

## PML nuclear bodies in the pathogenesis of acute promyelocytic leukemia: active players or innocent bystanders?

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### 1. ABSTRACT

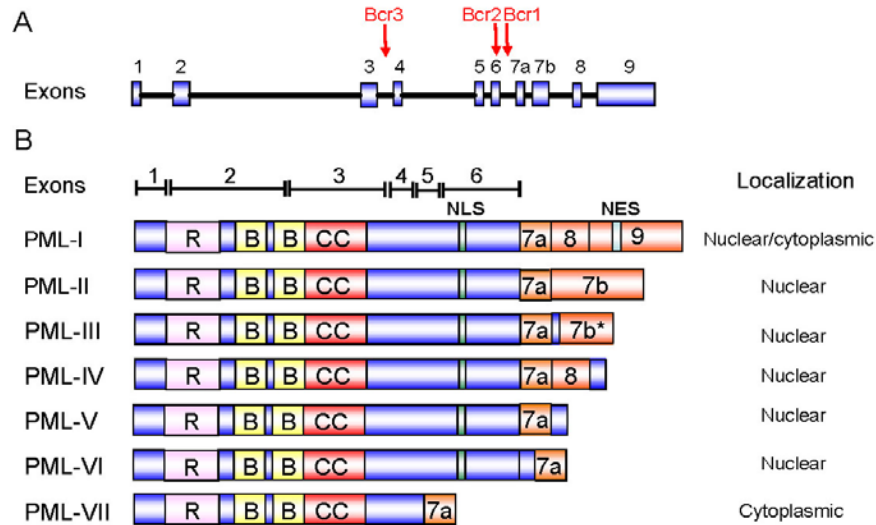
The promyelocytic leukemia gene (*PML*) encodes a protein which localizes to PML-nuclear bodies (NBs), sub-nuclear multi-protein structures, which have been implicated in diverse biological functions such as apoptosis, cell proliferation and senescence. However, the exact biochemical and molecular basis of PML function up until now has not been defined. Strikingly, over a decade ago, PML-NBs were found to be disrupted in acute promyelocytic leukemia (APL) in which *PML* is fused to the gene encoding retinoic acid receptor alpha (*RARA*) due to the *t*(15;17) chromosomal translocation, generating the PML-*RARA* chimeric protein. The treatment of APL patients with all-*trans*retinoic acid (ATRA) and arsenic trioxide which target the PML-*RARA* oncoprotein results in clinical remission, associated with blast cell differentiation and reformation of the PML NBs, thus linking NB integrity with disease status. This review focuses on the current theories for molecular and biochemical functions of the PML-NBs, which would imply a role in the pathogenesis of APL, whilst also discussing the intriguing possibility that their disruption may not be in itself a significant oncogenic event.

### 2. INTRODUCTION

*PML* was originally identified through characterization of the *t* (15;17) (q22;q21) chromosomal translocation, the diagnostic hallmark of acute promyelocytic leukemia, which results in its fusion to the gene encoding the retinoic acid receptor alpha (*RARA*) (1-6). Given that *RARA* was already known to function as a ligand-dependent (i.e. retinoic acid, RA) transcription factor involved in myelopoiesis, from an early stage the resultant PML-*RARA* fusion protein was perceived as a dominant negative inhibitor of retinoid signaling underlying the differentiation block that characterizes this subtype of acute myeloid leukemia (AML). A key question has been to establish the extent to which deregulation of PML-dependent pathways contribute to the process of leukemic transformation.

Although the exact biochemical functions of the PML protein remain to be elucidated, it has been implicated in a plethora of biological processes including tumor suppression, senescence and apoptosis (7). PML is ubiquitously expressed in mammalian cells, forming multi-protein aggregates termed PML nuclear bodies (PML-NBs)

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**Figure 1.** The *PML* gene and exons encoding the protein isoforms. A The *PML* gene is located on chromosome 15q22 and contains nine exons. The positions of the three translocation breakpoint cluster regions (Bcr1-3) within the *PML* gene are indicated in red. B Alternative splicing of the C terminal region results in the translation of different PML isoforms. All isoforms contain the first three exons, which encode the RBCC motif, a tripartite motif that contains a zinc-finger RING domain (R), two additional zinc finger motifs (B-boxes) and a coiled-coil domain (CC) (195). The RBCC domain promotes dimerization and the formation of the NB structures. The alternative splicing of the transcripts results in proteins with different protein-protein interaction surfaces and markedly different functional specificity, for example PML-IV binds to p53 inducing premature senescence and PML-III interacts with the centrosome. The cellular localization of the isoforms is governed by the presence or absence of the nuclear localization signal (NLS) and nuclear export signal (NES) encoded by exon 6 and 9, respectively. The majority of PML isoforms show a predominant nuclear localization, yet PML-I possesses both the NLS and NES and consequently can be expressed in both compartments dependent on the cellular context. Alternative splicing of exons 4, 5 and 6 has been reported leading to the production of a cytoplasmic isoform of PML which has been implicated in the regulation of TGF- $\beta$  signaling.

(8-11). Disruption of these structures has been observed in a variety of disease processes such as neurodegenerative disorders, viral infections and in the cells of patients with acute promyelocytic leukemia harboring the t (15;17) translocation, forming a micro-particulate pattern in the nucleus and cytoplasm (10-12). In the latter condition, PML-NBs are reformed after treatment with all-transretinoic acid (ATRA) or arsenic trioxide (ATO) correlating with achievement of clinical remission in patients, linking NB integrity with disease status (8, 9, 13-15). Nuclear bodies may serve as the sites of many critical metabolic activities, including transcription, DNA replication, pre-mRNA processing, ribonucleoprotein (RNP) assembly, protein modification, DNA condensation and fragmentation associated with apoptosis, cell cycle control, and ribosome production (16-27). The precise roles for the PML-NB in these biological processes remain to be established; however, remarkable progress has been made in understanding the individual constituents of the nuclear bodies and their potential biological functions. In this review we shall attempt to evaluate the role of PML-NB disruption in APL pathogenesis through a critical assessment of the possible functions of PML.

### 2.1 The *PML* gene

Understanding the function of PML has stimulated great interest since it was first discovered to be

involved in APL. The *PML* gene is located on chromosome 15 and consists of nine exons spread across a locus of 35Kb (Figure 1). Early predictions of the functional role of PML relied on the primary structure of the protein. PML is a member of the family of proteins harboring a tripartite structure that contains a cysteine rich zinc-finger called the RING motif (R), two additional zinc-finger motifs (B-boxes; B) and a coiled-coil domain (CC) (see review by Freemont and colleagues in this issue (28)). The RBCC motif is located at the N-terminus, promotes homo-multimerization and the formation of macromolecular complexes. The C-terminus of PML includes a nuclear localization signal (NLS) and an acidic serine-proline-rich region, which varies in length due to alternative splicing (29). The variability in the C-terminal regions is likely to be an important mechanism for generating the diverse PML-binding interfaces necessary to facilitate protein-protein interactions (30, 31).

### 2.2. PML Nuclear Bodies (PML-NBs)

One of the most striking features of PML is that it localizes to punctuate nuclear structures, previously known as nuclear domains-10, Kremer bodies and PML oncogenic domains, which are now simply referred to as PML-nuclear bodies (PML-NBs) (32). These are present in most mammalian cells typically numbering 1-30 bodies per nucleus, depending on the cell type, cell-cycle phase and

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**Table 1.** PML-body-associated proteins and their known or putative functions

Cellular Function	Activity	PML interacting Protein	PML isoform	Ref
Tumor Suppression	Transcriptional regulation	p53 <sup>1,2,3</sup>	PML IV	(116)
		CBP <sup>1,3</sup>	PML II	(178)
	DNA helicase	BLM <sup>1,2</sup>	?	(118)
	Initiation of translation	eIF-4 <sup>1,2</sup>	?	(26)
Apoptosis	Transcriptional regulation	Daxx <sup>1,3</sup>	PML VI	(19),
Post-translational modifications	Ubiquitination and SUMOylation	Ubc9 <sup>1</sup>	PML VI	(189)
		SUMO <sup>1</sup>	PML IV	(35)
Immune response	Transcriptional regulation	Sp100 <sup>1</sup>	?	(40)

Examples of the many diverse cellular functions attributed to PML-NBs and proteins implicated in those processes, which are found to localize within NBs or associate with PML directly. The listed proteins may either associate with a subset of PML-NBs or be distributed both throughout the nucleoplasm and within PML NBs. Due to the inherent problems associated with the detection of the interaction partner the methodology employed is listed for each component with <sup>1</sup> denoting endogenous, <sup>2</sup> GST pull down and <sup>3</sup> over expression.

differentiation state (33). NBs measure between 0.2 and 0.3  $\mu\text{m}$  and are associated with the nuclear matrix, which plays an important role in the trafficking of molecules and organization of chromatin within the nucleus. PML-NB formation requires the modification of PML by members of the SUMO (small ubiquitin-related modifier) family of ubiquitin-like proteins (34) (see review by Miller and colleagues this issue (35)). SUMO modification is not found to be essential for the formation of PML aggregates, yet is required for higher order NB formation and the recruitment of certain proteins to the PML-NB (33).

