The role of Alpha4 in regulating the PP2A family of serine/threonine phosphatases:

a structural, biochemical and cell-based approach

By

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CHAPTER 1

Background

Overview of the Protein Phosphatase 2A (PP2A) Family of Serine/Threonine Phosphatases

Reversible protein phosphorylation plays a key role in regulating signal transduction and cellular functions via modulating protein-protein interactions, as well as protein activity and localization. Protein kinases and protein phosphatases act in opposition to control protein phosphorylation levels. Proteins are phosphorylated on three major residues; serine, threonine and tyrosine, with approximately 98% of phosphorylation occurring on serine/threonine residues [1]. Serine/threonine phosphatases can be classified into three major families: the metal-dependent protein phosphatases (PPMs), such as PP2C, the aspartate-based phosphatases, and the phosphoprotein phosphatases (PP1, PP2A, PP2B, PP5, PP7) (PPPs) [2]. PP1 and PP2A are the most abundant protein phosphatases, accounting for >90% of serine/threonine dephosphorylation in the cell [3]. These studies focus on the PP2A family of serine/threonine phosphatases (PP2A, PP4 and PP6).

The PP2A family of serine/threonine phosphatases play an instrumental role in development, homeostasis and basic cellular functions including; cell growth, proliferation, apoptosis, migration, adhesion, and nutrient sensing [4–6]. The PP2A family catalytic subunits (PP2Ac, PP4c, PP6c) share approximately 60% sequence identity and are highly conserved throughout evolution, from yeast to mammals (Table 1)[5]. They share a conserved catalytic core region, a conserved C-terminal –YFL motif that is subject to post-translational modifications [7–10], and a conserved N-terminal region that regulates binding to the common PP2A- family regulator, Alpha4/Tap42 [11] (Figure 1). The conserved catalytic cores of the PP2A family of

1

	H. sapiens	*D. melanogaster*	*S. cerevisiae*
PP2Ac	PP2Acα/β	MTS	Pph21/Pph22
PP4	PP4	PP4	Pph3
PP6	PP6	PPV	Sit4
Ppg1	No known ortholog	No known ortholog	Ppg1

Table 1: Orthologs of PP2A family catalytic subunits.

A

Serine/Threonine Phosphatase
Catalytic Domain

PP1	REIFLSQPILL		KAKK
PPG1	KEMLVKESNVI		DYFL
PPH3	QELLMNEGNVT		DYFL
PP4	REILVEESNVQ		DYFL
PPH21	VDVLQFEENVK		DYFL
PP2A	KEILTKESNVQ		DYFL
MTS	KEILSKESNVQ		DYFL
SIT4	KELLMEESNIQ		GYFL
PP6	CDLLLEESNVQ		PYFL
PPV	CDILLEETNIL		PYFL

B

E42
N44
C-terminal tail

C-terminal tail
Catalytic core

C

Catalytic metal ions
E42
N44
L39
C-terminal tail
(Y307, F308, L309)

Figure 1: The PP2A family has high conservation across species and family members. A) PP2A family conservation showing residues strictly conserved in PP2A family members (red) but not in the closely related phosphatase, PP1. B) Surface rendering of level of conservation across PP2A family members with magenta being most conserved and cyan being the least conserved mapped onto the structure of PP2Ac (PDB code 2NPP). Two views rotated 180°. Left view highlights residues implicated in binding the PP2A shared regulatory subunit Alpha4 (E42 and N44) and the conserved C-terminal tail. Right view highlights the catalytic core. C) Cartoon depiction of PP2Ac shows strictly conserved residues in green and active site Mn^+ metals in red (based on PDB 2NPP). Sequence alignments done in MUSCLE [12]and structural rendering done in Chimera [13].

phosphatases are Zn^{2+} and Fe^{2+} containing metalloenzymes and extraction of these metal ions leads to inactivation and Mn^{2+} dependence [14,15]. In addition to sequence similarities, the PP2A family members are characterized by a sensitivity to enzymatic inhibition by okadaic acid and microcystin [5,6]. Substrate specificity in the PP2A family is generated through the formation of varying complexes with a wide array of regulatory subunits that modulate enzymatic activity of the catalytic subunits and subcellular localization [2–6]. Despite the high levels of sequence identity, all three of these phosphatases are essential in mammals, and the catalytic subunits have distinct roles within the cell [6,16–18].

PP2A

PP2A plays a key role in a number of cellular pathways, including cell growth, signaling, transformation, replication, transcription, protein synthesis, differentiation, DNA damage repair and apoptosis [4,19–22]. Dysregulation of PP2A has been identified in a number of human diseases, including cancer, Alzheimer's disease, and diabetes [20,21,23–27]. PP2Ac activity or expression levels or the expression levels of its regulatory subunits are downregulated in a variety of cancer cell lines and has led PP2A to be classified as a tumor repressor [20,21,28,29]. In Alzheimer's disease and other tauopathies, PP2A is commonly found to be downregulated and it is one of the primary phosphatases involved in dephosphorylating *tau* [30–33].

<u>PP2A holoenzyme composition and assembly</u>

As stated previously, substrate specificity and activity in the PP2A family is modulated by association with regulatory subunits. PP2A is the most well studied of these family members and it canonically forms a trimeric complex consisting of a catalytic subunit (PP2Ac, C subunit), a structural or scaffolding subunit (A subunit), and a variable B-type regulatory subunit that is termed the heterotrimeric holoenzyme (Figure 2A,B) [4,34]. Monomeric catalytic subunit isolated from *in vivo* holoenzymes has high nonspecific activity [35] with dimer or trimer

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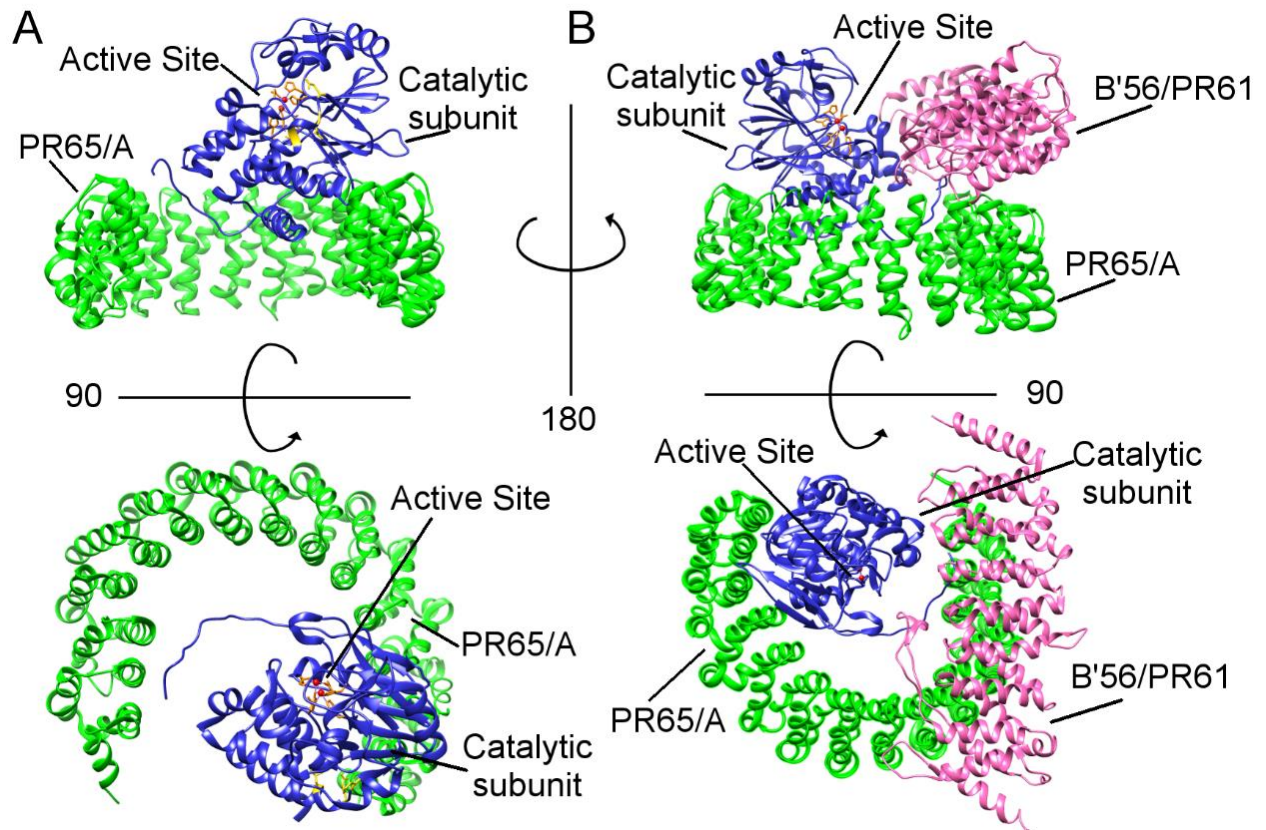
Figure 2: PP2A complex formation and regulatory subunits. A) Two views of core dimer rotated 90° out of the plane of the paper showing interface between A (green) and C (blue) subunits along with active site residues (orange), metal ions (red) B) Two views of example trimer with A (green), C (blue), and B (pink-PR61γ) (PDB 2NPP).

formation suppressing nonspecific activity and increasing substrate affinity [36]. Although,

canonically viewed as forming a trimeric holoenzyme, PP2A also is found as a dimeric complex,

termed PP2A$_D$, which accounts for approximately 30% of the total PP2A within the cell [37].

PP2A$_D$ consists of one of two catalytic subunit isoforms (PP2Acα or PP2Acβ) that share 97%

sequence identity [38,39] bound to one of two A-type subunit isoforms (PR65α or PR65β) [4].

The A subunits are all alpha-helical proteins composed of 15 terminal HEAT/ARM repeats that

form a horseshoe shaped scaffold [40] (Figure 2A). The catalytic subunit binds to the lateral

surface of this scaffold on the face opposite from its catalytic pocket [41–43]. The B-type

subunits bind to this core dimer and serve to regulate activity as well as substrate specificity and

subcellular localization [3,4,20,44,45] (Figure 2B). Disruptions in heterotrimer formation are

linked to cancer with mutations in the A subunit that affect binding to PP2Ac or the B-type

regulatory subunits found in approximately 15% of colon and lung tumors [46]. The binding of

these B subunits is regulated, at least in part, by post-translational modifications of the catalytic

subunit or the B-subunits themselves [3,8,9,47–49].

