IDENTIFICATION AND CHARACTERIZATION OF PROMYELOCYTIC LEUKEMIA (PML)-ISOFORM 1 SPECIFIC PROTEIN-PROTEIN INTERACTIONS

by

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ABSTRACT

Loss of the promyelocytic leukemia (PML) protein is associated with genomic instability and cancer of various histological origins. There are several isoforms of the PML protein that localize in the multiprotein subnuclear structures known as PML nuclear bodies (PML NBs). How each individual PML isoform contributes to the functions of PML NBs, through its specific protein-protein interactions, has yet to be elucidated. The aim of the present study was to identify and characterize PML isoform-I (PML-I) specific protein-protein interactions. Using yeast two-hybrid screens, several interacting partners of PML-I were identified that play roles in translational regulation, including eukaryotic initiation factor 3 subunit K (eIF3K). As a component of the mammalian eIF3 complex, eIF3K plays a significant role in regulating translation initiation. Using various experimental approaches such as yeast interaction mating assay, *in vitro* pull-down, co-immunoprecipitation, and immunofluorescence, eIF3K was shown to interact with PML-I *in vitro* and *in vivo*. Through its interaction with eIF3K, overexpression of PML-I resulted in the concomitant increase in eIF3K protein levels in both normal and cancerous mammalian cells. This suggests that PML-I may be involved in regulating eIF3K protein translation or stability, which in turn could affect translation of specific mRNAs or global translation in cancer cells that have reduced expression of PML-I. Therefore, this study has provided new insight into how PML-I may regulate translation in normal and malignant cells via its interaction with eIF3K.

LIST OF ABBREVIATIONS USED

4E-BP	Eukaryotic initiation factor 4E-binding protein
aa	Amino acid
Ad5	Adenovirus type 5
ADE2	Adenine biosynthetic reporter gene
AML1	Acute myeloid leukemia 1
APL	Acute promyelocytic leukemia
ATP	Adenosine triphosphate
ATRA	All-*trans* retinoid acid
AUR1C	Aureobasidin A-resistant gene
C/EBPε	CCAAT/enhancer-binding protein ε
CBP	CREB-binding protein
$CDK11^{p46}$	Cyclin-dependent kinase 11
CDK4 or 6	Cyclin-dependent kinase 4 or 6
CDKN1B	Cyclin-dependent kinase inhibitor 1B
Co-IP	Co-immunoprecipitation
COPS6	COP9 constitutive photomorphogenic homolog subunit 6 (Arabidopsis)
CREB	Cyclic adenosine monophosphate response element binding
DAPI	4',6-diamidino-2-phenylindole
Daxx	Death-domain-associated protein
DEAD	Aspartic acid (D), glutamic acid (E), alanine (A), aspartic acid (D)
DKC1	Dyskeratosis congenita 1

DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
E4 Orf3	E4 gene open reading frame 3
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
eIF	Eukaryotic initiation factor
EXOIII	Exonuclease III
FBS	Fetal bovine serum
FKBP12	FK506-binding protein-12
GAL4 AD	*GAL4* activation domain
GAL4 DBD	*GAL4* DNA binding domain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
K8/K18	Keratin 8 and 18
HA	Hemagglutinin
HAM	HEAT analogous motif
HCl	Hydrochloric acid
HDAC	Histone deacetylase
HEAT	Huntington, elongation factor 3, A subunit of protein phosphatase 2A, target of rapamycin
HEK-293T	Human embryonic kidney 293T cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α	Hypoxia inducible factor-1 alpha
HIPK2	Homeodomain-interacting protein kinase 2
HIS3	Histidine biosynthetic reporter gene
HRP	Horseradish peroxidase
ICD	Interchromosomal domain
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRES	Internal ribosome entry site
ITAF	IRES trans-acting factor
LacZ	β-galactosidase colorimetric reporter gene
LB	Luria-Bertani
Leu	Leucine
MBP	Maltose-binding protein
MEF	Murine embryonic fibroblast cells
MEL1	α-galactosidase colorimetric reporter gene
Met-tRNAi	Methionyl-transfer RNA initiator
MMTV	Mouse mammary tumor virus
MPN	Mpr1-Pad1 N-terminus
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target-of-rapamycin
NBS1	Nijemegen breakage syndrome 1
ND10	Nuclear domain 10
NES	Nuclear export signal
NHDF	Normal human diploid fibroblast cells

NLS	Nuclear localization signal
ODC	Ornithine decarboxylase
p300	Protein 300 kDa
p53	Protein 53 kDa
PABP	Poly (A)-binding protein
PBS	Phosphate-buffered saline
PCI	26S proteasome lid, COP9 signalosome, eukaryotic initiation factor 3
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PEI	Polyethylenimine
PI	Protease inhibitor
PI3K	Phosphatidylinositol 3-kinase
PML	Promyelocytic leukemia
PML NB	Promyelocytic leukemia nuclear body
PMSF	Phenylmethanesulfonylfluoride
POD	PML oncogenic domain
PTEN	Phosphatase and tensin homolog
PVDF	Polyvinylidene fluoride
Q-RT-PCR	Quantitative reverse transcriptase PCR
RARα	Retinoic acid receptor α
RBCC	RING-finger, two B-boxes and a predicted α-helical coiled-coil domain
RFP	Ret finger protein

RHEB	Ras Homolog Enriched in Brain
RING	Really interesting new gene
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
S	Svedberg unit
S6K1	S6 kinase 1
SD	Synthetic defined
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
shRNA	Short hairpin RNA
SIM	Small ubiquitin-like modifier-interacting motif
siRNA	Small interfering RNA
Sp100	Speckled 100 kDa
SUMO	Small ubiquitin-like modifier
TBST	Tris-buffered saline tween
TE/LiAC	Tris ethylenediaminetetraacetic acid/lithium acetate
TERT	Telomerase reverse transcriptase
TIF1	Transcriptional intermediary factor 1
tRNA	Transfer ribonucleic acid
Trp	Tryptophan

TSC 1 and 2	Tuberous sclerosis 1 and 2
U2OS	Osteosarcoma cells
UV	Ultraviolet
VSV	Vesicular stromatitis virus
WH	Winged-helix-like
X-α-gal	5-bromo-4-chloro-3-indolyl alpha-D-galactopyranoside
YNB	Yeast nitrogen base
γ-IFN	Gamma-interferon

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CHAPTER 1: INTRODUCTION

1.1: PROMYELOCYTIC NUCLEAR BODIES AND CANCER

The nucleus of a mammalian cell is highly organized and consists of DNA organized into chromatin and various subnuclear domains with distinct cellular functions, including the nucleolus (involved in ribosome biogenesis), the splicing speckle domain (involved in pre-mRNA splicing), and the promyelocytic leukemia nuclear body (PML NB).[1] The PML NB is involved in a wide spectrum of cellular functions, including tumour suppression, DNA repair, apoptosis, antiviral response, cellular senescence and transcriptional regulation (Figure 1).[2-6] PML NBs, also commonly referred to as PML oncogenic domains (PODs), Kremer bodies or nuclear domain 10 (ND10), range in size from 0.3-1.0 μm in diameter, and there are typically 10-20 bodies present per cell.[7, 8] These dynamic heterogeneous multiprotein structures are found in virtually every mammalian cell type, and they reside in the space between chromosomes known as the interchromosomal domain (ICD).[9-12] The major structural component of the PML NB is the PML protein, and PML (-/-) knockout mice are incapable of forming these discrete nuclear foci.[13, 14] It has long been debated as to how PML NBs can play a role in so many different cellular functions.[6, 12] The current consensus is that their function relates to the localization of a wide array of proteins at these bodies, either transiently (e.g., the tumour suppressor p53)[15] or constitutively (e.g., the transcriptional repressor Sp100),[16] and these proteins themselves are involved in the various functions ascribed to the PML NB.[2-4, 17] In total, there are over 80 proteins known to be associated with PML NBs,[18] and it is believed that

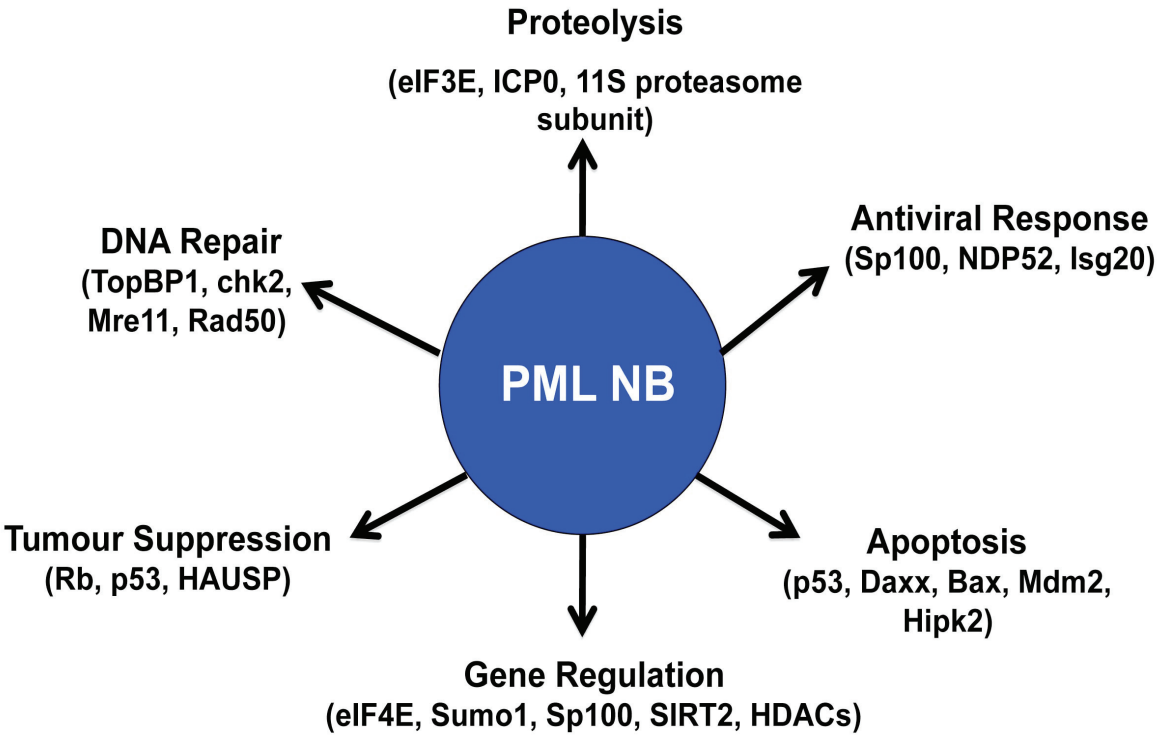


Figure 1: PML NBs are plurifunctional structures. A number of proteins co-localize with PML in PML NBs, thereby implicating PML NBs in diverse cellular functions including proteolysis, antiviral response, apoptosis, gene regulation, tumour suppression and DNA repair. Due to its multifaceted functions, PML NBs have been proposed to play a critical role in cancer development and progression. Adapted from Dellaire and Bazett-Jones (2004).[6]

their association with PML NBs facilitates either their sequestration/release or their post-translational modification, which in turns promotes or inhibits their functions in various cellular processes.[6, 12] Since many of the processes that are regulated by PML NBs play key roles in tumour suppression, including DNA repair and apoptosis, this suggests that PML NBs may play a critical role in preventing cancer development and progression.[12]

1.1.1: THE ROLE OF PML IN CANCER

The gene encoding the human PML protein was first linked to cancer by its identification as the fusion partner of the retinoic acid receptor α (*RARα*) gene as a result of the t(15;17) reciprocal chromosomal translocation in patients with acute promyelocytic leukemia (APL).[19-23] This translocation event creates a PML-RARα oncoprotein that is capable of initiating aberrant cell proliferation, gene expression and apoptosis, thus contributing to the onset and pathogenesis of the disease.[24, 25] PML-RARα fusion proteins are also responsible for disrupting PML NBs into microspeckle structures, a phenomenon that is considered a cytological hallmark of APL.[26] However, administration of all-*trans* retinoid acid (ATRA) can restore normal PML NB structure and function, leading to remission in patients with APL.[27, 28] This illustrates not only the importance of the PML gene in APL, but that the integrity of PML NBs is pivotal in maintaining normal cellular functions that prevent cancer.

Resembling many classical tumour suppressors such as p53,[29] loss of PML protein expression correlates with cancer progression. For example, loss of

the PML protein has been implicated in various solid tumours including carcinomas of the colon, lung, breast and prostate.[30] Additional evidence that supports the role of PML in tumour suppression came from the observation that PML is associated with the regulation of proteins that are involved in several tumour suppressor pathways, including p53,[31] phosphatidylinositol 3-kinases (PI3K/Akt),[32] transforming growth factor-beta (TGF-β),[33] and casein kinase II (CK2).[34] Despite its important function as a tumour suppressor, the PML gene encoding the PML protein is not essential for viability since PML (-/-) mice can still survive; however, they exhibit a weakened immune response, chromosome instability and increased susceptibility to various carcinogens.[13, 14]

1.2: PML STRUCTURE, ISOFORMS, AND ISOFORM SPECIFIC PROTEIN-PROTEIN INTERACTIONS

The PML genomic locus is ~35 kb and is composed of nine exons, of which exons 6-9 are spliced alternatively to yield at least seven known isoforms (designated PML-I to -VII) that are characterized by their varying C-terminal amino acid sequences.[35, 36] Exons 1-3, which are retained in all PML spliced variants, as well as in the PML-RARα fusion protein, encode a domain known as the RBBC, which contains a **R**eally **I**nteresting **N**ew **G**ene (RING)-finger, two B-boxes and a predicted α-helical coiled-coil domain (Figure 2).[37] The cysteine-rich RING finger is the most biologically active domain in the RBCC motif. One of the functions attributed to this domain is the regulation of homo- and heterodimer

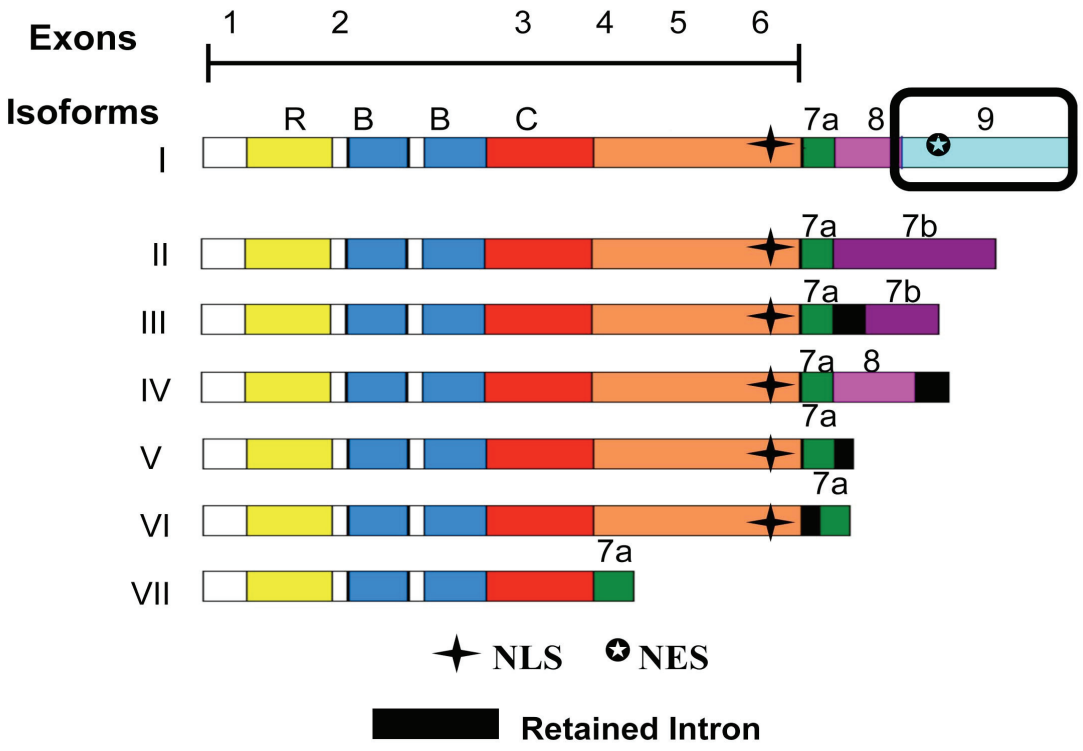


Figure 2: Schematic representation of the PML isoforms. The PML transcript is alternatively spliced at the C-terminus to yield seven known isoforms. Exons 1-3 encode a RBCC motif that is present in all PML isoforms. All PML isoforms with the exception of PML-VII harbour a nuclear localization signal (NLS) in their C-terminal domain. PML-I possesses a discrete nuclear export signal (NES) in the C-terminus, which results in PML-I being localized in both the nucleus and the cytoplasm. Adapted from Jensen *et al.* (2001).[36]

protein-protein interactions between the various PML isoforms. [38-40] *In vivo* studies demonstrated that mutations in this RING domain are capable of disrupting PML NB structure and tumour suppressor activity,[41, 42] suggesting that interactions via the RING domain are important, as they may regulate PML NB formation and function. Furthermore, characterization of the RBCC domain in several other proteins revealed that the RING-finger confers ubiquitin E3 ligase activity to these proteins.[43, 44] However, in the case of the PML protein, the RING-finger may actually confer small ubiquitin-like modifier (SUMO) E3-ligase activity.[45] SUMO is a small protein that is covalently conjugated to various intracellular proteins during post-translational modification to regulate diverse cellular processes including nuclear-cytosolic transport, transcriptional regulation, protein stability and apoptosis.[46] PML is one of a number of proteins that are modified by SUMO isoform 1 (SUMO-1), and this modification event plays an essential role in the formation of PML NBs.[47, 48] In fact, SUMOylated PML has been proposed to act as an organizer of PML NBs by recruiting a variety of other SUMOylated proteins into these nuclear domains, which facilitate the functions of PML NBs.[26]

1.2.1: PML ISOFORMS

As previously mentioned, PML isoforms are produced by alternative splicing of the C-terminal exons within the PML transcript, thereby generating several isoforms with varying C-terminal domains (Figure 2).[36] Similarly, several other RBCC proteins, including the Ret Finger Protein (RFP), Midline-1 and

Transcriptional Intermediary Factor 1 (TIF1), display well-characterized functional domains in their C-termini.[36, 49] A common feature of all PML isoforms with the exception of PML-VII is a nuclear localization signal (NLS) encoded within exon 6.[50] Consequently, all PML isoforms reside primarily in the nucleus during interphase except for PML-VII, which localizes to the cytoplasm.[51, 52] The largest PML isoform, PML-I is unique in that it also possesses a nuclear export signal (NES) encoded by exon 9, which is not present in any of the other PML isoforms (Figure 2).[36, 53] As a consequence, the presence of the NES allows nuclear PML-I to shuttle to the cytoplasm. The exon 9 region of the PML-I protein also contains an exonuclease III (EXOIII) domain (Figure 2), which may indicate a role for this isoform in chromatin tethering/remodeling by binding to DNA and/or facilitating interactions with RNA.[54, 55] Relative to the other PML isoforms, PML-I is also the most highly expressed,[52] suggesting that this isoform is a significant contributor to PML NB functions. In addition to the different subcellular distributions, overexpression of the individual PML isoforms results in PML NBs with distinct morphology and number in both transformed cell lines and in normal diploid fibroblast cells.[52, 56] Currently, the specific functions of the individual PML isoforms are unknown, and elucidating their function is complicated by the fact that multiple isoforms of the PML protein are expressed concurrently in mammalian cells and they form homo- and heterodimers with each other, which are likely mediated by their RBCC domains.[38, 29, 57] Since all PML isoforms share the same N-terminus, they are predicted to share some related functions; however, the variable C-terminal sequences may confer specific biological

functions to each isoform.[36] Indeed, this speculation is supported by a number of studies showing the existence of PML isoform specific protein-protein interactions as discussed below.

1.2.2: PML ISOFORM-I SPECIFIC PROTEIN-PROTEIN INTERACTIONS AND FUNCTIONS

Despite the fact that PML-I is the most abundant isoform, the biological function of this protein is highly elusive and very few proteins have been reported to interact with PML-I in an isoform specific manner. The hematopoietic transcription factor, acute myeloid leukemia 1 (AML1) is one of the few proteins that have been shown to interact specifically with PML-I.[58] AML1 plays an important role in promoting normal development of hematopoietic lineages and regulating gene expression by binding to specific promoter and enhancer elements.[59-61] Nguyen *et al.*[58] demonstrated that AML1 interacts specifically with the exon 9 domain of PML-I. Subsequently, PML-I promotes the association of AML1 and its co-activator p300 at PML NBs, which results in the activation of AML1-dependent transcription and myeloid cell differentiation.

Upon exposure to various genotoxic stresses, such as UV-C irradiation, gamma ionizing radiation, treatment with doxorubicin (topoisomerase II inhibitor), actinomycin D (RNA polymerase I inhibitor) or MG132 (proteasome inhibitor), PML-I along with PML-IV and -V are targeted to the nucleolus where transcription and maturation of ribosomal RNA (rRNA) occurs.[62] An evolutionary conserved C-terminal domain identified in PML-I, -IV and -V was hypothesized to play a role in targeting these isoforms to nucleolar caps. Owing to its natural abundance, PML-

I was the most efficient in localizing to the nucleolar caps in senescent cells compared to PML-IV and -V. Furthermore, the EXOIII domain in PML-I was shown to aid in targeting PML-I to the nucleolus. Conversely, knockdown of PML-I protein expression by small interfering RNA (siRNA) was sufficient to block endogenous PML recruitment to the nucleolus.[62] Collectively, these data support the role of PML-I in targeting PML to nucleolar caps in response to genotoxic stresses.

