6</sub>-tagged PML and PMLΔKR mutant were described previously and used for stable
expression (Lallemand-Breitenbach et al., 2008; Jeanne et al., 2010). HA-tagged versions of PML and mutants were used for Western blot analyses of NMs prepared from transduced pml−/− MEFs. HA tag was fused in frame with PML N terminus. PMLCC corresponds to the deletion of the coiled-coil motif [aa 216–333]. Unless otherwise indicated, PML refers to the PML N terminus. PML CC corresponds to the deletion of the coiled-coil motif. Unless otherwise indicated, PML refers to the PML N terminus. PML CC corresponds to the deletion of the coiled-coil motif.

FRAP and photoconversion
FRAP was performed using a confocal microscope (LSM510 Meta; Carl Zeiss). For SUMO3GG-GFP and GFP-SIM fusions, FRAP was performed on 2-μm regions of interest (ROI) surrounding NMs using the 480-nm laser, at zoom 20, maximum speed scan (0.05 s between acquisitions). 10 images were acquired before bleach, 3 iterations were used to bleach, and time of recovery was between 15 and 30 s. Dendra-DAXX was photo-converted using a confocal microscope (LSM510 Meta; Carl Zeiss). Dendra-DAXX localization was followed using a 488-nm laser and ROI corresponding to Dendra-DAXX associated with NMs was then exposed to 405-nm laser for 2 s at zoom 20. After conversion, acquisitions were then performed at both 488 and 594 nm.

Cell culture, transfections, and treatments
Cells, cell cultures, treatments, and siRNA silencing have been described previously (Lallemand-Breitenbach et al., 2008; Jeanne et al., 2010). In brief, HeLa, H1299, MRC5, CHO, COS, SaOS cells, and pml−/−MEFs were cultured in 10% FCS DMEM medium (Gibco). SV40 T-immortalized CHO cell lines, expressing PMLIII isoform, was obtained by cotransfection of pSgs-P5 and the DspHygro vector followed by selection for 2 wk in 800 μg/ml hygromycin-containing media. pml−/−MEFs were similarly transduced with MSCV-PML or PML mutants using viruses produced as described previously (Lallemand-Breitenbach et al., 2008). The cDNA encoding human SP100A isoform was also inserted in a MSCV retroviral vector, used to produce virus and to infect immortalized pml+/− and pml−/−MEFs. Viruses encoding RNF4 or the RNF4-DN dominant-negative mutant have been described previously (Lallemand-Breitenbach et al., 2008). Cellular ROS levels were quantified by CellROX Deep red reagent (Life Technologies). Probes were incubated for 20 min after arsenic exposure and quantified by FACs according to manufacturer's guidelines.

Immunoprecipitations
Cells were washed in ice-cold PBS supplemented with 10 mM Nethylmaleimide before lysis in 2% SDS and 50 mM Tris, pH 8. After brief sonication, cell lysates were diluted 10-fold in immunoprecipitation (IP) buffer containing 50 mM Tris, pH 8, 150 mM NaCl, 0.05% NP-40, 10% glycerol, and protease inhibitors. Lysates were incubated for 2 h at 4°C with the appropriate antibody, followed by incubation with protein A–agarose for 2 h. Beads were washed three times in the IP buffer before elution of immunoprecipitated proteins in sample buffer. For detection of His-tagged ubiquitin conjugates of endogenous SP100, His-tagged protein purification on Ni-NTA resin (QIAGEN or Invitrogen) was performed 24 h after transfection with His-ubiquitin or His-SUMO1 or 2–encoding vector as described previously (Lallemand-Breitenbach et al., 2008). Cells were lysed in denaturing buffer (6 M guanidium-HCl, 0.1 M NaH2PO4, NaOHPO4, and 10 mM imidazole, pH 8) and lysates were incubated with Ni-NTA resin (QIAGEN or Invitrogen) for 2 h. Three subsequent washes were performed with decreasing amounts of guanidium-HCl before elution in Laemmli buffer with 200 mM imidazole.

In vivo treatments
Experiments were performed in accordance with the French guidelines of institutional animal care committees, using protocols approved by the Comité Régional d’Ethique (protocol no. 4). The mouse APL model was described previously (Lallemand-Breitenbach et al., 1999). FVB-Nico mice were transplanted with leukemic blasts obtained from leukemic hMRP8-PML/RARA transgenic mice. Transplanted mice were treated with 150 mg/kg paraquat (paraquat dihydrochloride, PESTANAL, Sigma-Aldrich), 5 mg/kg arsenic, or 300 mg/kg APAP (acetaminophen [N-acetyl-p-aminophenol]) through peritoneal injection, or with 20 mg/kg doxorubicin (Adcar) through i.v. injections. Animals were sacrificed by cervical dislocation, organs were collected, and
protein extracts analyzed by direct Western blot or after purification on Ni-NTA resin.