It has become apparent that PML-NBs are dynamic structures whose constituents can assemble to form NBs, or disassemble and disperse, or redistribute to other nuclear structures. It appears that specific proteins are likely to be recruited to NBs in response to a variety of physiological and pathological conditions, such as the cell cycle, disease state, viral infection, or external stimuli, through signaling cascades mediated by membrane-bound and nuclear receptors (9, 36-38). Different isoforms of PML (Figure 1), which are generated by alternative splicing, have been shown to associate differentially with certain proteins; therefore, altering the PML isoform content of NBs may not only affect the factors that they recruit, but also modulate their nuclear positioning and functional interaction with chromatin (30). The PML-NBs are comprised of stable and transient components: for example, PML and Sp100 are consistently found in NBs, but CBP is recruited to a subset of NBs only under certain conditions, such as exposure of cells to alpha-interferon (39, 40). The heterogeneity of the constituents found in NBs may not only influence their size and shape, but also their functional capabilities at any given time (41). It has yet to be determined if PML-NBs interact with other types of nuclear bodies leading to an integrated and organized nuclear response to specific stimuli. Although the discrete biological functions of either PML or PML bodies remain unknown, the dynamic nature of PML-NBs suggests that they may serve as a flexible protein-based scaffold to control protein interactions.

Most studies aimed at understanding the function of PML-NBs have primarily focused on the identification of proteins localized within these structures (Table 1). To date, 78 naturally occurring components of PML-NB have been identified, inferring a role for PML in numerous

biological functions (see the Nuclear Protein Database <http://npd.hgu.mrc.ac.uk/>). Elucidation of the natural components of PML NBs has been complicated by many reports of proteins that are detected in association with PML only after high-level exogenous expression. A possible explanation of this phenomenon is the role of PML NBs in the unfolded protein response and protein degradation pathways leading to the false identification of NB associated proteins. The results of these strategies and their inherent limitations should be taken into account when inferring PML-NB function. However, the diverse nature of the constituent proteins suggests that the NBs may not be a site of active cellular metabolism but rather serve as a storage site for nuclear proteins which require tight control of expression. Of the established PML NB associated proteins only a few are known to bind to the PML protein directly (42).

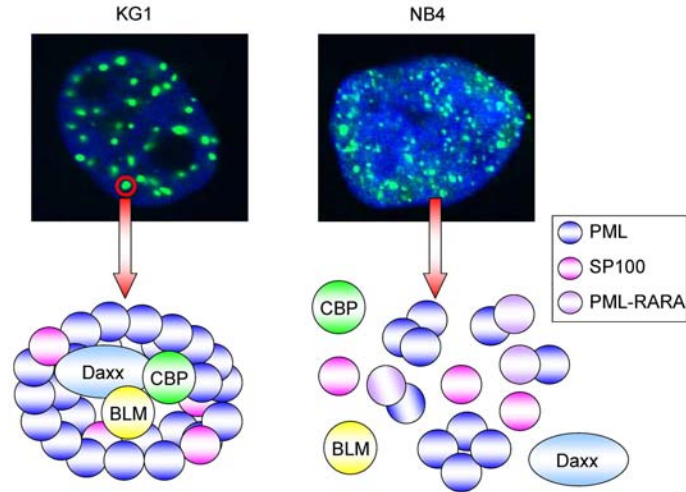
### 3. PML-NB DISRUPTION AND ACUTE PROMYELOCYTIC LEUKEMIA (APL)

#### 3.1. t (15;17) associated APL and PML-NB disruption

The t (15;17) most commonly disrupts the *PML* gene within intron 3 (bcr3) or intron 6 (bcr1), while approximately 5% of chromosome 15 breakpoints occur within exon 6 (bcr2) (3, 4, 43-45) (Figure 1). Breakpoints in the *RARA* locus occur within intron 2, such that all resulting PML-RARA isoforms retain the RBCC domain of PML and DNA-binding, co-repressor/co-activator, ligand-binding and retinoid-X-receptor (RXR)-binding domains of RARA (46).

In the presence of the PML-RARA fusion protein, nuclear architecture is disrupted with delocalization of PML from NBs into numerous (>100) micro-speckles (0.1  $\mu\text{m}$ ) (Figure 2) (10, 47, 48). The associated loss of PML-NB structure is thought to be due to hetero-dimerization of PML with PML-RARA through their respective coiled-coil domains (44, 49, 50). This motif has been shown to be essential for the biological activity of PML-RARA in blocking myeloid differentiation (51). The APL associated PML micro-speckles appear to have no formal structure and a number of studies demonstrated that a large proportion of the PML-RARA protein localizes within the cytoplasm. Therefore, the key functions of the PML-NB in tumor suppression, cell proliferation, differentiation and survival, which will be discussed later,

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**Figure 2.** PML-Nuclear Body Disruption in APL. Immunofluorescent staining of promyelocytic leukemia (PML) protein (green) over 4',6-diamidino-2-phenylindole (DAPI) staining of chromatin (blue) in the leukemic cell lines KG1 and NB4. KG1 shows the typical pattern of PML nuclear bodies (PML-NBs); whereas in the APL cell line NB4, in which PML-RARA is formed as a result of the t (15;17) chromosomal translocation, PML-NBs are disrupted associated with a microspeckled staining pattern. Below left is a schematic representation of a PML-NB with examples of the many diverse proteins found to localize to these structures or associate with PML directly. Below right is a schematic representation of the loss of PML-NB integrity due to the interaction between PML-RARA and PML in APL and the subsequent release of PML-NB constituents.

could be impaired in APL, potentially contributing to the proliferation and survival advantage of the leukemic clone. Addition of ATRA and arsenic trioxide (ATO) reverses this aberrant phenotype, resulting in the reformation of PML NBs, which correlates with remission in patients (7). ATRA treatment results in the degradation of PML-RARA through the action of the proteasome while leaving the wild-type proteins intact (47, 48, 52-54). Once PML-RARA is cleaved the resulting protein is predicted to be unable to bind wild-type PML, which is therefore free to form its usual macromolecular complexes i.e. the NB (47, 48, 52-54). ATO also results in the degradation of PML-RARA, facilitates reformation of PML NBs and induces apoptosis of APL cells in culture, relevant to its therapeutic activity (55, 56).

### 3.2 Alternative APL Fusions

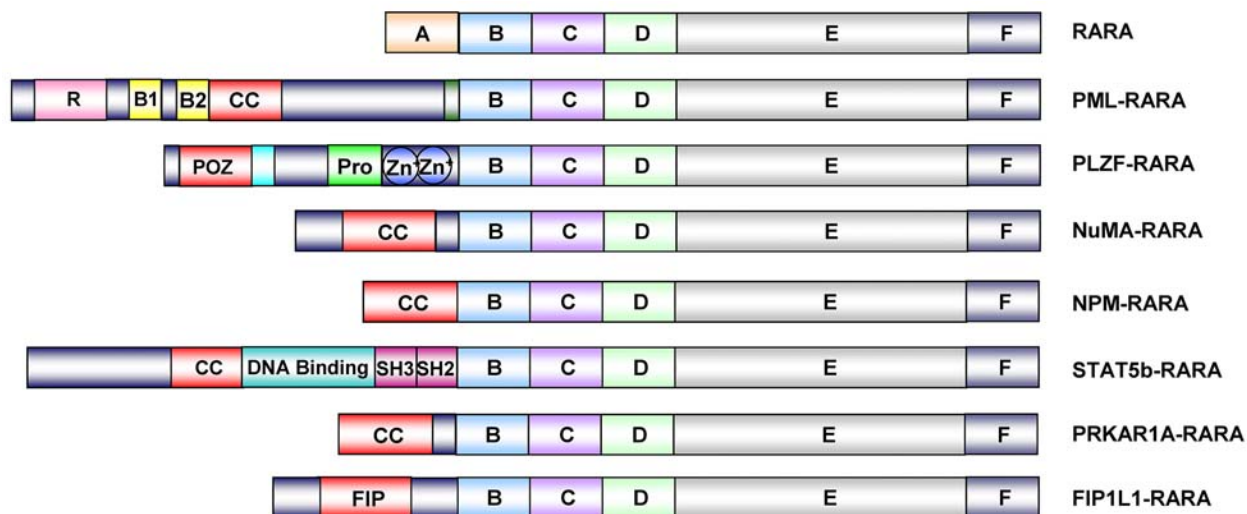
While the vast majority of APL cases (>98%) are due to an underlying *PML/RARA* rearrangement, rare cases have been identified in which *RARA* is fused to an alternative partner (designated "X"). These include the Promyelocytic Leukemia Zinc Finger (*PLZF*) and Nucleophosmin (*NPM1*) genes as a result of t (11;17) (q23;21) and t (5;17) (q35;q21), respectively (57-59). In addition, isolated cases have been identified involving the Nuclear Mitotic Apparatus Protein (NuMA), Signal Transducer and Activator of Transcription (Stat5b), cAMP-dependent protein kinase, regulatory subunit alpha 1 (PRKAR1A) and the *FIP1L1* gene (Figure 3) (57, 58, 60-64). In each of these alternative molecular subtypes of APL, in accordance with the t (15;17), *RARA* was found to be disrupted within intron 2 leading to retention of the whole of the *RARA* moiety, excluding the N-terminal ligand-independent transactivation domain (A region), within the X-RARA chimeric proteins. This common

feature provides strong evidence that deregulation of retinoid signaling is critical to the development of the APL phenotype. While the different molecular subtypes of APL share many common biological and clinical characteristics, the nature of the fusion partner has a critical bearing on the response to ATRA and ATO. APL blasts with PML-RARA, NPM-RARA, NuMA-RARA or FIP1L1-RARA differentiate in response to the former agent, while those harboring PLZF-RARA or STAT5b-RARA are relatively resistant (60, 65-68). The ATRA sensitivity of the PRKAR1A-RARA subtype remains unknown. Response to ATO has only been documented in PML-RARA+ APL, while cases with the PLZF-RARA fusion are known to be resistant to this agent (69-73).