1.2.3: PML ISOFORM-II SPECIFIC PROTEIN-PROTEIN INTERACTIONS AND FUNCTIONS

During early viral infections, some DNA viruses hijack PML NBs and use them as initial sites for the transcription and replication of their genomes.[63-65] Human adenovirus type 5 (Ad5) is one of a number of viruses that has the propensity to associate with and disrupt PML NBs.[66] More specifically, the Ad5 protein known as E4 gene open reading frame 3 (E4 Orf3) was shown to interact with PML isoform II *in vivo* and *in vitro*.[66] The isoform-specific protein interaction was validated by the isolation of the 40 amino acid region in PML-II that is essential for binding to E4 Orf3.[67] Furthermore, E4 Orf3 was shown to rearrange PML NBs in cells ectopically expressing only PML-II, causing the bodies to change from their normal spherical appearances to track-like structures.[66] As a result, part of the cellular pathology associated with human Ad5 infection may arise from the disruption of the structure and function of PML NBs.

1.2.4: PML ISOFORM-III SPECIFIC PROTEIN-PROTEIN INTERACTIONS AND FUNCTIONS

PML-III has been shown to localize to the centrosome where it may function to regulate centrosome replication and stability by suppressing Aurora A kinase activation.[68] The primary function of Aurora A is to facilitate centrosome maturation and separation, thereby regulating spindle assembly and stability during mitosis and meiosis.[69] In a normal cell cycle, the centrosome is duplicated once during the late G1 to S phase and this duplication checkpoint is tightly regulated as excessive amplification of the centrosome can cause genomic instability.[70, 71] Since centrosome amplification was observed in cells that are PML-III deficient, it has been hypothesized that PML-III may help prevent genomic instability through its association with Aurora A kinase and the centrosome.[68] However, this data remains controversial as another group studying the cellular localization of the various PML isoforms failed to observe the localization of PML-III at the centrosomes.[52] Finally, PML-III may also play a role in viral defense as PML (-/-) mice and primary embryonic fibroblast cells are more susceptible to vesicular stromatitis virus (VSV) infection,[72] and overexpression of PML-III can induce partial resistance to VSV.[73]

1.2.5: PML ISOFORM-IV SPECIFIC PROTEIN-PROTEIN INTERACTIONS AND FUNCTIONS

PML-IV is the most well characterized PML isoform to date and through its specific protein-protein interactions, PML-IV has been implicated in the regulation of cell senescence, apoptosis, transcription, and cell proliferation.[36] For example,

PML-IV is implicated in the regulation of cell senescence and apoptosis through its interaction with the tumour suppressor protein p53.[15, 74] Often dubbed as the "guardian of the genome," p53 plays an important role in the DNA damage response pathway by initiating cell-cycle checkpoints that allow time for the cell to repair the DNA damage.[75] Consequently, loss of p53 correlates strongly with poor prognosis in various types of cancer.[75, 76] The p53 and PML-IV protein interaction has been mapped to the p53 core domain and the C-terminal region of PML-IV.[15] Overexpression of PML-IV in wild-type p53 cells reduced cell viability by inducing premature cell senescence.[77] The mechanism of PML-IV-induced senescence involves stabilization and activation of p53 through phosphorylation at Ser46 by homeodomain-interacting protein kinase 2 (HIPK2) and acetylation at Lys382 by CREB binding protein (CBP).[78] In addition to p53, PML-IV as well as PML-I also interact with HIPK2.[79] This interaction is believed to be mediated via protein sequences in PML that are encoded by exon 8a, which is a region common to both PML-I and PML-IV. The SUMO-interacting motifs (SIMs) in PML-IV and HIPK2 are required for targeting HIPK2 to PML NBs, and for HIPK2-mediated p53 phosphorylation and apoptosis. Thus, the impairment of p53-dependent apoptosis in PML (-/-) mice may arise as a result of loss of PML-IV expression.[79]

PML-IV also participates in both the activation and repression of transcription. For example, PML-IV can repress transcription by interacting with histone deacetylase 1 and 2 (HDAC1/2).[80] This was postulated to be a specific function of PML-IV, since PML-VI and PML/RARα were shown to interact poorly

with HDAC1/2.[80] HDACs are involved in repressing transcription by catalyzing the removal of acetyl groups from the histone (main protein component of chromatin) tails, thereby increasing their positive charge. These positive charge groups can interact with and bind to the negatively charged phosphate groups on the DNA backbone which condenses the DNA structure, thus blocking transcription.[81] PML-IV transcription suppressor function is predicted to be lost in APL, which may lead to altered chromatin remodeling and gene expression, thus initiating transcriptional changes that may regulate the onset of leukaemia.[82]

PML-IV also plays a role in transcriptional activation via its interaction with the PU.1 transcription factor.[83] One of the functions of PU.1 is to activate upstream target genes by binding to a purine-rich PU box such as the CCAAT/enhancer-binding protein ε (C/EBPε) gene, which is an essential gene for granulocytic differentiation.[83, 84] PML-IV was shown to interact with PU.1 and augmented PU.1 association with its co-activator p300, leading to enhanced PU.1-induced transcription of C/EBPε and granulocytic differentiation. However, PML-RARα chimeric protein can dissociate the PU.1/p300/PML-IV complex and inhibit PU.1-induced transcription.[83] Thus, in addition to the deregulation of the transcriptional repressor function of PML-IV, this study provides an alternative mechanism in which PML-RARα may contribute to APL by inhibiting PU.1 transcriptional activation.

Finally, PML-IV plays a role in cellular proliferation through interaction with both the catalytic subunit of telomerase and the oncoprotein Myc.[85, 86] Telomerase is an enzyme that promotes chromosome stability by adding tandem

hexameric repeats to the ends of chromosomes, thus preventing loss of DNA from chromosome ends. The catalytic component of telomerase, telomerase reverse transcriptase (TERT), is recruited to PML NBs through its interaction with PML-IV,[86] suggesting that PML-IV may be involved in regulating telomerase activity. This is supported by the observation that overexpression of PML-IV reduced telomerase activity without altering TERT mRNA levels. This indicates that PML-IV may act as a negative regulator of telomerase through its interaction with TERT.[86] PML-IV also regulates cell proliferation by interacting with Myc oncoprotein. In fact, multiple PML isoforms (e.g., PML-I, -II and –IV) have been shown to interact with Myc; however, PML-IV is the only isoform that is capable of destabilizing Myc to prevent aberrant cell proliferation that is caused by uncontrolled Myc activity.[85]

Therefore, together these data indicate that the expression of the individual PML isoforms plays an important role in both the structure and function of PML NBs. Through their specific protein-protein interactions, PML isoforms can alter the biochemical composition of PML NBs and contribute to their distinct cellular functions.

1.3: EUKARYOTIC TRANSLATION INITIATION AND PML

Although PML is predominately a nuclear protein, many emerging studies indicate that PML may be involved in regulating global translation or translation of a specific subset of mRNAs through its interaction with eukaryotic initiation factor 4, subunit E (eIF4E)[87-89] and/or with the conserved serine/threonine protein

kinase, mammalian target-of-rapamycin (mTOR),[90] which will be discussed in greater detail later on in the chapter.

Translation in eukaryotes is a fundamental and sophisticated biochemical process by which proteins are synthesized at the ribosome from messenger RNAs (mRNAs) that are decoded to produce a specific amino acid sequence according to the genetic code. Due to the importance of proteins in executing and sustaining normal cellular functions, and thus maintaining cellular life, cells invest a great amount of energy in synthesizing proteins. There are three phases associated with translation: initiation, elongation and termination. Initiation is the rate-limiting step of translation; therefore the greatest level of regulation occurs during this stage, especially in response to various stimuli that can compromise the health of the cell such as nutrient deprivation, hormonal imbalance and exposure to various mitogenic growth factors.[91]

The process of translation initiation occurs in the cytoplasm; therefore, mRNAs that are synthesized in the nucleus must be exported to the cytoplasm where the ribosomes are located. However, before being exported to the cytoplasm, the nascent mRNAs are post-transcriptionally modified by the addition of a 7-methylguanosine cap (m^7GpppN where N is any nucleotide) to the first base pair at the 5' end and a poly (A) tail to the 3' end of the mRNA. Furthermore, the non-coding RNA encoded by introns is normally spliced out before the mRNAs are exported to the cytoplasm. Once in the cytoplasm, most eukaryotic mRNAs are translated in a cap-dependent manner; however, translation can also occur via a cap-independent mode.[92]

1.3.1: CAP-DEPENDENT TRANSLATION

Translation initiation in eukaryotic cells involves the formation of the 80S ribosomal complex containing the initiator methionyl transfer RNA (Met-tRNAi) bound to the AUG start codon in the mRNA. This process is highly dependent on the interplay and cross talk between various eukaryotic initiation factor (eIF) subunits. Translation commences when eIF4E binds to the m^7GpppN cap at the 5' end of the mRNA and facilitates nuclear export of the selected transcripts.[92] In the cytoplasm, eIF4E associates with the eIF4G scaffolding protein and this interaction is pivotal for translation to proceed. For example, eIF4E and eIF4G interaction can be inhibited by members of the eIF4E binding protein (4E-BP) family of translational repressor proteins which can compete with eIF4G for binding to eIF4E, thereby rendering capped mRNAs into a non-functional complex.[93] This occurs when 4E-BP is hypophosphorylated, which leads to tight binding with eIF4E and as a result, cap-dependent translation is blocked since eIF4G cannot bind to eIF4E.[94-96] In the next step of translation, eIF4E/eIF4G binds to eIF4A (collectively eIF4E, eIF4G and eIF4A form the eIF4F complex) and poly (A) binding protein (PABP) to form the cap-binding complex. eIF4A is a DEAD-box RNA helicase whose main function is to unwind secondary structures in the mRNA in an ATP-dependent manner, thus allowing efficient attachment of the cap complex to the mRNA. PABP binds to the poly (A) tail in the 3' end of the mRNA and promotes recruitment of the ribosome to the mRNA at the 5' end. The mRNA and cap complex then connects to the 40S ribosomal subunit that is equipped with the eIF2-GTP-Met-tRNAi ternary complex and multiple eIFs (eIF1,

eIF1A, eIF3 and eIF5), through an interaction between eIF4G and eIF3 (Figure 3). This forms a 43S preinitiation complex that then scans along the mRNA toward the 3' end until an AUG codon encoding methionine is recognized. The 43S preinitiation complex is then joined by the 60S large ribosome subunit to form the 80S initiation complex that is then ready for the next phase of translation, elongation. During elongation, transfer RNA (tRNA) bring specific amino acids to the ribosome based on their anti-codon base pairing with the corresponding codon in the mRNA to form a polypeptide chain and this occurs until a stop codon is recognized (termination) (Figure 4).[97]

1.3.2: CAP-INDEPENDENT TRANSLATION

Cap-independent translation differs from cap-dependent translation in that initiation of translation does not require ribosome scanning from the 5' end of the mRNA cap until a start codon is recognized.[98] Instead cap-independent translation relies on an Internal Ribosome Entry Site (IRES) for initiation where the ribosome is immediately directed to the start site by IRES trans-acting factors (ITAFs), which circumvents the need to scan the 5' end of mRNA for an initiator codon. The exact mechanisms by which cap-independent translation occurs are still vague; however, this method of translation has been found to be important for translating specific mRNAs during stress conditions, and offers an alternative mechanism for translation in cases where there are interferences with initiation cap formation.[98]

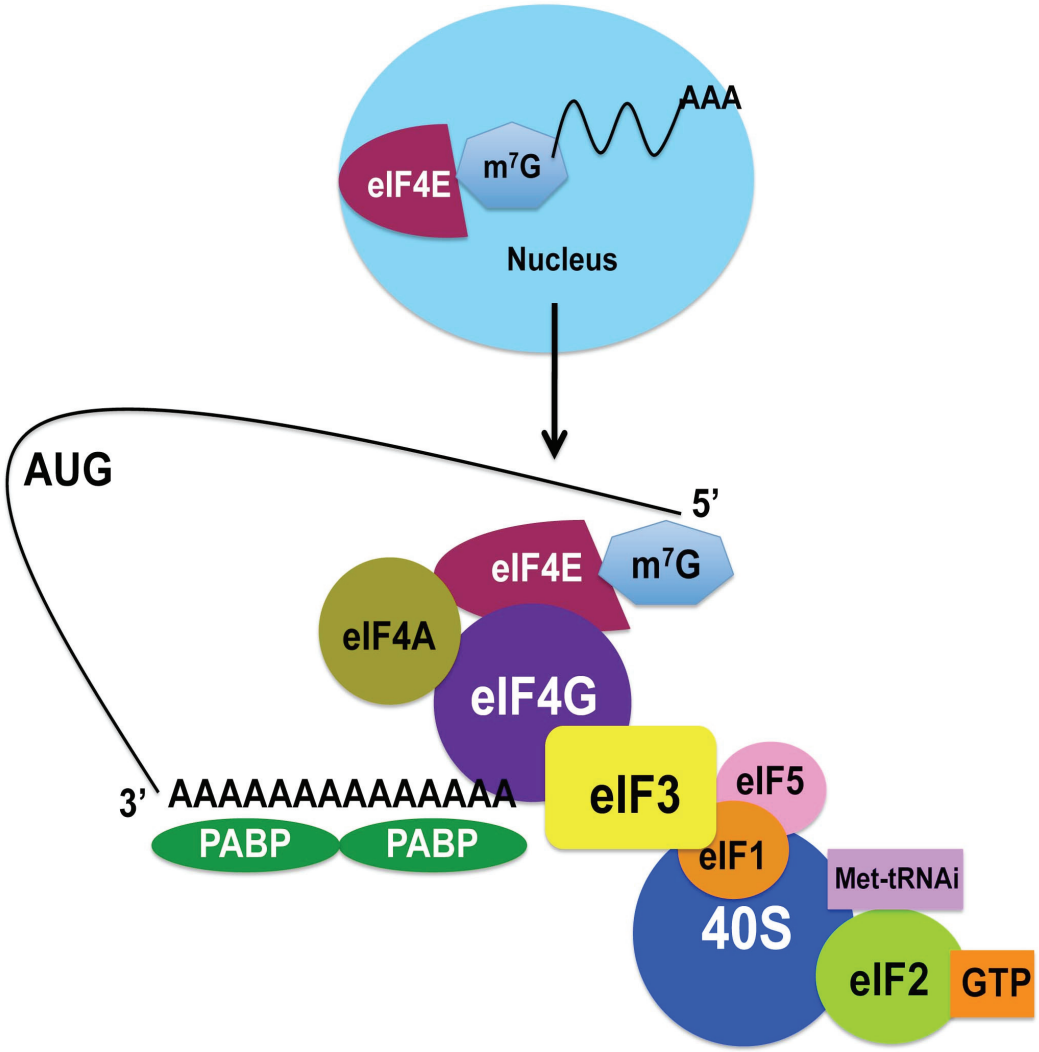


Figure 3: Schematic model of eukaryote cap-dependent translation initiation. The binding of eIF4E to the 7-methylguanosine cap at the 5' end of the mRNA promotes the export of the mRNA to the cytoplasm. In the cytoplasm, the mRNA/eIF4E form a larger complex with eIF4G, eIF4A and PABP. Through the interaction of eIF4G and eIF3, the capped mRNA is brought to the 40S ribosomal subunits containing the eIF2-GTP-Met-tRNAi ternary complex and multiple eIFs (eIF1, eIF1A, eIF3 and eIF5). This forms a 43S preinitiation complex that scans along the mRNA toward the 3' end until a start codon is recognized.

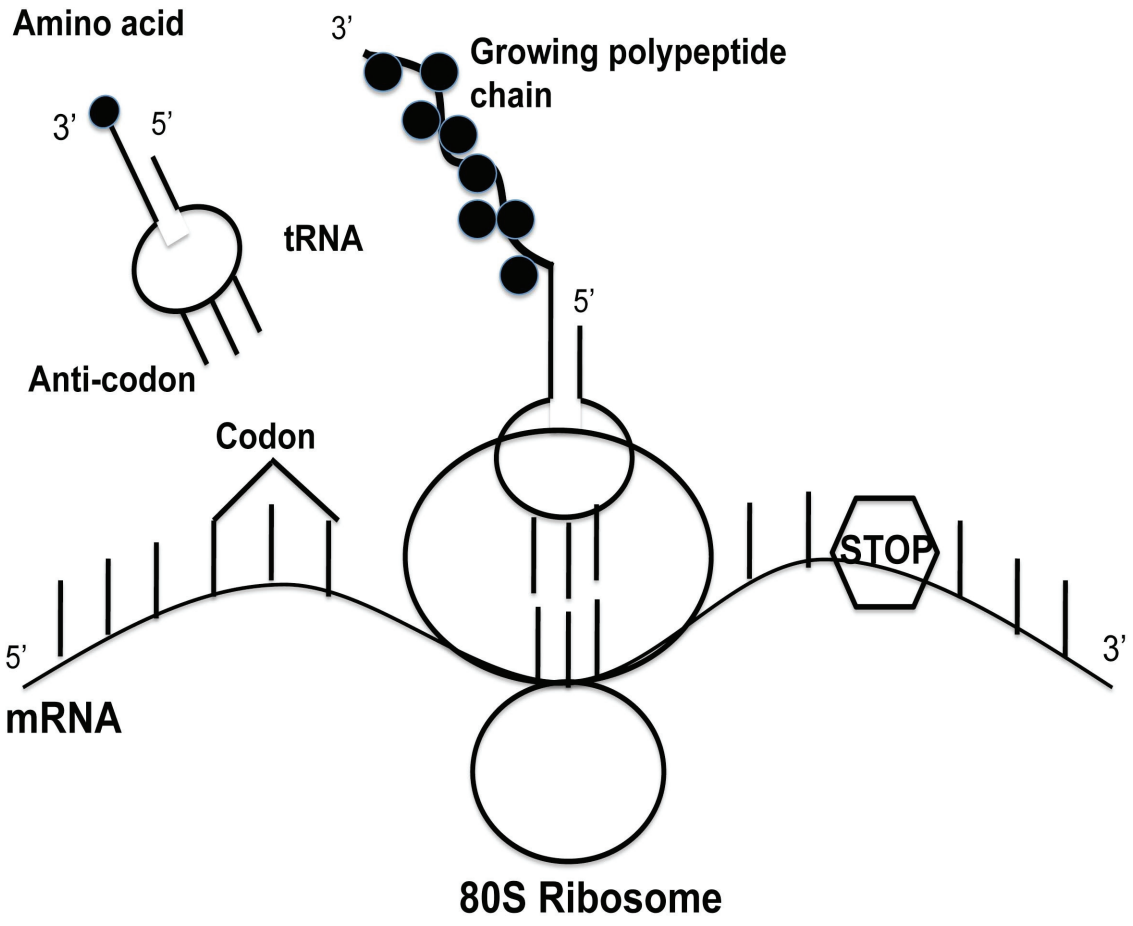


Figure 4: Eukaryotic mRNA translation. Proteins are synthesized from mRNA by the ribosome. Specific amino acids are brought to the ribosome by transfer RNAs based on the complementary codon and anti-codon sequence. The amino acids are added to the growing polypeptide chain as the ribosome travels along the mRNA. This event occurs until a stop codon is recognized after which the polypeptide chain is cleaved and folded properly to form a functional protein.

1.3.3: REGULATION OF TRANSLATION BY mTOR SIGNALING

The mTOR signaling pathway can regulate cap-dependent mRNA translation through several distinct but overlapping mechanisms.[99] One of the important signal transduction pathways that signals through mTOR is the PI3K/AKT pathway.[100] PI3K, and its subsequent downstream target AKT, can be activated by various stimuli such as nutrient, hormone and mitogenic growth factors. Activated AKT can phosphorylate mTOR directly or indirectly through the association with the tuberous sclerosis 1 and 2 (TSC1/2) complex.[101-103] The functional TSC1/2 complex is capable of inhibiting mTOR, and it achieves this inhibition through inactivation of a Ras family small GTPase known as Ras Homolog Enriched in Brain (RHEB). The TSC1/2 complex inactivates RHEB by catalyzing its GTP hydrolysis; therefore, preventing RHEB from activating mTOR. However, AKT can phosphorylate TSC2 and destabilize the TSC1/2 complex, thus allowing RHEB to activate mTOR.[103] Furthermore, loss of the phosphatase and tensin homolog (PTEN) tumour suppressor protein can also lead to activation of mTOR.[104, 105] Subsequently, activated mTOR mediates translation by promoting phosphorylation of the ribosomal protein S6 kinase (S6K1) and 4E-BP1 (Figure 5).[106] Phosphorylation of S6K1 plays an important role in ribosome biogenesis.[106, 107] As mentioned previously, phosphorylation of 4E-BP blocks binding to eIF4E, which allows eIF4E to interact with eIF4G.[95, 96, 108] This interaction is critical for the formation of the cap complex during translation initiation; therefore regulation of mTOR signaling is important for controlling the rate of mRNA translation.

