[Frontiers in Bioscience 14, 2293-2306, January 1, 2009]

Expanding PML's functional repertoire through post-translational mechanisms

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1. ABSTRACT

Post-translational modifications, such as acetylation and ubiquitination, can greatly expand the functionality of a particular protein. The promyelocytic leukemia (PML) protein is a functionally promiscuous protein with proposed roles in many cellular processes. Its cellular headquarters are the macromolecular structures termed PML nuclear bodies. Post-translational modification of PML is emerging as a defining feature of this protein that regulates its physiological consequences. This review will highlight the expansion of our knowledge about the post-translational modifications of PML.

2. INTRODUCTION

The promyelocytic leukemia protein (PML) is a tumour suppressor ubiquitously expressed in mammalian cells and is distinctly localized to punctate nuclear structures known as PML nuclear bodies (NBs). Understanding PML function has become an area of intense research because PML, along with the retinoic acid receptor-alpha (RAR-alpha), forms the fusion protein responsible for the development of Acute Promyelocytic Leukemia (APL), a distinct variant of myeloid leukemia. However, although PML has been implicated in numerous physiologically important functions, including DNA repair,

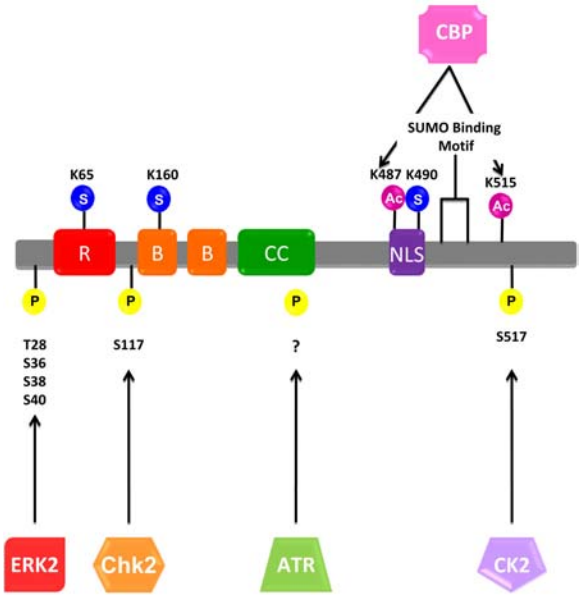

Figure 1. A schematic representation of the post-translational modifications of the promyelocytic leukaemia (PML) protein discussed in the text. The three sumoylation sites (S) of PML are indicated, together with the SUMO-binding domain that mediates non-covalent interactions with other SUMOylated proteins. The kinases that are known to phosphorylate (P) PML are shown, including extracellular signal-regulated kinase (ERK), CHK2, ATR, and casein kinase-2 (CK2), along with the amino-acid residues on PML that they phosphorylate. The two recently elucidated acetylation (Ac) sites of PML are also depicted. R: RING finger domain, B: B-boxes, CC: coiled-coil domain, NLS: nuclear localization signal.

senescence and apoptosis, its exact biological role remains enigmatic.

In 2004, Ciechanover, Hershko, and Rose were awarded the Nobel Prize in Chemistry for their elucidation of ubiquitin-mediated protein degradation, highlighting the fundamental role of post-translational modifications in essential cellular processes. These modifications can involve (1) the addition of functional groups or other proteins or peptides, (2) the alteration of the chemical nature of amino acids, or (3) structural changes to the protein. As such, these modifications are ultimately what govern the stability, localization and function of a protein. The purpose of this review is to explore the intriguing spectrum of post-translational modifications of the PML protein and how these provide insight into the role this protein plays within the cell. At the end, a particular emphasis will be given to the post-translational modifications of the PML fusion protein, PML/RAR-alpha, responsible for the pathogenesis of APL.

3. SUMOYLATION OF PML

Without doubt, the best characterized post-translational modification of PML is covalent conjugation by SUMO (also known as PIC1 and sentrin). Many substrates of SUMO have been identified, but PML was the first protein ever found to associate with SUMO (1). SUMO belongs to the family of ubiquitin-like proteins (ULPs), which mirror ubiquitin in their structure, their ability to be conjugated to other proteins, as well as their mechanism of conjugation. Both ubiquitination and SUMOylation result in the formation of an isopeptide bond between the C-terminal glycine residue of the modifier and the ε-amino group of a lysine residue in the substrate (2). Also like ubiquitin, SUMO is covalently attached to target proteins using the concerted activity of three enzymes, although there is no overlap in the enzymes that are used in their respective conjugation cascades (2). However, in contrast to ubiquitination, SUMOylation does not usually target proteins to the proteasome. In fact, there is evidence that SUMO conjugation can defend targeted proteins against degradation by blocking ubiquitination of the same lysine residue (3).

3.1. SUMO conjugation pathway

To date, four distinct SUMO proteins have been identified in mammalian cells: SUMO1, the highly related proteins SUMO2 and SUMO3 (4), and SUMO4 (5). PML colocalizes with and is modified by SUMO1, SUMO2 and SUMO3 (6), but no evidence yet exists for modification by the recently characterized SUMO4. PML was first associated with SUMO modification through its interaction with SUMO1 in yeast two-hybrid interaction screens, in which Boddy (1) and co-workers used PML as bait and isolated a small ubiquitin-related protein that they called PIC1 (for PML Interacting Clone-1). While they could not demonstrate a direct protein-protein interaction, immunocytochemical analysis demonstrated that PML and PIC1 co-localize in nuclear bodies.