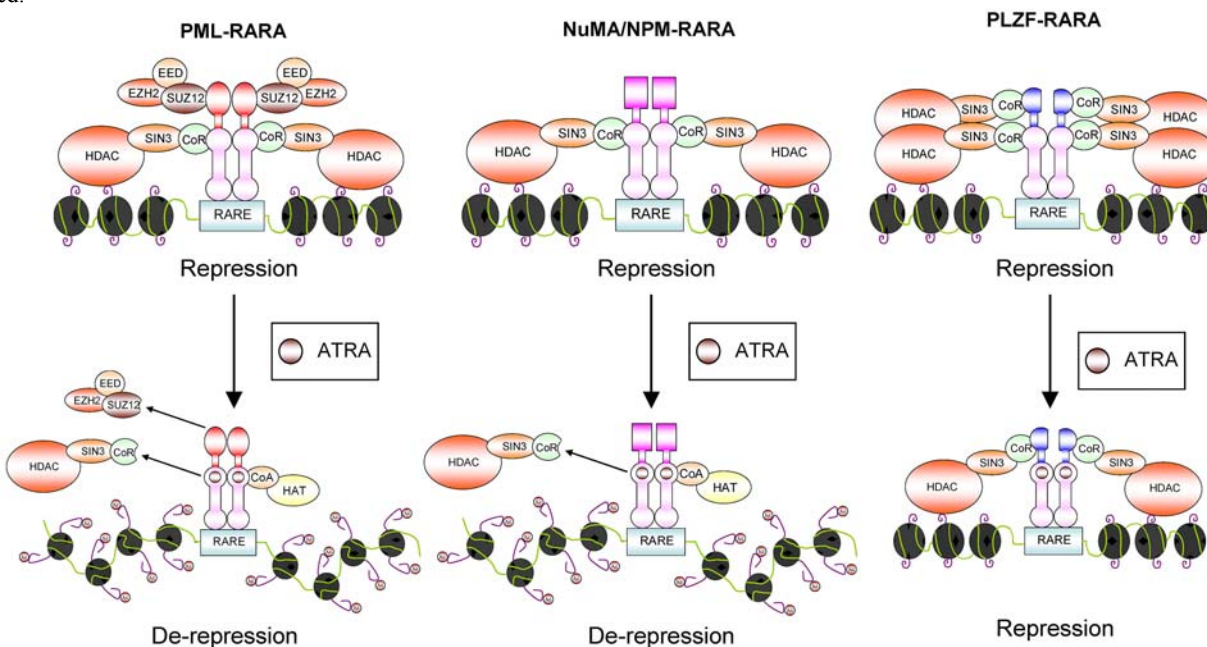
### 3.3 Homodimerization of the APL fusion proteins

The fusion partners of *RARA* (X-proteins) in APL are structurally diverse and exert very distinct biological functions, yet these leukemias can be recognized as similar clinical entities. This suggests that either the *RARA* fusion partner is irrelevant to the leukemic transformation process or the various fusion partners should share some common biological properties. To date all X-RARA variants have been shown to bind to consensus retinoic acid response elements (RARE) leading to transcriptional repression at physiological levels of RA (74-76). Interestingly, each of the respective X-proteins has been shown to be nuclear and normally function in homodimeric complexes. Under normal conditions, *RARA* requires to heterodimerize with RXR to bind to the RARE consensus sequences in DNA. Unlike *RARA*, the X-RARA fusion proteins overcome this dependence, homodimerize and bind to DNA, which is thought to be essential to oncogenic activation (Figure 4) (77). This homodimerization is mediated through the fusion partner,

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**Figure 3.** Schematic representation of RARA and its associated APL Fusion Proteins. Chromosomal rearrangements result in fusion proteins in which the B-F domain of RARA including the DNA binding and ligand binding domains are linked to amino-terminal regions of seven different nuclear proteins. All associated nuclear proteins contain a self-association domain shaded in red.



**Figure 4.** Dimerization of APL Fusion Proteins and Response to All-*trans* Retinoic Acid (ATRA). APL fusion proteins can efficiently dimerize and bind to retinoid response elements (RAREs), recruiting repressor complexes. Repression is released with pharmacological concentrations of retinoids in ATRA sensitive subtypes of APL (i.e. with PML-RARA, NPM1-RARA, NuMA-RARA and FIP1L1-RARA fusions), leading to recruitment of co-activator (CoA)-HAT complexes and gene expression. The PLZF-RARA fusion protein generated by the t(11;17)(q23;q21) recruits corepressor complexes including N-CoR through the amino-terminal POZ domain of the PLZF moiety in addition to the E-region of RARA; the former interaction is insensitive to ATRA contributing to the retinoid resistance that characterizes this subset of APL.

altering the transcriptional properties of RARA. Interestingly, homodimerization can both enhance the binding of pre-existing protein interactions, cofactors and downstream effectors of transcriptional target genes as well as leading to the creation of novel binding interfaces

between the protein and DNA (48, 75, 78-82). For example, while RARA/RXR heterodimers bind one molecule of the SMRT co-repressor, PML-RARA homodimers can bind two SMRT molecules (83). In addition, the fusion of PML to RARA results in an



allosteric change in the molecule, allowing binding to novel protein complexes involved in chromatin remodeling and retention of co-repressor complexes at physiological concentrations of RA. For example, the polycomb group (PcG) of proteins have been recently identified to associate with PML-RARA (84). PcG proteins were initially identified in *Drosophila* as epigenetic silencers of the homeotic genes (85). They have since been demonstrated to be required for X chromosome inactivation, germline development, stem cell renewal, hematopoiesis and cell proliferation (86-94). PcG-mediated histone modifications were found to contribute to gene silencing in APL, with the PML moiety of PML-RARA exhibiting stronger affinity for the PcG complexes than wild-type PML allowing and/or stabilizing the interaction between PML-RARA and PcG complexes at physiological concentrations of ATRA (84). However, the structural basis of the changes necessary to allow the recruitment of PcG complexes remains unknown.

Recently the role of RXR within the DNA-bound X-RARA oncogenic complex has been demonstrated through a variety of *ex vivo* and *in vivo* experiments. Whilst RXR is dispensable for immortalization of primary hematopoietic progenitors *ex vivo*, it is absolutely required for APL development in transgenic mice (82). The presence of RXR in the PML-RARA complex was found to not only facilitate DNA binding, but was also required for retinoid-induced differentiation, demonstrating that RXR is not a silent partner, but plays an active role in leukemic transformation (82). Interestingly, deletion mutant analysis of the STAT5-RARA fusion protein, demonstrated that only the STAT5-RARA constructs that conserve the capacity to bind DNA as hetero-oligomeric complexes with RXR retain the ability to transform primary murine hematopoietic progenitors *in vitro*. Moreover, shRNA-mediated silencing of RXR prevented immortalization of murine hematopoietic progenitors by STAT5-RARA, while inducing apoptosis in APL-derived PML-RARA expressing NB4 cells (95). These findings make a case for the relevance of RXR recruitment in the pathogenesis of APL and in the maintenance of the leukemic phenotype (96). However controversy still remains as to the role of the fusion partners to leukemogenesis, in particular PML.

### 3.4. Is forced dimerization of RARA and transcriptional repression sufficient for leukemogenesis?

One common feature of all X-RARA fusion proteins is the presence of a homodimerization/homooligomerization domain within the X-moiety resulting in aberrant recruitment of the transcriptional repression machinery to RAREs. Consequently, transcriptional repression at RAREs appears to represent a key event in APL pathogenesis. In order to dissect this property from other biological activities conferred by the respective RARA fusion partners, Sternsdorf *et al* fused the common RARA part to two different heterologous dimerization domains (97). Due to the lack of a *bona fide* APL fusion partner in the constructs it was possible to directly assess the contribution of RARA dimerization from other putative activities of the fusion protein in APL leukemogenesis. In the first construct, the dimerization domain of the NF $\kappa$ B p50 subunit was fused to RARA (p50-RARA) and in the

second, a constitutively dimerizing mutant of the FK506 binding protein (FKBP12) was employed (FKBP12F36M-RARA) (81). The dimerization of the latter mutant could be reversibly regulated by rapamycin. In these “fantasy” fusion proteins, the RARA region was identical to that found in the various naturally occurring X-RARA fusions. Expression of the fantasy fusion proteins did not lead to PML NB disruption, but was found to recapitulate the oncogenic properties of APL fusion proteins *in vitro* and this activity was dependent on the ability of the proteins to dimerize. Expression of p50-RARA and FKBP-RARA in myeloid progenitors of transgenic mice induced a myeloproliferative disorder; however, rates of transformation to acute leukemia were much lower than observed with PML-RARA (1.5-3% vs. 10-15%) (98). Therefore, these data would indicate that transcriptional repression of the RAREs through forced dimerization is not sufficient for oncogenic transformation and lends support for active involvement of the various fusion partners including PML (97, 99).

Collectively, evidence from the literature supports the hypothesis that aberrant recruitment of transcriptional repression complexes to RAREs represents a key event in the development of APL. Given the role of HDAC1 in this process it raises the question, as to whether aberrant HDAC-dependent transcriptional repression is necessary and sufficient for APL pathogenesis. Matsushita *et al* addressed this issue by generating a number of transgenic mice harboring the following RARA mutants: HDAC1-RARA expressing the full-length HDAC1 coding sequence fused to RARA, mHDAC1-RARA carrying a point mutation that abrogates HDAC1 enzymatic activity and  $\Delta$ RARA lacking the N-terminal A-domain of RARA (100). The latter deletion mimics one aspect of the various X-RARA fusion proteins, removing the region conferring ligand-independent transcriptional activation function. Initial experiments using luciferase assays revealed that HDAC1-RARA acted as a potent transcriptional repressor. However, HDAC1-dependent blockade of RARA function was neither sufficient to cause leukemia nor to block myeloid differentiation *in vivo* (100).