Post-translational modifications of the catalytic subunit: phosphorylation and methylation

The C-terminal conserved tail of the PP2A family of phosphatases undergoes two forms

of post-translational modifications, tyrosine phosphorylation and carboxymethylation [4] (Figure

3). In PP2Ac, phosphorylation of Y307 by various tyrosine kinases (i.e. Src, TNF-α) is reported

to lead to decreased PP2A activity [4,7,50], though this decrease in activity may be due to

subsequent alterations in the methylation state of PP2Ac and changes in B subunit binding [51].

As treatment with okadaic acid or other PP2A inhibitors block dephosphorylation, it appears that

PP2Ac undergoes auto-dephosphorylation [50,52]. Although, PP4c and PP6c have the C-

terminal tyrosine phosphorylation site conserved in PP2Ac, the role of phosphorylation has not

been extensively examined. One study looked at a phosphomimetic mutant of PP4c, Y305E,
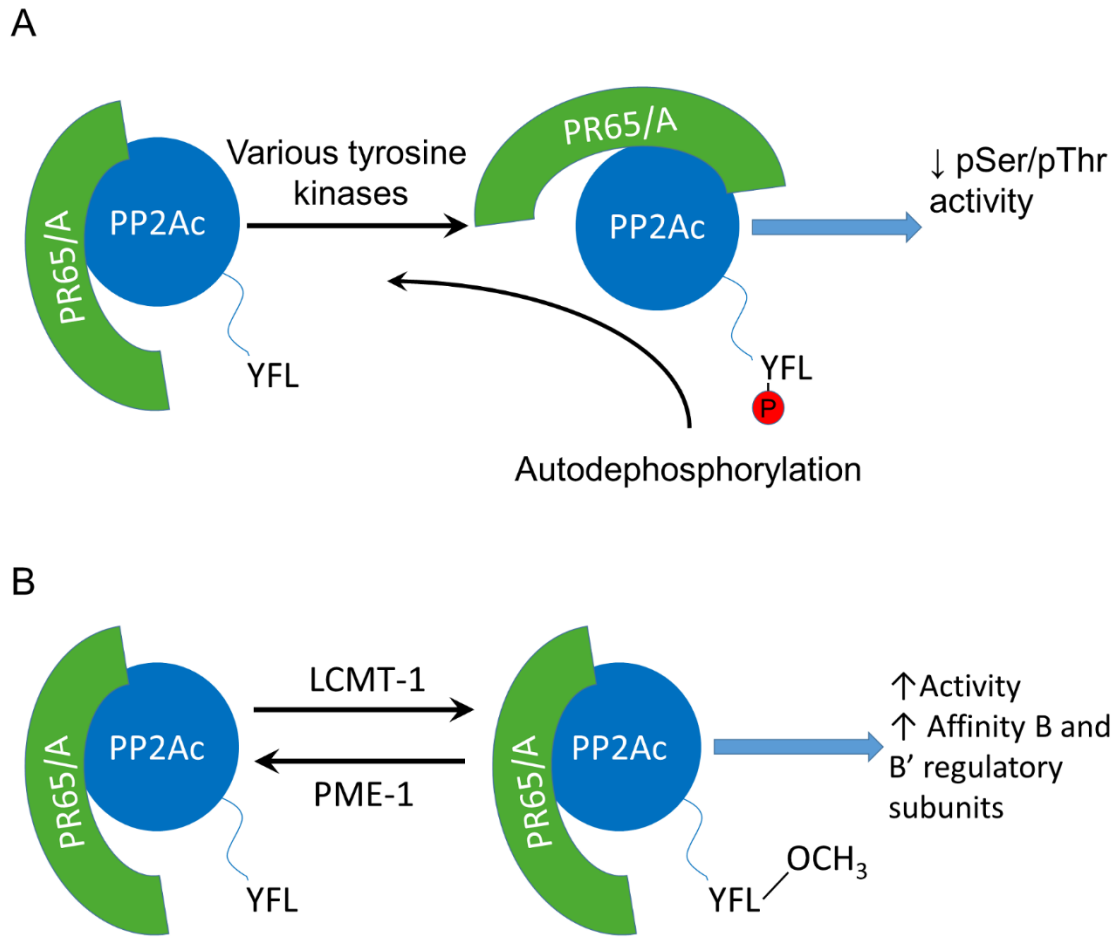
Figure 3: Common post-translational modifications of PP2A family members. Both Y307 and L309 are strictly conserved in PP4c and PP6c. A) Schematic showing phosphorylation and dephosphorylation of PP2Ac Y307. B) Schematic showing methylation and demethylation of L309 in PP2Ac.

and found that it did not affect interaction with regulatory subunits, nor did it lead to defects in DSB repair [53].

Whereas the importance of tyrosine phosphorylation of the PP2A family members is not clear, carboxymethylation of the C-terminal residue, L309, does play a critical role in regulating holoenzyme assembly. Methylation of this residue regulates interaction with regulatory subunits in both PP2A [8,9] and PP4 [53,54] and is a conserved means of regulation from yeast to mammals [55]. It has not been demonstrated that PP6c undergoes a similar methylation, but given the strict conservation of this leucine residue carboxymethylation may play a role in PP6c regulation.

Effects of catalytic subunit methylation on regulatory subunit association

The B-type regulatory subunits of PP2A have been classified into four families based upon sequence homology and structural similarity (PR55/B, PR61/B', PR72/B'', Striatin/B''') [4,49,56–59] (Figure 4, Table 2). Methylation of PP2Ac alters the affinity of certain regulatory subunits for the catalytic subunit [47] and thus the methylation state of L309 of PP2Ac can alter holoenzyme composition and PP2Ac activity [8,9,48,60–62]. [63]. The affinity of B regulatory subunits to methylated PP2Ac varies between families with B' having a 5-fold greater affinity for methylated PP2Ac than PR55α [8] and methylation being unnecessary for complexes containing striatin [48,61,62]. However, the requirements of various B-type regulatory subunits for methylation of the catalytic subunit to promote binding are controversial. Some studies state that association of the B (PR55) subunits with $PP2A_D$ requires methylation [51]; whereas others find that formation of this trimer is independent of methylation [8,64,65]. Similar discrepancies are evident in the literature with other regulatory subunits of PP2A. Methylation also plays a critical role in regulating PP4c association with its regulatory subunits (R1, R2, R3) and is induced by DNA damage [53]. A methylation deficient mutant of PP4c, L307A, PP4c is

B/PR55

B'56/PR61

B''/PR48/PR72
PR130/G5PR

Ca^{2+}

B'''/Striatin/SG2NA
PR93/PR110

STRIPAK Complex

PP2A A

PP2A C

Coiled-coil

P

GCKs

Adaptor

striatins

WD40
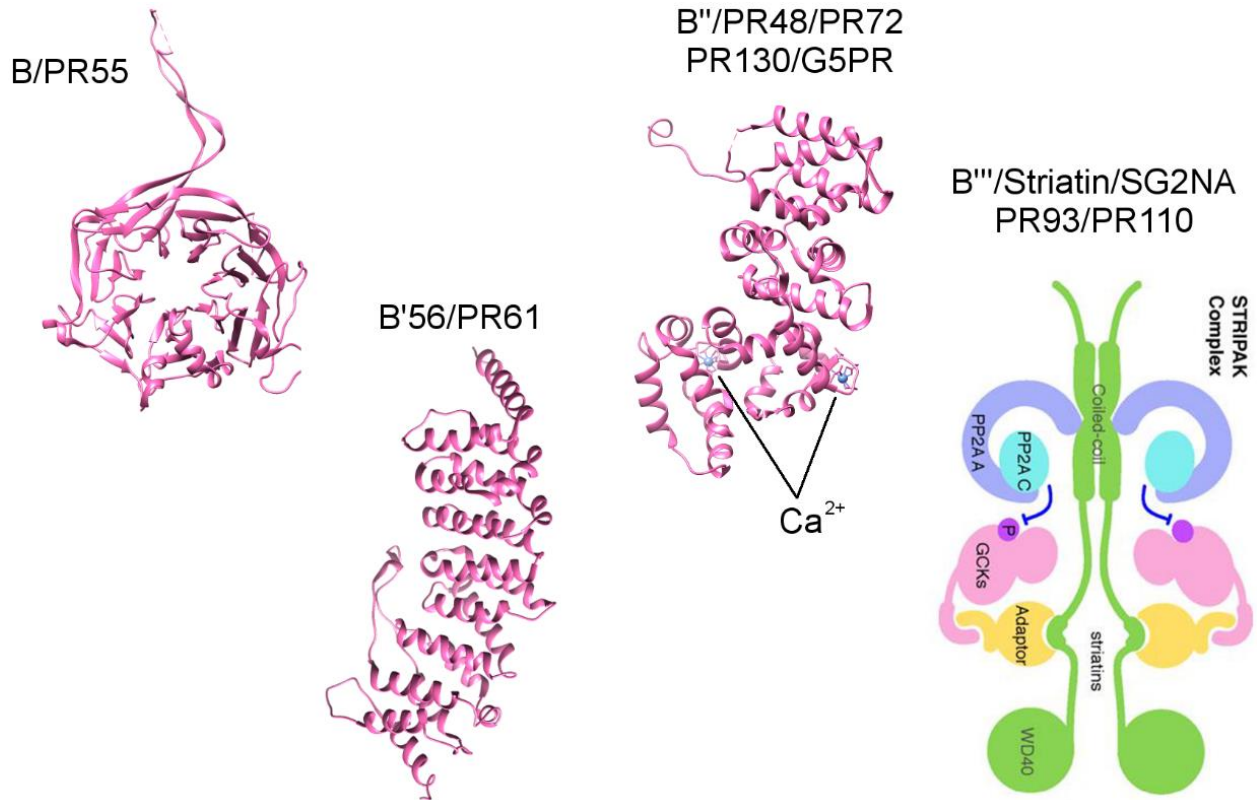
Figure 4: The four major families of B-type regulatory subunits of PP2A. B-type regulatory subunits showing structural diversity (PDB 3DW8 (B55), 2NPP(B'56), 4I5N (PR72)) and striatin(image from [56]). Structural rendering done in Chimera [13].

