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Perspectives in PML: a unifying framework for PML function

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1. ABSTRACT

The promyeloctyic leukemia protein (PML) has established activities as a potent repressor of proliferation, and oncogenic transformation, a promoter of apoptosis, an inducer of senescence, and may act as an inhibitor of angiogenesis in mammalian systems. Loss of PML or its nuclear bodies is associated with many human disease states. At the molecular level, the PML protein, and its associated nuclear bodies, play roles in diverse events ranging from mRNA export to DNA repair. PML expression impacts on Akt survival signaling, p53/Mdm2 activity, and cell cycle progression, to name a few. However, there is no discrete set of molecular activities associated with the PML protein that underlie its biochemical and physiological effects. In this review, we postulate a possible molecular model of PML function that could provide a unifying underpinning for many of its disparate activities. In particular, we explore how the ability of PML to coordinately and combinatorially regulate gene expression post-transcriptionally, enables PML to have such broad ranging effects on cellular physiology.

2. INTRODUCTION

2.1. Brief overview of PML and PML nuclear bodies

Over the last two decades, the attention of many groups has focused on the structure and function of the promyelocytic leukemia protein PML and its associated nuclear structures referred to as PML nuclear bodies (PML NBs), Kremer bodies, PML oncogenic domains (PODs) or ND10s (nuclear dot 10) (1-3). PML was the first identified component of these nuclear structures and remains their defining feature. Interest originally emerged because the integrity of PML nuclear bodies is disrupted in several pathogenic conditions including acute promyeloctyic leukemia (APL)(4) and polyglutamine neurodegenerative diseases (5-8), as well as a wide variety of viral infections including HIV (9-11). The PML protein appears to be conserved in mammals but seems to be absent in lower eukaryotes and in plants (9). The lack of evolutionary conservation leaves open the question of whether the NBs associated with PML are also present in these lower organisms, and if so, do they have similar functions.

The physiological activities of PML reveal that in mammals, the PML protein acts in growth control, transformation suppression, apoptosis and Ras induced senescence and may even play roles in the suppression of angiogenesis thereby impacting on metastases. How does PML achieve these cellular effects? At the molecular level, PML has been attributed roles in mRNA export (12-15), DNA repair (16-19), DNA replication (20-22), transcription (23-25), and post-translational modifications (including SUMO modification and phosphorylation) (26-28), to name a few. Aside from nuclear bodies, PML is also found diffusely throughout the nucleus, in the cytoplasm and under certain conditions, even in the nucleolus (29-31). There is not yet a unifying theory for PML NBs that could provide a molecular basis for these discrete sets of biochemical functions and unite these disparate functionalities. This may be difficult as PML NBs are dynamic structures, which position, size, number and composition vary during the cell cycle and under different stress conditions (9, 32, 33). Thus all PML NBs are unlikely to be functionally equivalent in all contexts.

The critical "missing link" for the PML field is to provide a molecular and biochemical basis for the physiological activities associated with PML and PML NBs. Ultimately, the goal should be to determine PML NB function to the same level that one understands how the mitochondria and other subcellular organelles work. Only with such a molecular and mechanistic understanding would be possible to get to the heart of how PML potently impacts on such wide-ranging physiological activities and to truly understand the impact of PML dysregulation in cancer.

As we have stated in previous reviews (9, 34), studies into PML and PML NB function have relied on answering the following questions: what nuclear structures are next to the bodies, what other macromolecules co-localize with the NBs, and what are the effects of disrupting the NBs? These strategies have not changed significantly in the last 5 years. The major means (understandably) of defining biochemical and molecular functions for PML has relied on the identification of PML partner proteins. So far, over 70 proteins were shown to localize with PML NBs (Nuclear Protein Database http://npd.hgu.mrc.ac.uk) (24, 35). Typically, PML function is then assigned based on the "known" functions of these partner proteins. Direct protein-protein contacts may provide a clearer molecular snapshot of how PML and thus PML NBs work. To date, there are only a few components of PML NBs that are known to directly interact with the PML protein, for example: eIF4E, SUMO/Pic1, Ubc9 and PRH/Hex (the proline rich homeoprotein) (12, 36-38). There have been other proteins suggested to directly bind PML, but these assays are typically done in reticulocyte lysates, rather than using purified proteins, and thus, are not established direct targets. However, subsequent experiments with purified proteins may demonstrate they are also direct partners. Another important feature of this system is that the integrity of PML nuclear bodies is associated with its physiological functions. However, the precise role these nuclear structures play beyond this is not entirely clear.

When considering PML NB function, a few facts should be kept in mind. These will be discussed in greater detail throughout this review. First, $PML^{-/-}$ mice develop normally and have only slightly increased cancers in later life (39). Second, PML is not an evolutionarily conserved gene in eukaryotes, being absent from *Drosophila melanogaster, S. cervisiae and Arapodopsis thaliana* (9). This is in contrast to some known partner proteins, such as eIF4E and SUMO, which are conserved from plants to humans. Finally, the PML protein has been suggested to be required for the organization of PML nuclear bodies; however, this is not the case for at least endogenous eIF4E (12, 40, 41) (discussed in more detail below) and overexpressed SP100 (Hans Will and Hannah Staege, personal communication). These last findings suggest that other evolutionarily conserved proteins may also be in nuclear structures in the absence of PML.