### 4. EVIDENCE SUPPORTING PML-NB DISRUPTION AS AN “ACTIVE PLAYER” IN LEUKEMOGENESIS

Given that forced dimerization of RARA and transcriptional repression of RAREs *per se* are not sufficient to initiate leukemogenesis, it could imply that deregulation of the normal function of PML may be a potent initiator of leukemic transformation. However the relative contribution of PML-NB disruption to this process remains undetermined. When attributing a role for PML-NB disruption in the pathogenesis of APL it is important to consider the fact that *PML* is not an essential gene, since *Pml*<sup>-/-</sup> mice are viable appearing to conflict with the accepted notion of PML as a tumor suppressor. However, it is not complete unexpected that *Pml* deficient mice are viable, as a common misconception when trying to understand the role of tumor suppressor genes is to suppose that their function is essential to survival. This point can be most easily rationalized by the finding that *p53*<sup>-/-</sup> mice are

viable, which was surprising given p53's role as the "guardian of the genome" (101). Like p53 null mice, *Pml*<sup>-/-</sup> mice are more susceptible to tumor formation when exposed to carcinogens and *Pml*<sup>-/-</sup> cells are less likely to undergo apoptosis under certain types of cellular stress (16, 17). However, unlike *p53*<sup>-/-</sup> mice, *Pml*<sup>-/-</sup> mice are not subject to early development of spontaneous tumors at rates higher than wild-type littermate controls (17, 101).

Interestingly the *PML* gene is not evolutionarily conserved among eukaryotes, being absent in *Drosophila melanogaster* (<http://www.fruitfly.org>), *Saccharomyces cerevisiae* (<http://genome-www.stanford.edu/Saccharomyces>), and *Arabidopsis thaliana*. In addition, although RING domain containing proteins can be found in *S. cerevisiae* and *A. thaliana* genomes, no RBCC motifs, nor combined RING/B-Box motifs are present (<http://smart.embl-heidelberg.edu>) (42). Moreover, in contrast to other nuclear organelles, there appear to be no PML bodies in *Xenopus laevis*. This apparent lack of PML bodies is intriguing, since these structures are thought to underlie basic cellular processes in mammals and their disruption apparently contributes to human disease. These features and their potential clues to PML-NB function in the pathogenesis of APL are discussed below.

### 4.1. PML-NBs, tumor suppression and cancer

*In vitro* experiments in cell lines of various histological origins have shown that PML can act as a potent growth suppressor (102, 103). Analysis of *Pml*<sup>-/-</sup> cells indicates that Pml may exert this activity in part through the regulation of cell cycle progression (17). Mouse embryonic fibroblasts (MEFs) derived from *Pml*<sup>-/-</sup> mice appear morphologically indistinguishable from those with *Pml*<sup>+/+</sup> and *Pml*<sup>+/-</sup> genotypes, however *Pml*<sup>-/-</sup> cells proliferate more rapidly, associated with an increase in the number in S-phase and a decrease in the G<sub>0</sub>/G<sub>1</sub> population (17). Furthermore loss of Pml is associated with increased capacity for MEFs to form colonies in soft agar. However, unlike fully transformed cells, they are unable to grow in semi-solid media. These findings demonstrate that PML can function as a negative regulator of cell proliferation (17).

In addition to its role in suppression of cell proliferation, *in vitro* studies show that PML can act as a tumor suppressor. Transfection of PML into NB4 APL cells suppresses their ability to form colonies in soft agar (104). Furthermore, when over expressed in HeLa cells, PML inhibits colony formation in agar and tumor growth in nude mice (105). Although the incidence of spontaneous tumors in the *Pml*<sup>-/-</sup> knock out mice was not found to be increased during the first year of life, they developed more carcinomas compared to the wild type controls when challenged with mutagens, namely dimethylbenzanthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (17).

The genetic lesions underlying the pathogenesis of leukemia have historically been regarded as distinct from those involved in solid tumors, as hematopoietic

malignancies are more often characterized by balanced chromosomal rearrangements. However, over recent years it has become apparent that the genes involved in these recurrent abnormalities are also expressed in non-hematopoietic tissues regulating key cellular processes such as proliferation and survival pathways. Given the involvement of PML in APL it has been tempting to investigate its role in solid tumors. Aberrant PML expression was first described by the Dejean group, who showed that PML was increased during inflammation and altered in tumor derived tissues suggesting that it may be involved in the pathogenesis of malignancies other than APL (9). In addition Gurrieri *et al.*, reported that the PML protein product is frequently lost in human cancers (106). In this study, loss of PML protein was found to be associated with disease progression in prostate, breast and central nervous system tumors. Interestingly, although PML was frequently lost in advanced cancers, it was also found to be down-regulated in the early stages of tumorigenesis (106). These observations raise the question of whether PML loss is an important event in tumor initiation and/or progression. In these cases, sequence analysis of the *PML* gene and promoter methylation studies revealed no inactivating mutations or aberrant methylation while mRNA transcripts were consistently detected, suggesting that the PML protein is aberrantly translated and/or degraded in human cancer (104, 106). Casein kinase 2 (CK2), a protein frequently up-regulated in human tumors, has been implicated in the loss of PML. *In vitro* studies demonstrated that CK2 regulated PML protein levels by promoting its ubiquitin-mediated degradation and importantly, found an inverse correlation between CK2 kinase activity and PML protein levels in human lung cancer-derived cell lines and primary specimens (107). Further studies are required to ascertain the survival advantages that the loss of PML protein would confer to non-hematopoietic tissues in order to fully characterize the role of PML in solid malignancies.

Of particular interest, neuroblastoma, a solid tumor of embryonal neural crest origin, is one of the most common pediatric malignancies, and like APL responds at least in part to differentiation therapy with RA (108). N- and I- type neuroblastoma cells *in vitro* and undifferentiated neuroblastomas *in vivo* have been observed to lack PML-NBs, but interestingly, as with APL cells, differentiation was accompanied by restoration of the PML-NBs (109). In addition, transient transfection of PML into neuroblastoma cells enhanced the responsiveness of these cells to RA (109). While in patients with advanced neuroblastoma who achieved remission, treatment of minimal residual disease with RA was found to reduce the risk of disease recurrence (108).

### 4.2. Further evidence supporting a role for the PML protein in the pathogenesis of APL

It has been suggested that the oncogenic activity of PML-RARA rests in its ability to act as a double dominant-negative protein, inhibiting the normal activity of both RARA and PML (32). As discussed previously, "fantasy APL fusion proteins" engineered to induce dimerization of RARA were limited in their capacity to

induce leukemia *in vivo*, implying a role for *bona fide* fusion partners such as PML beyond mere provision of a dimerization interface (97). To investigate this issue further, Sternsdorf *et al* crossed line p50-RARA 1182 with *Pml* null mice. It was hypothesized that lack of Pml might mimic the disruption of NBs mediated by the PML-RARA oncoprotein and cooperate with p50-RARA; however no increase in the rate of leukemia development or reduction in disease latency was observed in the absence of Pml (97). This suggested that the PML-RARA fusion does not simply block PML and RARA function, but rather represents a gain-of-function mutation, with deregulation of wild type PML contributing to the process of leukemic transformation, possibly by altering the regulation of apoptosis and senescence.

Interestingly, a recent study investigating the mechanism of ATRA resistance in a cohort of APL patients identified two PML mismatch mutations (1272delAG) and (IVS3-1G-A) in the remaining normal allele, which were found to be associated with aggressive disease (110). Both mutations generated a premature stop codon upstream of the NLS, resulting in the translation of a truncated protein which accumulates in the cytoplasm, thus implicating cytoplasmic PML in the pathogenesis of APL. Bellodi *et al* went on to discover that these cytoplasmic PML mutants form novel cytoplasmic structures (PML-CB), which closely resembled the PML-NB in electron microscopy analyses (111). Interestingly, a number of PML-NB components such as CBP were found to re-localize to the PML-CB in the PML mutant expressing cells, whereas Daxx and SP100 did not, therefore implying cytoplasmic sequestering of protein may alter cellular processing leading to poor prognosis (111). Moreover, since PML-RARA can be detected in the cytoplasm of APL cells, this represents a further compartment in which PML function could be deregulated through interference with its interaction partners and altered localization (112).

### 4.3. How does PML-NB disruption affect their constituent proteins and what are the possible downstream consequences?

Several models have been proposed to account for the many possible functions of the PML-NBs. Firstly, the structural model suggested that PML-NBs' main function is to recruit and act as storage areas for nuclear proteins (113, 114), modulating biochemical processes by adjusting the levels of the components in the nucleoplasm and providing catalytic surfaces for the assembly of protein-protein interactions (113). However, to date, only limited evidence has emerged demonstrating the impact of protein sequestration in these bodies and this structural model, can not account for all the properties assigned to PML, as the induction of senescence and modulation of TGF- $\beta$  signaling appear to be independent of PML localization to NBs (discussed later in this review) (115).

There are several potential models hypothesized for the oncogenic potency of PML-NB disruption in leukemogenesis. A number of studies have shown that PML is involved in regulation of apoptosis (16, 19, 116) and senescence (23). Disruption of these postulated PML

activities could very well establish a pre-malignant state that facilitates the acquisition or tolerance of additional genetic changes (117). Second, recruitment of PML-associated factors to the PML-RARA/RXR complex and its target genes may have an important impact on gene expression. Such PML-RARA mediated alterations in the transcription of genes that regulate apoptosis, proliferation, or differentiation may also underlie the ability of PML-RARA to initiate leukemia (118, 119). Another possibility is that the PML domain confers a specific conformational architecture to RARA DNA binding complexes, for instance by the formation of higher-order multimers as proposed previously (78). Therefore, the PML component of the PML-RARA oncoprotein could feasibly contribute to the pathogenesis of APL, perhaps through more than one molecular mechanism. Given the fact that the exact role of PML-NBs remains unclear, it is important to consider how their disruption may affect a variety of biological pathways also pertinent to the development of APL.