	B/PR55	B'/PR61/B56	B''	B'''
Isoforms	4 (α,β,γ,δ)	7 (α, β, γ1, γ2, γ3, δ, ε)	PR48, PR70, PR72, PR130	SG2NA, Striatin, PR93/PR110
Defining structure	WD40 repeat	Alpha-helical, highly acidic concave face	Ca^{2+} binding EFX -domain	Multi-domain scaffold (WD40 repeat, Calmodulin binding, membrane binding, caveolin binding, coiled-coil domains)
Regulation		Phosphorylation	Calcium	Dimerization
Effects of methylated PP2Ac	Increased binding	Increased binding	No change	No change
Targets	vimentin, EDD, tau, Akt, c-Jun, Raf, CaMKIV, others	MDM2, p53, paxillin, AMPKα, cyclin G	Rb, p107, cdc6, Naked	MOB1

Table 2: Characteristics of B-type regulatory subunit families

incapable of interacting with its substrates KAP1 or 53BP1 *in vivo* and impairs DSB repair both by HR and NHEJ, but retains its capacity to dephosphorylate these substrates *in vitro* [53]. These data indicate that methylation effects complex formation, rather than the inherent catalytic capacity of PP4c.

Regulation of catalytic subunit methylation by cellular processes and disease

Due to the role of methylation in regulation of heterotrimer composition, methylation of PP2Ac is important to a number of cellular processes. Methylation of PP2Ac is regulated during the cell cycle with cytoplasmic PP2A being demethylated at the G_0/G_1 boundary and remethylated when cells entered S phase, while nuclear PP2Ac is demethylated when cells enter S phase [66]. The methylation state of PP2Ac is also dysregulated in a number of diseases. In Alzheimer's disease, estrogen deficiency and Aβ production both decrease PP2Ac methylation which corresponds to an increase in tau hyperphosphorylation [67]. PP2Ac also undergoes demethylation during ischemic reperfusion leading to increased phosphorylation of ERK1/2 [68]. In diabetes, PP2Ac methylation and activity play an integral role in glucose homeostasis. Inhibition of carboxymethylation and PP2Ac activity increases glucose-induced insulin secretion in islet cells [27], and increased carboxymethylation leads to islet dysfunction [69]. In addition, glucose metabolites or high glucose levels (10-25mM) decrease PP2Ac methylation, but basal levels of glucose (5mM) increase methylation [70]. Clearly, regulation of PP2Ac methylation is involved in glucose homeostasis and diabetes, both in regulating insulin production and being regulated by glucose levels.

Regulation of methylation

It is apparent that methylation of PP2Ac and PP4, and possibly PP6, play a key role in regulating cellular pathways and alterations in methylation state can have dramatic consequences. In mammals, leucine carboxyl methyltransferase 1/PP2A methyltransferase

(LCMT-1/PPMT), an S-adenosylmethionine dependent methyltransferase [71], is responsible for methylation of the terminal leucine residue in PP2Ac and presumably PP4c [60]; while protein phosphatase methylesterase 1/PP2A-methylesterase 1 (PME-1/PPME) removes the methyl group [72] (Figure 3B). The *in vivo* substrate for both LCMT-1 and PME-1 is the dimeric form of PP2A, $PP2A_D$, [8] but $PP2A_D$ is primarily unmethylated due to rapid demethylation by PME-1 [64]. Alteration in the levels of these enzymes has profound effects upon cell growth, proliferation and apoptosis [9,60]. PME-1 negatively regulates PP2A activity and its overexpression promotes both cellular proliferation and anchorage-independent growth [73]. Conversely, knockdown of PME-1 decreases cellular proliferation due to increased rates of cellular senescence, as evidenced by increased β-galactosidase levels, not via increased apoptotic cell death [73]. The role of LCMT-1 in cellular growth is less clear. Knockout of LCMT-1 is embryonic lethal [60], but knockdown of LCMT-1 simultaneously increases levels of apoptosis [60,62] and increases anchorage-independent growth [62]. The dual role of LCMT-1 in promoting both death and proliferation pathways could be due to opposing roles of the various PP2A family members or to dysregulation of cell cycle checkpoints that lead to decreased DNA damage repair and thus increases in apoptosis at the same time as increased proliferation. The oncogenic proteins, polyomavirus middle (PyMT) and small (PyST) tumor antigens and SV40 small tumor antigen (SVST) all bind PP2Ac in a methylation insensitive manner and replace select B regulatory subunits, thereby blocking PP2Ac function and leading to cellular transformation [62]. The effects of PyMT and PyST oncogenic proteins on PP2Ac function and cellular transformation can be mimicked by altering the balance between LCMT-1/PME-1 expression levels thus changing the methylation state of PP2Ac and its association with B regulatory subunits [62]. .In addition to the direct effects on PP2Ac methylation state, LCMT-1 also alters holoenzyme composition by upregulating expression of the Bα family of regulatory subunits [62,69,74].

PP2A endogenous inhibitory proteins

In addition to modulation by post-translational modifications and regulatory proteins, PP2Ac function is also controlled by the endogenous inhibitory proteins, cancerous inhibitors of PP2A (CIP2A) [75] and inhibitor 2 of PP2A (I_2PP2A/SET) [76]. These proteins are upregulated in a number of human malignancies [75,77–82] and drugs that target these PP2A inhibitory proteins have been shown to be effective in reducing cell growth, proliferation, and metastasis [79–81]. Upregulation of CIP2A increases c-myc expression [81], as well as playing a role in regulating Akt activity and the mTOR pathway [78,83–85]. Knockdown of CIP2A or SET decreases cell proliferation and colony forming potential [81,85], but does not cause apoptosis [82]. Knockdown of CIP2A and SET proteins also sensitize cells to oxidative stress [82], as well as cisplatin and other genotoxic drugs [85,86]. In addition to these effects on cellular growth and proliferation, SET overexpression and mislocalization has been implicated in tau hyperphosphorylation via inactivation of PP2A [30,87–89].

PP4

PP4 modulates a number of important cellular processes and pathways, including NF-κB activity [90,91], B-cell development [92], T-cell development and signaling [18,93], DNA damage repair [92,94–99] , TNF-α signaling [100], JNK activation[101], hematopoietic progenitor kinase-1 activation and expression [102], microtubule organization [103,104], and apoptosis [18,92,99,105,106]. The catalytic subunit of PP4, PP4c, is ~65% identical to PP2Acα and PP2Acβ. PP4c is ubiquitously expressed in all tissues with 3-fold higher levels in the testis [107]. It is present throughout the nucleus and cytoplasm, but is especially localized to the centromeric region [100,104,107]. Knockout of PP4 is lethal in mice due to defects in neocortex development caused by misorientation of the mitotic spindle [108] and increased severing of microtubules due to katanin p60 centrosomal mislocalization [103]. Knockdown of PP4c by 70% leads to cellular arrest at the pro-metaphase/metaphase boundary and increases the

incidence of chromosomal abnormalities[109]. Similar to PP2Ac, PP4c forms complexes with

regulatory subunits, with each complex having its own substrate specificity and subcellular

localization (Figure 5).

PP4 regulatory subunits and holoenzyme complexes

Five regulatory subunits of PP4 have been identified that are distinct from those of PP2A

(PP4R1, PP4R2, PP4R3α, PP4R3β, and PP4R4) [110–112], in addition to subunits shared with

other PP2A family members (Alpha4, TIP, and PTPA) [6,110,112] (Figure 5). The PP4/PP4R2

complex is involved in p53 checkpoint signaling via dephosphorylation of DBC-1 leading to

inhibition of apoptosis [99] and activation of KAP1 which represses p21 transcription [113].

Inhibition of CHK1 prevents recruitment of PP4/PP4R2 complex to replication forks contributing

to replication protein A2 (RPA2) hyperphosphorylation and apoptosis [114]. The heterotrimeric

complex containing PP4/PP4R2/PP4R3 localizes to centrosomes and regulates centrosome

maturation [109,111,115] and meiosis [95,116,117]. The PP4 complex of PP4c/PP4R2/PP4R3

is also involved in conveying cisplatin resistance and knockdown of any of these subunits

confers an increased sensitivity to cisplatin [112,118,119]. Cisplatin is a genotoxic agent often

used in cancer therapies, however several cancer cell lines show resistance to cisplatin based

therapies. This cisplatin resistance phenotype may be in part due to the integral role that PP4

has in regulating DNA damage repair and cell cycle checkpoints.

PP4 and DNA damage repair

PP4 plays a critical role in regulating the repair of damaged DNA that results from

normal DNA replication events and from exposure to toxic, exogenous agents. It is integral in

regulating both homologous recombination (HR) [95,96] and non-homologous end joining

(NHEJ) [92,98] of double-stranded DNA breaks (DSBs), as well as checkpoint signaling

Shared PP2A family subunits

- Phosphatase Inhibition

- Centrosomal/Nuclear
- Centrosome maturation
- Mitotic Spindles
- Checkpoint Signaling
- Spliceosome assembly
- SMN complex

- Phosphatase activation
- Transcription regulation

hTIP41

PTPA

PP4R2

PP4c

- Cisplatin sensitivity
- DNA damage repair
- γH2AX

CCT/ TRiC

Alpha4

PP4R3α PP4R3β

- TOR signaling

- DNA damage repair
- 53BP1, KAP-1

PP4R1

PP4R4

- NF-κB activation
- IRS-4 downregulation
- HPK-1
- Apoptosis
- Proliferation

- Cell Cycle regulation
- NF-κB inhibition
- JNK activation
- HDAC3, NDEL1

- Highest in brain
- Cytoplasmic localization
- Unknown substrate

Specific PP4 subunits

Figure 5: PP4 regulatory subunits show specificity and differential localization. PP4c catalytic subunit is shown as a blue circle. Solid arrows denote experimentally verified interactions in mammals. Blue dotted arrows show interactions shown with yeast homologs. Subunits in peach ovals are PP4c specific, subunits in green hexagons are shared with other PP2A family members. Light yellow ovals denote known trimeric complexes. Also included are bullet points regarding what is known about the indicated protein complexes.

