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REVIEW

New insights into the role of PML in tumour suppression

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The *PML* gene is involved in the t(15;17) translocation of acute promyelocytic leukaemia (APL), which generates the oncogenic fusion protein PML (promyelocytic leukaemia protein)-retinoic acid receptor alpha. The PML protein localises to a subnuclear structure called the PML nuclear domain (PML-ND), of which PML is the essential structural component. In APL, PML-NDs are disrupted, thus implicating these structures in the pathogenesis of this leukaemia. Unexpectedly, recent studies indicate that PML and the PML-ND play a tumour suppressive role in several different types of human neoplasms in addition to APL. Because of PML's extreme versatility and involvement in multiple cellular pathways, understanding the mechanisms underlying its function, and therefore role in tumour suppression, has been a challenging task. In this review, we attempt to critically appraise the more recent advances in this field and propose new avenues of investigation.

***Keywords*:** PML, cancer, PML nuclear body, cell growth, apoptosis, senescence
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Introduction

The promyelocytic leukaemia protein (PML) has been the subject of intense research for over a decade because of its involvement in acute promyelocytic leukaemia (APL), a subtype of acute myeloid leukaemia (AML). The *PML* gene was originally identified through its location at the breakpoint of the t(15;17) translocation that characterises most cases of APL [1]. This translocation is reciprocal and balanced and fuses the *PML* gene on chromosome 15 with the retinoic acid receptor alpha (RARα) gene on chromosome 17 [1]. The result of this is the generation of two fusion genes, *PML-RARα* and *RARα-PML*, each encoding a fusion protein [1, 2]. PML-RARα, which encompasses the majority of the coding regions of the two genes, is the main oncogenic event of APL, while RARα-PML acts as a facilitator in the transformation process [3-6]. RARα belongs to the nuclear hormone receptor superfamily and activates transcription in the presence of its ligand, retinoic acid (RA) [1, 7] to induce many target genes involved in differentiation [1, 7]. The general view is that PML-RARα acts a dominant negative mutant for both RARα and PML, thus leading, respectively, to maturation arrest and inappropriate survival [1, 8]. However, recent data suggest that the picture is more complex and that PML-RARα can also act as a gain of function mutant [9] (discussed below). Treatment of leukaemic blasts with pharmacological doses of all-trans-retinoic acid (ATRA) results in terminal differentiation and causes remission in patients [1, 10]. For this reason, APL has become the paradigm of differentiation therapy. Nevertheless, a large number of APL patients acquire resistance to ATRA and undergo relapse [1, 10].

Although there are basic aspects of PML biology that remain enigmatic, research in the PML field has moved forward in the last few years, and accumulating evidence suggests that PML has tumour suppressive functions beyond APL in non-haematopoietic tumours.

PML structure

The human *PML* gene is expressed as at least 12 (and potentially more) alternatively spliced mRNAs, each of which encodes a distinct protein [11-13]. Each PML isoform shares an identical N-terminal region, consisting of 570 amino acids, encoded by exons 1-6. All nuclear PML isoforms contain a nuclear localisation signal (NLS) in exon 6, and localise both to the nucleoplasm and to a nuclear oncogenic domain (the PML-ND), a large protein complex tethered by high-order PML multimers. The varied nomenclatures used to describe the PML-ND (PML-body,

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PML-NB, POD, ND10) were generated from disparate fields of research and should be considered as interchangeable. There also exist cytoplasmic PML isoforms deficient in exon 6 and, as such, lacking a functional NLS [14]. A single PML isoform, PML-I, also expresses a nuclear export signal (NES), along with its NLS, and hence is able to shuttle between the nucleus and the cytoplasm.

The N-terminal region of PML contains a Really Interesting New Gene (RING), B-box, coiled-coil (RBCC) motif [11], thereby defining PML as a member of the tripartite motif (TRIM) family of proteins [15]. There are more than 60 TRIM family proteins encoded by the human genome, at least five of which are known to be involved in viral restriction (including PML, which is also known as TRIM19), but many others remain functionally uncharacterised [12]. Interestingly, the RING domain is well established as having E3 ligase activity [16] and this activity appears to extend to other TRIM family members [17], although whether all TRIM proteins can act as E3 ligases remains to be ascertained. Coiled coils are well established for their role in oligomerisation [18], but the function of the B-box is less well understood. The recently solved structure of the first human B-box domain revealed a striking similarity with RING domains and suggests a potential role for this domain as an E4 ligase [19]. However, in the context of the RBCC motif, the B-box is known to assist the oligomerisation mediated by the coiled-coil region [20] and so its precise role remains enigmatic. It is known, however, that the RBCC motif as a whole is important for oligomerisation; for example the RBCC motif of the KAP-1 protein has been shown to exist as a trimer *in vitro* [21]. Also, the spacing, relative to one-another, of the constituent domains of the RBCC motif is precise and highly conserved across the whole TRIM family, suggestive of an important role in defining the overall structure and in coordinating oligomerisation [11]. Indeed, each of these domains is known to be essential for PML-ND formation [22-25]. Despite this knowledge, structural analysis of the PML protein has, thus far, been limited to the individual domains that compose the RBCC motif and there exists only a three-dimensional structure of the isolated RING domain [24]. No structural information is available for any complete RBCC motif or for the exact mechanism of self-association used by the TRIM proteins, which appears so vital for their individual functions.

A further layer of structural and functional complexity is added by small ubiquitin-like modifier (SUMO) modification of PML, which is implicated in multiple aspects of PML function. SUMOylation of PML occurs on three lysine residues (K65 in the RING domain, K160, and K490 in the NLS) [26]. These SUMOylations are critical for the formation of the PML-ND, since PML homo-oligomerises through its RBCC and SUMO-interacting motifs [27], and are critical for the interaction with many PML-ND cargo proteins, including the transcriptional repressor DAXX [28, 29]. In fact, several members of the RBCC/TRIM protein family act as SUMO, rather than ubiquitin, E3 ligases, and there is some evidence that PML may act in a similar manner [30], possibly mediated by interaction with the SUMO E2 enzyme UBC9 [31]. SUMOylation is a highly dynamic process that is efficiently regulated by the members of the ubiquitin-like protein-specific protease family [32]. Interestingly, PML can be deSUMOylated by the SUMO protease SuPR1 and SUMO proteases in general can affect PML subcellular distribution [32, 33]. This finding would suggest that PML SUMOylation is also a very dynamic process.

The cellular roles of SUMOylation are multiple and still not fully understood [34], but this modification is known to be important for regulating subcellular localisation and protein complex formation, both of which are also integral to PML function [35]. The intricate and dynamic nature of PML can be highlighted by various observations relating to its SUMOylation. PML can be modified by any of the three SUMO isoforms, 1, 2 and 3 [31, 36, 37]. SUMO 1 modification tends to be monomeric, but recent evidence suggests that *in vivo* SUMO2/3 oligomers may be covalently linked to PML and that these poly-SUMO chains are integral to PML-ND formation [32, 38]. It is also known that, whilst the majority of SUMO-1 is constitutively conjugated to its substrates, there exists a free pool of SUMO2/3 that becomes substrate-linked on the receipt of various environmental stresses [39], an observation likely to impact on PML biology.

PML isoforms

PML function depends on its ability to interact with, hold as cargo or even directly modify various cellular components. This promiscuous protein is best known for its interactions with other nuclear proteins, but it can also form complexes with ssDNA [40, 41], specific chromatin sites [41-45], viral genomes [46] and plasmid DNA [47]. PML-NDs have been found associated with many other nuclear organelles or domains such as the nucleolus, telomeres, DNA damage foci and Cajal bodies, and are often described as being attached to the nuclear matrix. This promiscuity, in turn, leads to the association of the PML protein with a wide variety of biological processes, including apoptosis, senescence, protein turnover and the regulation of gene expression.