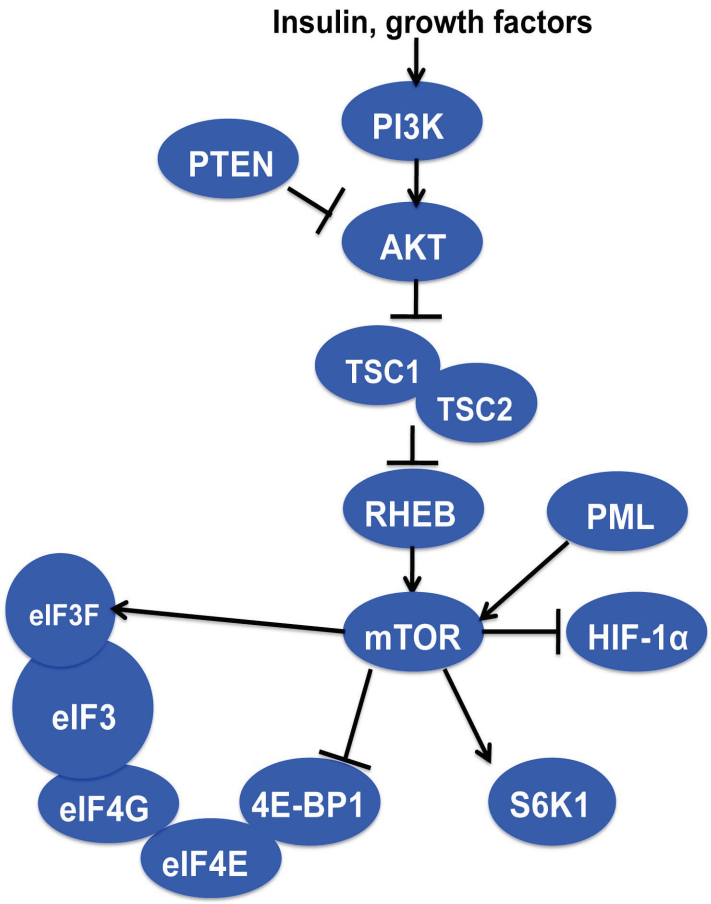


Figure 5: Regulation of translation initiation by the mTOR signaling pathway. Insulin and growth factors can activate the PI3K/AKT signaling cascade. Activated AKT stimulates mTOR activity by promoting the destabilization of the TSC1/2 complex. Activated mTOR can affect translation via four mechanisms: 1) mTOR phosphorylates S6K1 to promote ribosome biogenesis; 2) mTOR phosphorylates 4E-BP, allowing increased association between eIF4E and eIF4G, which is critical for the formation of the cap complex during translation initiation; 3) mTOR interacts directly with eIF3F and increases the association of eIF3 with the docking protein eIF4G; and 4) mTOR signaling can be suppressed by interaction with PML, leading to decrease HIF-1α protein synthesis. Adapted from Mamane *et al.* (2006).[99]

Activation of mTOR through insulin signaling can also stimulate translation initiation by increasing the amount of eIF4G that is bound to eIF3. This is achieved by direct binding of mTOR with eIF3F, which in turn promotes the association of the eIF3 complex with the docking protein, eIF4G. The activation of translation through this pathway can be abolished by treatment with rapamycin.[109] Rapamycin inhibits mTOR through association with its intracellular receptor, FK506-binding protein-12 (FKBP12), which binds directly to the FKBP12-rapamycin binding domain of mTOR.[110] This suggests that mTOR may regulate translation by binding directly to one of the eIF3 subunits, eIF3F, which promote the association of eIF3 with the eIF4G scaffolding protein.

1.3.4: TRANSLATION AND CANCER

Since proteins are linked to virtually every cellular function, deregulation of translation plays a critical role in cancer growth. For example, translation has been implicated in cancer development by controlling the rate of global protein synthesis as well as the synthesis of a select set of mRNAs that are involved in promoting tumour cell survival, angiogenesis, transformation, invasion and metastasis.[91] A paradigm has been proposed that under normal cell conditions, there are weak or inactive mRNAs (e.g., usually GC rich mRNAs with long 5' untranslated regions and complex hairpin structures)[111] that are not normally translated efficiently enough to generate a detrimental effect on the cell. However, when translation is hyper-activated (e.g., in response to viral infection, hormonal changes, growth factor stimulations, etc.), these weak mRNAs can be

translated more successfully, leading to an unbalanced production of cellular proteins that can cause cell proliferation and malignant transformation.[112, 113] Indeed, the survival of various cancer cells appears to depend highly on an enhanced global translation rate and increased binding to ribosomes.[114, 115] Furthermore, cancer cells often exhibit a greater number of nucleoli, which are enlarged in appearance in comparison to normal cells, suggesting that nucleoli can be utilized as a cancer biomarker.[116, 117] Since the nucleolus is the site of rRNA biogenesis and ribosome assembly, this further implicates translation with cancer. In support of the relationship between ribosome biogenesis and cancer, mutations in the dyskeratosis congenita 1 (DKC1) or S19 gene, which is required for ribosome biogenesis, result in an enhanced susceptibility to tumour development.[118-120] Lastly, further validation of the role of translation in cancer comes from the observations that mutations in tumour suppressor genes such as TSC1/2 or PTEN, both which are involved in regulating protein synthesis, can increase susceptibility to cancers.[121-123] Consequently, these studies demonstrate a clear role of translation and protein synthesis in cancer development.

1.3.5: EVIDENCE IMPLICATING PML IN TRANSLATIONAL REGULATION AND CANCER

One of the earliest pieces of evidence linking PML to translational regulation was from a study which showed that PML interacts with the ribosomal P proteins (P0, P1, P2) in murine fibroblast cells.[124] These ribosomal P proteins form part of the larger ribosomal subunit that is required for protein synthesis,

thus implicating PML in translation. More recently, several studies have indicated that PML can regulate translation by mechanisms that affect both individual mRNAs as well as global cap-dependent translation.

One of the best characterized examples of the connection of PML with translation is the interaction of PML with eIF4E. This initiation factor is one of the numerous eIF subunits that are involved in the initiation step of translation. Katherine Borden's group was one of the first to demonstrate that PML, through its RBCC domain, can co-immunoprecipitate and co-localize with the eIF4E initiation factor.[87] Overexpression of eIF4E can promote oncogenic transformation and enhance nuclear export of a subset of mRNAs including those encoding cyclin D1, ornithine decarboxylase (ODC) and c-Myc in a m^7GpppN cap-dependent manner but are independent of the ongoing RNA and protein synthesis rate.[87-89, 125-127] The export of these mRNAs appeared to be specific, as overexpression of eIF4E did not affect glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *PML* or *eIF4E* mRNA export.[126] The oncogenic transformation and mRNA export functions of eIF4E are inhibited when PML is overexpressed, thus implicating PML in translational regulation and tumour suppression.[87, 88] It is worth noting that overexpression of eIF4E alone is not sufficient to elicit an increase in the global protein synthesis rate suggesting that eIF4E must act in concert with other eIF subunits for the initiation of translation.[95, 128]

Overexpression of eIF4E has also been shown to promote cell survival via the AKT signaling pathway, which is involved in a myriad of cellular functions

including cell proliferation, apoptosis and transcription.[100] The multifunctional eIF4E protein appeared to mediate AKT activation by up-regulating the expression of the AKT upstream activator, Nijemegen breakage syndrome 1 (NBS1). Supporting the hypothesis that PML is a negative regulator of eIF4E, overexpression of PML can suppress AKT activation and its apoptotic rescue function by inhibiting eIF4E-mediated NBS1 mRNA export.[129]

More recently, PML was demonstrated to regulate cap-dependent translation through interaction with mTOR.[90] As discussed earlier, the mTOR signaling pathway plays a central role in translational regulation (Figure 5). Under hypoxic conditions, PML was shown to act as a negative regulator of mTOR by promoting its nuclear retention, thereby preventing its interaction with the cytoplasmic GTPase protein RHEB, which is involved in stimulating mTOR activity. Consequently this leads to inhibition of hypoxia inducible factor 1 alpha (HIF-1α) protein synthesis (Figure 5).[90] HIF-1α has been implicated in tumour angiogenesis and embryonic vascularization. Conversely, in cells that are PML deficient, translation of HIF-1α is up-regulated which leads to enhanced neoangiogenesis and tumour vascularization.[90] While these data implicate PML in the regulation of HIF-1α translation through inhibition of mTOR, PML may also broadly affect global translation through the same mechanism.

1.4: THE ROLE OF MAMMALIAN EUKARYOTIC INITIATION FACTOR 3 (eIF3) IN TRANSLATION INITIATION AND CANCER

As discussed earlier, the eIF3 protein complex plays an imperative role in the initiation of translation. However, mammalian eIF3 complex is also the largest

and least understood of the translation initiation factors. Previous studies have implicated the eIF3 complex in multiple steps in the initiation of translation, including bridging the gap between the capped mRNA and the 40S ribosomal subunit, stabilizing the binding of the Met-tRNAi and eIF2-GTP ternary complex to the 40S subunit, and preventing the re-association of the free 40S and 60S ribosomal subunits long enough for initiation to occur.[92] Mammalian eIF3 complex is comprised of 13 subunits (A-M); however, the mechanisms by which each individual eIF3 subunit contributes to translation initiation is not well understood and the challenge in elucidating the mechanisms lies in the fact that many subunits interact with one another to form the functional eIF3 complex.[130-132] For example, in humans, five of the subunits (eIF3A, B, C, G and I) form a stable core in the eIF3 complex (Figure 6) and are highly conserved among eukaryotes.[133] The other eIF3 subunits (eIF3D, E, F, H, J, K and M) are less conserved and are not required for the formation of an active eIF3 complex; therefore, these subunits have been proposed to play regulatory roles instead.[134, 135] The eIF3 complex is much simpler in the budding yeast *Saccharomyces cerevisiae*, which only encodes six subunits, all which are homologous with the mammalian subunits of eIF3A, B, C, G, I and J.[130, 134, 136] This suggests that the other additional eIF3 subunits may have evolved to accommodate the complex translation in higher organisms and/or mediate specific protein-protein and/or protein-RNA interactions to provide tighter translational control. Furthermore, mass spectrometry analysis revealed that human eIF3 complex is relatively

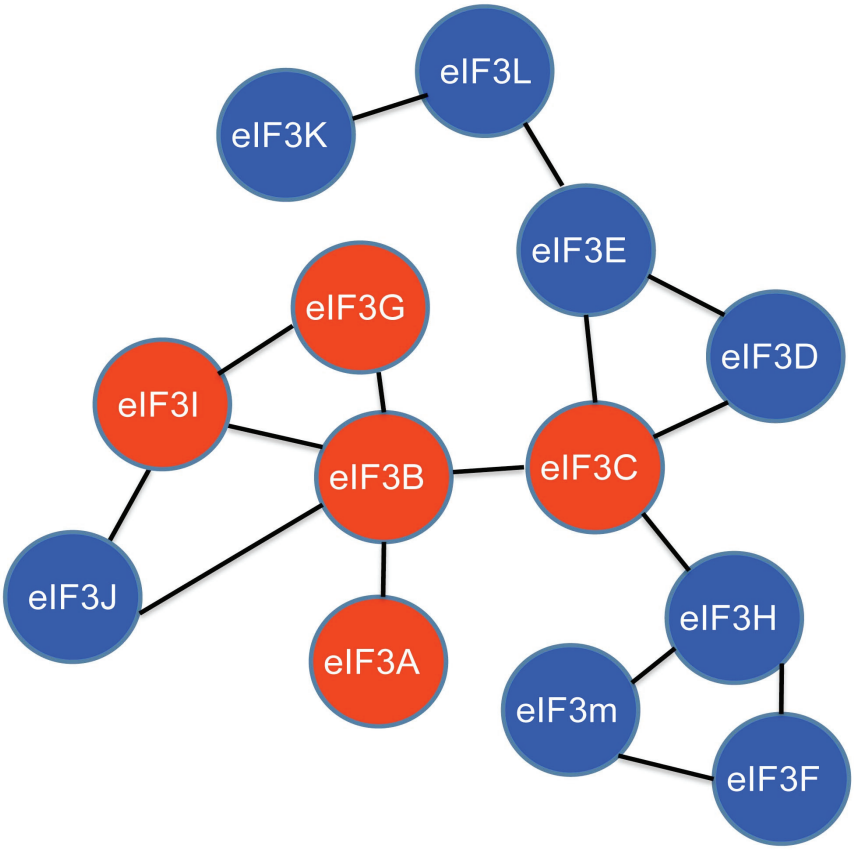


Figure 6: Schematic representation highlighting subunit rearrangement and selective subunit interactions in the mammalian eIF3 complex. The eIF3 subunits in red are conserved subunits that form the core of the eIF3 complex and the remaining subunits are non-essential subunits that play regulatory roles in facilitating the function of the eIF3 complex. Adapted from Pick *et al.* (2009).[139]

stable compared to the yeast version and that the eIF3I, J, K and M subunits are more easily dissociated from the eIF3 core, suggesting that these subunits are situated on the periphery of the eIF3 complex (Figure 6).[137]

Many eIF3 subunits are characterized by the presence of a PCI (26S proteasome lid, COP9 signalosome, eukaryotic initiation factor 3) or MPN (Mpr1-Pad1 N-terminus) signature motif.[138] These motifs are commonly found in the subunits of large protein complexes (e.g., COP9 signalosome,19S proteasome lid and eIF3), and are implicated in mediating and stabilizing protein-protein interactions. This suggests that the eIF3 subunits that contain either PCI or MPN domains (e.g., eIF3A, C, E, F, H, and K) may facilitate protein interactions by acting as structural scaffolds or docking sites for other proteins.[139, 140] Interestingly, one of the eIF3 subunits, eIF3E, has been shown to interact with subunits of the COP9 signalosome (e.g., COPS4 and COPS6), the 26S proteasome lid (e.g., Rpt4) and other eIF3 subunits (e.g., eIF3C).[141, 142] This suggests that eIF3, COP9 signalosome and 26S proteasome lid subunits may interact with one another to form a larger complex that are involved in regulating the life span of proteins, since eIF3 plays a critical role in the initiation step of cap-dependent translation, COP9 signalosome is involved in regulating the ubiquitin-conjugation pathway, and the proteasome is responsible for the degradation of proteins.

1.4.1: THE ABERRANT EXPRESSION OF eIF3 SUBUNITS ARE IMPLICATED IN CANCER

Translation is a tightly regulated process, because even a modest change in the concentration or function of eIF3 subunits can have a profoundly detrimental effect on protein expression, which may ultimately lead to cancer.[143] Despite the widely accepted view that the subunits of the eIF3 complex act in conjunction with one another to regulate translation, overexpression of specific eIF3 subunits (e.g., eIF3E and eIF3F) alone inhibits the global protein synthesis rate,[143, 144] thus implying that each individual subunit may have a distinct contributory role in cancer development.

Indeed, many studies have linked overexpression or loss of specific eIF3 subunits to cancer. For example, ectopic overexpression of eIF3A, B, C, H and I in normal diploid fibroblast cells has been shown to cause cell transformation. This is postulated to occur through stimulation of the global protein synthesis rate, which may augment the translational efficiency of specific weak mRNAs that are involved in cell proliferation (e.g., cyclin D1, c-Myc, and ODC).[143] Enhanced translation of these mRNAs may be responsible for the oncogenic malignancy of the transformed cells.

Overexpression of eIF3A is commonly observed in breast, cervical, esophageal, lung and gastric cancer.[96] The role of eIF3A in cancer is further supported by studies which showed that knockdown of eIF3A protein expression by antisense RNA is capable of eliminating the malignant phenotype in cultured cells.[145] In addition, eIF3A was shown to down-regulate translation of a subset of mRNAs that are implicated in inhibiting cell proliferation, such as cyclin-

dependent kinase inhibitor 1B (CDKN1B).[146] This provides a possible molecular mechanism in which overexpression of eIF3A can cause cell transformation.

Loss of eIF3E mRNA or protein expression is found to correlate with breast and lung carcinomas, frequently as a result of loss of heterozygosity at the *eIF3E* locus.[96, 147, 148] Furthermore, eIF3E may act as a prognostic biomarker for breast cancer, as increased eIF3E protein expression correlates strongly with increased survival of breast cancer patients treated with tamoxifen.[149] Lastly, mouse mammary tumor virus (MMTV) is often integrated into the *eIF3E* locus, which creates a truncated form of eIF3E that is capable of causing transformation of mouse mammary epithelia cells.[150-152] Moreover, when this truncated form of eIF3E was expressed in mice mammary alveolar epithelium cells, persistent hyperplasia and tumorigenesis was observed.[153] Since loss of eIF3E protein expression leads to cancer development and progression, eIF3E has been proposed to act as a tumour suppressor.

Loss of eIF3F protein expression correlates with several human cancers including pancreas, breast and melanoma cancer,[96, 154] which suggests that eIF3F may also play a role in tumour suppression. Indeed, ectopic expression of eIF3F in melanoma cells inhibits cell proliferation and the global protein synthesis rate, and induces apoptosis. Overexpression of eIF3F was thought to decrease global protein synthesis by promoting rRNA degradation.[154] The role of eIF3F in global translation is further supported by studies in the fission yeast *Schizosaccaromyces pombe*, which showed that the *eIF3F* gene is essential for viability, and that depletion of eIF3F can cause a marked decrease in global

protein synthesis.[155] Consequently, this may lead to decrease synthesis of various proteins that are involved in promoting cell survival. Furthermore, the role of eIF3F in translation and apoptosis is dependent on phosphorylation of eIF3F by cyclin dependent kinase 11 ($CDK11^{p46}$), which increases the association of eIF3F with the eIF3 core complex during apoptosis.[156]

In summary, loss or overexpression of eIF3 subunits correlates substantially with various types of cancer; however, it is not known whether aberrant expression of eIF3 subunits is a cause or a consequence of malignant transformation.

1.4.2: CHARACTERIZATION OF THE eIF3K SUBUNIT

Of particular interest to this study is the non-conserved eIF3 subunit, eIF3K. As one of the smallest subunits of the eIF3 complex, eIF3K is expressed ubiquitously in human tissues, with the highest expression concentrated in the brain, kidney, and testis.[157] eIF3K is highly conserved among higher eukaryotic organisms, including human, cow, dog, mouse and frog (Figure 7); however, eIF3K sequence is more divergent in organisms that are in the lower evolutionary scale (e.g., worm and fruit fly).[157] Furthermore, eIF3K is not expressed in yeast, including *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, suggesting that eIF3K may have evolved to play specialized and critical regulatory role in multicellular organisms.[157, 158] Glutathione S-transferase (GST) pull-down assays have revealed that eIF3K interacts directly with multiple eIF3 subunits such as eIF3C, G and J *in vitro*, and forms a stable

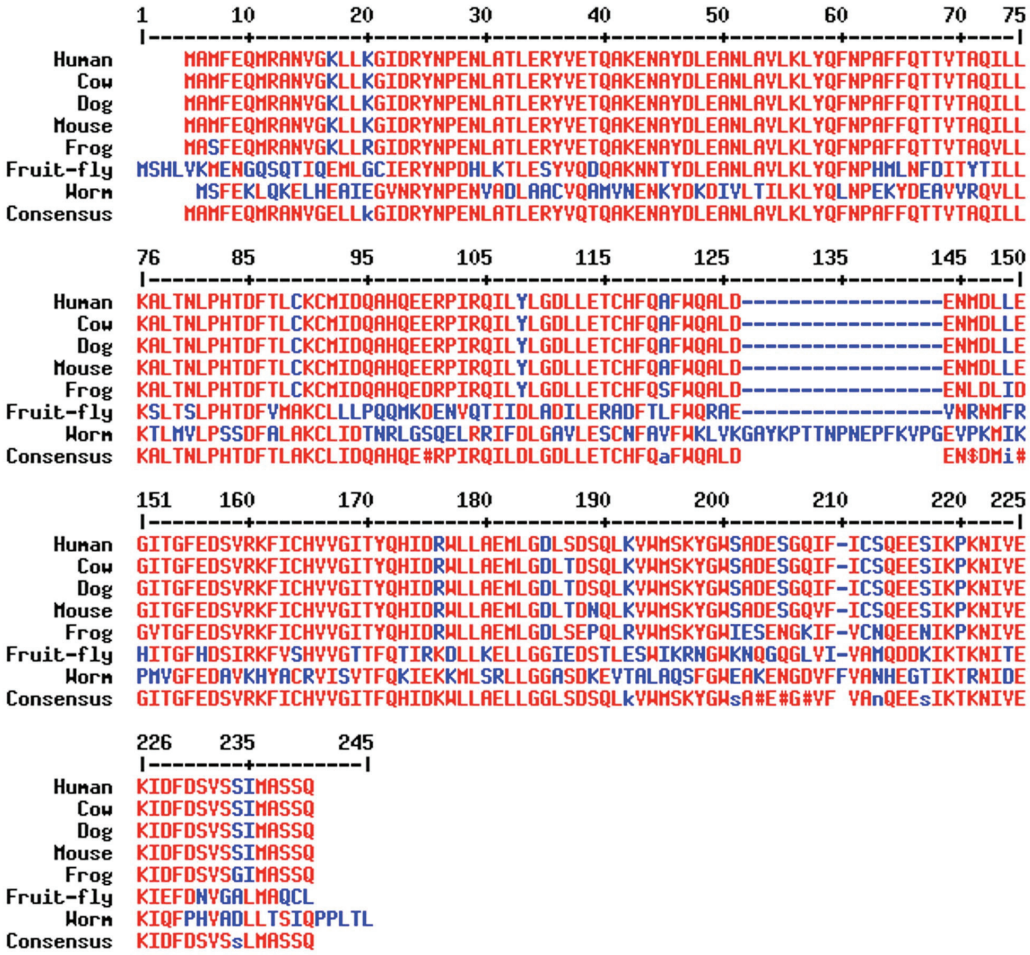


Figure 7: eIF3K is conserved among higher eukaryotic organisms. Amino acid sequence alignment of eIF3K revealed that eIF3K is highly conserved among human, cow, dog, mouse and frog and less conserved in worm and fruit fly. Conserved residues are indicated in red and non-conserved residues are indicated in blue.

immunoprecipitatable complex with the eIF3 core.[157] However, eIF3K is not required for the formation of a functional eIF3 complex, suggesting that eIF3K may facilitate translation but is not essential.[157] The non-essential role of eIF3K in the formation of an active eIF3 complex is supported by the discovery that eIF3K is one of the most easily dissociated subunits, suggesting that this subunit is situated on the periphery of the eIF3 complex.[137, 159] Furthermore, studies in *Caenorhabditis elegans* demonstrated that eIF3K is not essential for viability.[157] Currently, the molecular mechanism by which eIF3K contributes specifically to initiation of translation is unknown, but eIF3K has been found to interact with the 40S small ribosomal subunit.[157]

The crystal structure of eIF3K has been resolved, and it revealed that eIF3K contains two well-defined domains: a HEAT (Huntington, Elongation factor 3, A subunit of protein phosphatase 2A, Target-of-rapamycin) repeat-like HAM (HEAT analogous motif) domain in the N-terminus and a winged-helix-like (WH) domain in the C-terminus.[160] Studies of the HEAT repeat in other proteins suggest that this motif is involved in mediating protein-protein interactions.[161, 162] For example, eIF4GII, which contains a HEAT domain, interacts with eIF1, eIF4A and eIF5.[163, 164] This suggests that eIF3K may interact with the other eIF3 subunits (e.g., eIF3C, G, J) via the HEAT motif. The WH domain was proposed to mediate protein-RNA interactions because other proteins that contain the WH domain such as the elongation factor, SelB, bind to RNA.[165, 166] Furthermore, previous studies indicated that multiple eIF3 subunits, such as eIF3A, B, C, D, F

and G, bind to RNA; therefore, making it likely that eIF3K may bind RNA directly, or indirectly via other eIF3 subunits.[167-171]

Immunofluorescence studies demonstrated that eIF3K localizes to the nucleus and to the cytoplasm.[172] As translation initiation occurs in the cytoplasm, a majority of the eIF subunits are localized to this subcellular compartment; however, there are a few selected eIFs (e.g., eIF3E,[173] eIF3F,[174, 175] eIF4E,[176] eIF4G[177] and eIF5A[178, 179]) that are localized in the nucleus as well. Shen *et al.* [172] have demonstrated that eIF3K interacts with cyclin D3 *in vivo* and *in vitro* both in the cytoplasm and in the nucleus. The main function of cyclin D3 is to associate with cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) to drive cells through the G1 restriction point in the cell cycle. Therefore, eIF3K may play a role in cell cycle regulation through its interaction with cyclin D3. In addition, overexpression of cyclin D3 resulted in enhanced global translation in a dose-dependent manner, possibly due to its interaction with eIF3K.[172]

The eIF3K protein was also found to co-localize with keratin 8 and 18 (K8/K18) filaments in non-apoptotic cells and with K8/K18 in the insoluble cytoplasmic inclusions in apoptotic cells.[180] K8/K18 are structural components of the intermediate filaments in simple epithelial cells. The interaction between K18 and eIF3K has been mapped to the HAM domain, which, as previously mentioned, is the domain involved in mediating protein-protein interactions. The significance of eIF3K interaction with K8/K18 is that endogenous eIF3K sensitizes K8/K18-containing epithelial cells to mitochondrial and death-receptor mediated apoptosis by promoting the release of active caspase 3 from the

cytoplasmic inclusions.[180] Caspase 3 is a member of the cysteine-aspartic acid protease family that is involved in cell apoptosis. Conversely depletion of endogenous eIF3K desensitizes simple epithelial cells to apoptosis through a K8/K18 dependent manner and promotes the retention of active caspase 3 in the cytoplasmic inclusions.[180] This indicates that eIF3K may regulate apoptosis in epithelial cells through the actions of caspase 3.