[94,113,120]. Overexpression of PP4c and the resulting increased dephosphorylation of 53BP1 during mitosis leads to mitotic defects evidenced by lagging chromosome and micronuclei which can be blocked by inhibiting NHEJ [97]. Conversely, PP4c knockdown increases basal levels of histone 2A variant phosphorylated at S139 (γH2AX), a marker of DSBs, and delays resolution of γH2AX positive foci induced by DNA damaging agents [94,96]. This leads to cell cycle arrest and an increase in the number of cell in G1 phase [92,94,95,113]. The complex of PP4c/PP4R2/PP4R3β is involved in HR repair of DSBs that occur during DNA replication and knockdown of any of these components impairs DSB repair [96].

PP4 and cellular growth and proliferation

PP4 is upregulated in a number of cancers [119,121] and overexpression of ectopic PP4 in ECC-1, an endometrial cancer cell line, leads to increased cell proliferation [73]. In addition, conditional knockout of PP4c in thymocytes leads to increased apoptosis and decreased cell proliferation [73], as does knockdown of PP4c or various regulatory subunits in a variety of different cell lines [96,109,119,122]. Most studies indicate that PP4 plays an anti-apoptotic role, pro-growth role in cells, but some studies in leukemic and peripheral T-cells do show a proapoptic role [105,106]. These studies indicate that while knockdown of PP4 increases resistance to apoptosis, it also increases the number of mutations when exposed to UV [106]. This increase in mutations is likely due to the role of PP4 in DNA damage repair.

_PP6_

Protein phosphatase 6 is ubiquitously expressed [17,123] and knockout of the catalytic subunit, PP6c, is embryonic lethal [124,125]. In yeast, Sit4 (the PP6c homolog) plays a critical role in G1 to S phase progression [126–129], response to mtDNA damage [130], TOR signaling [131], and ER-to-Golgi transport [132]. In higher eukaryotes, PP6 is involved in regulating DNA damage repair [133–136], mitosis [134,137,138], autophagy [139], cell death [123,133,140–

142], and inflammatory signaling [17,124,125,136,143]. As is the case for the other PP2A family members, PP6 forms holoenzyme complexes consisting of a catalytic subunit, PP6c, and at least one regulatory protein. In yeast, four regulatory proteins have been identified that are unique to PP6 (SAP155, SAP185, SAP190, and SAP4) [126,128]. The yeast SAPS have been classified into two groups based on sequence homology and the ability to rescue defects caused by deletion of genes [126]. In mammals, two different classes of regulatory proteins have been identified, SAPS regulatory domain containing subunits (PP6R1, PP6R2, PP6R3) that bind directly to the catalytic subunit [17], and ankyrin repeat domain containing subunits (ANKRD28, ANKRD44, ANKRD52) that bind to the C-terminal domain of the SAPS subunits [144] (Figure 6A). The human PP6R2 and PP6R3 proteins and to a lesser extent PP6R1 can rescue growth defects in SAP deficient yeast mutants, but do not restore all functions [145].

Knockdown or loss-of-function mutations of PP6 lead to increased mutation rates and increased tumor progression. Conditional knockout of PP6c increases tumor induction and progression when cells are exposed to either dimethylbenzanthracene or UVB radiation [124,142] and overexpression of ectopic PP6c leads to a decrease in cell proliferation in ECC-1 cells [73]. Knockdown of PP6 also leads to defects in mitosis causing micronucleation and chromosome instability [137,138]. PP6 activity is important in repairing DSBs via both HR and NHEJ through interactions with different SAPS-containing regulatory subunits [133–136]. In addition to its roles in mitosis and DNA damage repair, PP6 suppresses cancer metastasis through regulation of E-cadherin surface expression[146]. Large scale screens for mutations associated with tumors found that PP6c is mutated in approximately 10% of melanomas [147–149]. Further investigation of these mutations reveal that they disrupt the interaction of PP6c with its canonical regulatory subunits [136]. This disruption leads to decreased PP6c activity, manifesting in increased Aurora A kinase activity and increases in γH2AX [133,134,136,142]. Paradoxically, these mutations lead to increases in expression from the non-mutated PP6c allele and increases in PP6c activity towards certain substrates, such as GCN2 which increases
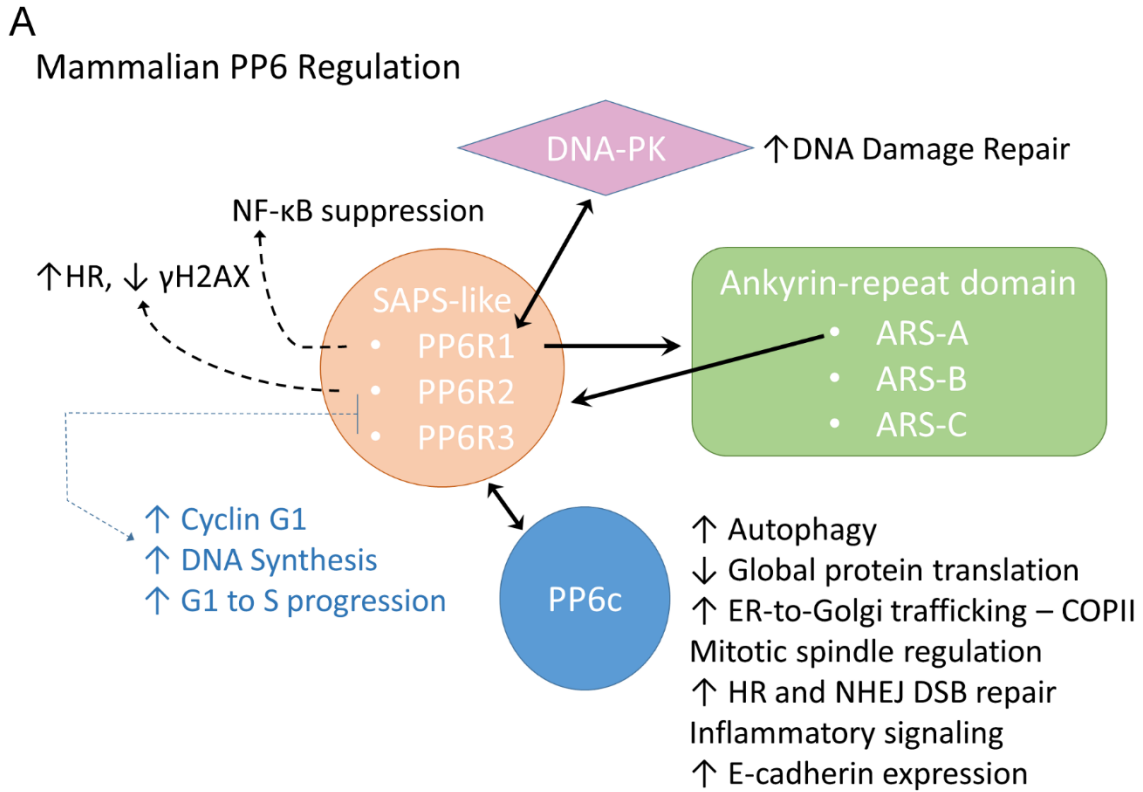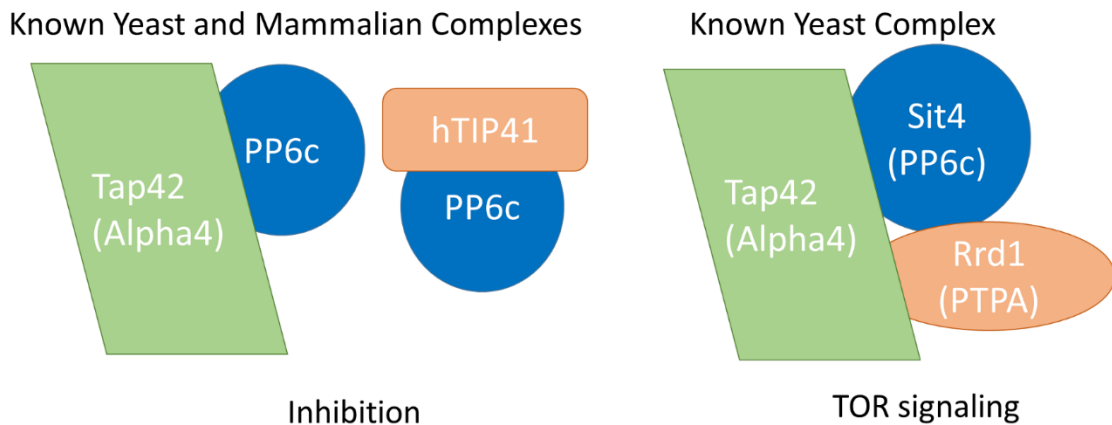
Figure 6: PP6 regulatory subunits show substrate specificity. A) PP6 specific regulatory subunit complexes. Solid arrows denote experimentally verified interactions in mammals. Effects of particular subunit complexes shown adjacent to specific complexes (black mammals and blue yeast) or with dotted arrows. B) Complexes formed with the PP6 catalytic subunit and regulatory subunits shared with other PP2A family members. Both Tap42 and Tip41 complexes inhibit PP6c activity. The Tap42-Sit4-Rrd1 complex has been implicated in regulating TOR signaling.

autophagy [139]. Although PP6 itself is a tumor repressor, knockdown of PP6 can lead to increased radiation sensitivity in cancer cells due to the decreased ability to repair ionizing radiation (IR) induced DNA damage [135]. Therefore, knockdown or inhibition of PP6 could prove beneficial in cancer therapies when combined with IR based therapies.