The analysis of covalent modifications by ULPs is difficult, and represents a challenge in the field that is not unique to SUMO but is common to ubiquitin and all ULPs. When harvesting cellular extracts, target substrates may be demodified by functional isopeptidases present in the soluble fraction, leading to loss of the signal. The confirmation of PML SUMOylation has been further complicated by very low levels of PML expression, the existence of several PML isoforms and the presence of multiple distinct SUMO-PML conjugates. Despite these hurdles, three groups (6-8) were able to confirm the presence of SUMO-PML conjugates. Sternsdorf et al. (7) provided the most compelling evidence for covalent modification when they showed that a SUMO1 antibody reacted with PML even after boiling the extracts in SDS under reducing conditions. Kamitani et al. (6) used a COS cell expression system to show that PML is covalently modified by SUMO1, 2 and 3. Significantly, this group demonstrated specificity of SUMO as the member of the ubiquitin-like family for PML, as neither NEDD8 nor ubiquitin could modify PML (6). Mutational analysis identified the existence of at least three lysine residues within PML that can serve as SUMO1 acceptor sites (Figure 1): Lysine65 (K65) in the RING finger domain, Lysine160 (K160) in the B1 Box, and Lysine490 (K490) located within the nuclear localization signal (NLS) (9, 10). A distinguishable feature of SUMO conjugation is that the

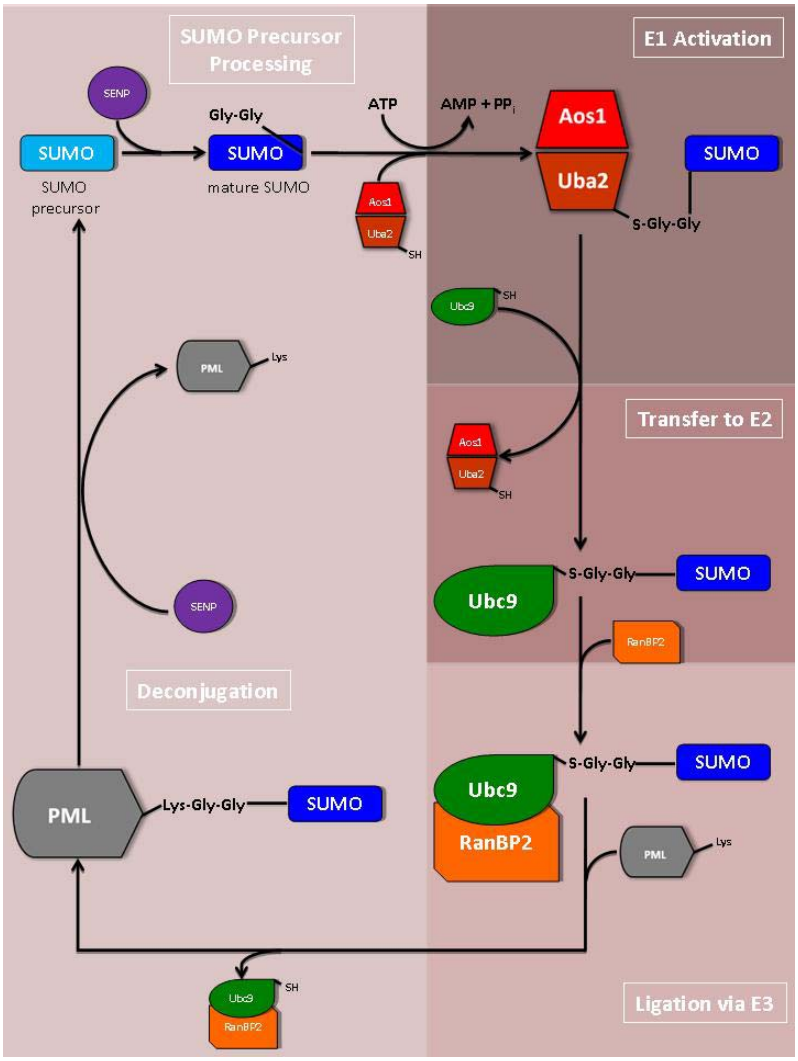

Figure 2. The SUMOylation of PML. SUMO is covalently attached to PML via the concerted activity of a cascade of enzymes. SUMO is first synthesized as a precursor which is cleaved by the C-terminal hydrolase activity of SENPs (Sentrin/SUMO-specific proteases) to expose a di-glycine motif. The mature SUMO is then activated in an ATP-dependent process that results in the formation of a thioester conjugate with the SUMO E1 activating heterodimer, Aos1/Uba2. SUMO is then transferred to the E2 conjugating enzyme Ubc9. From Ubc9, SUMO is conjugated to PML via an isopeptide bond with the target lysine residue (K65, K160 or K490). RANBP2 acts as an E3 ligase which has been shown to stimulate transfer of SUMO from Ubc9 to PML *in vitro*. SUMO modification may be removed from PML by the isopeptidase acitivity of several members of the SENP family.

target lysine residues are often found within a consensus modification sequence, psi-KXE (where psi is a hydrophobic residue) (3, 11). The K65 residue of the RING finger domain is not in fact part of such a SUMO consensus tetrapeptide, and thus its designation as a functional SUMO conjugation site has been disputed (10).

The initiating step in PML modification is the ATP-dependent activation of SUMO at its C-terminus by the SUMO-specific E1 activating enzyme heterodimer AOS1–UBA2 (12) (Figure 2). Next, SUMO is passed to the E2 conjugating enzyme Ubc9, which conjugates SUMO to PML with the aid of an E3 protein ligase (10). Unlike ubiquitination, where substrate specificity is determined in large part by the E3 ligases, Ubc9, the E2, is responsible, at least in part, for the recognition of SUMO targets (13). Indeed, a series of simple, but well-designed studies established that the modification of PML by SUMO1 is mediated by the direct interaction of Ubc9 with PML through its RING finger domain (10). There are three classes of SUMO ligases; the PIAS family, which have a RING finger like structure, RanBP2/NUP358, a component of nuclear pore complexes, and Pc2, a member of the Polycomb group (12). RanBP2 appears to be the E3 ligase mediating SUMO1 and 2 modification of PML through its interaction with Ubc9 (14). However, to date, RanBP2 has been shown to enhance SUMOylation of PML, as well as other proteins such as histone deacetylase HDAC4 (15) and Sp100 (16), only *in vitro*. *In vivo* targets for RanBP2 are not yet known. It has also been suggested that the RING

domain of PML might function as a SUMO E3 ligase for itself and other SUMO targets (17). In support of this, Quimby *et al.* (18) found that PML expression in yeast lacking endogenous E3 enzymes was able to restore the SUMOylation of yeast proteins and that this activity was dependent upon the RING domain of PML. However, more evidence is needed to confirm this theory.