2.2. Scope of this review

We have covered many important issues relevant to determining the biochemical and molecular basis for PML function in previous reviews (9, 34). In particular, we described several of the general experimental limitations for many areas of PML-ology previously (9). Thus, we do not describe these here. We will not cover the important role of PML in APL as this is being discussed by two other reviews in this issue (David Grimwade and colleagues), or senescence (covered by Gerardo Ferbeyre), nor its role in DNA repair (covered by David Bazett Jones and colleagues), or structural organization and modification of PML NBs (covered by Alexander Ishov, Wilson Miller, and Paul Freemont and colleagues).

In this review, we will present a potential molecular mechanism that unifies at least a large subset, of PML's biochemical and physiological activities. This involves the role of PML in network level control of mRNA expression through modulation of the eIF4E RNA regulon. Many observations with regard to PML's effects on gene expression, and subsequent physiological effects, can be understood using this model. For instance, it provides a molecular means to understand how PML potently modulates so many apparently unrelated pathways. Further, we will discuss other means by which PML coordinately modulates gene expression post-transcriptionally. These models may provide a starting point for understanding how PML carries out its physiological functions in growth and tumor suppression.

3. PML's ROLE IN POST-TRANSCRIPTIONAL REGULATION

3.1. General features

There are several observations that have led to the idea that PML acts as a post-transcriptional regulator of gene expression. For instance, previous studies have indicated that PML can carry out, at least some of its activities, in the absence of new RNA synthesis. For instance, the pro-apoptotic function of PML and PML's

effects on cell cycle progression and angiogenesis are likely post-transcriptional (see below, (42-44)). Observations from several years ago also indicated that PML post-transcriptionally represses several genes involved in growth promotion and cell cycle progression including cyclin D1, cyclin E1, cyclin A2, cyclin B1, and c-Myc (to name a few)(12, 14, 45, 46). In these cases, PML overexpression leads to reduced levels of these proteins but does not modulate the total level of the corresponding mRNAs. Thus, PML does not modulate transcription or steady state stability of these transcripts. The effects of PML on gene expression are specific, as PML does not modulate the expression of housekeeping genes such as GAPDH or beta-actin. Further, PML overexpression does not globally change the composition of the proteome, as observed by ^{35}S-Met incorporation experiments (14).

Consistent with the above observations, there are several circumstantial lines of evidence that support a role in post-transcriptional regulation of gene expression for PML. First, PML is localized adjacent to sites of RNA processing, including cleavage bodies, Cajal bodies and splicing speckles (9, 47). Further, PML interacts with several proteins implicated in post-transcriptional regulation. Several groups have independently shown that PML interacts with the eukaryotic translation initiation factor eIF4E (12, 14, 48, 49). In the nucleus, eIF4E is involved in nucleo-cytoplasmic mRNA export (this is discussed in detail below). PML is also associated with eIF3/Int6 (a component of the translation initiation complex) and the polysomal P-proteins (50, 51). Furthermore, PML associates with GAPDH, in an RNA dependent manner, where the nuclear fraction of GAPDH has been attributed roles in RNA binding to AAUAA rich segments and shuttling specific tRNAs into the nucleus (52-54).

Although it is not direct evidence of PML involvement in post-transcriptional processes, it is interesting that all-trans-retinoic acid (ATRA) treatment of APL cells leads to post-transcriptional regulation of a number of genes that are themselves involved in post-transcriptional processes (like proteins involved in mRNA splicing and export: hnRNP A, K, F, UP2, C1/C2, snRNP D3 and E and proteins involved in regulation of initiation and elongation of translation: IF2, eIF4AI, eIF5, eEF1A-1, etc). ATRA treatment also induces expression of PML isoform 1 (see below) and PML NBs re-formation (55). Direct involvement of PML and PML NBs in these changes has to be further investigated.

Interestingly, under certain experimental conditions, PML is also found in the nucleolus. The nucleolus is a site for ribosomal biogenesis in the cell. The appearance of PML here further implicates it in post-transcriptional regulation. Initial studies indicated that treatment with the proteosomal inhibitor MG132 led to re-localization of PML from the nucleus to the nucleolus (29). Similar effects are observed upon treatment with doxorubicin and mitomycin C (30). Shut off of rRNA synthesis also caused re-localization of PML to the nucleolus, in a cell type specific manner (31). Recently, it was proposed that this re-localization could be linked to senescence and proteolysis (56). The molecular meaning of PML's re-localization to the nucleolus and its precise impact on ribosomal biogenesis and related nucleolar activities are yet to be elucidated. However, this seems to be an interesting future direction for PML research.