How, then, is it possible for one protein to have such a wide functionality? Part of the answer to this question lies in the fact that human PML is not really a single protein, but,

as mentioned above, it exists as a collection of markedly different protein isoforms, each of which may have highly distinct functions. As has been discussed, the N-terminal region governs PML homo-multimerisation, but it is the variable C-terminal region that appears to determine PML's interactions. The C-terminal region of human PML shows remarkable variety across its various isoforms and since murine cells only express two almost identical isoforms this probably represents recent evolutionary diversification. The expression of a limited number of PML splice forms in mice versus multiple variants in higher vertebrates may reflect an ever-specialised role for PML protein in regulating cell stress pathways [48]. The question arises as to whether a driving force for an evolved suite of PML functions has been to overcome the spontaneous transformation events that are so prevalent in murine cells [49]. The standardised nomenclature for the human isoforms is outlined in Figure 1, which indicates how each protein is spliced from the 13 exons present in the human *PML* gene. PML isoform 1 (PML-I) has the longest C-terminal region, containing more than 300 amino acids, including an exonuclease (EXOIII) domain, and is the only isoform with an identifiable globular domain outside of the RBCC motif. PML-I is also the most highly expressed [48] and probably the oldest isoform, sharing the highest homology with the murine isoforms. It is probable, therefore, that PML-I plays a key structural role in PML-ND formation, but the presence of the EXOIII domain suggests that this isoform may also be important for chromatin tethering and remodelling and for interactions with nucleic acid [41, 47]. Indeed, one specific role of PML is in the direct control of gene expression through associations with particular gene loci such as the MHC gene locus [45, 50]. Apart from the EXOIII domain, the remainder of the PML-I C-terminus is predicted to be unstructured, a common theme amongst the other PML isoforms also (Figure 2). The identification of such unstructured regions in the PML protein is consistent with the promiscuity of its interactions. It is becoming established that natively unstructured or disordered regions are common amongst proteins involved in protein/protein and protein/DNA interactions. It is estimated that 40% of proteins encoded by the human genome contain such regions and this percentage is higher amongst proteins implicated in transcriptional regulation and cell signalling pathways [51]. Structural flexibility provides several advantages to protein interactions, all of which can be applied to PML functionality. For example, disordered polypeptides interact with faster kinetics and with a wider range of binding partners than rigid, globular protein domains [51], and these represent traits that are clearly advantageous to PML.

In support of the idea that individual isoforms have distinct roles, there is growing evidence for isoform-specific protein/protein interactions. PML-II, for example, is known to interact specifically with the adenoviral E4 Orf3 protein [52], whilst PML-IV [53, 54] has been claimed to interact specifically with p53. PML-IV also interacts with

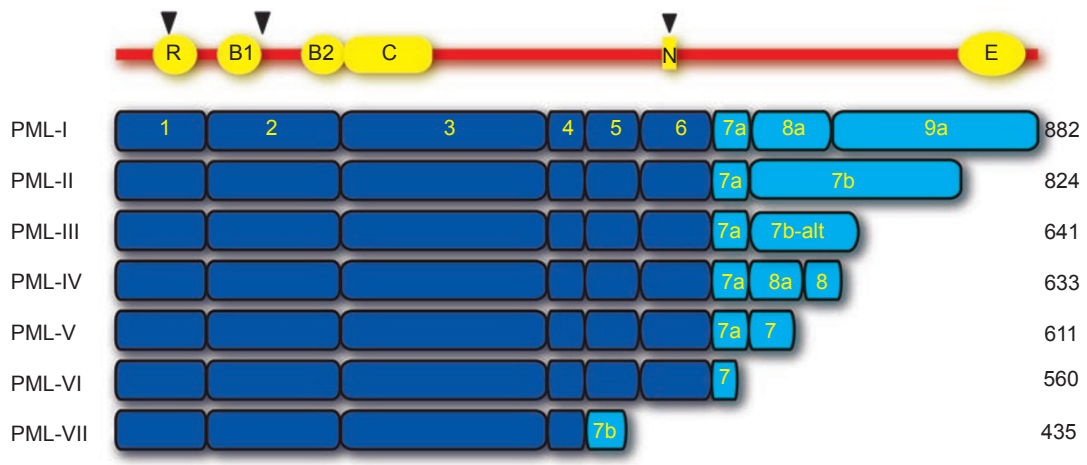

Figure 1 Human cells express a repertoire of PML isoforms. PML protein comprises a set of six nuclear isoforms I-VI encoded by a single gene via alternative RNA splicing. The generalised domain structure is shown (top), indicating positions of the Ring domain (R – amino-acid residues 45-105), Beta box 1 (B1 – residues 124-166), Beta box 2 (B2 – residues 184-230), Coiled-coil (C – residues 229-323) and Exonuclease domain (E – residues ~600-750, exclusive to isoform I). Positions of the nuclear localisation sequence (N) and SUMOylation sites (black arrowheads) are also indicated. The lower panel indicates exon usage for the PML isoforms. Exons 1-6 (dark blue) are common to all nuclear isoforms, the variability in their C-termini being encoded by several alternatively spliced exons as indicated (light blue). The omission of exon 6, which carries the nuclear localisation signal, generates cytoplasmic PML (isoform VII).

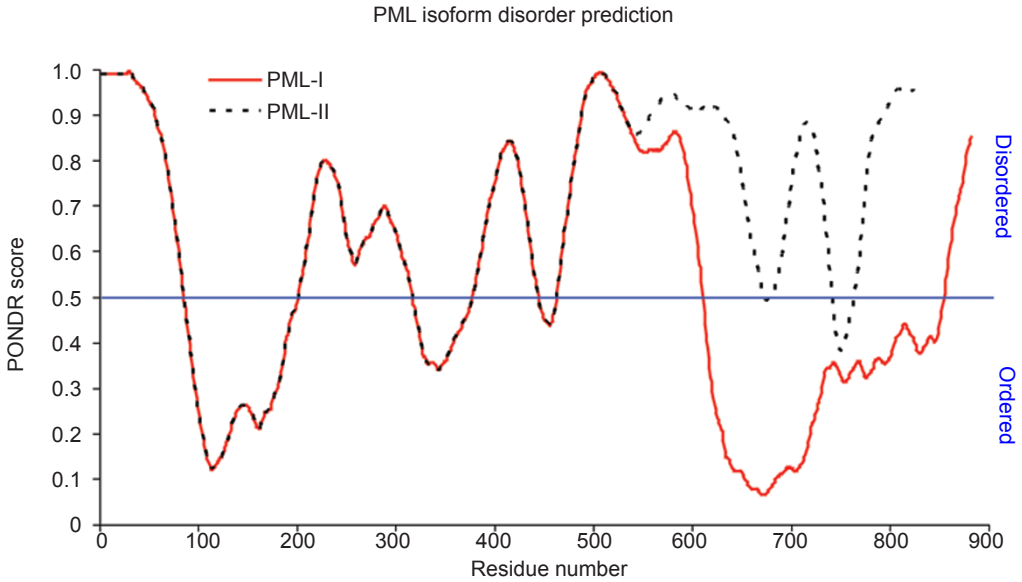

Figure 2 PML isoforms display differential propensities for structural order at their C termini. The PONDR algorithm (http://www.pondr.com/pondr-tut2.html) uses a neural network predictor trained to identify naturally disordered regions in proteins. PONDR is the acronym for 'Predictor Of Naturally Disordered Regions'. PONDR scores below the boundary (0.5) signify a propensity for order, with scores above 0.5 indicating a high likelihood of disorder. The PONDR VL3 disorder prediction for PML isoforms I and II is shown. Extensive regions of disorder occur throughout the C-terminus of PML-II, whereas PML-I has a structured C-terminal region which corresponds to the EXOIII domain.

hypophosphorylated retinoblastoma protein (pRb) [55] and the histone deacetylases (HDACs) [56] and it is through such interactions that PML-IV has also been implicated in specific cellular processes such as the induction of senescence [57]. The C-terminus of PML-I was recently shown to be responsible for its interaction with the nucleolus [58] although the precise specificity and function of this interaction are unknown. Cytoplasmic PML isoforms, meanwhile, have been implicated in TGFβ signalling through direct interactions with the SMAD2 and SARA proteins [59].