SUMOylation regulates a number of diverse biological functions and thus the removal of SUMO from, as well as the attachment to, target substrates is essential. SUMO is returned to the free cellular pool through the activity of deconjugating enzymes. A single gene family of eight Sentrin/SUMO-specific proteases (SENPs) has been described (12). Even though the products of these genes probably function as proteases for other ubiquitin-like proteins, only five have been confirmed to encode actual SUMO proteases: SENP1 (19), SENP2 (20) (also denominated Axam, SuPr-1, SSP3, SUSP1 and SMT3IP2), SENP3 (21) (SMT3IP1), SENP5 (22) and SENP6 (23). Substrate specificity appears to be primarily mediated through partitioning of the enzymes into distinct subcellular locations. SENP2 contains a nuclear localization signal (NLS) (24) and has been shown to localize to the nuclear side of the nuclear pore complex (NPC) (25). Therefore, it was the most likely candidate enzyme to desumoylate PML, although its activity is restricted to SUMO1 and SUMO3 conjugates. SENP5 was recently shown to have specificity for the removal of SUMO2/3 conjugates from PML specifically at K160 and K490 and for removal of SUMO1,2 and 3 from K65 (22).

3.2. Nuclear body assembly

PML nuclear bodies (NBs) are discrete subnuclear structures approximately 0.5μm in diameter that are closely associated with the nuclear matrix. They are highly dynamic structures to which more than fifty proteins have been found to transiently localize. Current evidence implies that PML NBs are functionally, as well as structurally, heterogeneous, and many biochemical and molecular functions have been ascribed to them. Consequently, characterization of the NB constituents has been a major area of research, which may provide valuable insights into the cellular functions of PML NBs.

PML and Sp100, a protein involved in transcriptional regulation, are considered to provide the fundamental framework of these structures. GG Maul's group (26) established that PML is the essential protein for proper NB assembly under physiological conditions. Their work showed the dispersal of all other NB-associated proteins in the absence of PML and that NBs could be reconstructed by the introduction of wild-type PML into $PML^{-/-}$ cells. Accordingly, one of the earliest questions about the mechanism of NB formation concerned the necessity of SUMOylation for the recruitment of PML itself to the NB. Work done by the Maul lab (26) and others (27) found that a SUMOylation deficient mutant did still accumulate in the NBs, suggesting that SUMO modification was not required for PML localization. However, these experiments were not done in a $PML^{-/-}$ background, therefore permitting the possibility that the mutant formed heterodimeric complexes with the endogenous wild-type PML, which then recruited the mutant to the NB. Subsequent reports by others (28) using the same PML mutant in the context of a $PML^{-/-}$ background showed that PML SUMOylation is essential for PML to localize to the NBs. Additionally, biochemical fractionation experiments demonstrated that only NB-associated PML is modified with SUMO1 (8).

SUMOylation of PML is considered necessary for recruitment of all other NB proteins. The SUMO unmodifiable PML mutant fails to recruit classical PML-NB components such as Sp100, Daxx, CBP and ISG20 (28). Significantly, many of these proteins are also SUMOylated when they are associated with the NB (7, 29). In addition to the traditional covalent modification sites, a SUMO binding domain located within exon seven of PML has been reported (17). This motif enables PML to form non-covalent interactions with other SUMO-modified proteins, including itself. Interestingly, neither the SUMOylation-deficient PML nor the PML lacking the putative SUMO binding motif can form normal NBs, suggesting that both of these SUMO motifs are required for proper NB aggregation. These data support a new model for NB formation proposed by Shen and colleagues in a recent article (17). In interphase, SUMO modified PML interacts with nearby SUMOylated PML molecules through its SUMO binding motif, thereby forming structured PML networks. Higher order protein networks are then formed through the recruitment of additional proteins through binding of their SUMO moieties.

Components of the SUMOylation machinery are also found in the NBs (30). Dysregulation of these enzymes highlights the importance of PML SUMOylation on NB body formation. Cultured cells deficient for the SUMO E2-conjugating enzyme Ubc9 showed profound defects in nuclear structure and function (30). Additionally in these cells, PML remained unSUMOylated, and PML nuclear bodies were completely disrupted. Overexpression of SuPr-1, a SUMO protease, induces both a reorganization of PML-NBs into fewer, larger aggregates and the redistribution of several PML-NB associated proteins, including DAXX and CBP (31).

Distinguishable roles for the different SUMO isoforms have been demonstrated. While most of the above studies pertain to modification by SUMO1, a recent report demonstrates that the nuclear localization of PML is also regulated by SUMO3 (32). Importantly, the decrease in NB number and redistribution of PML from the cytoplasm to the nucleus that was observed upon knockdown of SUMO3 could not be rescued by overexpression of SUMO1 and was only partially rescued by overexpression of SUMO2. Furthermore, the formation of polymeric SUMO3 chains, an ability that is unique to the SUMO2 and SUMO3 isoforms, was found to be important for regulating PML retention in the nucleus and the structure of PML-NBs.

A final word of caution must be said about the necessity of PML for NB formation. One PML body component, eIF4E, forms nuclear bodies in the absence of

PML cells (33). Additionally, overepxression of sp100 in PML$^{-/-}$ cells leads to the formation of nuclear structures similar to NBs (34) and these bodies still recruit the classical NB components, p53 and Daxx. Therefore, it remains controversial whether a SUMOylated PML is the component necessary and sufficient for NB formation.

3.3. Transcriptional role

PML NBs do not contain nucleic acid (35) and so are not active sites of transcription. Nonetheless, PML NBs have been shown to modulate transcription, an activity that primarily stems from their ability to recruit and sequester cofactors necessary for transactivation or transrepression. As SUMO1 modification of PML is necessary for proper NB formation, this post-translational modification ultimately affects responses on different promoters thereby implicating PML SUMOylation as a critical step in transcriptional control.

It has been suggested that NBs serve as molecular reservoirs, controlling the availability of certain transcription factors. Consistent with this idea, PML was shown modulate the transrepression activity of Daxx by the sequestration of Daxx within the NB (27). Specifically, a PML mutant that cannot undergo modification by SUMO1 is defective in reversing Daxx repressive activities, as measured by transient transfection assays with a reporter construct. To give this finding biological relevance, it was subsequently shown that this same sequestration of Daxx by PML activates Pax3 dependent transactivation (36). Pax3 is a transcription factor that plays a major role in a variety of developmental processes. In the presence of SUMO1-modified PML, Daxx is recruited to the NBs, relieving the repression Daxx normally exerts on Pax3.