Biogenesis and activation of PP2A

In addition to the role of PME-1 in demethylation, PME-1 is associated with an inactive pool of PP2A, PP2Ai, that requires activation by the phosphoprotein tyrosine phosphatase 2A activator (PTPA) [63,150] (Figure 7). This inactive pool of PP2A can also be activated by incubation with 1 µM Mn^{2+} [151], this metal-dependent form has increased phosphotyrosyl activity [14,15,152–156] and metal-free apoenzyme is less stable and tends to partially unfold allowing binding of Alpha4 [152] and preventing binding of the A subunit. PME-1 associates stably with two inactive mutants of PP2Ac, H59Q and H118Q, both residues located in or near catalytic site of PP2Ac [72]. These mutants bind to A and PyMT, but poorly to B subunits [72], indicating that a B subunits have lower affinity for these inactive forms. The ability of PME-1 to bind to the inactive mutants indicates that PME-1 might serve a role in regulating PP2Ac stability after loss of one or more catalytic metal ions, preventing its unfolding and association with Alpha4 (Figure 8A).

PTPA is a highly conserved, essential protein [156–162] that was initially described as activating the phosphotyrosyl phosphatase activity of PP2A via a process that requires Mg^{2+} and ATP [63,163]. More recent investigations have uncovered an essential role for PTPA in proper folding and activation of the phosphoserine/phosphothreonine phosphatase activity of PP2A [156]. PTPA binds to PP2A$_D$, but not the heterotrimeric holoenzymes [164]. PTPA also stably binds to a catalytically inactive mutant of PP2Ac, H59S [156]. The PTPA-PP2A complex forms a composite ATPase with ATP hydrolysis being required for PP2Ai activation [151,163,165] (Figure 8B). The ATP head group binds within a conserved deep pocket on PTPA, while the
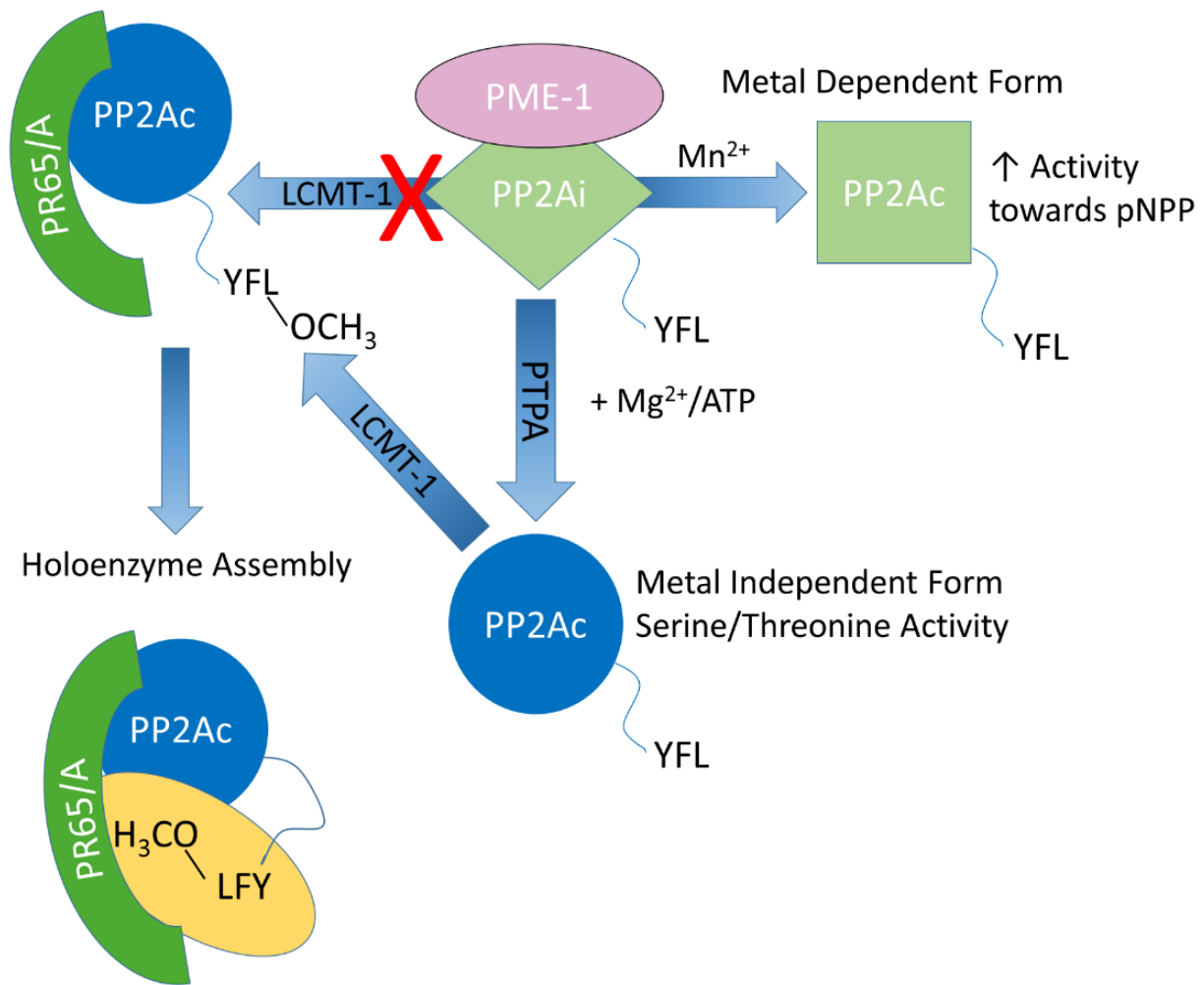
Figure 7: PME-1 and PTPA act to stabilize and reactivate an inactive pool of PP2Ac.

Schematic showing stabilization of inactive pool of PP2Ai by PME-1 and reactivation by Mn^{2+} or PTPA, but not by LCMT-1.
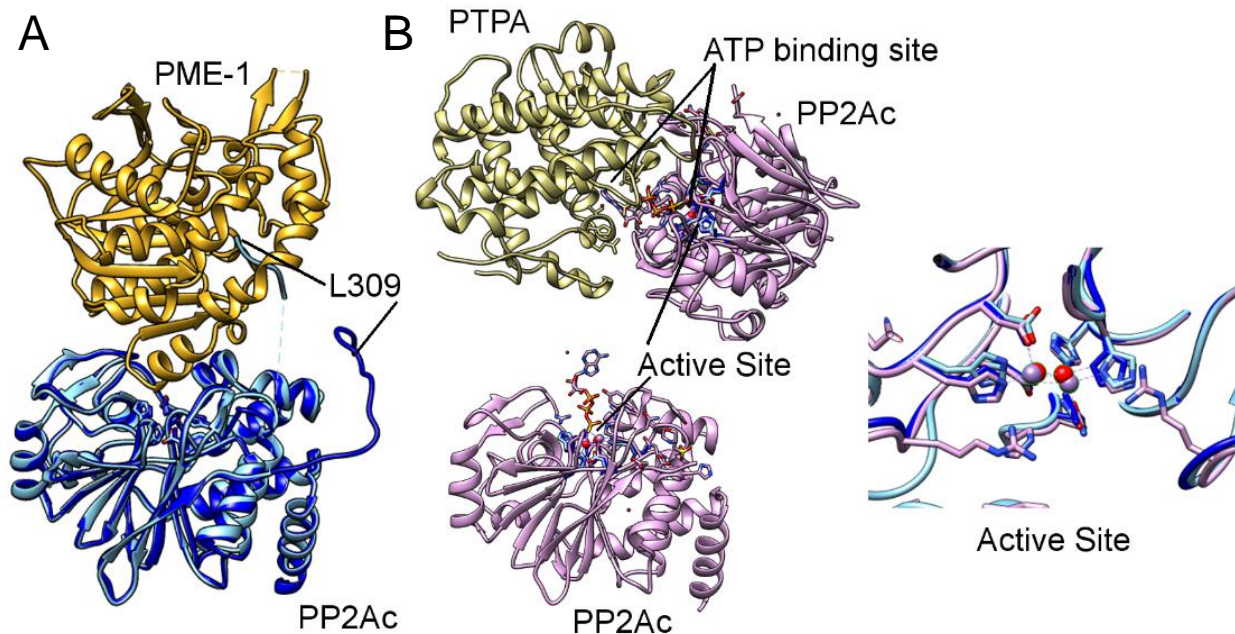
Figure 8: Interaction of PME-1 and PTPA with the active site of PP2Ac. A) Cartoon showing structure of PME-1/PP2Ac complex showing insertion of C-terminal tail of PP2Ac (cyan) into pocket within PME-1 (gold) and interaction of PME-1 with both C-terminal tail and active site residues of PP2Ac. PP2Ac has been overlaid with apoform of PP2Ac (blue) from trimeric holoenzyme (PDB 2NPP). B) Cartoon of PTPA/PP2Ac complex (top) with PTPA (beige) and PP2Ac (light purple) showing shared ATP binding site at interface. View of ATP bound into active site of PP2Ac (bottom). Close-up of active site residues with apoform (blue), PME-1 bound form (cyan) and PTPA bound form (light purple) of PP2Ac showing variation in orientation of residues involved in binding the catalytic metal ions (red/lavender spheres). Structural rendering done in Chimera [13]

γ-phosphate of ATP makes contacts with the metal binding residues within the PP2Ac active site [165]. The interface between PTPA and PP2Ac is highly conserved across the PP2A family. Loss of PTPA leads to a less stable form of PP2A that has increased tyrosine phosphatase capabilities and dependence on Mn^{2+} [63,156,166].

PTPA and its yeast homologs also have peptidyl prolyl cis/trans isomerase activity that is stimulated by the addition of Mg^{2+} and ATP [10,167]. Proline 190 of PP2Ac has been shown to be an *in vitro* substrate of PTPA and mutation of this residue renders PTPA incapable of activating PP2Ac phosphatase activity [10]. Crystal structures of Ypa1 (a yeast homolog of PTPA) in complex with a prolyl containing peptide revealed a homodimer with the peptide bound at the interface [168]. The residues that form this interface are highly conserved across species and mutations lead to a loss of function both in *in vitro* isomerase assays and in the ability to activate PP2Ac phosphatase activity [10,160,168]. Interestingly, PTPA-activated PP2A has properties similar to forms of PP2A bound to the viral antigens that lead to cell transformation [169].

