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PML nuclear bodies as sites of epigenetic regulation

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1. ABSTRACT

The protein-based core of a promyelocytic leukemia nuclear body (PML NB) accumulates numerous factors involved in many nuclear processes, including transcription and DNA repair. We suggest that these proteins could act on chromatin in the vicinity of the bodies. The physical dependence of PML NB structure on the integrity of the surrounding DNA implies a functional connection between the bodies and chromatin. Indeed, some genetic loci are non-randomly associated with PML NBs, indicating that nuclear bodies organize at specific loci, or are able to recruit specific genetic loci to their periphery. Since many of the factors that accumulate in PML NBs and PML-containing structures in acute promyelocytic leukemia cells are known histone methyltransferases, histone deacetylases or DNA methyltransferases, we suggest that PML NBs may have a role as epigenetic regulators. Down-regulation of normal PML protein, observed in a variety of cancers, may impair epigenetic regulation in early tumorigenesis, which ultimately leads to genetic instability and cellular transformation.

2. INTRODUCTION

The most fundamental level of gene regulation occurs through interactions between trans-acting factors and cis-elements in gene promoters and enhancers. An additional level, however, is required because DNA is complexed with histones as nucleosomal chromatin. Regulation at this level is directed to overcoming the generally repressive action of chromatin, to provide access of transcription factors to the DNA (1). Post-translational modifications of the histone N-terminal tails play a central role in relieving chromatin-based repression, including phosphorylation, methylation, acetylation, sumoylation and ubiquitination (2). Histone modifications, however, are linked to CpG methylation within the DNA itself (3). Dynamic variations in histone modifications at particular genetic loci can reflect fine-tuning of chromatin structure required for various cellular processes. Such dynamic changes would not necessarily qualify as epigenetic regulators. If, however, specific modifications of DNA or histone modifications are maintained through mitosis, these modifications would constitute a component of the epigenetic code.

Histone modifications that confer both transient and/or epigenetic signals for gene regulation are dependent on the action of large multi-subunit complexes which include specific kinases, transferases or ligases. A common theme that is emerging in molecular cell biology is that such macromolecular complexes can self-assemble in spatial compartments. This principle is especially applicable to the cell nucleus, where such enzyme complexes localize into foci or sub-nuclear organelles, rather than distributing uniformly in a nuclear-diffuse manner. Such compartmentalization has a profound implication for epigenetic regulation. Since the enzymes that create and maintain epigenetic marks on specific loci are spatially restricted, then the gene loci themselves must also be non-randomly positioned. Sub-nuclear organization, therefore, has the potential to contribute prominently in epigenetic regulation.

The nuclear lamina along the inner nuclear membrane illustrates how a nuclear sub-compartment could contribute to gene regulation. A number of co-repressors interact directly or indirectly with the lamins at the nuclear envelope, including LBR, HP1, LAP2B, GCL, emerin, BTF and BAF (4). As a result, the lamina becomes a site that is enriched in silent chromatin. The establishment of a gene-silenced compartment may involve a feedback loop through co-operative mechanisms leading to a further expansion of the co-repressor pool, along with an expanding domain of silenced chromatin along the nuclear envelope. Moreover, the nuclear envelope would provide one architectural component to restrict the position a chromosome territory in a radial or relative orientation. Similarly, active loci transcribed by RNA polymerase II can congregate into local spatial domains called transcription factories, foci that are enriched in elongation-competent forms of the polymerase (5-7).

It is conceivable that epigenetic marks are partly responsible for a defined chromatin architecture (8) and the resulting sub-nuclear organization further enhances and maintains these marks. This architecture could contribute to gene regulation and be inherited between successive cell generations. (Refer to the article by P. Freemont in this issue for a discussion of PML and nuclear architecture.) In this review, we discuss the potential role of promyelocytic leukemia nuclear bodies (PML NBs) in establishing local compartments that participate in epigenetic regulation. We propose that PML NBs could participate in these functions in both normal tissue differentiation and tumorigenesis.

3. STRUCTURAL INTEGRITY OF PML NBS DEPENDS ON CHROMATIN

PML NBs could participate in gene expression according to two models. In one model, the bodies could serve as platforms where regulatory molecules assemble into complexes and where post-translational modifications lead to their activation. Following assembly and activation, the complexes would then move into the nucleoplasm where they function. A second, though not mutually exclusive model, is that PML NBs interact via adaptor components with specific gene loci. These bodies would thereby bring otherwise distant loci into close proximity. Co-activators or co-repressors that concentrate in PML NBs could then service the chromatin at the immediate periphery of the bodies. PML NBs could thereby function as a nuclear matrix component that serves as attachment points for chromosome loop domains. This model could be adapted to explain how PML could function as an epigenetic regulator by bringing specific loci into the proximity of high concentrations of epigenetic modifiers. To determine whether such a function is plausible, we will first discuss the structure of PML NBs.

PML NBs are protein based nuclear compartments (Figure 1), also known as PODs, ND10 or Kremer bodies. The number of PML NBs is cell-type, differentiation state, and cell cycle dependent. Some cells have as few as 4-5 bodies, whereas other cell types have over 30 bodies. PML NBs usually exhibit radial symmetry, appearing spherical or doughnut-shaped, and range from approximately 0.1-1 micron in diameter. PML NBs are positionally stable over extended periods of time in interphase. They also exhibit a rapid, energy-dependent oscillatory movement of less than a body diameter (9). The positional stability could be based on interaction of the bodies with other stationery protein-based structures, or with relatively immobile gene loci.