1.5: CONCLUDING REMARKS

There is considerable interest in the study of PML due to the strong correlative relationship between loss of PML and the development of various types of cancer.[30] The regulation of PML protein interactions in response to cell stress is implicated in the proper functioning of PML NBs, which have been demonstrated to act as cellular nodes for co-accumulation of proteins and their associated catalytic enzymes.[6, 12] In fact, a vast number of proteins have been found to co-localize with PML in the NB, thereby implicating PML in multiple cellular functions, including tumour suppression and gene regulation.[2, 4, 17] There are at least seven known isoforms of the PML protein that contribute to the biochemical compositions of PML NBs.[36] While some PML NB-associated proteins interact with PML without preference for a particular isoform, other proteins, such as p53[15] and HDAC1/2,[80] interact specifically with one isoform (PML-IV), which suggests that individual PML isoforms through their specific protein-protein interactions may alter PML NB function in a tissue-specific manner. Currently, the cellular functions of the various PML isoforms are

undefined, and the mechanisms in which they contribute to the functions of PML NBs, through their specific protein-protein interactions, have yet to be elucidated. To gain a better understanding of the function of PML-I, a yeast two-hybrid screen was used to discover potential interacting partners of PML-I. Using this approach, eIF3K was identified as a novel interacting partner of PML-I. eIF3K is a non-essential subunit of the eIF3 complex which plays a pivotal role in the initiation of translation. In this dissertation, I present data supporting eIF3K interaction with PML-I *in vitro* and *in vivo*. Through its interaction with eIF3K, PML-I can up-regulate eIF3K protein expression both in normal and cancerous cells, suggesting that PML-I may be involved in regulating eIF3K protein translation or stability, which in turn could affect the global translation initiation or the stability of specific mRNAs in cancer cells that have reduced expression of PML-I.

CHAPTER 2: MATERIALS AND METHODS

2.1: PLASMIDS

The human PML isoform I exon 9 (PML-I-E9) sequence encoding amino acids 604-882 was amplified by polymerase chain reaction (PCR) using Phusion™ High-Fidelity DNA polymerase enzyme (Finnzymes, Ottawa, ON) and the primers; PMLI-E9-forward (5'-CCAAGCAGAAGACAGACCTCTGGT-3') and PMLI-E9-BamHI-reverse (5'-TTTGGATCCTCAGCTCTGCTGGGAGGCCCT-3'). Introduction of the restriction site indicated is underlined. PML-I-E9 PCR product was generated using mammalian expression vector encoding FLAG tagged PML-I (GenBank Accession NM_033238.2) as template. PCR conditions were set up as follows: 1X Phusion buffer, 2 mM $MgCl_2$, 0.2 mM dNTPS, 0.5 µM each of forward and reverse primers, 50-100 ng template DNA, and 1 U Phusion enzyme (see Appendix I for PCR profile). The amplified PCR product was resolved on agarose gel and gel purified using a gel purification kit (Qiagen Inc., Mississauga, ON) followed by digestion with BamHI restriction enzyme at 37^{o}C for 2 hr. The amplified PML-I-E9 coding sequence was subcloned into the BamHI site of the yeast two-hybrid vector pGBT9, in-frame with the coding region for the DNA binding domain of the yeast Gal4 transcription factor (Gal4 BD). The pGBT9 vector was prepared by digesting pGBT9 plasmid encoding PRP4 kinase with EcoRI (all restriction enzymes used for molecular cloning were purchased from Invitrogen Canada Inc., Burlington, ON or from New England Biolabs Ltd., Pickering, ON) followed by treatment of the digested pGBT9 vector with Klenow fragment of DNA polymerase I (Invitrogen Canada Inc.) according to manufacturer's protocol. Subsequently, pGBT9 vector was digested with BamHI,

purified by agarose gel using a gel purification kit (Qiagen Inc.) and 5'-dephosphorylated with antarctic phosphatase (New England Biolabs Ltd.). Digested pGBT9 vector and PML-I-E9 insert was incubated with T4 DNA ligase (New England Biolabs Ltd.) at 16^{o}C overnight. The ligation reaction was transformed into *Escherichia coli* XL-Blue competent cells using standard chemical transformation protocol and plated on Luria-Bertani (LB) solid medium (BioShop Canada Inc., Burlington, ON) supplemented with 50 µg/mL of ampicillin. DNA sequencing was employed to confirm that the PML-I-E9 coding region was cloned in-frame with the Gal4 DBD of pGBT9. All subsequent cloning procedures were performed in a similar manner.

The human PML-I-E9 sequence encoding residues 604-882 was also amplified using PMLI-E9-NdeI-forward (5'-GCGTATCGCATATGCAAGCAG AAGACAGACCTCTGGT-3') and PMLI-E9-BamHI-reverse (5'-GTCGACGG ATCCTCAGCTCTGCTGGGAGGCCCTCT-3') primers. The amplified PCR product was digested with NdeI and BamHI and cloned in-frame with the Gal4 DBD in the yeast two-hybrid vector pGBKT7 that had been digested with the same restriction enzymes. A smaller fragment of PML-I-E9 (750-882 aa) (termed PML-I-E9Δexo) was amplified using primers; PMLI-E9-EcoRI-forward (5'-GTATC TGAATTCATGCGTGACCTGTGCCGCCTCCT-3') and PMLI-E9-NotI-reverse (5'-TTTAATGCGGCCGCTCAGCTCTGCTGGGAGGCCCT-3'). The resulting PCR product was cloned into pGBKT7 vector via EcoRI and NotI.

An alternative splice isoform of human eIF3K (eIF3K isoform 2, unpublished sequence, see Appendix II) was reverse transcribed from total HeLa

RNA using the primers; eIF3K-EcoRI-forward (5'-TCGAGGGAATTCGAATGGC GATGTTTGAGCAGATGAGA-3') and eIF3K-XhoI-reverse (5'-GCGTTGCTCGAG CTGGGAGGAGGAGGCCATGATGCT-3'). All subsequent eIF3K cDNA manipulations were performed with this DNA template unless otherwise indicated. The resulting eIF3K cDNA was cloned into the EcoRI and XhoI site of pcDNA3.1 (-) mammalian expression plasmid encoding a human influenza hemagglutinin (HA) tag to generate N-terminally HA-tagged eIF3K. In a similar manner, an HA tag was added to the C-terminus of eIF3K by amplifying the target gene with the following primers; eIF3K-HindIII-forward (5'-TCGAGGAAG CTTATGGCGATGTTTGAGCAGATGAGA-3') and eIF3K-ClaI-reverse (5'-TCA ACTATCGATCTGGGAGGAGGCCATGATGGAGGTGGA-3'). The resulting PCR product was digested with HindIII and ClaI and inserted into pcDNA3.1 (-) vector digested with the same restriction enzymes. Full length eIF3K coding sequence (GenBank Accession No. NM_013234) was amplified using the same primers and incorporated into HA tagged pcDNA3.1 (-) vector.

GST-eIF3K recombinant plasmid was created by amplifying eIF3K coding sequence using the primers; eIF3K-EcoRI-forward (5'-TCGAGGGAATTCATGG CGATGTTTGAGCAGATGAGA-3') and eIF3K-XhoI-reverse (5'-GCGTTGCTCG AGCTGGGAGGAGGAGGCCATGATGCT-3'). Amplified PCR product was cloned into the EcoRI and XhoI sites of pGEX-5X1 vector. Maltose binding protein (MBP)-PML-I recombinant plasmid was generated by amplifying PML-I (773-882 aa) using PML-BamHI-forward (5'-ACCCCGGATCCAGCCTGCAGTGC TTTGCCTCCCT-3') and PML-SalI-reverse (5'-GGTCACCCCGTCGACTCAGCT

CTGCTGGGAGGCCCT-3') primers, which was then inserted into the pMal-p4G vector via BamHI and SalI restriction sites. The resulting fusion protein is termed MBP-PML-I-E9Δexo.

2.2: YEAST TWO-HYBRID SCREENS

Yeast two-hybrid screens were performed following instructions described in the Matchmaker™ Gold Yeast Two-Hybrid System User Manual (Clontech Laboratories, Inc., Mountain View, Ca).

2.2.1: YEAST TRANSFORMATION

Plasmids were transformed into yeast using lithium acetate transformation. Yeast competent cells were produced by growing the yeast strain of interest in 3 mL YPDA rich medium (2% glucose, 2% bactopeptone, 1% yeast extract, 120 μg/mL adenine) (see Appendix III for other yeast media and solutions) at 30°C overnight. Cells were harvested by centrifugation at 14,000 rpm for 2 min and washed with 1.5 mL sterile water. Cells were centrifuged as before, and washed with 1.5 mL 1X TE/LiAC (10 mM Tris-HCl pH 8, 1mM EDTA, 100 mM lithium acetate). Following 5 min incubation at room temperature (RT), cells were centrifuged and resuspended in 1 mL of 1X TE/LiAC. For one transformation reaction, 100-500 ng of plasmid DNA, 1 μg of denatured single strand salmon sperm carrier DNA, and 700 μL of 50% polyethylene glycol (PEG) was added to 100 μL yeast competent cells. The transformation reactions were incubated at 30°C with shaking at 200 rpm for 30 min, followed by heat shock at

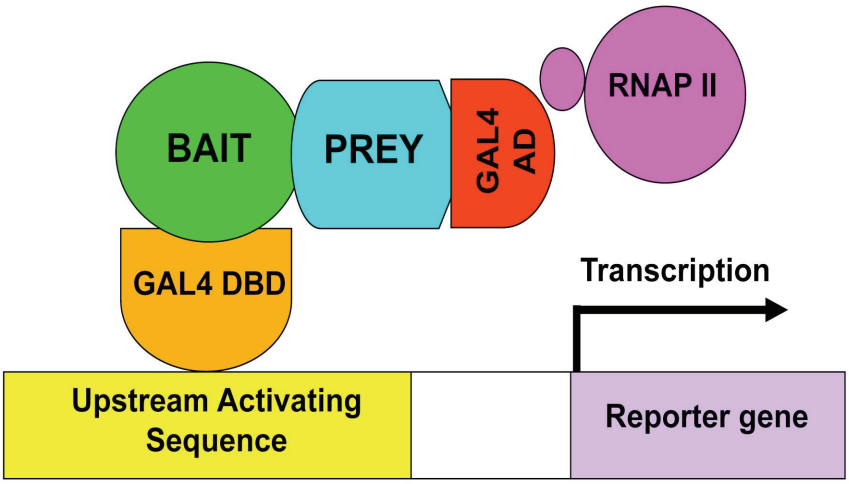
42°C for 15 min. To enhance yeast transformation efficiency, 80 μL of 95% ethanol was added to the transformation reaction and incubated at 42°C for 5 min. Cells were centrifuged at 14,000 rpm for 30 sec, resuspended in 200 μL of 1X TE and plated on synthetic defined (SD) medium lacking the amino acid tryptophan (SD Trp^-) (2% bacto-agar, 2% glucose, 0.67% yeast nitrogen base without amino acids, 1X amino acid dropout supplement, 1X L-leucine, 1X L-adenine hemisulfate salt, 1X L-histidine HCl monohydrate). All amino acids and reagents used for the yeast two-hybrid assays were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON) or BioShop Canada Inc. Transformation of plasmids cloned into pACT2 or pGADT7-RecAB vectors containing *LEU2* selection marker were plated on SD medium lacking the amino acid leucine (SD Leu^-) (2% bacto-agar, 2% glucose, 0.67% yeast nitrogen base without amino acids, 1X amino acid dropout supplement, 1X L-tryptophan, 1X L-adenine hemisulfate salt, 1X L-histidine HCl monohydrate). Yeast (strain Y2HGold) transformed with PML-I-E9 bait plasmid was mated with yeast (strain Y187) transformed with a plasmid encoding a empty Gal4 AD for testing auto-activation of various reporter genes (*HIS3*, *ADE2*, *LACZ*, *MEL1* or *AUR1C*) by plating the mated yeast cells on SD solid medium lacking the relevant amino acids or nucleotides and in the presence of 70 ng/mL auerobasidin A (Clontech Laboratories, Inc.) and 40 μg/mL 5-bromo-4-chloro-3-indolyl alpha-D-galactopyranoside (X-α-gal) (Clontech Laboratories, Inc.), as outlined in the Clontech yeast two-hybrid manual.

2.2.2: YEAST PROTEIN EXTRACTION

Relevant yeast strains expressing PML-I-E9 bait protein were inoculated in 50 mL SD Trp- medium and cultured at 30^{o}C for 2 days. Cells were harvested by centrifugation at 4000 rpm for 15 min and resuspended in 400 μL disruption buffer (20 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 0.3 M ammonium sulfate, 4 mM PMSF, 2X protease inhibitor). Cells were disrupted by vortexing with glass beads for 6 min at 4^{o}C. The cell lysates were subjected to centrifugation at 14,000 rpm for 10 min and the supernatant containing soluble proteins was analyzed by Western blotting.

2.2.3: YEAST TWO-HYBRID ASSAY

The scheme of the yeast two-hybrid assay is described in Figure 8. PML-I-E9 bait protein was used to screen two different cDNA libraries [human fetal brain (Clontech Laboratories, Inc.) or human normalized universal libraries (Clontech Laboratories, Inc.) cloned into pACT2 and pGADT7-RecAB, respectively, and in-frame with the Gal4 activation domain (AD)] as outlined in Table 1. An overnight culture of the PML-I-E9 bait strain was used to inoculate 300 mL of SD Trp^{-} medium, and cells were cultured at 30^{o}C until an OD_{600} of 0.5 was achieved. The pretransformed cDNA library was resupended in 50 mL YPDA medium and incubated at 30^{o}C for 30 min with shaking at 200 rpm. The library culture was mixed with the bait culture and collected on a 0.22 μm GS-Millipore filter (20 mL culture was collected per filter). The filters were overlaid on



BAIT = PML-I-E9-pGBT9 or pGBKT7 (GAL4 DBD)
PREY = Human fetal brain cDNA library (pACT2-GAL4 AD) or
Human normalized universal cDNA library (pGADT7-RecAB-GAL4 AD)

Reporter genes = *HIS3*, *ADE2*, *LACZ, MEL1*, *AUR1C*

Figure 8: Overview of the yeast two-hybrid system. PML-I-E9 bait protein coding region was cloned in-frame and downstream from the sequence encoding the Gal4 DNA binding domain (Gal4 DBD) in the yeast two-hybrid vectors pGBT9 and pGBKT7. The PML-I-E9 and Gal4 BD fusion protein binds specifically to the upstream activation sequence of the targeted reporter gene. The human fetal brain and normalized universal cDNA libraries used in the yeast two-hybrid screens contained cDNAs cloned in-frame with the GAL4 activation domain (GAL4 AD). When the “bait” and “prey” proteins interact, RNA polymerase II (RNAPII) is recruited and drives the Gal4-inducible promoter to activate transcription of various reporter genes.

Table 1: Summary of the PML-I-E9 yeast two hybrid screens

Bait	Bait yeast strain	Human cDNA library	Initial media for identifying yeast two-hybrid interactions from mated yeast cells	Media for assaying two-hybrid interaction
Screen #1				
PML-I-E9 cloned into pGBT9 vector (GAL4 DBD and *TRP1* selection marker)	PJ696α	Yeast (strain AH109) pretransformed with human fetal brain cDNA library cloned into pACT2 (*GAL4* AD and *LEU2* selection marker)	SD $Leu^-Trp^-His^-$	1) SD $Leu^-Trp^-His^-$ (with 2 mM 3-AT) 2) SD $Trp^-Leu^-Ade^-$ 3) Filter β galactosidase assay
Screen #2				
PML-I-E9 cloned into pGBKT7 vector (GAL4 DBD and *TRP1* selection marker)	Y2HGold	Yeast (strain Y187) pretransformed with mate & plate normalized universal human cDNA library cloned into pGADT7-RecAB vector (*GAL4* AD and *LEU2* selecton maker)	SD Leu^-Trp^- (with 125 ng/mL aureobasidin A and 40 μg/mL X-α-gal)	1) SD $Leu^-Trp^-His^-$ (with 2 mM 3-AT) 2) SD $Trp^-Leu^-His^-$ Ade- (with 70 ng/mL aureobasidin A and 40 μg/mL X-α-gal

(Note: 3-AT is 3-Amino-1,2,4-triazole used to increase the stringent selection of the *HIS3* reporter gene).

YPDA solid medium for 5 hr at 30°C to allow mating between the two cell types to occur. Cells were then rinsed off the filters with yeast nitrogen base (YNB) medium (2 mL YNB per filter) and plated on selective SD solid medium (see Table 1 for summary of the two yeast two-hybrid screens). Plates were incubated at 30°C for 4-6 days. False protein interactions were eliminated by plating the yeast two-hybrid colonies on various SD media for assessing activation of the reporter genes (Table 1).

Expression of the β-galactosidase reporter gene was detected using a filter assay. Yeast colonies were replica-plated to a Whatmann filter placed on SD Leu^-Trp^- solid medium and incubated at 30°C for 3 days. Cells were lysed by freezing the filter at -80°C for 10 min followed by 5 min at RT. The freeze/thaw cycle was repeated two times. The filter was soaked in 2.5 mL Z buffer (60 mM Na_2HPO_4 ($7H_2O$), 40 mM NaH_2PO_4 (H_2O), 10 mM KCl, 1 mM $MgSO_4$ ($7H_2O$) pH 7) supplemented with 0.25% β-mercaptoethanol and 1 mg/mL x-β-galactoside for 1-2 hr at 30°C. Alpha-galactosidase activity was assayed by including 40 μg/mL of X-α-gal in the SD selective solid medium (see Appendix III).

