Research Article

The translation initiation factor 3 subunit eIF3K interacts with PML and associates with PML nuclear bodies

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ABSTRACT

The promyelocytic leukemia protein (PML) is a tumor suppressor protein that regulates a variety of important cellular processes, including gene expression, DNA repair and cell fate decisions. Integral to its function is the ability of PML to form nuclear bodies (NBs) that serve as hubs for the interaction and modification of over 90 cellular proteins. There are seven canonical isoforms of PML, which encode diverse C-termini generated by alternative pre-mRNA splicing. Recruitment of specific cellular proteins to PML NBs is mediated by protein–protein interactions with individual PML isoforms. Using a yeast two-hybrid screen employing peptide sequences unique to PML isoform I (PML-I), we identified an interaction with the eukaryotic initiation factor 3 subunit K (eIF3K), and in the process identified a novel eIF3K isoform, which we term eIF3K-2. We further demonstrate that eIF3K and PML interact both in vitro via pull-down assays, as well as in vivo within human cells by co-immunoprecipitation and co-immunofluorescence. In addition, eIF3K isoform 2 (eIF3K-2) colocalizes to PML bodies, particularly those enriched in PML-I, while eIF3K isoform 1 associates poorly with PML NBs. Thus, we report eIF3K as the first known subunit of the eIF3 translation pre-initiation complex to interact directly with the PML protein, and provide data implicating alternative splicing of both PML and eIF3K as a possible regulatory mechanism for eIF3K localization at PML NBs.

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Introduction

The human promyelocytic leukemia (PML) protein is an important tumor suppressor protein, and mice in which the PML gene is disrupted are more susceptible to carcinogen-induced cancers [5,13,56]. The PML protein acts as the molecular scaffold for the formation of promyelocytic leukemia nuclear bodies (PML NBs), which are discrete, dynamic subnuclear structures found within mammalian cells [3]. These heterogeneous multi-protein complexes are implicated in a wide spectrum of cellular functions including tumor suppression, DNA repair, apoptosis, antiviral response, cellular senescence, and transcriptional regulation [4,13,37,45,60]. While PML is the major structural component of the PML NB, more than 90 different proteins can associate with PML NBs, either transiently or constitutively [15,40,54] and many of these proteins themselves are intimately linked to the various functions ascribed to PML NBs. One of the prominent functions associated with PML and PML NBs is tumor suppression, since loss of the PML protein and/or PML NBs has been associated with acute promyelocytic leukemia [12,32] and various human solid...
tumors including carcinomas of the colon, lung, breast and prostate [25,55].

The PML gene transcript is generated from nine exons, of which exons 6–9 are spliced alternatively to yield at least seven PML isoforms with unique C-terminal sequences [20,29]. Exons 1–3 are conserved in all PML isoforms and encode a tripartite motif (TRIM) containing a RING finger, two B-boxes and an α-helical coiled-coil motif [44] which are involved in mediating homo- and hetero-association between PML isoforms [6,22]. Several motifs have also been identified in the C-terminus of PML, including a nuclear localization signal in exon 6 [30] and a nuclear export signal in exon 9 of PML isoform I (PML-I) [26,29]. Relative to the other PML isoforms, PML-I is more highly conserved between mouse and humans, and has the highest expression level [10], suggesting that this isoform is a significant contributor to PML NB functions. It is becoming increasingly clear that isoform-specific PML protein–protein interactions help regulate PML biology. For example, PML-IV has been shown to interact with p53 [21,24], whereas PML-II has recently been implicated in expression of the MHC class II gene cluster through specific recruitment of CIITA [53]. Additionally, PML-I interacts with acute myeloid leukemia 1 (AML1) [41] in an isoform-specific manner and PML-III interacts with TRF1 to facilitate the alternative lengthening of telomeres (ALT) in U2OS cells [58]. Collectively, these examples suggest that individual isoforms through their protein–protein interactions can alter PML NB function, which could occur in a tissue-specific manner or in response to various stimuli [10]. Thus, it is likely that the diversity of cellular functions attributed to the PML NB arise, at least in part, via equally diverse protein–protein interactions mediated by the various PML isoforms.

In this study, we used a yeast two-hybrid screen to identify a novel protein–protein interaction between peptide sequences unique to PML-I, encoded by exon 9 of the PML gene, and subunit K of the eukaryotic translation initiation factor 3 (eIF3K). In addition, we show that eIF3K also interacts with the PML protein in an exon 9-independent manner, and localizes to PML NBs. Furthermore, the recruitment of eIF3K to PML NBs appears to be regulated by alternative splicing, as eIF3K isoform 2, which we term eIF3K-2, is preferentially recruited to PML NBs and is further enriched at bodies by overexpression of PML-I. This is the first subunit of the eIF3 translation pre-initiation complex shown to interact directly with PML and, in addition to eIF3E, is the second subunit of eIF3 known to associate with PML nuclear bodies.