There is also evidence for SUMO1-PML-dependent sequestration of coactivators of transcription. In a screen to identify c-Jun activators, Zon and colleagues (31) discovered that SuPr-1, a SUMO1 protease, was able to positively regulate c-Jun dependent transcription independently of c-Jun phosphorylation. Deconjugation of SUMO1 from PML, mediated by SuPr-1, was required for c-Jun activation of transcription. One possible explanation for these results is that SUMO1-modified PML concentrates co-activators, like CBP, to the NB. The displacement of these cofactors, through the action of SuPr-1, stimulates c-Jun activity indirectly by increasing the availability of CBP.

Since many other NB constituents are also SUMO-modified, it can be difficult to distinguish the exact role of SUMO-modified PML. SUMOylation of the orphan receptor LRH-1 causes its compartmentalization in the NB away from its target genes (37). Overexpression of SuPr-1 results in release of LRH-1 from the NB. This could result from either the deSUMOylation and subsequent release specifically of LRH-1 from the NB, or the complete disruption of the NB by deSUMOylation of PML. However, these ideas are not necessarily mutually exclusive. In fact, these data lend support to the model proposed by Pandolfi (17) whereby SUMO-modified PML interacts with and sequesters other SUMO modified proteins through its SUMO binding motif. An unexpected and novel result emerged from the same paper. SuPr-1 overexpression does not result merely in the random relocalization of PML but causes PML to form aggregates containing RNA polymerase II (37). This leads to the hypothesis that PML may also regulate transcription directly by interacting with the RNA polymerase II transcription complex upon deSUMOylation.

3.4. Viral attack

PML NBs have been shown to be highly sensitive to viral infection, and PML itself is upregulated by interferon (IFN) (38, 39), suggesting an important role for this protein and these structures in the antiviral response. Indeed, targeting PML NBs may represent a viral strategy to circumvent IFN action. It is beyond the scope of this paper to review the entirety of the current knowledge on the interactions between viruses and PML. For more on this topic, readers are referred to a recent comprehensive review by Everett and Chelbi-Alix (40).

Dramatic structural alteration of PML NBs is an early event during infection with DNA viruses (41, 42). However, whether this nuclear shakeup is a cause or a consequence of PML deSUMOylation remains uncertain. The herpes simplex virus (HSV) ICP0 protein (41, 43) and the cytomegalovirus (CMV) IE1 protein (42) both induce a complete disruption of the NB structure. Therefore, Muller and Dejean (44) examined the ability of these viral proteins to abrogate the SUMO modification of PML. They observed a correlation between co-expression of these proteins with PML to a loss of PML-SUMO1 conjugates. Furthermore, a mutant 1E1 protein that leaves NBs intact also does not affect the SUMO modification of PML, further linking this protein's capacity to disassemble NBs with the ability to abolish PML SUMOylation (44). In addition to supporting a model in which ICP0 and IE1 disrupt the NBs, either by preventing the formation of or by degrading SUMO1-modified PML, these data reinforced the role for SUMO1 conjugation in maintenance of the structural integrity of the NBs.

Although both viral proteins have the same biological consequence, the mechanisms by which each exerts their effects appear to differ. It has been well documented that ICP0 promotes NB disruption by causing the proteasome-mediated degradation of PML (43, 45). SUMO1-modified PML is more sensitive to this degradation than the unmodified form and mutation of K160, one of the three SUMO1 conjugation sites, renders PML less sensitive to the action of ICP0 (46). However, despite possessing a RING finger domain that functions as an ubiquitin E3 ligase, ICP0 does not interact with or directly ubiquitinate PML or SUMO1-PML *in vitro*. This suggests either that PML degradation is an indirect consequence of ICP0's ligase activity on another NB constituent or that additional factors are required for the ICP0-mediated ubiquitination of PML *in vivo*. In accordance with the latter possibility, ICP0 was shown to recruit the SUMO protease, SENP1 to NBs. It is thus possible that upon SENP1 recruitment, PML is targeted for SUMO deconjugation and having lost SUMO1, PML

would then be degraded. There are two models through which this may occur: (1) PML is simultaneously modified by ubiquitin and SUMO1 (as has been recently demonstrated for ubiquitin and SUMO2 (47)), and that in order for PML to be susceptible to degradation, SUMO1 has to be first removed or (2) SUMO1 is removed prior to ubiquitin conjugation on the same (or different lysines). SENP1-deconjugation and ICP0 ligase activities could participate in either of these representations.

IE1 has been shown to physically interact with PML (48). However, IE1 does not interfere with the formation of SUMOylated forms of PML, nor does it possess intrinsic deSUMOylating activity, *in vitro* (49). Unlike ICP0, the disruption of NBs is proteasome independent and IE1 does not interact with SENP1. Collectively, these data are consistent with the idea that IE1 and ICP0 exploit distinct mechanisms to reduce SUMO1-PML levels. Intriguingly, IE1 was even able to completely disrupt SUMO-independent PML aggregates. This finding suggests that inhibition of PML oligomerization by IE1 may indirectly induce PML deSUMOylation by exposure of SUMO1-PML conjugates to cellular SUMO isopeptidases.

Similar results were found to be mediated by the Epstein Barr virus immediate early protein BZLF1 (50). Interestingly, BLZF1 itself is SUMO1 modified and competes with PML for limiting amounts of SUMO1. Although tempting to speculate that this competition is the method employed by this virus to disrupt NBs, it does not appear to be the primary mechanism, as loss of the BLZF1 SUMO1 modification site did not prevent PML body disruption.

3.5. Cell cycle

At the beginning of cell division the nucleus disassembles, a striking process involving chromosome condensation, nuclear envelope breakdown and the disappearance of subnuclear compartments, such as the nucleolus. All of these processes are reversed at mitotic exit into G1. Although many mechanisms of nuclear re-formation have been well characterized, the mechanisms governing the restoration of subnuclear structures, such as PML NBs, are just beginning to be elucidated. PML-NBs undergo dramatic changes in both their number and biochemical composition during cell cycle progression. There are few PML-NBs in G0 phase and their numbers begin to increase as cells enter G1. During S and G2 phase, there is a further increase in number by a factor of up to twofold (51). This may be due to alterations in the structural stability of PML NBs, such that a body undergoes several fission events during this time. As discussed previously SUMO modification of PML is essential for PML NB integrity, so it is not surprising that this modification plays a role in NB formation during cell cycle.