**Proximity ligation assays**

Duolink assays (Olink Bioscience) allowed the detection of individual proteins in close interaction (<40 nm), and were based on the in situ proximity ligation of DNA linked to secondary antibodies followed by PCR amplification. Assays were performed using anti-SUMO1 and -SUMO2, anti-S100 and -UBC9, anti-UBC9 and -PML, and anti-RNF4 and -PML primary antibodies.

**Online supplemental material**

Fig. S1 shows statistics on NB formation by PML mutants and upon SUMO siRNA knockdown, as well as efficient degradation of PML mutants upon arsenic exposure and controls for silencing efficiency. Fig. S2 shows SIMs and SUMO-conjugating sites on NB partners, as referenced in the supplemental references. Fig. S3 shows UBC9-GFP localization in soluble NB and SUMO-conjugating sites on NB partners, as referenced in the supplemental references. Fig. S4 shows in situ NB facilitated sumoylation and ubiquitination of SP100 induced by arsenic. Fig. S5 shows PML-dependent loss of high molecular weight SUMO conjugates ex vivo and in vivo, as well as quantification of NB formation in vivo in response to oxidative stress inducing agents. Supplemental references list publications showing NB association of proteins with functional SIMs and SUMO-conjugating sites, as schematically represented in Fig. S2. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201305148/DC1. Additional data are available in the JCB Data-Viewer at http://dx.doi.org/10.1083/jcb.201305148.dv.

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**References**


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Figure S1. Controls of PML sumoylation, degradation, and NB formation for PML mutants or in SUMO-deficient cells. (A) NB numbers per cell and relative importance of the diffuse nucleoplasmic fraction from cells shown in Fig. 1 B (means from ≥50 cells). (B, left) Western blot analysis showing expression levels of PML mutants stably expressed in pml−/− MEFs. (Right) Quantifications show reduced level of PML3KR and 3KR ΔSIM mutants. (C) Western blot analysis of arsenic effects on the degradation of PML mutants. Note that PMLΔSIM is efficiently degraded. (D) Immunofluorescence analysis performed using anti-PML and anti-SUMO1 or SUMO2/3 antibodies in HeLa cells transfected with the indicated siRNAs. (E) Western blot analysis of the same cells as in D. MDM2 is loading control. siCtr, control siRNA; siS1, siRNA targeting SUMO1; siS2/3, mix of siRNA targeting SUMO2 and siRNA targeting SUMO3; siS1/2/3, mixture of siRNAs targeting the three SUMO paralogues. RanGap+S denotes SUMO1-conjugated RanGAP1 protein. (F) Reduced cell extracts from the experiment shown in Fig. 1 D.
Figure S2. Schematic representation of NB partner proteins with both SUMO-conjugating site and SIMs (see list of references below). SUMO is represented in green and SIMs in yellow. sc denotes Saccharomyces cerevisiae, otherwise mammalian proteins are depicted.
Figure S3. **UBC9 localization inside PML NB soluble core and demonstration of ROS production induced by arsenic.** (A) Localization of PML and UBC9GFP in total or in situ nuclear matrix preparation from HeLa cells overexpressing UBC9GFP. Bar, 5 mm. (B) Confocal analysis showing localization of UBC9GFP inside PML bodies (zoom on a nucleus). Bar, 1 mm. (C) FACS analysis of ROS detection by CellROX in cells treated or not with 1 μM As₂O₃.
Figure S4. **Arsenic- and IFN-induced SP100 sumoylation in NBs.** (A) Western blot analysis of endogenous SP100 from HeLa cells transfected with the indicated siRNAs. (B) Western blot analysis of SP100 from HeLa cells treated with arsenic (time-course on IFNα-pretreated cells). (C) Duolink analysis of SP100/SUMO1 interactions (red dots) and localization of these interactions in PML NBs using anti-PML antibodies (green). (D) Western blot analysis of SP100 from HeLa cells treated with IFNα for 24 h, showing increased SP100 sumoylation. (E) Western blot analysis of SP100/SUMO1 dual purification, performed first by SP100 immunoprecipitation and a subsequent Ni-NTA pull-down, from cells transfected with His-SUMO1 or control vectors and treated as indicated. SP100-SUMO conjugates were subsequently probed with anti-ubiquitin antibody. Enhanced sumoylation/ubiquitination and subsequent degradation are indicated by arrows.
Figure S5. PML/ROS-dependent sumoylation profiles and ROS-induced NB formation. (A) Western blot analysis of endogenous SUMO profiles after time-course of arsenic exposure of pml+/+ or pml−/− MEFs. Bracket indicates high molecular weight conjugates. (B) Western blot analysis of endogenous sumoylated proteins of bone marrow (BM) from pml+/+ or pml−/− mice. (C) Quantification analysis of NB formation in mice treated with the indicated oxidative stress inducers, as shown in Fig. 7A. The number of NBs per nucleus was calculated from 40 cells. The same cells were also used to calculate mean NB areas; ratios (treated vs. untreated mice) show that oxidative stress increases NB size in vivo.
As indicated in Fig. S2, PML NB partners with functional SIMs and SUMO-conjugating sites are referenced below.

References