PML protein itself has the capacity to self-assemble *in vitro* into spherical macromolecular complexes that resemble PML NBs formed *in vivo* (10). Electron spectroscopic imaging (ESI, also referred to as energy filtered transmission electron microscopy (EFTEM)) demonstrates that PML NBs in interphase are composed of a radially symmetric, protein-based core. This core is typically surrounded by chromatin and sometimes by accumulations of ribonucleoproteins (RNPs) as well (Figure 1). It is tempting to suggest that the mobility of PML NBs is restricted by contacts with this surrounding chromatin, which often appears to make direct physical contacts with the protein core. Size alone is not the basis for the positional stability, since similarly sized Cajal bodies can display a high degree of mobility (11). Although PML bodies are positionally stable through periods of interphase, under normal conditions, their integrity and stability is lost upon disruption of chromatin by cellular stress, transcriptional repression, early apoptotic events, or following exposure of live cells to nuclease treatment. Chromatin and PML NB integrity are intimately related. PML NBs re-organize by fission mechanisms into numerous smaller PML-protein-based microstructures following heat or heavy metal stress (12) or into microbodies following transcription inhibition, again by a fission mechanism. The biochemical composition of microbodies is identical to the parental PML bodies, whereas microstructures lack some of the typical PML NB-associated proteins, such as Sp100 or SUMO (12, 13). (For a more detailed discussion of Sp100-PML interactions and SUMO modifications of PML, see the accompanying articles by H. Will and Miller, respectively.) The fission process could result from the retraction of chromatin away from the body, due to topological changes in the DNA. If DNA makes strong physical contacts with the PML NBs,

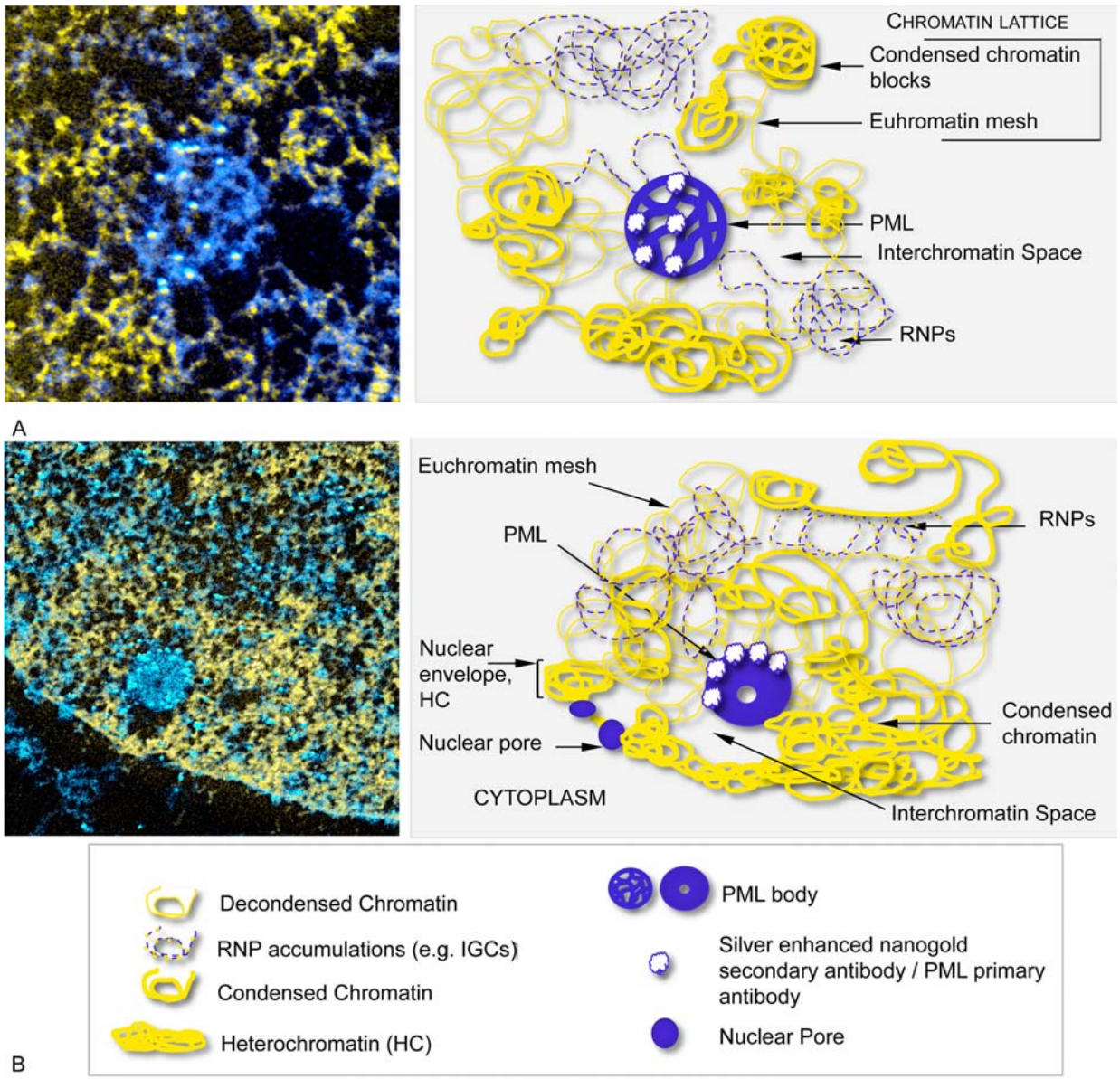

Figure 1. PML NBs are protein based structures surrounded by chromatin loops. Electron Spectroscopic Imaging (ESI) of a PML NB in a Hep2 cell. Protein-based structures are false-coloured in blue, nucleic acid based structures in yellow. PML protein was immunocytochemically labelled with nanogold conjugated secondary antibody and silver-enhanced, represented by bright white spots. An interpretive drawing at the right illustrates important structures. A. ESI image of a single PML NB. Full field corresponds to 800 nm. B. A PML NB, imaged by ESI, is surrounded by a block of chromatin. Full field corresponds to 1.7 micron.