Initial immunofluorescence staining showed an absence of colocalization of both PML and Sp100 with SUMO1 in mitotic cells, implying that SUMO modification of PML is cell-cycle dependent, although these data were not verified directly via protein-protein interactions (7). Orr's group (52) consequently undertook an extensive study of the intracellular distribution and biochemical characteristics of PML, as well as Sp100, throughout the cell cycle. They established that during mitosis, PML NBs become depleted in Sp100 and SUMO1 concomitantly with the appearance of a novel PML isoform of distinct electrophoretic mobility. This mitosis-specific isoform was stabilized by inhibition of phosphatases, leading to the hypothesis that this isoform corresponded to a phosphorylated version of the PML protein. The authors proposed a model whereby disruption of nuclear bodies that occurs at the beginning of mitosis is a consequence of deconjugation of SUMO1 from both PML and Sp100, followed by increased phosphorylation of PML. Reversal of this process leads to reestablishment of NBs as cells leave mitosis and re-enter G1.

Bazett-Jones's group (53) extended these earlier studies and showed that during mitosis, PML accumulates in structures called mitotic accumulations of PML protein (MAPPs), that are distinct from PML NBs in their dynamics, biochemistry and structure. These structures appear coincident with nuclear envelope breakdown and do not contain SUMO1, Daxx or Sp100. Importantly, the PML protein within MAPPs is not degraded but instead is recycled to form the basal structure of the new PML nuclear bodies in daughter nuclei. As the nuclear envelope reforms in late anaphase, nascent PML nuclear bodies accumulate components sequentially, recruiting those first that are essential for NB integrity, specifically SUMO1 and Sp100.

4. PML PHOSPHORYLATION

PML is phosphorylated on threonine and serine residues and some of the kinases that phosphorylate PML have recently been identified (Figure 1). However, the functional implications of this modification are much less extensively studied than the previously discussed SUMO modification.

4.1. Tumor suppression

Beginning with a report in 1995 linking PML mislocalization to hepatoma (54), a significant literature has developed showing that PML functions as a tumor suppressor that mediates apoptosis, growth arrest and cellular senescence. PML protein loss occurs frequently in human tumors of various histologic origins (55). Additionally, this loss, at least in prostate, breast and CNS tumors, is associated with tumor progression or tumor grade (55). Interestingly, the loss of PML occurs through post-translational mechanisms, as has been demonstrated for other tumor suppressive proteins, such as p27 (56). Thus, it is important to elucidate the molecular mechanisms underlying the aberrant degradation of PML in tumor cells.

Scaglioni *et al.* (57) demonstrated that the serine-threonine casein kinase 2 (CK2) negatively regulates PML levels by phosphorylating PML and targeting it for degradation via the ubiquitin-proteasome pathway. CK2 is a pro-survival kinase that is frequently overexpressed and

aberrantly active in lung tumors. In addition, PML protein levels and CK2 kinase activity are inversely correlated in non-small cell lung cancer cell lines. Loss of phosphorylation by CK2 at Serine517, the primary CK2 phosphorylation site, results in stabilization of PML and enhancement of PML's tumor-supressive properties. Of note, Serine517 is located within the previously discussed SUMO binding motif (17), which has been shown to mediate non-covalent interactions with other SUMO-modified proteins. CK2 phosphorylation may therefore prevent PML's association with other SUMO-conjugated proteins, including itself, thereby rendering PML more vulnerable to ubiquitination and degradation. The enforced expression of activated Ras induces more aggressive lesions in the lungs of PML-deficient mice compared to wild-type animals. CK2 may inhibit oncogene-induced senescence and promote tumor formation by mediating PML loss. Pharmacologic inhibition of CK2 could therefore provide a therapeutic means towards restoring the cellular PML supply.

4.2. DNA damage

Phosphorylation of PML is strongly implicated in the DNA damage response. In response to cellular stress, PML has been shown to be an inducer of both p53-dependent and independent apoptosis. Importantly, the choice of mechanism of PML's pro-apoptotic effects depends upon the apoptotic stimuli.

$PML^{-/-}$ mice and cells are protected against γ-radiation-induced apoptosis, suggesting that PML plays an important role in the response to this genetic assault (58). After γ-radiation, PML is phosphorylated by the DNA damage checkpoint kinase, Chk2, *in vitro* and *in vivo* at Serine117 (59). Furthermore, Chk2 and PML were shown to interact and Chk2 co-localizes with PML in the NBs. Exposure to γ-radiation decreased the interaction, suggesting that phosphorylation of PML by Chk2 causes the release of Chk2 from the PML-NB. The downstream molecular mechanism by which this mediates apoptosis remains unknown. However, the pathway is p53-independent; over-expression of wild-type PML, but not a non-phosphorylatable mutant, in p53 null cell lines increased apoptosis after irradiation (59).

In contrast, although the essential residues of PML have not been elucidated, the mechanism by which PML mediates p53-dependent apoptosis is clearer. Phosphorylation of PML by ATR in response to topoisomerase inhibition is proposed to trigger PML translocation to the nucleolus (60). This is followed by nucleolar sequestration of the p53 ubiquitin-ligase, MDM2, through direct interaction between the two proteins. Thus, phosphorylation of PML leads to p53 stabilization and p53-dependent apoptosis by antagonizing MDM2 function.

4.3. SUMO crosstalk

Evidence for cross-talk between individual post-translational modifications is abundant. It is especially well documented in epigenetics, where interplay between phosphorylation, methylation and acetylation of histones establishes the so-called "histone code (61)." Similarly, phosphorylation of PML is clearly linked to its SUMOylation, however, findings related to the exact nature of the relationship between these modifications have been contradictory.