2.2.4: IDENTIFYING AND RESCUING YEAST TWO-HYBRID PLASMIDS

Library plasmids that activated multiple reporter genes (e.g., two or more) when mated with PML-I-E9 bait were amplified by yeast colony PCR as follows: a fresh yeast colony was resuspended in 3 μL of 0.02 M NaOH and boiled for 10 min at 95°C. PCR conditions were set up as follows: 1X PCR buffer (100 mM Tris-HCl pH 8.8, 250 mM KCl), 2 mM $MgCl_2$, 0.1 mM dNTPs, 2 μM forward and 2

μM reverse primers, 1 U Taq polymerase (see Appendix I for PCR profile used). Primers used for amplifying library inserts are as follows: pACT2 forward (5'-CGC GTTTGGAATCACTACAGGGATG-3') and pACT2 reverse (5'-GAAATTGAGATG GTGCACGATGCAC-3') or pGADT7-RecAB forward (5'-GATGATGAAGATACC CCACCAAACCCA-3') and pGADT7-RecAB reverse (5'-TCTATAGATCAGAGGT TACATGGCC-3') (see Table 1 for primer references). The resulting PCR products were purified by agarose gel extraction and the identity of the library plasmids were obtained by DNA sequencing the PCR product with pACT2 forward or pGADT7-RecAB forward primers listed above. The identity of the positive clones were identified by comparing the DNA sequences to all known sequences in the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in the National Center for Biotechnology Information (NCBI) gene database. Clones that contained cDNA coding regions cloned in-frame with the GAL4 AD were rescued. To extract plasmids from yeast, yeast colonies were inoculated in 3 mL SD Leu^- media and cultured at 30^oC for 1-2 days. Cells (1.5 mL culture) were harvested by centrifugation at 14,000 rpm for 1 min and resuspended in 300 μL breaking buffer (2% Triton X, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8) and 300 μL phenol:chloroform:isoamyl (25: 24:1 ratio) solution. Yeast cells were lysed by vortexing with glass beads for 6 min and the aqueous/phenol layer was separated by centrifugation at 14,000 rpm for 8 min. The aqueous layer was transferred into a new eppendorf tube and mixed with equal volume of chloroform:isoamyl (24:1), followed by centrifugation at 14,000 rpm for 3 min.

The aqueous layer was added to 800 μL of cold 95% ethanol. DNA was precipitated by incubation at -20°C overnight and resuspended in 30 μL of sterile water and 1 ng/μL of RNaseA. Subsequently, yeast plasmids were transformed into *Escherichia coli* KC8 competent cells by electroporation and plated on LB solid medium supplemented with 50 μg/mL of ampicillin. Transformed bacterial colonies were replica-plated onto M9 solid medium and plasmids were isolated using the mini-prep kit (Qiagen Inc.). Isolated plasmids were subsequently transformed into *Escherichia coli* XL-Blue competent cells to obtain high copy number of the plasmids.

2.2.5: CONFIRMING YEAST TWO-HYBRID INTERACTIONS

Library plasmids rescued from the yeast two-hybrid screen were re-transformed into yeast (strain Y187), which were then mated on YPDA medium with yeast (strain Y2HGold) expressing the PML-I-E9 bait protein. Mated colonies were selected by plating yeast cells on SD Leu^-Trp^- solid medium and interactions were evaluated by assessing growth on SD $Leu^-Trp^-His^-Ade^-$ supplemented with 70 ng/mL Auerobasidin A and 40 μg/mL X-α-gal.

2.3: CELL CULTURE, TRANSIENT AND STABLE TRANSFECTIONS

All cell lines used were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (complete DMEM) at 37°C in a 5% CO_2 atmosphere. All tissue culture reagents were purchased from Sigma Aldrich Canada Ltd. Mammalian cells used for

immunofluorescence studies were transiently transfected with relevant plasmids using TransIT-HeLaMONSTER® transfection kit (Mirus Bio LLC, Montreal, QC) or Lipofectamine 2000 (Invitrogen Canada Inc.) according to manufacturers' protocol. For co-immunoprecipitation (Co-IP) experiments, human embryonic kidney 293T (HEK-293T) cells were grown to 50-70% confluency in 100 mm plates and relevant plasmids were transfected using a calcium phosphate precipitation method as follows: 4 μg of total plasmid DNA was mixed with 500 μL of HEBS solution (275 mM NaCl, 42 mM HEPES, 9.6 mM KCl, 1.5 mM $Na_2HPO_4x7H_2O$, pH 7.1) and 500 μL of 250 mM $CaCl_2$. The calcium phosphate transfection solution/DNA mix was added to HEK-293T cells containing 8 mL complete DMEM medium. Eight hours post-transfection, cells were washed with 10 mL PBS two times and supplemented with fresh complete DMEM medium. Transfected cells were allowed to recover in complete DMEM medium for 48 hr prior to lysate preparations for Co-IPs.

Cell lines stably expressing PML-I or PML-IV were generated through retroviral transduction of PML (-/-) murine embryonic fibroblast (MEF) cells (gift from Dr. Paolo Salomoni, University College London, London, United Kingdom) using pBABE retroviral vectors encoding PML-I or PML-IV (gift from Dr. Oliver Bischof, Institut Pasteur, Paris, France). Retroviral transduction was performed by seeding approximately $4\text{-}5x10^6$ phoenix retroviral producer cells (gift from Dr. Patrick Lee, Dalhousie University, Nova Scotia, Canada) in a 100 mm tissue culture dish. Transfections were performed by mixing 6 μg of PML-I or PML-IV plasmid DNA with 500 μL of serum/antibiotic free DMEM medium. Likewise, 18

μg of polyethylenimine (PEI) transfection reagent (Polysciences Inc., Warrington, PA) was diluted in 500 μL of serum/antibiotic free DMEM medium. The diluted DNA and PEI solution was mixed together and incubated at RT for 20 min prior to packaging the DNA into phoenix cells. Six hours post-transfection, cells were washed with PBS and allowed to recover in complete DMEM medium for 48 hr. Virus-containing supernatant was collected from the phoenix cells and filtered through a 0.45 μM filter. Actively dividing PML (-/-) MEF cells (40-50% confluency) were incubated for 24 hr with the virus-containing supernatants in the presence of 4 μg/mL of polybrene (Sigma Aldrich Canada Ltd.). Following removal of the virus, cells were recovered in complete DMEM for 24 hr. Stable transduced clones were then selected with 1.5 μg/mL of puromycin. Human osterosarcoma cell lines stably expressing green fluorescent protein (GFP)-PMLI and GFP-PMLIV was obtained from the American Type Culture Collection (ATCC), Manassas, VA.

PML protein expression was knocked down in TERT-immortalized normal human diploid fibroblast (NHDF) cells using short hairpin RNA (shRNA) targeted against all isoforms of PML (shPML) or control luciferase (shControl), as described previously.[181] These PML knockdown stable cell lines were created by Dr. Kendra Cann (Dalhousie University, Nova Scotia, Canada).

2.4: TOTAL CELL LYSATE PREPARATION AND WESTERN BLOT ANALYSIS

For Western blot analysis of protein expression, total cell lysates were prepared by adding an equal volume of PBS and 2X SDS loading buffer (20%

glycerol, 125 mM Tris HCl pH 6.8, 4% SDS, 0.2% bromophenol blue, 10% β-mercaptoethanol) to the cell pellet. Lysates were boiled for 5 min followed by sonication at low output to shear genomic DNA (Misonix Sonicator 3000). Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Bio-Rad Laboratories, Ltd., Mississauga, ON) using the Bio-rad semi-dry transfer apparatus. After blocking with 5% skimmed milk for 1.5 hr at RT, the membrane was incubated with primary antibody at 4^{o}C overnight, followed by three washes (5 min each) with 0.1% TBST buffer (137 mM NaCl, 2.7 mM KCl, 25 mM Tris HCl, 0.1% Tween-20, pH 7.4). The membrane was then incubated with HRP-conjugated labeled secondary antibody (Sigma Aldrich Canada Ltd.) for 1 hr at RT and proteins were detected using an ECL detection kit (ThermoFisher Scientific, Nepean, ON) and Kodak BioMax film (Sigma Aldrich Canada Ltd.). Various antibodies used for Western blotting analysis includes mouse monoclonal eIF3K (Abcam, Cambridge, MA), rabbit polyclonal eIF3K (Novus Biologicals, LLC., Littleton, CO), chicken polyclonal HA (Abcam), rabbit polyclonal anti-PML (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), monoclonal mouse anti-FLAG M2 (Sigma Aldrich Canada Ltd.), rabbit polyclonal eIF3F (BioLegend, San Diego, CA), rabbit polyclonal eIF3E (Abcam), and rabbit polyclonal β-tubulin (Abcam). Sheep anti-PML-I IgG serum, raised against amino acids 751-883 of PML and sheep anti-PML-IV antibody raised against the C-terminal peptide sequence of PML-IV (SGFSWGYPHPFLI) was generated by Dr. Graham Dellaire (Dalhousie University, Nova Scotia, Canada).

2.5: PROTEIN EXPRESSION AND PURIFICATION AND *IN VITRO* BINDING ASSAY

GST-eIF3K and MBP-PML-I-E9Δexo fusion proteins were expressed in *Escherichia coli* Rosetta cells (DE3) (EMD Chemicals Inc., Gibbstown, NJ). Bacteria transformed with plasmids encoding the recombinant GST-eIF3K or MBP-PML-I-E9Δexo, were grown overnight in 5 mL LB broth (BioShop Canada Inc.) supplemented with 50 μg/mL each of ampicillin and chloramphenicol. The overnight culture was used to inoculate a 50 mL LB broth and cultures were incubated at 37°C until cells reached a density of OD_{600} 0.5. Protein expression was induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 hr at 37°C. Bacteria cells were collected by centrifugation at 4000 rpm for 20 min and lysed with 1 μg/mL of lysozyme in 1 mL PBS at 37°C for 30 min. Subsequently, 1 mM PMSF, 6 mM ethylenediaminetetraacetic acid (EDTA) and 1% Triton X-100 was added to the bacterial lysates and incubated on ice for 20 min. Genomic DNA was sheared by brief sonication at low output and protein supernatant was collected by centrifugation at 14,000 rpm for 10 min. Protein concentrations were determined by subjecting protein lysates to SDS-PAGE and Comassie Staining and using Bovine Serum Albumin (BSA) as a protein standard.

GST or GST-eIF3K proteins (2 μg) were precleared in 30 μL of amylose beads (New England Biolabs Ltd.) for 30 min at 4°C. Likewise, 2 μg of MBP or MBP-PML-I-E9Δexo proteins were precleared in 30 μL of gluthathione Sepharose 4B beads (GE Healthcare, Baie d'Urfe, QC) for 30 min at 4°C. Precleared GST or GST-eIF3K lysates were bound to 30 μL of glutathione

Sepharose beads for 2 hr at 4^{o}C with mixing, followed by a brief wash with 200 μL of ice cold PBS. Precleared MBP-PML-I-E9Δexo lysates were incubated with bead-bound GST or GST-eIF3K for 3 hr at 4^{o}C. To perform the reverse MBP or MBP-PML-I-E9Δexo IP, 2 μg of precleared MBP or MBP-PML-I-E9Δexo lysates were bound to 30 μL of amylose beads for 2 hr at 4^{o}C, followed by a brief wash with 200 μL of PBS. The precleared GST-eIF3K lysates were then incubated with bead-bound MBP or MBP-PML-I-E9Δexo for 3 hr at 4^{o}C. All the beads were washed three times with wash buffer (0.1% Triton and 50 mM NaCl in 1X PBS) and one time with PBS. The bound proteins were eluted by boiling in 2X SDS loading buffer and analyzed by Western blotting using GST rabbit immunoglobulin G (IgG) anti-serum (Sigma Aldrich Canada Ltd.), MBP rabbit anti-serum (New England Biolabs Ltd.), mouse monoclonal eIF3K and sheep anti-PML-I IgG serum.

2.6: *IN VIVO* INTERACTION ASSAY

Plasmids encoding FLAG-tagged PML-I (created by Dr. Graham Dellaire) and HA-tagged eIF3K (eIF3K-HA) were transfected in HEK-293T cells by calcium phosphate precipitation method as described previously and 48 hr post-transfection, cells were washed three times with ice-cold PBS and solubilized with lysis buffer (50 mM Tris-HCl pH 8, 300 mM NaCl, 0.25% Nonidet P-40, 5 mM EDTA, 10% glycerol, 2 mM PMSF, 1X protease inhibitor). Cells were mechanically disrupted by passing the lysates through a 22G needle, followed by a 26G needle. Detergent-insoluble proteins were removed by centrifugation at

14,000 rpm for 15 min. Mouse monoclonal anti-FLAG or anti-eIF3K antibody was pre-incubated with PureProteome^{TM} Protein G magnetic beads (Millipore Corp., Billerica, MA) for 2 hr at 4^{o}C. Following a brief wash with 1 mL PBS, the antibody-bound beads were incubated with cell lysates for 3 hr at 4^{o}C with mixing. The final concentration of Nonidet P-40 was reduced to 0.12% by adding an equal volume of PBS to the lysates after 3 hr of incubation and the Co-IPs were allowed to continue incubating overnight. The beads were washed three 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 off the beads by boiling in 2X SDS sample buffer and resolved by SDS-PAGE. Proteins interactions were analyzed via Western blotting.

2.7: IMMUNOFLUORESCENCE

Cells were plated onto coverslips in a 6-well plate and transfected with relevant plasmids. Forty-eight hours post-transfection, cells were washed with 2 mL PBS, fixed in 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS for 10 min at RT, followed by three 5 min washes with PBS. Cells were then permeabilized in 0.5% Triton X-100/PBS for 5 min, followed by another three 5 min washes with PBS. After blocking in PBS containing 5% donkey serum for 30 min at RT, cells were immunolabeled by incubating with the relevant antibodies for 2 hr at RT. Antibodies used for various immunofluorescence experiments included polyclonal rabbit anti-PML (1:300), polyclonal sheep anti-PML (1:200), polyclonal rabbit anti-eIF3K (1:500) and

monoclonal mouse anti-HA (1:1000) (Covance, Princeton, NJ). Coverslips were washed three times with PBS for 5 min each and then incubated with secondary antibodies conjugated to Alexa Fluor 488 (1:200), 549 (1:200) or 649 (1:100) for 1 hr at RT. All fluorescent-conjugated secondary antibodies used for immunofluorescence were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Following incubation, cells were washed two times with PBS, followed by 5 min incubation with 4',6-diamidino-2-phenylindole (DAPI) stain (1 μg/mL) in PBS. Coverslips were washed two times with PBS to remove DAPI stain and mounted onto slides containing 2% propyl gallate and 90% glycerol in PBS. Fluorescent images were captured with Zeiss Axiovert 200M Microscope and Hamamatsu Orca R2 Camera or with Zeiss Cell Observer Microscope under a 40X immersion oil objective lens. Images were processed with Slidebook and Adobe Photoshop 7.0. Line scan analysis on immunofluorescent images was performed with Image J v.1.42 software program (National Institute of Health).

CHAPTER 3: RESULTS

3.1: IDENTIFICATION OF eIF3K AS A NOVEL INTERACTING PARTNER OF PML ISOFORM I USING A YEAST TWO-HYBRID SCREEN

The functional differences between individual PML isoforms have been illustrated in numerous published studies.[36] Despite being the most abundant isoform, very little is known about the specific cellular functions of PML isoform I (PML-I) or how PML-I contributes to the biochemical composition of PML NBs.[52] Therefore, to acquire a deeper understanding of its functions, we used a yeast two-hybrid screen to identify potential interacting partners of PML isoform I.

The yeast two-hybrid screen involves the expression of two proteins in *Saccharomyces cerevisiae* budding yeast, one fused to the Gal4 DNA binding domain (DBD) ("bait") and the other fused to the Gal4 activation domain (AD) ("prey"). If the two fusion proteins interact, the Gal4 AD is brought to the promoters where the Gal4 DBD fusion protein is bound, resulting in transcription of the downstream reporter genes (Figure 8), subsequently allowing yeast growth on synthetic defined (SD) media lacking the relevant synthetic amino acids or nucleotides.[182] To identify proteins that interact specifically with PML-I, the exon 9 region of human PML-I (PML-I-E9), which encodes amino acids 604-882, was introduced into pGBT9 or pGBKT7 vector. The plasmid encoding PML-I-E9 fused to the GAL4 DBD was transformed into the relevant yeast strain (Table 1) and protein expression was confirmed by Western blot analysis of total yeast cell lysates with an anti-PML-I antibody (Figure 9). In agreement with previous literature reports that PML is not conserved in yeast,[183, 184] we did not detect

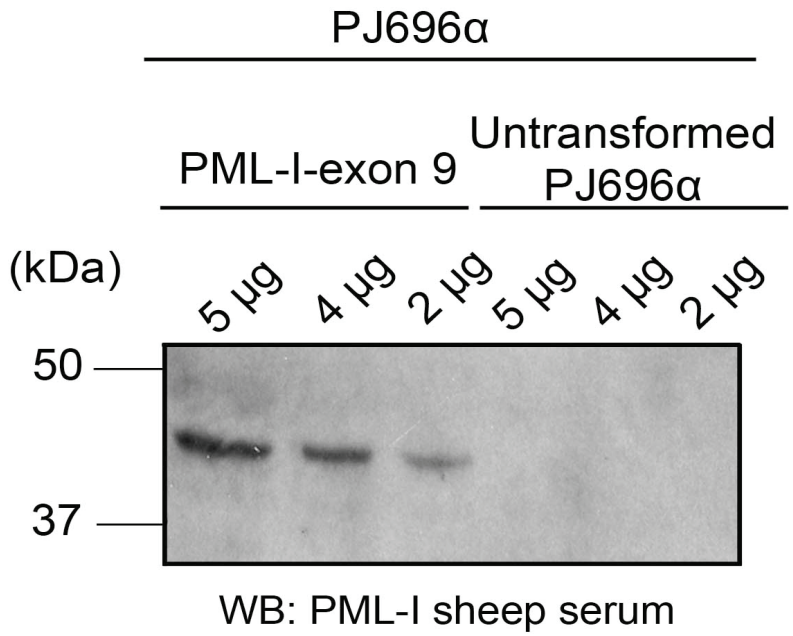


Figure 9: Confirmation of PML-I-exon 9 protein expression in yeast. Plasmid encoding PML-I-exon 9 was transformed into yeast (strain PJ696α). Total proteins were extracted from yeast cells and different amounts were resolved by SDS-PAGE and detected via Western blotting using a PML-I specific antibody. Untransformed PJ696α yeast strain was used as a negative control.

PML-I protein expression in untransformed PJ696α yeast strain (Figure 9) used in the yeast two-hybrid screen.

Prior to performing the yeast two-hybrid screen, the bait protein was tested for its ability to activate the biosynthetic (*HIS3*, *ADE2*), colorimetric (*LACZ* or *MEL1*) and antibiotic (*AUR1C*) gene reporters used in our yeast two-hybrid system when expressed in yeast in the absence of a Gal4 AD fusion protein. This is a crucial control as auto-activation by the bait protein can impair the feasibility of this assay. Having confirmed that PML-I-E9 bait did not auto-activate in the absence of the prey protein, we then used this bait to screen the human fetal brain and normalized universal human cDNA libraries for interacting proteins. Yeast colonies obtained from the yeast two-hybrid screen were initially assayed for activation of the *HIS3* and/or *ADE2* reporter genes (Table 1). Colonies that survived this first round of screening were subjected to a colorimetric assay for either beta-galactosidase (*LACZ* expression) or alpha-galactosidase (*MEL1* expression) activity coupled with aureobasidin A antibiotic selection (AUR1C reporter expression) (Table 1) to assess the relative strength of the protein interactions before prioritizing which library plasmids to rescue and sequence. The identities of the putative PML-I-E9 interacting proteins were determined by sequencing PCR products amplified from yeast colonies or rescued plasmids using primers specific to the library backbone. The resulting DNA sequences were compared to all known DNA sequences in the Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) gene database.

In our first yeast two-hybrid screen performed with the human fetal brain cDNA library, we screened approximately $1.1x10^6$ clones. Following stringent selections with the *HIS3*, *ADE2* and *LACZ* reporter genes, we uncovered 398 protein interactions, of which 175 putative interacting clones were verified by sequencing and 25 proteins were identified to be in frame with the Gal4 AD. We used higher stringent selections in our second yeast two-hybrid screen with the normalized universal human cDNA library. By screening $2.4x10^6$ clones in the second screen, we obtained 178 protein interactions following *HIS3*, *ADE2*, *MEL1* and *AUR1C* selections and we identified 89 putative interacting clones via DNA sequencing, of which only 14 were found to be in-frame with the Gal4 AD. The in-frame library plasmids should result in functional fusion proteins; therefore they are considered putative interacting partners of PML-I-E9. The prey plasmids that contain out-of-frame insertions of exons or untranslated regions were deemed false positives, because they produce truncated nonsense protein sequences that are fused to the Gal4 AD.