the retracting DNA would pull PML accumulations out of the parental body. Alternatively, if a dynamic equilibrium of PML NB components exists between the body core and the surrounding chromatin, loss of this chromatin through DNA damage or retraction away from the body would shift the equilibrium, favouring a fragmentation of the body by mass action (13).

PML NBs also undergo fission following induction of DNA double strand breaks (DSBs) with ionizing radiation or with genotoxic agents such as etoposides (14). The functional consequences of the disruption remains to be elucidated. After the induction of DSBs by various stimuli, PML NBs increase in number and co-localize with sites of DNA repair. Many DNA repair and checkpoint proteins localize to PML NBs (14). The increase in PML NB number is based on a supramolecular fission mechanism, and is inhibited by the loss of function of NBS1, ATM, Chk2 or ATR kinase (14). PML NBs participate in the DNA damage response on different levels by acting as storage sites of repair and checkpoint enzymes that are released after DNA damage, by actively regulating

DNA repair and by integrating sites of DNA repair with checkpoint responses. The fission of PML NBs results in an increase in the state of subdivision of body-associated proteins involved in the DNA damage response, including cell cycle arrest and DNA repair (15).

Since PML NBs seem to take their cues for positional stability and structural integrity from chromatin, an important question is whether their structural integrity is compromised when DNA replicates in S-phase, a process that is accompanied by major changes in local DNA topology. The number of PML NBs increase in early S-phase, when euchromatin is replicated. Importantly, the number does not just increase, but in many cell lines, increases by 2-fold (16). This result strongly supports a relationship, perhaps even a stoichiometric one, with a set of genomic loci. The new PML NBs do not arise primarily from *de novo* formation of bodies from nucleoplasmic PML protein. Instead, the structural stability of PML NBs is compromised in early S-phase resulting in body fission. Moreover, the protein cores of the bodies are disrupted, leading to the redistribution of PML along the chromatin fibres. It may be that PML NB dynamics and integrity in S-phase is a reflection of changes in chromatin topology as DNA is replicated. These observations provide more evidence for an intimate and dynamic link between PML NB integrity and chromatin in the interphase nucleus. The obvious question that arises is whether there is a direct functional relationship between the chromatin and the nuclear body, beyond the stabilising influence of chromatin on these bodies.

4. SPECIFIC GENE LOCI ASSOCIATE WITH PML NBS

The apparent connection between chromatin and PML NB integrity supports the hypothesis for a functional relationship. The disruption of PML NBs could release PML NB-associated proteins which become available for regulatory processes elsewhere in the nucleoplasm. On the other hand, PML NBs could bring specific gene loci together, loci that might be co-regulated, perhaps even co-regulated by PML NB components. An apparent non-random association of gene loci in the vicinity of PML NBs has been observed. In Jurkat T-cells a locus that includes the TP53 gene localizes to PML NBs in approximately 50% of the cells, whereas the BCL2 locus is never observed in association with PML NBs (17). In addition, the major histocompatibility (MHC) class I gene cluster is associated with PML NBs in human primary fibroblasts (18). Besides these specific loci, PML NBs may associate with more specific genes, since PML NBs are more frequently associated with transcriptionally active genes rather than with transcriptionally silent genes or gene-poor loci (19). (For a discussion of PML's role in post-transcriptional processes, see the accompanying review by K. Borden.)

In general, PML bodies associate with gene-rich chromosomal regions more frequently than gene-poor regions. Also, PML NBs are found in transcriptionally active regions of the nucleus. In some cases, the genes in these regions are not affected by PML levels, but whether this is generally true has not been addressed. However, PML protein can act both as a transcriptional co-activator (20) and a co-repressor (21). Such observations fail to establish whether such regulatory activity by PML protein occurs at PML NBs themselves, or through dispersed PML in the nucleoplasm. To address this question, a novel strategy was developed to target reporter gene plasmids to PML NBs. This was used to determine whether the "micro-environment" of the PML NB itself promotes transcription regulation (22). The targeting approaches were all based on fusion proteins that can localize to PML NBs and bind to a luciferase reporter plasmid. Transcription from a luciferase reporter plasmid driven by the SV40 early promoter was repressed when targeted to PML NBs. In contrast, the human CMV intermediate early promoter was upregulated when targeted to the bodies. A minimal promoter was not affected by targeting to bodies. The response of the targeted promoters was also sensitive to the biochemical composition of the PML NBs. This implies that though the PML NB could serve as a passive storage domain or platform for post-translational modifications of PML NB components, it also plays an active role in creating a transcription regulatory environment. Activation or repression of specific endogenous promoters at a PML NB may reflect the factors that are concentrated in that body and that act on near-by promoters.