#### 4.3.1. Myeloid Differentiation

Within the hematopoietic compartment, PML is highly expressed in cells of the myeloid lineage, whilst minimal or no expression is detected in mature circulating granulocytes and monocytes (120). Analysis of the peripheral blood from *Pml*<sup>-/-</sup> mice revealed a marked reduction in granulocytes, with an overall decrease in circulating myeloid cells (17). In the presence of RA, the number of myeloid colonies obtained from *Pml*<sup>+/+</sup> progenitors was increased as expected, but this effect was completely abrogated in *Pml*<sup>-/-</sup> cells. Thus, the presence of PML is crucial for the growth-inhibitory activity of RA, as well as for RA induction of myeloid differentiation. The mechanism by which PML is involved in myeloid differentiation remains to be defined, but interestingly, CCAAT/enhancer-binding protein  $\epsilon$  (C/EBP $\epsilon$ ) expression was found to be reduced in BM mononuclear cells derived from *Pml*-deficient mice (121).

C/EBP $\epsilon$  is expressed exclusively in granuloid cells and is essential for their terminal differentiation (reviewed in (122)). Granulopoiesis is a tightly regulated developmental process that begins with the commitment of myeloid precursor cells followed by their terminal differentiation, a process requiring the co-operative expression of lineage-specific transcription factors. In normal myeloid development, PML and in particular PML-NBs were found to co-operate with PU.1 to regulate C/EBP $\epsilon$  expression (121). PU.1 has been termed a master regulator of myeloid genes and directly activates the transcription of the C/EBP $\epsilon$  gene (123). Tight regulation of PU.1 is critical to normal myelopoiesis and its deregulation has been shown to predispose to leukemic transformation (124, 125). The relevance of the PML-NB body in PU.1 regulation has been investigated. PML was found to indirectly regulate PU.1 target genes, as PML isoforms IV and VI were found to co-operate to target PU.1 to PML-NBs, leading to the formation of a PU.1/PML/p300 ternary complex, resulting in an increase in C/EBP expression (121). *In vivo* sumoylation of PML is essential for the higher order architecture of NBs and in addition appears to be crucial for the formation of the transcriptional regulation



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complex PU.1/PML/p300. Furthermore, the two B box domains and the coiled-coil motif of PML which are necessary for NB formation were also found to be required for the interaction between PU.1 and PML isoform IV (121). These data suggest that structurally intact PML NBs may be required for the formation of PU.1/PML/p300 complex. Therefore PML-RARA may exert dominant negative effects on myeloid differentiation not just through the RA signaling pathway but by also disrupting the PML induced transcriptional activation of C/EBP<sub>E</sub> through disruption of the PU.1/PML/p300 complex.

### 4.3.2. RNA Processing and translation

PML disruption may lead to alterations in the global translation rate as PML-NBs are adjacent to known sites of RNA processing including splicing speckles, cleavage speckles and Cajal bodies. Furthermore, under normal conditions PML interacts with and negatively regulates the functions of the eukaryotic translation initiation factor eIF4E (25) (see review by Culjkovic and Borden in this issue (126)). eIF4E functions as a rate limiting step in cap-dependent protein synthesis, by facilitating the recruitment of a RNA helicase allowing binding of ribosomes (127). A substantial fraction of eIF4E (33-68%) is localized to eIF4E nuclear bodies yet PML-NBs may regulate its cellular availability, as eIF4E is a constituent component of PML-NBs (25, 128, 129). In contrast to PML, eIF4E expression is necessary for cellular growth and in excess, rather than altering global translation, selectively increases the translation and/or mRNA export of transcripts associated with cell proliferation, including vascular endothelial growth factor, and cyclin D1 (127, 130). EIF4E seems to selectively up-regulate protein translation by facilitating the transport of certain mRNA transcripts from the nucleus to the cytoplasm (127). PML protein negatively regulates the eIF4E dependent nuclear export of cyclin D1 (25). As a result, disruption of PML-NB would lead to increased levels of free eIF4E and subsequently the abrogation of PML-induced down-regulation of cyclin D1 protein synthesis (25). Of note unlike PML-NBs, eIF4E nuclear bodies are conserved in *S. cerevisiae*, *D. melanogaster* and *X. laevis*, implying that PML-NBs may serve as an additional level of translation control acting as a mammalian regulator of the evolutionarily older, eIF4E (25, 36, 127, 130).

### 4.3.3. Intracellular proteolysis

The conjugation of ubiquitin to proteins is a well-established modification for tagging proteins for degradation by the 26S proteasome (131). Proteasomes are large multisubunit proteases that are found both in the cytoplasm and the nuclei of eukaryotic cells that recognize, unfold and digest protein substrates that have been marked for degradation by ubiquitination. It had long been assumed that protein degradation predominantly occurred in lysosomes, but analysis of cellular proteolysis with proteasome inhibitors indicated that 80-90% of protein breakdown occurred through proteasome dependent means in most cultured cells under optimal growth conditions (132, 133). However, current knowledge of the processes governing nuclear proteolysis is very rudimentary. Initially the PML RING finger motif was proposed to act as an E3

ubiquitin ligase and consequently enable the protein to participate in protein degradation however, there is currently no evidence to support this hypothesis. (134). The association of PML-NBs with the ubiquitination/deubiquitination process has however been demonstrated through the presence of the ubiquitin-dependent hydrolase, HAUSP (135). HAUSP removes conjugated ubiquitin indicating PML-NBs may represent an intermediate reservoir for ubiquitinated proteins targeted for degradation.

The 11S complex could play a role in the recruitment and degradation of NB-associated proteins and this may account in part for their number and variety. However the exact role of the 11S proteasome complex remains poorly understood. Proteasomal degradation at PML-NBs may serve to end active gene expression by dissolving functional protein complexes and regulate the delivery of nuclear proteins for assembly of active protein complexes through the degradation of excess protein. Like PML, the 11S proteasome complex was found to be delocalized in the PML-RARA+ APL cell line NB4 (136). Moreover, RA treatment of NB4, which is known to lead to reformation of the PML-NBs, resulted in the relocalization of the 11S complex. Therefore, one consequence of the loss of PML-NB structure could be the deregulation of nuclear proteolysis (136). Interestingly, the 11S complex is involved in antigen processing and many cancer cells are not recognized by the immune system, possibly because of failure of normal antigen processing or presentation.

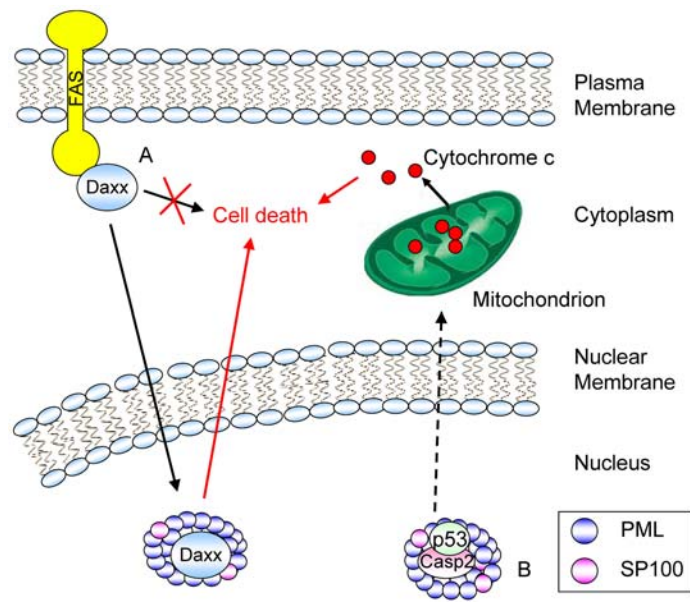
### 4.3.4. Post-translational modifications of proteins

To date, one of the most plausible hypotheses for the biological functions of PML is that it could serve as an E3 ligase for sumoylation (7). The PML protein itself is efficiently sumoylated, and several of its properties, including binding to MDM2, degradation by ATO and the transformation capacity of PML-RARA are dependent upon integrity of particular sumoylation sites (137, 138). Moreover, many proteins which accumulate in PML-NBs can themselves undergo sumoylation, and their recruitment requires the same critical sumoylation site in PML. Intriguingly, the CK2 phosphorylation site controlling PML degradation is located within a recently identified SUMO-interacting motif (SIM), a short sequence that interacts noncovalently with different SUMO isoforms (107, 139). *In situ* sumoylation assays, observed that PML-NBs, together with the nuclear rim and the nucleolus, are the main sites for active sumoylation in the cell (140). However the evidence supporting PML as a SUMO E3 ligase is based predominantly on analyses undertaken in yeast, as obtaining confirmatory data from mammalian cells has proven difficult (33, 141). As a result, further investigation of PML sumoylation could bring important insights into PML function.

### 4.3.5. Apoptosis

Apoptosis is a genetically controlled process for the elimination of the cell in which it occurs. The importance of PML for the regulation of apoptosis in response to cellular stress or DNA damage was demonstrated in studies conducted in *Pml*<sup>-/-</sup> mice. These

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**Figure 5.** Possible models for the involvement of PML in apoptosis. (A) Daxx interacts with the FAS receptor in the plasma membrane potentiating FAS mediated apoptosis. Translocation to the nucleus triggers apoptosis in cooperation with the PML protein inside the PML-NB bodies leading to cell death (B) Caspase-2 is recruited to the PML-NBs by SP100 via its CARD domain and it is then self-activated or activated by additional unknown signals. At this point, it could trigger apoptosis by functional coordination with p53 as an upstream regulator of caspase activity leading to loss of mitochondrial membrane potential. The mitochondrion is represented in green and cytochrome c is shown as red dots.

were found to be resistant to doses of gamma irradiation that were lethal in the wildtype controls (116). Furthermore, cells derived from *Pml*<sup>-/-</sup> mice were found to be defective in apoptosis induced by tumor necrosis factor (TNF), Fas, interferons and ceramide, suggesting a broad involvement in the death pathway and that PML may be a pro-apoptotic protein (16). In keeping with the dominant negative role of PML-RARA on the functions of PML, myeloid progenitors from PML-RARA transgenic mice were also found to be resistant to these apoptotic stimuli (16). As apoptosis induction was found to be reduced, but not abrogated, this implied PML was a modulator rather than an essential trigger for apoptosis. In addition, as described previously, studies on immunostained human carcinomas demonstrated a correlation between loss of PML expression and tumour invasion, consistent with loss of apoptotic control (106).