Treatment with phosphatase inhibitors results in SUMO1 de-modification of PML, suggesting that SUMO1 modification is negatively regulated by phosphorylation (8, 62). Additionally, co-treatment with phosphatase inhibitors and arsenic trioxide (ATO) abrogated the SUMO1 conjugation normally seen after administration of ATO alone (8). These reports are confused by more recent accounts showing that SUMO1 modification is positively regulated by phosphorylation. Hayakawa and Privalsky (63) proposed that phosphorylation of PML by the extracellular signal-related kinase (ERK), a mitogen activated protein kinase (MAPK), potentiated SUMOylation after ATO treatment. Additionally, both modifications are needed for full induction of PML-dependent apoptosis in response to ATO. This is consistent with the many published reports demonstrating that ubiquitination often relies on prior phosphorylation of the target substrate (64). This is also consistent with a subsequent publication showing that poliovirus infection induces PML phosphorylation through a MAPK pathway and increased PML phosphorylation was associated with increased PML SUMOylation (65). However, the mechanism of how phosphorylation leads to enhanced SUMOylation has not yet been determined. Of note, the N-terminal phosphorylation site is adjacent to the RING domain of PML where Ubc9 and SUMO bind, suggesting that phosphorylation of PML may make it more amenable to SUMO conjugation.

5. PML/RAR-ALPHA

The PML protein's discovery and fame can be attributed to its role as RAR-alpha's fusion partner after a t(15;17) rearrangement event producing the PML/RAR-alpha the driving oncogene behind acute promyelocytic leukemia (APL). This chimeric protein impedes terminal cell differentiation, which leads to an accumulation of promyelocytes. Most APL patients are treated with therapeutic doses of all-trans retinoic acid (ATRA), a vitamin A derivative that activates RAR-alpha and causes promyelocytes to differentiate. Arsenic trioxide (ATO) is also used clinically as a potent antileukemic agent and can induce prolonged remissions even in ATRA-resistant APL cases.

The traditional notion of the mechanism of action of ATRA in APL holds that PML/RAR-alpha functions as a potent silencer of RAR-alpha target genes. A pharmacologic dose of ATRA causes release of corepressors associated with PML/RAR-alpha and allows for the recruitment of coactivators, leading to reactivation of genes required for the cells to travel through the promyelocyte stage of differentiation. Thus, the bulk of past research has focused on the direct transcriptional mechanisms of ATRA. However, the importance of non-transcriptional changes mediated by ATRA, particularly post-translational modifications of various proteins, has

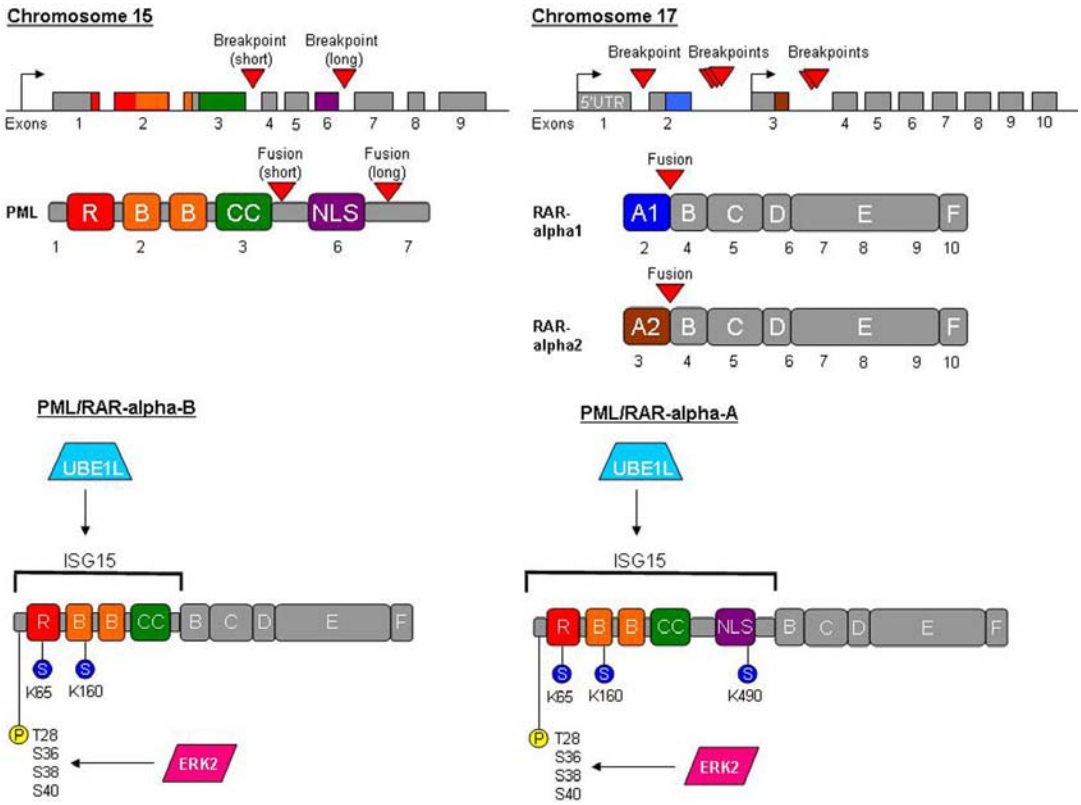

Figure 3. Schematic of the PML and RAR-alpha genes, the two PML-RAR-alpha fusion protein isoforms and their modifications. Red triangles denote fusion points between the PML and RAR-alpha sequences. Based on the genomic breakpoint in PML gene, either a short (PM/RAR-alpha-B) or long (PML/RAR-alpha-A) isoform is generated.

been recently highlighted. The following section will review post-translational modifications specifically of PML/RAR-alpha and their impact on the biology and pathology of APL.

5.1. ISG15

The interferon-stimulated gene 15 kDa (ISG15) protein belongs to the growing family of ubiquitin-like proteins (ULPs), including SUMO, whose biological functions are actively being pursued. As with the other characterized ULPs, ISG15 is covalently conjugated to target substrates using a cascade of three enzymes. The activating enzyme (E1) of the ISG15 conjugating system is the ubiquitin E1-like protein UBE1L (66). UBE1L is considered to be a putative tumor suppressor gene, as its expression in normal lung tissue is decreased or lost in the majority of lung carcinomas (67, 68). Similarly, reduced ISGylation is linked to a malignant phenotype, suggesting that ISG15 itself also serves a tumor suppressor function (69).