PML NBs could also participate in gene regulation by affecting long-range interactions. Long distance chromosomal interactions might both activate or repress gene activity as well. Classical examples for such long distance intrachromosomal interactions is the looping of beta-globin gene and the locus control region (LCR) (23) or the positioning of the LCRs of IL4, IL5 and IL13 in close proximity in T-cells (24). The long distance chromosomal interactions suggest the possible role for nuclear architecture in maintaining the stable state of gene expression or epigenetic regulation (25). Regulatory chromosomal domains may be positioned to nuclear subcompartments which might act as regulatory centres for co-ordinating gene activity. Since the surface of PML NBs directly interacts with chromatin loops, the bodies might play a role in bringing long distance chromatin domains into functional and physical proximity. In addition, the open chromatin configuration of the loops is approachable not only for gene transcription but also for regulation or changing the epigenetic code. Moreover, all reported long range interactions are cell-type specific and the timing of transient chromosomal domain co-localizations correlates with changes in cellular differentiation. This suggests a role for range chromosomal interactions as developmental regulators (26).

The propensity for PML NBs to associate with specific genetic loci is further demonstrated in some cancer cells that lack telomerase. These cells utilize a recombination-based alternative to lengthening of telomeres, and thus the cells are referred to as ALT cells. A subset of PML NBs in these cells is called ALT PML NBs or APBs. The presence of such APBs appear to serve as an important prognostic marker for the degree of tumor

malignancy (27). The characteristic of APBs is the co-localization of telomere binding proteins, such as TRF1 or TRF2, with PML NBs. Because these proteins bind telomeric DNA, it is assumed that telomeres and/or extrachromosomal telomeric sequences are found at or inside these unique PML NBs (28). The functional significance for the presence of telomeres at a fraction of PML NBs is not known. A final example of an association of PML NBs with a specific chromosomal domain is found in a pathological condition referred to as ICF syndrome (immunodeficiency, centromeric instability and facial dysmorphy) (OMIM 242860). In ICF cells in G2, a single giant and morphologically unusual PML NB associates with a satellite chromatin region of chromosome 1 (29). The relationship of PML and this genetic locus will be discussed later. The co-localization of these specific DNA elements in ICF syndrome or ALT cells may provide clues to the relationship between specific genetic loci and PML NBs, hypomethylated satellite DNA in ICF syndrome and aberrant telomere regions in ALT tumors. Although these represent pathological conditions, they may indicate how PML NBs have a propensity to associate with specific DNA elements and contribute to the epigenetic modification of these loci in the immediate periphery of the bodies.

The association of PML bodies with certain chromatin segments appears to be non-random and may be cell-type and differentiation state specific. This heterogeneity can be explained by the highly dynamic composition of the PML NBs. Over eighty proteins were reported to associate with PML NBs in different models, many of these associations being cell-type specific. Some PML NB components exchange rapidly between the PML NB and the nucleoplasm (30, 31). The composition of PML NBs within one cell is not necessarily uniform. Of the various possible co-activator and co-repressor components we discuss a few of those which might participate in the epigenetic regulation and maintenance of the integrity of the DNA code. PML NBs may affect the assembly and activation of complexes that contain these factors, or PML NBs may concentrate them so that they can act efficiently with chromatin in the surrounding environment.

5. SOME COMPONENTS OF PML NBS AFFECT GENOME INTEGRITY AND GENE EXPRESSION

Werner syndrome protein (WRN) is a RecQ DNA helicase mutated in Werner syndrome (OMIM 277700) patients. Werner syndrome is characterized by premature aging, scleroderma-like skin changes, cataracts, premature arteriosclerosis, diabetes mellitus and predisposition to malignancy. Mutations in WRN lead to chromosomal instability, with a propensity to develop chromosomal aberrations, including translocations, inversions and deletions. Werner syndrome fibroblast cell lines are especially sensitive to DNA damage. Loss of WRN helicase activity results in dramatic telomere loss from individual sister chromatids, so that the DNA instability that leads to cancer may depend directly on telomere dysfunction. WRN also enhances p53-dependent apoptosis. The WRN protein translocates to PML bodies upon DNA damage after acetylation. (32, 33)

Bloom's syndrome protein (BLM) is a RecQ DNA helicase mutated in Bloom's syndrome (OMIM 210900) patients. Bloom's syndrome is characterized by pre- and post-natal growth deficiency, sun-sensitive, teleangiectatic, hypo- and hyperpigmented skin. The predisposition to various malignancies is consistent with high levels of sister chromatid exchange (SCE) and chromosomal instability associated with this syndrome. The BLM protein co-localizes with PML NBs especially in S and G2 phase of the cell cycle or after ionizing radiation (32, 34).

Topoisomerase III alpha (TOPOIIIa) is recruited to PML bodies by the BLM protein. It may function as a tumor-suppressor together with BLM by preventing loss of heterozygosity (35).

CREB-binding protein (CBP) is a dynamic and cell-type specific component of PML NBs (30). CBP is a histone acetyl-transferase, but acetylates other proteins as well. CBP is involved in various aspects of regulation of gene transcription and differentiation. Mutations affecting the CBP gene cause Rubenstein-Taybi syndrome (OMIM 180849) (36), which is characterized by mental retardation, specific facial and skeletal features, and an increased risk of tumor formation, especially of the head and leukemias.

H2A.X is a core histone variant, which is phosphorylated after DNA damage and associates with PML NBs late in the repair process. Together with the BRCA1, HP1, Daxx, SUMO-1 and PML, H2A.X also participates in the condensation and silencing of the XY facultative heterochromatin body (37).