Results

eIF3K interacts with a peptide encoded by exon 9 of PML-I

In order to identify proteins that specifically interact with PML-I and not with other PML isoforms we performed a yeast two-hybrid screen using the unique exon 9 region of human PML-I [20,29],
encoding amino acids 604–882 as a bait to screen a human fetal brain cDNA library [16] (Fig. 1A). One of the interacting peptides identified was an N-terminal fragment of subunit K of the eukaryotic translation initiation factor 3, (eIF3K, aa 1–97) (Supplementary information Fig. 1). The human eIF3 complex contains at least 13 subunits, of which eIF3K is non-essential, and may therefore play a role in regulating the translation initiation of specific transcripts [2,7,27,36,38,42]. There are three well-defined domains within the eIF3K sequence (Fig. 1B). The first is the N-terminal HEAT (Huntington, Elongation factor 3, A subunit of protein phosphatase 2A, Target-of-rapamycin) repeat-like HAM (HEAT analogous motif) (aa 24–121) [57]. HEAT domains often mediate protein–protein interactions [1]. The second domain is the winged-helix-like (WH) domain in the C-terminus (aa 132–191), which could mediate protein–RNA interactions [57]. In addition, the HAM and WH domains form elements of the PCI (26S proteasome lid, COP9 signalosome, eukaryotic initiation factor 3) domain (aa 60–218). The fragment of eIF3K identified in the yeast two-hybrid screen (aa 1–97) encodes ~75% of the HAM domain (summarized in Fig. 1B).

To further characterize the interaction between PML-I and eIF3K, yeast two-hybrid interaction assays were performed using various truncated peptides encoding fragments of PML-I and eIF3K. The amino acid sequence encoded by exon 9 of PML-I includes a predicted exonuclease III-like fold (aa 604–750) required for targeting PML-I to nucleolar fibrillar centers during stress or senescence [9]. To determine whether the exonuclease III-like domain of PML-I was required for interaction with eIF3K, we tested a fragment encoded by exon 9 of PML-I (aa 750–882), which lacks the exonuclease III-like domain, for interaction with eIF3K using a yeast two-hybrid assay. This fragment of PML-I was found to interact with eIF3K, indicating that the PML exonuclease III-like domain was not required for interaction with eIF3K (Supplementary information Fig. 1).

In order to map the region of eIF3K required for interaction with amino acids 750–882 of PML-I, several eIF3K deletion mutants were also generated, and tested for interaction with PML-I (aa 750–882) in a yeast two-hybrid assay (Fig. 1C). The smallest fragment of eIF3K found to interact with PML-I contained residues 57–97 that correspond to a region within the HAM domain of eIF3K. The C-terminal region of eIF3K (aa 97–218) absent in the sequence identified from the yeast two-hybrid screen did not interact with PML-I (aa 750–882), suggesting that residues 57–97 may contain the only region required for interaction with this PML-I fragment.

During the cloning of the full-length human eIF3K cDNA from HeLa total mRNA, we isolated two different forms of eIF3K, one matching the previously reported isoform ([52]; NCBI Reference ID: NM_013234), as well as an unreported isoform (Supplementary information Fig. 2). The novel eIF3K isoform (eIF3K isoform 2; GenBank ID: JX870647) is similar to a human (EAW56803) and a mouse EST (AAH27638), suggesting that this isoform is conserved in mammals. Isoform 2 of eIF3K (eIF3K-2) contains an in-frame deletion of exon 2 (encoding aa 21–53) resulting in deletion of part of the HAM domain. Removing the residues encoded by exon 2 from the fragment of eIF3K isolated in the yeast two-hybrid screen (clone 1–20, 54–97) appeared to weaken but did not

Fig. 2 – In vitro interaction between PML-I and eIF3K. MBP, MBP-PML-I-E9Δexo (750–882 aa), GST and GST-eIF3K proteins were expressed in E. coli and purified with amylose (MBP) or glutathione Sepharose (GST) beads. (A) GST or GST-eIF3K proteins were bound to GST beads, followed by incubation with MBP–PML-I-750–882. Recovery of MBP–PML-I-750–882 with GST-eIF3K was detected by SDS-PAGE and Western blotting. (B) MBP or MBP–PML-I-E9Δexo proteins were bound to amylose beads, followed by incubation with GST-eIF3K. Recovery of GST-eIF3K with MBP–PML-I-E9Δexo was detected by SDS-PAGE and Western blotting. For (A) and (B) lanes 1–3 were loaded with the equivalent of 10% of the sample used for pull-downs. Lanes 4 and 5 show the eluted proteins after purification.
abolish interaction with PML-I (aa 750–882), suggesting both isoforms of eIF3K interact with this isoform of PML (Fig. 1C). This weaker two-hybrid interaction could reflect a preference of PML-I for isoform 1 over isoform 2 of eIF3K. We also observed that the eIF3K-2 fragment was not as highly expressed in yeast as the isoform 1 fragment (Supplementary information Fig. 3), thus expression level differences could also contribute to the differences observed in the reporter assay.

