

CHARACTERIZATION OF THE INTERACTION OF ALPHA4 PHOSPHOPROTEIN
WITH NOVEL BINDING PARTNERS: EDD E3 UBIQUITIN LIGASE AND
POLY(A)-BINDING PROTEIN

by

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DALHOUSIE UNIVERSITY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

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ABSTRACT

$\alpha 4$ phosphoprotein (also known as IGBP1) is a component of the mammalian target-of-rapamycin (mTOR) pathway that controls the initiation of translation and cell-cycle progression in response to nutrients and growth factors. Aberrant signaling of the mTOR pathway has been reported in many cancers. $\alpha 4$ interacts with the catalytic subunit of protein phosphatase 2A (PP2Ac) to mediate the dephosphorylation of eukaryotic initiation factor 4E-binding protein1 (4E-BP1) and p70S6 kinase (p70S6K). Our laboratory has reported that EDD E3 ubiquitin ligase (EDD/UBR5) and poly(A)-binding protein (PABP) are novel binding partners of $\alpha 4$ phosphoprotein. In the present study, the interaction of EDD and PABP with $\alpha 4$ was confirmed in human MCF-7 breast cancer and African green monkey COS-1 kidney cell lines, using immunoprecipitation and immunoblotting (IP/IB) analysis. However, co-IP of total MCF-7 cell lysates with anti-EDD antibodies revealed that EDD does not physically interact with PP2Ac. Several $\alpha 4$ deletion constructs, that contained either the N-terminal or C-terminal regions of $\alpha 4$, were transfected into MCF-7 and COS-1 cells. Co-IP studies with anti-EDD and PABP antibodies revealed that EDD interacts with the C-terminal region of $\alpha 4$ whereas PABP, like PP2Ac, binds to the N-terminal region. EDD and PABP were found to interact with $\alpha 4$ in both quiescent and actively growing cells. EDD is known to ubiquitinate poly(A)-binding protein-interacting protein 2 (Paip2), targeting it for proteosomal degradation. Paip2 is an antagonist of PABP activity. When $\alpha 4$ levels in MCF-7 cells were knocked down using small interfering RNA (siRNA), there was no effect on EDD protein levels. There was also no effect on Paip2 levels, indicating that $\alpha 4$ is not involved in the EDD-mediated ubiquitination of Paip2. Knockdown of EDD gene expression by siRNA did not alter mono-ubiquitination of $\alpha 4$, indicating that $\alpha 4$ is not a substrate of EDD. However, knockdown of EDD gene expression decreased poly-ubiquitination of PP2Ac and increased the overall cellular levels of PP2Ac, suggesting PP2Ac as a novel substrate of EDD. The present study suggests a potential role for $\alpha 4$ in PABP-mediated initiation of mRNA translation. Furthermore, this study suggests a role for EDD in regulating PP2Ac levels through its interaction with $\alpha 4$. In summary, the $\alpha 4$ partners EDD, PABP and PP2Ac interact at specific regions of $\alpha 4$. PP2Ac, but not $\alpha 4$, is a substrate of EDD. The interaction of PABP with $\alpha 4$ suggests a potential role for $\alpha 4$ in PABP-mediated initiation of mRNA translation.

LIST OF ABBREVIATIONS AND SYMBOLS USED

4E-BP1	eukaryotic initiation factor 4E binding protein 1
Akt	Akt kinase
AMPK	adenosine monophosphate activated protein kinase
APS	ammonium persulfate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
cDNA	complementary deoxyribonucleic acid
DMEM	Dulbecco's modified eagle's medium
EDD	E3 ubiquitin ligase discovered by differential display
eIF3	eukaryotic initiation factor 3
eIF4A	eukaryotic initiation factor 4A
eIF4E	eukaryotic initiation factor 4E
eIF4G	eukaryotic initiation factor 4G
ERK	extracellular signal-related kinase
FBS	fetal bovine serum
GβL	G-protein β-subunit-like protein
HEAT	Huntington, elongation, A subunit, TOR
HECT	homologous to the E6-AP C-terminus
HRP	horse-radish peroxidase
IgG	immunoglobulin G
IP	immunoprecipitation
MEK3	MAPK/ERK 3
MID1	Midline 1
MMLV-RT	Maloney murine leukemic virus reverse transcriptase
mSin1	mammalian stress-activated protein kinase-interacting protein 1
mRNA	messenger ribonucleic acid
mTOR	mammalian target-of-rapamycin
mTORC1	mammalian target-of-rapamycin complex 1
mTORC2	mammalian target-of-rapamycin complex 2
p70S6K	p70S6 kinase
PABP	poly(A) binding protein
PAGE	polyacrylamide gel electrophoresis
Paip1	poly(A) interacting protein 1
Paip2	poly(A) interacting protein 2
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PKD1	phosphoinositide kinase 1

PI3K	phosphoinositide 3 kinase
PIKK	phosphoinositide 3 kinase related kinases
PIP ₂	phosphatidylinositol-4,5-bisphosphate
PIP ₃	phosphatidylinositol-3,4,5-trisphosphate
PMSF	phenylmethylsulfonyl fluoride
PP2A	protein phosphatase 2A
PP2Ac	protein phosphatase 2A catalytic subunit
PP4	protein phosphatase 4
PP6	protein phosphatase 6
PRAS 40	proline-rich Akt substrate 40
PRR5	proline rich protein 5
PTEN	phosphatase and tensin homolog
Raptor	regulatory subunit associated protein of mTOR
Rheb	Ras-homology enriched in the brain
Rictor	rapamycin insensitive companion of mTOR
RING	really interesting new gene
RT	reverse transcriptase
RTK	receptor tyrosine kinase
SDS	sodium dodecylsulfate
siRNA	small interfering ribonucleic acid
TEMED	tetramethylethylenediamine
TOP	terminal oligopyrimidine-tract
TSC1	tuberous sclerosis complex 1
TSC2	tuberous sclerosis complex 2
TTBS	Tween-20 tris buffered saline solution
UBR1	ubiquitin-protein ligase E3 component n-recognin 1
UIM	ubiquitin interacting motif

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CHAPTER 1

Introduction

1.1 Mammalian Target-of-Rapamycin (mTOR) Pathway

1.1.1 The mTOR complexes

The yeast proteins Tor1 and Tor2 were originally identified in yeast as targets of the immunosuppressive agent rapamycin, a macrocyclic antibiotic with antifungal properties (Sehgal *et al.* 1975). The mammalian homologue of TOR (mTOR) was later identified as a 298-kDa protein which interacted with a complex formed between cytoplasmic receptor FKBP12 and rapamycin, and which increased G1-phase progression in mammalian cells (Siekierka *et al.* 1989). Further study showed that mTOR controls the initiation of translation, ribosome biosynthesis, and gene transcription in response to nutrients and growth factors (Gingras *et al.* 2001). Although predominately nuclear, the mTOR protein undergoes nuclear-cytoplasmic shuttling to elicit a signalling response (Kim and Chen 2000). mTOR itself, acts as two independent complexes, mTORC1 and mTORC2, which exhibit different cellular functions. mTORC1, the rapamycin sensitive complex consists of mTOR, Raptor (regulatory associated protein of mTOR), Proline-Rich Akt Substrate 40 kDa (PRAS 40) and G-protein β -subunit-like protein (G β L). This complex is responsible for the phosphorylation of downstream targets of mTOR, namely, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and S6 kinase (p70S6K) (Hara *et al.* 2002; Kim *et al.* 2003) (**Figure 1**). mTORC2, which is rapamycin insensitive, consists of mTOR, Rictor (rapamycin insensitive companion of mTOR),

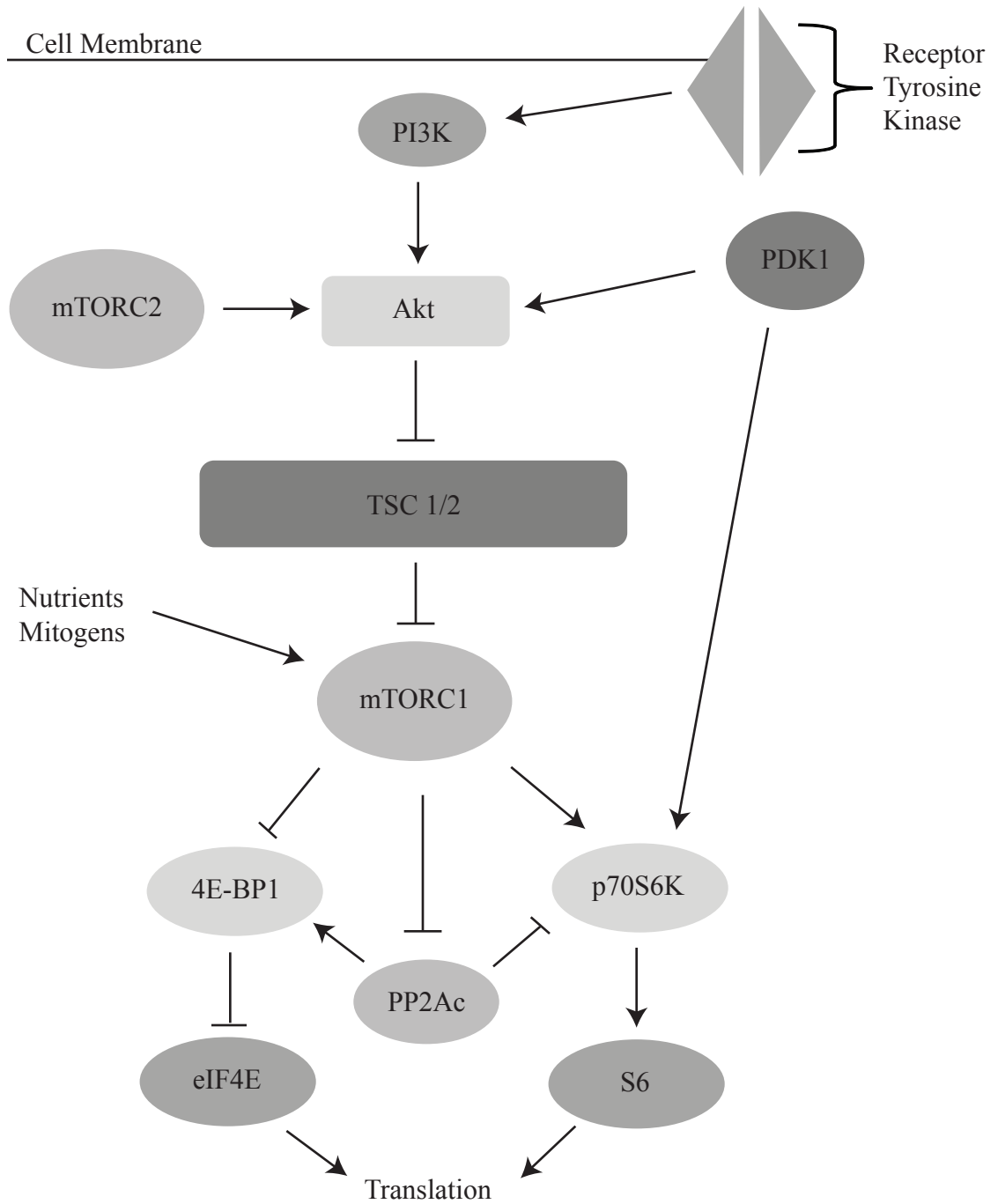


Figure 1. Mammalian Target of Rapamycin Pathway

The mTOR pathway responds to the presence of mitogens and nutrients to stimulate translation and cell cycle progression, through the downstream phosphorylation of 4E-BP1 and p70S6K (see text).

proline-rich p-protein (PRR5), protein observed with mTOR (protor), and mammalian stress-activated protein kinase-interacting protein 1 (mSin1). mTORC2 activates Akt kinase (Akt) by phosphorylating it on Ser473, and is involved in the regulation of the actin cytoskeleton through its association with other proteins, including Rho and Rac1 (Alessi *et al.* 1996; Jacinto *et al.* 2004; Jacinto *et al.* 2006). Despite being termed rapamycin insensitive, mTORC2 activity has recently been shown to respond to prolonged rapamycin treatment (Sarbasov *et al.* 2006).

1.1.2 Pathways of mTORC1 Regulation

Growth factors binding to receptor tyrosine kinases (RTKs) in the cell membrane lead to activation of phosphoinositide 3 kinase (PI3K) which, in turn, catalyzes the conversion of phosphatidylinositol (4,5)-bisphosphate (PIP₂) to phosphatidylinositol-3, 4, 5-trisphosphate (PIP₃). The increased level of PIP₃ leads to activation of Akt through binding to the pleckstrin homology (PH) domain. Activation of Akt also requires Ser/Thr phosphorylation by PDK1 and mTORC2 (Sarbasov *et al.* 2005) (**Figure 1**).

Activated Akt phosphorylates tuberous sclerosis complex 2 (TSC2) on multiple binding sites (Inoki *et al.* 2002). As a complex, TSC1/TSC2 catalyzes the conversion of a guanine nucleotide triphosphatase (GTPase), Ras-homology enriched in the brain (Rheb), from its GTP bound state to its GDP bound state (Garami *et al.* 2003). This hydrolysis of Rheb-GTP acts to inhibit mTOR, as Rheb-GTP binds directly to the C-terminal domain of FKBP38, the endogenous inhibitor of mTOR. Binding of Rheb-GTP to FKBP38 prevents the inhibition of mTOR activity through the dissociation of FKBP38 from mTOR. The TSC1/2 complex is inhibited by Akt phosphorylation, as well as, by

phosphorylation from other pathways, including adenosine monophosphate-activated protein kinase (AMPK), extracellular signal-regulated kinase (ERK), RSK, and glycogen synthase kinase 3 β (GSK3 β) (Inoki *et al.* 2006). Due to its ability to be phosphorylated through multiple pathways, the TSC1/2 complex acts as hub through which multiple pathways can activate the mTOR kinase (**Figure 1**).