To date, the most relevant candidate to transduce the FAS/TNF signal from the plasma membrane to the PML-NB, is the death-domain-associated protein (Daxx) which could be associated with both nuclear and cytoplasmic events during apoptosis (Figure 5) (see review by Ishov and colleagues in this issue). Daxx was originally identified as a protein that specifically binds to the death domain of the trans-membrane death receptor FAS (also called CD95) in the cytoplasm and potentiates FAS-induced apoptosis (142). PML has been shown to be required for both FAS-induced cell death and Daxx pro-apoptotic function leading to the proposal that the two proteins cooperate in a nuclear pathway for apoptosis (143, 144). In *Pml*<sup>-/-</sup> cells, Daxx is delocalized and accumulates

in chromatin-dense nuclear regions; moreover, the ability of Daxx to trigger apoptosis and potentiate the FAS pro-apoptotic signal is markedly impaired (144, 145). Daxx represses basal transcription and its sequestration in the PML-NB blocks this activity. Conversely, expression of PML-RARA results in the delocalization of Daxx from the PML-NB, in turn enhancing Daxx anti-apoptotic functions through transcriptional repression (146). Indeed, Daxx is known to repress several transcription factors, including Pax3, ETS1, E2F1, p53 and p73 (18, 144-146). Moreover, Daxx was reported to interact with several crucial proteins involved in transcriptional silencing, namely histone deacetylase II, core histones and the chromatin-associated protein DEK, providing a potential mechanism by which Daxx can repress transcription (147). Treatment of PML-RARA expressing APL cells with RA leads to relocation of Daxx within the reformed, PML-NBs, suggesting that the sub-cellular localization of Daxx dictates its role in apoptosis (144). Moreover, elegant experiments have demonstrated that mutation of K160 of the PML moiety, which corresponds to the major site of sumoylation, abrogates the ability of PML-RARA to initiate leukemia *in vivo* by affecting the recruitment of the Daxx repressor (138, 146). Finally, it has been recently demonstrated that PML contributes to Fas-induced apoptosis by an alternative mechanism. The protein FLICE-associated huge protein (FLASH), which functions as a positive regulator of Fas-induced apoptosis was also found to localize to the PML-NBs under steady-state conditions (148, 149). In response to Fas activation, FLASH is released from PML-NBs and accumulates in mitochondria where it promotes the activation of caspase-8 (148).

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The recently described co-localization of caspase-2 within the PML-NBs implies that these structures may also be involved in the caspase-dependent apoptotic pathway (Figure 5). Sequence analysis of SP100, a resident protein of the PML-NBs, identified a potential caspase recruitment domain (CARD) in its N-terminal region (150). However the tight regulation of apoptosis suggests that additional signals would be required to activate pro-caspase-2 in the nucleus; otherwise, the existence of an apoptosis mechanism that is constitutively activated by a simple recruitment process would be deleterious to the cell's survival. If caspase-2 is assembled into the PML-NB machinery, where SP100 is also interacting with additional proteins of the complex (i.e. p53), it could facilitate further apoptotic activities as a result of self-activation via dimerization. Subsequently, active caspase-2 might mediate MOMP and caspase-3 activation, resulting in possible mitochondrial permeability changes that would lead to apoptosis. In addition, Quignon *et al.* demonstrated that PML expression could trigger caspase-independent death in the absence of *de novo* transcription (19). Several cell death inducers, including BAX and MYC expression, ceramides and inhibition of the ubiquitin degradation pathway have been shown to induce apoptosis in the presence of broad range caspase inhibitors, suggesting that caspase-independent executors of cell death exist (152, 153).

### 4.3.6. Genome stability

Given the observation that *Pml*<sup>-/-</sup> mice are more susceptible to cancer when exposed to potent carcinogens, it could be envisaged that one of the mechanisms by which loss of PML could contribute to oncogenesis is by favoring the accumulation of further genetic insults (17). Several proteins involved in the maintenance of genetic stability are known to accumulate in the PML-NB, such as BLM, p95, MRE11 and the BLM-interacting protein topoisomerase III alpha (117, 154, 155). PML-NB disruption in APL could affect their function by loss of spatial regulation. In support of this hypothesis, while PML-NB morphology is normal in BLM-deficient cells, BLM does not form NBs in *Pml*<sup>-/-</sup> cells resulting in diffuse nuclear staining, as also observed in PML-RARA+ APL blasts (117). Moreover, the frequency of sister chromatid exchange, a hallmark of whole genome instability and a distinct feature of Bloom cells, is also increased in *Pml*<sup>-/-</sup> MEFs (117). These data strongly indicate that the localization of BLM to the PML-NBs is required for normal function however, the exact role of these interactions remains to be elucidated.

In most human tumors, telomere maintenance is essential for the unlimited proliferative potential of human cells, and hence immortalization. Telomerase, the enzyme responsible for maintaining telomeric DNA, prevents both telomere erosion and senescence, is active in cancer cells resulting in stabilization of telomeric arrays and presumably unlimited cell division potential (156). However, a number of tumors, tumor-derived cell lines and *in vitro* immortalized cell lines have been described that do not possess detectable telomerase activity (157, 158). In around 5% of telomerase-negative cells, the telomere-binding proteins hTRF1 and hTRF2 co-localize with PML-NBs and *in situ* hybridization studies indicate that telomeric

DNA repeats are present in these bodies (159). This appears to be the only time that DNA and PML bodies associate. In addition, the proteins Replication factor A and Rad52 have also been found to localize to these sites, indicating that these cells have developed a mechanism of telomere length maintenance in the absence of telomerase activity, now referred to as alternative lengthening of telomeres (ALT). Telomere length in these cell lines was highly heterogeneous, with repeats ranging in size from >20 kb to <5 kb. The occurrence of ALT is not correlated with the method of immortalization nor with mutations in any known oncogenes or tumor suppressor genes (160). The molecular mechanism for telomere maintenance in the absence of telomerase remains speculative but based on evidence from telomerase-null strains of the yeast *S. cerevisiae* it has been suggested that recombination between telomeric repeats is the underlying basis for telomere stabilization (161, 162). In contrast, in non-ALT cells (which can be either telomerase negative or telomerase positive), the telomere-binding proteins, Rad52, and replication factor A exist in discrete sub-nuclear domains which do not overlap with nor have any apparent spatial relationship to PML NBs. Nevertheless, PML-NBs are involved in the maintenance of genome stability through a variety of pathways, which could be subject to deregulation in APL.

### 4.3.7. Gene transcription

PML-NBs are positionally stable over long periods during interphase. Following stress, they are conserved in size and position after a cycle of disruption and re-formation (36), indicating that their location and size are non-random and might be dictated by chromatin, thereby restricting their mobility. Electron microscopy studies have revealed that the PML-NBs make extensive contacts with chromatin fibres through protein-based threads which extend from the core of the bodies. These contacts maintain the integrity and positional stability of PML-NBs in the nucleus; however, what determines their association with particular gene loci remains unclear (163). Evidence suggests a role for PML-NBs in transcriptional regulation, as immunofluorescence microscope studies have revealed their accumulation at sites of high transcriptional activity. In particular, PML-NBs lie near highly acetylated chromatin and many transcription factors and transcriptional regulators dynamically localize to PML-NBs. In addition, nascent RNA has been detected in the immediate vicinity of PML-NBs, especially in the G1 phase of the cell cycle (164, 165). To date, most of the data supporting the association of specific loci with PML-NBs rely on the interpretation of observations made using immunofluorescence. The lack of sufficient spatial resolution of the fluorescence microscope makes it difficult to define a non-random association. Finally, it is not clear whether PML-NBs are homogeneous, and to what extent biochemical heterogeneity would affect their function. However, further investigation is required to shed light on the possible function of PML-NBs in regulating gene activity. It will be interesting to determine whether the chromatin environment in the vicinity of the PMLNB dictates its biochemical composition and structure or *vice versa*.

These data do not provide a direct functional link between transcribing chromatin and PML-NBs *per se*, as they may be fortuitously located in regions of the nucleus where transcription occurs. However recent evidence does suggest that PML-NBs may control transcriptional activities indirectly by participating in chromatin-remodelling processes and establishing chromatin domains that are permissive for transcription. For example, in a study focusing on the MHC locus, it was recently suggested that PML-NBs regulate transcription by organizing the genes contained within the locus into distinct, high-order chromatin-loop structures that are more or less permissive to transcriptional activity (166). Moreover, a number of transcription factors are associated with PML-NBs further implying that NBs may act as transcriptional regulators. It has been postulated that PML-NBs influence gene expression by sequestering transcription factors away from their cognate gene sequences in the soluble nuclear fraction (167). For example, the transcription factors, Sp1, Fos, TIF alpha, PR, RARA and RXRA display a predominantly diffuse nuclear or microspeckled staining pattern (44, 168). However, when PML is over expressed, progesterone receptors and TIF alpha are sequestered to the PML-NB through their indirect interaction with PML (167-169). As a result it is possible that the PML-NBs are regulating transcription factors and co-factors by transiently recruiting them, indicating that disruption of these structures in APL could alter transcription through a number of possible mechanisms, even though transcription takes place outside the NBs themselves (167). However, it should be noted that PML-NBs have also been associated with transcriptional repression (7). Not only do some PML-NBs colocalize with transcriptional co-repressors and heterochromatin-bound proteins such as heterochromatin protein-1 (HP1), but they can also be found near centromeric regions, although only during the G2 phase of the cell cycle (167, 170-173).