**PML and eIF3K interact in vitro and in vivo**

To test whether the interaction between PML-I and eIF3K observed in our yeast assay was direct, we performed in vitro protein interaction assays using affinity-purified recombinant glutathione-S-transferase (GST)-tagged eIF3K-2 and maltose binding protein (MBP)-tagged PML-I (aa 750–882), which lacks the exonuclease III domain. This shorter exon 9 fragment was used in these assays due to the propensity of the full-length peptide encoded by exon 9 to form insoluble protein aggregates when expressed in *E. coli* (data not shown). In vitro pull-down assays revealed that MBP–PML-I<sub>750–882</sub> bound to GST-eIF3K but not GST alone (Fig. 2A). The reciprocal MBP co-purification assay demonstrated that GST-eIF3K bound to MBP–PML-I<sub>750–882</sub> but not MBP alone (Fig. 2B). Collectively these data support the interaction of PML-I directly with eIF3K in vitro, which is consistent with the observed yeast two-hybrid interaction assay (Fig. 1C).

To address the possibility that eIF3K could interact with other regions of PML-I, hexahistadine-tagged full length PML-I and PML-IV were expressed in insect cells and assessed for the ability to co-purify with GST-tagged eIF3K-1 or eIF3K-2 (Fig. 3A). PML-IV contains the same exon arrangement as PML-I, but lacks exon 9 and instead contains a short sequence of 13 aa at the C-terminus that are unique to PML-IV [10,29]. After GST-purification, both PML-I and PML-IV were recovered with eIF3K isoforms 1 and 2, but not with GST alone (Fig. 3A), suggesting that both isoforms of eIF3K also interact with PML independent of the sequence encoded by exon 9.

To determine whether PML and eIF3K interact in human cells we performed co-immunoprecipitation assays. Consistent with data obtained from the yeast two-hybrid and in vitro protein interaction assays, endogenous PML was recovered when endogenous eIF3K was immunoprecipitated from human HEK-293T cells (Fig. 3B), indicating that these two proteins can also associate in vivo.

**eIF3K colocalizes with PML NBs**

Although several reports indicate that eIF3K is primarily localized to the cytoplasm, the precise subcellular localization of eIF3K is not well established. Different reports indicate eIF3K can be found associated with intermediate filaments [35] or sites of cell–cell contact [31] in epithelial cells as well as adopting either perinuclear [31] or pan-cytoplasmic [35] localization in other cell types. Cytoplasmic localization is also consistent with the ability of eIF3K to associate with the translation pre-initiation complex. However, other translation initiation factors, such as eIF3E and eIF4E, also have nuclear functions and have been reported to associate with PML and PML NBs [17,33]. Similarly, although PML is primarily a nuclear localized protein, at least one isoform (PML-VIIb) lacks the nuclear localization signal [29] and is localized to the cytoplasm where it appears to play a role in TGF-β signaling [34]. PML isoforms also localize to both the nucleus and cytoplasm in early G1 [14,19]. In addition, PML-I contains a predicted nuclear export signal (NES), potentially allowing shuttling to and from the nucleus, although the precise regulation of
this shuttling and its functional consequences have yet to be determined [26]. Therefore, we wanted to determine the cellular compartment in which PML and elf3K interact, which may then affect the interpretation of the possible functional consequences of their interaction.

To determine the cellular localization of PML and elf3K, U2OS cells were immunostained for endogenous elf3K and PML (Fig. 4A). Endogenous elf3K is localized primarily to the cytoplasm in perinuclear structures consistent with a previous report [31]. The specificity of the antibody used in our studies for elf3K-1 and elf3K-2 was confirmed by the co-detection of ectopically expressed epitope-tagged elf3K isoforms by immunofluorescence and Western blotting techniques (Supplementary information Fig. 4). Conversely, PML was primarily found in the nucleus of

Fig. 4 – elf3K colocalizes with PML-I and PML-IV in PML NBs. (A) U2OS cells were fixed and immunostained for endogenous elf3K (green) and endogenous PML (red). (B) U2OS cells stably expressing GFP-tagged PML-I or PML-IV (green) were fixed and immunostained for endogenous elf3K (red). Examples of colocalization (merge) between elf3K and PML nuclear bodies are indicated with arrows. DNA is visualized with DAPI (blue, Merge + DAPI). Scale bars = 10 μm. (C) Bar graph indicating the percentage of cells displaying either strong (black) or partial (gray) colocalization between elf3K and PML in PML-I- or PML-IV-expressing cells as indicated. (D) Western blot of 293A, U2OS and HeLa cell lysates with anti-elf3K antibody. Arrow indicates a protein of ~20 kDa in U2OS and HeLa lysates observed with longer exposure (Long Exp) that is of similar molecular weight as elf3K-2. (E) U2OS cells were transfected with empty vector, elf3K-1 or elf3K-2 expression plasmids and cell lysates were analyzed by Western blot with anti-elf3K antibodies. Untransfected GFP-PML-I- and GFP-PML-IV-expressing U2OS cells were similarly prepared and analyzed.
cells within punctate nuclear domains known as PML nuclear bodies (NBs). Thus, PML and elf3K do not appear to colocalize to the same discrete structures under normal growth conditions in U2OS cells. However, with the exception of a small number of constitutively associated PML NB components such as SPF100, SUMO1 and the PML isoforms [10,51] the majority of PML NB-associated proteins interact with PML NBs in a transient fashion either during the cell cycle or in response to diverse cellular stimuli and cell stress [5,13]. Often these interactions can be enhanced by increased expression of PML, the putative interacting protein, or both. In order to enhance the probability of observing elf3K at PML NBs, we used U2OS cell lines that express N-terminally GFP-tagged PML-I or PML-IV [18]. PML NBs typically contain all nuclear PML isoforms, with PML-I being the predominant isoform [10]. In these cell lines, PML NBs increase in number and are enriched in the PML isoform that is over-expressed (Fig. 4B, Supplementary information Fig. 5).