These observations underlie several crucial and unanswered questions in PML biology, which must be addressed in order to correctly understand this system. Although it is now known that multiple PML isoforms must accumulate to create a single, functional PML-ND [48], it is not known whether PML's activities are associated with its presence in the nucleoplasm or at the nuclear domain. It is possible, for example, that the PML-ND acts merely as an inert storage site for inactive cargo proteins, such as DAXX, which can be shuttled in and out of the domain, probably by specific PML isoforms, as and when required [56, 60]. This model might explain why PML bodies can be found juxtaposed to other nuclear domains, such as DNA damage foci. Here, signalling proteins such as CHK2, which is known to be modified by ATM at the site of double-strand breaks and then further modified at the PML-ND [61, 62], may be efficiently shared between the two sites. However, PML's role in DNA and chromatin interactions appears to be centred inside the nuclear domain itself as it is here that quiescent viral genomes, ssDNA and telomeres are found [40, 46, 63-65]. These considerations lead to an alternative model, in which the PML-ND provides a microenvironment that fosters specific activities in the proteins with which it associates.

It is also apparent that PML functions differently and interacts with different partners in different cell lines, or even within the same cell population at different times. In ALT cells, for example, PML associates with telomere foci [64], whilst in senescent cells it often rings the nucleolus [58]. Studies of relative levels of PML-I RNA in various tissues, tumours and cell lines indicated a diverse level of expression for this isoform [48]. The loss of PML-I expression (as well as the loss of PML expression in general) is often associated with transformation [66]. It thus seems likely that isoform expression levels titrate PML function in different cell environments, including the adaptive response to various types of cell stress. Similarly, the intracellular localisation of PML varies between cell lines. The majority of PML in human diploid fibroblasts, for example, lies within PML bodies, whereas various transformed cell lines, such as HCT116 and HEK293, appear to contain a greater pool of nucleoplasmic PML (BF, TR, unpublished

observations).

The PML network

PML functionality, although enigmatic, remains intrinsically linked to its interactions with other cellular macromolecules. The detection of a wide range of cellular stresses alters the expression, modification and localisation of PML, which, in turn, alters its capacity for interaction. All of these factors combine to determine the function of PML.

In order to understand the function of PML, it is therefore essential to characterise its interactions both before and following specific stresses. So far attempts to do so have been hampered by weak-binding antibodies and by PML's dynamic nature, which results in rapid deSUMOylation following cell lysis [67]. Despite this, the identification of several mitogenic signalling proteins as novel PML interacting partners has helped to underline PML's role in growth and tumour suppression. Akt, mTOR (the target of rapamycin protein) and PLCγ [68-70] have all been recently identified as PML binding partners. Thus, as will be discussed later, PML has been shown to be a member of a signalling network, negatively regulating the nuclear content of pAkt via dephosphorylation by PP2A, and hence supporting the accumulation of nuclear FOXO3A and the resulting transcription of the cell cycle inhibitor $p27^{Kip}$ and the pro-apoptotic factor Bim [70]. The modulation of such signalling pathways is a novel aspect of PML biology, but fits well with the paradigm that PML, or PML bodies, provides the micro-environment for many varieties of protein modifications and so regulates a wide spectrum of cellular behaviour.

The PML-ND

The PML-ND is still something of *terra incognita* on the cell biology map. We do not yet have a blueprint for how or where in the nucleus to construct PML-NDs. On top of this, heterogeneity of PML-ND morphology and composition can exist within the same cell nucleus, which may be driven by contributions from different PML isoforms. From a biochemical perspective, the fabric of the PML-ND is SUMOylated PML, which imparts an exquisite sensitivity to cell injury. Stress deforms, fragments and disperses the PML-ND, sending its cargo proteins to varying fates that provide little chance of capturing their peptide signatures by classic biochemistry. PML-ND modifications appear to be driven by stresses that can sever the chromatin fibres that anchor PML-NDs to the matrix, as well as by activated injury response pathways that alter PML's phosphorylation or SUMOylation status [67]. The technical challenge posed by the frailty of PML-NDs has often been sidestepped by analysing more robust ectopic species. This approach revealed that all nuclear isoforms of PML, including a non-SUMOylatable mutant, could form homotypic PML-NDs [28]. A significant drawback to these experiments, however, was the realisation that ectopic human PML-NDs do not resemble their endogenous counterparts in size, stress sensitivity nor even, in many cases, morphology [71, 72]. Even the authenticity of an ectopic murine PML-ND is suspect given that nucleoplasmic PML is often drawn into the PML-ND, thereby skewing its molecular composition. This composition may be critical if macromolecular crowding plays any part in driving PML's effector functions [73]. Despite these caveats, mass spectrometry of PML co-immunoprecipitates has been used to identify PML partners and ND cargo proteins, one of which was PLCγ-1 [69]. This discovery is noteworthy given that PLCγ-1 shares a substrate (PIP_2) with PI3K, an upstream activator of pAkt. The identification of both Akt and PLCγ-1, bound to PML, illustrates the potency of PML and PML-NDs in intervening in multiple pathways involved in cellular homeostasis. This finding also illustrates the variety of lipid metabolising proteins that have been found in the nucleus, many with roles autonomous to those played out in the cytoplasm [74].

A significant conceptual advance in our understanding of PML-ND formation came with the identification of a SUMO interacting motif (SIM) in the PML protein. PML's SIM, together with its RING domain, is critical for PML-ND formation [27] and provides a mechanism to interweave SUMO modified PML and third party SUMOylated proteins. Within this higher-order PML structure other scaffold proteins are found. Thus, the transcriptional repressor DAXX uses a SIM to tether and silence SUMOylated transcription factors at PML-NDs [29]. These data reveal a hierarchy of signalling nodes, each providing a niche to drive or repress effector functions. A simplistic mechanism to compartmentalise repressive and activating PML-ND chaperone functions could be to partition these functions to the inner and outer surfaces of the domain, respectively. Exceptions to this segregation almost certainly occur, and may include the oncogene-driven acetylation of p53 [75]. The discovery of SUMO proteases targeting SUMOylated PML and the PML-NDs has added a further level of complexity [32, 33]. It is therefore plausible that changes in PML-ND composition or structure could be controlled by the balance between SUMOylation and deSUMOylation of PML and other PML-ND constituents. This area of research awaits further investigation.

PML-ND alteration by stress

Several endogenous and exogenous types of stress have been shown to alter PML-NDs. These observations led to

experiments that attempted to link cell fate with PML-ND morphology. Initially the emphasis was on using sources of exogenous stress to promote PML-ND modification; more recently PML-ND modification by endogenous stress has also been recognised [67]. Varying levels of endogenous stress may explain the regular and abundant PML-NDs that typify early passage fibroblasts [72] versus the low numbers of fragmented PML-NDs that typify many cancer cell lines. Collectively, data accrued for PML-ND function after stress recognition point to a protective homeostatic role. It is therefore not surprising that PML-ND distribution and composition following differing challenges reflect these variables. In telomerase negative cancer cells, for example, the imperative to maintain telomeres by recombination is supported by a subset of PML-NDs (in this case called ALT bodies – see sections below). Likewise, clastosomes, rich in ubiquitin conjugates and proteasome subunits, are PML-NDs that metabolise misfolded proteins [76].