## **5. PML-NB DISRUPTION MAY BE AN “INNOCENT BYSTANDER” EFFECT IN APL**

When attributing a role for PML-NB disruption in the pathogenesis of APL it is important to remember that the exact biological functions of PML have not been determined and crucially PML nuclear bodies appear to remain intact in the molecular subtypes of APL in which RARA is fused to other partners.

### **5.1. Lack of PML-NB disruption in the alternative APL fusions**

Immunofluorescence studies undertaken in primary cells from APL cases encompassing a range of alternative fusion partners including those expressing PLZF-RARA, NPM-RARA, and NUMA-RARA, have shown that PML exhibits a wild-type speckled staining pattern consistent with integrity of the nuclear bodies (75, 174-177). Furthermore, pull-down experiments have failed to indicate a direct interaction between NPM-RARA and PML (178). Any relationship between PML and PLZF remains controversial; similar to PML, PLZF is located in

nuclear structures that appear by immunofluorescence as punctuate 'dots' (176). PML and PLZF were found to colocalize in normal CD34<sup>+</sup> hematopoietic progenitors and in KG1 cells, while in the PLZF-negative U937 cell line transfected with PLZF, PML and PLZF were found to localize in adjacent but functionally distinct NBs (176). Such studies have not been reported as yet with the STAT5b-RARA fusion, but experiments to date with the other APL variants indicate that de-localization of PML from nuclear bodies is not necessary for maturation blockade. This poses the question whether there is some overlap in functionality between the other APL-associated fusion proteins and the PML-NBs, and whether PML-NB disruption is dispensable due to shared properties between PML and the other partners of RARA.

### **5.2. PML-NB formation is not required for PML induced premature senescence**

Eukaryotes have evolved at least two different mechanisms to combat proliferation of cells which are on the brink of oncogenic transformation; i) apoptosis (as discussed earlier in this review) and ii) cellular senescence (see review by Ferbeyre and colleagues in this issue). Cellular senescence was first recognized in 1961 by Hayflick, as a process that prevents normal fibroblasts from growing indefinitely in culture (179). Ectopic expression of PML has been shown to promote premature senescence in primary human and murine fibroblasts (23), with evidence to suggest that this is mediated by PML isoform IV (115). However, this isoform failed to induce growth arrest in *Pml*<sup>-/-</sup> cells, suggesting that it may be necessary but not sufficient to elicit the senescent phenotype (115).

Until recently, PML-NBs were regarded as the organizing centers, where such processes as senescence were executed or regulated (23, 115). However recent evidence has questioned this hypothesis. Bischof *et al* found that all seven isoforms of PML efficiently recruited endogenous p53 as well as CBP to the NBs in human fibroblasts and *Pml*<sup>+/+</sup> MEFs (115). However, their recruitment was not sufficient for establishing a premature senescence phenotype. Enforced localization of p53 to NBs via a fusion protein consisting of the SP100 and the p53-binding domain of HDM2 resulted in no noticeable effect on growth. Furthermore, in *PML*<sup>-/-</sup> cells, endogenous p53 remained diffuse upon ectopic expression of the different PML isoforms, whereas CBP relocalized to the PML-NBs. This implied that additional factors, such as multiple isoforms of PML, are required for proficient recruitment of p53 to PML-NBs (115). PML-NB disruption was not found to alter the outcome of premature senescence, as neither lack of sumoylation nor complete NB disruption by the virally expressed protein IE1 influenced PML IV-induced senescence. This suggests that functional pro-senescent PML/p53/CBP complexes are assembled and act outside of PML-NBs (121). Together these data suggest that functionally active PML may exist outside the NB, thus challenging the current dogma of PML-NBs being the major sites of PML function.

### 5.3. Lack of a major impact of PML-NB disruption on gene expression profiles

Given the postulated involvement of the PML-NB in the regulation of free transcription factors in the nucleus as discussed previously, one would expect PML-NB disruption to lead to a significant change in gene expression profile in APL cells. However, expression profiling of promyelocytes harboring PML-RARA from pre-leukemic mice identified less than 2% of genes with significant changes in expression when compared with normal promyelocytes (180). Importantly, expression of the PML-RARA fusion associated with disruption of the PML-NBs was confirmed in these cells. Only after a latent period of 3–9 months, when overt leukemia developed, was a major change in gene expression noted (119). Due to the changes in expression of relatively few genes, conceivably the disruption of PML-NBs has little effect on global transcription. This would imply that it is not the disruption of PML-NBs which is important for the altered gene transcription, but rather it is a gain of function provided by the PML-RARA fusion which is critical for the initiation of the pre-leukemic phenotype. Recently chromatin immunoprecipitation (ChIP)-chip experiments identified 372 direct genomic PML-RARA targets. A subset of these was confirmed in primary APL samples (118). Direct PML-RARA targets were found to include regulators of global transcriptional programs as well as critical regulatory genes for basic cellular functions such as cell-cycle control and apoptosis (118).

Given the similar clinical phenotypes of X-RARA induced APLs one would expect that the different fusion proteins may deregulate similar pathways. Following expression of different APL fusion proteins in the U937 cell line, 57 genes were commonly down-regulated by both PML-RARA and PLZF-RARA, whereas 37 and 31 genes were down-regulated by either PML-RARA or PLZF-RARA alone, respectively (181). In addition, 16 genes were found to be commonly up-regulated by both fusion proteins, whereas 16 and 37 genes were up-regulated by only PML-RARA or PLZF-RARA, respectively (181). These subtle changes in genes involved in self-renewal, DNA repair and apoptosis might be required to initiate the leukemic process, rendering the target progenitor vulnerable to the acquisition of additional mutations underlying progression to frank leukemia (119). However, it is not yet clear which target genes are most important for the disease phenotype and direct comparison of all the X-RARA variants should be performed which may potentially establish common biological pathways that underlie the pathogenesis of APL.

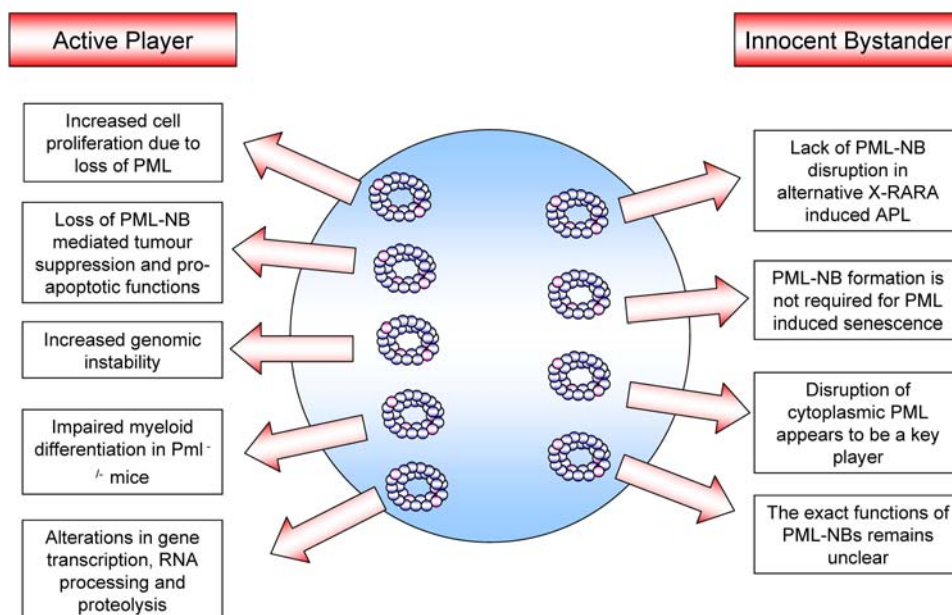
### 5.4. The role of cytoplasmic PML

PML-RARA has been shown to undergo alternative splicing to produce several isoforms, some of which are predicted to localize to the cytoplasm (44, 47, 50). Through studies employing a PML-RARA mutant lacking the NLS, it has now been demonstrated that cytoplasmic PML-RARA is less sensitive to RA-induced proteasomal degradation, indicating that the cytoplasmic localization may promote stabilization, which may result in increased resistance to RA-based therapies (111).

Despite knowledge of the existence of a number of cytoplasmic isoforms of PML, their functional characterization has been limited until recently (182). Initial observations conducted using a mutant PML-IV lacking the NLS, led to a significant reduction in PML-NBs, suggesting a potential dominant negative function over wild-type nuclear PML (183). A number of functions have been proposed for cytoplasmic PML (cPML), which include mediation of transforming growth factor beta (TGF-B) signaling. TGF-B is a ubiquitously expressed cytokine that has varied roles, affecting cellular processes including proliferation, differentiation, apoptosis, fibrosis and tumorigenesis. APL blasts have been shown to respond poorly to TGF-B. However, until recently the molecular mechanisms underlying this phenomenon were unknown (184). Similar results were obtained in *Pml*<sup>-/-</sup> MEFs inferring an essential role of PML in this process (22).

TGF-B signaling is initiated when ligand bound TGF-B type II receptor (TBR2) binds to, and phosphorylates, the TGF-B type I receptor (TBR1) (185-187). This phosphorylation, in the TBR1's characteristic SGSGSG sequence called the GS domain, causes it to activate the receptor-regulated Smads (R-Smads), Smad2 and Smad3, by COOH terminal serine phosphorylation. Once phosphorylated, the R-Smads form a heteromultimeric complex with the common mediator (Co)-Smad (Smad4) and accumulate in the nucleus to regulate transcriptional responses (185-187). TGF-B1-induced Smad2/3 phosphorylation and Smad3 nuclear translocation was found to be defective in the APL cell line NB4 (22). Interestingly the TGF-B1-dependent induction of cPML was also found to be impaired. Importantly, PML-RARA was shown to exert a dominant-negative function over cPML, interrupting its association with Smad3 either in the presence or absence of TGF-B. Upon RA treatment, which triggers PML-RARA degradation, the interaction between cPML and Smad2/3 was rescued (22). These data therefore demonstrate that TGF-B1 signaling is impaired in APL blasts due to the PML-RARA oncoprotein antagonizing cPML function, resulting in TGF-B unresponsiveness.