In contrast to untransfected U2OS cells, endogenous elf3K associated with PML NBs in a subset of cells expressed GFP-tagged PML-I or PML-IV (Fig. 4B). The percentage of cells in which endogenous elf3K colocalized with PML nuclear bodies was quantified (Fig. 4C). Colocalization was scored as either strong, partial or negative (Supplementary information Fig. 6). In cells with strong colocalization every PML nuclear body had an obvious elf3K punctum associated with it (Supplementary information Fig. 6). Partial colocalization was defined as a cell having either some, but not all PML bodies associated with elf3K or having poorly defined elf3K-PML body colocalization (Supplementary information Fig. 6). In U2OS cells expressing GFP—PML-I, 71% of cells (29% strong, 43% partial colocalization) showed some degree of endogenous elf3K localized to PML-I enriched NBs (Fig. 4C). Similarly, in U2OS cells expressing GFP—PML-IV, 91% of cells (47% strong, 44% partial colocalization) showed some degree of elf3K association with PML-IV enriched NBs (Fig. 4C). These results indicate that endogenous elf3K isoforms can associate with PML NBs enriched in either PML-I or PML-IV, which is consistent with our ability to co-purify full-length recombinant PML-I and PML-IV with elf3K in vitro (Fig. 3A).

While the data presented in Fig. 4B indicate that endogenous elf3K interacts with PML-I or PML-IV enriched PML NBs, it is unclear which elf3K isoform(s) might be involved in the association. To begin to identify which isoform(s) of elf3K might be associated with PML NBs we first analyzed cell lysates from U2OS, 293 and HeLa cells by Western blotting (Fig. 4D). In all three cell lysates, a protein of ~24 kDa was detected with the anti-elf3K antibody (Novus Biologicals, NB100-93304, Fig. 4D). There is some discrepancy about the molecular weight of elf3K, with various reports detecting elf3K at between 24 and 28 kDa [31,48]. Since this antibody was confirmed to detect epitope-tagged elf3K-1 and elf3K-2 by Western blotting (Supplementary information Fig. 4C) we also tested the ability to detect untagged elf3K-1 and elf3K-2 by expressing these proteins in U2OS cells (Fig. 4E). Compared to vector-transfected cells, elf3K-1—transfected cells showed a substantial increase in the ~24 kDa band, suggesting that elf3K-1 is the predominant isoform in the cell lines we tested (Fig. 4E). Expression of untagged elf3K-2 revealed a protein of ~20 kDa (Fig. 4E) which is similar in size to proteins identified in U2OS and HeLa lysates upon longer exposure (Fig. 4C, arrow), suggesting that elf3K-2 may comprise only a small fraction of total elf3K in cells. In addition, detection of elf3K in GFP—PML-I and GFP—PML-IV expressing U2OS cells (Fig. 4E) was similar to untreated U2OS (Fig. 4D) or vector-transfected U2OS cells (Fig. 4E), suggesting that elf3K-1 is the predominant isoform in these cells and that expression of PML-I or PML-IV does not significantly alter the elf3K isoform distribution. Given that only a portion of the endogenous elf3K immunoﬂuorescence signal is associated with PML-NBs (Fig. 4B) it remained unclear which elf3K isoform(s) was being detected at the PML NB. It is possible that only elf3K-2, a minor component of elf3K in the cell, is specifically recruited to PML NBs. Alternatively, only a small portion of total elf3K-1 may be localized to bodies. Finally, since both isoforms of elf3K interact with PML in vitro, it is possible that both isoforms of elf3K associate with PML NBs.