Previous research has associated ATRA-induced differentiation of APL cells with several steps in the ISGylation pathway. UBE1L is induced in ATRA-sensitive APL cells but not in those that are resistant to ATRA-mediated differentiation (70). Of note, ATRA is unable to induce expression of E1, an activating enzyme that shares homology with UBE1L, indicating that ATRA specifically induces UBE1L. These data suggest that ATRA may target ISG15 to specific substrates within the cell by induction of enzymes responsible for ISG15 conjugation. Significantly, overexpression of UBE1L triggered PML/RAR-alpha degradation and caused a rapid induction of apoptosis in ATRA-sensitive APL cells. The inverse relationship between PML/RAR-alpha and UBE1L protein expression has fueled speculation that ATRA, via UBE1L, promotes PML/RAR-alpha ISGylation and degradation (70). Recently, a direct protein-protein interaction between ISG15 and the PML portion of PML/RAR-alpha (Figure 3) was shown (71). However, while the functional consequences of ISGylation remain mysterious, considerable evidence to date indicates that ISG15 does not target proteins for proteasomal degradation (72, 73). Notwithstanding these uncertainties, these data are still consistent with an ATRA-mediated posttranscriptional mechanism playing a key role in the APL retinoid response.

5.2. SUMOylation

There is a puzzle of conflicting data concerning PML/RAR-alpha SUMOylation and its functional consequences. Initial attempts failed to demonstrate SUMOylation of the two reported PML/RAR-alpha isoforms, PML/RAR-alpha-A and PML/RAR-alpha-B (12). This was not due to a postulated lack of the PML SUMOylation sites, as a follow up study on the PML/RAR-alpha amino acid sequence revealed that PML/RAR-alpha-

A retained all three PML SUMOylation sites and PML/RAR-alpha-B lacked only the K490 site located within the NLS (9). Indeed, overexpression of SUMO1 is reported to induce SUMOylation of both PML/RAR-alpha isoforms (9). In 1999, a study by Ruthardt's group was the first to show that PML/RAR-alpha has a basal level of SUMO1 conjugation, but this study relied on extracts from cells which expressed the fusion protein only transiently (74). de Thé's group was able to demonstrate that PML/RAR-alpha is SUMOylated in an established APL cell line as well as in human primary APL cells.

In APL, PML/RAR-alpha expression causes the disruption of NBs and results in the delocalization of NB-associated proteins to nuclear microspeckles. Treatment with ATRA or ATO degrades the aberrant PML/RAR-alpha fusion protein and relocalizes NB components, linking NB disruption to APL pathogenesis. Furthermore, since SUMOylation of PML is intimately linked to NB stability, many studies have focused on SUMOylation after treatment with these two agents. ATRA-induced degradation is dependent upon the AF2 domain of RAR-alpha and occurs in the microspeckles. Consistently, ATRA treatment was unable to induce any appreciable SUMOylation of PML/RAR-alpha-A or –B.

ATO degradation of PML/RAR-alpha has a distinct mechanism from that of ATRA. ATO degradation of PML/RAR-alpha appears to take place in the NBs and is dependent on its PML moiety, which may be critical for its clinical efficacy. Dejean's group showed that ATO induced SUMOylation and rapid degradation of PML/RAR-alpha (8). Ruthardt's group was also able to observe that ATO caused PML/RAR-alpha to become highly modified (74), verifying the earlier observations made by Dejean's group. However, Ruthardt's group postulated that ATO does not degrade PML/RAR-alpha, but rather results only in its post-translational modification by SUMO1, a theory that was not supported by further research. Ruthardt's group also concluded that PML/RAR-alpha could be poly-SUMO conjugated at multiple sites . de Thé's group would later build on this by providing evidence implying that PML/RAR-alpha is in fact SUMO1 conjugated at two sites in response to ATO: K160 and K490 (62).

It has been proposed that mature NBs are the sites of intranuclear proteolysis of PML/RAR-alpha, as this is where the fusion protein localizes after ATO exposure. de Thé's group was able to demonstrate that mutating the K160 residue in PML/RAR-alpha not only altered the SUMOylation pattern of the fusion protein but also abolished it's ATO-induced degradation (62). While the wild-type shuttles towards the NBs after ATO exposure, the treatment does not affect localization of a PML/RAR-alpha-K160R mutant, implicating SUMOylation, at least at residue 160, as being necessary for ATO-induced localization. In addition, mutation of the K160 residue failed to recruit components of the 11S proteasome to the NB. These two results suggest (1) that SUMOylation at K160 is important to direct PML/RAR-alpha to PML NBs, where it is degraded; and (2) SUMOylation is essential for PML/RAR-alpha degradation because it leads to recruitment of the 11S proteasome. Although de Thé's group never shows a direct recruitment of the 11S proteasome complex to PML/RAR-alpha, the evidence compiled in favor of this model using PML is nonetheless persuasive enough to peg the 11S protease complex as the culprit protease responsible for ATO induced PML/RARα degradation.

K160 SUMOylation is by no means the entire story. A study by Kim *et al.* suggests that the RING finger domain along with PML's coiled-coil domain plays a role in sumoylation at K160 and K490 (75). Mutations impacting the structural integrity of either of these domains resulted in a complete loss of PML/RAR-alpha SUMOylation. Further investigation by Kim *et al.* revealed that the PML/RAR-alpha coiled-coil domain mutant was unable to bind Ubc9, the SUMO conjugating E2 enzyme, thereby explaining this PML/RAR-alpha mutant's inability to be sumoylated. However, the PML/RAR-alpha RING finger domain mutant was able to bind Ubc9 as well as the intact PML/RAR-alpha. Altogether the work of Kim *et al.* implies that SUMOylation of PML/RAR-alpha is dependant on the intact structural integrity of the RING finger domain along with a coiled-coil domain capable of Ubc9 binding (75).