As a result, the disruption of cytosolic PML function appears to be a key feature of APL pathogenesis. However, it is important to note, that nuclear isoforms of PML (nPML) (Figure 1) can shuttle between the nucleus and the cytoplasm after viral infection and it is possible that nPML isoforms could function in the cytosol under physiological conditions. In agreement with this notion, nPML isoforms, when artificially targeted to the cytoplasm, are also capable of modulating TGF-B signaling. When attributing a function to PML, it would be of interest to assess whether cytoplasmic isoforms evolved before nPML, and if the PML-NB was a later evolutionary development providing a more specialized role (22). This discrete oncogenic function of cytosolic PML-RARA involving prevention of cPML from participating in the transduction of the TGF-B1 pathway is independent of PML-NB disruption and implies that although PML-NB disruption is important in the pathogenesis of APL the



**Figure 6.** PML NBs as Active Players or Innocent Bystanders in the pathogenesis of APL?

impairment of other isoforms of PML could also contribute to leukemogenesis.

**5.5. PML-NB biological functions: “guilt by association”**

A plethora of proteins have been identified to co-localize with PML, which possess divergent activities making it difficult to assign a distinct biological function to the PML-NBs. Currently, only four proteins have been shown to directly interact with PML, namely Ubc 9, SUMO1, eIF4E and the proline-rich homeodomain protein (PRH) (42, 188). Interestingly, PML-NBs appear to be associated with foreign or mis-folded proteins; therefore, data obtained suggesting co-localization with an over-expressed protein must be viewed with caution and validated to apply also to the respective endogenous proteins. This could be achieved by immunofluorescence showing that the protein of interest colocalizes with PML within NBs in untransfected cells and can be co-immunoprecipitated with endogenous PML. Not surprisingly, the association of PML-NBs with over-expressed proteins has led to the hypothesis that the bodies themselves are not functionally active compartments, but are simply storage facilities for the cells.

Due to the higher concentration of NB constituent proteins in PML-NB compared to the rest of the nucleus, one would naturally expect a higher activity of the respective proteins in these structures. However, evidence suggests this may not be the case. For example, the large subunit of RNA Polymerase II (PolIIa) is active throughout the nucleus and found at increased concentration at the PML-NB, which does not however result in an increase in RNA associated with the body (189-192). In fact the results suggest the reverse, with less RNA being detectable than in

the space surrounding the PML-NBs (189). However, the association of PolIIa within PML-NBs remains hotly contested as consequently does the role of these structures in the transcription of RNA (193, 194). On first inspection, this would imply that disruption of the PML-NB would lead to an increase in the availability of active protein; however the relative amount of protein contained within the NB as compared to the nucleus as a whole is expected to be relatively low. Due to the inherent problems associated with the study of PML-NBs, it is unlikely if not impossible to quantify this exactly.

**6. PERSECTIVE: IS PML-NB DISRUPTION AN “ACTIVE PLAYER” OR “INNOCENT BYSTANDER” IN THE PATHOGENESIS OF APL?**

Studies have revealed that the PML protein is involved in many essential cellular functions such as cell proliferation, differentiation, survival and tumor suppression. Such a wide variety of effects highlights similarities with the classical tumor suppressor protein p53. However, unlike p53, the majority of functions attributed to PML-NBs are indirect and inferred from studying constituent proteins. Yet evidence from the literature does suggest that the PML protein and/or the PML-NB may potentially be one of a select class of regulatory factors implicated in biological processes at multiple levels. In this review, we have discussed the evidence for and against a role for PML-NB disruption in the pathogenesis of APL (Figure 6).

Since the discovery of the additional chromosomal rearrangements involving the *RARA* gene, the significance of the fusion partners in the promotion of APL



development has been a matter of great debate. Although the X-RARA fusion partners lack an obvious functional resemblance, all introduce a homo-oligomerization domain into the fusion protein. Experiments utilizing artificial fusion proteins capable of dimerization, although biologically active *in vitro*, obviously lacked the capacity to induce leukemia in an efficient fashion, implying that this is provided by the APL fusion partner moiety. Further evidence for the importance of PML in APL pathogenesis was suggested by the lack of leukemic progression in transgenic animals expressing a homodimerizing HDAC-RARA fusion protein.

One of the most compelling pieces of evidence supporting a role for deregulation of PML in the pathogenesis of APL has been provided by the characterization of its tumor suppressor function in *Pml*<sup>-/-</sup> mice. These animals are viable and do not exhibit an excess of spontaneous tumors, but are highly susceptible to physical- or chemical-induced carcinogenesis (17). These findings clearly indicate that PML can act as a tumor suppressor *in vivo* and that its functional inactivation may be critical in APL leukemogenesis (17). However, the extent to which PML inactivation and/or PML-NB disruption leads towards transformation remains unclear, as the precise biochemical function of PML remains unknown. However, given that the functions of PML-NB components are so diverse, PML could potentially regulate oncogenic progression at multiple levels. Therefore, in addition to disrupting the normal function of PML, loss of the NB structure may potentially disrupt the growth regulatory activities of a large number of PML components. Several studies have highlighted the role of PML in regulation of apoptosis and senescence (17, 19, 23, 116). Disruption of these postulated PML activities could very well establish a premalignant state that facilitates the acquisition of, or tolerance to additional genetic changes. A role for the PML-NB in the modulation of transcription has also been postulated, considering the capacity for PML to interact with corepressors and histone deacetylases (HDAC), as well as histone acetyltransferases (HATs) within these structures. Additional hypotheses concerning the biochemical function of PML and the NBs can be entertained in view of the fact that proteins involved in seemingly distinct biochemical pathways have been found to localize in the PML-NB, such as proteasome subunits (136). It remains to be established whether the localization of all proteins associated with the PML-NB is of functional relevance and conversely caution should be exercised in attributing potential roles to PML-NBs extrapolated from what is known about particular constituents. In this respect, the purification of the PML-NB, the isolation of its core components, and a critical evaluation of its accessory molecules remain key goals of PML research in the years to come.

Recent evidence suggests that the PML-RARA oncoprotein has complex activity, which extends beyond simple disruption of the NBs and RARA functions, implying additional activities of the PML domain, and that these gains of function critically contribute to leukemic transformation. Furthermore, recruitment of PML-associated factors to the PML-RARA/RXR complex may

impact on expression of critical target genes. Such PML-RARA-specific alterations in the transcription of genes that regulate apoptosis, proliferation, or differentiation may underlie the ability of PML-RARA to initiate leukemia complexes, for instance by the formation of higher-order multimers as proposed previously. Taken together, evidence to date suggests the PML domain plays a very significant role in leukemogenesis, perhaps through more than one molecular mechanism. Given that multiple isoforms of PML are coexpressed in the cell and that some of them accumulate in the cytoplasm, PML could also potentially exert PML-NB independent cytosolic functions whose disruption may contribute further to the pathogenesis of APL.

In conclusion, there is strong evidence to support the idea that PML-RARA induced leukemia is a multi-step process, requiring co-operating events that together lead to oncogenic transformation. Moreover, it appears highly likely that the PML-RARA oncoprotein has a complex activity operating at multiple levels, extending beyond simple disruption of PML and RARA. The disruption of PML nuclear bodies themselves seems likely to play an active role in this multi-step process. However in order to fully understand the molecular role of both PML protein and PML-NBs in the pathogenesis of APL, greater understanding of the biochemical processes in which they are involved is required, including careful dissection of activities that are NB dependent or independent and the impact of delocalization of particular constituents on cellular function. Biochemical purification of the PML-NB and their constituents remains a key goal in PML research which could provide further insights into the molecular pathways that contribute to acute promyelocytic leukemia.

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**Abbreviations:** PML: promyelocytic leukemia; NBs: nuclear bodies; APL: acute promyelocytic leukemia; AML: acute myeloid leukemia; RARA: retinoic acid receptor alpha; ATRA: all-*trans*retinoic acid; RA: retinoic acid; RNP: ribonucleoprotein; R: ring motif; B: B-Boxes; CC: coiled-coil domain; NLS: nuclear localization signal; NES: nuclear export signal; Bcr: break point cluster region; TGF- $\beta$ : transforming growth factor-beta; SUMO: small ubiquitin-related modifier; SP100: Speckled 100 kDa; CBP: CREB binding protein; RB: retinoblastoma; BLM: Bloom; eIF-4: eukaryotic translation initiation factor 4; Daxx: death-associated protein; PLZF: promyelocytic leukemia zinc finger; NPM: nucleophosmin; NUMA: nuclear mitotic apparatus; Stat5b: signal transducer and activator of transcription; PRKAR1A: protein kinase, cAMP-dependent, regulatory, type I, alpha; FIP1L1: FIP1-like 1; X-RARA: fusion partners of RARA; RARE: retinoid acid response element; RXR: retinoid X receptor; PcG: poly comb group; N-CoR: nuclear receptor co-repressor; NF $\kappa$ B: nuclear factor kappa B; HDAC1: histone deacetylase 1; DMBA: dimethylbenzanthracene; TPA: tetradecanoylphorbol-13-acetate; CNS: central nervous system

**Key Words:** PML, Promyelocytic Leukemia, Nuclear-Bodies, RARA, Review

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