In order to more directly test which isoform(s) of elf3K can associate with PML bodies, C-terminally FLAG-tagged elf3K isoforms 1 and 2 were expressed in U2OS cells stably expressing GFP—PML-I or GFP—PML-IV, and the ability to associate with PML NBs was assessed (Fig. 5A). Isoform 1 of elf3K showed a pan-cellular localization pattern and was generally not observed to associate with PML NBs in either the PML-I or PML-IV expressing cells. Isoform 2 of elf3K adopted a similar pan-cellular localization pattern, however in contrast to isoform 1, it could additionally be found at PML NBs (Fig. 5A). In contrast to endogenous elf3K, this association was more apparent in PML-I—enriched bodies than PML-IV enriched bodies (Fig. 5A). Similar localization experiments performed with untagged or HA-tagged elf3K-1 and elf3K-2 yielded similar results, with elf3K-2 showing increased association with PML-I—enriched PML NBs (Supplementary information Figs. 7 and 8). The percentage of transfected cells showing strong or partial colocalization with PML NBs was determined (Fig. 5B) using the same scoring criteria as in Fig. 4 (Supplementary information Fig. 9). When FLAG-tagged elf3K-1 was expressed in PML-I or PML-IV—expressing U2OS cells, there was no colocalization between elf3K-1 and PML NBs. (Fig. 5B). In contrast, FLAG-tagged elf3K-2 was partially (20.5%, gray bars) or strongly (0.7%, black bars) associated with PML-IV enriched NBs in 21.2% of the elf3K-2—transfected cells (Fig. 5B). In PML-I expressing U2OS cells, elf3K-2 was partially (35%) or strongly (26.1%) associated with nuclear bodies in 61.1% of transfected cells (Fig. 5B). A similar trend was observed with untagged and HA-tagged elf3K (Fig. 5B). These results suggest that elf3K-2, but not elf3K-1, associates with PML NBs enriched in PML-I to a greater degree than those bodies enriched in PML-IV. Unlike the localization pattern observed for endogenous elf3K (Fig. 4B), in which the isoform(s) of elf3K being detected are uncertain, these results identify elf3K-2 as a specific isoform of elf3K that interacts with PML NBs. Furthermore, these results implicate the exon 9 encoded peptide, found only in PML-I, as an important determinant of elf3K-2 interaction with PML NBs in cells. Similar colocalization of elf3K-2 and PML-I was also observed when HA-tagged elf3K-2 and FLAG-tagged PML-I were expressed in U2OS cells (Supplementary information Fig. 10). The association of elf3K-2 and PML-I was also confirmed by co-immunoprecipitation from cellular lysates from cells expressing FLAG—tagged PML-I and HA—tagged elf3K-2 (elf3K-2—HA) in transfected HEK-293A cells (Fig. 5C). elf3K-2—HA was not recovered from cell lysates when FLAG—PML-I was not present or in the rabbit IgG control IP samples (Fig. 5C), indicating that elf3K-2—HA was specifically recovered with PML-I. Taken with the experiments in Fig. 3B, these data also indicate that elf3K-2 and PML can be reciprocally immunoprecipitated, further supporting the specificity of this interaction in cells.
Fig. 5 – eIF3K isoform 2 colocalizes with PML-I-containing nuclear bodies. (A) FLAG-tagged eIF3K was expressed in U2OS cells stably expressing GFP–PML-I or GFP–PML-IV. Cells were fixed and immunostained for eIF3K (anti-FLAG). Examples of colocalization between eIF3K and PML is indicated by arrows. DNA is visualized with DAPI (blue, merge with DAPI). Scale bars = 10 μm. (B) Bar graph indicating the percentage of transfected cells displaying either strong (black bars) or partial (gray bars) colocalization between eIF3K and PML in GFP–PML-I- or GFP–PML-IV-expressing U2OS cells. For each cell line, eIF3K-1 or eIF3K-2 was expressed with either no epitope tag (-/-) or with an N-terminal FLAG (F) or HA (H) tag as indicated. Values represent the mean +/− standard error, n = 3–5. (C) FLAG-PML-I and eIF3K-2-HA expression plasmids were transiently expressed, alone or in combination, in 293A cells. Following immunoprecipitation (IP) with anti-FLAG resin or mouse IgG control beads, recovered proteins were identified by Western blotting (WB) for PML-I-FLAG and eIF3K-2-HA as indicated. Input lanes were loaded with the equivalent of 10% of the sample used for immunoprecipitation and 80% of the post-IP elution was loaded in the IP lanes.
Discussion

Loss of the PML protein is associated with genomic instability and cancer of various histological origins [25,59]. There are multiple isoforms of the PML protein that localize to PML NBs [10,29] yet how each individual PML isoform contributes to the various functions attributed to PML NBs is poorly understood. The distinct C-terminal domains of PML isoforms can coordinate specific protein–protein interactions and contribute to the regulation of PML NB function. Although more than 90 proteins are confirmed to associate with PML NBs [15], the majority of interactions are transitory in nature [13], and only a few isoform-specific protein–protein interactions have been reported. Furthermore, the majority of PML NB interactions reported to date have been described in relation to PML-IV. In this study, we have identified the eukaryotic translation initiation factor 3 subunit K (eIF3K) as a novel interacting partner of PML. At least two other translation factors, eIF3E and eIF4E [17,33] have been reported to colocalize with PML NBs; however, no PML isoform-specific interactions have been described for these proteins nor have specific isoforms of translation factors been shown to preferentially interact with PML. The interaction between PML and eIF3K appears to be multifactorial with one interaction domain unique to PML-I (aa 750–882, encoded by exon 9) and at least one other interaction domain found in exons 1–8, which are conserved between PML-I and PML-IV. Yeast two-hybrid assays indicated that the association of eIF3K with exon 9 of PML-I requires a portion of the HAM domain of eIF3K. The HAM domain of eIF3K mediates other protein interactions, such as with cytoplasmic keratin 18 and the 5-HT7 receptor [11,35]. Although the PCI domain of eIF3K may also mediate protein-protein interactions, as has been seen for other PCI domain-containing proteins [43], this domain was not required for association with aa 750–882 of PML-I in a yeast two-hybrid assay (Fig. 1B and C).

In the course of our experiments we also identified a novel human isoform of eIF3K, which we have designated as isoform 2 (eIF3K-2). eIF3K-2 is encoded by an alternatively spliced mRNA that results in an in-frame deletion of exon 2, and which can also be identified in cDNA libraries of humans and mice (Supplementary information Fig. 2). In addition, analysis of expression tagged sequences (ESTs) within Genbank encoding eIF3K suggests there may be several other isoforms of eIF3K that could vary in their ability to interact with PML.