PTPA is a ubiquitous protein that is expressed in all tissues tested [151]. In yeast, PTPA homologs (Ypa1/Ypa2) play a role in cell cycle progression [157–159], transcription [170] and rapamycin resistance [158,171]. These two related proteins exhibit different, but overlapping functions. Ypa1 associates preferentially with Ppg1, Sit4 and Pph3; whereas, Ypa2 prefers binding to Pph21 and Pph22 [164,171]. Ypa1 expression can reactivate mammalian PP2Ai, but ypa2 cannot; denoting a difference in the activities of these two proteins [171]. Mammalian PTPA has six splice variants, yielding four functional proteins with two of these PTPAα and PTPAβ shown to expressed in tissues [172]. Its transcription is downregulated by p53 indicating that it may play a role in apoptosis regulation [162], as many of the proteins regulated by p53 are involved in cell survival and apoptosis. Overexpression of PTPA decreases PP2Ac phosphorylation at Y307, increases phosphotyrosine phosphatase 1B expression, increases methylation of PP2Ac and decreases tau phosphorylation, whereas knockdown of PTPA has

the opposite effects [173]. Both knockdown [156] and overexpression of PTPA [174] lead to increased apoptosis, indicating that proper regulation of this protein is essential to cell function.

Tip41 and Alpha4 – Shared PP2A family regulatory subunits

Canonical subunits of the PP2A family of phosphatases (e.g., PR65, PR55, PP6R1, PP6R2, PP4R1, PP4R2) interact with a specific PP2A family member and these subunits are not shared between family members. Two non-canonical subunits have been identified that interact directly with the catalytic subunit of all members of the PP2A family in the absence of canonical regulatory subunits [112,175,176]. TIP41 was identified in yeast as a Tap42 interacting protein that regulates TOR signaling by interacting with Tap42 and suppressing its interaction with the PP2A family phosphatase catalytic subunits [177,178]. In yeast, TIP41 regulates transcription through repression of RNA polymerase III [179] and activates Sit4 and PP2A activity towards Gln3 [177] and Msn2 [180]. Tip41 also forms complexes with Pph3 and Ypa1 [179], though these interactions have yet to be demonstrated in higher eukaryotes.

In higher eukaryotes, TIP41 only appears to interact directly with the catalytic subunit of the PP2A phosphatases to inhibit activity rather than enhancing activity by relieving Alpha4 inhibition [94,175]. TIP41 has been implicated in regulating DNA damage repair through regulating phosphorylation of an ATM/ATR substrate [175], and in regulating mTORC1 signaling by repressing PP2A activity through disassociation of the PP2A/PR65 complex [181]. It is unclear whether TIP41 forms trimeric complexes with the catalytic subunit of PP2A family phosphatases and Alpha4. Some studies have found no interaction between TIP41 and Alpha4 [112,175], whereas other have detected the formation of a heterotrimer [181,182]. Interestingly, in higher eukaryotes TIP41 appears to favor association with the methylated (activated) form of PP2Ac, whereas Alpha4 appears to favor the unmethylated form [181]. This argues for two separate complexes and for TIP41 to play a role in suppressing promiscuous activity of active monomeric catalytic subunit, whereas Alpha4 could play more of a role in biogenesis or

reactivation of the proper catalytic activity. The role of Alpha4/Tap42 will be explored more

thoroughly in later sections.

Alpha4: A Shared PP2A family regulatory protein

Alpha4 was first described as a B-cell receptor (BCR) interacting protein of 52 kDa from

murine cells [183,184]. It was shown to be phosphorylated *in vivo* by PKC and to associate with

a tyrosine kinase upon BCR cross-linking [184]. Identification of the human Alpha4 gene

revealed a 45 kDa protein with high similarity to murine Alpha4, yeast Tap42, and *Drosophila*

Tap42 [185] (Figure 8). This protein is ubiquitously expressed in a wide range of tissues

[110,185] with its expression pattern generally paralleling the expression patterns of PP2Ac,

PP4c and PP6c [110]. The Alpha4 gene is located at Xq13.1-13.3 and the 5' flanking region of

DNA contains several potential binding sites for regulatory proteins [186]. A closely related

gene, Alpha4-b, has been identified and the gene product found to be highly expressed in the

brain and testis [187]. In the brain, the expression is confined to neuronal cells and is especially

concentrated in the hippocampus [187]. Alpha4-b is 66% identical to Alpha4 and has been

shown to bind to PP2Ac [187]. Alpha4 contains a number of evolutionarily conserved predicted

phosphorylation, N-glycosylation and myristylation sites, as well as a predicted SH3-binding

motif [185] (Figures 9, 10A). The carboxy-terminal 21 amino acids of Alpha4 are also very

highly conserved across higher eukaryotes, but the function of this region of the protein has not

been determined [185] (Figure 9, 10A).

Alpha4/Tap42 has been shown to bind directly to all of the PP2A family catalytic

subunits [110,176,188]. Initial studies using yeast two hybrid screens and direct binding assays

found that the PP2A family of phosphatases all contain a conserved N-terminal motif that is

essential for binding to Alpha4/Tap42, which in Sit4 were L35, E38 and N40 [11,176] (Figure 1).

Mutational analysis showed that an L35A mutation highly reduced binding of Alpha4 to Sit4 and

that a double mutation to alanine of residues E37 and E38 completely abolished binding, as did
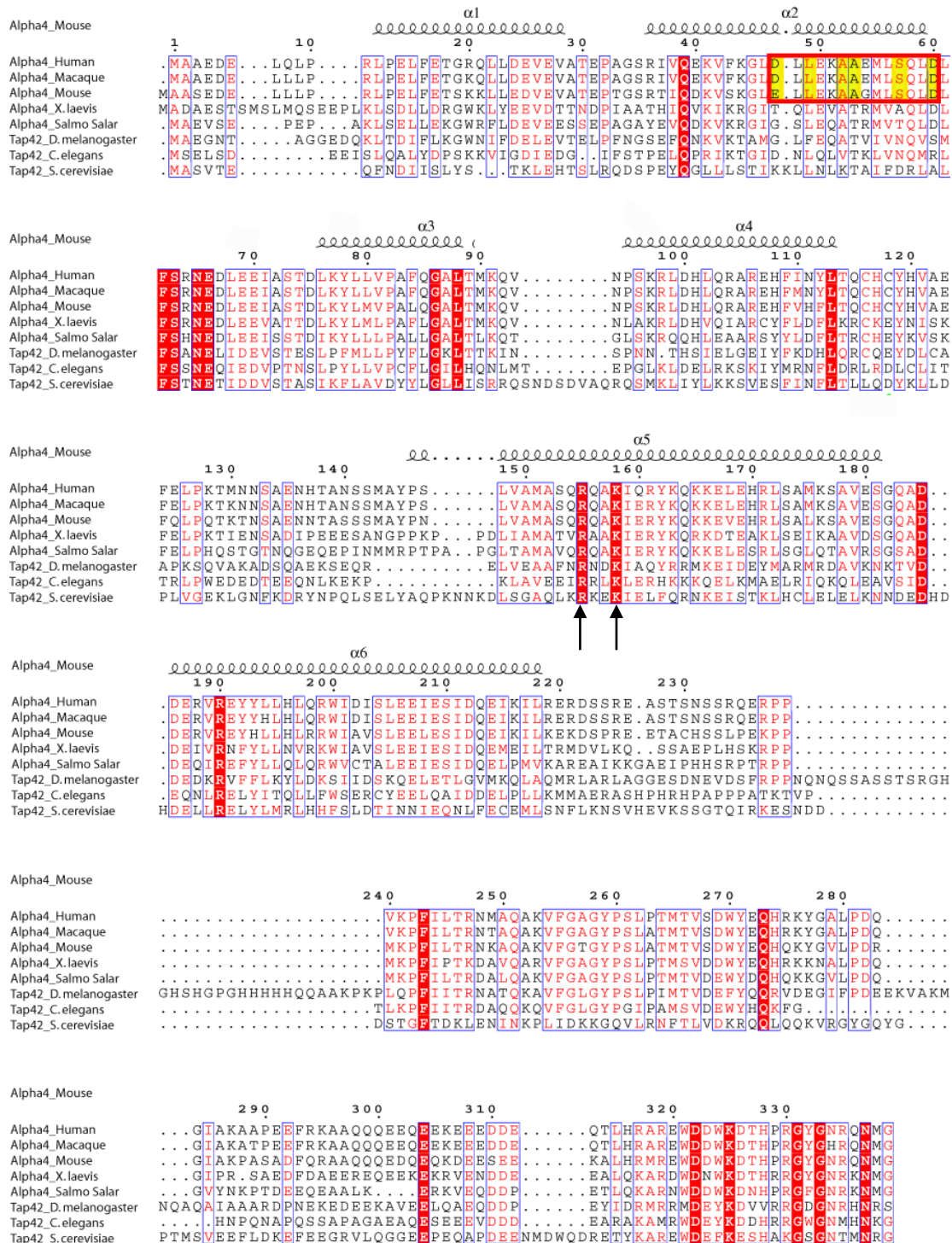
Figure 9: Multiple sequence alignment of Alpha4/Tap42. Conserved residues (as determined by %Equivalent set at 0.7 in ESPRIPT [189]) are colored red and invariant residues are white on a red background. The black arrows indicate the residues essential for binding to PP2Ac. UIM consensus in mammalian species with conserved residues highlighted in yellow and the motif boxed in red. Figure was created using CLUSTALW [190] and ESPRIPT [189]. Sequence database IDs are as follows: *H. sapiens* - CAG33063.1, *M. mulatta* - *NP_001182718*, *M. musculus* - EDL14183.1, *X. laevis* - NP_001084735, *S. salar* - NP_001140137, *D. melanogaster* - NP_723811.1, *C. elegans* - NP_497591.1, and *S. cerevisiae* - NP_013741.
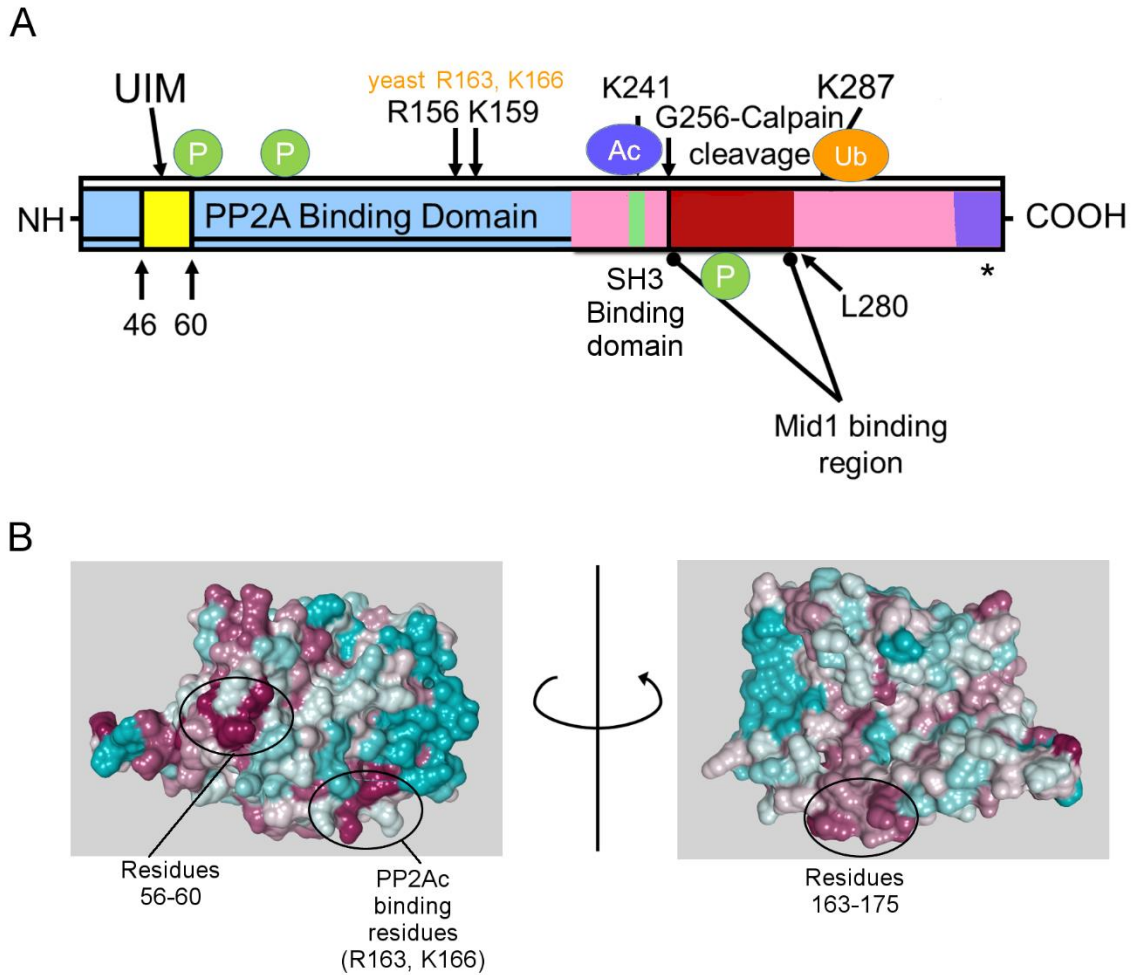
Figure 10: Alpha/Tap42 domain structure and conserved residues. A) Schematic representation of Alpha4 domain structure showing sites of predicted post-translational modifications [185]. PP2Ac binding region is in light blue with arrows indicating residues shown to be critical. UIM consensus region is highlighted in yellow. A conserved SH3-binding region is shown in light green. The C-terminal intrinsically disordered region is shown in pink with the area mapped to bind to Mid1 in maroon. The highly conserved C-terminal tail is indicated in purple. The calpain cleavage site between F255-G256 [191]is identified by a black arrow. Conserved consensus sites for phosphorylation are shown with green circles. Experimentally determined acetylation (blue)[192] and ubiquitination [193] (orange) site are shown with ovals. The asterisk (*) denotes a conserved possible myristylation site. B) Surface rendering of level of conservation in Alpha4 across species with magenta being most conserved and cyan being the least conserved mapped onto the structure of Tap42 (PDB code 2V0P) using MUSCLE multiple sequence alignment [12] and Chimera [13], two views rotated 180°.

