Modulation of H3.3 chromatin assembly by PML: A way to regulate epigenetic inheritance

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Abstract
Although the promyelocytic leukemia (PML) protein is renowned for regulating a wide range of cellular processes and as an essential component of PML nuclear bodies (PML-NBs), the mechanisms through which it exerts its broad physiological impact are far from fully elucidated. Here, we review recent studies supporting an emerging view that PML’s pleiotropic effects derive, at least partially, from its role in regulating histone H3.3 chromatin assembly, a critical epigenetic mechanism. These studies suggest that PML maintains heterochromatin organization by restraining H3.3 incorporation. Examination of PML’s contribution to H3.3 chromatin assembly in the context of the cell cycle and PML-NB assembly suggests that PML represses heterochromatic H3.3 deposition during S phase and that transcription and SUMOylation regulate PML’s recruitment to heterochromatin. Elucidating PML’s contributions to H3.3-mediated epigenetic regulation will provide insight into PML’s expansive influence on cellular physiology and open new avenues for studying oncogenesis linked to PML malfunction.

KEYWORDS
epigenetic inheritance, heterochromatin, histone H3.3, PML, PML-NB, S phase, SUMOylation

INTRODUCTION

The promyelocytic leukaemia (PML) protein is the master organizer of the PML nuclear body (PML-NB), one of the most investigated organelles of the eukaryotic nucleus.[1,2] PML-NBs are dynamic spherical structures that vary in size (0.1–1.0 µm in diameter) and number depending on the cell type.[2] Composed of an outer shell of PML protein, over 170 proteins localize constitutively or transiently to PML-NBs.[3] PML-NB composition varies both within cells and between cell types and changes in response to development, carcinogenesis, senescence, viral infection, and metabolic stress, which suggests that their structure and assembly mechanisms are functionally significant.[4,5]

PML, also known as TRIM19, is a member of the tripartite motif (TRIM) family, one of the largest subfamilies of E3 ubiquitin ligases some of which conjugate the Small Ubiquitin-like Modifier (SUMO) to target proteins.[1] TRIM proteins are characterized by an N-terminal RING-finger, B boxes, coiled-coil (RBCC)/TRIM motif, which is composed of a RING-finger, one or two B-boxes and an alpha-helical Coiled-Coil domain. In humans, there are seven main PML isoforms all of which share the N-terminal RBCC/TRIM motif but have different C-terminal regions generated by alternative splicing of a single gene.[6]

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chromosome translocation that fuses the PML N-terminus to the C-terminus of the retinoic acid receptor alpha in acute promyelocytic leukemia (APL).[7] The resulting PML-RARA oncoprotein blocks myeloid differentiation and redistributes PML-NBs into nuclear and cytoplasmic microspeckles.

PML has been reported to be a tumor suppressor as well as a regulator of many cellular processes including: DNA repair, DNA replication, senescence, apoptosis, transcription, translation, viral suppression, heterochromatin organization, stem cell renewal, differentiation, and angiogenesis.[12] However, despite extensive investigation, the molecular mechanism through which PML wields its broad regulatory influence has long eluded researchers. Because many PML-NB proteins regulate chromatin and transcription, it is thought that PML also participates in these processes.[1,2,8] This idea is supported by the finding that PML-NBs accumulate at genomic loci including: telomeres,[9] centromeres,[10] the major histocompatibility (MHC) class I gene cluster,[11] satellite DNA in patients with immunodeficiency, centromeric instability and facial dysmorphism (ICF) syndrome,[12] foreign DNA associated with virus-like particles (VLPs),[13] a heterochromatic transgene array,[14,15] a herpes simplex virus 1 (HSV-1) genomic DNA,[16,17] and Y chromosome repeats.[18]

The discovery in the last decade that several key PML-NB proteins regulate histone H3.3 chromatin assembly (discussed below) has focused attention more specifically on the theory that these bodies regulate H3.3-mediated epigenetic inheritance.[19] Attempts to discern PML’s role in this pathway, however, have been stymied by technical challenges. First, PML-NBs are associated with the nuclear matrix,[20] which made it difficult to analyze PML’s genome-wide associations using standard chromatin immunoprecipitation (ChIP) protocols. It has also been difficult to decipher the relationship between PML-NB assembly and H3.3 deposition due to the difficulty of tracking protein dynamics at specific genomic loci in single living cells. Recently, we and others have used newly developed tools and optimized protocols to (i) identify PML’s genomic associations, (ii) investigate PML’s impact on H3.3 incorporation and histone post-translational modifications (hPTMs), and (iii) examine PML recruitment to heterochromatin in the context of the cell cycle. Our findings suggest that PML functions to restrain the deposition of H3.3 into heterochromatin during DNA replication and consequently, to preserve the epigenetic inheritance of transcriptional silencing. Here, we consider PML’s role in H3.3 chromatin assembly in light of our current understanding of PML-NB dynamics and function and the impact that dysregulation may have on cellular physiology and oncogenesis.