Mutations in the ATRX gene cause the alpha-thalassemia/mental retardation syndrome (OMIM 301040). The ATRX-Daxx complex may play a role in ATP-dependent chromatin-remodelling activities. Both ATRX and Daxx are present in PML NBs, and therefore might have a role in chromatin remodelling (38).

Death associated protein 6 (Daxx) was shown to bind to the Fas death domain and enhance Fas-mediated apoptosis. Daxx also acts as a transcriptional co-repressor of certain anti-apoptotic genes, such ascIAP2 (39). A more detailed review of Daxx and PML is found in this issue by A. Ishov.

Sp100, another PML-body component, also functions as a transcriptional co-repressor. This function may be related to its interaction with the heterochromatin protein 1 (HP1) family of non-histone chromosomal proteins (40).

Beta-catenin may represent a class of molecular partners that could link PML to the epigenetics of both normal differentiation and tumorigenesis. Beta-catenin is a key component of the Wnt signalling pathway, which controls body patterning, aberrantly activated beta-catenin contributes to cancer progression. Beta-catenin was reported to upregulate the PML promoter and, as a positive feedback loop, PML augmented the beta-catenin responsive

promoters (41). Although beta-catenin is an integral component of E-cadherin complexes at intercellular adherent junctions, it also recruits chromatin remodelling complexes to activate the transcription of various genes. PML forms complexes with beta-catenin in PML-bodies. PML, p300 and beta-catenin cooperate in transactivation of a subset of beta-catenin responsive genes. Whether beta-catenin simply recruits chromatin remodelling complexes or also regulates remodelling activity is not known (42).

6. PML BODIES CONTRIBUTE TO THE CONSERVATION OF EPIGENETIC INFORMATION

Post-translational modifications of histones that are inherited contribute to epigenetic regulation of gene activity. These chromatin marks are often related to methylation of DNA bases, which provide an additional level of information. The activity of DNA methyltransferases and demethylases play major roles in epigenetic regulation. PML may contribute to epigenetic regulation by modulating the activity of these enzymes at specific loci. NB4 cells, promyelocytes from acute promyelocytic leukemia (APL) patients, are arrested in differentiation, resulting from a reciprocal translocation between the genes encoding PML and retinoic acid receptor alpha (RARalpha). The dramatic characteristic of these cells is that typical PML NBs are largely absent. Instead of a few large PML NBs, PML forms many small foci distributed throughout the nucleus. However, between 1 and 3 apparently normal PML NBs are retained in some cells. Following retinoic acid (RA) treatment, the numerous PML foci re-organize into approximately 10 morphologically typical PML NBs. The PML-RARalpha fusion protein causes aberrant silencing of RA target genes by epigenetic mechanisms, resulting in the differentiation block (43). The fusion protein binds to retinoic acid response elements (RAREs) and represses transcription by recruiting co-repressor complexes, such as the NCo-R-HDAC complex (44), repressive histone methyltransferases (SUV39H1), DNA methyltransferases (45), and Polycomb Repressive Complex 2/3/4 (PRC2/3/4) (46). The fusion protein, however, also recruits the DNA methyltransferases DNMT1 and DNMT3a through a direct interaction (47, 48). As a result, the PML-RARalpha fusion protein increases methylation of CpG islands in retinoic acid responsive gene promoters, i.e. containing RAREs, such as the RARbeta2 gene, likely leading to epigenetic silencing. Indeed, microarray analysis of DNA methylation of CpG islands and histone acetylation state of NB4 cells revealed extensive aberrant cytosine methylation in APL cells, although much of this methylation pattern remained unaffected by all-*trans* retinoic acid (ATRA) treatment (47). Treatment with in combination with chemotherapy frequently leads to remission and cure through the release of co-repressor complexes, degradation of the PML-RARalpha fusion protein and the re-formation of a few normal (i.e. composition and morphology) PML NBs from many dispersed PML-containing foci, or micro-PML NBs, a characteristic of NB4 cell nuclei (49).

The ability of PML NBs to affect the DNA methylation status is clearly demonstrated in another pathological condition referred to earlier as ICF syndrome. Though the core of PML NBS is generally devoid of DNA (50), a morphologically distinct and very large PML-containing structure encapsulates a specific chromosomal element. Fluorescence microscopy indicates that the centre of this giant body contains the disease-specific hypomethylated satellite DNA of chromosome 1 (29). ICF syndrome is caused by a mutation in the DNMT3b enzyme, which is responsible for the *de novo* methylation of GC-rich satellite DNAs. On the chromatin level it causes hypomethylation and decondensation of DNA. Cytogenetic abnormalities mostly occur on chromosomes 1 and 16. In G2 phase a single giant body is formed in ICF syndrome cells. Its composition resembles that of normal PML NBs, but is much larger and contains DNA, the disease-specific hypomethylated satellite chromatin region of chromosome 1. Clearly a change in the epigenetic code results in a change of the PML NB organization of the cells. A specific function for PML in G2 that was proposed was the re-establishment of condensed heterochromatin state on late-replicated satellite DNA. In their model PML protein creates a spherical compartment into which other proteins are packaged and interact in a coordinated manner (29). Though details of the interactions of PML and the mutated DNA methylase have yet to be provided, the potential for PML to modulate an epigenetic mark is well demonstrated in ICF. It is clear that a PML NB either has the ability to interact with a specific chromosomal locus or is created at a hypomethylated DNA site.