The responses of PML-NDs to different types of DNA damage illustrate some of the complexities of PML-ND function. The activation of the ATM-CHK2 and ATR pathways following ionising radiation plays an important role in the reorganisation of PML-NDs after genotoxic damage [72, 77]. Activated ATR also regulates a different stress signal that controls MDM2 sequestration by PML at the nucleolus [78]. The demonstration that caspase-2 can also reside within PML-NDs [79] has led to the fresh speculation that this initiator caspase is part of a nuclear apoptosome at the PML-ND [80]. Though the finding that Sp100 also contains a CARD domain may be taken as supporting evidence of this [81], it is equally likely that caspase-2 is sequestered in the PML-ND and that Sp100 plays some part in this sequestration. Note that caspase-2 is ordinarily expressed at the Golgi, and stimuli that would cause its re-localisation to PML-NDs have yet to be identified. The striking juxtaposition of PML-NDs with irradiation induced foci (IRIF), together with the delayed timing of this event and its relationship to the delivered radiation dose, may point to a role for PML in the auditing of DNA damage prior to selecting cell fate [72], or to its participation in chromatin modification. A precedent for PML-ND function in chromatin regulation has been provided by patients with centromeric instability and facial dysmorphy (ICF) syndrome, which is caused by defective DNA methylation [41, 44]. The expression of the EXOIII domain in PML-I may even be a relic of PML activity in metabolising nucleic acid, though it is likely that this function has now been lost [58].

Different types of genotoxic stress affect the PML-NDs differently. For instance, upon UV irradiation or cisplatin treatment PML-NDs dramatically reorganise into microspeckles, where the transcription factor c-Jun accumulates [8, 82, 83]. Unlike ionising radiation, UV damage tends to elicit a response mediated by ATR, which promotes MDM2 sequestration by PML at the nucleolus [78]. Overall, these findings suggest that PML-NDs have a close relationship with the DNA damage machinery and may integrate DNA damage signals with other stress signals (e.g. through c-Jun). Microspeckle formation contrasts with the PML response to other stresses such as heat shock and viral infection. Heat shock also reorganises PML, but by an almost instantaneous deSUMOylation event which, if prolonged, disperses PML along with SP100 and DAXX [84].

Studies from the fields of neurodegeneration and virology have also illuminated different aspects of PML-ND function. There is an extensive literature showing PML-ND disruption by protein aggregates and viruses. Collectively these data show that, in general, PML-NDs detect unusual cellular events, including unscheduled accumulations of proteins (aggregates or viral capsids) or nucleic acids (naked viral material, open chromatin or plasmid DNA). Interestingly, exposure to interferon, which is part of the cellular response to viral infection, greatly enhances PML expression and results in increased numbers of enlarged PML bodies and, depending on the interferon used, higher levels of nucleoplasmic protein [85, 86]. The question remains as to how PML-NDs perceive protein aggregates or nucleic acids. Elegant experiments from the Everett laboratory have shown that PML-NDs readily detect the ingress of virus and that PML protein re-localises to the site of viral entry [87]. Experiments based on fluorescence recovery after photo-bleaching demonstrate the rapid exchange of PML protein between matrix-bound PML-NDs and the nucleoplasm and may point to a mechanism for stress detection [87]. PML shuttling between compartments may serve to alert the PML-ND as to local concentrations of protein and nucleic acid, and these transitions may be accelerated by the detection of pathogen-associated molecular patterns (PAMPs) or the activation of injury recognition pathways.

In the case of protein folding diseases, nuclear inclusions of aggregated protein rapidly recruit PML-NDs [88] and in some circumstances may seed within PML-NDs, eventually rupturing the domain [89]. Whilst the propensity for PML-NDs to detect and sequester ectopic protein often leads to the false positive designation of cargo proteins, this intrinsic property of PML clearly demonstrates its important homeostatic function. Interestingly, *in vitro* reconstruction of the genesis of aggregates of ataxin-1 within nuclei demonstrated not only the avidity of PML-NDs for these aggregates, but also that PML-NDs bound to the aggregates completely lost their capacity to respond to other stress stimuli such as heat shock or ionising radiation [90]. This observation thus adds defective PML-ND

function to the list of mechanisms that might be ultimately responsible for the neuronal cytotoxicity associated with protein aggregate diseases.

Biologic functions suggested by PML-ND modification

PML-NDs have, by virtue of their modifications after stress, given tantalising clues as to their biologic role, though the observational nature of these data has often led to their neglect. Thus, ten years ago it was reported that senescent fibroblasts express unusually large fibrillar PML-NDs [91], but only recently has it been shown that enlarged PML-NDs in senescent cells can sequester nucleolar proteins [58, 92] and that the C terminus of PML-I and its EXOIII domain, promote the stress-induced nucleolar redistribution of PML [58]. The degradation of PML-I by excessive casein kinase II activity in cancer cells would disrupt this homeostatic function and could exacerbate cellular injury [93] (see below). It is now clear that PML can be distributed within cells in several patterns other than classical PML-NDs, potentially causing, or reflecting, different biological and pathological functions.

PML and the nucleolus

The link between nucleoli and PML-NDs is a fascinating one. In an early report, PML and PML-ND cargo proteins were seen to translocate to the nucleolus in cells treated with proteasome inhibitors [94]. We noted that PML may re-localise to the area of chromatin immediately surrounding the nucleolus after the delivery of other stresses including heat shock or IR. The role of this translocation is unknown though several possibilities exist. Proteome analyses of the nucleolus have revealed a surprisingly large number of DNA repair and tumour suppressor proteins [95]. Many of these are mobilised both into and out of the nucleolus after stress which may accompany the partitioning of nucleolar compartments [96]. Condemine [58] proposed that PML-NDs actively involved in proteolytic regulation are recruited to nucleoli. PML translocation to the periphery of the nucleolus may also deliver effectors that repress ribosome biogenesis, as part of cellular shutdown. Alternatively, the perinucleolar expression of PML may signify repairs to nucleolar DNA.

PML-NDs and fibril tracks

One of the principal un-explored observations of PML-ND function is 'tracking' of PML fibrils: sinuous threads decorated with PML microbodies that can extend the length of the nucleus (Figure 3). In our hands (TR, unpublished observations), the most prominent examples of tracking have been seen in senescent diploid fibroblasts. The existence of a fibrillar nuclear matrix is contentious [97] though elegant descriptions of aberrant matrices in cancer cells would suggest that a normal matrix exists and supports a tumour suppressive function [98]. The extent to which domains of PML-NDs would traverse the matrix tethered to fibrils is open to debate. The dispersal of monomeric or oligomeric PML from PML-NDs into the nucleoplasm shows none of the linearity that would be expected of protein traversing a fibrillar network. Though mobile domains of PML have been reported, their movement may have reflected the action of Sp100 protein rather than PML [99]. Of relevance to our observations of tracks in senescent cells are the ubiquitin bridges described by Condemine [58], which are especially prominent in cells treated with proteasome inhibitors. These ubiquitin bridges resemble the 'comets' of PML that we have seen between PML-NDs and polyglutamine aggregates [100]. As proteasome inhibition can arise in cells expressing polyglutamine proteins as well as in senescent cells (discussed later) [101], we could suggest that a directionality for PML and PML-ND movement may be provided by concentration gradients of ubiquitinated substrates. The possibility that both poly-ubiquitin and PML attach to a shared structure in the nuclear matrix has been made [58]. Perhaps this arrangement would allow

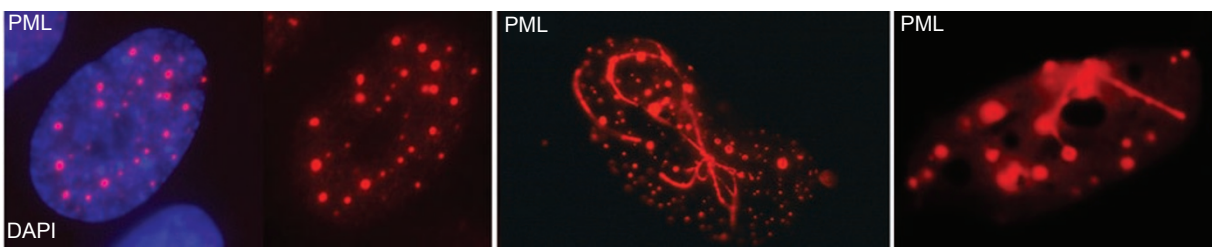

Figure 3 PML tracking in senescent fibroblasts. Immunofluorescence in actively growing normal diploid human fibroblasts (left panel) exhibits the standard, punctate PML staining common to many cell types both *in vitro* and *in vivo*. Following the transition to senescence achieved by degradation of the SV40 large T antigen, and without recourse to proteasome inhibitors, these cells reveal endogenous PML tracks (right panels).