HISTONE H3.3 REGULATES THE EPIGENETIC INHERITANCE OF TRANSCRIPTIONAL SILENCING

The nucleosome, which consists of DNA wrapped ~1.5 times around an octamer of histone proteins (i.e. two each of H2A, H2B, H3, and H4), is the basic structural unit of chromatin and an important repository of epigenetic information.[21] Specifically, the addition of post translational modifications (PTMs) to the histone N-terminal tails correlates with the stable inheritance of different states of chromatin compaction and transcriptional activation.[21] The majority of the histones in a cell (i.e. the canonical histones) are synthesized during S phase when they are needed to package the newly replicated DNA.[22] However, histone variants are also expressed and incorporated into chromatin throughout the cell cycle.[21] Histone variants have emerged as important epigenetic regulators because they impact events including gene expression, chromatin organization, development, and cellular reprogramming.[23,24] Currently, it is not fully understood how the epigenetic functions of the canonical and variant histones are coordinately regulated.

H3.3 is a variant of the H3 histones, which, in mammals, differs from the canonical H3.1 and H3.2 by five and four amino acids, respectively.[21] H3.3 is incorporated into both generic and repetitive regions of the genome by distinct chromatin assembly proteins that specifically associate with H3.3 through its unique amino acid sequence.[25,26] Death-domain associated protein (DAXX) is an H3.3 specific chaperone that together with the ATP-dependent chromatin remodeling factor, alpha-thalassemia/mental retardation syndrome X-linked (ATRX), incorporates H3.3 into heterochromatic regions including: telomeres, pericentromeric heterochromatin, endogenous retroviral elements (ERVs), imprinted differentially methylated regions and intragenic methylated CpG islands.[27] DAXX and ATRX-mediated H3.3 chromatin assembly is required for the heterochromatic hPTM, H3 lysine 9 tri-methylation (H3K9me3), to be deposited at these regions which indicates that this pathway regulates the establishment and maintenance of transcriptional silencing.[27]

Histone cell cycle regulator (HIRA), which forms a complex with Ubinucin 1 (UBN1) or Ubinucin 2 (UBN2) and Calcineurin-binding protein 1 (CABIN1),[26] is required for H3.3 incorporation into generic regions, including promoters, gene bodies, and enhancers,[28–31] as well as sites of DNA damage repair.[32] The anti-silencing function 1a (ASF1a) protein delivers H3.3-H4 dimers to the HIRA complex and is considered an auxiliary component.[26] The HIRA complex regulates H3.3 deposition at both transcriptionally active and silent genes.[23] HIRA-mediated H3.3 chromatin assembly promotes the deposition of the heterochromatic hPTM, H3 lysine 27 tri-methylation (H3K27me3), by polycomb repressive complex 2 (PRC2) which suggests that HIRA also regulates transcriptional silencing.[28,29] In Hira-/- ESCs, H3K27me3 levels are reduced at the promoters of developmentally regulated genes, which are typically silenced or expressed at low levels.[28,29] Differentiation is also dysregulated, which indicates that Hira-mediated H3.3 chromatin assembly is required for development.[28,29]
were known to localize to PML-NBs, it also provided a new avenue for investigating PML and PML-NBs. DAXX and ATRX are constitutive PML-NB components, and the functional significance of their PML-NB targeting is underscored by the fact that many herpesviruses must displace and/or degrade them, as well as PML, in order to establish lytic infections and to overcome latency. Importantly, we and others have reported that a subpopulation of newly synthesized H3.3 is targeted to PML-NBs prior to being incorporated into chromatin (Figure 1A), which supports a model in which the targeting of DAXX and ATRX to PML-NBs plays an important role in regulating H3.3 chromatin deposition (Figure 1B).[34–37]