a reverse of charge mutation E38K [11]. Prickett *et al.* found that mutation E42A (homologous to E38 in Sit4) in PP2Ac to have a similar negative effect on binding to Alpha4 [194]. In addition, fusion of an N-terminal 55 amino acid construct of *Drosophila* PPV (Sit4/PP6) containing the proposed Alpha4 binding residues to the catalytic domain of *Drosophila* PP1 was able to complement the Sit4-102 mutant in yeast [195]. Another study found that residues 19-22 and 150-165 in PP2Ac are required for binding to Alpha4 using deletion mutants, it is possible that PP2Ac contains multiple interaction sites with Alpha4 that all contribute to binding [196].

Both Alpha4 and Tap42 contain a highly conserved and structurally stable N-terminal domain that binds to the PP2A family catalytic subunits and an unstructured C-terminal domain [197]. Analysis of Tap42 and Alpha4 by circular dichroism reveal a predominantly alpha-helical structure with the C-terminal domain appearing to be an intrinsically disordered protein that may undergo induced folding upon binding [197]. This type of protein is more common in eukaryotes [198] and is more often found in regulatory and cancer related proteins [199], which fits the proposed role of Alpha4, as both a regulatory protein of the PP2A family and its upregulation in cancer. Interestingly, thermal unfolding studies of both Tap42 and Alpha4 indicate that the full-length version of these proteins adopt a beta-sheet like structure upon unfolding, whereas the truncations containing only the structured N-terminal portions aggregate [197]. This may have implications as to the role of Alpha4 in PP2A phosphatase degradation and stabilization, especially in response to heat shock. This beta-sheet structure upon partial unfolding of the full length, but not the truncated form is also interesting given the possible relationship between Alpha4 and Alzheimer's disease. Especially, in light of evidence that cleavage of Alpha4 is increased in the brains of patients with Alzheimer's disease [191]. Further structural characterizations and analysis of Alpha4 are included as part of this study.

Lessons from Yeast Tap42

In yeast, Tap42 (Two A and related phosphatase-associated protein of 42 kDa) interacts directly with the catalytic subunit of all members of the yeast Type 2A phosphatase family (Pph21,Pph22, Pph3, Sit4, Ppg1), and has been found to be essential to their function [11,200–203]. Tap42 was discovered as a 42 kDa protein, 24% identical and 37% similar in sequence to human Alpha4, that is part of the Target of Rapamycin (TOR) signaling pathway [200]. Tap42 is an essential protein whose deletion leads to growth arrest [200,202]. The interaction between Tap42 and phosphatase catalytic subunits is regulated by nutrient growth signals via TOR activation and inhibition [200]. Tap42 is phosphorylated by TOR and phosphorylated Tap42 interacts with the phosphatase catalytic subunits [200,201]. Rapamycin is a TOR inhibitor that leads to growth arrest in yeast [204,205]. Mutations in TOR or in Tap42 lead to a rapamycin resistant phenotype due to a failure of TOR to regulate Tap42 phosphorylation and thereby association with the PP2A phosphatase catalytic subunits [200,201,203,206]. Under normal conditions, rapamycin and nutrient deprivation lead to dissociation of Tap42 from the phosphatase catalytic subunit and increased activity of the phosphatases [200,201,203]. This reduces overall protein translation, but increases production of stress response genes [200,207,208]. The manner in which TOR inactivation and/or rapamycin treatment induces phosphatase activation and/or dissociation of the Tap42/PP2Ac complexes is up for debate.

A portion of Tap42 interacts with TOR at membrane surfaces and this complex includes PP2A family phosphatase catalytic subunits [203]. A closer look at the kinetics of PP2A family activation by rapamycin reveals that phosphatase activity increases much more quickly than dephosphorylation of Tap42 or dissociation of the Tap42/PP2Ac complex [203]. This observation led to the current model in which rapamycin or nutrient starvation causes dissociation of the Tap42/phosphatase complex from the TOR complex and this dissociation is sufficient to allow for phosphatase activity (Figure 11) [203,206]. The dephosphorylation and
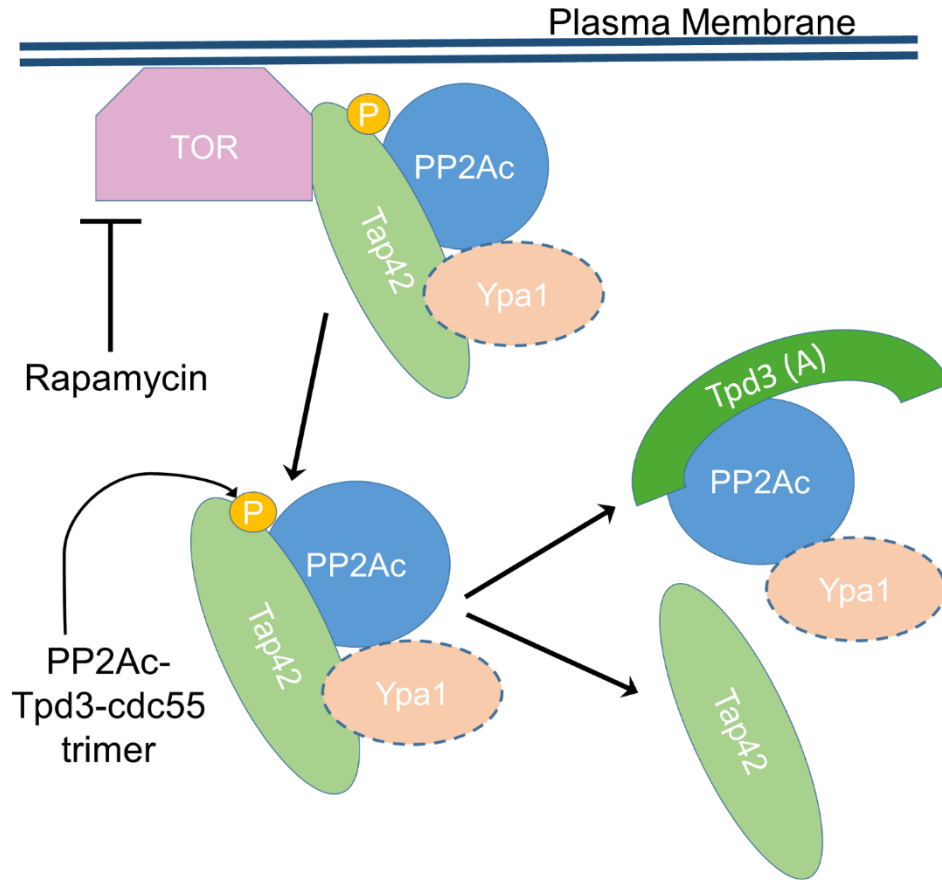
Figure 11: Model of membrane association and activation of PP2A. This model proposes that Tap42 forms a complex with TOR and PP2A family catalytic subunits (PP2Ac) at the membrane when kept in a phosphorylated state by TOR. This complex may also contain Ypa1 (yeast PTPA). Treatment with rapamycin inhibits TOR activity and promotes release of the Tap42-PP2A complex. Once released, PP2Ac is active, although still associated with Tap42. Full activation and formation of canonical heterotrimer occurs once Tap42 is dephosphorylated by a PP2Ac-Tpd3-cdc55 complex. Ypa1 may play a role in this process by activating PP2Ac after release of Tap42, as treatment with rapamycin leads to release of Tap42 from the Ypa1-PP2Ac-Tap42 trimer.