PML protein to monitor the contents of the interchromosomal space in a fashion analogous to another TRIM protein in the cytoplasm, TRIM5α [102]. One constant in the study of PML-NDs is that the observation of atypical distributions of these domains, in uninfected cells, has led to significant discoveries concerning stress pathways and homeostasis. To overlook PML-ND distribution is to lose a critical clue as to the prevailing effector functions active in each cell.

PML and the centrosome

Several lines of evidence suggest that PML isoforms can have specialised functions. In particular, a recent report demonstrated a role for PML-III in centrosome duplication and genome stability [103]. *Pml*$^{-/-}$ MEFs were shown to have amplified centrosomes in more than 35% of cells examined. In normal cells centrosome duplication occurs in the G1/S phase of the cell cycle. In primary and immortalised MEFs lacking PML, the mechanism that regulates centrosome reduplication is impaired, allowing unscheduled duplication in the absence of DNA replication. Isoform-specific siRNAs were then used to demonstrate that PML-III was the isoform responsible for the control of this cellular process. Furthermore, PML-III was shown to bind to and inhibit Aurora A, a serine/threonine protein kinase associated with the centrosome and implicated in the regulation of mitotic microtubules and centrosome maturation [103]. Interestingly, co-localisation of PML-III and γ-tubulin (a central component of the centrosome) was not impaired in APL cells, as shown using a PML-III specific antibody. This indicated that, although other PML isoforms form heterodimers with PML-RARα and become diffusely distributed in the nucleus of APL blasts, PML-III apparently retains its normal functions through its interaction with the centrosome in the cytoplasm [103]. Aurora A phosphorylates and destabilises p53 [104], hence inhibiting the transcription of p21 and so permitting activation of the Cdk2/cyclin E complex, which is essential for regulating centrosome duplication. Taken together, these data suggest that PML-III controls centrosome duplication by regulating Aurora A activation, a potent demonstration of PML isoform-specific function in genomic stability. These conclusions have recently been challenged, as colocalisation of PML-III with the centrosome was not observed in Chinese hamster ovary cells or *Pml*$^{-/-}$ MEFs that stably expressed PML-III, or in HeLa cells, MRC5 primary human fibroblasts, the APL-derived cell line NB4 or APL primary cells [48]. Discrepancies between the two studies may be explained by the use of different antibodies or immunofluorescence conditions. Clearly, PML localisation to centrosomes needs reassessment.

Cytoplasmic accumulation of PML

Whilst the nuclear function of PML has been the focus of much research in the past decade, several lines of evidence suggest that PML plays additional roles in the cytoplasm. As mentioned above, splice variants of PML localise to the cytoplasm and PML isoform I contains a functional NES [11, 58]. Interestingly, cytoplasmic PML isoforms are induced by TGFβ and mediate its growth suppressive functions [59, 83] (extensively discussed in [14]). During the cell cycle, the number and shape of PML-NDs are dramatically altered at the S and M phases [105-108]. In particular, during mitosis, PML re-distributes to cytoplasmic structures called mitotic accumulation of PML proteins (MAPPs), which are retained in the cytoplasm in early G1 phase [77, 109]. MAPPs have been proposed to regulate the nuclear formation of PML-NDs after mitosis [109]. Overall, these findings suggest that the balance between nuclear and cytoplasmic PML could be involved in regulating its tumour suppressive functions. Indeed, cytoplasmic accumulation of PML has been found in human neoplasia, such as in APL and skin tumours due to mutation or other mechanisms, thus suggesting it could play a role in tumorigenesis [58, 66] (see the paragraphs below).

PML tumour suppressive functions

Although the tumour suppressive role of PML was immediately suggested by the identification of the RARα fusion protein in APL, clear evidence for its mechanism has emerged more slowly. PML has been shown to interact and co-localise with a very large number of proteins, but the physiological role of many of these interactions remains unproven as they have been observed only under conditions of enforced expression of ectopic PML. Also, many such interactions have not been shown to be relevant for tumour suppression. Here we focus only on PML interacting partners and regulated pathways with a clear role in oncogenesis.

Cell cycle and cellular senescence

Cellular replicative senescence is a permanent exclusion from the cell cycle. It is considered to be one of the main barriers against tumorigenesis, and there is good evidence that it is caused by induction of the DNA damage response [110-112]. Most data on the induction and regulation of senescence have been obtained using cell culture systems [110-112] and, more often than not, study damage-induced senescence that may still be reversible. The term 'senescence' seems to be applied to a vast number of disparate cell states and this usage, as well as the absolute assumption of a G1 arrest, is problematic. However, senescence-associated markers have also been demonstrated *in vivo* in normal

tissues and in pre-neoplastic lesions, thus suggesting that senescence can occur *in vivo* and is activated in the early phases of tumorigenesis [110-112]. Depending on the cell type, it requires activation of p53 alone or both p53 and pRb [112, 113] and it can be initiated by inappropriate activation of oncogenes. Several studies have clearly shown that overexpression of PML induces cell cycle arrest in cancer cell lines [114-116]. This was associated with increased levels of pRb, and an arrested cell cycle, principally at the G1 phase [116-118]. Interestingly, PML potentiates the repressive function of pRb [119]. Moreover, endogenous PML expression is augmented both in G1 arrested cells and when p53 is over-expressed [116]. More recently, it has been shown that expression of the PML-IV isoform induces replicative senescence in primary human and mouse fibroblasts [57, 120]. This requires endogenous PML [57] though, perhaps surprisingly, not the PML-ND [57]. In human fibroblasts, pRb, but not p53, is required for PML-IV-induced senescence [120].

One of the problems associated with PML over-expression studies is that PML tends to form high molecular weight aggregates that could have non-specific effects on the cell cycle. For this reason *Pml–/–* cells and mice represent a better model with which to study PML function [121]. Knockout of PML results in increased proliferation in mouse embryonic fibroblasts and a reduced sensitivity to the growth suppressive effects of RA [121, 122]. PML is also required for senescence induced by expression of the mutated G12V H-ras oncogene and modulates p53 activation through acetylation in a CBP-dependent mechanism [75] (Figure 4). This acetylation is inhibited by PML-RARα because of its dominant negative effect on PML [123]. Interestingly, PML is induced by p53 activation [124] and is a target of p53, suggesting the existence of a positive feedback that may lock affected cells into permanent replicative arrest [125]. Both in humans and mouse, PML interacts with and colocalises with pRb [119, 120, 126], but the non-murine isoform, PML-IV, induces a senescence which is pRb dependent [120] (Figure 4). PML over-expression results in the accumulation of hypophosphorylated pRb [116-118] and potentiates pRb function through a HDAC-dependent mechanism [119]. Finally, PML-RARα blocks pRb function, thus suggesting that the inactivation of pRb may be involved in APL pathogenesis [119]. These findings demonstrate that PML is involved in the induction of cellular senescence, and that this is achieved through regulation of the tumour suppressors, pRb and p53. It remains to be addressed how PML action is directed towards either tumour suppressive pathway.