In contrast to DAXX and ATRX, HIRA is diffusely distributed throughout the nucleus in most proliferating somatic cells.[35,36,38] HIRA accumulates in PML-NBs, however, (i) at the onset of senescence, (ii) in response to anti-viral interferons (IFNs) and (iii) following the introduction of naked DNA through plasmid transfection and viral infection.[16,35,36,39–42] The finding that HIRA deposits H3.3 onto naked plasmid and viral DNA[16,41] is consistent with its ability to fill nucleosome gaps in a sequence-independent manner.[31] Nevertheless, as a PML-NB component, HIRA’s contributions to transcriptional and epigenetic regulation are complex and context dependent. For example, IFN, which triggers the innate immune response, can independently induce HIRA’s translocation to PML-NBs.[41,42] In cells infected with replication-defective herpesviruses, PML-NB-targeted HIRA likely promotes the chromatin assembly of viral genomes for subsequent epigenetic silencing.[16,41,42] In cells infected with wild-type HSV-1, which degrades PML and disrupts PML-NB organization, HIRA is dispersed away from viral genomes, although it may initially promote the onset of viral gene expression by contributing to viral chromatin formation.[42,43] At later time points, as a PML-NB component in sensory neurons, HIRA may promote viral latency.[16] In vivo, both H3K27me3 and H3K9me3 have been detected on latent HSV-1 genomes.[44–46] Therefore, it will be important to decipher how HIRA and DAXX/ATRX-mediated H3.3 chromatin assembly are coordinated during the establishment and maintenance of viral latency. Taken together, these studies suggest that to fully understand PML-NB’s contributions to H3.3-mediated epigenetic inheritance, it will be necessary to unravel their dynamic interactions with distinct genomic loci in different cellular contexts.

**FIGURE 1**  Histone variant H3.3 is found at PML-NBs. (A) Immunofluorescence detection of H3.3-Flag at PML-NBs in a MEF nucleus and quantification of fluorescence corresponding to PML (green line) and H3.3-Flag (red line) along the corresponding trace (graph). Arrow heads show examples of PML-NBs harboring H3.3 accumulation (merge image) and corresponding fluorescence intensities (graph). DNA is stained with DAPI. a.u.: arbitrary units. Bar, 5 µm. (B) A non-nucleosomal pool of H3.3-H4 dimers is targeted to PML-NBs by DAXX (1). PML-NBs also contain DAXX and ATRX, suggesting the formation of an H3.3 pre-deposition complex in these structures. The other fraction of soluble H3.3-H4 dimers binds ASF1A to be subsequently deposited into chromatin by the HIRA complex (2).

**PML-NB-TARGETING MECHANISMS REGULATE H3.3-MEDIATED EPIGENETIC INHERITANCE**

Given the correlation between PML-NBs, H3.3 deposition, and transcriptional silencing, it is likely that PML-NB-assembly mechanisms also regulate epigenetic inheritance. SUMOylation is required for PML-NB formation; when UBC9, the only SUMOlyating E2 enzyme, is depleted, PML-NBs do not coalesce, and chromosome defects ensue.[47,48] Both DAXX and PML are SUMOylated and contain SUMO-interacting motifs (SIMs), which suggests that SUMOylation regulates their functions and interactions within PML-NBs.[47,49–51] DAXX requires both SUMO-2 and its SIM to interact with centromere protein B (CENP-B) and to accumulate at centromeres, and when DAXX targeting fails, H3.3 is not incorporated into centromeric and pericentromeric heterochromatin.[52] Additionally, UBC9 depletes DAXX and PML from accumulating at H3.3 from being incorporated into a heterochromatic transgene array indicating that SUMOylation is required for H3.3 chromatin assembly at some genomic loci.[15] Nevertheless, PML does not require its SIM or SUMOylation sites to accumulate in PML-NBs,[15,50] making SUMOylation’s contribution to PML targeting less straightforward. It is possible that SUMOylation is needed to establish interactions between PML’s binding partners within PML-NBs, including heterochromatin protein 1 (HP1)[52] and DAXX,[51] and repetitive DNA (discussed further below).

Intermolecular interactions also play essential roles in targeting H3.3 chromatin assembly proteins to PML-NBs and chromatin. The direct interaction between DAXX and PML is required for ATRX to localize to PML-NBs.[1,54] On the chromatin side, formation of the DAXX-ATRX complex ensures that both proteins are properly targeted to telomeres and centromeres.[52,55–57] ATRX also requires its N-terminal ADD (ATRX-Dnmt3-Dnmt3L) domain, which binds...
H3K9me3, to localize to pericentric heterochromatin suggesting that its targeting is modulated by the epigenetic mechanism it regulates.\cite{58} Non-covalent self-interactions between PML RBCC domains are also essential for PML-NB nucleation.\cite{50} Remarkably, both H3.3 depletion and mutations in DAXX that prevent it from interacting with H3.3, reduce DAXX stability which suggests that H3.3 may also impact PML-NB composition.\cite{59}