We have shown by cell-free and tissue culture methods that eIF3K and PML can interact with each other. In vitro, eIF3K-1 and eIF3K-2 both interact with the C-terminal domain of PML-I (exon 9, Fig. 1) and with full-length recombinant PML-I and PML-IV (Fig. 3). The interaction between aa 750–882 of PML-I and eIF3K is likely directed based on our in vitro pull-down assays using purified recombinant proteins (Fig. 2). In addition, we demonstrate that eIF3K and PML form protein complexes in vivo that can be co-immunoprecipitated from human HEK-293 cell lysates (Figs. 3 and 5C).

Although both eIF3K isoforms studied here can interact with PML in vitro, we observed differential abilities to associate with PML NBs in cells. Expression of GFP-tagged PML-I or PML-IV in U2OS cells promoted an association of endogenous eIF3K with PML NBs (Fig. 4). Although eIF3K-1 appears to be the predominant isoform in these cells (Fig. 4E) it is not clear which endogenous eIF3K isoform is detected at PML NBs. However, when specific isoforms of eIF3K were introduced in these cells, only eIF3K-2 was found to be strongly associated with PML NBs, and eIF3K-2 was associated with PML-I-enriched NBs to a greater degree than PML-IV-enriched NBs. Our in vitro data also indicated that eIF3K isoforms can interact with PML-I through the unique exon 9 encoded C-terminus, and through another domain that might be common between all PML isoforms. However, in cells PML-I is more efficient at recruiting eIF3K-2 to PML NBs. The discrepancy between the in vitro and cell line data may reflect the different secondary and tertiary structure of PML within nuclear bodies in cells versus soluble PML used for in vitro studies. PML NBs form through SUMO-dependent heteromultimerization of nuclear body associated proteins like DAXX and SP100 that are SUMOylated with several PML protein isoforms via their SUMO interaction motif (SIM) domains encoded in exon 7 of isoforms 1 to VI [28,47,50]. In addition, multimerization of PML protein isoforms is driven by interactions between the RING finger B-box coiled coil (RBCC) domain found in all PML isoforms [29]. The highly multimeric state of PML within PML NBs is one factor in their resistance to solubilization, which is in contrast to recombinant soluble PML protein which is either monomeric or in a reduced multimeric state, and is not associated with other proteins such as DAXX and SP100. Thus, PML protein within PML NBs is likely to form tertiary structures that may partly occlude protein interactions that might occur between eIF3K isoforms and the common N-terminus (encoded by exons 1–7) that encodes the RBCC and SIM domains found in PML isoforms I to VI. As such, the unique C-terminus of PML-I may serve as a more accessible docking site for eIF3K isoforms in cells. Nonetheless, it remains unclear why only eIF3K-2, but not eIF3K-1, colocalizes to PML bodies since both isoforms of eIF3K were able to interact with PML exon 9 in vitro. It is possible that the inclusion of exon 2 in eIF3K-1 could facilitate an interaction with a cellular factor, not present in our in vitro interactions studies, that excludes it from interacting with PML bodies. Alternately, secondary structure changes catalyzed by exclusion of peptide sequences encoded by exon 2 within eIF3K-2 may facilitate better interaction with PML-I within nuclear bodies. Another eIF3 subunit, eIF3E, also colocalizes with PML NBs indirectly via interaction with the Ret finger protein in an isoform-independent manner [39]. Therefore, a third possibility, although less likely given our in vitro data demonstrating direct interaction between eIF3K and the C-terminus of PML-I, is that an adapter protein might interact with eIF3K-2, but not eIF3K-1, to facilitate association with PML-NBs. Alternatively, while eIF3K-2 clearly showed stronger association with PML NBs than eIF3K-1 when these proteins were ectopically expressed (Fig. 5), it is possible that endogenous eIF3K-1 (the more abundant eIF3K isoform) might also have an affinity for PML NBs when it is expressed at endogenous levels (Fig. 4B). However, since only a small proportion of total eIF3K is at PML bodies, it is possible that eIF3K-2, which represents a small proportion of total eIF3K, is still the major endogenous eIF3K isoform at PML bodies. This latter interpretation would be consistent with the observation that eIF3K-2 is more strongly associated with PML NBs when ectopically expressed.

Finally, in response to various cellular stresses (e.g. DNA damage) many proteins transiently interact with PML bodies and post-translational modifications often regulate or result from these PML body interactions [5,13]. In the case of another eIF3
subunit, eIF3K, its localization to the nucleus is regulated via phosphorylation by the cyclin dependent kinase 11 (CDK11) [49]. Although our in vitro data suggests that eIF3K interacts with PML in the absence of post-translational modifications, we cannot exclude the possibility that the association of eIF3K isoforms with PML NBS in cells may be regulated by post-translational modification of eIF3K and/or the PML protein itself. Nonetheless, given our novel finding that eIF3K-2 localizes to PML NBS in an isoform-dependent manner, it is tempting to speculate that alternative splicing of eIF3K transcripts may regulate the localization of eIF3K at PML NBS, for example between different cell types or under various physiological conditions that promote the alternative splicing of eIF3K.