Recent studies have added a further level of complexity by proposing that PML-NDs are involved in chromatin reorganisation during senescence [127-129]. The senescent phenotype necessitates the formation of specialised domains associated with transcriptionally silent chromatin. These domains, called senescence associated heterochromatic foci (SAHF), are thought to repress the expression of genes involved in cell cycle progression [130]. Interestingly, a number of E2F target genes acquire heterochromatic features during senescence, and active pRb binds their promoters [130]. Furthermore, pRb is required for SAHF formation and the repression of E2F target genes during senescence [130]. Both SAHF and PML-NDs are induced during cellular senescence. The temporal and physical relationship between the two structures has been,

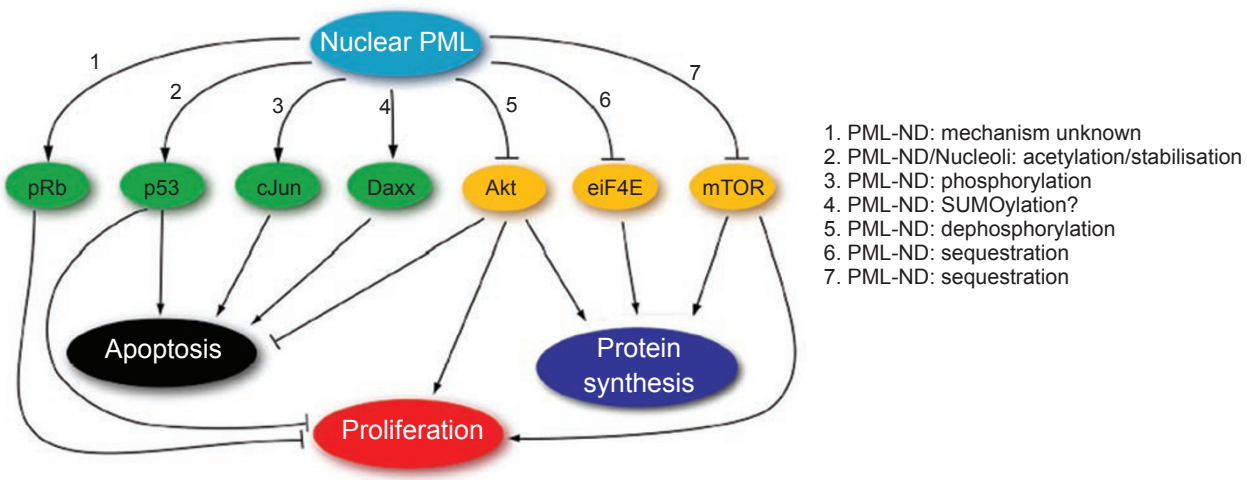

Figure 4 PML interacting proteins and their roles in tumorigenesis. PML can activate several tumour suppressors and inhibit oncogenic pathways. Boxed in green are proteins with tumour suppression activity, while proteins boxed in yellow positively affect metabolism/proliferation and inhibit cell death. The text summarises current knowledge of the underlying mechanisms.

in part, elucidated by the work of Peter Adams' laboratory [127, 128, 131]. These studies showed that, prior to SAHF formation, a known chromatin regulator, HIRA, enters PML-NDs and transiently colocalises with heterochromatin-1 (HP1) proteins, which are involved in the formation of heterochromatin. Disruption of either HIRA or PML function results in impaired SAHF formation, thus suggesting that transient accumulation of HP1 in PML-NDs is required for SAHF generation [127]. As mentioned above, the modified PML-NDs found in the centromeric ICF syndrome also contain multiple HP1 isoforms, in addition to the chromatin remodelling α-thalassemia/mental retardation X-linked syndrome (ATRX) protein [41, 44]. The identification of giant PML-NDs in ICF cells at the G2 phase of the cell cycle has been used to propose a model for PML-ND function in the epigenetic control of chromatin during cell cycle [41]. A similar role could be proposed for chromatin modelling during senescence.

One possible mechanism for these changes in chromatin would be that chromatin modifiers undergo post-translational modifications as they transit through PML-NDs or ICF bodies, which are in turn required for SAHF formation. In this respect, phosphorylation of one of the HP1 proteins, HP1γ, is known to be required for its localisation to SAHF but not to PML-NDs, a circumstance easily explained if its phosphorylation occurs in PML-NDs to promote its loading into SAHF [131]. Perhaps the colocalisation of pRb in the PML-NDs of senescent cells [118, 120] also permits the post-translational modifications necessary for the repression of E2F release and subsequent chromatin alteration. Overall, these findings support the notion that PML-NDs are credible candidate sites for the modification of tumour suppressors and other proteins involved in senescence.

Apoptosis

Pml and p53 In addition to the inhibition of cell cycle, several tumour suppressors initiate apoptosis, and there is good evidence that PML also activates this process. Thus, PML over-expression induces apoptosis by a process blocked by the pan-caspase inhibitor zVAD [132], and splenic lymphocytes and thymocytes from Pml–/– mice show barely half the capacity of wild-type cells to initiate apoptosis. It is notable that this applies to apoptosis induced by both ionising radiation (a stimulus whose lethality depends largely on p53 in these cell types) [54, 133, 134] and the cytokine death-receptor pathway [135]. These observations suggest that the modulation of apoptosis by PML affects a step in the apoptosis effector pathway that is common to both stimuli. At present it is far from clear what this might be. More recent studies have proposed that PML-dependent p53 regulation during apoptosis is multifaceted. For instance, PML can regulate p53 degradation through the inhibition of Mdm2, which is the major p53 E3 ubiquitin ligase [78, 136-140] (Figure 4). In particular, PML has been shown to co-localise with and sequester Mdm2 into nucleoli, thus promoting p53 activation upon DNA damage [78].

Overall, the evidence presented in this and the previous section suggests that PML can activate p53 by different means, acetylation and stabilisation. However, it is not clear what triggers either of the two mechanisms. It could be hypothesised, for example, that different PML isoforms could exert different functions. In this respect, PML-IV is the main isoform able to interact with p53, but it is not clear whether a specific isoform is required to sequester Mdm2 to nucleoli. As different types of DNA damaging agents have been used in the papers discussed above, it is plausible that specific isoforms could be induced or activated depending on the stimulus used. For instance, PML-I contains a nucleolar localisation sequence that promotes its accumulation into nucleoli upon stress and cellular senescence [58]. It remains to be determined whether PML-I is required for p53 stabilisation upon cellular stress.

Additional mechanisms Another PML-ND component is DAXX, a protein originally cloned as a CD95-associated factor [141-143]. However, the cytoplasmic localisation of endogenous DAXX and its association with CD95 are not clear and more recent studies have doubted the relevance of this association for the induction of cell death ([144] and references cited therein). The requirement of PML for DAXX-induced cell death suggests the existence of a novel nuclear complex for apoptosis induction [144] (Figure 4). Possible mechanisms, for which there is some evidence, include the activation by DAXX of stress-associated kinases such as the apoptosis signal-regulating kinase ASK-1 [145] or zip kinase [146, 147]. Many aspects of this pathway remain poorly defined, including the implied transfer of DAXX from nucleus to cytoplasm, although several candidate mechanisms have been described [145, 148-150]. Nonetheless, DAXX can have both pro- and anti-apoptotic functions, depending on different factors [144]. It has been shown to act as a chromatin modifier through its interaction with HDACs and other proteins involved in epigenetic mechanisms [29, 144]. DAXX is recruited to PML-RARα through its ability to bind SUMO, and this has been proposed to be required for transformation [151]. In addition, DAXX has been found to promote the Mdm2-mediated degradation of p53, which would support its anti-apoptotic function [152]. It would be tempting to speculate that PML could block DAXX-mediated p53 degradation by sequestering DAXX into PML-NDs. These findings suggest that, depending on different partners or localisation patterns, DAXX may exert either tumour-promoting or

tumour-suppressive functions. Clearly, the field is in need of animal models to test this and other hypotheses.

PML has also been shown to interact with c-Jun upon UV irradiation and to modulate its pro-apoptotic function through c-Jun-N-terminal kinase (JNK)-dependent phosphorylation [83, 153] (Figure 4). JNK activation provokes dramatic PML-ND re-organisation, which leads to the formation of multiple microspeckles positive for both c-Jun and PML [153]. This may be another manifestation of the function of PML-NDs as sensors of DNA damage. Formation of UV-induced microspeckles has been shown to be dependent upon p53, although this has not been confirmed in vivo [82]. Finally, as described above, PML can limit the availability of phosphorylated Akt through its targeting to PML-NDs and its PP2A-dependent dephosphorylation [70] (Figure 4). This in turn results in activation of forkhead transcription factors and the transcription of proapoptotic Bim and the cell cycle inhibitor $p27^{kip1}$. This connection has functional implications in vivo, as Pml inactivation promotes tumour formation in the prostate in a mouse heterozygous for Pten, which is the major cellular inhibitor of the PI-3K pathway (see below).