Less is known about how HIRA is targeted to PML-NBs as well as how HIRA functions with PRC2 to effectuate H3K27me3 deposition.\cite{28} However, for IFN-mediated PML-NB-targeting of HIRA, both PML and the core PML-NB protein, Sp100, are required but DAXX and ATRX are not; Janus-Associated Kinase (JAK) signaling is also required for HIRA to localize to PML-NBs in response to IFN.\cite{42} Notably, HIRA is refractory to IFN-mediated PML-NB-localization in many cancer cells lines; currently, it is not known whether this deficiency is a cause or an effect of carcinogenesis.\cite{41,42} Taken together, these reports suggest that H3.3 deposition is a dynamic regulator of transcriptional silencing because its chromatin assembly proteins are targeted to genomic loci through mechanisms that are cooperative, reversible, and responsive to changing stimuli.\cite{50}

**HYPOMETHYLATED REPETITIVE DNA IS SILENCED BY THE DAXX-ATRX-H3.3 PATHWAY AND MAY SEED PML-NB ASSEMBLY**

Because PML-NBs have been investigated using a variety of genomic loci and cell types, it has been difficult to elucidate a simple assembly mechanism. The fact that H3.3’s contributions to transcriptional silencing change during development and have been correlated to changes in PML-NB protein organization also contributes to the regulatory complexity.\cite{9,29,34,56,60} In ESCs, the DAXX-ATRX-H3.3 pathway is required to silence hypomethylated repetitive DNA that, in adult somatic tissue, is silenced by DNA methylation (e.g. telomeres and a subset of ERVs).\cite{56,61} Vertebrate telomeres are composed of terminal tandem TTAGGG repeats that are separated from chromosomespecific regions by CpG-rich sub-telomeres, which are hypomethylated in ESCs and heavily methylated in differentiated cells.\cite{62,63} DAXX, ATRX, H3.3, and PML all accumulate at ESC telomeres but cannot be visually detected at somatic cell telomeres, which suggests that PML-NB assembly is curtailed by the epigenetic changes that occur during differentiation (i.e. DNA methylation and H4K20me3).\cite{29,64}

Nevertheless, H3.3 continues to be incorporated into telomere chromatin after differentiation and DAXX and ATRX are required for genomic stability. Indeed, loss of DAXX and ATRX is a hallmark of cancer cells that use the alternative lengthening of telomere (ALT) pathway to maintain telomere length (discussed below) although depleting DAXX or ATRX alone is insufficient to induce ALT, which indicates that other changes must also occur.\cite{65–69} Interestingly, PML accumulates at ALT cell telomeres in structures known as ALT-associated PML bodies (APBs).\cite{63} As ALT-cell sub-telomeres are hypomethylated, this finding also supports the idea that PML-NB proteins have an affinity for hypomethylated DNA.\cite{63} Likewise, DAXX and ATRX do not accumulate at telomeres in pre-implantation embryos until the morula stage when DNA methylation has been drastically reduced.\cite{56} The hypomethylated status of pericentric satellite repeats in cells from ICF patients that carry autosomal recessive mutations in the de novo DNA methyltransferase, DNMT3B, may also explain why DAXX, ATRX and PML accumulate at these sites.\cite{12,63}

The DAXX-ATRX-H3.3 pathway is also required to maintain transcriptional silencing at a subset of ERVs (i.e. IAPs and ETn ERVs) in ESCs.\cite{70} However, in contrast to telomeres, which continue to incorporate H3.3 and H3K9me3 after differentiation, H3.3 and H3K9me3 levels are dramatically reduced at the ERVs after differentiation, which suggests that (i) H3.3 regulates the embryonic establishment but not the somatic maintenance of their silenced chromatin state and (ii) DAXX and ATRX are displaced by the epigenetic changes that occur.\cite{70} In ESCs, DAXX, ATRX and the co-repressor, KRAB-associated protein 1 (KAP1) (i.e. TRIM28), are recruited to ERVs in a co-dependent manner.\cite{70} As KAP1, like PML, is a TRIM family protein, it will be interesting to determine whether DAXX and ATRX regulate H3.3-mediated silencing at different genomic loci with different TRIM proteins.\cite{70} The differences in how H3.3 is regulated at telomeres and ERVs further highlights the regulatory complexity of this epigenetic mechanism. Factors including DNA sequence and repeat number, DNA methylation status, transcription factor binding, hPTM deposition, co-repressor associations and the presence or absence of PML all likely contribute to the intricacy.