PML's ability to affect the methylation state of DNA is also illustrated in ALT cells. Mouse sub-telomeric regions are heavily methylated and the lack of DNMTs result in increased telomeric recombination and formation of ALT-bodies (51). It is not known whether the methylation state of sub-telomeric regions in human tumors determines the telomere maintenance mechanism. It remains to be determined whether PML directly affects the methylation status of the sub-telomeric DNA or the methylation causes the seeding of a PML NB, or the telomeric DNA appears as large repair foci that associates with PML. In the last case, the association of PML and methylated chromatin would be fortuitous.

7. A MODEL FOR PML NB FUNCTION IN EPIGENETIC REGULATION BASED ON APL PATHOLOGY

In the previous section, we have seen how the PML fusion protein can affect the localization of regulatory factors in nuclei of promyelocytes from APL patients. The fusion protein that results from a reciprocal translocation also affects the methylation status of DNA of retinoic acid response genes. (See the accompanying article by D. Grimwade on the activity of this fusion protein.) The recruitment of co-repressors such as histone lysine methyltransferases (KMTs) or DNMTs may reflect an activity of PML or PML NBs in normal cells. If PML-RARalpha foci form at or recruit RAREs, then the possibility that PML NBs could associate with genetic

regulatory elements is further supported. Through the PML component, the fusion protein has many properties that distinguish it from RARalpha itself, including its ability to recruit histone deacetylase complexes (HDACs), KMTs, and DNMTs (45). Some of RARalpha's new functions are based on its new capacity to homodimerize via the N-terminal RBCC domain of PML, the basis for PML's self-assembly properties (10). But co-repressor activity and recruitment of DNMTs likely occur through Daxx (52), which itself is a partner of PML and a major component of PML NBs. Hence, PML could affect epigenetic regulation of other genes through Daxx in both normal and leukemic cells.

NB4 cells could provide clues to how PML contributes to compartmentalization of both regulatory machinery and specific gene loci, thereby affecting epigenetic mechanisms of regulation. NB4 cells are characterized by having many micro-PML containing structures instead of a few large bodies, typical of most cell types. Some proteins that are generally found in normal PML NBs, such as CBP and Daxx, are found in the micro-PML accumulations (53, 54), whereas other PML NB components, such as Sp100, are not found in the micro-PML accumulations (55). The micro-PML structures are likely localized at RAREs or perhaps clusters of RAREs, though this has not been shown. In APL only one allele is translocated, thereby allowing for the expression of both PML and PML-RARalpha proteins. Although not demonstrated, the numerous PML accumulating foci could contain PML-RARalpha homodimers or PML:PML-RARalpha heterodimers. Interactions with the heterodimers and homodimers may explain the new activities of PML at RAREs. Surprisingly, between one and three "normal" PML NBs can be observed in untreated NB4 cells. These bodies are more compositionally typical of PML NBs than the micro-accumulations since they contain the standard components of PML NBs, including Sp100, and CBP (Figure 2), and are more typical in terms of their size. The question arises, how do these PML NBs retain their integrity whereas the other PML NBs that would be found in normal differentiating promyelocytes are lost? Our hypothesis is that (i) either these bodies form in a unique genetic environment with a specific set of genes, or (ii) these bodies recruit particular genes to this locale. Regardless of whether (i) or (ii) is correct, the critical point of the hypothesis is that the association between the PML NB and these specific loci is far stronger than the association of PML and PML-RARalpha accumulations with RAREs. That is, the presence of the fusion protein and its interaction with RAREs is not strong enough to disrupt these particular PML NBs, but can disrupt the others, which constitute the majority. The gain of function at RAREs by PML-containing complexes could lead to oncogenic transformation. Loss of function could also be postulated, where the function of normal PML NBs to create clusters of otherwise dispersed gene loci has been greatly impaired.

8. POSSIBLE ROLE OF PML NBS AS EPIGENETIC CONTRIBUTORS TO EARLY TUMORIGENESIS

The classical link between PML and carcinogenesis is the PML-RARalpha fusion protein in APL (56). The formation of PML-RARalpha oncogenic fusion protein *per se* is sufficient for malignant transformation, in addition this transformation is specific to APL. Whereas in humans, expressing the PML-RARalpha fusion protein is sufficient for leukemogenesis, transgenic mice expressing this fusion protein require additional oncogenic factors to exhibit the cancer phenotype (57, 58). This difference could depend on the fact that human PML or human RAR do not completely mimic the functionality of the endogenous mouse forms of these proteins. Regardless, PML-RARalpha fusion protein causes aberrant epigenetic silencing as part of the tumorigenic process, as discussed above. Interestingly, in several tumor types, PML is under-expressed and fewer PML NBs are observed compared to the corresponding normal tissues (59). Importantly, the decrease in PML expression correlates with the grade of malignancy (in prostate carcinoma) or metastasis formation (breast carcinoma). Furthermore, induction of differentiation in neuroblastoma cells correlates with an increase in the number of PML NBs, analogous to the re-establishment of PML NBs in APL promyelocytes following treatment with ATRA (60-62). The loss of PML in tumors was shown to be the result of protein degradation (63). However, the loss of PML on its own is not sufficient to promote tumor formation, but it increases tumor predisposition in PML knock-out mice (64, 65). Based on these findings PML was proposed to be a tumor-suppressor. Many of its anti-tumor effects could depend on interactions between PML NBs and epigenetic regulators.