3. 2. Network level control of growth promoting genes by PML: modulation of the eIF4E RNA regulon

Several years ago, we identified eIF4E as a partner protein for PML and as a component of PML NBs (12, 14). Since then, other groups have verified this association (48, 49, 57). eIF4E acts at two distinct levels of gene expression: its nuclear fraction is involved in mRNA export and its cytoplasmic function in m^7G cap dependent translation. For both of these activities, eIF4E must bind the m^7G cap found on the 5' end of mRNAs. PML is a key negative regulator of both eIF4E functions. In particular, the RING domain of PML directly binds eIF4E, reducing its affinity for its substrate, the m^7G cap by over 100 fold (12, 58). In this way, PML inhibits eIF4E dependent mRNA export and is positioned to inhibit the mRNA translation functions of eIF4E as well. This is one of the few discrete biochemical functions that have been identified for PML, the other being its role in SUMO modification (37, 59, 60) (discussed by Miller and colleagues in this issue). It is worth noting that PML expression does not modulate levels of eIF4E protein or RNA (15). Importantly, the impact of eIF4E on gene expression is network wide, where it simultaneously promotes the expression of many growth-promoting genes (61, 62). This type of coordinated expression, and its impact on cell physiology suggest that eIF4E is a central node in an RNA regulon (defined below). PML appears to play a key role in negatively modulating this regulon. We describe below the regulon hypothesis and its potential for providing a unified framework for PML function and how this is related to the physiological effects of PML.

3.3. What is an RNA regulon?

An RNA regulon is a theoretical construct designed to explain the coordinated regulation of post-transcriptional events in eukaryotes (63, 64). To achieve coordinated gene expression, mRNAs contain common elements known as USER codes in the UTRs of transcripts, which allow for their coordinate and combinatorial control at certain levels of gene expression, for instance at the level of mRNA export or translation. Thus, all the mRNAs acting in a given pathway could have their nucleo-cytoplasmic export coordinated so they arrive in the cytoplasm in a coordinated fashion. Equally well, once in the cytoplasm, their stability and translation can be modulated by the presence of different USER codes. In this way, the combination of USER codes present in the given mRNAs contributes to the fate of the cell.

It is clear why such a coordinated level of gene expression is required. Even for a simple biochemical pathway, one would want to coordinate transcription of all the relevant enzymes in the pathway (through the use of common promoter elements for instance). If these mRNAs have markedly different stabilities, export rates from the

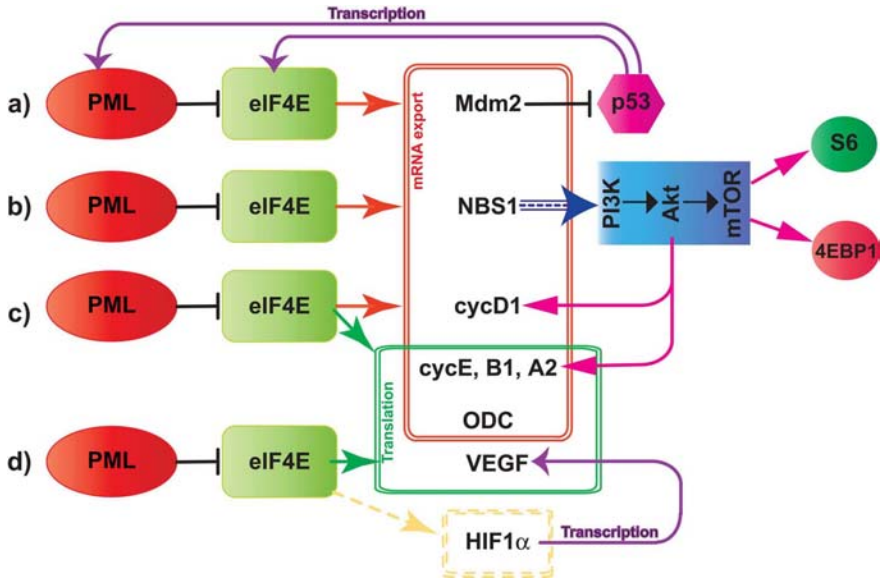

Figure 1. PML modulates the eIF4E regulon. This diagram demonstrates how PML could impact on different pathways by acting as an inhibitor of the eIF4E regulon. Note that the figure is not intended to be all-inclusive (see section 3.3), but rather sets out to provide a framework for explaining how a subset of disparate PML activities could be related.

nucleus or translatabilities, then not all of the enzymes in the given biochemical pathway would be produced in a coordinated manner. Thus, the required biochemical reactions would not occur. The presence of different USER codes enables both coordinated and combinatorial modulation of gene expression. In this way, the regulon provides a rheostat enabling more rapid responses to extra-cellular stimuli than transcriptional reprogramming, for instance.

Previous studies indicate that eIF4E is a central node in an RNA regulon that governs proliferation and apoptosis (45, 61). Below, we outline some of the previous observations with PML and its impact on certain pathways, in order to place these into the context of PML's role as an inhibitor of the eIF4E regulon. In this way, we propose the RNA regulon model as a unifying framework to understand at least some of the many diverse activities observed for PML.

3.3.1. Example 1: The PML-p53-Mdm2-eIF4E axis

Previous studies indicate that PML could be a modulator of p53 activities by a number of mechanisms. Under certain conditions, PML and p53 co-localize in PML NBs. It was shown that PML can act downstream and upstream of p53 in Ras–induced and replicative senescence (see review in this issue by Gerardo Ferbeyre and colleagues). Earlier studies had suggested that PML could modulate the acetylation and phosphorylation state of p53 and thereby its stability and activity (27, 28, 46, 65, 66), but more recent studies suggest that PML is itself a p53 target gene that acts downstream of p53, besides acting as an upstream regulator of p53 (67).