**PML-NBS PROVIDE AN ESSENTIAL PLATFORM FOR H3.3 CHROMATIN ASSEMBLY AT SOME GENOMIC LOCI**

Telomeres are an important model for studying the nuclear organization of the DAXX-ATRX-H3.3 pathway because the high copy number of their repeats makes it possible to correlate protein recruitment with chromatin regulation. Indeed, a study of ESC telomeres provided one of the first indications that PML, itself, regulates H3.3 deposition by showing that PML is required for ATRX to be recruited to ESC telomeres and for H3.3 to be incorporated.\cite{34} Additionally, H3K9me3 and H4K20me3 levels increase at ESC telomeres when PML is depleted, which suggests that the DAXX-ATRX-H3.3 pathway modulates hPTMs and that its presence may prevent other silencing mechanisms from being established.\cite{34} PML is also required for DAXX and ATRX to co-localize with latent HSV-1 genomes which also supports the idea that PML-NBs facilitate H3.3-mediated epigenetic silencing.\cite{16,34}

**PML REGULATES THE EPIGENETIC INHERITANCE OF HETEROCHROMATIN BY RESTRAINING H3.3 DEPOSITION AND MAINTAINING H3K9ME3 LEVELS**

In contrast to reports showing that PML’s essential function in DAXX and ATX-mediated H3.3 chromatin assembly is ensuring the chromatin-targeting of DAXX and ATRX, our laboratories recently
reported that PML also directly regulates H3.3 chromatin assembly by repressing its deposition.\cite{15,37,71} Using both genomic techniques and a single-cell imaging model, we showed that H3.3 deposition increases and H3K9me3 levels decrease in the absence of PML.\cite{15,37,71} Importantly, these results also show that DAXX and ATRX can be targeted to heterochromatin independently of PML further demonstrating the complexity of PML’s regulatory impact on H3.3 chromatin assembly.

Specifically, we used ChIP followed by high-throughput sequencing (ChIP-Seq) to investigate PML’s contribution to H3.3-mediated epigenetic regulation in wild type and Pml$^{-/-}$ mouse embryonic fibroblasts (MEFs).\cite{37,71} First, we determined that PML associates with multiple gene-poor, H3K9me3-enriched transcriptionally silent regions throughout the genome, which we named PML-associated domains (PADs).\cite{37} (Figure 2A and 2B). Notably, in Pml$^{-/-}$ MEFs, H3.3 deposition into PADs is increased and accompanied by a decrease in H3K9me3 (Figure 2B), which suggests that PML maintains H3K9me3 levels by restraining H3.3 incorporation.\cite{37} Similar results were seen at subtelomeres in Pml$^{-/-}$ MEFs (Figure 2C), which is consistent with reports that they continue to be regulated by the DAXX-ATRX-H3.3 pathway in somatic cells.\cite{70,71}

PML depletion also increased H3.3 and decreased H3K9me3 levels at a transgene array stably integrated into HeLa cells (Figure 2D).\cite{15} The transgene includes an inducible transcription unit and repetitive regions of the tetracycline response element (TRE) (~6 kb) and lac operator (~10 kb) (Figure 2D). The inclusion of the lac operator repeats allows the integration site of the transgene array to be visualized in single living cells when lac repressor (LacI) fused to an auto-fluorescent protein is expressed.\cite{14} PML, DAXX, ATRX, SUMO-1, and SUMO-2/3 all accumulate at the transgene array (Figure 2E).\cite{15,72} As shown in Figure 2D, H3.3 is specifically enriched in the lac operator and TRE repeats of the transgene, which is consistent with reports that DAXX and ATRX regulate H3.3 deposition at tandem repeats.\cite{29,57} The finding that H3K9me3 levels decrease across the transgene following PML depletion also suggests that PML’s role in restraining H3.3 incorporation into repeats regions is essential for H3K9me3 to spread (Figure 2D).