As previously stated, transcriptional repression through enhanced recruitment of co-repressors to the RAR-alpha portion of PML/RAR-alpha oligomers has long been the dogma of APL pathogenesis. However, de Thé's group challenged this principle when they demonstrated that without the K160 SUMOylation site, PML/RAR-alpha is unable to efficiently induce immortalization or a differentiation block either *ex vivo* or *in vivo* (76). Importantly, the PML/RAR-alpha mutant behaves exactly as PML/RAR-alpha in terms of dimerization, DNA binding and affinity for the SMRT co-repressor but still fails to significantly impair differentiation of primary hematopoietic progenitors. Furthermore, although PML/RAR-alpha-K160R transgenic mice present with myeloproliferation, they never develop typical APL. Collectively, these observations led de Thé's group to postulate that the APL differentiation block is not solely due to RARα-moiety dimerization, but somehow must also involve a PML-moiety K160-SUMOylation dependant mechanism. The model they propose is one whereby the PML moiety of PML/RAR-alpha, via its K160 SUMOylation site, acts to recruit the transcriptional repressor Daxx. This, coupled with PML/RAR-alpha's ability to strongly recruit the SMRT co-repressor complex, results in a strong repression of ATRA-target genes, ultimately resulting in APL. Compelling evidence in favor of this model was provided when the fusion of PML/RAR-alpha-K160R to the repression domain of Daxx (thereby artificially recreating PML/RAR-alpha-Daxx recruitment) recapitulated all the features of cell transformation by the wild-type fusion protein. However, an obvious caveat remains: the importance of the K160 site might not be due to its modification by SUMO but rather due to another post-translational modification, such as ISGylation or acetylation.

5.3. Phosphorylation

The link between PML/RAR-alpha phosphorylation and SUMOylation remains controversial. The debate stems from a lack of data focusing specifically on the PML/RAR-alpha fusion protein, along with conflicting evidence from different groups. The only certainty here is that the two are interconnected. Dejean's group were the first to hint at a relationship between PML/RAR-alpha phosphorylation and SUMOylation in response to ATO. They found that ATO treatment combined with a potent phosphatase inhibitor (calyculin A) caused hyperphosphorylation of PML and abrogated PML SUMOylation (8). In a later study, de Thé's group also suggests that ATO can control PML SUMOylation indirectly through its phosphorylation. Using the phosphatase inhibitor okadiac acid, they determined that ATO induced PML dephosphorylation is necessary for its initial recruitment to the nuclear matrix, whereas PML SUMOylation is necessary for the formation of a mature NB (62). Thus, in the context of PML localization in response to ATO, the work of both groups (Dejean's and de Thé's) suggest a coordinated sequence where PML dephosphorylation allows for PML to be trafficked to the nuclear matrix. The now dephosphorylated PML can subsequently be SUMOylated, which is necessary for the formation of mature NBs. However, whether or not the same mechanism is true for ATO induced PML/RAR-alpha localization to NBs remains unclear. de Thé's group only presents data showing that the PML/RAR-alpha-K160R SUMOylation deficient mutant is unable to move to NBs in response to ATO (62). Since a hyperphosphorylated PML is incapable of being SUMOylated, it would have been interesting to see if the phosphatase inhibitor could have prevented PML/RAR-alpha NB localization.

A subsequent study by Hayakawa and Privalsky (63) would challenge the idea that a phosphorylated PML cannot be SUMO conjugated. The work of Dejean and de Thé's groups both made use of potent phosphatase inhibitors, possibly yielding an artificially hyper-phosphorylated form of PML. The subsequent work of Hayakawa and Privalsky took a less crude approach, employing site-specific mutagenesis to study PML and PML/RAR-alpha phosphorylation. As previously discussed, Hayakawa and Privalsky determined that in response to ATO the N-terminus of PML could be phosphorylated by ERK2 (Figure 1) at four possible sites (Threonine28 and Serines36, 38 and 40) but not at a fifth predicted site (Serine8). As PML's N-terminus is conserved in the PML/RAR-alpha fusion protein, PML/RAR-alpha mutants lacking the native PML phosphorylation sites were constructed. Indeed, Hayakawa and Privalsky determined that PML/RAR-alpha could be phosphorylated (Figure 3) in response to ATO through the MAP kinase cascade (63). While Hayakawa and Privalsky revealed that increased PML phosphorylation led to increased PML SUMOylation, they were unable to demonstrate this same relationship for PML/RAR-alpha. However, this stems from an inability to detect any PML-RAR-alpha SUMOylation, which suggests a technical problem rather than a biological certainty.

6. CONCLUDING REMARKS

PML is a functionally cryptic protein. There is an overwhelming amount of published information on PML's involvement in many critical cellular pathways. The importance of this protein is highlighted by the pathology associated with PML disruption, as evidenced in the disease APL, and the observation that $PML^{-/-}$ mice are more vulnerable to tumourigenesis and bacterial infection. However, in apparent contradiction to these data, PML is not essential for viability.

Most proteins are subjected to post-translational modifications (PTMs), which represent a way to reversibly regulate cellular location and biological activity. Here we have summarized the various PTMs of the PML protein. Single PTMs, such as SUMOylation and phosphorylation, are capable of regulating PML function and since PML is multiply modified, these modifications can expand PML's repertoire of functions by acting combinatorially. Furthermore, novel PTMs of PML and their consequences are continuously being elucidated. For instance, Hayakawa *et al.* (77) just recently demonstrated that PML can be acetylated, which is associated with enhanced PML SUMOylation (Figure 1). In this review, we have tried to present what is known about the consequences of PTMs of PML in the hopes that dissecting the current literature will give an appreciation for the complexities associated with this protein.

7. ACKNOWLEDGEMENTS

We are indebted to Suzan McNamara for thoughtful discussions of this review. W. H. Miller, Jr., is a Chercheur National of Fonds de la Recherche en Santé du Québec. J. Nichol and L. Petruccelli are supported by the MCETC/ FRSQ / CIHR strategic training program in Cancer Experimental Therapeutics and through Cole Foundation Fellowships.

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Abbreviations: PML: promyelocytic leukemia, NBs: nuclear bodies, RAR-alpha: retinoic acid receptor-alpha, APL: acute promyelocytic leukemia, ULPs: ubiquitin-like proteins, PIC1: PML interacting clone 1, SENPSs: Sentrin/SUMO-specific proteases, NLS: nuclear localisation signal, NPC: nuclear pre complex, IFN: interferon, HSV: herpes simplex virus, CMV: cytomegalovirus, MAPPs: mitotic accumulations of PML protein, CK2: casein kinase 2, ATO: arsenic trioxide, ERK: extracellular signal-related kinase, ATRA: all-trans retinoic acid

Key Words: PML, Post-translational, SUMO, Phosphorylation, ISG15, PML-RAR-alpha, Review

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