Regulation of protein synthesis

In the last few years several seminal studies have demonstrated that regulation of translation appears to be a crucial step in tumorigenesis [154, 155]. A number of papers have proposed that PML can regulate translation through its ability to interact with the eukaryotic initiation of translation factor 4 E (eIF4E) [156, 157], which leads to the nuclear retention of eIF4E and inhibition of its role in mRNA export. This results in the sequestration of a number of transcripts involved in cell cycle regulation and a reduced proliferative capacity. In particular, cyclin D1 mRNA has been shown to be one of the major targets of the PML/eIF4E complex [156, 157] (Figure 4).

Another twist in this area of investigation came from a recent paper by the Pandolfi group, which showed that PML regulates translation through its interaction with mTOR [68] (Figure 4). In particular, PML regulates the rate of translation of the hypoxia inducible factor 1 alpha (HIF1α) through its ability to alter mTOR function and its localisation to PML-NDs. Consequently, in PML-deficient mice HIF1α translation is increased, which augments neoangiogenesis and increases tumour vascularisation. As already mentioned, previous work from the same group demonstrated that Akt was inhibited by PML through a PP2A-dependent dephosphorylation [70], which would suggest that this could also inhibit Akt-dependent mTOR activation (Figure 4). However, mTOR inactivation appears to be tuberous sclerosis 2 (TSC2)-independent, indicating that Akt is not a main player in this context [68]. This could be proven by testing whether hyperactivation of mTOR in PML-deficient cells is blocked upon inhibition of Akt. It would also be of interest to determine whether mTOR and Akt are present in the same or different subsets of PML-NDs. Finally, it is not clear whether increased translation of HIF1α in *Pml* null cells relies on eIF4E, which is activated by mTOR and inhibited by PML [156], and if PML induces nuclear retention of HIF1α mRNA. In summary, increasing evidence suggests that PML is involved in the regulation of translation and potentially metabolism through nuclear accumulation of different components of the PI3K/Akt pathway. However, little effort has been made to establish the connections between the different components of this pathway in the context of PML-dependent regulation.

Another way by which PML could regulate cellular metabolism is through its connection with p53. For instance, p53 regulates metabolism and autophagy through its target gene TIGAR [158, 159]. Therefore, it would be plausible that PML also regulates this novel p53 function.

Genomic stability

Tumour suppressors can limit tumour progression by controlling genomic stability. PML has been implicated in this cellular function through its ability to target effectors of the DNA damage pathways to PML-NDs or other structures. For instance, the DNA helicase Bloom protein (BLM) localises to the PML-NDs, and PML loss results in increased sister chromatid exchange, which is normally repressed by BLM [160]. It appears that BLM can shuttle between PML-NDs and sites of DNA damage, and that this process is regulated by its SUMOylation [161]. PML also co-localises and interacts with MRE11, which is involved in the regulation of the DNA damage response [162, 163]. MRE11 is released from PML-NDs upon DNA damage and localises to DNA damage foci, thus suggesting that PML regulates the availability of MRE11 [162]. The impact of PML inactivation on MRE11 function is, however, unclear. PML/MRE11 localisation is also observed in cancer cells that maintain their telomeres by a telomerase-independent 'alternative mechanism' (ALT; see below). Interestingly, two other DNA helicases localise to PML-NDs, Werner (WRN) and RecQL4, which, like BLM, are mutated in human syndromes that display increased genomic instability [164, 165]. Again, the functional involvement of PML-NDs in processes regulated by these helicases is currently unknown.

Another potential role for PML in the regulation of genomic stability is suggested by the observation that the chromatin-remodelling ATRX protein can localise to PML-NDs [166, 167]. ATRX appears to shuttle between heterochromatin and PML-NDs, and this movement is altered by mutations found in ATR-X patients [168]. Another

PML-ND component, DAXX, has also been found associated with ATRX, in both PML-NDs and heterochromatic foci ([144] and references cited therein). It is, however, still unclear if and how DAXX and PML can regulate ATRX function.

Anti-viral functions

A vast literature has detailed the disruption of PML-NDs wrought by virus infection, particularly by the oncogenic DNA viruses [reviewed in depth in reference 168]. Though extensive coverage of this literature is beyond the scope of this review, we should point out that we still have very little idea as to the PML-ND-centric pathways that are activated in order to protect the cell from infection. PML-ND detection of viral ingress [64] and inhibition of viral replication are clearly functions that are countered by several viral proteins and promoted by the interferons. Future work will almost certainly show that viral alterations to PML-ND function serve the virus throughout its lifecycle and that the efficacy of the PML-ND response to other stresses may be titrated by the activities of viral proteins.

A role for PML in cancer

Most research in the PML field focused for several years on studying the impact of the APL translocation on the function of PML and other PML-ND components. To date, it is not completely clear whether PML inactivation or disruption of PML-NDs is an essential step in the pathogenesis of APL. Moreover, only recently have a number of studies attempted to determine if PML acts as a tumour suppressor in cancers other than APL. In this section, we attempt to critically discuss the available data on the role of PML in tumorigenesis.

APL

One of the open questions in the field of PML and APL research is whether PML inactivation is required for APL leukaemogenesis and to what extent the interaction between PML and PML-RARα is instrumental for transformation. PML-RARα homo-oligomerises to form high molecular weight complexes, which are required for transformation both *in vitro* and *in vivo* [9, 169, 170]. In addition, PML-RARα is able to interact with PML ([8] and references cited therein). Loss of PML in a mouse model of APL causes acceleration and increased incidence of the disease, thus suggesting that inactivation of PML is important for disease progression [171]. In contrast, *in vitro* studies have shown that a PML-RARα mutant unable to bind PML or disrupt the PML-NDs is capable of transforming haematopoietic cells [9, 169, 170]. Thus, clear differences exist between *in vivo* and *in vitro* studies. This is confirmed by the study from Sternsdorf and co-workers [9] that clearly showed that fusing an unrelated homodimerisation domain to RARα creates a chimaeric protein (p50-RARα) with transforming potential *in vitro* but not *in vivo*. This finding indicates that the PML portion of PML-RARα, and its potential to regulate the PML-ND, is required for transformation *in vivo*. However, loss of PML in cells expressing p50-RARα does not result in full-blown leukaemia, indicating that combined PML inactivation and RARα homodimerisation cannot phenocopy the activity of PML-RARα. Given these findings, how does one explain the observation that PML inactivation accelerates PML-RARα-mediated APL progression [172]? One possibility would be that through binding to PML, PML-RARα could induce growth suppression, and therefore lack of PML would unleash the full transforming potential of PML-RAR. PML-RARα is growth suppressive in most haemopoietic cell lines, although it is not known if this is dependent on its interaction with PML [173]. On the other hand, it is not clear why PML-RAR would require its PML moiety to induce transformation *in vivo*, other than for its oligomerisation capacity. One possibility is that PML-RARα, through binding to PML, could hijack a number of PML-ND components required for chromatin remodelling and transcriptional repression. In this fashion PML-RARα may act both as a dominant negative and gain-of-function mutant. Supporting evidence for this hypothesis is the finding that the PML-ND component DAXX, which is involved with PML in the regulation of cell death, appears to be required for transcriptional repression and (potentially) transformation by PML-RARα? [151]. Interestingly, a recent report showed that the *PML* gene is found translocated to the *Pax5* locus in B-Acute lymphoblastic leukaemia [174]. This is the first report of a translocation involving PML, other than in APL. The main question would be whether similar mechanisms are exploited by this fusion protein to promote transformation. Overall, these findings support the notion that disruption/modification of PML and the PML-ND can be instrumental in disease progression in APL and possibly B-ALL.