Strikingly, in Pml$^{-/-}$ MEFs, the increase in H3.3 and decrease in H3K9me3 at PADs (Figure 2D) was accompanied by an increase in H3K27me3, which is consistent with our finding that these regions remain silent despite the loss of H3K9me3 (Figure 2B).\cite{37} Because HIRA is required for PRC2-mediated H3K27me3,\cite{28} it also suggests
FIGURE 3 PML is recruited to heterochromatin during S phase and restrains transcription-induced deposition of H3.3 by DAXX to ensure maintenance of the epigenetic state. The cartoons on the left propose a model for how PML regulates DAXX-mediated H3.3 deposition during S phase. During G1, DAXX, ATRX, and HP1 are present at heterochromatin but PML is not. H3.3 is not incorporated into the heterochromatin during G1 due to its highly condensed state. During the process of DNA replication, heterochromatin decondenses, allowing temporary transcription events. During S phase, SUMO-1 may be conjugated to DAXX and/or HP1, which was shown to be recruited by RNA and conjugated with SUMO-1 during de novo heterochromatin assembly by Maison et al. (2011). HP1 and possibly DAXX are conjugated with SUMO-1 during S phase, which may be the signal that recruits PML to heterochromatin via its SIM domain. After PML’s initial association with the array, it becomes SUMOylated with both SUMO-2/3 and SUMO-1 and through protein-protein and SUMO-SIM interactions, SUMOylated PML eventually encapsulates the heterochromatic site throughout G2. PML may repress further, H3.3 deposition by attenuating transcriptional events or promoting the degradation of the RNA signal that recruits H3.3. Images of YFP-PML IV (green) recruitment to the transgene array corresponding to different points in the cell cycle taken from a time series of a single cell are shown on the right and are from Shastrula et al. (2019). Different steps of the model are illustrated on the right side. Images are maximum projections of confocal image stacks. Merged images include YFP-PML IV (green), Cherry-lac repressor (red), which marks the transgene array, and the differential interference contrast (DIC) taken from a time series of a single cell containing a heterochromatic transgene array image (visualized in red). Arrows indicate the transgene array. Insets show cropped enlarged images of the array. Large scale bar = 5 µm. Small bar in insets = 1 µm. Adapted with permission from Shastrula et al. (2019)

that, in the absence of PML, HIRA contributes to H3.3 deposition at PADs. Taken together, these results indicate that when evaluating PML’s impact on H3.3-mediated epigenetic inheritance, it is essential to identify the H3.3 chromatin assembly proteins recruited in the presence and absence of PML as well as to consider the regulatory impact of cell type, differentiation state, and the DNA sequence.[9,15,55,56]

PML IS RECRUITED TO HETEROCHROMATIN DURING S PHASE

Currently, the mechanism through which PML restrains H3.3 incorporation into heterochromatin is unknown. However, our study using the transgene array system, which permits H3.3 incorporation to be correlated with protein recruitment (Figure 2D and 2E), suggests that PML represses H3.3 deposition during DNA replication because PML is specifically recruited to the site during S phase (Figure 3).[15] This result is also consistent with reports that PML-NB numbers increase during the S and G2 phases of the cell cycle.[9,12,34,38,73–75] Although we did not detect PML at PADs in MEFs using immunofluorescence and DNA FISH, this analysis is not dispositive because it was done using confluent cells.[37] In the future, PML localization in relation to PADs should be investigated using cycling cells to ensure that cells undergoing DNA replication are being evaluated.

Chromatin assembly is broadly divided into replication-coupled and replication-independent mechanisms.[21] As H3.3 is expressed and incorporated into chromatin throughout the cell cycle, its deposition was thought to be exclusively replication independent. However, the DAXX-ATRX-H3.3 pathway has also been associated with DNA replication which suggests that it is also linked to replication-coupled chromatin assembly. For example, in ESCs, ATRX is recruited and H3.3 is incorporated into telomeres during S phase.[9,64] In zygotes, DAXX and ATRX accumulate at pericentric DNA during de novo heterochromatin assembly, which coincides with DNA replication.[76,77] Additionally, ATRX-null cells exhibit an increase in stalled replication forks, fragile telomeres and mitotic catastrophe, which suggests that ATRX is needed to resolves the secondary structure of repetitive DNA during synthesis.[78–83] ATRX also works with Fanconi anemia Complementation Group D2 (FANCD2), which is mutated in the chromosome instability syndrome Fanconi anemia (FA), to restart stalled replication forks through a process that requires H3.3 chromatin assembly.[84] Taken together, these studies support the hypotheses that PML modulates DAXX and ATRX-mediated H3.3 chromatin assembly during DNA replication.
DO TRANSCRIPTIONAL EVENTS RECRUIT PML TO HETEROCHROMATIN DURING S PHASE?