From our yeast two-hybrid screens, we identified several PML-I-E9 interacting proteins that have roles in diverse cellular processes, including translational regulation (Figure 10). One of the clones identified which plays an important role in the initiation of translation is the eukaryotic initiation factor 3 subunit K (eIF3K). This protein was isolated from our yeast two-hybrid screen with the human fetal brain cDNA library, and was chosen for further study

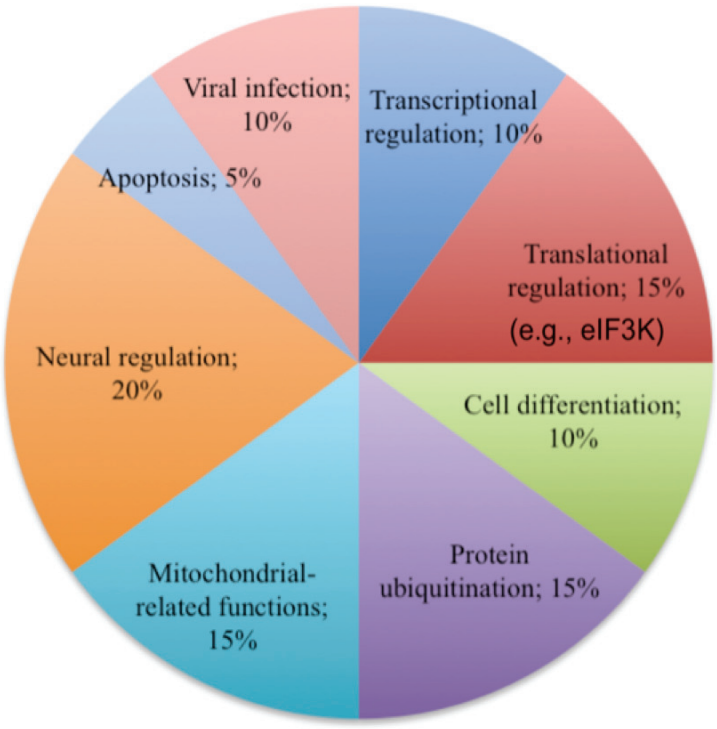


Figure 10: PML-I interacting proteins identified from the yeast two-hybrid screens. PML-I putative interacting partners identified from the yeast two-hybrid screens played roles in diverse cellular functions. One of the proteins, eukaryotic initiation factor 3 subunit K (eIF3K) functions as a translational regulator.

because previous reports have indicated that other eIF proteins (e.g., eIF3E and eIF4E) interact with PML.[87, 185]

The PML-I-E9 two-hybrid protein interaction with eIF3K was confirmed by re-transforming the rescued eIF3K library plasmid back into yeast (strain Y187) and mating it with the yeast (strain Y2HGold) strain of opposite mating type expressing PML-I-E9 bait protein. Relative strengths of protein interactions were measured by growth of yeast colonies on SD medium lacking histidine, and adenine and supplemented with aureobasidin A and X-α-gal (i.e., indicating expression of *HIS3, ADE2*, *AUR1C and MEL1* reporter genes, respectively) (Figure 11). This system was also used to identify the region of interaction between PML-I-E9 and eIF3K. From analyzing the sequence of the eIF3K plasmid that was rescued from the yeast two-hybrid screen, we saw that only the N-terminal 97 amino acids of eIF3K were present and in-frame with the Gal4 AD (Figure 11A, see Appendix II for amino acid sequences), and this eIF3K fragment was sufficient for interaction with PML-I-E9 (Figure 11B, row 1). Conversely, to investigate the minimal region of PML-I-E9 that is essential for interaction with eIF3K, PML-I-E9 bait plasmid encoding amino acids 750-882 but lacking the exonuclease III domain (PML-I-E9Δexo) was generated (Figure 11A), and, indeed, this region was sufficient for interaction with the N-terminus of eIF3K (Figure 11B, row 2). In the absence of protein-protein interactions (i.e., bait or prey proteins interacting with Gal4 AD or DBD alone, respectively), eIF3K prey (Figure 11B, row 3) and both PML-I-E9 bait proteins (Figure 11B, row 4 and 5)

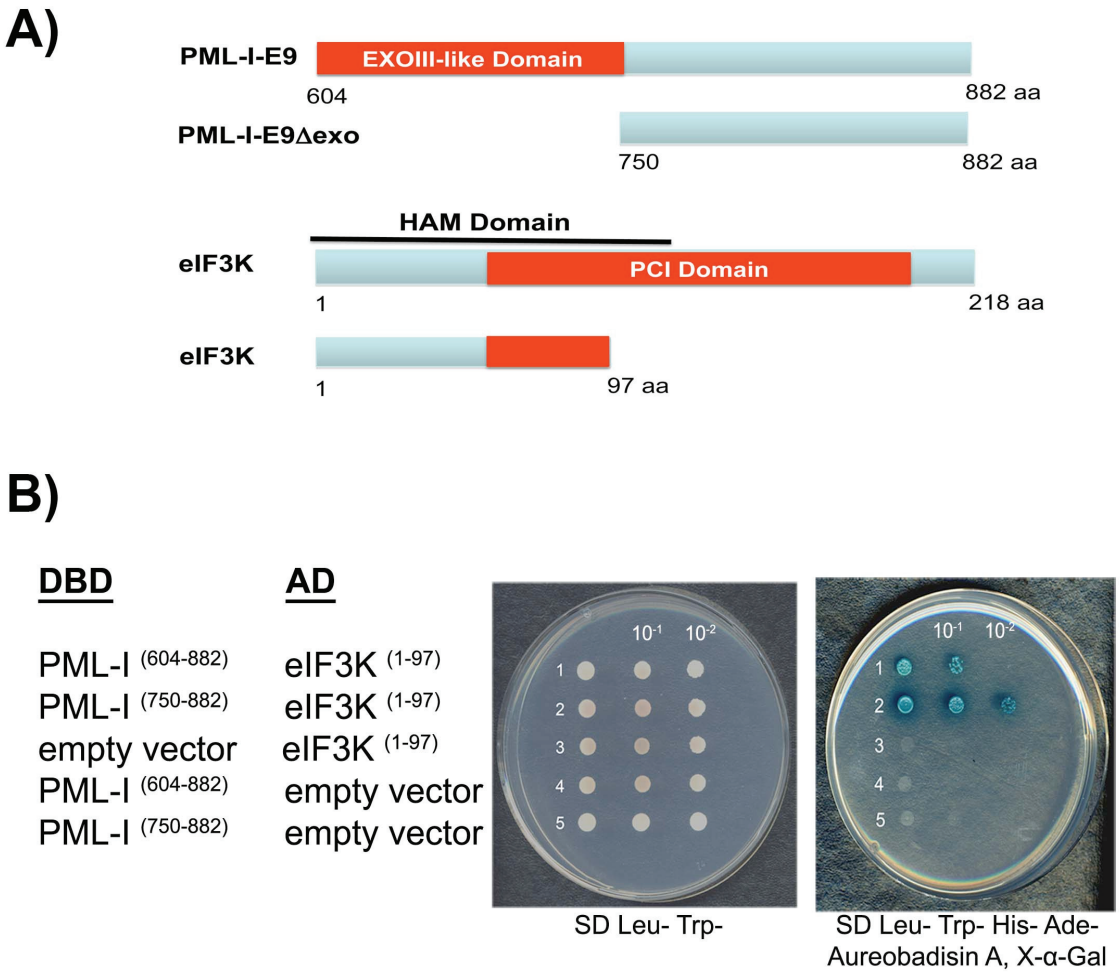


Figure 11: PML-I interacts with eIF3K in the yeast two-hybrid system. (A) Schematic diagram of PML-I-E9 and eIF3K fragments used for assessing protein interaction in the yeast two-hybrid mating assay. The full length PML-I-E9 (604-882 aa) and eIF3K (1-218 aa) are indicated above and the truncated fragment of PML-I-E9 (750-882 aa) and eIF3K (1-97 aa) are indicated below. (B) Relevant yeast strains transformed with the indicated plasmids were mated and selection was maintained on SD Leu^- Trp^- solid medium (as indicated by the panel on the left). Protein interactions were assessed by plating serial dilutions of the mated yeast colonies on SD $Leu^-Trp^-His^-Ade^-$ supplemented with 70 ng/mL of the antibiotic Aureobasidin A and 40 µg/mL X-α-Gal medium (right panel). Growth of yeast colonies on this medium required activation of the *HIS3*, *ADE2* and *AUR1C* reporter genes and the blue color indicates expression of the *MEL1* reporter gene.

did not induce expression of the reporter genes in yeast cells. Therefore, these results suggest that the N-terminus of eIF3K interacts with the C-terminus of PML-I-E9.

3.2: PML-I INTERACTS WITH eIF3K *IN VITRO*

To investigate whether PML-I interact with eIF3K *in vitro*, glutathione-S-transferase (GST) or maltose binding protein (MBP) pull-down assays were performed. The full length PML-I-E9 is very difficult to express and purify from bacteria because of its size and propensity to form insoluble protein aggregates (Dr. Graham Dellaire, personal communication); therefore, we expressed a shorter fragment of PML-I-E9 (750-882 aa) lacking the exonuclease III domain (PML-I-E9Δexo) as a MBP-fusion protein. *In vitro* pull-down assays were then performed using bacterial-expressed recombinant GST-eIF3K or MBP-PML-I-E9Δexo fusion proteins. The GST pull-down experiments revealed that MBP-PML-I-E9Δexo interacts with GST-eIF3K but not GST alone (Figure 12A). The reciprocal MBP pull-down assay demonstrated that GST-eIF3K interacts with MBP-PML-I-E9Δexo but not MBP alone (Figure 12B). Collectively these data confirm that PML-I-E9Δexo interacts with eIF3K *in vitro*, which is consistent with the observed yeast two-hybrid interaction (Figure 11B, row 2).

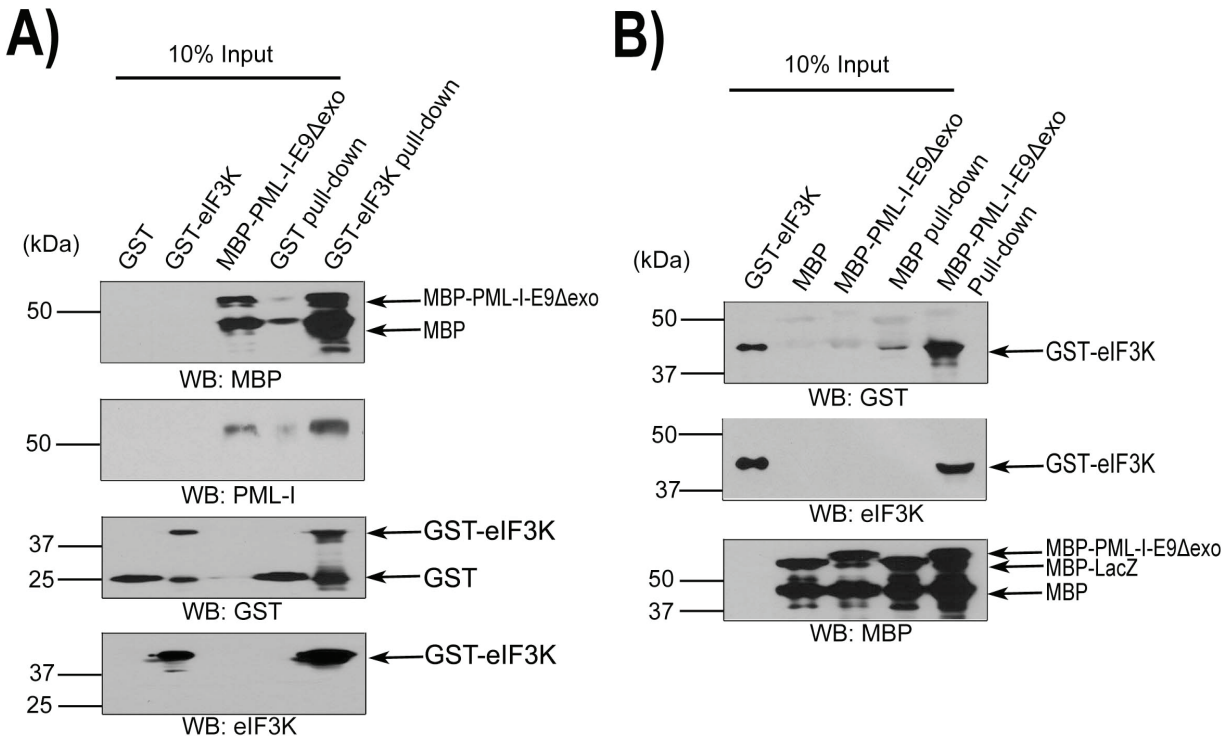


Figure 12: *In vitro* interaction between PML-I and eIF3K. MBP, MBP-PML-I-E9Δexo (750-882 aa), GST and GST-eIF3K proteins were expressed in bacteria and purified with amylose or glutathione Sepharose beads for MBP and GST containing proteins, respectively. (A) GST or GST-eIF3K proteins were bound to GST beads, followed by incubation with MBP-PML-I-E9Δexo. (B) MBP or MBP-PML-I-E9Δexo proteins were bound to amylose beads, followed by incubation with GST-eIF3K. After incubation, the beads were washed three times with wash buffer and resolved by SDS-PAGE and Western blotted with the indicated antibodies. pMal-p4G vector encoding MBP-LacZ fusion protein was detected in the MBP Western blot. The above experiments were repeated twice, and data were reproducible.

3.3: PML-I ASSOCIATES WITH eIF3K *IN VIVO*

Although our GST/MBP pull-down data indicated that the C-terminal exon 9 region of PML-I can interact with eIF3K *in vitro*, we wanted to investigate whether the full length PML-I could also interact with eIF3K *in vivo*. To achieve this objective, we performed co-immunoprecipitation (Co-IP) experiments using the human embryonic kidney cell lines (HEK-293T) and antibodies directed against eIF3K (Figure 13A). Consequently, we found that endogenous PML protein could be Co-IPed with endogenous eIF3K (Figure 13A). Due to the lack of available commercial antibodies, we could not efficiently immunoprecipitate (IP) the endogenous PML-I protein; however, we were able overcome this limitation and further confirm the *in vivo* interaction of PML-I with eIF3K by co-expressing C-terminally hemagglutinin (HA)-tagged eIF3K (eIF3K-HA) and FLAG-epitope- tagged PML-I (FLAG-PML-I) in HEK-293T cells followed by IP using antibodies directed against eIF3K (Figure 13B) or the FLAG epitope (Figure 13C). Subsequent SDS-PAGE and Western blot analysis of the immunoprecipitated protein complexes revealed that eIF3K and PML could be Co-IPed together in a reciprocal fashion. Taken together, these results demonstrated that full length PML-I can interact with eIF3K *in vivo*.

3.4: CHARACTERIZATION OF MAMMALIAN CELL LINES WITH STABLE ECTOPIC EXPRESSION OR KNOCKDOWN OF THE PML PROTEIN

The PML protein is expressed in mammalian cells as several spliced isoforms.[52] Therefore, to characterize PML isoform-specific protein-protein, various mammalian cell lines were used in this study that are stably

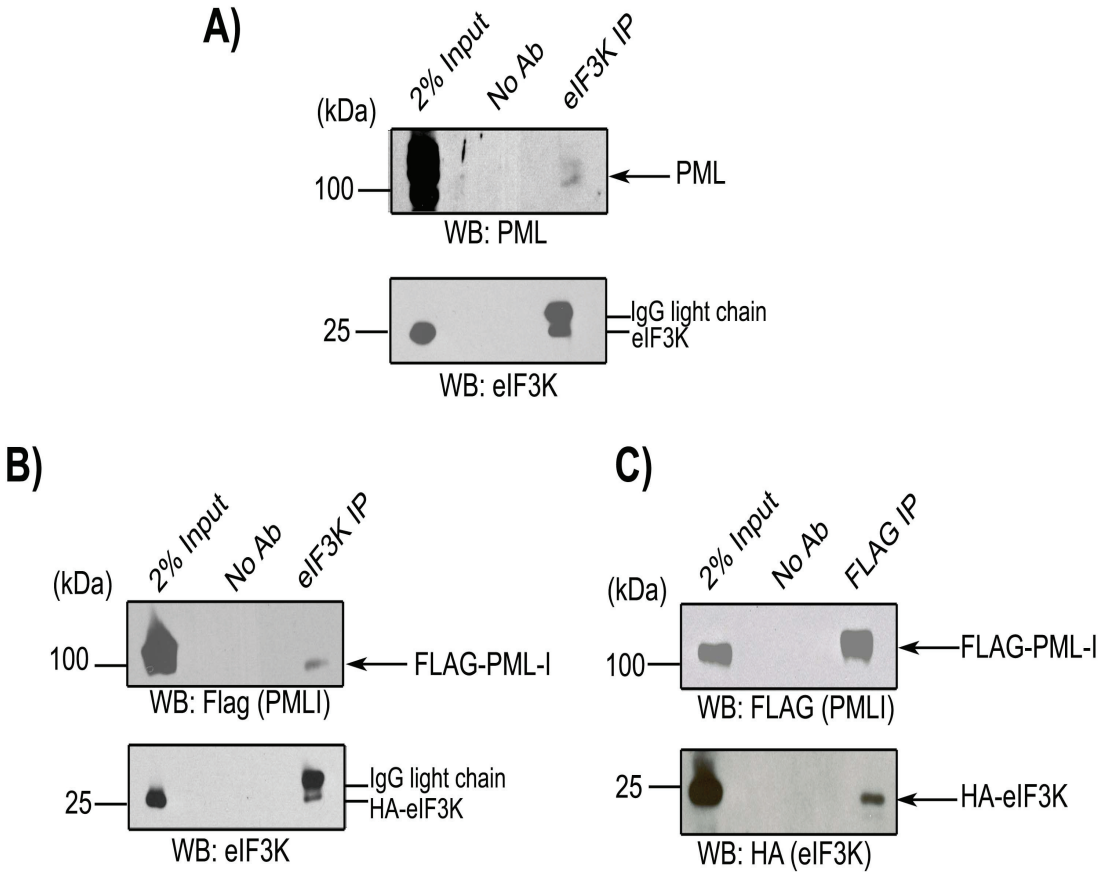


Figure 13: PML-I interacts with eIF3K *in vivo*. (A) HEK-293T cell lysates were used for co-immunoprecipitation of endogenous PML and eIF3K proteins with mouse anti-eIF3K monoclonal antibody. Immunoprecipitated proteins were separated by SDS-PAGE and the co-immunoprecipitated PML protein was assessed by Western blotting analysis with rabbit anti-PML polyclonal antibodies. (B) FLAG-PML-I and eIF3K-HA expression plasmids were transiently transfected into HEK-293T cells. Endogenous and HA-tagged eIF3K were immunoprecipitated with mouse anti-eIF3K monoclonal antibody and the co-immunoprecipitated PML proteins were analyzed by Western blotting with mouse anti-FLAG antibody. (C) HEK-293T cells co-expressing FLAG-PML-I and eIF3K-HA was immunoprecipitated with mouse anti-FLAG antibody and immunoprecipitated proteins were probed with chicken anti-HA antibody. As controls for all Co-IPs, relevant antibodies were omitted. Experiments were repeated twice, and data were reproducible.

overexpressing the different PML isoforms or where PML gene expression was stably knocked-down. The level of PML protein in these cell lines was determined by Western blotting and/or immunocytological analysis prior to use for later studies. The first cell lines we characterized were variants of the human osteosarcoma (U2OS) cell line expressing green fluorescent protein (GFP)-tagged PML isoform I or IV. The expression of the GFP-tagged proteins was confirmed by Western blotting analysis of total cell lysates with a pan-PML antibody that recognizes all PML isoforms. We detected both ectopically expressed GFP-tagged PML-I and -IV (red arrow) as well as endogenous PML expression (blue arrow) in the U2OS cell lines (Figure 14A). The higher molecular weight bands detected in the Western blot represent post-translationally modified (e.g., sumoylated and/or phosphorylated) forms of PML. As evident in our Western blot, the GFP-tagged PML-I and -IV proteins were substantially overexpressed in the U2OS cell lines compared to the endogenous PML protein levels in the wild-type U2OS cells (Figure 14A). To ensure equal amounts of protein were loaded onto our protein gel, we also analyzed the total protein lysates for β-tubulin as a loading control.

A TERT-immortalized normal human diploid fibroblast cell line (NHDF) with stable knockdown of PML gene expression and its corresponding control cell lines were generated by transducing these cells with retrovirus encoding short hairpin RNAs (shRNAs) that are directed against all isoforms of PML (shPML) or luciferase (shControl).[181] Western blot analysis of total protein isolated from these clonal cell lines indicated a significant knockdown of PML protein

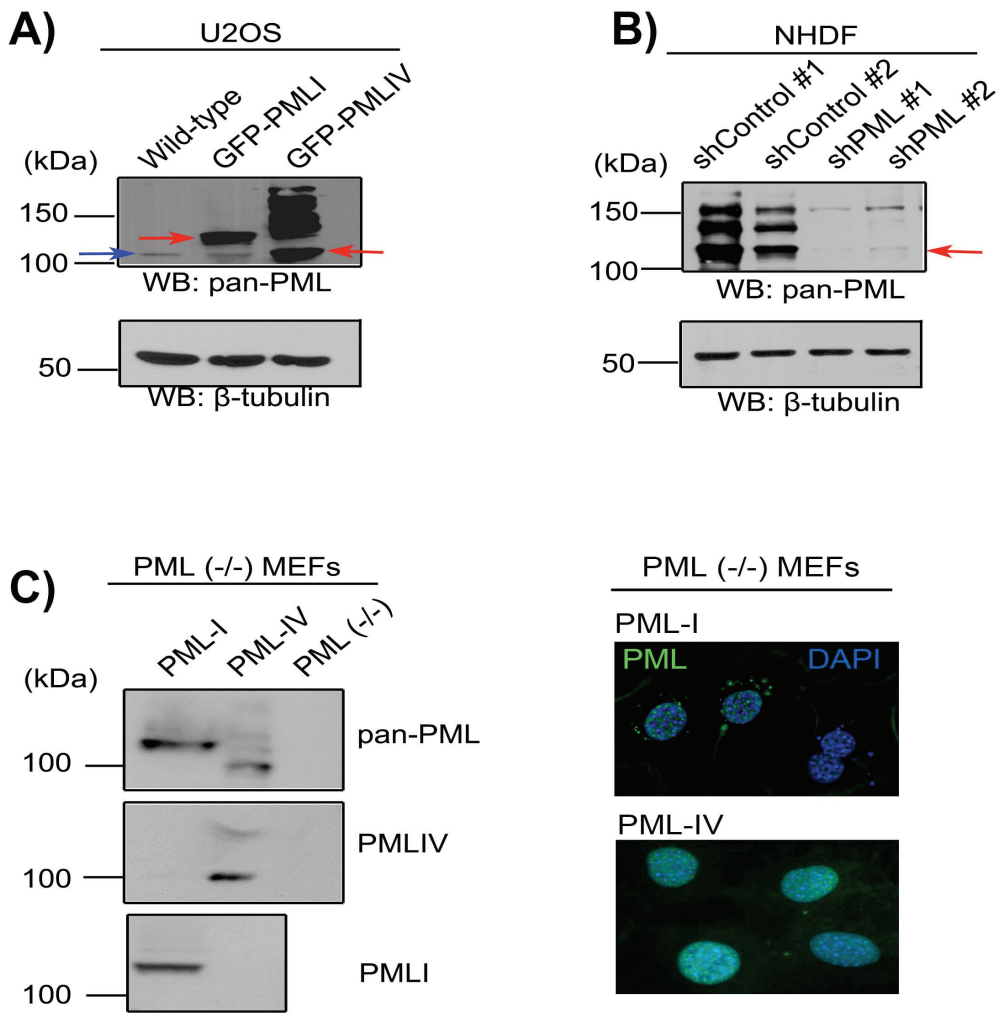


Figure 14: Characterization of mammalian cell lines with stable overexpression or knockdown of the PML protein. (A) Overexpression of GFP-tagged PML isoform I or IV in U2OS cells was confirmed by Western blotting with pan-PML polyclonal antibodies. β-tubulin was used as a loading control. The red arrow indicates the overexpression of the different PML isoforms and the blue arrow indicates endogenous PML protein expression. (B) PML protein expression was stably knocked-down in NHDF cells via retroviral transduction of shRNA targeting all PML isoform gene expression (shPML) or a control luciferase gene (shControl). Different biological replicates were analyzed for efficient PML protein knockdown. (C) PML isoform I or IV were introduced into a PML (-/-) MEF background by retroviral transduction with pBABE retroviral mammalian expression vector encoding PML-I or PML-IV. The expression of PML in these cell lines was confirmed by Western blotting analysis and immunocytology with anti-PML-I or anti-PML-IV specific antibodies.

expression in PML shRNA expressing cells compared to the control cell lines (Figure 14B). Of the various NHDF clones expressing shPML analyzed, clone number one appeared to have the most efficient knockdown of PML protein level (Figure 14B). Finally, using PML (-/-) murine embryonic fibroblast (MEF) cells derived from the PML knockout mouse (gift from Dr. Paolo Salomoni, University College London, London, UK), we generated MEF cell lines expressing either PML isoform I or IV by retroviral transduction. Western blot (Figure 14C, left panel) and immunofluorescence (Figure 14C, right panel) analysis of these cell lines using anti-PML-I or anti-PML-IV specific antibodies confirmed successful introduction of PML-I and -IV into PML (-/-) MEF cells.