1.1.3 Molecular and Cellular Targets of mTORC1

As a member of the phosphoinositide 3-kinase related kinases (PIKK), mTOR functions as a serine/threonine kinase to phosphorylate its downstream targets (Brown *et al.* 1995). The two main targets for mTOR phosphorylation are translational repressor 4E-BP1 and p70S6K. 4E-BP1, in its hypophosphorylated state, competes with the eukaryotic initiation factor 4G (eIF4G) for binding to eIF4E. mTOR targets 4E-BP1 for phosphorylation at several serine and threonine residues, leading to its dissociation from eIF4E, thereby allowing eIF4E to form a complex with eIF4G (Pause *et al.* 1994). eIF4G acts as a scaffold for the association of other initiation factors, including the RNA helicase eIF4A. The association of eIF4E with eIF4G also allows for interaction of eIF3, which recruits the 40S ribosome to the 5' end of mRNA. Formation of this complex leads to an increase in the translation of 5'-cap mRNAs (Marcotrigiano *et al.* 1999)(**Figure 2**).

mTOR phosphorylates p70S6K on Thr389 within its hydrophobic region. This creates a docking site for phosphoinositide kinase 1 (PDK1) allowing it to phosphorylate p70S6K on Thr229 leading to the activation of p70S6K (Saitoh *et al.* 2002) (Figure 1). S6K phosphorylates the 40S ribosomal protein S6 to promote the translation of 5' terminal oligopyrimidine-tract (5'-TOP) mRNAs. Recent studies have shown that in its

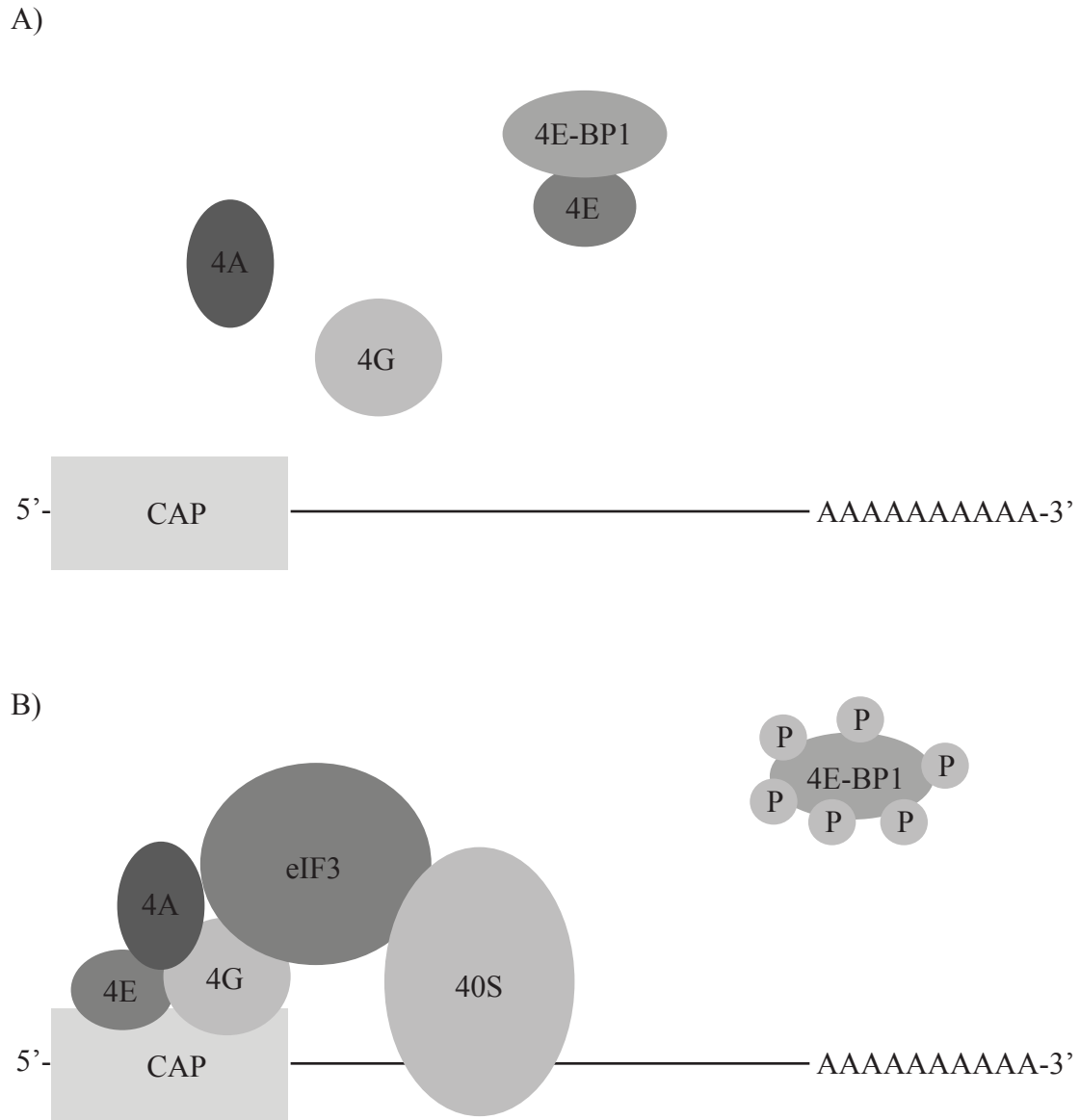


Figure 2. Formation of the 5'-cap Complex

A) Unphosphorylated 4E-BP1 binds to eIF4E preventing its interaction with the 5'-cap. B) Phosphorylation of 4E-BP1 by mTORC1 induces its dissociation from eIF4E. Released eIF4E can associate with eIF4A, eIF4G, and eIF3 at the 5'-cap. eIF3 recruits the 40S ribosome to the 5'-cap. Formation of this complex promotes translation of 5'-cap mRNAs (see text).

hypophosphorylated state S6K1 associates with eIF3 (Holz *et al.* 2005). Activation of p70S6K by mTOR and PDK1 leads to its dissociation from eIF3, allowing it to phosphorylate its targets. Rapamycin treatment and subsequent inactivation of the TOR complex leads to dephosphorylation of Thr389 and Thr229 within S6K (Choo *et al.* 2008).

mTOR also targets protein phosphatase 2A (PP2A) for phosphorylation, thereby inactivating it and decreasing its phosphatase activity. Rapamycin treatment of mammalian cells has been shown to decrease PP2A phosphorylation and to increase its activity (Peterson *et al.* 1999).

1.1.4 mTOR in Tumourigenesis

Due to the large number of oncogenes and tumour suppressors that lie upstream of mTOR, activation of the mTOR pathway has been implicated in many types of cancer, including breast (Pang and Faber 2001), pancreatic (Grewe *et al.* 1999), prostate (van der Poel *et al.* 2003), ovarian and lymphomas (Muthukkumar *et al.* 1995). These cancers show increased phosphorylation of 4E-BP1 or S6K leading to an increased cell cycle progression. Mutations in TSC1/2 can lead to development TSC syndrome, which results in tumours in multiple organs. In addition, the tumour suppressor, LKB1 serine/threonine kinase, activates AMPK, which phosphorylates TSC1/2 to enhance its GTPase activating protein (GAP) activity and leads to decreases in mTOR kinase activity (Shaw *et al.* 2004). AMPK also directly inhibits mTOR kinase activity through phosphorylation of Raptor. In lung cancer as well as Peutz-Jaghers syndrome, which is characterized by colon polyps, LKB1 mutations increase mTOR kinase by inactivation of AMPK and

TSC1/2. The resulting decrease in AMPK activation limits mTOR sensitivity to energy stress (Shaw *et al.* 2004).

Phosphatase and tensin homolog (PTEN) inhibits the PI3K activation of Akt (Stambolic *et al.* 1998). Loss-of-function mutations in PTEN lead to aberrant Akt activity and decreased activity of TSC1/2 (Manning *et al.* 2002). Cowden's syndrome, caused by dominant mutations in the PTEN gene and characterized by increased risk of breast, colorectal and other cancers, leads to Akt activation independent of growth factors as well as an increase in mTORC1 activity (Neshat *et al.* 2001).

Currently, rapamycin is being tested as a cancer therapeutic for its ability to inhibit aberrant mTORC1 activity in cases where TSC1/2 or PTEN function is altered (Rachdi *et al.* 2008; Squarize *et al.* 2008). Despite initial decreases in tumour size following rapamycin treatment, these tumours regain their original size when rapamycin treatment is halted (Franz *et al.* 2006). Therefore, fully understanding the complexity of the mTOR signalling pathway and delineating the activities of all effectors of the signalling network are important to the development of therapies to treat aberrant mTOR signalling in cancer.

1.2 Protein Phosphatase 2A

1.2.1 Structure of PP2A Holoenzyme

PP2A is a member of a subfamily of serine/threonine phosphatases that includes protein phosphatase 4 (PP4) and protein phosphatase 6 (PP6). The PP2A holoenzyme exists as a core dimer made up of the 36-kDa catalytic subunit of PP2A (PP2Ac), or C-

subunit, and the 65-kDa structural subunit PR65, or A-subunit. The holoenzyme also consist of a regulatory B-subunit, which is comprised of 4 families, B, B', B'', and B''' (Janssens and Goris 2001) (**Figure 3**). The currently proposed models for the assembly of the PP2A holoenzyme suggest that the core dimer of the A- and C-subunits is formed prior to recruitment of any one of the regulatory B-subunits. Binding of the regulatory subunits determines the target substrate for catalytic activity as well as the subcellular localization of the holoenzyme (Xu *et al.* 2006).

1.2.2 PP2A A and B Subunits

The A-subunit of PP2A consists of 15 tandem repeats of a 39 amino acid HEAT domain (Huntington, elongation, A subunit, TOR), with each repeat being composed of two superimposed α helices (Groves *et al.* 1999). The successive α -helix structure forms an elongated hook that acts as a scaffold for the complete holoenzyme by tightly associating to PP2Ac, and as a docking site for the B- subunits to interact with the holoenzyme. The docking sites for each B-subunit have been shown to bind to overlapping sites, making the interaction between the A-subunits and specific B-subunits mutually exclusive (Prickett and Brautigan 2004).

The B subunit of PP2A exists in a wide variety of isoforms, which are separated into 4 families based on the molecular weight of the proteins. Each member of particular B-subunit family may exhibit different tissue specificity as well as cellular localization, leading to a large variety of cellular targets for PP2Ac activity. For instance, PR61 α , PR61 β , and PR61 ϵ of the B' family localize to the cytoplasm, whereas PR61 γ , PR61 γ , PR61 γ are found specifically in the nucleus (McCright *et al.* 1996). The B-subunits of

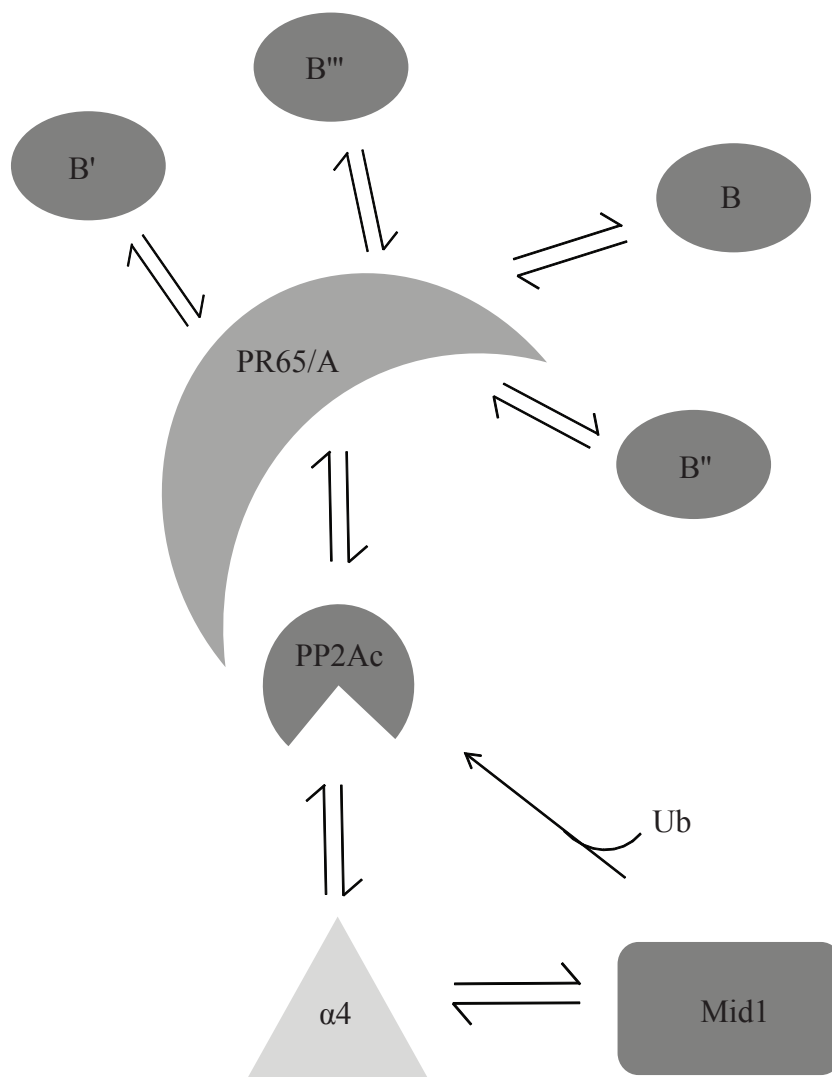


Figure 3. Structure of PP2A Heterotrimer

The PP2A heterotrimer consists of a structural A-subunit (PR65/A), a regulatory B-subunit (B, B', B'', or B'''), and a catalytic C-subunit (PP2Ac). $\alpha 4$ phosphoprotein interacts with PP2Ac and mediates phosphatase activity. The Opitz Syndrome associated Mid1 ubiquitinates PP2Ac and targets it for proteasomal regulation. $\alpha 4$ phosphoprotein acts as an adaptor protein to mediate the binding of PP2Ac and Mid1 (modified from Janssens et al. 2001).

PP2A can also differ in their post-translational modifications as some, but not all, members of the B' family can be phosphorylated. *In vitro* studies reveal that phosphorylation of PR61 δ alter the substrate specificity of PP2A without affecting the composition of the heterotrimer (Usui *et al.* 1998).

1.2.3 PP2A Catalytic Subunit

Mammalian PP2Ac exists in 2 isoforms, α and β , which are ubiquitously expressed and, while they maintain a high level of sequence identity, do not exhibit redundant functionality within the cell (Stone *et al.* 1987). The phosphatase activity of PP2Ac has been shown to target proteins involved in a wide variety of cellular processes, including G2/M-phase cell cycle transition, apoptosis, and translation termination (Picard *et al.* 1989; Andjelkovic *et al.* 1996; Deng *et al.* 1998). Reversible phosphorylation is important in controlling protein activation in signalling cascades, hence, PP2Ac and its regulation are critical for proper cellular function.

PP2Ac activity has also been implicated in translational control of the mTOR pathway. Our laboratory has previously demonstrated formation of a complex between PP2Ac and S6K in NB2 lymphoma cells (Bishop *et al.* 2006). In Jurkat T leukemic cells, PP2Ac immunoprecipitates with S6K, and *in vitro* studies showed that dephosphorylation of S6K can be blocked by the addition of inhibitors specific for the PP2A subfamily of phosphatases (Peterson *et al.* 1999). Our laboratory has also shown an interaction between PP2Ac and 4E-BP1 in Nb2 lymphoma cells (Bishop *et al.* 2006). In addition, *in vitro* studies revealed that PP2A could dephosphorylate 4E-BP1 that was phosphorylated by mTOR (Nanahoshi *et al.* 1998). Inactivation of PP2Ac by okadaic acid leads to the

hyperphosphorylation of tumour suppressor p53, implicating the phosphatase activity of PP2Ac in the dephosphorylation of the p53 tumour suppressor (Yatsunami *et al.* 1993).

Similar to the B-subunit, PP2Ac also undergoes post-translational modification, which affects its catalytic function. Post-translational phosphorylation of PP2Ac occurs on Tyr307 of the conserved C-terminal region, leading to inactivation of the enzyme (Chen *et al.* 1992). Phospho-Tyr307 dependent inhibition of PP2Ac may arise from inhibition of binding between PP2Ac and the B-subunit. In addition to phosphorylation on Tyr307, PP2Ac can also be phosphorylated on threonine residues, leading to the inactivation of PP2Ac phosphatase activity towards phosphoserine/threonine residues. Interestingly, PP2Ac can auto-activate itself by dephosphorylating these threonine residues. Post-translational methylation of PP2Ac also occurs within the conserved C-terminal domain (Lee and Stock 1993). Methylation of Leu309 has been shown to vary during cell cycle progression. Transient decreases in PP2Ac methylation have been observed during the transition between G₀ and G₁ phases as well as between G₁ and S phases of the cell cycle (Turowski *et al.* 1995). In addition to the effects on the cell cycle, methylation of the C-terminal Leu309 is necessary for binding of certain B-subunits, including some members of the B' and B'' families (Bryant *et al.* 1999).

A fourth member of the PP2A holoenzyme has been identified as $\alpha 4$ phosphoprotein. $\alpha 4$ is the mammalian homologue of the yeast Tap42, which interacts with PP2Ac independently of the A and B subunits (Inui *et al.* 1995).