The significance of the association of eIF3K with PML NBS is unclear since the biological role(s) of eIF3K remain poorly characterized. In the cytoplasm, the main function of eIF3K is presumably the regulation of translation initiation; however, eIF3K does not appear to be an essential subunit of the eIF3 complex [36] and general translation does not appear to be affected by eIF3K silencing [35]. As well, eIF3K, along with the eIF3E and eIF3F subunits, are not conserved in budding yeast [2,38,42]. Even in the cytoplasm, eIF3K has been reported to play various physiological functions are shared between eIF3K isoforms, and what specific consequences of the localization of this isoform to PML NBs can be weakly associated with PML NBs in cells, or endogenous eIF3K, where the isoform(s) being studied are demonstrated to be weakly associated with PML NBS in cells, or endogenous eIF3K, where the isoform(s) being studied are unclear [31,35]. Therefore, it will be important to determine what specific functions can be attributed to eIF3K isoforms, before the functional consequences of the localization of this isoform to PML NBS can be fully explored.

Materials and methods:

Plasmid construction

The human PML isoform I, exon 9 (PML-I-E9) sequence encoding amino acids 604–882 (GenBank Accession NM_033238.2) and a smaller fragment of the human PML-I-E9 sequence lacking the exonuclease III domain (amino acids (aa) 750–882) was cloned into pgBT9 or pbGBK7 in frame with the coding region for the DNA binding domain of the yeast Gal4 transcription factor (Gal4DBD). Fragments of eIF3K were cloned into pACT2 (Clontech) in frame with the coding region for the activation domain of the yeast Gal4 transcription factor (Gal4AD). An alternative splice isoform of eIF3K (eIF3K isoform 2, see Supplementary information Fig. 2) was isolated from total HeLa RNA extracts, reverse transcribed and cloned into the mammalian expression plasmid pcDNA3.1 (−) containing an influenza hemagglutinin (HA) epitope tag on the C-terminus (eIF3K-iso2-HA). For expression of GST-eIF3K in E. coli, the coding sequence for eIF3K was cloned into pGEX-5 × 1 vector (GE Life Sciences). Maltose binding protein (MBP)–PML-I recombinant plasmid was created by cloning the sequence encoding PML-I-E9 lacking the exonuclease III domain (750–882 aa) into pMal-p4G vector (New England Biolabs).

Yeast strains and media

Yeast strains and media—Standard methods were used for growth and manipulation of yeast and bacteria [8,46]. Yeast were cultured in YPDA (1% yeast extract (BioShop Canada), 2% Peptone (BioShop Canada). 0.003% adenine, 2% glucose, synthetic defined (0.67% yeast nitrogen base without amino acids (BioShop Canada), 2% glucose, 0.003% adenine, 0.002% uracil, and all required amino acids). Yeast were transformed by the LiAc/SS-DNA/PEG method [23].

Cell culture

HEK-293A, U2OS and HeLa cell lines were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal calf serum, 1% penicillin/streptomycin at 37 °C with 5% CO₂. Human osteosarcoma (U2OS) cell lines stably expressing green fluorescent protein (GFP)–PML-I and GFP–PML-IV [18] were maintained as above with the addition of 800 (PML-I) or 1600 (PML-IV) μg/ml of G418.

Yeast two-hybrid assay

Yeast two-hybrid screens were performed according to instructions described in the Matchmaker Gold yeast two-hybrid manual (Clontech). PML-I-E9 bait plasmid (pbGBK7) was introduced into Saccharomyces cerevisiae PML-I-E9 bait plasmid (pGBKT7) was introduced into Saccharomyces cerevisiae P696 yeast two-hybrid reporter strain and mated with an isogenic yeast strain transformed with a human adult brain cDNA library cloned into pACT2 (Clontech). For domain mapping, plasmids directing expression of the indicated Gal4AD-tagged fragments of eIF3K and Gal4DBD-tagged PML-I (aa 750–882) were transformed into yeast strains Y2HGold and Y187, respectively. Transformants were mated, and diploids were isolated on selective medium lacking leucine and tryptophan and subsequently patched to solid rich medium containing a limiting amount of adenine (YPDA).

Expression and purification of recombinant eIF3K and PML

GST-eIF3K and MBP–PML-I (aa 750–882) recombinant proteins were expressed in Escherichia coli Rosetta (DE3) cells (Novagen), while His₆-tagged full-length PML-I and PML-IV were expressed in insect cells.

For expression of GST-tagged eIF3K in E. coli, the coding sequence for eIF3K (isoform 1 or 2) was cloned into the GST expression vector pGEX-5X-1, and the plasmid transformed into Rosetta cells. Transformants were grown to OD₆₀₀ 0.5–1.0 in LB medium and were induced with 0.1 mM IPTG for 3 h at 37 °C. Cells were harvested, aliquoted and stored at −80 °C. Cell suspensions were sonicated for five rounds of 10 s each at 30% output on a Misonex Sonicator 3000 and centrifuged at 21 000 g for 15 min at 4 °C. Protein concentration in the supernatant was quantified by a Bradford assay and diluted to a final volume of 750 μl in purification buffer. 60 μl of glutathione
Sepharose 4B beads (GE Life Sciences) were added to the soluble fraction and the suspensions rocked for 30 min at room temperature. Beads were washed three times in purification buffer.