Several mechanisms were proposed for PML regulation of p53. PML has been proposed to organize CBP, an acetyltransferase, into NBs enabling it to acetylate p53 in a PML dependent manner (28). PML may act in sequestration of Mdm2 after DNA damage thereby resulting in stabilization of p53 (30, 68, 69). PML may act in the recruitment of Chk2 or HIPK2 kinases and p53 into PML NBs under stress conditions, which could facilitate phosphorylation and activation of p53 (27, 66, 70). There is a clear link between PML expression and the cellular levels of p53. For instance, there is less p53 in PML$^{-/-}$ cells than in wild type controls both under steady state conditions and upon gamma-irradiation (46, 68), while introduction of PML into PML$^{-/-}$ cells leads to increased p53 levels (68). These changes in gene expression are paralleled by the expected alterations in cell physiology.

The RNA regulon model provides another theoretical construct to understand the relationship between PML and p53 expression (Figure 1a). It was shown that eIF4E enhances the expression of a negative regulator of p53, Mdm2 (45, 71). eIF4E modulates the mRNA export of mdm2 mRNA when overexpressed (but not its translation (71)) and this occurs in response to extracellular stimuli (71, 72). Further, PML suppresses this eIF4E function, leading to reduced Mdm2 levels (45). This is consistent with observations that increased PML expression leads to increased p53 protein levels (46, 68). Interestingly, the ties between these pathways become more intricate. p53 overexpression leads to transcriptional repression of eIF4E (73). These studies also showed that Mdm2 overexpression led to reduced p53, and increased eIF4E levels. This could lead to a feedback loop whereby increased eIF4E levels lead to increased export of mdm2 mRNA etc. In addition, co-expression of Mdm2 with PML caused a redistribution of PML from the nucleus to the cytoplasm leading to p53 destabilization (74), thereby providing another possible feedback in this network. Adding to the intricacy of these

connections, increased p53 levels leads to reduced translation initiation via dephosphorylation of 4EBP-1 (a critical cytoplasmic negative regulator of eIF4E), the increased formation of translationally impaired 4EBP-1-eIF4E complexes and reduced formation of translationally active eIF4E- eIF4G complexes (75).

In summary, modulation of the RNA regulon provides a possible mechanistic basis that could explain how the biochemical activities of PML impact on gene expression in the p53 pathway and PML's roles in the promotion of apoptosis and regulation of senescence.

3.3.2. Example 2: The PML-Akt connection and PML induced apoptosis

Recent observations suggest that PML overexpression leads to decreased Akt phosphorylation and also decreased phosphorylation of S6K and 4EBP-1 (76) (Culjkovic et al, 2008, in press), two downstream effectors of Akt. Consistently, it has been observed that $PML^{-/-}$ cells have increased phosphorylated Akt levels. These observations suggest that PML could play a key role as a negative regulator of Akt survival signaling. But how?

Several export targets of eIF4E are downstream regulators of Akt. In addition, an upstream regulator of Akt, NBS1 is also modulated by eIF4E. NBS1 plays a key role in DNA repair (see review by David Bazett Jones in this issue, (17)). However, new studies indicate that NBS1 also functions in the activation of PI3K, and subsequently, Akt (77, 78). Overexpression of eIF4E leads to enhanced NBS1 mRNA export and protein expression (45). This is correlated with increased Akt phosphorylation and increased phosphorylation of BP1 and S6 (Culjkovic et al 2008, in press). These activities of eIF4E are both PI3K and NBS1 dependent. Interestingly, eIF4E upregulates the mRNA export of several downstream effectors of this pathway, including c-Myc, Mdm2 and cyclin D1 (45). Thus, eIF4E can impact on the Akt pathway both upstream, via NBS1, and downstream, by increasing the concentration of Akt effector proteins (Figure 1b).

PML substantially abrogates eIF4E dependent mRNA export of NBS1 transcripts and thereby decreases the levels of NBS1, and thus, phosphorylation of Akt and phosphorylation of S6 and BP-1, as well as the levels of the downstream effectors of Akt such as c-Myc and cyclin D1. Thus by turning off/down the eIF4E regulon, PML can impair Akt survival signaling.

The physiological effects of these changes in gene expression are striking. eIF4E rescues cells from serum deprivation induced apoptosis, at least in part, via this pathway (Culjkovic et al 2008, in press). PML antagonizes eIF4E's rescue function (Culjkovic et al 2008, in press). Clearly different apoptotic pathways will engage different genetic programs. Thus, this serves as an example of how PML's inhibition of the RNA regulon impacts on PML mediated apoptosis in the serum deprivation context. Importantly, PML requires its RING domain for its pro-apoptotic functions and to directly bind and impair eIF4E function (12, 79). These data provide a potential explanation for the observation that the pro-apoptotic function of PML is independent of on-going transcription (43).