1.3 $\alpha 4$ Phosphoprotein

1.3.1 PP2Ac Interaction

The $\alpha 4$ cDNA was first cloned from murine bone marrow and was originally identified as a component of immunoglobulin receptor-mediated signal transduction (Inui *et al.* 1995). Later, studies revealed the interaction of $\alpha 4$ with PP2Ac as well as with the catalytic subunits of protein phosphatases 4 and 6 (PP4 and PP6), which belong to the same family of protein phosphatases (Murata *et al.* 1997; Chen *et al.* 1998). Studies involving the interaction of $\alpha 4$ with PP2Ac, have shown $\alpha 4$ as an essential gene product that plays a role in the targeting of PP2A to substrates (Di Como and Jiang 2006). The interaction between PP2Ac and $\alpha 4$ may produce opposing effects. For example, PP2Ac-dependent dephosphorylation of 4E-BP1 was shown to be inhibited by the formation of the PP2Ac/ $\alpha 4$ complex. In contrast, $\alpha 4$ acts as an essential regulator of apoptosis in murine cells through its ability to promote the PP2Ac-dependent dephosphorylation of p53 and c-Jun (Kong *et al.* 2004). Early studies implicating a role for $\alpha 4$ in the stability PP2Ac, show that $\alpha 4$ knockout mice have drastically decreased levels of PP2A (Kong *et al.* 2009). Knockout of $\alpha 4$ also decreases the stability of PP4 and PP6 (Kong *et al.* 2009). Mouse embryonic fibroblasts with $\alpha 4$ deletion contain altered morphology compared to control cells. In contrast, overexpression of $\alpha 4$ leads to an increase in cell spreading and motility (Kong *et al.* 2007). $\alpha 4$ overexpression was found to increase the GTP-bound state of Rac-1, which controls actin polymerization and is essential for cell migration. It was proposed that $\alpha 4$ affects the levels of GTP bound Rac-1 levels through an mTORC2-mediated fashion (Kong *et al.* 2007).

$\alpha 4$ exists as a highly α -helical protein that resembles 14-3-3 proteins that act as scaffolding and adapter proteins. The interaction between $\alpha 4$ and PP2Ac occurs at the N-terminal region of $\alpha 4$ which maintains a high level of structural ordering. In contrast, the C-terminal region of $\alpha 4$ has been shown to lack a stable secondary structure and acts as a docking site for proteins that undergo dephosphorylation (Yang *et al.* 2007). The proposed model for $\alpha 4$ as a modifier of PP2Ac activity suggests that it acts as a scaffold, recruiting to its C-terminus various proteins, which can then undergo PP2Ac-, PP4-, or PP6-dependent dephosphorylation. As an example, MAP2K3 interacts with the C-terminal region of $\alpha 4$, and this interaction is critical for the PP2Ac-dependent dephosphorylation of MAP2K3 (Prickett and Brautigan 2007). Interestingly, the binding site of $\alpha 4$ and A/PR65 overlap at the N-terminus of PP2Ac and formation of the $\alpha 4$ /PP2Ac complex has been shown to be independent of both the A and B subunits of PP2A. H118N mutations at the N-terminus of PP2AC have been shown to disrupt binding of A/PR65, but have no effect on binding of $\alpha 4$ (Lizotte *et al.* 2008).

1.3.2 Novel Binding Partners of $\alpha 4$

Recently, the Mid1 ubiquitin ligase, a mutated gene in Opitz Syndrome, has been identified as a binding partner for $\alpha 4$ (Troddenbacher *et al.* 2001). Mid1 poly-ubiquitinates PP2Ac, through its interaction with a $\alpha 4$, and targets it for degradation by the proteasome. The $\alpha 4$ protein contains a ubiquitin interacting motif (UIM) towards its N-terminus, which is distinct from the region which binds to PP2Ac (McConnell *et al.* 2010). UIMs act as links between ubiquitin ligases and their targets (Wooten *et al.* 2005). Deletion of the UIM domain within $\alpha 4$ leads to a decrease in PP2A poly-

ubiquitination, but has no effect on the interaction between PP2Ac and $\alpha 4$ (McConnell *et al.* 2010). Mutation of *Mid1* has been proposed to lead to an increase in PP2Ac levels and increased dephosphorylation of microtubule associated proteins. This model suggests that the increased dephosphorylation of microtubule-bound proteins leads to the disruption of microtubules found in Opitz Syndrome (Troddenbacher *et al.* 2001).

Previously, our laboratory reported that when rat Nb2 lymphoma cells were pulsed with [32 P]-orthophosphate in the presence of phorbol esters and prolactin, a number of unidentified phosphoproteins formed an immunocomplex with $\alpha 4$ (Boudreau *et al.* 2002). To determine the identity of these proteins a yeast 2-hybrid analysis was performed using $\alpha 4$ as bait to screen a human K562 erythroleukemia library. The result of this study revealed E3 Ubiquitin Ligase Discovered by Differential Display (EDD/UBR5) as a novel binding partner of $\alpha 4$ (McDonald *et al.* 2010). These results were confirmed by using anti- $\alpha 4$ antibodies to immunoprecipitate EDD in Nb2-cell lysates. Thus, EDD is the second E3 ubiquitin ligase shown to interact with $\alpha 4$ (McDonald *et al.* 2010)

1.4 E3 Ubiquitin Ligase Discovered by Differential Display (EDD/UBR5)

Originally isolated as a progesterone upregulated gene in human T47-D breast cancer cells, EDD is the 300 kDa human ortholog of the *Drosophila melanogaster* protein HYD which regulates cell proliferation during development (Mansfield *et al.* 1994; Callaghan *et al.* 1998). Mutations in the *hyd* gene have been shown to result in hyperplasia suggesting a role for EDD as a tumour suppressor. In addition EDD has been shown to be overexpressed in ovarian and breast cancers and is frequently mutated in colorectal and gastric cancers, suggesting a role in cancer progression (Mori *et al.* 2002;

Clancy *et al.* 2003). EDD has also been implicated as a key component in the DNA-damage response pathway that is activated by double-stranded breaks in DNA, through its interaction with CHK2 kinase and DNA topoisomerase II-binding protein (Honda *et al.* 2002; Henderson *et al.* 2006). Depletion of EDD leads to failure of cells to properly regulate the cell cycle in response to DNA-damage. EDD is also involved in the WNT signalling pathway that controls various processes during development (Ohshima *et al.* 2007). Hyperactivation of the WNT pathway has been linked to the development of colorectal cancer (Korinek *et al.* 1997; Morin *et al.* 1997). Control of the pathway is exhibited by regulation of β -catenin levels. β -catenin translocates to the nucleus and increases transcription of genes such as cyclin D1. EDD targets β -catenin for poly-ubiquitination. The poly-ubiquitination of two lysine residues by EDD leads to enhanced stability and nuclear localization of β -catenin (Hay-Koren *et al.* 2011). EDD has also been shown to interact with MAPK1, which is involved in multiple signalling cascades, and the de-ubiquitinase USP49, implicating a role for EDD in a variety of cellular processes (Eblen *et al.* 2003; Sowa *et al.* 2009).

EDD is a large 300-kDa protein and it belongs to the family of ubiquitin ligases which contain HECT (homologous to E6-AP carboxyl terminus) domains (Callaghan *et al.* 1998) (**Figure 4**). EDD has a C-terminal HECT domain that reversibly binds ubiquitin on a cysteine residue found within the HECT domain to form an E3-ubiquitin intermediate, and it then catalyzes the transfer of ubiquitin lysine residues within the substrate (see figure 3). In addition to the HECT domain, EDD has 3 other domains: a putative zinc finger domain that was originally discovered in the E3 ubiquitin ligase UBR1, a really interesting new gene (RING) domain found in some E3-ubiquitin ligase,

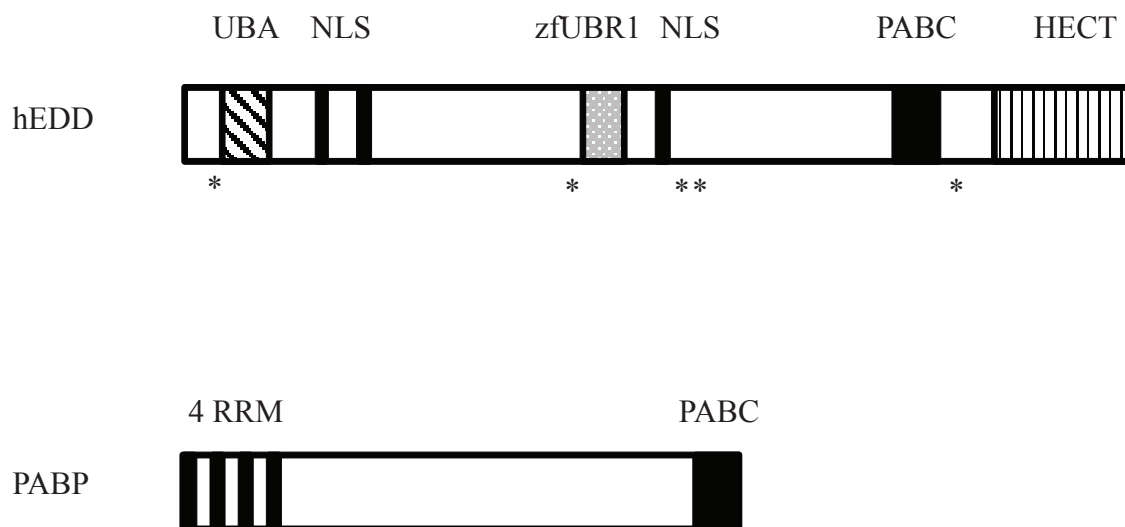


Figure 4. EDD and PABP domain structure.

EDD domain structure: UBA domain which binds to mono/poly-ubiquitin chains; zfUBR1 is a RING like zinc finger found in UBR1 ubiquitin ligase; HECT domain exhibits ubiquitin ligase activity; LXXL (*) is a potential steroid binding site; NLS are putative nuclear localization signals. PABP domain structure: the 4 RRMs recognizes the poly (A) tail of mRNA; PABC is the PABP C-terminal domain and is also found in EDD. (Henderson et al. 2002; Mangus et al. 2003)

thus, EDD may have both HECT and RING E3 function, and a UBA domain (Henderson *et al.* 2002)(**Figure 4**). In contrast to HECT E3 ubiquitin ligases, RING domain E3 ubiquitin ligases catalyze the direct transfer from the E2 ubiquitin conjugating enzyme to the target lysine residue. The putative zinc finger domain of EDD has been suggested to play a role in protein-protein interactions between EDD and substrates as it is distinct from the HECT domain within EDD (Henderson *et al.* 2002). The UBA is found at the N-terminal end of EDD. The UBA domain interacts with both poly- and mono- ubiquitin and may be responsible for the protein-protein interactions of EDD (Kozlov *et al.* 2007).

1.5 Poly(A) Binding Protein (PABP)

PABP is a conserved acidic protein that acts as a scaffold for the ribonucleic acid protein complex at the poly(A)-tail of mRNA (Mangus *et al.* 2003). PABP isoforms are localized within the nucleus and cytosol of the cell, and they have a variety of functions. Nuclear PABP is involved in polyadenylation of newly synthesized mRNA and is also thought to be required for export of the mRNA from the nucleus (Keller *et al.* 2000). The binding of PABP to the poly(A) tail leads to recruitment of translation factors, primarily the eukaryotic initiation factor 4G (eIF4G) which binds to the 5' cap of mRNAs. In the currently proposed model, it is suggested that circularization of the mRNA stimulates translation initiation (Tarun *et al.* 1997; Wells *et al.* 1998) (**Figure 5**).

The N-terminal region of PABP contains four conserved RNA recognition motifs (RRM) which are required for binding to the poly(A) tail of mRNA (Sachs *et al.* 1987). At the C-terminal region of PABP there is the C-terminal domain of poly(A) binding

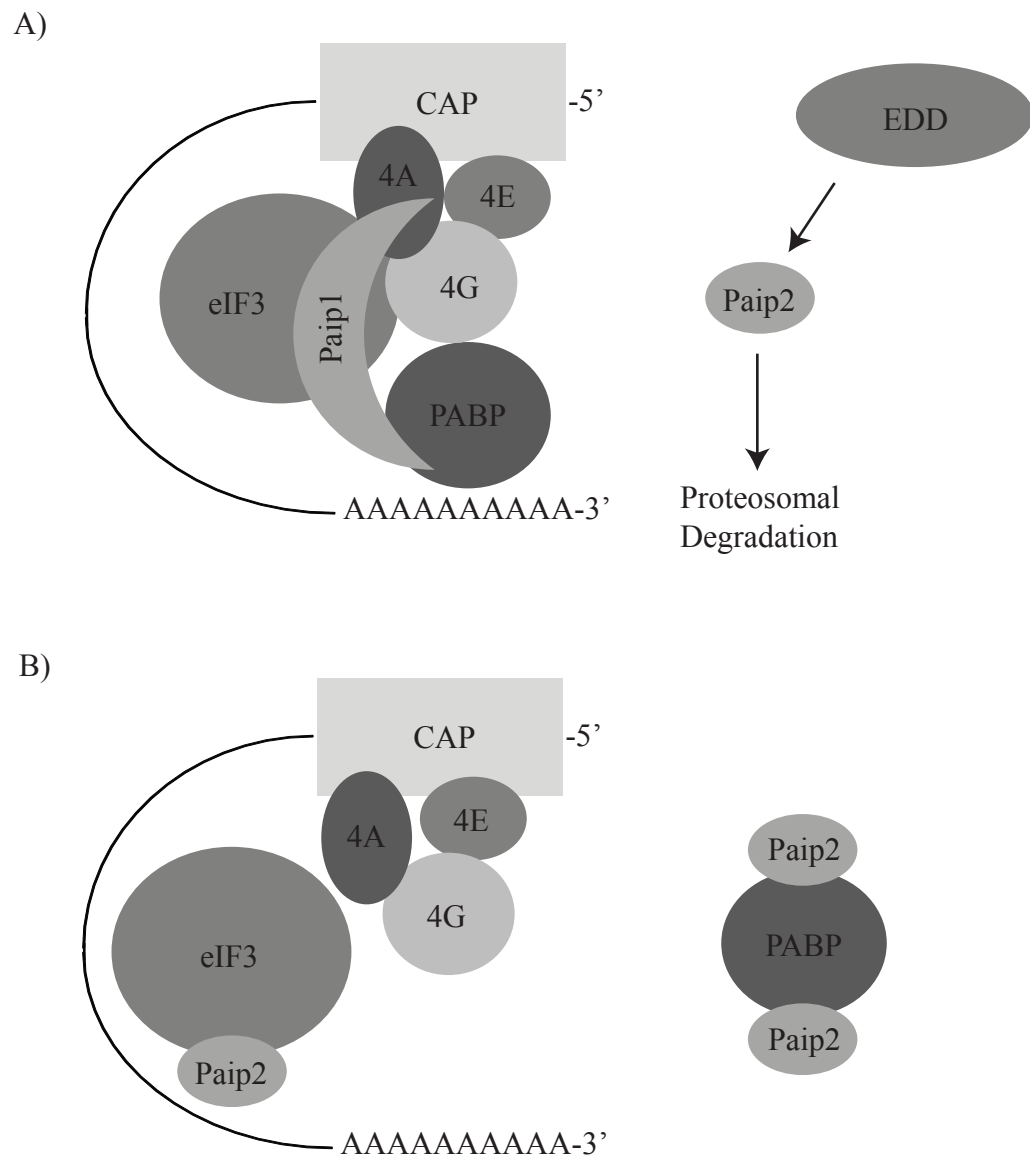


Figure 5. PABP Circularization of mRNA

A) Translation: PABP binds to the poly(A) tail of mRNA and eIF4G at the 5' CAP. Paip2 is polyubiquitinated by EDD and is targeted for proteosomal degradation. Paip1 stabilizes circularization by interacting with PABP and eIF3. B) Translation Inhibition: Paip2 binds to PABP reducing the interaction between PABP and the poly(A) tail of mRNA. Paip2 also reduces the interaction of Paip1 with eIF3 and PABP (modified from Derry et al. 2006)

protein (PABC) which recruits various proteins that affect the function of PABP. A PABC domain has also been identified in EDD (Henderson *et al.* 2002).