For expression of full-length PML-I and PML-IV protein in insect cells, the Bac-to-Bac baculovirus expression system was used according to the manufacturer’s instructions (Life Technologies). The coding sequence for PML-I and PML-IV were cloned into Fastbac expression vectors and transformed into Max efficiency DH10Bac competent cells (Life Technologies). Bacmids were transfected into sf-9 cells with Cellfectin (Life Technologies) to produce recombinant baculovirus particles. Virus was collected from the culture medium and used to infect sf-9 cells for 48 h. Infected cell lysates were prepared by resuspending cell pellets in ten volumes of insect cell lysis buffer (50 mM Tris pH 7.4, 250 mM sucrose, 150 mM NaCl, 0.1% Triton X-100, and 2 mM PMSF). Suspensions were sonicated for six rounds of 10 s at 30% output.

In vitro binding assays

In vitro affinity purification assays were performed by pre-clearing 2 μg of protein lysates with glutathione Sepharose 4B or amylase resin for GST and MBP tagged proteins, respectively and incubated with GST, GST-eIF3K or MBP and MBP-PML-I (750–882 aa) bound beads for 3 h at 4 °C. The beads were washed three times with wash buffer (PBS supplemented with 0.1% Triton X-100 and 50 mM NaCl) and once with PBS. The co-purified proteins were eluted by boiling in 2 × SDS loading buffer and analyzed by Western blotting.

For testing interaction of GST-eIF3K with His6-PML-I and His6-PML-IV, 400 μL of diluted insect cell lysate was added to 20 μL of glutathione Sepharose beads containing bound GST or GST-eIF3K and rocked for 1 h at room temperature. Beads were washed four times for 5 min at room temperature in 1 mL purification buffer containing 300 mM NaCl. Co-purified proteins were eluted by boiling for 5 min in 60 μL of protein gel loading buffer.

Co-immunoprecipitation assay and western blotting

For co-immunoprecipitation of endogenous proteins, untransfected human embryonic kidney (HEK-293T) cells were lysed with ice-cold lysis buffer (50 mM Tris–HCl pH 8, 300 mM NaCl, 0.25% Nonidet P-40, 1 × protease inhibitor cocktail). Cleared lysates were incubated overnight at 4 °C with mouse anti-eIF3K antibody that was pre-bound to PureProteome™ Protein G magnetic beads (Millipore). The beads were washed 3 times with wash buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA) followed by one wash with PBS. The bound proteins were eluted by boiling in 2 × SDS sample buffer and analyzed by Western blotting. For co-immunoprecipitation of tagged PML and eIF3K, HEK-293A cells were transfected with N-terminal FLAG tagged PML-I and C-terminal HA-tagged eIF3K expression plasmids using Lipofectamine 2000 (Life Technologies) following the manufacturer’s instructions. At 48 h post transfection, cells were lysed with ice-cold lysis buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 0.5% Nonidet P-40, 1 × protease inhibitor). Cleared lysates were mixed with mouse monoclonal anti-FLAG M2 magnetic beads (Sigma) or mouse IgG bound to PureProteome™ Protein G magnetic beads (Millipore) and incubated overnight at 4 °C. Beads were washed four times with lysis buffer and eluted with 2 × protein sample buffer and boiling. Recovered proteins were analyzed by SDS-PAGE and Western Blotting with rabbit anti-ECS (DDDDK) antibody (Bethyl Laboratories, A190–102A) and rabbit anti-eIF3K (Novus Biologicals, NB100-93304).

Immunofluorescence

Protein colocalization was visualized by fluorescence microscopy. Cells were plated onto coverslips in a 6-well plate and transfected with the relevant plasmids. Forty-eight hours post-transfection, cells were washed with PBS and fixed in 3% paraformaldehyde. Cells were permeabilized with 0.5% Triton X-100 in PBS, and blocked with 4% BSA in PBS. Cells were then immunolabeled with primary antibodies (1 h, diluted in blocking buffer), washed with PBS and incubated with fluorescently labeled secondary antibodies (45 min, diluted in blocking buffer). Following washes in PBS, cells were incubated with 1 μg/mL of 4',6-diamidino-2-phenylindole (DAPI) stain to visualize DNA. Fluorescent images were captured with Zeiss Axiovert 200M Microscope and Hamamatsu Orca R2 Camera or with Zeiss Cell Observer Microscope under a 63 × immersion oil objective lens. Images were processed using only linear adjustments (e.g. brightness/contrast) with Slidebook (Intelligent Imaging Innovations, Boulder, CO) and Adobe Photoshop CS5. Quantification of eIF3K colocalization with PML nuclear bodies was determined by counting 50–100 eIF3K positive cells per experimental condition and calculating the percentage of cells with no, partial or complete colocalization between eIF3K and PML nuclear bodies. The means and standard errors from at least three independent experiments were calculated and statistical significance was determined using the student’s t-test in Microsoft Excel 2007.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2013.09.001.

R E F E R E N C E