Parallel explanations have been suggested for how PML modulates mTOR and Akt function directly. mTOR and Akt may co-localize with PML at PML NBs (42, 76). For instance, it was suggested that PML inactivates Akt by recruiting pAkt together with its phosphatase PP2a into PML NBs (76). This, of course, would invoke a unique mode of action of both mTOR and Akt, which are usually associated with the plasma membrane. Further, it is unclear how these would be organized in a PML null context. Note that we describe the regulation of eIF4E in the PML null context below (see section 3.4.).

3.3.3. Example 3: PML mediated growth suppression and transformation suppression

It has bee reported that PML overexpression promotes apoptosis (43, 79, 80), and leads to decreased proliferation and G1/S arrest in several cell types (9). These effects depend on the structural integrity of the RING domain of PML and correspondingly the presence of intact nuclear bodies (9). In contrast, eIF4E overexpression is associated with increased proliferation and oncogenic transformation in animal models and in tissue culture lines (reviewed in (62, 81-84)). PML overexpression is associated with decreased levels of proteins involved in cell cycle progression such as cyclin D1, cyclin E1, cyclin A2 (as well as others) (14, 45, 85, 86). Importantly, these mRNAs are eIF4E mRNA export targets and their mRNA export is inhibited by PML (Figure 1c). Cyclin D1 mRNA export and protein levels are elevated in $PML^{-/-}$ cells and unlike in wild type control cells, gamma-interferon treatment (IFN-gamma , increases transcription of PML) does not reduce cyclin D1 mRNA export or cyclin D1 protein levels in $PML^{-/-}$ cells in contrast to wildtype cells (15). These data show that PML is a key regulator of interferon dependent G1/S growth arrest and demonstrate its importance to the regulation of these cellular growth effectors.

Our previous studies indicate that the mRNA export function of eIF4E contributes to its transformation potential. Particularly, the presence of the USER code for export of cyclin D1 mRNA (the 4E-SE) substantially augments the ability of eIF4E to transform cyclin $D1^{-/-}$ fibroblasts (13). PML suppresses eIF4E mediated transformation in a wide variety of cell types, suggesting that its role in inhibition of eIF4E dependent mRNA export is important to its ability to suppress eIF4E mediated transformation (9, 12, 61).

3.3.4. Example 4: PML and suppression of metastatic disease

PML expression is suggested to be lost as part of metastatic disease either through reduced levels of PML or its relocalization to the cytoplasm (87-90). Tumor angiogenesis is increased in a prostate cancer model using $PML^{-/-}$ animals (42). It has been suggested that PML, via its ability to impact on mTOR, can downregulate HIF1-alpha

protein synthesis and thus decrease VEGF levels (42). Increased VEGF levels correlate with increased metastatic disease (91, 92). Importantly, PML does not modulate the transcript levels of HIF1-alpha. Typically, the type of translational control suggested by the authors involves the 5'UTR of transcripts. However, deletion of the 5'UTR of HIF1-alpha did not modulate production of HIF1-alpha protein in the presence or absence of PML (42). The authors suggested a model where PML impacts translation of HIF1-alpha through mTOR inhibition, showing sequestration of mTOR into PML NBs under hypoxia. It seems equally likely (or in addition to control at the translation level) that PML could negatively regulate the mRNA export of HIF1-alpha. Further, PML inhibits Akt phosphorylation (that directly phosphorylates and activates mTOR) via its effects on the eIF4E regulon (see Example 2). Determining how PML modulates gene expression of HIF1-alpha awaits a direct test of translation (i.e. polysomal analysis) and analysis of the ability of PML to modulate the mRNA export of HIF1-alpha (via eIF4E). Further, VEGF and ODC, two genes important for the progression of metastatic disease, are both sensitive to eIF4E levels (84). Thus, PML could reduce VEGF levels independently of HIF1-alpha, via its effects on eIF4E, or through its effects on other gene products such as ODC (Figure 1d).

3.4. Why isn't the eIF4E regulon disrupted in PML$^{-/-}$ mice?

Clearly one of the most confounding issues for "PML-ologists" is the lack of a severe phenotype for PML$^{-/-}$ mice (39, 80, 93). Obviously, the *PML* gene is not essential for survival. These mice develop normally although they have impaired IFN, ceramide, Fas, TNF-alpha and TGF-beta responses. Further, PML$^{-/-}$ mice are more resistant to gamma-irradiation than normal mice (~2 fold) (80, 93). PML$^{-/-}$ mice treated with DMBA and tetradecanoyl phorbol acetate developed slightly more papillomas than controls (~2 fold) (39) and have a reduced TNF-alpha response (~2 fold) (80). However, the literature is replete with reports of a wide variety of activities that absolutely require PML. Thus, one is faced with a choice, either that activity is not critical for the health of the cell/animal, or PML is one of many redundant regulators that can modulate a given process.