The interaction between PABP and the 5' cap is primarily regulated by two proteins, designated Paip1 and Paip2. Each of these proteins contains a PAM1 domain, a 25 amino acid acidic region that binds to the RRM2 domain of PABP (Roy *et al.* 2002), and a highly conserved 15 amino acid PAM2 domain which interacts with the PABC domain of PABP (Kozlov *et al.* 2001; Derry *et al.* 2006) (**Figure 6**). Under conditions favourable for translation, Paip1 acts to stabilize the circular mRNA by binding to PABP as well as the eukaryotic initiation factor 3 (eIF3) (**Figure 5**). Direct interaction of eIF4G at the 5' CAP of the mRNA and PABP at the 3'tail was confirmed in yeast and mammalian systems (Tarun *et al.* 1997; Kessler and Sachs 1998). In contrast, Paip2 destabilizes the circular mRNA through its interaction with the PABP RRM domains, preventing its interaction with the poly(A)-tail of the mRNA (Yoshida *et al.* 2006). Paip2 inhibition of PABP is regulated by degradation of Paip2 by the proteasome-ubiquitin pathway. EDD interacts with the PAM2 site of Paip2 at its PABC domain and targets Paip2 for degradation through polyubiquitination. Paip2 is protected from EDD degradation through its interaction with PABP, as the interaction between PABP and Paip2 is stronger than the interaction between EDD and Paip2 (Deo *et al.* 2001). Although Paip1 also contains the PAM2 site, it is not degraded by EDD, indicating that the PAM2 site may not be sufficient for EDD binding and that another factor may be required to elicit this response (Roy *et al.* 2002).

Based on the presence of PABC domain within EDD and PABP, our laboratory proposed that EDD and PABP may both be a binding partner of $\alpha 4$. Immunoprecipitation

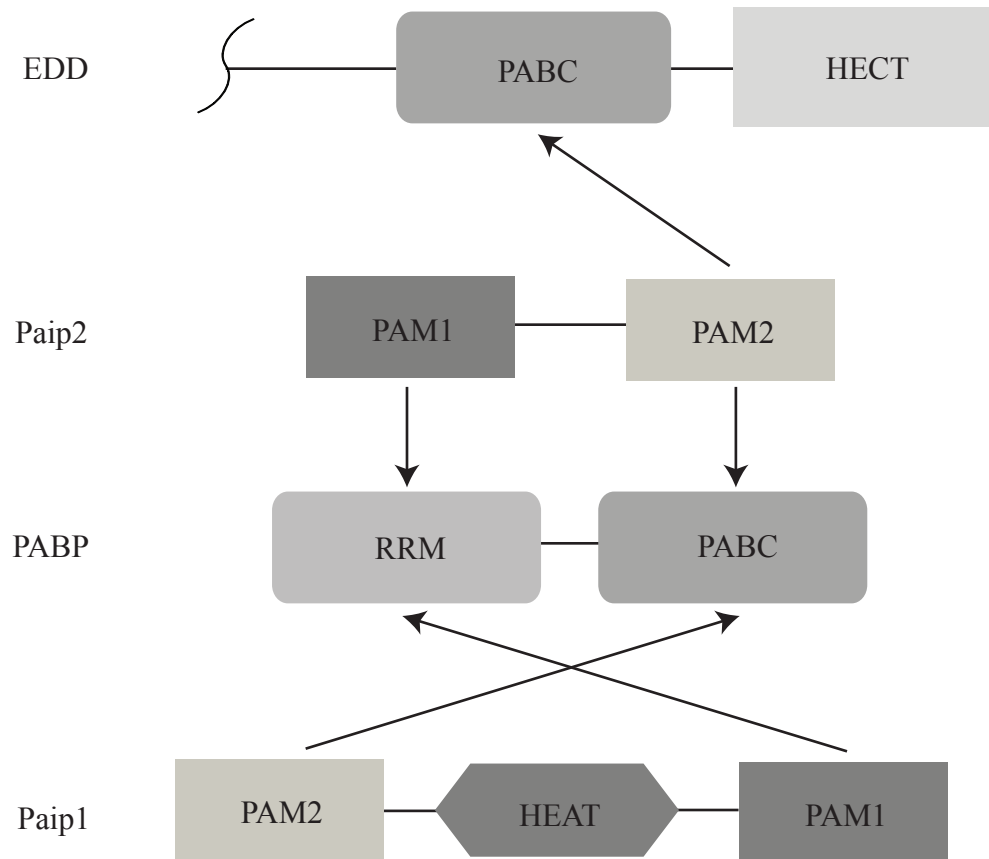


Figure 6. Interactions of Paip1 and Paip2 Domains

The PAM1 domains of Paip1 and Paip2 interact with the RRM domain of PABP. The PAM2 domains of Paip1 and Paip2 interact with the PABC domain of PABP. The PAM2 domain of Paip2 also interacts with the PABC domain in EDD (see text) (modified from Derry et al. 2006).

of PABP by anti- $\alpha 4$ antibodies in Nb2 cells confirmed this interaction (McDonald *et al.* 2010). This interaction suggested a potential role for $\alpha 4$ in the initiation of translation involving PABP catalyzed mRNA circularization.

1.6 Objective of Study

The interaction of $\alpha 4$ with its novel binding partners, EDD and PABP, suggests that the complex may play a role in protein ubiquitination and the initiation of translation. The purpose of this study was to first determine the binding sites on $\alpha 4$ for EDD and PABP. Second, the interaction between $\alpha 4$, EDD, and PABP was studied in both arrested and growing cells. Thirdly, this study investigated the role of $\alpha 4$ in EDD-mediated protein ubiquitination.

CHAPTER 2

Materials and Methods

2.1 Antibodies

Anti- $\alpha 4$ polyclonal antibodies have been described previously (Boudreau *et al.* 2002). Primary antibodies, used at the indicated concentrations, were: rabbit anti-EDD (1:500), Abcam, Inc (Cambridge, Massachusetts); rabbit anti-PP2Ac (1:2000), Cell Signalling (Danvers, Massachusetts); mouse anti-ubiquitin (1:1000) Cell Signalling; mouse anti-Xpress (1:1000), Invitrogen (Burlington, Ontario); goat anti-Paip2 (1:200), Santa Cruz (Santa Cruz, California); rabbit anti-PABP (1:500), Santa Cruz; rabbit anti-actin (1:5000), Sigma-Aldrich (Oakville, Ontario). Secondary antibodies used were donkey anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma-Aldrich), donkey anti-goat IgG HRP conjugate (Santa Cruz), and goat anti-mouse IgG HRP conjugate (Santa Cruz).

2.2 Cell Culture

The human MCF-7 breast cancer cell line was maintained in Dulbecco modified Eagle's medium (DMEM) (Invitrogen) containing 10% (v/v) fetal bovine serum (FBS), and was supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, and 50 U/mL of penicillin/streptomycin. In certain experiments, MCF-7 cells were growth-arrested in 1% charcoal-stripped FBS for a period of 48 h prior to treatment with normal growth medium in order to stimulate cells into the cell-cycle.

African green monkey COS-1 SV40-transformed kidney cells were maintained in DMEM containing 5% heat-inactivated FBS. Suspension cultures of rat Nb2-11C lymphoma cells were maintained in Fischer's medium supplemented with 10% (v/v) heat-inactivated FBS and 10% (v/v) lactogen free horse-serum. Nb2-11C cells are critically dependent on prolactin for cell proliferation (Tanaka *et al.* 1980). For certain experiments Nb2-11C cells were growth-arrested in Fisher's medium containing 10% horse serum for a period of 48h prior to treatment with 10 ng/mL of prolactin in order to stimulate cell growth.

2.3 Deletion Mutants of $\alpha 4$

Deletion mutants D3, D4, and D5 were generated by PCR using full-length pcDNA3- $\alpha 4$ as a template by Lori D. Moffat (Bsc Honours student, Too Laboratory) and Shirley Sangster (technician, Too Laboratory). Products from PCR reactions were excised from a 1% agarose electrophoresis gels and purified using UltraClean 15 DNA Purification Kit (Medicorp Inc, Montreal, Quebec). Purified DNA was subcloned into pcDNA3 or pcDNA4/HisMax TOPO (Invitrogen) and sequenced. D3 contains the N-terminus of $\alpha 4$, including the PP2Ac binding site, and an N-terminal Xpress-tag. D4 lacks the PP2Ac binding site and consisted primarily of the C-terminal region of $\alpha 4$ that contains the epitope that was used to raise polyclonal $\alpha 4$ antibodies (Boudreau *et al.* 2002). D5 contains an N-terminal Xpress tag and the C-terminal epitope.

2.4 Transfection of Deoxyribonucleic Acid (DNA)

COS-1 or MCF-7 cells in growth medium, containing 5% or 10% (v/v) FBS, respectively, were seeded at a density of 3×10^6 cells per 10 cm dish. After 24 h, the medium was removed, cells were washed with 5 mL of phosphate-buffered saline (PBS), and reincubated in 5 mL of serum free DMEM. Plasmids were prepared for transfection by incubating 4 μ g of pcDNA3- α 4 mutant (D4/D5) or 8 μ g of pcDNA3- α 4 mutant (D3) with 20 μ L of LipofectAMINE PLUS™ (Invitrogen) in 750 μ L of serum free DMEM for 15 min at room temperature. 30 μ L of LipofectAMINE™ (Invitrogen Life Technologies) in 750 μ L serum-free DMEM was mixed with the LipofectAMINE™ PLUS mixture and then incubated at room temperature for 15 minutes. The complete transfection mixture was added to COS-1 or MCF-7 cells in 5 mL of serum-free DMEM, giving an approximate final volume of 6.5 mL. After 24h at 37°C, cells were given 5 mL of complete growth medium. Cells lysates were harvested after 48 h for immunoprecipitation or Western analysis.

2.5 Transfection of Small Interfering Ribonucleic Acid (siRNA)

Predesigned Silencer® Select siRNAs and Silencer® Select Negative Control #1 were purchased from Applied Biosystems (Streetville, Ontario). The siRNAs used were si α 41 (5'-GAG CAU AGG UUG UCU GCA Att-3') or si α 42 (5'-AGG CUA AAA UAC AGA GAU Att-3'). siEDD1 (5'-AGA CAA AUC UCG GAC UUG Att-3') or siEDD2 (5'-GCG UGA ACG UGA AUC CGU Utt-3'). siMid1 (5'-GGC UGA UAG CUG GAU GAU Att-3'). A final concentration of 10 nM of siRNA was incubated in 500 μ L of Opti-MEM medium (Invitrogen) and 7.5 μ L RNAiMAX (Invitrogen) for 20 min in 6 well plates.

During the time, cells were being prepared for transfection by seeding them on six well plates at a density of 3×10^5 cells per well in complete transfection medium. Following transfection, cells were incubated for 48 hr at 37°C prior to harvesting for Western analysis or immunoprecipitation.

2.6 Preparation of Total Cell Lysates

Cells were harvested in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL/octylphenoxypolyethoxyethanol (Sigma), 1 mM phenylmethylsulphonyl fluoride (PMSF), containing protease inhibitor cocktail P8340 (Sigma)). Lysates were homogenized by passaging cells through a 21-gauge needle and incubation on ice for 30 min. This was followed by a centrifugation at 1.3×10^4 rpm for 20 min at 4°C to remove cellular debris. The supernatant was removed (total cell lysate) and used in immunoprecipitation studies or frozen at -20°C for further analysis.

2.7 Protein Assay

Protein concentrations from total cell lysates were determined using DC™ Protein Assay (Bio-Rad Laboratories, Mississauga, Ontario) according to the manufacturer's instructions. Samples were prepared for the assay by diluting 5 µL of cell lysate with 20 µL of distilled water. A standard curve was prepared using bovine serum albumin (BSA) of known concentrations (i.e., 1.41 µg/µL, 0.705 µg/µL, 0.353 µg/µL, 0.176 µg/µL, 0.088 µg/µL, and 0.044 µg/µL). Absorbances were measured at 655 nm.

2.8 Immunoprecipitation

Total cell lysates were centrifuged at 13000 rpm for 20 minutes at 4°C and then precleared with 0.25 µg of pre-immune IgG and 20 µL of protein A/G PLUS agarose (Santa Cruz). After 1 h at 4°C, lysates were centrifuged at 300 rpm for 5 min and the supernatant was collected. Precleared lysates were incubated at 4°C with 1 µg of a specific antibody for 1 h at 4°C and then with 40 µL of protein A/G PLUS agarose. Precipitated agarose beads were washed 4 times with (PBS) and centrifuged at 3000g for 5 min at 4°C. The washed beads were resuspended in 3x sample buffer (0.1875 M Tris, pH 6.8, 30% glycerol, 15% β-mercaptoethanol, 6% SDS, 0.75% bromophenol blue).

2.9 Sodium Dodecylsulphate and Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 10-40 µg total protein diluted with 50% volume of 3X SDS-PAGE sample buffer. Samples were incubated at 95°C for 5 min and loaded onto an SDS-polyacrylamide gel (resolving gel: 0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS), and 0.05% tetramethylethylenediamine (TEMED); stacking gel: 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05% APS, and 0.05% TEMED). Samples were resolved at 200 V in SDS-PAGE running buffer (0.02 M Tris-HCl, pH 8.3, 0.2 M glycine, and 0.1% SDS) prior to transfer onto nitrocellulose membranes.

2.10 Western Analysis

After SDS-PAGE, protein samples were transferred to Biotrace™ NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida) in Western transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol) at 100V at 4°C for at least 1h. After protein transfer was completed, nitrocellulose membranes were blocked in a solution containing 10% skim milk powder (w/v) in 0.05 % Tween-20 tris-buffered saline solution (TTBS; 0.02 M Tris-HCl, pH 7.6, 0.2 M NaCl) for 1 h at room temperature. The blots were then incubated in primary antibody diluted to appropriate concentration in 10% skim milk (w/v) in 0.05% TTBS for 2 h at room temperature or for 16 h at 4°C. Excess primary antibody was removed by washing blots 3 times in 0.05% TTBS at 10 min intervals. The blots were then incubated in secondary antibody diluted to the appropriate concentration in 10% milk solution (w/v) in 0.05% TTBS for 1 h at room temperature. Excess secondary was removed by washing the blots in 0.005% TTBS in 3 x 10 min intervals. A 1:1 mixture of Immun-Star™ WesternC™ Chemiluminescence reagents (Bio-Rad) was spread over the blot for 5 min prior to autoradiography.

2.11 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from cells using GenElute™ Mammalian Total RNA Prep Kit (Sigma). DNA was removed from RNA samples by incubating 1µg of total RNA with 1µL DNase1 and 1µL of 10X Reaction Buffer (Fermentas, Burlington, Ontario) in a total volume of 10 µL for 30 min at 37°C. One microlitre of EDTA solution was added to stop the DNase reaction and the RNA samples were then incubated at 65°C for 10 minutes. Complementary DNA (cDNA) was synthesized in a reaction mixture

containing 100 U of Maloney murine leukemia virus reverse transcriptase (MMLV-RT), 0.04 nM pdN6 random primers, 0.2 mM of deoxynucleotide triphosphates (dNTPs), and 10 U of RNaseOUT™ (ribonuclease inhibitor) and dithiothreitol. Synthesized cDNA was amplified by PCR in a reaction mixture containing 12.5 µL of GoTaq™ Green Master Mix (Promega, San Luis Obispo, California), 0.5 µL of forward primers, 0.5 µL of reverse primers, and 3 µL of synthesized cDNA mixture, in a total volume of 25 µL. Amplification of cDNA was performed by incubating samples at 94°C for 75s followed by 25 or 35 cycles at 94°C for 45s, 57°C for 45s, and 72°C for 60s. Further elongation of PCR products was performed at 72°C for 10 min. PCR products were visualized in a 1.4% (w/v) agarose gel in Tris-acetate EDTA buffer (TAE) (0.04 M Tris-acetate, pH 8.0, and 1 mM EDTA) containing 0.005% ethidium bromide (v/v). Primers for human β -actin were: 5'-AAA CTG GAA CGG TGA AGG TG-3' and 5'AGA GAA GTG GGG TGG CTT TT-3' (172 bp amplicon). Sequences for Mid1 primers were taken from the Harvard PrimerBank and were: 5'-TCC TAG TAT CAC ACT GTG CCA-3' and 5'- GAT GTT CTG TAG GGT GAC GTT G-3' (137 bp amplicon).