3.5: eIF3K CO-LOCALIZES WITH PML *IN VIVO*

To determine whether eIF3K co-localizes with PML *in vivo*, we performed indirect immunofluorescence detection of endogenous and HA-epitope-tagged eIF3K (green) and endogenous PML (red) proteins in human HeLa cervical cancer cells (Figure 15). DAPI staining of the nuclear DNA (blue) shows the position of the nucleus. Due to the fact that tagging a protein on either the N- or C-terminus may affect the ability of the protein to fold properly and retain its native functions, we observed the subcellular distribution of eIF3K tagged with the HA-epitope at either the N- or C-terminus by immunofluorescence microscopy (Figure 15A and 15B). The N-terminal tagged HA-eIF3K was found to localize both in the nucleus and cytoplasm, which is consistent with a previous

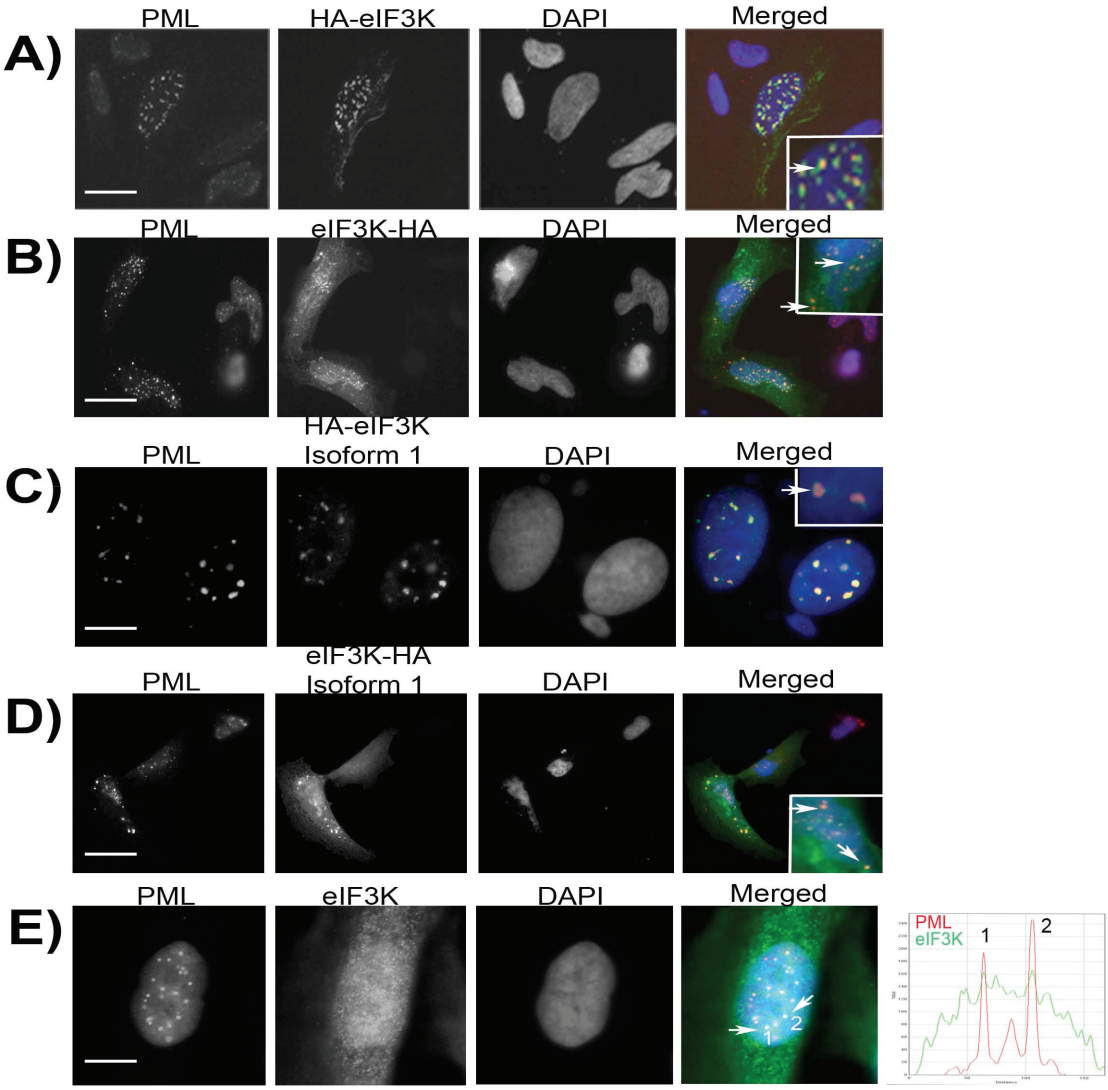


Figure 15: eIF3K co-localizes with PML in PML NBs in HeLa cells. HeLa cells were fixed onto coverslips and stained for PML (red), eIF3K (green) and DAPI indicating the nucleus (blue). (A) Co-localization of endogenous PML and exogenous HA-eIF3K proteins (unless indicated, all immunofluorescence was performed with the alternative splice isoform 2 of eIF3K as outlined in Materials and Methods). White arrows indicate PML NB sites where both proteins co-localize together. (B) Co-localization of endogenous PML and exogenous eIF3K-HA proteins. (C-D) Co-localization of ectopic HA-eIF3K isoform 1 (C) and eIF3K isoform 1-HA (D) with endogenous PML. (E) Co-localization of endogenous eIF3K and PML proteins. Line scan analysis was used to demonstrate co-localization of eIF3K and PML in two PML bodies (i.e., 1 and 2). Scale bars = 10 μm

published study.[172] Moreover, HA-eIF3K was found juxtaposed with endogenous PML as indicated by the corresponding yellow colour dots that resulted from superimposing the two fluorescent images (white arrow, Figure 15A). However, tagging eIF3K at the C-terminus with the HA-epitope resulted in eIF3K-HA co-localizing to a greater extent with endogenous PML at PML NBs in the nucleus and also in the cytoplasm of G1 cells (Figure 15B), which coincides with the published cellular distributions of both eIF3K and PML-I.[52, 172] Therefore, the C-terminally tagged eIF3K-HA expression plasmid was used for all subsequent immunofluorescence analyses and in the Co-IP experiments described in section 3.3 (Figure 13B, 13C). Furthermore, the *in vitro* and *in vivo* experiments described in section 3.2 and 3.3 respectively, were conducted with the alternative splice isoform of eIF3K, eIF3K isoform 2, but for the purpose of this thesis, referred to as eIF3K (see Materials and Methods, section 2.1). However, we were interested in elucidating whether PML interact specifically with eIF3K isoform 2 or whether it can interact with both isoforms. To assess this, the localization of exogenous eIF3K isoform 1 and endogenous PML in HeLa cells was investigated. Regardless of which end of eIF3K isoform 1 was tagged with a HA-epitope, this isoform of eIF3K also co-localized with PML (Figure 15C, 15D). The co-localization pattern of eIF3K isoform 1 with PML was reminiscent of the co-localization pattern between eIF3K isoform 2 with PML, thus supporting the notion that PML interacts with both eIF3K isoforms.

To determine whether PML interaction with eIF3K is physiologically relevant, the localization of endogenous eIF3K and PML proteins was examined

in HeLa cells. Endogenous eIF3K co-localized with endogenous PML and eIF3K appeared to be enriched in the nucleus (Figure 15E). Line scan analysis of the endogenous eIF3K and PML immunofluorescence images provided additional evidence that a subset of eIF3K protein co-localizes with PML in PML NBs, as indicated by the overlapping fluorescence intensity profiles for the detection of PML (red) and eIF3K (green) (Figure 15E).

Finally, to determine the specificity of the interaction between eIF3K and PML isoform I, the localization of eIF3K and PML was investigated in wild-type MEFs, PML (-/-) MEFs and PML (-/-) MEFs complemented with expression vectors encoding either PML-I or PML-IV. eIF3K was observed to co-localize with PML predominately in PML NBs in wild-type MEF cells (Figure 16A), which is consistent with the immunofluorescence localization observed in HeLa cells (Figure 15). As expected, we did not see any PML staining and very little nuclear eIF3K staining in PML (-/-) MEF cells (Figure 16B). PML isoform specific co-localization with eIF3K was studied by stably transfecting eIF3K-HA into PML (-/-) MEFs expressing either PML-I or PML-IV. Surprisingly, eIF3K co-localized with both PML-I (Figure 16C) and PML-IV (Figure 16D); however eIF3K appeared to be preferentially sequestered in PML NBs of PML-I expressing MEFs compared to PML-IV expressing cells (Figure 16C and 16D, indicated by the white arrows). Similarly, eIF3K-HA exogenous proteins also co-localized with PML NBs in U2OS cells expressing both GFP-tagged PML-I (Figure 16E) and PML-IV (Figure16F).

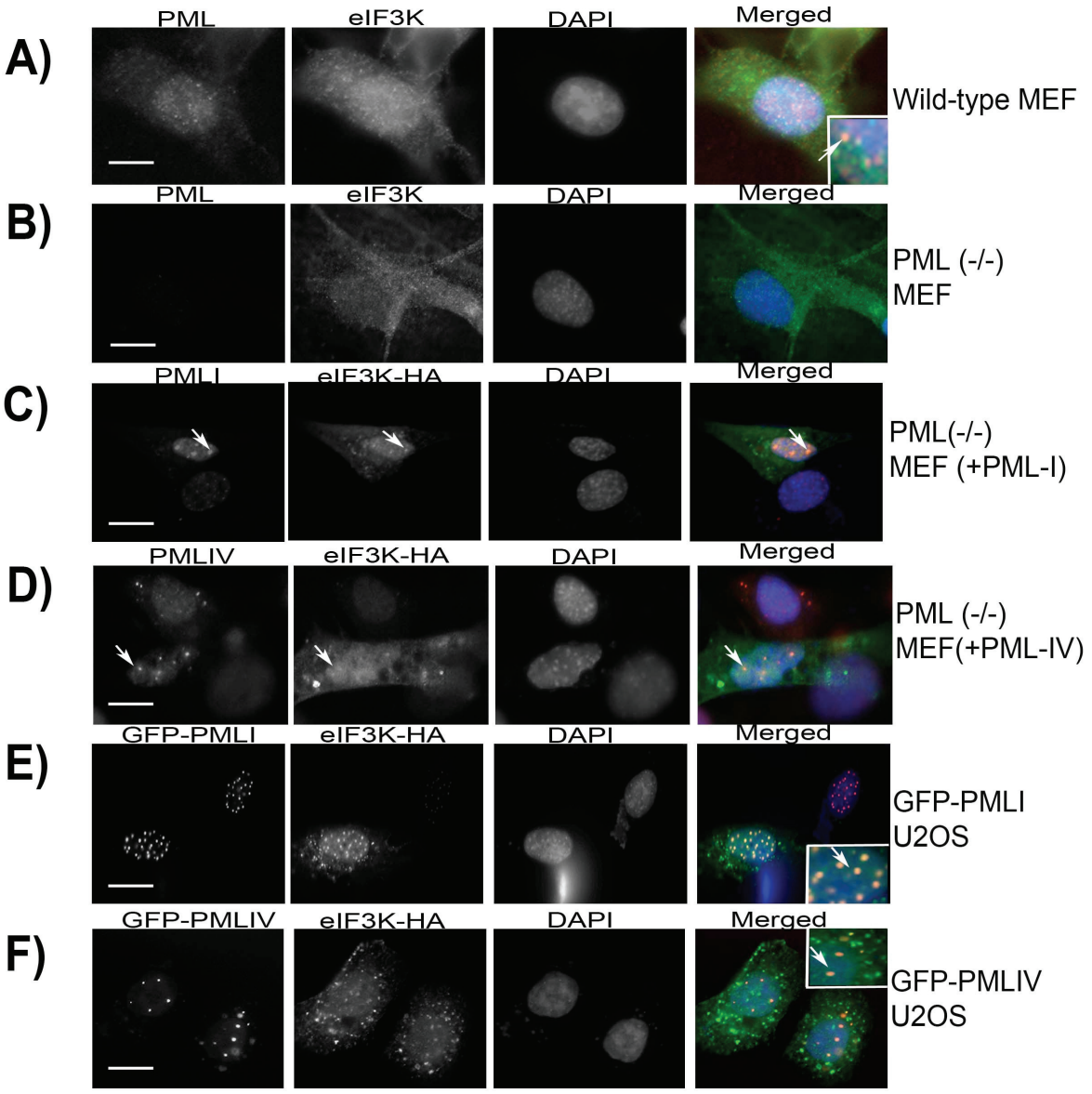


Figure 16: Co-localization of eIF3K with PML isoform I and IV. Cells were fixed onto coverslips and stained for PML (red), eIF3K (green) and DAPI indicating the nucleus (blue). (A) Endogenous eIF3K co-localize with endogenous PML proteins in wild-type MEF cells. White arrows indicate PML NB sites where both proteins co-localize together. (B) When PML proteins are absent (i.e., in PML (-/-) MEFs), there is less nuclear eIF3K protein. (C-D) PML isoform specific co-localization with eIF3K was investigated by transfecting eIF3K-HA into PML (-/-) MEFs overexpressing either PML-I or PML-IV. PML-I bodies preferentially sequester eIF3K-HA compared to PML-IV bodies (white arrows). (E-F) Exogenous eIF3K co-localizes with PML isoform I (E) and IV (F) in U2OS cells expressing GFP-tagged PML-I or PML-IV. White arrows indicate PML NB sites where both proteins co-localize together. Scale bars = 10 μm

3.6: PML INCREASES eIF3K STEADY-STATE PROTEIN LEVELS

With extensive data supporting eIF3K interaction with PML, we next sought to investigate the biological implications of this protein interaction. To address this aim, we performed Western blot analysis of total cell lysates from human and mouse cell lines with stable overexpression or knockdown of PML protein expression to determine whether PML may be involved in regulating eIF3K protein expression or stability. In U2OS cells overexpressing GFP-tagged PML-I or PML-IV, eIF3K protein levels were substantially elevated compared to eIF3K levels in wild-type U2OS cells (Figure 17A), which expressed very low levels of endogenous PML protein (Figure 14A). In contrast, the levels of other eIF3 subunits such as eIF3E and eIF3F were unaffected by PML overexpression in the U2OS cells (Figure 17A), suggesting that PML specifically regulates eIF3K protein expression or stability. A similar phenomenon was observed in the MEF cell lines where the eIF3K protein level was clearly lower in PML (-/-) MEFs compared to wild-type MEFs expressing PML. Furthermore, complementation of PML (-/-) MEFs with PML-I partially restored eIF3K protein levels to that observed in wild type MEFs, whereas expression of PML-IV did not (Figure 17B). To further confirm that PML is involved in regulating eIF3K protein expression or stability, PML protein expression was knocked down in TERT-immortalized NHDF cells. Consistent with our observations in wild-type and PML (-/-) MEFs, eIF3K protein levels were decreased in NHDF cells expressing a shRNA directed against all isoforms of PML (shPML) compared to NHDFs expressing a control shRNA directed against luciferase (shControl) (Figure 17C). Furthermore, the

protein level of eIF3E and eIF3F also remained constant between the PML knockdown and control cell lines (Figure 17C), again supporting the hypothesis that PML specifically regulates the steady-state protein level of eIF3K.

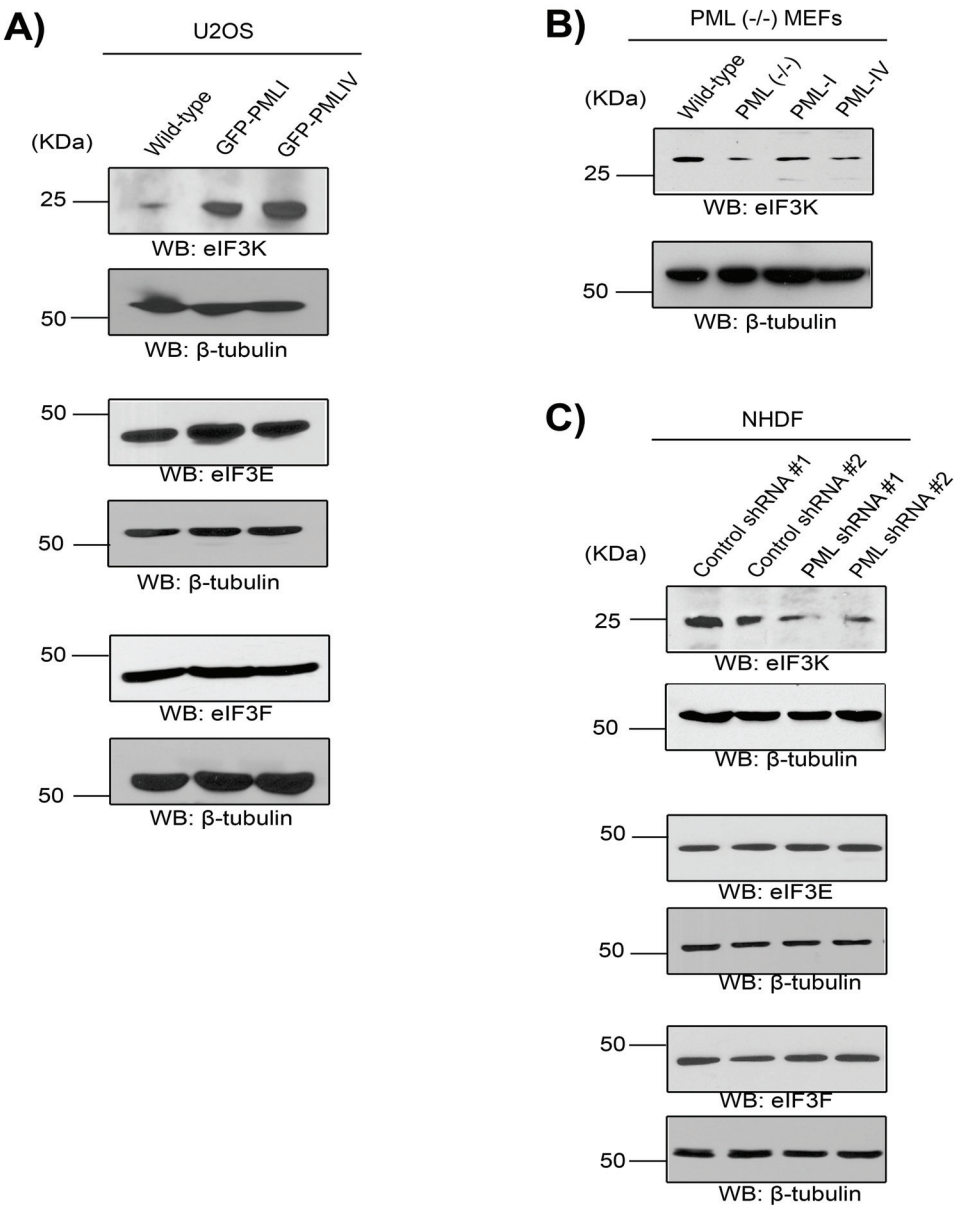


Figure 17: PML increases eIF3K protein steady-state levels. (A) eIF3K steady-state protein levels were significantly increased in U2OS cells overexpressing either PML isoform I or IV; however, the levels of other eIF3 subunits including eIF3E and eIF3F remained unchanged. (B) The steady-state level of eIF3K was reduced in PML (-/-) MEFs compared to wild-type MEFs. Complementation of PML (-/-) MEFs with PML isoform I partially restored eIF3K protein levels but PML-IV did not. (C) Reduced PML protein expression in NHDF cells resulted in decreased eIF3K protein levels whereas the levels of eIF3E and eIF3F were unaffected. The above experiments were repeated twice to demonstrate that the data were reproducible.