Tumors are populations of heterogeneous cells, composed of tumour progenitor cells and cells derived from them. If transplanted, only some have the potential to grow into a new, identical tumor. Whereas the potential for generating a tumor is relatively high, the probability that a tumor precursor cell will succeed in producing a full tumor is, fortunately, very low. A process of "micro-evolution" operates to select viable tumor cells, cells with the best "fitness landscape". These will represent cells with higher genetic and epigenetic variability, having lost their genetic and epigenetic gatekeepers, and which can thereby acquire new characteristics that are advantageous for tumor survival. This is reflected by the higher tumor rates in several syndromes with genetic instability (e.g. ataxia telangiectasia, retinitis pigmentosa) (66, 67). Likewise, aberrant epigenetic regulation may define important early steps in tumor progression, even at the level of tumor stem cell formation. Loss of epigenetic gatekeepers could open a cascade of events leading to self-augmenting epigenetic and genetic aberrations (68). Loss of PML may represent the loss of one of several epigenetic regulators or regulatory pathways. Loss of only one would typically be insufficient for a malignant transformation. Hence, in the PML knockout mouse model, though overly sensitive to tumorigenic agents and exhibiting a mild level of chromosome instability, this loss alone does not lead to tumorigenesis. The loss, however, could place stress on other epigenetic pathways, which could begin the cascade (68), which eventually leads to genetic changes such as chromosome instability. Whether there is any evidence that chromatin organization is changed in PML knockout mice

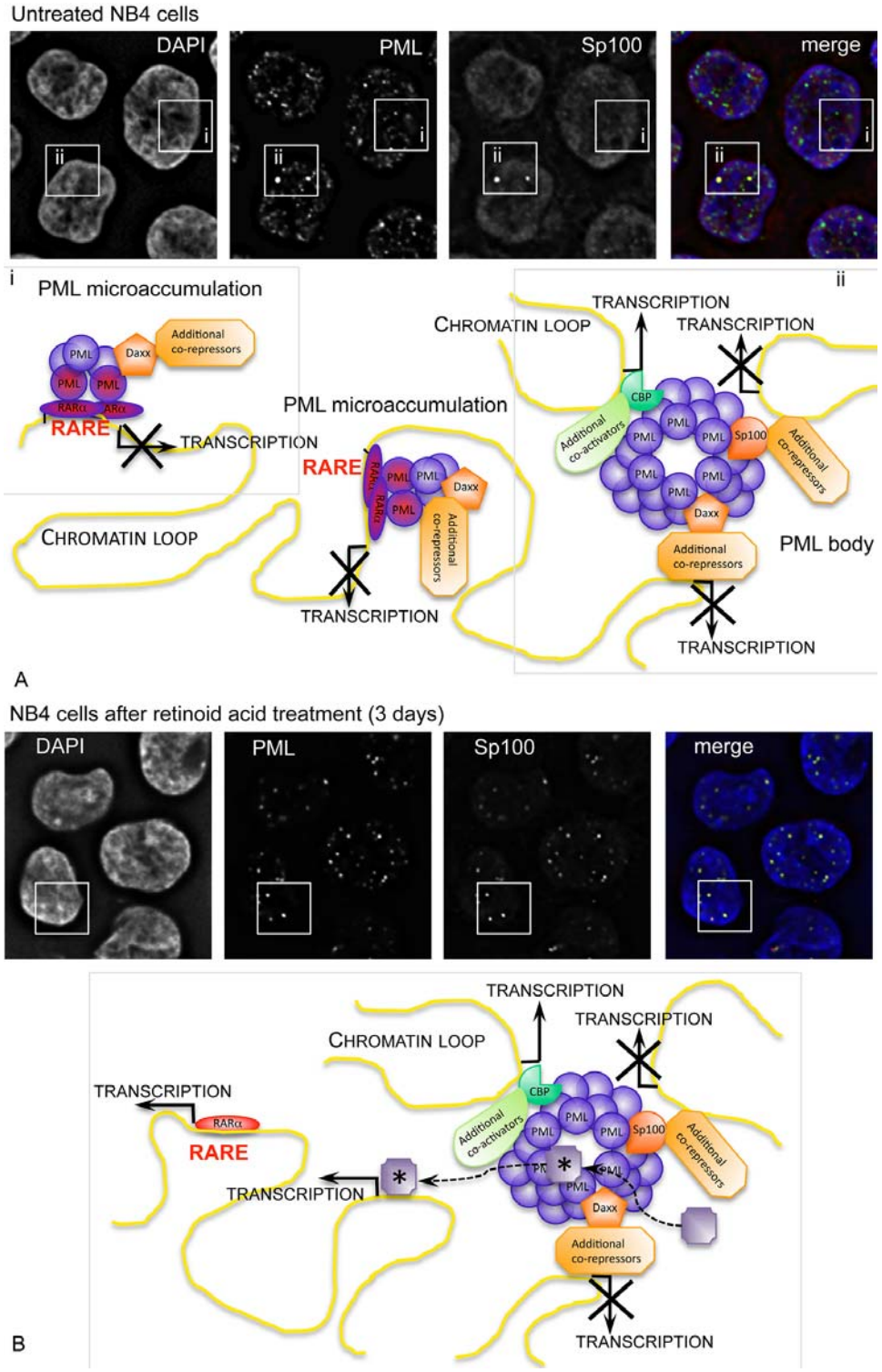

Figure 2. PML NBs are disrupted by the PML-RARalpha fusion protein, but are structurally and functionally restored by retinoic acid treatment. Immunofluorescence images of APL-derived NB4 cells before (A) and after (B) retinoic acid treatment. The nuclei contain numerous small accumulations of PML (field i), though between 1 and 3 apparently normal PML NBs are forms frequently observed (field ii). Sp100 does not localize in the small PML accumulations. After retinoic acid treatment both PML NB structure and composition (Sp100 localization) are restored. A hypothetical model of the PML-containing micro-structures and PML NB composition, chromatin binding and effect on transcription are shown in the schematics. See text for details.

needs to be systematically addressed at various levels, from promoter-specific nucleosome modifications to whole chromosome territory arrangements.