CHAPTER 3

RESULTS

McDonald, W. J., S. M. Sangster, L. D. Moffat, M. J. Henderson and C. K. L. Too (2010) alpha4 phosphoprotein interacts with EDD E3 ubiquitin ligase and poly(A)-binding protein. *J Cell Biochem.* **110**, 1123-9.

3.1 Interaction of $\alpha 4$ with EDD and PABP

3.1.1 $\alpha 4$ Phosphoprotein binds to EDD and PABP in MCF-7 and COS-1 cells

Using yeast-two hybrid analysis to screen a human K562 erythroleukemia cDNA library, our lab had previously identified EDD E3 ubiquitin ligase as a binding partner of $\alpha 4$ phosphoprotein (McDonald *et al.* 2010). This interaction was confirmed in Nb2 cells using co-immunoprecipitation/immunoblotting (IP/IB) analysis. An immunocomplex was detected between $\alpha 4$ and EDD in Nb2 cell lysates immunoprecipitated with anti- $\alpha 4$ antibodies. Based on the difficulty in transfecting suspension cells, it was important to confirm the interaction of EDD with $\alpha 4$ in adherent cell lines that would allow for higher transfection efficiencies. Western analysis of cell lysates immunoprecipitated by anti-EDD antibodies confirmed the interaction between $\alpha 4$ phosphoprotein and EDD in MCF-7 breast cancer cells. The $\alpha 4$ phosphoprotein is 39-kDa in size and a single immunoreactive band of 39-kDa and was detected in the EDD immunocomplex (**Figure 7, left lane**). As a control, MCF-7 cell lysates were immunoprecipitated with pre-immune IgG and neither EDD nor $\alpha 4$ was detected in the IgG immunocomplex (**Figure 7, right lane**). Interaction between EDD and $\alpha 4$ was also confirmed in COS-1 cell lysates following immunoprecipitation with anti-EDD or $\alpha 4$ antibodies (**Figure 8, lanes 1 and 2 respectively**).

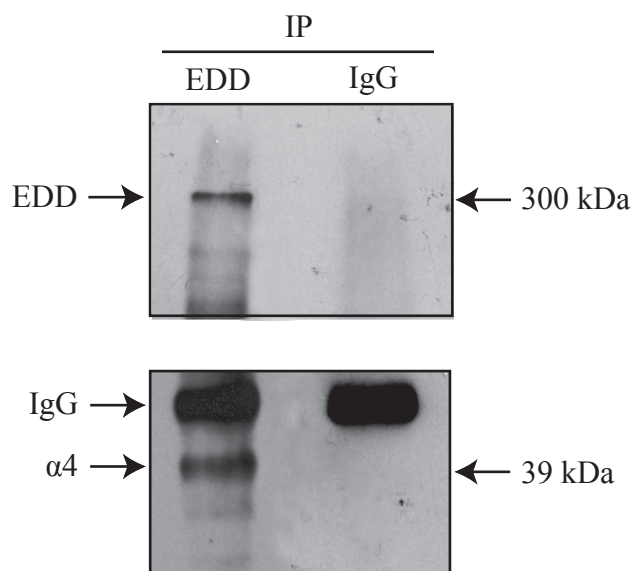


Figure 7. EDD Interacts with $\alpha 4$ in MCF-7 cells

Total lysates of MCF-7 cells were immunoprecipitated with 1 μ g of anti-EDD antibodies or pre-immune IgG. Cell lysates were resolved using SDS-PAGE, followed by immunoblotting for EDD (top panel) or $\alpha 4$ (bottom panel). Samples were resolved in a 12% polyacrylamide gel.

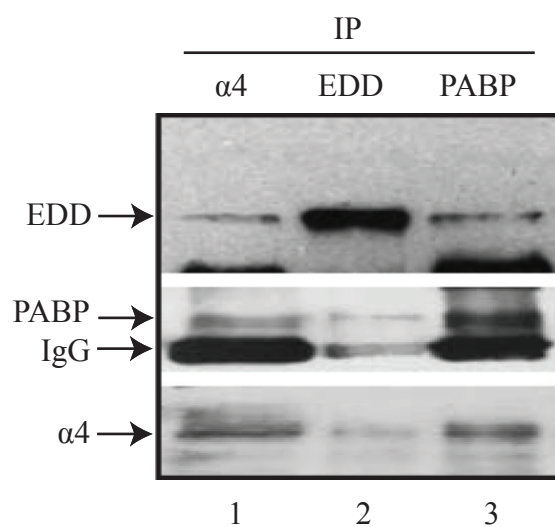


Figure 8. $\alpha 4$ interacts with EDD and PABP in COS-1 cells

Total cell lysates from COS-1 cells were used for immunoprecipitation with anti- $\alpha 4$, EDD, or PABP antibodies. Cell lysates were resolved by SDS-PAGE, followed by immunoblotting for $\alpha 4$, PABP, or EDD. Representative of three independent experiments. Samples were resolved in a 12% polyacrylamide gel.

Previously, our lab had also identified PABP as a binding partner of $\alpha 4$ in Nb2 lymphoma, Jurkat, and K652 leukemic cell lines (McDonald *et al.* 2010). For the purpose of this study, the interaction between $\alpha 4$ and PABP was also confirmed in COS-1 cells immunoprecipitated with anti- $\alpha 4$ or PABP antibodies (**Figure 8, lanes 1 and 3 respectively**). An interaction between EDD and PABP was also detected in COS-1 cells immunoprecipitated with anti-EDD or PABP antibodies (**Figure 8, lanes 2 and 3 respectively**).

3.1.2 Generation of Deletion Mutants of $\alpha 4$ Phosphoprotein

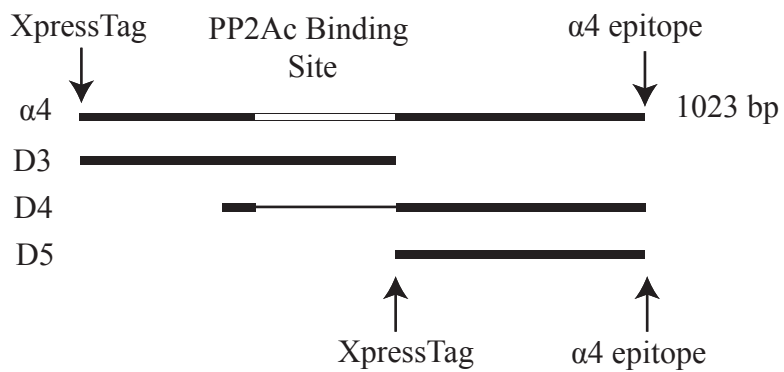
To determine the binding sites for EDD and PABP on $\alpha 4$ phosphoprotein, $\alpha 4$ deletion mutants were constructed that contained either the N-terminal or C-terminal regions of the $\alpha 4$ protein (**Figure 9A**). The D3 deletion mutant has an Xpress-tag at its N-terminus. The C-terminus of the D4 deletion mutant contains the $\alpha 4$ -epitope that was used to raise polyclonal anti- $\alpha 4$ antibodies (Boudreau *et al.* 2002). The D5 deletion mutant contains both the N-terminal Xpress-tag and the C-terminal $\alpha 4$ epitope. The D4 and D5 deletion mutants of the $\alpha 4$ phosphoprotein were transfected into MCF-7 breast cancer cells, and Western analysis showed that the expressed proteins were approximately 33 and 23 kDa respectively. The expressed D4 was detected using anti- $\alpha 4$ antibodies. The expressed D5 protein was detected using either anti-Xpress tag or anti- $\alpha 4$ antibodies. The D3 plasmid was also transfected into MCF-7 cells, but the protein was either poorly expressed or not detectable using Western analysis despite several attempts and repurification of the cDNA construct.. Instead, the D3 plasmid was transfected into

Figure 9. Deletion Mutants of $\alpha 4$ Phosphoprotein

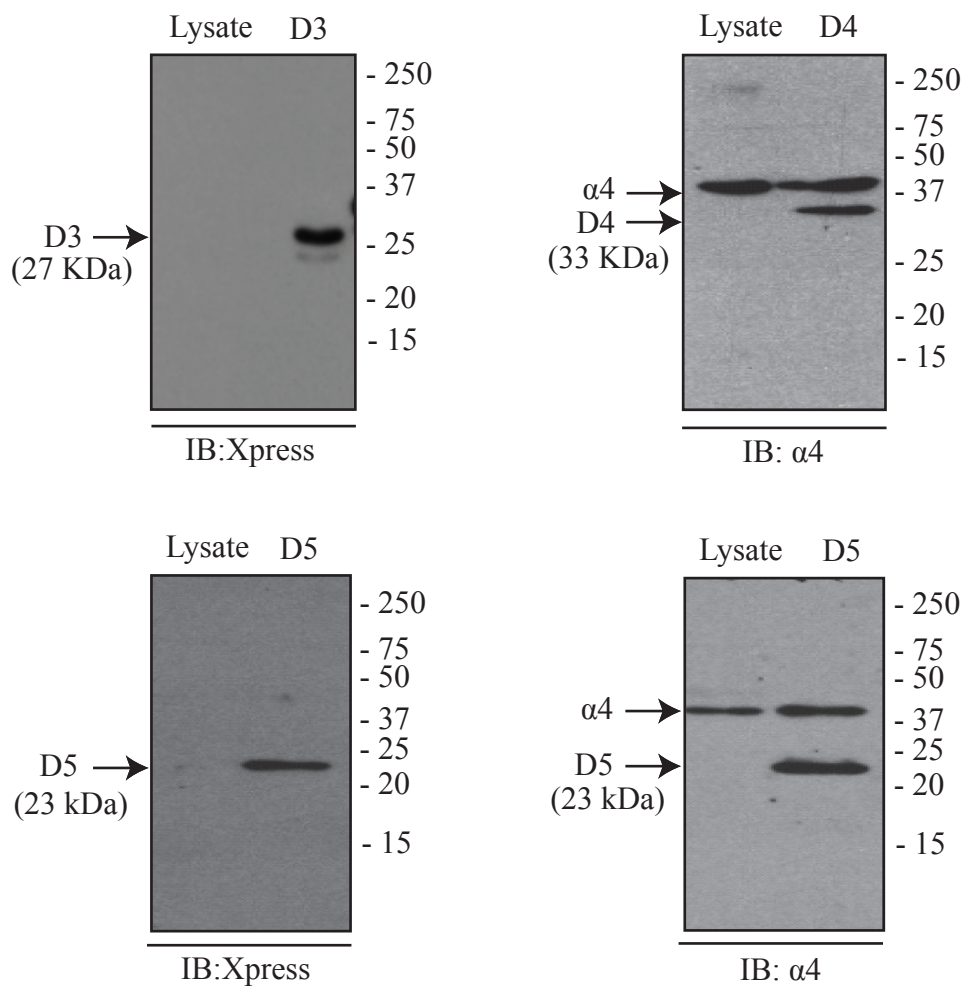
A) The full length $\alpha 4$ phosphoprotein contains a PP2Ac binding site. Polyclonal anti- $\alpha 4$ antibodies recognize the $\alpha 4$ epitope at the C-terminus. Deletionmutant D3 contains an N-terminal Xpress tag, D4 contains the $\alpha 4$ epitope, and D5 contains both the Xpress tag and $\alpha 4$ epitope. The thin line in D4 refers to the deletion of the PP2Ac binding site.

B) MCF-7 cells were transfected with plasmids containing the D4 or D5 constructs, whereas COS-1 cells were transfected with D3 construct. Cell lysates were harvested for 48 h post-transfection and Western analysis was performed to detect the expressed proteins. Untransfected cell lysates were used as controls. D5 samples were resolved in 15% polyacrylamide gels. D3 and D4 samples were resolved in 12% polyacrylamide gels.

A)



B)



COS-1 cells, it produced an abundant D3 protein of 27-kDa. The D3 deletion mutant was detected using the anti-Xpress tag antibodies only.

3.1.3 EDD Binds to the C-Terminal Region of $\alpha 4$

To determine the binding site of EDD on $\alpha 4$ phosphoprotein, MCF-7 cells were transfected with the D4 or D5 constructs, or were left untransfected (controls). Total cell lysates were used for IP/IB analysis. Western analysis of control lysates transfected with the D5 construct showed the presence of the 39-kDa endogenous $\alpha 4$ and the 23 kDa D5 protein, both detected using anti- $\alpha 4$ antibodies (**Figure 10A left panel**) or the presence of D5 alone using Xpress antibodies (**Figure 10A left panel**). IP/IB analysis of D5 transfected cell lysates with anti-EDD antibodies revealed the formation of an immunocomplex between EDD, D5 and the endogenous $\alpha 4$. (**Figure 10A**).

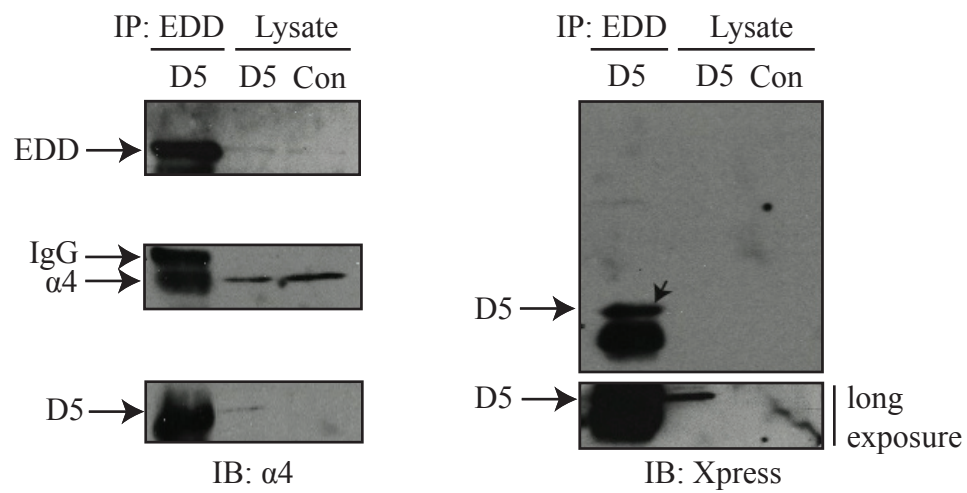
Similarly, the 33 kDa D4 protein was detected only in D4 transfected cells and not in the untransfected control cell lysates (**Figure 10B**). IP/IB analysis of D4 with anti-EDD antibodies showed the formation of an immunocomplex consisting of EDD with D4 and the endogenous $\alpha 4$ (**Figure 10B**). The interaction of EDD with D4 and D5 confirms that EDD with $\alpha 4$, specifically at the C-terminal region.

To confirm these results, further experiments were performed using COS-1 cells transfected with the D3 construct which consists of the N-terminal region of $\alpha 4$ and the PP2Ac binding site (**Figure 9A**). The 27 kDa D3 protein was detected in control lysates prepared from COS-1 cells, but not in lysates from cells transfected with the D3 construct and immunoprecipitated with EDD antibodies (**Figure 10C**). The results show that EDD

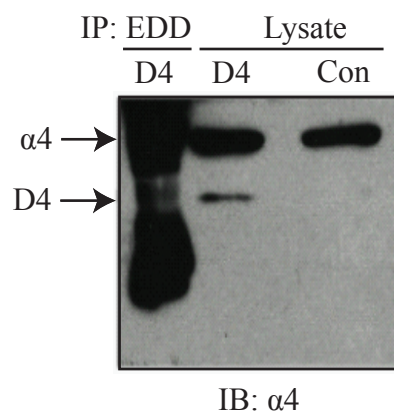
Figure 10. EDD interacts with D4 and D5

MCF-7 cells were transfected with D4 or D5 plasmids. Control cells were not transfected. Total cell lysates were immunoprecipitated with anti-EDD antibodies followed by A) immunoblotting using anti- $\alpha 4$ (left panel) or anti-Xpress (right panel) or B) immunoblotting for anti- $\alpha 4$ antibodies. In the right panel of A, D5 in the lysate lysate was detected after increased exposure of the autoradiograph. C) COS-1 cells were transfected with 8 μg of D3 plasmid. Cell lysates were used for IP analysis using 1 μg of anti-EDD antibodies followed by immunoblotting for Xpress tag. The right panel is a darker exposure of the left panel. Representative of at least two experiments. Control lysates consisted of 5% of the original cell lysate.