As mentioned above, in APL one *PML* allele is involved in the t(15;17) translocation of APL, while the other allele is apparently unaffected. However, a recent study has shown that the second *PML* allele can also be mutated [175]. The first mutation, Mut1, is a deletion, 1272delAG, in exon 5. The second DNA variant, Mut2, is a splice site mutation that causes a frameshift and splices out exon 4 from the mature transcript. Both mutations produce a stop codon upstream of the NLS. Consequently, the two mutant proteins accumulate in the cytoplasm [175]. It is worth noting that both mutations were associated with very aggressive disease and poor prognosis [175]. A recent paper has also demonstrated that cytoplasmic PML mutants inhibit ATRA-

dependent degradation of PML-RARα and potentiate its inhibitory function toward differentiation [176] (reviewed in [14]). PML-RARα itself localises to the cytoplasm, and a constitutively cytoplasmic form of the fusion protein retains its anti-differentiation properties [14, 176]. Other studies have shown that forcing PML to the cytoplasm results in nuclear PML-ND disruption [177, 178], and that this negatively affects p53 function and p53-dependent cellular senescence [179]. Overall, these findings suggest that localisation of PML and PML-RARα to the cytoplasm may contribute to leukaemogenesis. Despite this progress, the mechanisms involved and the description of an innate transforming capacity for cytoplasmic PML-RARα have yet to be shown.

Non-APL human tumours

Loss of PML A recent study has employed tissue micro-arrays to analyse PML expression in cancers of multiple histological origins [66]. PML expression appears to be lost or reduced in many different human neoplasms, from haematopoietic tumours to carcinomas: PML expression is lost in 17% of colon adenocarcinomas, 21% of lung tumours, 27% of prostate adenocarcinomas, 31% of breast adenocarcinomas, 49% of CNS tumours (100% medulloblastomas and over 90% oligodendroglial tumours), 49% of germ cell tumours and 68% of non-Hodgkin's lymphomas (83% diffuse large cell lymphomas and 77% follicular lymphomas). Other studies have shown that PML expression is lost in breast carcinomas [180], gastric cancer [181], small cell lung carcinoma [182] and in invasive epithelial tumours [107]. Another interesting finding of the work by Gurrieri *et al.* is that loss of PML correlates with higher tumour grading in breast adenocarcinomas, prostate carcinomas and CNS tumours, which confirmed the data from gastric cancers

Table 1 Summary of the *in vivo* models available to study PML tumour suppressive function

***In vivo* model to study PML tumour suppressive function**	**Results and principle conclusion**	**Refs**
DMBA/TPA-induced carcinogenesis	Skin exposure: increased papilloma/carcinoma incidence Salivary gland injection: T/B lymphomas and fibrohistocytomas *Conclusion*: PML acts as a tumour suppressor in two models of chemically induced carcinogenesis	[120]
APL pathogenesis: PML-RARa expressed in murine myeloid progenitors	APL-like disease in mice with long latency and incomplete penetrance. Reduced *Pml* gene dosage decreases latency and increases penetrance. *Conclusion*: PML plays a tumour suppressive role in a tumour model of APL	[171]
Tumorigenesis in nude mice: transformed *Pml–/–* MEFs injected into nude mice fibrosarcomas scored	*Pml–/–* MEFs generate larger fibrosarcomas and tumours with greater microvessel density and more haemorrhagic lesions *Conclusion*: suggests role for PML in angiogenesis	[67, 69]
Prostate and colon cancer: reduce PML gene dosage in *Pten+/–* mice	Invasive adenocarcinoma of the colon in *Pten+/–*; *Pml–/–* animals. *Pml* gene dosage-dependent increase in the number and size of polyps. Highly invasive prostate cancers in *Pten+/–*; *Pml+/–* and *Pten+/–*; *Pml–/–* mice *Conclusion*: establishes tumour suppressive link for PML in the PI-3K/Akt pathway and supports the notion that *Pml* is haploinsufficient in cancer development.	[65, 69]
Lung cancer: *Pml* null mice were crossed with a doxycycline (DOX)-inducible compound transgenic mutant model of K-Ras^{G12D}-induced non-small cell lung cancer (NSCLC)	*Pml–/–* mice have increased tumour burden and more malignant phenotype *Conclusion*: genetic evidence that loss of PML in the lung plays an important role in NSCLC pathogenesis	[92,186]

[183]. The only caveat of this work is its dependence on a single anti-PML antibody, the monoclonal PGM3 antibody. PGM3 has been reported to be partially resistant to fixation [180], indicating that low levels of expression could be missed. Nevertheless, western blot analyses of PML expression shows that it is lost in two colon carcinoma cell lines, confirming the data obtained using tissue sections. Despite the lack of expression at the protein level, PML mRNA is normally expressed and no mutations are found in any of the samples analysed [66]. Interestingly, the use of a proteasome inhibitor causes PML re-expression in PML-negative colon carcinoma and gastric cancer cell lines, suggesting that PML could be actively degraded in cancer cells [66, 183]. Indeed, the same group demonstrated that PML is degraded by the ubiquitin-proteasome system [93]. Interestingly, PML ubiquitination is controlled by casein kinase 2-dependent phosphorylation, and this mechanism is important for tumorigenesis [93]. However, a PML ubiquitin ligase(s) has not yet been identified. In this respect, it would be interesting to determine whether the expression of a PML ubiquitin ligase(s) inversely correlates with PML expression in cancer.

PML-ND modification Another way by which PML could be altered in cancer is through modifications of its localisation pattern and of PML-ND composition/integrity. A number of studies have shown that PML-NDs are altered in tumour cells that maintain their telomeres by an alternative mechanism (ALT), which is independent of telomerase. ALT tumours represent approximately 15% of human cancers [184]. A novel PML body is present in 1-5% of interphase nuclei of ALT-positive cells and not telomerase-positive or normal cells [184]. These structures were termed ALT-associated PML bodies (APBs). The composition of APBs is significantly different from that of PML-NDs. APBs are unique due to the presence of TTAGGG telomeric repeat sequences, telomere binding proteins TRF1 and TRF2, PML and several proteins involved in DNA repair and recombination, such as replication factor A, homologous recombination gene products RAD51 and RAD52, components of the MRN complex and others [184]. In addition, APBs have also been demonstrated to contain proteins found in PML-NDs, such as BLM and Sp100 [184]. It has become evident that these structures are specific to ALT-positive cells, since other cell types examined show no evidence of such aggregates [184]. APBs were also detected in ALT tumour tissues [64, 184]. APBs have been proposed to play a role in recombinatory mechanisms for telomere elongation in ALT cells [184]. A recent study suggests that APBs are sites of accumulation of single-stranded telomeric DNA originated from telomere processing [185]. However, it is still unclear whether these ssDNAs are simply stored in APBs or are instead used in the recombination process. What remains to be addressed is the role of PML in ALT cells and telomere elongation. In this regard, a recent report has postulated that PML is involved in transduction of growth arrest signals following alterations of the shelterin complex in ALT cells [186]. Another study has proposed that PML is required for the formation of APBs [187], although the functional implications of its down-regulation have not been determined.

Collectively, these studies suggest a tumour suppressive role for PML in human cancer, which is supported by *in vivo* data collated from animal models (Table 1). Future work must establish the different levels at which PML regulates tumorigenicity and cell fate selection, as well as the regulators of PML itself.

Concluding remarks

PML plays a tumour suppressive role in a wider spectrum of human neoplasms than previously thought. In cancer cells its concentration may be down-regulated by the ubiquitin/proteasome system. The fact that PML loss on its own is not sufficient to promote tumour formation probably reflects its role in stress responses and indicates that further changes are needed to promote tumorigenesis. For instance, in animal models PML inactivation cooperates with activation of the PI-3K pathway to promote carcinoma formation in the prostate. Versatile PML function, combined with the complex signalling microenvironment provided by each PML-ND, makes it difficult to predict how different tissues will respond to PML loss and hence its role in different varieties of cancer. The recent work reviewed here has expanded our knowledge of PML and added further layers of complexity to an already intricate field. There still exist major biological questions and technical challenges in the field of PML biology. One significant test in this field will be the development of rigorous biological models for the analysis of the PML/PML-ND proteome following stresses and at different stages of tumorigenesis along with examination of the role of specific PML isoforms in these processes.

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