CHAPTER 4: DISCUSSION

4.1: SUMMARY

Loss of the PML protein is associated with genomic instability and cancer of various histological origins.[30] There are multiple isoforms of the PML protein that localize to the discrete subnuclear structures known as PML NBs. These bodies play a complex role in diverse biological processes, including tumour suppression, apoptosis and gene regulation.[6, 12] How each individual PML isoform contributes to the various functions attributed to PML NBs, and ultimately cancer, is poorly understood. Different PML isoforms harbour distinct C-terminal domains, which may play a role in regulating isoform specific protein-protein interactions. In this study, we have identified eIF3K as a novel interacting protein of PML isoform I (PML-I), thereby possibly implicating this isoform in translational regulation.

4.2: IDENTIFICATION OF eIF3K AS A NEW INTERACTING PARTNER OF PML-I VIA A YEAST TWO-HYBRID SCREEN

Protein-protein interactions are important for the regulation of diverse cellular functions including DNA replication, cell cycle control, transcription, translation and intermediary metabolism.[186] Although PML-I is the most abundant isoform,[52] and therefore most likely contributes to a wide range of protein-protein interactions in PML NBs, very few isoform-specific protein-protein interactions have been reported. PML-I was used as a focal point for this research not only because it is the most abundant isoform expressed in mammalian cells but because it is also one of only two PML isoforms that are known to be conserved between human and mouse. As such, by working with PML-I, we expected that

the interactions we observed would be conserved between mouse and human and would have wide biological relevance in a variety of cell types. Furthermore, PML-I contains a unique protein sequence at its C-terminus encoded by exon 9, which is absent from all other isoforms (Figure 2). In our yeast two-hybrid assays, we determined protein interactions with exon 9, therefore, the protein interactions we identified from our screens are mostly likely unique to PML-I (Figure 2 and Figure 10).

The yeast two-hybrid screen is an *in vivo* technique that is commonly used to screen large scale protein-protein interactions and offers the advantages of being able to detect weak and transient, as well as binary and ternary protein interactions. Despite these advantages, the yeast two-hybrid screen also has some limitations, including a high rate of false positive protein interactions. For example, overexpression of the “bait” and “prey” fusion proteins can sometimes result in non-specific interactions, or the prey library fusion proteins may auto-activate the reporter genes in the absence of the bait protein. In addition, some protein interactions may not be detected in the yeast two-hybrid screen because the proteins are not folded properly in yeast or the proteins themselves are toxic to yeast. Lastly, some protein interactions that are detected in yeast may not actually occur in mammalian cells, either because the interacting proteins are not expressed in the same cell type or reside in different cellular compartments.[187] Therefore, the results obtained from the yeast two-hybrid screen must always be verified by other biochemical techniques. This may include: GST pull-down experiments to determine if the proteins interact *in vitro* and whether the

interactions are direct versus being mediated by other proteins; immunofluorescence analysis to confirm that the two proteins co-localize together to the same subcellular compartment; and co-immunoprecipitation of endogenous proteins, which indicates that the protein interactions occur *in vivo* and are most likely physiologically relevant.

The yeast two-hybrid assay allows the strength of the protein interactions to be assessed based on the ability of the protein interactions to activate reporter genes. From my study, PML-I interaction with eIF3K was demonstrated to be relatively strong since the mated colonies carrying the PML-I bait and eIF3K library plasmids activated four reporter genes simultaneously (*HIS3*, *ADE2*, *MEL1*, *AUR1C*), which is the most stringent selection possible with this screening system (Figure 11). We also confirmed that PML-I interacts with eIF3K *in vitro* (Figure 12) and *in vivo* (Figure 13) as demonstrated from our GST/MBP pull-down and co-immunoprecipitation experiments, respectively. Furthermore, by co-immunofluorescence, eIF3K was shown to co-localize in the nucleus with PML in PML NBs, and with PML-containing structures in the cytoplasm (Figure 15). Collectively these data suggests that the interaction between the C-terminus of PML-I and eIF3K identified from the yeast two-hybrid screen is genuine and occurs within mammalian cells, which indicate that this protein interaction may have biological relevance.

4.3: ANALYSIS OF THE REGION OF INTERACTION BETWEEN PML-I AND eIF3K

The region of interaction between eIF3K and PML-I was determined using a number of complementary approaches. The yeast two-hybrid assays indicated that the N-terminal HEAT analogous motif (HAM) domain of eIF3K (1-97 aa) (Appendix II) is capable of interacting with the C-terminal 750-882 aa of PML-I. This is consistent with previous reports that the HAM domain (1-121 aa) is involved in mediating protein-protein interactions.[160, 180] Since the N-terminal region of eIF3K may be involved in interacting with PML-I, all Co-IP and immunofluorescence experiments were performed with eIF3K-HA expression plasmid because tagging the protein at the C-terminus does not disrupt the N-terminal region of eIF3K. There are at least two human eIF3K isoforms that have been identified, which differ by the 33 amino acids encoded by exon 2, which are present in eIF3K isoform 1 (GenBank Accession No. NM_013234) and absent in eIF3K isoform 2 (unpublished report; see Appendix II). As mentioned in the Materials and Methods (section 2.1), we identified the human eIF3K isoform 2 from mRNA isolated from human HeLa cervical carcinoma cells, and a very similar eIF3K isoform was also identified in dog (GenBank Accession No. XP_867485) and in rat (GenBank Accession No. NP_001099712), suggesting that this isoform is conserved between mammals. Our immunofluorescence studies showed that PML co-localized with both isoforms of eIF3K (Figure 15). Furthermore, we demonstrated that the second isoform, which likely arises by alterative splicing of exon 2 of eIF3K, interacted with PML both *in vitro* and *in vivo*. This suggests that the 33 amino acids that are present in eIF3K isoform 1

are dispensible for protein interaction with PML-I. Therefore, by deduction, the eIF3K region that may be necessary for interaction with PML-I is either the first 21 amino acids in the N-terminus (highlighted in green in Appendix II) or the region that occurs after the skipping of the 33 amino acids (i.e., 55-97 aa, highlighted in red in Appendix II). Interestingly, the region encoding the PCI domain is conserved between the two isoforms. Since previous studies have implicated the PCI domain in mediating protein-protein interactions,[139] this provides an interesting possibility that the PCI domain of eIF3K, or a portion of this domain, may also be involved in interacting with PML-I. However, we have not ruled out the possibility that the C-terminal region of eIF3K (98-218 aa) does not interact with PML-I. Therefore, to map the minimal region of interaction between eIF3K and PML-I, we will need to create deletion mutants of eIF3K encoding the PCI domain and the C-terminus alone to test for their interaction with PML-I either by yeast two-hybrid and/or *in vitro* GST pull-down assays.

Even though we isolated eIF3K as a PML-I interacting protein by using the unique exon 9 region of this isoform as a bait in our yeast two-hybrid screen, we also observed that eIF3K could co-localize with PML NBs in human osteosarcoma (U2OS) cells expressing PML-I and PML-IV. This result suggests that PML-IV in addition to PML-I may interact with eIF3K. However, one of the greatest difficulties in studying PML isoform specific protein-protein interactions is that several PML isoforms are usually expressed in any given cell, and these isoforms have the propensity to form heterodimers with one another.[12, 52] Since the U2OS cell lines we used in these experiments expresses endogenous PML-I,

this may explain why we saw co-localization of eIF3K with PML NBs in U2OS cells ectopically expressing PML-IV. Therefore, the co-localization we observed in these cells may not be mediated by PML-IV alone but by heterodimers of PML-I and PML-IV. Another approach to circumvent this problem is to study individual PML isoforms expressed in PML (-/-) murine embryonic fibroblasts (MEFs). These cells were derived from PML knockout mice where the exon 2 of the PML gene was disrupted by homologous recombination in murine embryonic stem cells.[13] Using this approach, we saw eIF3K co-localizing with PML-I and PML-IV in PML (-/-) MEF cells overexpressing PML-I or PML-IV, however, eIF3K appeared to be preferentially sequestered in PML-I bodies compared to PML-IV bodies (Figure 16C and 16D). Moreover, by simple visual observation, eIF3K appeared to be enriched in the nucleus in the wild-type MEF cells compared to PML (-/-) MEF cells (Figure 16A and 16B), suggesting that PML may be involved in recruiting eIF3K to the nucleus. In addition, not all PML NBs contain eIF3K, which supports the view that PML NBs are heterogenous structures with distinct biochemical compositions.[9-12] From these observations, we hypothesized that eIF3K may have higher affinity for interaction with PML-I compared to PML-IV. To test this theory, we will need to perform co-immunoprecipitation from PML (-/-) MEFs overexpressing either PML-I or PML-IV to see whether eIF3K preferentially co-immunoprecipitates with PML-I.

4.4: POSSIBLE IMPLICATIONS OF PML-I INTERACTION WITH eIF3K

In the cytoplasm, the main function of eIF3K is presumably the regulation of translation initiation; however, eIF3K co-localizes with PML predominately in the nucleus, suggesting that eIF3K may have nuclear functions as well. Several eIF3 subunits, such as eIF3E[176, 188] and eIF3F,[175] also localize to the nucleus. In fact, eIF3E has been demonstrated to localize to PML NBs via interaction with the Ret Finger protein,[185] and eIF3F distributes mainly to the nucleus upon phosphorylation by the cyclin dependent kinase 11 ($CDK11^{p46}$).[156] Currently, the nuclear functions of the eIF3 subunits are unknown. However, in fission yeast there is some evidence suggesting that nuclear eIF3 may play a role in regulating ribosome biogenesis.[189-193]

In our studies, we have shown that PML-I positively regulates eIF3K protein levels in a subunit-specific manner, because the levels of eIF3E and eIF3F were not affected by PML overexpression (Figure 17). This suggests that PML-I may be involved in mediating eIF3K protein translation or stability. As a consequence, global translation or translation of a subset of mRNAs may be affected, especially in cancer cells with reduced expression of PML-I. Interestingly, we also saw increased eIF3K protein levels in U2OS cells expressing ectopic PML-IV. However, this was not seen in murine embryonic fibroblast (MEF) cells overexpressing PML-IV (Figure 17). One possible reason for this difference may be that U2OS cells are cancerous cells; therefore, they may exhibit different biochemical behaviour from the noncancerous MEF cells. Furthermore, there are endogenous PML proteins in U2OS cells, the bulk isoform

being PML-I; therefore, the heterodimers formed between endogenous PML-I and ectopic PML-IV may be sufficient to elicit an increase in eIF3K protein levels.

4.5: FUTURE DIRECTIONS

In this study, we have shown that eIF3K interacts with PML, and overexpression of PML resulted in a concomitant increase in eIF3K protein levels. However, to fully characterize eIF3K protein interaction with PML, we will need to map the minimal region of eIF3K that is required for interaction with PML-I and determine by co-immunoprecipitation whether eIF3K interacts specifically with this isoform or whether it can also interact with another isoform (e.g., PML-IV). Furthermore, there are a number of ways in which protein levels may be augmented, including increased mRNA synthesis, mRNA export and protein stability. Therefore, we are greatly interested in elucidating the mechanism(s) by which PML regulates eIF3K protein levels. In the following sections, I have outlined several experiments that together will address the possible mechanisms by which PML-I regulates eIF3K protein levels.

4.5.1: DOES OVEREXPRESSION OF PML BY GAMMA-INTERFERON TREATMENT ALSO RESULT IN ELEVATED eIF3K PROTEIN LEVELS?

There are many studies illustrating that upon treatment with gamma-interferon (γ-IFN), expression of PML and PML-associated proteins such as Sp100 increases dramatically.[194] As part of the immune response, γ-IFN is released by cells in response to pathogenic (e.g., viruses, bacteria or tumour cells) invasions.[195] Therefore, it would be interesting to determine whether

overexpression of PML via γ-IFN treatment would also lead to an increase in eIF3K protein levels. To test this hypothesis experimentally, we can treat normal or cancerous cells with γ-IFN and use Western blotting analysis to monitor whether eIF3K protein levels are increased.

4.5.2: DOES OVEREXPRESSION OF PML PROMOTE AN INCREASE IN eIF3K mRNA SYNTHESIS OR STABILITY?

As mentioned previously, there are a number of ways in which PML may up-regulate eIF3K protein levels, and one of these ways is through promoting eIF3K mRNA synthesis. There are several lines of evidence that indicate a possible role of PML NBs in transcriptional regulation. First, PML NBs are located in the nucleus close to sites of active transcription, and they associate preferentially with gene-rich regions of the genome.[196] Second, nascent RNAs are found in close vicinity to PML NBs.[197] Third, PML NBs are located adjacent to Cajal bodies and splicing speckles, which suggests that PML may play a role in RNA processing, although this role is not well defined.[198] Since PML NBs do not contain RNA polymerase II,[3, 87, 196] this indicates that PML NBs may not play a direct role in transcription. Instead, PML may regulate transcription by interacting with various transcriptional co-activators such as CREB-binding protein (CBP)[199] and p53.[200] Therefore, to determine whether PML augments eIF3K protein expression by promoting an increase in eIF3K mRNA synthesis, quantitative reverse transcriptase PCR (Q-RT-PCR) can be performed on total RNA isolated from mammalian cells with stable overexpression or knockdown of PML. If eIF3K mRNA levels are constant between our cell lines with varying levels of PML, this

would suggest that PML does not up-regulate eIF3K protein expression by increasing the synthesis of its transcript.

Instead of increasing the transcription of eIF3K, another mechanism by which PML-I can elevate eIF3K protein expression is through enhanced translation of eIF3K mRNA. To test this hypothesis, we can perform sucrose gradient fractionation of cell lysates extracted from mammalian cells with varying levels of PML expression. This will enable us to isolate polyribosomes containing highly translated mRNAs. The abundance of eIF3K mRNA in these polyribosome fractions can then be determined by either Northern blotting or Q-RT-PCR. If eIF3K mRNAs are enriched in the polyribosome fraction isolated from PML-I overexpressing cells, this would suggest that PML-I is affecting the translational efficiency of eIF3K mRNAs.

4.5.3: DOES OVEREXPRESSION OF PML ENHANCE eIF3K mRNA EXPORT?

Previous studies have shown that overexpression of PML inhibits eIF4E-mediated export of a subset of mRNAs such as cyclin D1 and significantly decreases eIF4E association with the methylguanosine cap on the 5' end of the mRNA, thereby decreasing protein synthesis.[87-89, 126] Since we saw a concurrent increase in eIF3K protein levels when PML was overexpressed, this suggests that eIF4E may not play a role in mediating the nuclear export of eIF3K mRNA. Moreover, since overexpression of PML only inhibits nuclear export of a subset of mRNAs in an eIF4E-dependent manner, the same mechanism may not apply for eIF3K mRNA export. Therefore, to assess whether overexpression of PML

leads to an increased in the export of eIF3K mRNA, RNA *in situ* hybridization may be performed using RNA probes directed against the eIF3K mRNA in mammalian cell lines with stable overexpression of PML-I or knockdown of total PML protein expression. This technique relies on the use of a biotin-labeled mRNA probe that is specific for eIF3K. The biotin-labeled probe, once hybridized to eIF3K mRNA, can be detected by fluorescent labeled streptavidin. Therefore, the intracellular distribution of the eIF3K mRNA can be determined at high resolution by fluorescence microscopy. If PML is involved in promoting eIF3K mRNA export, this could certainly lead to an increase in eIF3K mRNA accumulation in the cytoplasm in PML-I overexpressing cells and an accumulation of eIF3K mRNA in the nucleus of cells in which PML has been knocked-down by RNA interference.

4.5.4: DOES OVEREXPRESSION OF PML REGULATE eIF3K PROTEIN STABILITY?

Lastly, to determine whether PML is involved in regulating eIF3K protein stability, wild-type and PML (-/-) MEF cells can be treated with cyclohexamide which is a drug that is commonly used to inhibit global translation. If PML is involved in regulating protein stability, we would expect to see an increase in eIF3K protein turnover in PML (-/-) MEF cells compared to wild-type. The “turnover” of a protein refers to its degradation, which proceeds via the ubiquitin-mediated proteasomal pathway. Subsequently, if we treat wild-type and PML (-/-) MEF cells with a 26S proteasome inhibitor such as MG132, and there is no change in eIF3K protein expression both in wild-type and PML (-/-) MEF cells,

this would suggest that PML may be involved in preventing the proteasomal degradation of eIF3K. This would support the notion that PML may be involved in regulating eIF3K protein stability by sequestering the protein in PML NBs, thus preventing its degradation via the proteasome.

4.6: CONCLUSIONS

There are multiple isoforms of the PML protein that heterodimerize and localize in the nucleus within multiprotein structures known as PML NBs. The functions of these PML isoforms and their contributions to cancer development are poorly understood. In this study we have identified eIF3K as a novel interacting protein of the largest and most abundant PML isoform, PML-I. As a subunit of the eIF3 complex, eIF3K is implicated in translation initiation. Overexpression of PML-I resulted in concurrent increase in eIF3K protein levels both in normal and cancerous mammalian cells, suggesting that PML-I may be involved in regulating eIF3K protein translation or stability. Ultimately, this may lead to altered translation initiation for either specific mRNAs or globally. This is an important finding, as PML protein expression is often lost in solid tumours from various tissues. Consequently, the protein levels of eIF3K may be negatively regulated in these cancer cells. Therefore, through this study we have shed new light on how PML isoform I, through its protein-protein interaction with eIF3K, may regulate translation in normal and malignant cells.

APPENDIX I
(PCR Cycle Profile)

Phusion PCR profile:
Initial denaturation: 95^{o}C for 5 minutes
29 cycles
Denaturation: 95^{o}C for 30 seconds
Annealing: 58^{o}C for 30 seconds
Extension: 72^{o}C for 45 seconds
Final extension: 72^{o}C for 10 minutes

Yeast colony PCR profile:
Initial denaturation: 95^{o}C for 5 minutes
30 cycles
Denaturation: 95^{o}C for 30 seconds
Annealing: 55^{o}C for 30 seconds
Extension: 72^{o}C for 1.5 minutes
Final extension: 72^{o}C for 10 minutes

APPENDIX II
(Human eIF3K protein sequences)

Human eukaryotic initiation factor K (eIF3K) isoform 1 protein sequence (GenBank Accession No. NM_013234). The amino acids underlined are not present in eIF3K isoform 2 (unpublished sequence). The sequences highlighted in green and red are possible PML-I interacting region:

MAMFEQMRANVGKLLKGIDRYNPENLATLERYVETQAKENAYDLEANLAVLKL
YQFNPAFFQTTVTAQILLKALTNLPHTDFTLCKCMIDQAHQEERPIRQILYLGDLL
ETCHFQAFWQALDENMDLLEGITGFEDSVRKFICHVVGITYQHIDRWLLAEMLG
DLSDSQLKVWMSKYGWSADESGQIFICSQEESIKPKNIVEKIDFDSVSSIMASSQ

Human eukaryotic initiation factor 3 subunit K (eIF3K) isoform 2 protein sequence:

MAMFEQMRANVGKLLKGIDRYQFNPAFFQTTVTAQILLKALTNLPHTDFTLCKC
MIDQAHQEERPIRQILYLGDLLETCHFQAFWQTLDENMDLLEGITGFEDSVRKFI
CHVVGITYQHIDRWLLAEMLGDLSDSQLKVWMSKYGWSADESGQIFICSQEESI
KPKNIVEKIDFDSVSSIMASSQ

The region of eIF3K identified as required for interaction with PML-I by yeast two-hybrid analysis:

MAMFEQMRANVGKLLKGIDRYNPENLATLERYVETQAKENAYDLEANLAVLKL
YQFNPAFFQTTVTAQILLKALTNLPHTDFTLCKCMIDQAHQEER

APPENDIX III
(Yeast solutions and media)

YPDA broth
20 g/L Glucose
20 g/L Bactopeptone
10 g/L Yeast extract
Sterilized by autoclave
Supplemented with 120 μg/mL adenine

Amino Acids:
Individual amino acids (100X)
2 mg/mL Adenine hemisulfate salt
2 mg/mL L-Tryptophan
2 mg/mL L-Histidine HCl monohydrate
10 mg/mL L-Leucine

10X dropout supplement
300 mg/L L-Isoleucine
1500 mg/L L-Valine
200 mg/L L-Arginine HCl
300 mg/L L-Lysine
200 mg/L L-Methionine
500 mg/L L-Phenylalanine
2000 mg/L L-Threonine
300 mg/L L-Tyrosine
200 mg/L Uracil

All amino acids were filter sterilized

YNB
20 g/L Glucose
6.7 g/L Yeast nitrogen base without amino acids
Sterilized by autoclave

Aureobasidin A stock concentration
Dissolved Aureobasidin A at 500 μg/mL in absolute ethanol

X-α-Gal stock concentration
Dissolved X-α-gal at 20 mg/mL in dimethylformamide

SD Leu- Plate
20 g/L Bacto-agar
20 g/L Glucose
6.7 g/L Yeast nitrogen base without amino acids
Sterilized by autoclave
Added (final concentration):
1X DO supplement
1X Adenine
1X Histidine
1X Tryptophan
(Note: other SD dropout plates are made using the same ingredients except with the required amino acids and/or nucleotides added)

SD Leu-Trp- + Auerobasidin A + X-α-gal
20 g/L Bacto-agar
20 g/L Glucose
6.7 g/L Yeast nitrogen base without amino acids
Sterilized by autoclave
Added (final concentration):
1X DO supplement
1X Histidine
1X Adenine
70 ng/mL of aureobasidin A
40 μg/mL X-alpha-Gal

5X M9 Salts:
64 g/L Na_2HPO_4
15 g/L KH_2PO_4
2.5 g/L NaCl
5.0 g/L NH_4Cl
Sterilized by autoclave.

M9 agar plate:
20 g/L Bacto-agar
1X M9 salts
2 mM $MgSO_4$
100 mm $CaCl_2$
0.4% glucose
1 mM Thiamine-HCl
1X SD-Leu
50 μg/mL Ampicillin

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