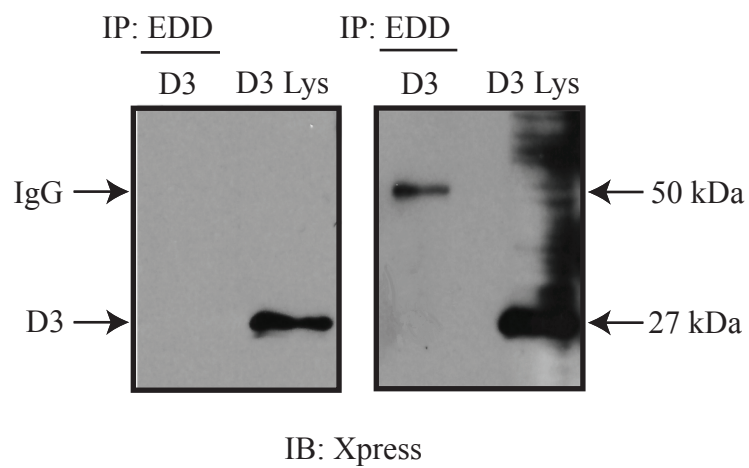
A)



B)



C)



binds to the C-terminal region of the $\alpha 4$ phosphoprotein, independent of the PP2Ac binding site. EDD does not bind to the N-terminal region of the $\alpha 4$ phosphoprotein.

3.1.4 PABP and PP2Ac bind to the N-terminal region of $\alpha 4$

To determine the $\alpha 4$ -binding site for PABP, COS-1 cells were first transfected with the D5 plasmid, followed by IP/IB analysis. D5 transfected cell lysates contained a single immunoreactive band corresponding to the 23 kDa D5 protein, detected using anti-Xpress tag antibody (**Figure 11A**). IP/IB analysis using anti-PABP antibodies or pre-immune IgG did not immunoprecipitate the D5 protein (**Figure 11A**). These results suggest that PABP, unlike EDD, does not bind to the C-terminal region of $\alpha 4$ -phosphoprotein.

Secondly, COS-1 cells were transfected with the D3 plasmid and were prepared for IP/IB analysis. IB with anti-Xpress antibodies showed the presence of the D3 protein in the cell lysates (**Figure 11B**). IP of cell lysates with anti-PABP antibodies revealed an immunocomplex that contained PABP and the D3 protein (**Figure 11B**). The D3 protein was not detected in cell lysates immunoprecipitated with pre-immune IgG (**Figure 11B**). Therefore PABP binds to the N-terminal region of $\alpha 4$ phosphoprotein.

The immunoreactive signal for D3 in the immunocomplex was faint. D3 contains the PP2Ac binding site. Therefore, to further show that the expressed D3 is folded properly enough to bind PP2Ac, COS-1 cell lysates were transfected with the D3 plasmid and immunoprecipitated with either anti-PP2Ac antibodies or pre-immune IgG. The D3 protein was immunoprecipitated with anti-PP2Ac antibodies, but not immunoprecipitated

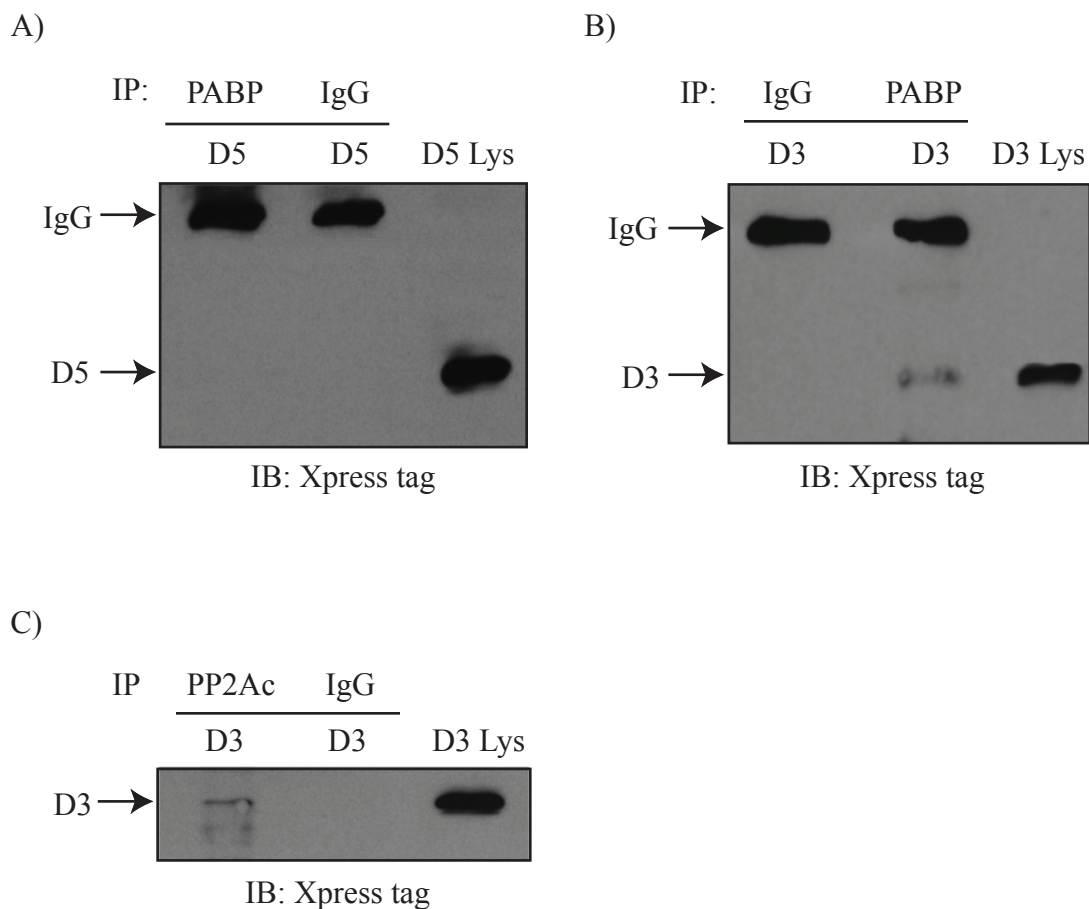


Figure 11. PABP Interacts with the N-terminal region of $\alpha 4$

COS-1 cells were transfected with either A) 4 μ g of the D5 plasmid or B) 8 μ g of the D3 plasmid. Cell lysates were immunoprecipitated with 1 μ g of anti-PABP antibodies or 1 μ g of IgG control. C) COS-1 cells were transfected with 8 μ g of the D3 plasmid and cell lysates were immunoprecipitated with 1 μ g of anti-PP2Ac antibodies or 1 μ g of preimmune IgG. Immunocomplexes were resolved by SDS-PAGE and immunoblotted using anti-Xpress antibodies. Representative of at least two independent experiments. Control lysates consisted of 5% of the original cell lysate.

with the pre-immune IgG (**Figure 11C**). D3 is able to bind PP2Ac and PABP. These results validate the structural integrity of the expressed D3.

3.2 Interaction of $\alpha 4$ with EDD and PABP in Relation to Translation

3.2.1 Interaction of $\alpha 4$ With PABP and EDD is Independent of the Cell Cycle

The interaction between $\alpha 4$ and PABP suggests a functional relationship for $\alpha 4$ in PABP controlled mRNA processing. Based on the involvement of PABP in the initiation of translation, it is likely that its activity is increased in growing cell compared to its activity in quiescent cells. To determine if $\alpha 4$ could be involved in regulation of PABP activity the interaction between PABP and $\alpha 4$ phosphoprotein was investigated in cells that were growth- arrested in G_0 phase and then allowed to re-enter into the cell cycle. Therefore, human MCF-7 cells were made quiescent in 1% charcoal stripped FBS. After 48 h the cells were given complete growth medium containing 10% FBS and cell lysates were prepared at 0, 4, 8, and 24 h. IP analysis was performed using anti- $\alpha 4$ antibodies followed by immunoblotting using anti- $\alpha 4$ or anti-PABP antibodies. In both quiescent and growing cells, $\alpha 4$ was able to form an immunocomplex with PABP (**Figure 13A**), indicating that the interaction between these proteins is independent of cell cycle.

Similarly, prolactin-dependent rat Nb2 lymphoma cells were made quiescent in medium containing 10% prolactin-free horse serum and then treated with prolactin to stimulate cell cycle progression. Cell lysates were prepared at 0, 4, 8, and 24 h. IP of cell lysates with anti- $\alpha 4$ antibodies once again showed formation of an immunocomplex of $\alpha 4$

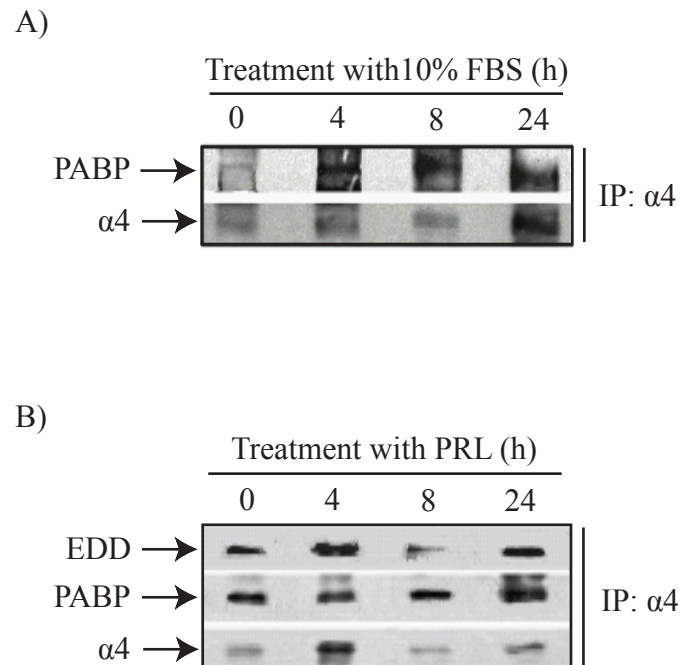


Figure 12. Interaction of $\alpha 4$ with PABP and EDD is cell cycle independent

MCF-7 and Nb2 cells were made quiescent as described in the Materials and Methods. Quiescent MCF-7 cells (A) or Nb2 cells (B) were treated with 10% FBS or 10 ng/mL PRL, respectively, for the indicated times. Cell lysates were immunoprecipitated with anti- $\alpha 4$ antibodies, followed by immunoblotting for $\alpha 4$, PABP, or EDD. Samples were resolved in 10% polyacrylamide gels. Representative of at least two independent experiments.

and PABP in both quiescent and growing cells (**Figure 12B**). In addition to PABP, immunoblotting for EDD also showed an interaction between EDD and $\alpha 4$ over the 24 h period. Although the study was not quantitative, nevertheless, it demonstrated that $\alpha 4$ can bind EDD and PABP, independent of the cell cycle.

3.2.2 $\alpha 4$ is Not Involved in EDD-Mediated Ubiquitination/Degradation of Paip2.

Circularization of mRNA by PABP is regulated by the interaction of PABP with two regulatory proteins, Paip1 and Paip2 (see **Figure 6 in Introduction**). Whereas Paip1 acts as a translation enhancer, the antagonist Paip2 competes with Paip1 for binding to PABP to inhibit formation of the 80S ribosomal complex (Khaleghpour *et al.* 2001; Derry *et al.* 2006). However, EDD targets Paip2 for ubiquitination and subsequent degradation by the proteasome. EDD does not ubiquitinate Paip1, even though both Paip1 and Paip2 contain the PAM2 domain that is necessary for interaction with the PABC domain of EDD (Roy *et al.* 2002). To determine whether or not $\alpha 4$ acts as an adapter protein regulating EDD-dependent degradation of Paip2, the effect of knocking down $\alpha 4$ phosphoprotein gene expression on Paip2 steady state protein levels was determined in MCF-7 cells. MCF-7 cells were transfected with siRNA directed against the $\alpha 4$ phosphoprotein (si $\alpha 4$) or non-targeting siRNA (siNT). Western analysis confirmed a decrease in $\alpha 4$ protein levels following si $\alpha 4$ treatment (**Figure 13A**). In contrast, there was no effect on EDD or actin protein levels. Immunoblotting with anti-Paip2 antibodies showed the continued presence of Paip2 in control cells and cells transfected with si $\alpha 4$ (**Figure 13A**). Immunoblotting with anti-EDD antibodies showed no difference in EDD

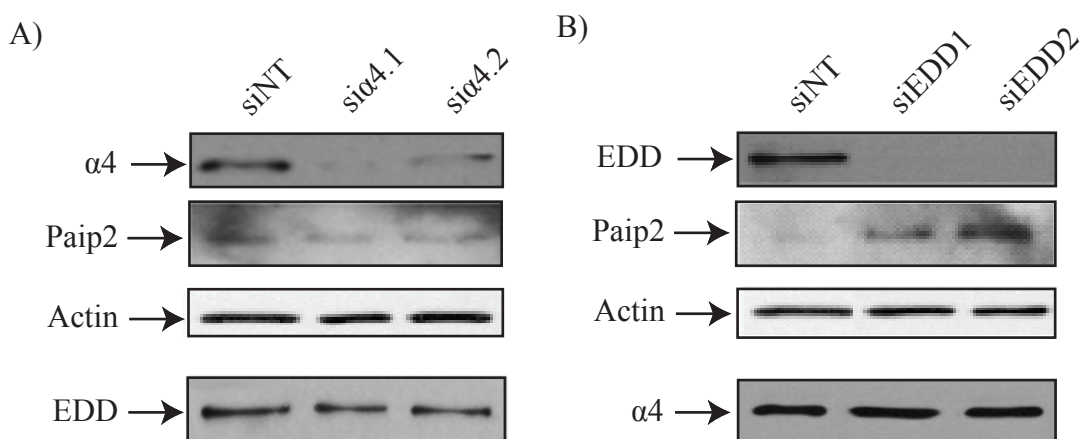


Figure 13. $\alpha 4$ is not Involved in Paip2 Ubiquitination by EDD

A) MCF-7 cells were transfected 2 sets of sia4 (sia4.1, sia4.2) or siNT. Cells lysates were harvested 48 h post-transfection, followed by immunoblotting for $\alpha 4$ or Paip2. B) MCF-7 cells were transfected with 2 sets of siEDD (siEDD1, siEDD2) or siNT. Cells lysates were harvested 48 h post-transfection, followed by immunoblotting for EDD or Paip2. Representative of at least two independent experiments. Samples were resolved in 10% polyacrylamide gels.

protein levels between control cells and cells transfected with si α 4 (**Figure 13A**).

Therefore, decreased levels of α 4 do not affect EDD or Paip2 protein levels

As a positive control, MCF-7 cells were transfected with siRNA targeting EDD (siEDD) or siNT. The decrease in EDD protein levels following siEDD transfection was confirmed by Western analysis (**Figure 13B**). A decrease in EDD levels resulted in an increase in cellular levels of Paip2 (**Figure 13B**), indicating that EDD is not required for the degradation of Paip2. Taken together, these results show that α 4 is not involved in the EDD mediated ubiquitination and subsequent proteasomal degradation of Paip2.