The finding that PML is recruited to the transgene array during S phase\(^{15}\) also raises the question as to the identity of its recruitment signal. Here, we discuss the possibility that PML is recruited to heterochromatin by transcriptional events that occur during DNA replication. Indeed, transcription of heterochromatin during S phase has been previously linked to the regulation of transcriptional silencing.\(^{85–88}\) In both fission yeast, \textit{Schizosaccharomyces pombe}, and budding yeast, \textit{Saccharomyces cerevisiae}, RNA pol II transcribes pericentric and centromeric regions in both directions during DNA replication; both increased and decreased expression disrupts heterochromatin organization and causes chromosome segregation defects.\(^{85,87,88,90,91}\)

If PML is recruited to heterochromatin by transcription, it also raises the possibility that PML represses H3.3 recruitment and/or deposition by modulating a transcriptional or regulatory mechanism. Such a finding would be consistent with our previous report that Rpp29, a protein component of the endonucleases RNase P and RNase MRP best known for their roles in tRNA and rRNA processing\(^{92}\) represses H3.3 deposition.\(^{93,94}\) Initially, we showed that H3.3 co-localizes with Rpp29 and antisense RNA at the activated transgene array, and subsequently, we found that Rpp29 depletion increases H3.3 incorporation at the lac operator and TRE repeats as well as the promoters and bodies of genes.\(^{93,94}\) It is possible that H3.3 is recruited to its incorporation sites by an RNA signal that is degraded by Rpp29.\(^{93–95}\) Currently, we do not know whether PML and Rpp29 function in the same pathway to repress H3.3 chromatin assembly.

If restraining transcription is the mechanism through which PML represses H3.3 chromatin assembly, it could also help to explain why H3.3 levels increase and H3K9me3 levels decrease when PML is depleted.\(^{15,37,71}\) Nucleosomes are more fully disassembled during transcription than DNA replication, thus providing greater opportunity for H3 histones in pre-existing nucleosomes (i.e. those bearing hPTMs such as H3K9me3) to be replaced with newly synthesized H3.3 during reassembly.\(^{15}\) Nucleosomes consist of an H3-H4 tetramer ([H3-H4]\(_2\)) core to which two H2A-H2B dimers are added.\(^{21}\) The H3-H4 tetramers in pre-existing nucleosomes rarely split during replication-coupled chromatin assembly\(^{97}\) but often do at highly transcribed genes.\(^{15}\) Therefore, if heterochromatin transcription increases during S phase when PML is depleted, it could increase H3-H4 tetramer splitting, which in turn could cause the canonical H3s (i.e. H3.1 and H3.2) in the pre-existing nucleosomes to be replaced with newly synthesized H3.3. Consequently, existing H3K9me3 patterns would also be lost as hPTMs are typically copied from pre-existing nucleosomes onto adjacent newly assembled nucleosomes.\(^{96–98}\)

Future studies are needed to determine the impact of PML depletion on heterochromatin transcription and nucleosome splitting during S phase as well as to determine whether and how RNA regulatory events impact H3.3-mediated silencing.

It will also be important to elucidate the role of SUMOylation in H3.3-mediated epigenetic inheritance because UBC9 depletion (i) prevents DAXX, PML, SUMO-1, and SUMO-2/3 from accumulating at the transgene array and (ii) reduces H3.3 incorporation, which indicates that SUMOylation is required for these events.\(^{15}\) Like PML, SUMO-1, and SUMO-2 accumulate and co-localize with PML at the transgene array during S phase, which suggests that PML is their main conjugation target.\(^{15}\) This idea is supported by the finding that SUMO-2/3 does not accumulate when PML is depleted.\(^{15}\) Although SUMO-1 does not encapsulate the array in the absence of PML, it is detectable at the site following PML knockdown, which suggests that SUMO-1 is conjugated to one or more proteins upstream of PML\(^{15}\) any of which could also serve as the PML recruitment signal. Heterochromatin protein 1 (HP1), which is a key component of the constitutive heterochromatin architecture that specifically binds to H3K9me3,\(^{90}\) is a strong candidate to be a SUMO-1-conjugation target at the array (Figure 3) because its de novo targeting to pericentric heterochromatin was previously shown to require transient SUMO-1 conjugation and cis transcription of the non-coding RNA.\(^{53}\) Other factors supporting SUMOylated HP1 being a PML recruitment signal include the findings that PML and HP1 interact\(^{12,99–101}\) and HP1 is enriched at the transgene array.\(^{102}\)

Based on these observations, we suggest a tentative model in which the transcriptional activity occurring in heterochromatic regions during replication\(^{85–89}\) initiates the SUMOylation of HP1, and possibly DAXX,\(^{51}\) which in turn recruits PML via its SIM. Subsequently, PML is conjugated with SUMO-1 and SUMO-2/3 and eventually encapsulates the transgene array functioning to repress H3.3 deposition and maintain H3K9me3 by abrogating transcriptional events initiated during DNA replication or facilitating RNA degradation, thus preventing H3-H4 tetramer splitting and incorporation of new H3.3.\(^{15,37,71}\) (Figure 3).