We think that PML NBs have the potential to contribute to the integrity of the epigenetic regulation on several levels. First, the number of PML NBs in the nucleus is cell type- and differentiation state-specific, i.e. body number is an inherited characteristic. Second, PML NBs are positionally stable over longer periods in interphase, likely due to interactions with specific genetic loci (12). Indeed, PML NBs frequently contact decondensed, open chromatin fibres at the periphery of their protein-based core. Either the bodies establish an environment favourable to transcription, or recruit transcriptionally active loci to these sub-nuclear domains, or form in this environment, perhaps reinforcing the transcriptionally favourable environment. Third, several chromatin remodelling enzymes, transcriptional co-activators and co-repressors, have been identified as components of PML NBs, many associating with bodies in a cell type- and differentiation state-specific manner. Fourth, the integrity of PML bodies contributes to genetic stability and the conservation of the genetic and epigenetic codes over multiple cell divisions by sensing DNA damage and participating in DNA repair. Failure to fully repair DNA could accelerate the deterioration of both genetic and epigenetic integrity, as well as compromise apoptotic signalling, through abrogated p53 activation for example (69). Failure to compartmentalize or modify DNA helicases at PML NBs could also contribute to downstream loss of genetic and epigenetic integrity, resulting in malignant transformation. In normal cells, PML NBs appear to form specific associations with genetic loci. Compromising these associations through the loss of early epigenetic marks could lead to an erosion of the size and stability of PML NBs. These further compromise the ability of PML NBs to sense damage (14, 15), and also lead to a reduction in the cell's ability to position loci into spatially restricted domains. This in turn leads to fewer opportunities for maintaining epigenetic regulation and further losses of genetic integrity in a cascading tumorigenic process.

9. PERSPECTIVE

PML NBs contribute to the physical and functional integrity to the genome through maintaining the stable state of gene expression in several ways. There are emerging evidences that PML bodies can cooperate with epigenetic mechanisms in chromatin organization and transcriptional regulation in several cellular processes (e.g. proliferation, apoptosis, differentiation) (70). This might strongly contribute to the tumor-suppressor roles of PML bodies. Further elucidation of the importance of the three dimensional nuclear landscape and PML's contribution to nuclear architecture will expand our understanding of healthy tissue differentiation and tumorigenesis. Such knowledge may also reveal new diagnostic markers and targets for therapeutic intervention in a variety of cancers.

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Abbreviations: PML NB: promyelocytic leukemia nuclear body; DNA: deoxyribonucleic acid; LBR: lamin B receptor; HP1: heterochromatin protein 1; LAP2B: lamin-associated polypeptide 2 beta; GCL: germ cell-less; BTF: bcl-2-associated transcription factor; BAF: barrier to autointegration factor; RNA: ribonucleic acid; PODs: promyelocytic leukemia oncogenic domains; ND10: nuclear domain 10; ESI: electron spectroscopic imaging; EFTEM: energy filtered transmission electron microscopy; RNP: ribonucleoprotein; SUMO: small ubiquitin-like modifier; Sp100: speckled 100 kDa; DSBs: double strand breaks; NBS1: Nijmegen breakage syndrome protein 1; ATM: ataxia-telangiectasia mutated gene; Chk2: checkpoint kinase 2; ATR: ataxia-telangiectasia and RAD3-related; TP53: tumor protein p53; BCL2: B-cell leukemia 2; MHC: major histocompatibility complex; SV40: simian virus 40; CMV: cytomegalovirus; LCR: locus control region; IL: interleukin; ALT: alternative lengthening of telomeres; APBs: ALT-associated promyelocytic leukemia nuclear body; TRF1: telomeric repeat-binding factor 1; TRF2: telomeric repeat-binding factor 2; ICF: immunodeficiency-centromeric instability-facial dysmorphy syndrome; WRN: Werner syndrome; BLM: Bloom syndrome; SCE: sister chromatid exchange; TOPOIIIa: topoisomerase III alpha; CBP: CREB-binding protein; H2A.X: histone 2A variant X; BRCA1: breast cancer 1; Daxx: death-associated protein 6; ATRX: alpha thalassemia/mental retardation x-linked syndrome; Fas: tumor necrosis factor receptor superfamily, member 6; Wnt: wingless-type MMTV integration site family member; ATP: adenosine triphosphate; APL: acute promyelocytic leukemia; RARalpha: retinoic acid receptor alpha; RARE: retinoic acid response elements; NCo-R: nuclear repressor corepressor; PRC: polycomb repressive complex; HDAC: histone deacetylase; RA: retinoic acid; ATRA: all-trans retinoic acid; DNMT: DNA methyltransferase; KMT: K-methyltransferase; RBCC: RING-finger, B-box, coiled-coil motif

Key Words: PML Nuclear Bodies, Epigenetic Regulation, Nuclear Structure, Chromatin Structure, Review

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