This issue becomes somewhat more complicated if one considers that many factors associated with PML do not form nuclear bodies in the PML null background e.g. Daxx (3). However, other factors are present in body type structures in PML$^{-/-}$ cells such as endogenous eIF4E and overexpressed Sp100 ((12) and Staege and Will, personal communication). In fact, eIF4E bodies are even present in Drosophila, where there is no *PML* gene (9, 12)}. Thus, nascent forms of these structures may well be present, at least for some PML partner proteins in the null cells. Intriguingly, eIF4E is more evolutionarily conserved than PML, which has not been reported beyond mammals, leaving open the possibility that there are nascent PML bodies comprised of evolutionarily conserved proteins and that PML is a later arrival (in the evolutionary context) to this structure. Whether these bodies are functionally equivalent to their PML-positive counterparts is an open question.

3. 4. 1. So how would the eIF4E RNA regulon be regulated in the absence of PML?

We determined that PML was one of many factors that could inhibit the eIF4E regulon. Over 200 homeoproteins were found positioned to modulate both the nuclear and cytoplasmic functions of eIF4E (38, 94, 95), and thus, to modulate eIF4E dependent changes in gene expression. These homeoproteins contain conserved eIF4E binding sites and use these to bind the same region of eIF4E (its dorsal surface) that is recognized by PML RING (38). In some cases, they repress eIF4E's activity in mRNA export and in others they stimulate export (38, 94, 95). This further adds to the complexity of how the regulon could be modulated to achieve certain physiological outcomes. Note that homeoprotein regulators of eIF4E are much more phylogenetically conserved than PML, permitting regulation of the eIF4E regulon in a wide variety of evolutionary contexts. In terms of these homeoproteins providing redundancy in regulation, we previously determined that one key negative regulator of eIF4E, which also associates with PML, the proline rich homeodomain PRH, negatively regulates eIF4E in APL cells (where PML is displaced from nuclear bodies- see review in this issue by David Grimwade and colleagues). Further, PRH and eIF4E form nuclear structures indistinguishable from PML NBs in APL cells prior to ATRA treatment. This is a clear case where a redundant regulator ensures that eIF4E activity is kept in check, even in the absence of PML. Consistently, our previous studies indicated that APL cells do not have dysregulated eIF4E activity (12), but of course, they do have a variety of other unrelated problems that contribute to their oncogenic nature. Clearly, redundancy in control of the regulon is unlikely to be complete, and thus under certain conditions such as interferon treatment, the redundant factors may not inhibit the eIF4E regulon as effectively as PML.

4. NUCLEAR PML AND PML NBs

4.1. The physical function of the PML NB

One must decouple the importance of the presence of the physical PML NB with the presence of the PML protein itself. Disruption of PML bodies is associated with the loss of the physiological functions of PML in growth suppression and apoptosis (9). For instance, mutation of the RING domain or B-boxes in PML leads to disruption of PML NBs and loss of its physiological activities in growth and suppression of transformation as well as loss of its pro-apoptotic effects (9). It was also proposed, that some PML functions are independent of its ability to form NBs (65).

Our previous biophysical studies indicate that RING domains can self assemble into structures, and that these structures can promote specific biochemical activities of the associated proteins (96, 97). Thus, RINGs self assemble into spherical catalytic surfaces that lead to increased efficiency of the biochemistries associated with the given RING domain (96). For instance, BRCA1-

BARD1 heterodimers rapidly assemble into bodies visible by EM, and these bodies more efficiently catalyze the formation of poly-ubiquitin chains than BARD-BRCA1 heterodimers (96). Similar types of phenomenon have been seen for the RING domain from mdm2 for its E3 ligase activity (98). PML, and its viral homologue Z, provides another example. Efficiency of inhibiting the eIF4E-m7G cap interaction is 10 fold higher in bodies than in monomers (96). Thus PML bodies could serve as catalytic surfaces to enhance a subset of cellular biochemistries. This could, in part, explain the relative normal physiology of $PML^{-/-}$ mice i.e. that the efficiencies of certain chemistries are impaired- but not lost altogether- by the loss of NBs and its ability to physically organize certain chemistries in an optimal way. This is likely to be the case, but also likely and very important is that PML may impact on pathways that have redundant regulation (as discussed above), which allows cells to utilize alternative means to achieve their biological endpoints in the PML null context.

An associated theory (which is not mutually exclusive with the catalytic surface theory) is that PML nuclear bodies act as nuclear depots (10, 99). Here, the active component of PML is considered to be the nucleoplasmic fraction, whereas the nuclear bodies themselves are depots or "nuclear closets" (99). In fact, there is no reason why bodies could not act as catalytic surfaces with interiors used as storage sites at the same time. Once again, one would assume that such nuclear storage is not absolutely critical for the cell, since in $PML^{-/-}$ mice, many PML body components are not organized into bodies but rather are found throughout the nucleoplasm. This redistribution of components does not seem to seriously imperil the health of the cell.

From the functional perspective of PML and eIF4E nuclear bodies, it is important to note that there are at least two types of eIF4E nuclear bodies found in the nucleus (13, 45). First, those bodies that co-localize with PML. Second, those bodies that associate with mRNA export targets of eIF4E. In this way, it appears that PML nuclear bodies are RNA negative for eIF4E export targets, and thus represent bodies of inhibited eIF4E. Presumably, these bodies could respond to physiological stresses enabling PML bodies to have other activities or to enable eIF4E mRNA export activity by the physical removal of PML from these eIF4E bodies. However, these issues need to be resolved with more experiments. Furthermore, it is interesting that some of the protein products of mRNAs that eIF4E is known to enhance the mRNA export of (and conversely, that PML is known to impede the mRNA export of), actually associate with PML. These include NBS1 and Mdm2 (68, 100). In this way, these proteins may serve as some sort of feedback mechanism. Precisely how such a mechanism would work is yet to be elucidated.