3.3 Role of EDD in Ubiquitination of α 4 and PP2Ac

3.3.1 EDD does not ubiquitinate α 4

It has been reported that α 4 phosphoprotein is mono-ubiquitinated within its ubiquitin interacting motif (UIM, residues 46-60), and deletion of this motif increases binding of α 4 to poly-ubiquitinated proteins (Fisher *et al.* 2003; McConnell *et al.* 2010). The enzyme involved in the mono-ubiquitination of α 4 has not been discovered. To determine whether or not EDD mono-ubiquitinates α 4 phosphoprotein, MCF-7 cells were transfected with siEDD and siNT. The decrease in EDD protein levels was confirmed by Western analysis (**Figure 14**). Cell lysates were immunoprecipitated with anti- α 4 antibodies and then immunoblotted with anti-ubiquitin or anti- α 4 antibodies. Immunoblotting with anti-ubiquitin antibodies revealed a single immunoreactive band of approximately 46 kDa (**Figure 14**). This band is distinct from the immunoreactive α 4,

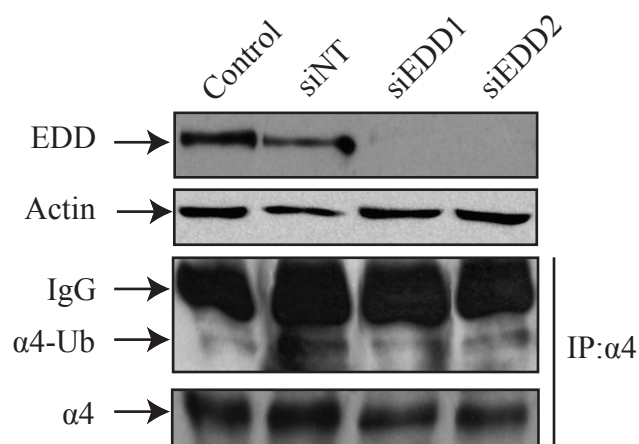


Figure 14. EDD does not Mono-Ubiquitinate $\alpha 4$

MCF-7 cells were transfected with 2 sets of siEDD (siEDD1, siEDD2) or siNT. Cell lysates were harvested 48 h post-transfection and were used for Western analysis (top 2 panels) or immunoprecipitated with anti- $\alpha 4$ antibodies (bottom 2 panels). Cell lysates resolved by SDS-PAGE and immunocomplexes were immunoblotted for $\alpha 4$, ubiquitin, or EDD. Control cells were not transfected. Samples were resolved on 10% polyacrylamide gels. Representative of three independent experiments.

detected at 46 kDa, and represented the mono-ubiquitinated $\alpha 4$. The mono-ubiquitinated $\alpha 4$ was detected in both control cells (no transfection) and in cells transfected with siNT. Mono-ubiquitinated $\alpha 4$ was also detected in cells with decreased EDD indicating that EDD does not mono-ubiquitinate $\alpha 4$. Similar to previous reports, no other immunoreactive bands were detected by immunoblotting with the anti-ubiquitin antibody, confirming that $\alpha 4$ is mono-ubiquitinated only (McConnell *et al.* 2010).

3.3.2 Knockdown of EDD Gene Expression Increases PP2Ac Protein Levels

Previously, it has been reported that PP2Ac protein levels are regulated by the ubiquitination pathway and proteasomal degradation mediated by the Mid1, an E3 ubiquitin ligase mutated in Opitz Syndrome (Troockenbacher *et al.* 2001; McConnell *et al.* 2010). Due to the cytoplasmic localization of Mid1, it is possible that it is only responsible for the ubiquitination and subsequent degradation of a pool of PP2Ac that is in the cytosol. In contrast, PP2Ac localized elsewhere in the cell may be targeted by a completely different ubiquitin ligase, such as the nuclear localized EDD. Therefore, to test this possibility, the effect of depleting cellular levels of EDD on PP2Ac levels was determined. MCF-7 cells were transfected with siEDD or siNT. Once again, a decrease in EDD protein levels was confirmed using Western analysis (**Figure 15A,B**).

Immunoblotting with PP2Ac antibodies revealed high levels of the PP2Ac protein in cells treated with siEDD1 or siEDD2, as compared to low levels in cells transfected with siNT or control cell (**Figure 15A**). When MCF-7 cells were treated with the proteasomal inhibitor MG132 (10 μ M) for a period of 2 h, PP2Ac protein levels seen were again high in cells with depleted levels of EDD (**Figure 15B**). In the presence of MG132, PP2Ac

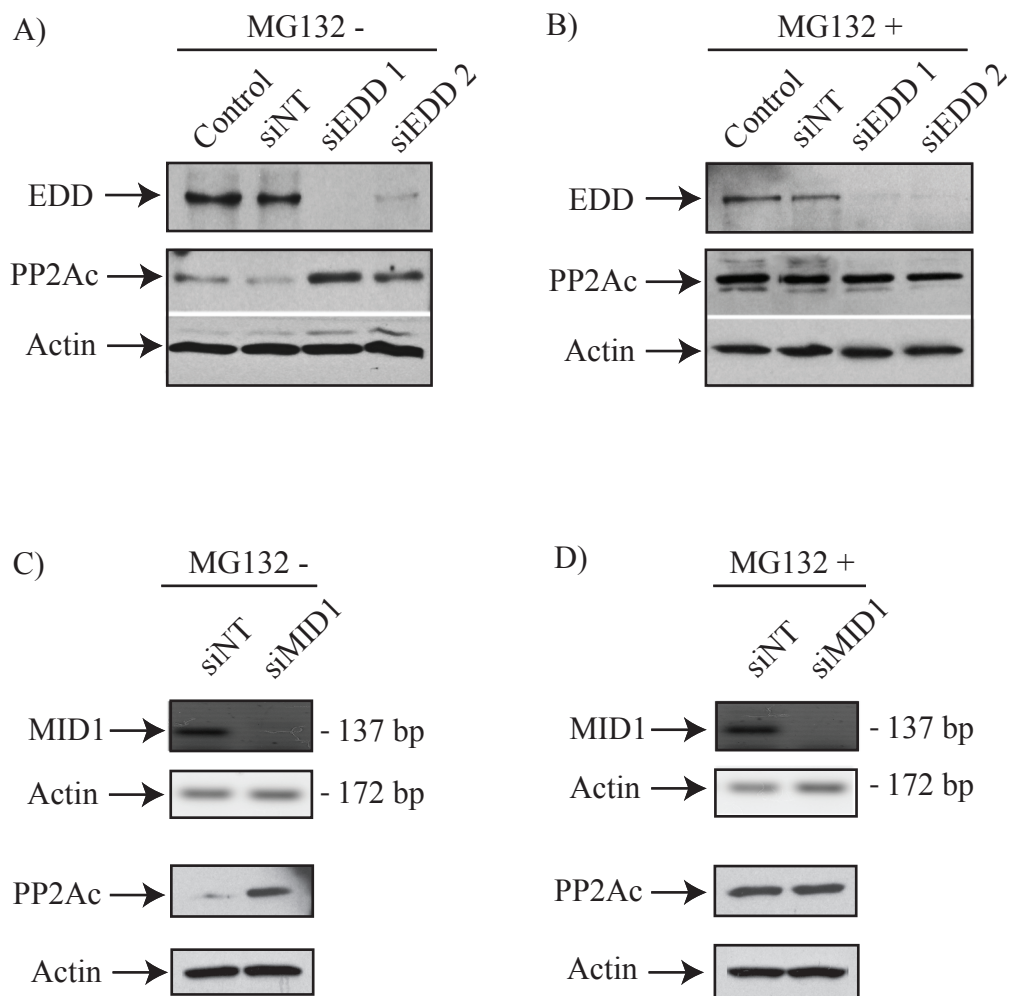


Figure 15. EDD Knockdown by siRNA Increases PP2Ac Protein Levels

MCF7 cells were transfected with siEDD1, siEDD2, siNT or left untransfected (Control). Cells were treated without (A) or with (B) 10 μ M MG132. After 2 h, cells were harvested for the preparation of cell lysates. Lysates (40 μ g protein/lane) were resolved using SDS-PAGE and then immunoblotted using anti-PP2Ac or anti-EDD antibodies. MCF-7 cells were transfected with siMid1 or siNT. Cells were treated without (A) or with (B) 10 μ M MG132. After 2 h, cells were harvested for the preparation of cell lysates. Lysates (40 μ g protein/lane) were resolved using SDS-PAGE and were immunoblotted using anti-PP2Ac antibodies. Mid1 expression was measured by RT-PCR. Representative of at least two independent experiments.

protein levels were equally high in cells transfected with siNT or control cells (**Figure 15B**). These results suggest that in the absence of MG132, poly-ubiquitinated proteins are not protected from undergoing proteasomal degradation. As a result, PP2Ac protein levels are low in control cells or cells transfected with siNT, but high in cells with EDD knocked down. This suggests a role for EDD in the poly-ubiquitination of PP2Ac.

Furthermore, as a control, PP2Ac protein levels were measured in MCF-7 cells transfected with siRNA directed against Mid1 (siMid1). Since the commercially available anti-Mid1 antibodies were not able to detect any immunoreactive bands (data not shown), Mid1 expression was determined by RT-PCR. Cells transfected with siMid1 were shown to have decreased expression of the Mid1 mRNA (**Figure 15C,D**). Similar to the previous experiments, MCF-7 cells were treated with or without 10 μ M MG132 for a 2 h. In the absence of MG132, PP2Ac levels were high in siMid1 treated cells, but low in cells transfected with siNT (**Figure 15C**). In the presence of MG132, PP2Ac protein levels were equally high in cells transfected with siMid1 and siNT (**Figure 15D**). Taken together, these results indicate a role for EDD in the regulation of PP2Ac levels within the cell.

3.3.3 Knockdown of EDD Gene Expression Decreases PP2Ac Ubiquitination

The increase in PP2Ac protein levels following knockdown of EDD may be accompanied by changes in the poly-ubiquitination of PP2Ac. Therefore MCF-7 cells were transfected with siRNA targeted towards EDD. Cells were treated with 10 μ M MG-132 for 2 hours prior to harvesting and lysates were immunoprecipitated with anti-PP2Ac antibodies. Immunoblotting for anti-ubiquitin antibodies revealed a smear in the non-

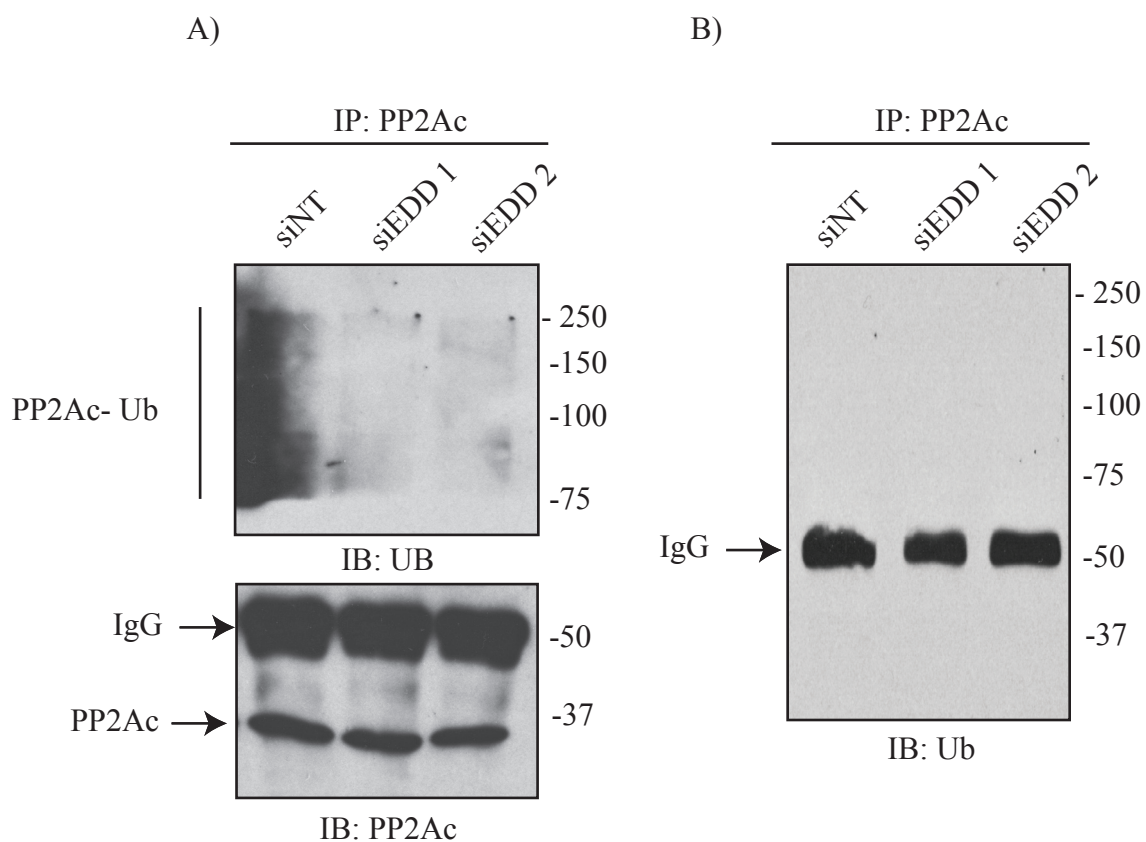


Figure 16. EDD Knockdown Decreases PP2Ac Poly-Ubiquitination

MCF-7 cells were transfected with 2 sets of siEDD (siEDD1, siEDD2) or siNT and were incubated A) with or B) without 10 μ M MG132. Lysates were used for immunoprecipitation with anti-PP2Ac antibodies, followed by immunoblotting for A) PP2Ac (lower panel), ubiquitin (upper panel) or B) ubiquitin. Samples were resolved in 10% polyacrylamide gels. Representative of two independent experiments.

targeting siRNA control lane corresponding to the presence of poly-ubiquitinated PP2Ac (**Figure 16**). Ubiquitin signals were decreased in lanes containing lysates from cells that were treated with siEDD1 or siEDD2. PP2Ac levels were shown to be similar between EDD siRNA lanes and the control lane. These results show EDD dependent poly-ubiquitination of PP2A.

3.3.4 EDD does not Interact with PP2Ac

Studies on the ubiquitination of PP2Ac have shown the formation of an immunocomplex consisting of PP2Ac and Mid1 or PP2Ac, Mid1 and $\alpha 4$ phosphoprotein (Liu *et al.* 2001; Trockenbacher *et al.* 2001). The physical interaction between $\alpha 4$ and Mid1 is required for the recruitment of Mid1 to PP2Ac. To determine if there is a physical interaction between EDD and PP2Ac, MCF-7 lysates were immunoprecipitated with anti-PP2Ac, EDD, or $\alpha 4$ antibodies (**Figure 17**). Immunoblotting for PP2A showed successful immunoprecipitation of PP2Ac (**Figure 17, lane 3**). Immunoblotting for PP2Ac also revealed interaction of PP2Ac with $\alpha 4$ (**lane 1**), but not EDD (**lane 4**) nor IgG (**lane 2**). Furthermore, immunoblotting for EDD showed the successful immunoprecipitation of EDD (**Figure 17, lane 4**). Therefore, although EDD interacts with $\alpha 4$ phosphoprotein, a regulatory subunit of PP2Ac, there is no physical interaction between EDD and PP2Ac in MCF-7 cells.

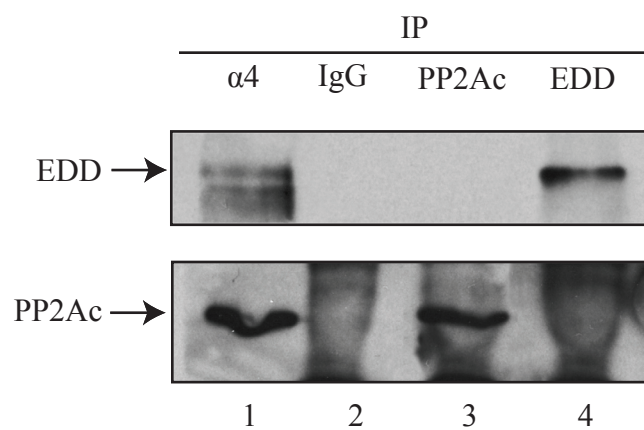


Figure 17. EDD does not Interact with PP2Ac in MCF-7 cells.