IS PML A COMPONENT OF THE DAXX-ATRX-H3.3 CANCER AXIS?

PML first gained prominence in the field of cancer biology when it was identified at the breakpoint of the chromosomal translocation that drives APL.\(^{103}\) Subsequently, PML was categorized as a tumor suppressor because \textit{Pml} \(-/-\) mice are prone to tumorigenesis upon mild oncogenic stimulation,\(^{104}\) and PML expression is decreased in many human cancers.\(^{105}\) Although, notably, PML is up regulated in triple negative breast cancer.\(^{106}\) Despite reports that PML impacts signal transduction pathways (e.g. Rb, p53, Akt, PTEN, Ras),\(^{107}\) the mechanisms through which PML contributes to tumorigenesis are largely unknown. Our finding that PML modulates H3.3 chromatin assembly suggests that changes in its expression could drive tumorigenesis by rewiring the epigenome, which, in turn, could alter oncogene and tumor suppressor expression and/or cause genomic instability. Notably, \textit{Pml} \(-/-\) MEFs are resistant to oncogenic ras-induced senescence.\(^{108}\) As H3.3 and its chromatin assembly proteins regulate senescence,\(^{109–111}\) disruption of PML could also drive tumorigenesis by dysregulating senescence.
Considering PML’s contributions to H3.3 chromatin assembly could also provide insight into how PML-RARA drives APL. PML-RARA is targeted to the all-trans retinoic acid (ATRA) response elements throughout the genome by the RARA-DNA binding domain; PML-RARA is thought to interfere with gene expression programs involved in differentiation, apoptosis and self-renewal by functioning as a constitutive transcriptional repressor. It is possible that the PML region of the PML-RARA onco-fusion protein promotes H3K27me3 and H3K9me3 deposition and histone deacetylation by recruiting the proteins involved in H3.3-mediated transcriptional silencing. Because PML-RARA redistributes wild-type PML from PML-NBs into micro speckles, it may also drive tumorigenesis by blocking H3.3-mediated silencing and cellular senescence through a dominant negative mechanism. Future studies are needed to determine PML-RARA’s impact on H3.3 chromatin assembly.

Cancer-causing mutations have also been identified in DAXX, ATRX, and H3.3, which has established this pathway as an important cancer axis. Specifically, loss-of-function mutations in DAXX and ATRX have been identified in brain and pancreatic neuroendocrine tumors as well as the majority of cancer cells that use the alternative lengthening of telomere (ALT) pathway instead of telomerase to maintain telomere length. Compared to telomerases in wild-type somatic cells, ALTcell telomerases are less compact, hypomethylated and have lower H3K9me3 — features which are thought to make them more conducive to the homologous recombination through which telomere length is maintained. If PML is targeted to heterochromatin by transcription, then the persistent expression of TERRA in ALT cells may also contribute to PML accumulation in APBs.

Somatic driver mutations located in the N-terminal tail of H3.3, and to a lesser extent H3.1 and H3.2, have also been identified in pediatric high-grade gliomas (HGGs), chondroblastoma, giant cell tumors of the bone, chondrosarcoma, pediatric soft tissue sarcoma, head and neck squamous cell carcinoma and leukemia. Among the most frequent H3 mutation is the conversion of lysine 27 to methionine (K27M), which globally decreases H3K27me3 by inhibiting Enhancer of Zeste 2 (EZH2), the methyltransferase subunit of PRC2. Notably, all pediatric HGGs with G34R mutations show the ALT phenotype, and the G34R and K27M mutations are frequently accompanied by loss of ATRX. Therefore, a better understanding of PML’s function in H3.3 chromatin assembly could also provide insight into ALT cell pathogenesis in H3.3-oncohistone driven cancers.

CONCLUSION

Ever since PML was discovered, it has been the focus of intense research efforts that have revealed both its broad physiological impact and the functional significance of PML-NB assembly and organization. Nevertheless, PML’s mechanism of function remained elusive. Recent studies showing that PML expression impacts H3.3 chromatin assembly now suggest that PML has broad and diverse effects on cellular processes because it regulates H3.3-mediated epigenetic inheritance. Based on these analyses, we believe that considering PML’s function in the context of H3.3 function will provide important insight into how its dysregulation contributes to diseases including cancer.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing not applicable – no new data generated.

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