4.2. Functional PML: bodies or free protein?

The precise physical role that PML NB plays relative to PML is still not clear. To truly exploit what we know about the effects of PML NBs on cell physiology, we need to have a molecular level understanding of PML NB organization, ultra-structure and degree of heterogeneity. For instance, at the microscopic level, we have only a gross understanding of PML organization. Ultimately, we need to understand the organization in much greater detail (at the structural level) to reveal how biochemically active parts of the body are organized and how this organization permits/enhances certain types of biochemistries accomplished by PML and PML NBs. Eventually, this will enable to us to understand how remodeling of PML bodies (e.g. by changing protein composition in response to extracellular stresses) effects functionalities both in terms of types of functions and efficiencies of functions. With atomic type resolution, one would finally have an idea of how the few distinct biochemical activities of PML are achieved. Of course these goals are difficult to achieve. PML bodies have been resistant to purification, and they are heterogeneous in nature. Thus, one is unsure which components are in the same body at the same time or whether components identified are actually nucleoplasmic PML partners rather than nuclear body PML partners.

5. CYTOPLASMIC FUNCTIONS FOR PML

In clear support of potential roles for PML in post-transcriptional gene regulation, is the observation that PML is normally found in the cytoplasm as well as the nucleus. Up to 20% of cells have some PML in the cytoplasm (101, 102). In fact, there are isoforms of PML which lack the NLS (nuclear localization signal), but retain their RBCC domains, and these appear to form cytoplasmic structures (89, 101-103). These studies suggest that wild type PML may well have some cytoplasmic, as well as nuclear, functions. It was shown that PML1, the longest isoform, also contains a NES (nuclear export signal), present in exon 9, which allows shuttling between nucleus and the cytoplasm (89). The same study showed that PML1 is the isoform expressed at the highest level in a number of different primary and immortalized cell types. Further, the PML1 isoform was found to be upregulated after ATRA of APL cells (55). A recent report also showed that PML redistributes to cytoplasmic structures called mitotic accumulation of PML protein (MAPPs) during mitosis (104), and that even in the early G1 phase of the cell cycle, a large portion of PML still reside in MAPPs. It was shown that MAPPs are different in structure and composition from PML NBs. Additionally, PML is found in the cytoplasm in certain pathogenic conditions. For instance, cells infected with arenaviruses or with HIV have PML translocated to the cytoplasm (11, 105). This may be a viral mechanism to evade host cell apoptosis. Cytoplasmic PML (cPML) has also been found in hepatocellular carcinomas (87, 90) and in some APL patients prior to ATRA treatment (4). In some APL patients with retinoic acid resistant disease, two mismatch mutations in the remaining PML allele have been identified. These mutations generate a premature stop codon that result in a truncated form of the PML protein that is found mainly in the cytoplasm. The combination of PML-RARalpha and these mutations is correlated with particularly aggressive disease (106). Further, a mutant form of PML has been identified in a murine plasmocytoma cell line, which also leads to a premature stop codon, and also results in cytoplasmic PML (107). These mutants lack the NLS. It is not clear in these cases if

PML has a gain of function in the cytoplasm, and/or if the pathogenesis of these diseases is due solely to a loss of function in the nucleus.

Recent studies suggest that the mutant cytoplasmic form of PML may impact on ATRA responsiveness. A PML-RARalpha mutant that accumulates in the cytoplasm (cPML-RARalpha) appears less sensitive to ATRA-dependent proteosomal degradation, indicating that cytoplasmic localization may promote stabilization that can cause increased resistance to ATRA therapies. Intriguingly, cPML-RARalpha retained the ability to inhibit ATRA dependent transcription and differentiation (108). The precise mechanism by which this works is not clear, and one assumes here that the effects must be indirect. It was suggested that cPML-RARalpha could sequester necessary factors from the nucleus, thereby impairing transcription.

Overexpression of mutant forms of PML that are largely cytoplasmic have been shown to inhibit p53 function as measured by luciferase activity (109). It is unclear whether this mutant leads to alterations in p53 levels, but it does not lead to relocalization of p53. Instead, it is hypothesized that this cytoplasmic mutant of PML relocates important p53 co-activators to the cytoplasm, thus inhibiting its transcriptional activity. However, direct involvement of PML in p53 transactivation in the nucleus is not completely resolved (46, 67, 110). Also, Bellodi et al. suggested the possibility that cPML could also affect transcription-independent functions of p53. Given previous studies showing that PML could inhibit translation of reporter genes in reticulocyte lysates (58), it would be interesting to know if the inhibition of p53 function by cytoplasmic PML is due to an indirect translational effect (of some unidentified gene(s)).