Total cell lysates were harvested from MCF-7 cells maintained in normal growth medium and immunoprecipitation was performed with anti-EDD, anti-PP2Ac, and anti- $\alpha 4$ antibodies. Immunocomplexes were resolved by SDS-PAGE and immunoblotted for PP2Ac. Pre-immune IgG was used as a control. Samples were resolved in 10% polyacrylamide gels.

CHAPTER 4

Discussion

4.1 $\alpha 4$ Phosphoprotein as an Adaptor/Scaffold

$\alpha 4$ phosphoprotein is an essential component of the mTOR pathway that responds to the presence of nutrients and growth factors to control the initiation of translation, ribosome biosynthesis, and gene transcription (Gingras *et al.* 2001). Studies on the structure of $\alpha 4$ have suggested that $\alpha 4$ may serve as a scaffold/adaptor for other proteins to facilitate their cellular function (Yang *et al.* 2007). The C-terminal region of $\alpha 4$ is important for its role as an adaptor protein in PP2Ac-mediated dephosphorylation since it is a site for protein recruitment of potential PP2Ac substrates. This region of $\alpha 4$ has a flexible secondary structure, and depending on its conformation, is capable of interacting with a variety of proteins. While the N-terminus of $\alpha 4$ binds to PP2Ac, the C-terminus binds to proteins that affect or are affected by PP2Ac (Yang *et al.* 2007). The present study showed interaction of $\alpha 4$ with EDD and PABP, in addition to PP2Ac, in human MCF-7 breast cancer and African green monkey COS-1 kidney cells. It is possible that $\alpha 4$ may serve as an adaptor/scaffold to facilitate the cellular action of EDD and PABP. The interaction of EDD with the C-terminal region of $\alpha 4$ also suggests a possible role for EDD in the modulation of PP2Ac activity in the cell.

4.2 PP2Ac- α 4 Complex

α 4 phosphoprotein interacts with PP2Ac (Chen *et al.* 1998; Boudreau *et al.* 2002) and regulates PP2Ac activity (Kong *et al.* 2009). Deletion of the α 4 gene in mouse embryonic fibroblasts leads to progressive loss of PP2A, PP4 and PP6 phosphatase complexes (Kong *et al.* 2009). PP2A is a key regulator of multiple signal transduction pathways within the cell, hence, regulation of PP2A is important to facilitate normal cell signalling. The formation of the α 4/PP2Ac complex has been implicated in the regulation of the mTORC1 signalling pathway, particularly on its downstream effectors, 4E-BP1 and p70S6K (Nanahoshi *et al.* 1998; Peterson *et al.* 1999). PP2Ac serves to dephosphorylate 4E-BP1, thus increasing its interaction with eIF4E and preventing formation of the 5'-cap complex (4E, 4G, and 4A) of mRNA that is required for the initiation of translation. PP2Ac dephosphorylates p70S6K to decrease its kinase activity and, thus, inhibits translation of 5'-TOP mRNA. Alterations in PP2Ac protein levels and/or activity could either increase or decrease the phosphorylation state of 4E-BP1 and S6K. Decreasing the phosphorylation of 4E-BP1 and p70S6K would lead to a decrease in translation and cell cycle progression. Previous studies have shown that the predominant medium for the regulation of PP2Ac levels within the cell is not at the level of transcription, but at the protein level thorough an auto-regulatory mechanism (Baharians and Schonthal 1998). Although this translational control of PP2Ac maintains a constant protein level in the cell, evidence has been provided demonstrating changes in PP2Ac levels during differentiation of adipocytes and HL-60 cells (Tawara *et al.* 1993; Altiook *et al.* 1997). From the recent studies on the poly-ubiquitination and subsequent proteasomal

degradation of PP2Ac, it is evident that PP2Ac levels can be regulated post-translationally. For example, Mid1, the Opitz Syndrome related protein binds to the C-terminal region of $\alpha 4$, allowing it to interact with PP2Ac. Mid-1 targets PP2Ac for poly-ubiquitination and degradation (Trockenbacher *et al.* 2001). Similarly in our studies, we observed that siMid1 transfected MCF-7 showed increased levels of PP2Ac (**Figure 15C**). Furthermore, we observed that knockdown of EDD by siRNA also increased PP2Ac protein levels in MCF-7 cells (**Figure 15A**) and depletion of EDD corresponded to a decrease in the poly-ubiquitination of PP2Ac (**Figure 16**). Therefore, we have provided evidence of a novel mechanism by which PP2A levels may be regulated. We have also provided evidence that PP2Ac is a target of EDD as well as Mid-1.

4.3 EDD- $\alpha 4$ Complex

The present study showed that EDD E3 ubiquitin ligase binds to the C-terminal region of $\alpha 4$, an essential component of the mTOR pathway. EDD is the only known E3 ubiquitin ligase that contains both RING and HECT domains (Henderson *et al.* 2002). As a family, HECT domain-containing ubiquitin ligases do not require adapter proteins to mediate the ubiquitination of substrates, but instead bind directly to their targets. In contrast, some RING domain E3 ubiquitin ligases, such as APC/C or Cull1, require the recruitment of adapter proteins for substrate binding and direct transfer of ubiquitin from the E2 ubiquitin conjugating enzyme (Rudner and Murray 2000; Cope and Deshaies 2003). Based on the interaction of EDD with $\alpha 4$, we hypothesized that $\alpha 4$ could act as an adapter protein for the EDD-mediated ubiquitination of its substrates.

Similar to EDD, Mid1 also binds to the C-terminal domain of $\alpha 4$ (Troockenbacher *et al.* 2001). Unlike the formation of the Mid-1-PP2Ac- $\alpha 4$ complex observed in human fibroblasts (McConnell *et al.* 2010), the present study showed formation of an EDD- $\alpha 4$ complex, with no physical interaction observed between EDD and PP2Ac in MCF-7 cells. We have previously reported formation of an EDD- $\alpha 4$, but not EDD-PP2Ac, immunocomplex in Nb2, Jurkat, and, K652 cells (McDonald *et al.* 2010). This lack of physical interaction between EDD and PP2Ac is puzzling, and it suggests that the mechanism by which EDD regulates PP2Ac levels in the cell does not require direct physical interaction for the ubiquitination of PP2Ac by EDD. While it is possible that the physiological conditions for EDD-PP2Ac interaction were not met in the immunoprecipitation experiments, it is also possible that EDD binding to $\alpha 4$ interferes with formation of an $\alpha 4$ -PP2Ac complex. In fact, deletion of $\alpha 4$ decreases PP2Ac protein stability resulting in a loss of PP2Ac (Kong *et al.* 2009). Furthermore, it is possible that the degradation could involve binding of the A/PR65 and B subunits of PP2A, which can bind to PP2Ac in the absence of $\alpha 4$. Indeed, it has been reported that the dissociation of $\alpha 4$ from PP2Ac correlates directly with increased binding of A/PR65 to PP2Ac (Prickett and Brautigam 2004). The decrease in the level of poly-ubiquitinated PP2Ac observed in the EDD deficient MCF-7 cells, as compared to that of control cells, suggests that the mechanism by which PP2Ac levels are maintained in these cells is based on its ubiquitination by EDD. Although the post-translational modification of EDD was not examined during this study, it is of note that EDD is phosphorylated by ATM/ATR in response to DNA damage (Daub *et al.* 2008). The dephosphorylation of these sites has yet to be determined but may involve PP2Ac activity since PP2A has been shown to

dephosphorylate auto-phosphorylated ATM (Goodarzi *et al.* 2004). Thus, EDD and PP2Ac may act on each other, EDD ubiquitinating PP2Ac and PP2Ac dephosphorylating EDD.

Further evidence for the action of EDD as a RING domain E3 ubiquitin ligase is that EDD ubiquitinates and targets Paip2 for degradation, but is unable to ubiquitinate Paip1, despite both proteins containing a PAM2 domain, EDD does not target PAM1 for degradation (Roy *et al.* 2002). It has been suggested that an additional factor is required for the ubiquitination of Paip2. Analogous to $\alpha 4$ acting as an adaptor protein for the Mid-1-dependent degradation of PP2Ac, we hypothesized that $\alpha 4$ may be an adaptor for EDD-mediated ubiquitination of its substrates. However, this study showed that levels of Paip2 remain constant in $\alpha 4$ -depleted cells. In contrast, EDD depleted cells showed a marked increase in Paip2 levels. Although $\alpha 4$ does not act as an adaptor protein to facilitate the EDD-mediated ubiquitination of Paip2, $\alpha 4$ may act as an adaptor protein in EDD ubiquitination of other proteins.

It is also of note that the knockdown of EDD has no effect on $\alpha 4$ protein levels and vice versa (**Figure 13A,B**). In this study, we observed that $\alpha 4$ remained mono-ubiquitinated in EDD depleted cells (**Figure 13A**). This is in agreement with previous reports that $\alpha 4$ is mono-, but not poly-, ubiquitinated. The means by which $\alpha 4$ becomes mono-ubiquitinated remains to be discovered. However, it has been determined that it is the UIM domain of $\alpha 4$ that contains the ubiquitin monomer, which is thought to promote interaction between $\alpha 4$ and ubiquitin chains (McConnell *et al.* 2010). Taken together, the present study suggests that $\alpha 4$ acts as a scaffold for EDD and PP2Ac, whereby EDD can affect the stability of PP2Ac without affecting the stability of $\alpha 4$ phosphoprotein.

4.4 PABP- α 4 Complex

In this study, it was observed that PABP interacts with the N-terminal region of α 4. Originally, we hypothesized that EDD interacts with PABP to form the α 4-EDD-PABP complex in COS-1 cells. However, the binding of PABP to the D3 protein (N-terminal region of α 4) and not to the D5 protein (C-terminal region of α 4) indicates that PABP has the potential to bind to α 4 independently of EDD (**Figure 11B,A**).

Interestingly, D3-specific immunoreactivity in immunocomplexes was much weaker than in control lysates consisting of 5% of the protein content from the pre-immunoprecipitation lysate. Most frequently, the interaction between PP2Ac and α 4 is quite strong, and immunoprecipitation studies give strong immunoreactive signals for both PP2Ac and α 4 (Boudreau *et al.* 2002). The weak interaction observed between D3 and PP2Ac may arise from improper folding of the N-terminal protein encoded by the D3 construct, and/or the absence of key residues in the C-terminus which are required for maintaining structural integrity of the N-terminal region of α 4. In fact, C-terminal residues are required for establishing the highly ordered secondary structure of the N-terminal region of α 4 (Yang *et al.* 2007). Similarly, the apparently weaker interaction observed between D3 and PABP, as compared to that of full-length α 4 and PABP, suggests that the highly ordered secondary structure of the N-terminal region of α 4 is necessary for the binding of PABP.

Since PABP and PP2Ac do not share any similarity in primary sequence or secondary structure, they are unlikely to share the same binding site on α 4. Unfortunately, this study could not determine whether or not PABP binds to the N-terminal region of α 4

independently of the PP2Ac-binding site. Although two $\alpha 4$ constructs were created, denoted as D1 and D2, that contained only part of, or none of, the PP2Ac binding site, we were unable to express them at a concentration high enough for immunoprecipitation experiments in COS-1 cells. The interaction of $\alpha 4$ with PP2Ac involves a conserved region of $\alpha 4$ that consists of a large number of positively charged residues. These residues, in the PP2Ac-binding site are suitable for the interaction with the positively charged N-terminal region of PP2Ac (Yang *et al.* 2007). The interaction of PABP with its binding partners involves either its RRM or PABC domain region. The RRM domain of PABP interacts with the PAM1 domain of Paip2 and Paip1. The PABC domain of PABP interacts with the PAM2 domain of Paip2 and Paip1 (**See Figure 6 in the Introduction**). The $\alpha 4$ phosphoprotein itself does not contain a PAM1 or a PAM2 domain. Therefore, it is possible that PABP interacts with other unknown motifs in $\alpha 4$. We have previously observed the interaction of PP2Ac with PABP in human Jurkat T-leukemic cell lysate co-immunoprecipitation analysis (McDonald *et al.* 2010). PABP undergoes phosphorylation on multiple residues, and its phosphorylation is increased during cell cycle progression (Dephoure *et al.* 2008). It is possible that phosphorylation of PABP coincides with the changes in PP2Ac activity that can occur during cell-cycle progression (Turowski *et al.* 1995).

Although the present study established the interaction between PABP and $\alpha 4$, no functional relationship could be inferred from cell-cycle experiments. The specific interaction between $\alpha 4$ and PABP is maintained throughout the cell cycle in MCF-7 and Nb2 cells, even though the actual level of interaction could not be quantified. It is possible that the ratio of PABP-bound $\alpha 4$ changes at different stages of cell growth. In

fact, $\alpha 4$ levels decrease when Nb2 lymphoma cells are stimulated by prolactin (Boudreau *et al.* 2002), suggesting that less $\alpha 4$ is available to interact with PABP.

CHAPTER 5

Conclusion

5.1 Future Studies

The present study has provided insight into the interaction of PABP and EDD with $\alpha 4$ phosphoprotein, however, many questions still remain unanswered. A functional role for the interaction of PABP and $\alpha 4$ was not determined in this study. It will be of interest to determine if $\alpha 4$ has any effect the formation of the 5' cap or the association of PABP with the poly(A) tail of mRNA by either overexpressing or knocking down the $\alpha 4$ phosphoprotein gene. As PABP is a phosphorylated protein, it would also be of interest to determine if PP2Ac could dephosphorylate PABP, and if so, what the physiological implications of this dephosphorylation would be. Furthermore, it would be of interest to determine whether or not PABP binds to the N-terminal region of $\alpha 4$ independent of the PP2Ac. It is possible that PP2Ac has a functional interaction with PABP, and $\alpha 4$ is simply acting to bring about the interaction of these proteins.

Although $\alpha 4$ is not involved as an adaptor protein in the EDD-mediated degradation of Paip2, it may be involved in EDD-mediated ubiquitination of other proteins. Based on this hypothesis, ubiquitination levels of EDD substrates, such as β -catenin, could be determined in cells treated with si $\alpha 4$ to determine whether or not $\alpha 4$ phosphoprotein has a role in mediating the ubiquitination of these proteins.

Finally, because the $\alpha 4$ -PP2Ac complex plays a role in mTOR signalling, the effects of increasing or decreasing levels of EDD may have an effect on the phosphorylation state of 4E-BP1 or p70S6K. Similarly, the effects of either EDD gene

knockdown or overexpression on other targets of the $\alpha 4$ -PP2Ac complex, such as p53 or c-Jun, and the resulting biological implications, if any, could be determined.

5.2 Summary of Study

In summary, this study has identified that EDD and PABP interact with $\alpha 4$ phosphoprotein in MCF-7 and COS-1 cells. Through the use of $\alpha 4$ deletion mutants it was determined that EDD binds to the C-terminus of $\alpha 4$, whereas PABP binds to the N-terminus. The formation of an EDD-PABP- $\alpha 4$ immunocomplex was observed in quiescent and normal growth cells indicating that these proteins interact independently of cell cycle progression. Furthermore, it was observed that depletion of EDD in MCF-7 cells leads to an increase in PP2Ac protein levels with no corresponding change in $\alpha 4$ phosphoprotein levels. The EDD-mediated increase in PP2Ac corresponded to a decrease in the poly-ubiquitination of PP2Ac. This study provides evidence that EDD regulates the ubiquitination and stability of PP2Ac. In summary, the $\alpha 4$ partners EDD, PABP and PP2Ac interact at specific regions of $\alpha 4$. PP2Ac, but not $\alpha 4$, is a substrate of EDD. The interaction of PABP with $\alpha 4$ suggests a potential role for $\alpha 4$ in PABP-mediated initiation of mRNA translation.

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