The cPML mutant seems to have a dominant effect on wild type PML, leading to a net relocalization of PML to the cytoplasm. Interestingly, cPML does not lead to growth arrest, as wild type PML does (108). In another study, overexpression of mutant PML lacking the NLS, resulted in cytoplasmic accumulation of this protein, and revealed that this mutant was impaired in the ability to suppress *neu*-mediated oncogenic transformation of NIH3T3 cells (111). Expression of this mutant also led to reduction of PML NBs, suggesting the dominant-negative effect over wild type nuclear PML. Also, in another study, overexpression of cytoplasmic isoform referred to as PML3-4-7 (based on exon composition) failed to induce growth suppression in colony forming assay (112). These different isoforms could have different functions in the cytoplasm and thus different physiological effects.

A recent study suggested that cPML might function in TGF-beta signaling (113). $PML^{-/-}$ mice appear to be impaired in their TGF-beta response by about 2 fold, and this defect was restored by expression of a cytoplasmic isoform of PML (3-4-7), but not the nuclear PML IV isoform. These studies propose that cPML directly binds Smad2/3 and SARA (Smad anchor for receptor activation), and is essential for efficient recruitment and assembly of the TGF-beta receptors/SARA/Smad complex. Another study has demonstrated that TG-interacting factor (TGIF), a negative regulator of TGF-beta pathway, blocks cPML function (and leads to nuclear sequestration of cPML), which results in inhibition of Smad2 activation (114). Interestingly, TGF-beta is an eIF4E target gene (84). Thus, there could be another possible feedback loop given that mRNA levels of cPML isoforms were induced by TGF-beta treatment in different cell types (113). If PML is indeed required for this, one would think that the $PML^{-/-}$ mice would have a much more severe effect. Notably, there is absence of phenotypic overlap between animal models lacking different components of TGF-beta pathway and $PML^{-/-}$ mice (115-118), suggesting that the role of cPML could be confined to specific tissues or pathological conditions, or redundancy in regulation of TGF-beta pathway.

As discussed above, PML binds eIF4E in both the nuclear and cytoplasmic compartments. In the nuclear compartment, eIF4E functions in mRNA export and in the cytoplasmic compartment, eIF4E functions in cap dependent translation. Interestingly, eIF4E modulates the export and translation of only a subset of growth promoting mRNAs. For both of these functions, eIF4E binds the m^7G cap found on the 5' end of mRNAs. The RING domain of PML directly binds eIF4E, which leads to a reduction of eIF4E's affinity for the m^7G cap by over 100 fold (12). PML uses site I of the RING to bind the dorsal surface of eIF4E, as determined from biochemical and mass spectrometry studies. PML does not affect protein levels of eIF4E. Furthermore, PML is positioned to block association with eIF4G, and thus to impair translation of eIF4E sensitive mRNAs. PML's ability to so substantially reduce the ability of eIF4E to associate with the cap, both impairs eIF4E's mRNA export function (see above) and in reticulocyte lysates, eIF4E's translation functions (58). It would be important to carry out studies demonstrating the effects of PML expression on polysomal loading of eIF4E sensitive targets, particularly when substantial amounts of PML are found in the cytoplasm. Consistently, recent studies suggest that PML impacts on the translation of HIF1-alpha (discussed above). Together these data suggest, that in conditions where PML is found substantially in the cytoplasmic fraction (as described above), PML could directly modulate translation of at least a subset of growth promoting mRNAs.

To date, three models for PML function in the cytoplasm have been suggested. None of these models are mutually exclusive. 1. cPML sequesters factors away from the nuclear compartment, disabling key PML functions, 2. cPML directly impacts on translation of a subset of transcripts through its association with eIF4E and 3. PML binds directly to receptors on the plasma membrane to influence signaling. Intriguingly, eIF4E is known to impact on TGF-beta and VEGF signaling, and thus could provide an alternative model to point 3's.

6. CONCLUSIONS AND PERSPECTIVES

The RNA regulon model provides a model for how coordinated and combinatorial modulation of gene

expression could be achieved in eukaryotes. eIF4E post-transcriptionally regulates expression of a certain subset of genes and PML is a key negative regulator of this network. Existence of nuclear and cytoplasmic isoforms provides multiple levels of control for the eIF4E regulon, and the existence of isoforms that are differentially expressed under different conditions or in different tissues suggests a means for stress/cell-type specificity. Different protein modifications to PML under different stress stimuli could also affect its activity. Of course, it is likely that other, yet to be described, actors play key roles in these processes, giving it even more complexity. Further, PML is implicated in several levels of post-transcriptional regulation (see section 3.). The plasticity of PML expression in terms of isoform specific activity and localization means that PML may be a key actor in both the nuclear and cytoplasmic compartments, enabling it to effect the eIF4E regulon at multiple fronts simultaneously**.**

We have attempted to provide a theoretical framework via the regulon model for understanding how PML can have such wide-ranging and apparently disparate effects on gene expression. In particular, prior to this model, many PML dependent activities seemed to be completely unrelated, and thus difficult to understand the molecular basis of. Clearly, the theoretical framework we suggest is limited. For instance, the molecular nature in terms of the nuts and bolts physicality of these interactions is not yet clear. However, we hope that this model can provide a starting point for a more biochemical and molecular description of the events, which underpin PML associated physiology.

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