dissociation of the Tap42/phosphatase complex follows at a slower rate and is dependent upon active pph21/22 complexes with cdc55 and Tpd3 [9,201,203].

In addition to the association with phosphatase catalytic subunits, Tap42 also interacts with Ypa1 and Ypa2 (yeast PTPA) [164]. This interaction is enhanced but not dependent on the presence of the phosphatase, and is also rapamycin sensitive with rapamycin treatment leading to release of the PTPA-phosphatase complex from Tap42 [164]. In fact, deletion of PTPA increases Tap42 interaction with Pph21, but has no effect on interaction of Tap42 with Sit4 [171]. Whether the PTPA-phosphatase-Tap42 complex also exists at the membrane with TOR has not been clearly elucidated. It has been postulated that rapamycin acts indirectly through promoting Tip41 interaction with Tap42, leading to dissociation of the Tap42/phosphatase complex [177]. Whether these same interactions hold true in higher eukaryotes remains to be seen, but nevertheless important information on phosphatase regulation has been gained through the study of yeast homologs and their protein-protein interactions.

Alpha4 regulation of the PP2A phosphatase family

Alpha4 interacts with PP2A family catalytic subunits and plays a key role in regulating overall phosphatase activity within the cell, but the mechanism of that regulation and the actual effect of the Alpha4-phosphatase complex remains controversial. Methylation of PP2Ac does not affect association with Alpha4, in fact Alpha4 association may be enhanced in its absence [9]. Mutation of the two conserved residues in the C-terminal tail of PP2Ac, Y307F and L309Q, resulted in Alpha4-PP2Ac complex formation, while restricting formation of the canonical heterotrimer [209]. The insensitivity to PP2Ac methylation status may lead to Alpha4 altering the balance of heterotrimeric holoenzymes in the cell by either displacing select B-type regulatory subunits or promoting their association and thereby modulating PP2A activity and substrate specificity.

Initial studies of Alpha4 did not agree on whether Alpha4 was an inhibitor of PP2A, an activator of PP2A, or a regulatory subunit that altered activity and substrate specificity. In COS1 cells, studies show that overexpression of Alpha4 increases PP2Ac methylation, activity towards a peptide substrate, and increases dephosphorylation of 4E-BP1 [210] indicating upregulation of PP2Ac activity. Other studies found that PP2Ac complexed with Alpha4 have a decreased ability to dephosphorylate 4E-BP1 [211,212]. Studies using phosphorylated myelin basic protein (pMBP) yield similar contradictory results, with one study showing the PP2Ac-Alpha4 complex having increased activity [213] and another showing decreased activity of the complex with a shift in substrate specificity towards pMBP from phosphorylase a [214]. Others have found that the Alpha4-phosphatase complex is essentially inactive [188,215]. Given that Alpha4 itself is a highly regulated protein that is capable of undergoing numerous post-translational modifications [192,214,216,217], it is possible that this variability arises due to modifications of Alpha4 that differ in a cell type or context dependent manner.

Alpha4 may also regulate PP2A phosphatases, separately from their catalytic activity, by regulating their stability and expression levels. It was initially postulated that Alpha4 enhanced PP2Ac degradation based on the discovery that Alpha4 promoted interaction between Mid1, an E3 ubiquitin ligase associated with microtubules, and PP2Ac [218]. This study found that mutations in Mid1, including those that led to disruption of the Mid1/PP2Ac complex led to increased dephosphorylation of microtubule associated proteins [218]. The necessity of Alpha4 for this association has been confirmed in a number of studies, but the role of Alpha4 in this complex appears to be more complex [217–219]. Although originally thought to promote PP2Ac degradation, several studies have now shown that association of Alpha4 with PP2Ac protects PP2Ac from polyubiquitination and that knockout of Alpha4 has a negative effects upon phosphatase expression and stability [215,217]. These findings led to two competing models for the role of the PP2c-Alpha4-Mid1 heterotrimer (Figure 12). McConnell *et al.* [217] found that deletion of the UIM within the N-terminus of Alpha4 abrogated the ability of Alpha4 to protect
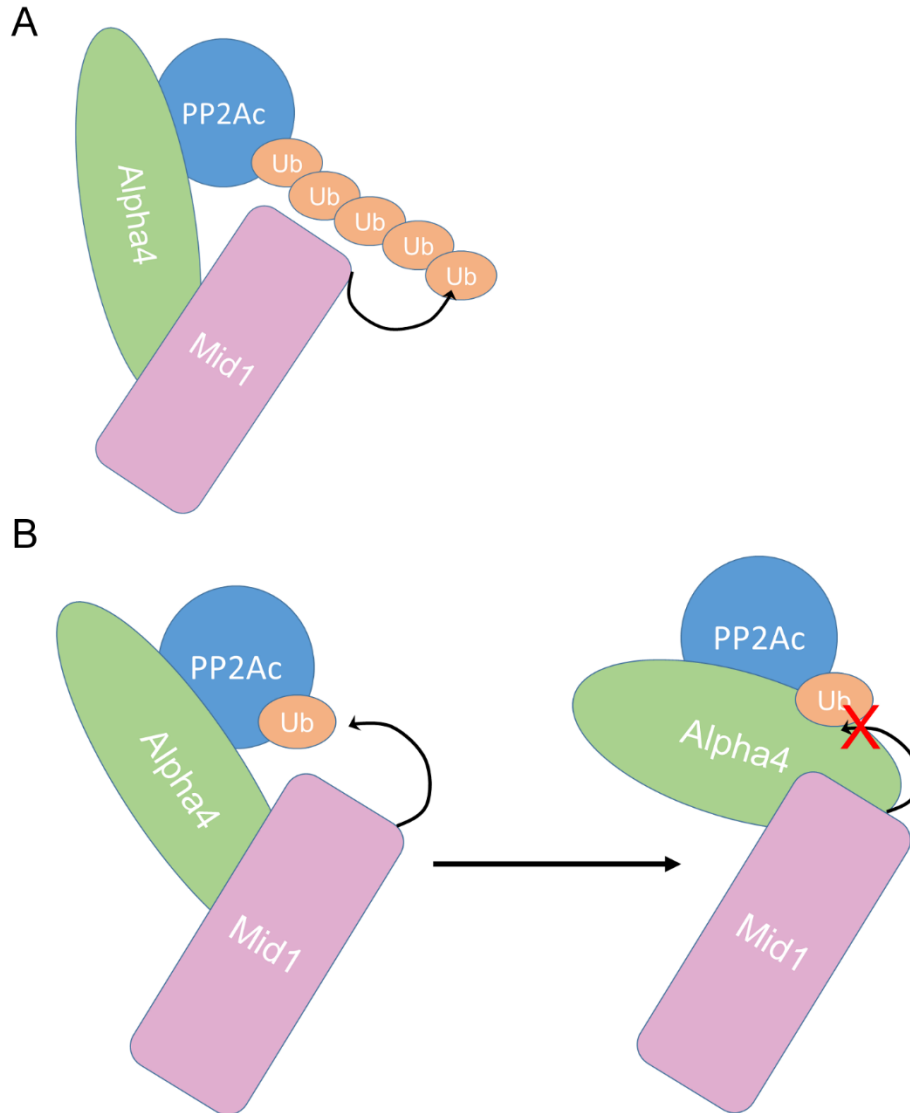
Figure 12: Initial models of PP2Ac-Alpha4-Mid1 interactions. A) Degradation model: Alpha4 scaffolds PP2Ac to its E3 ubiquitin ligase, Mid1, leading to increased PP2Ac polyubiquitination and degradation. B) Protective model: After an initial ubiquitination event, the UIM of Alpha4 binds to the ubiquitin moiety on PP2Ac and prevents further ubiquitination, thus leading to increased stability of PP2Ac.

PP2Ac from polyubiquitination, which led to the hypothesis that this UIM motif blocked ubiquitination of PP2Ac by Mid1 (Figure 12B). The C-terminal domain of Alpha4 has been less well studied due in part to its intrinsically disordered nature, but this regions serves as the binding domain for both Mid1 and another E3 ubiquitin ligase, EDD [217–221]. Recent studies show that Alpha4 itself is the subject of Mid1 monoubiquitination that leads to Alpha4 cleavage by calpains [191,217,222]. Alpha4 also may serve as a substrate for Mid1 polyubiquitination, but the exact role and extent of Alpha4 polyubiquitination is still in question [193]. Later chapters will discuss how our findings elucidate the mechanism and protein domains involved in the protective effect of Alpha4 on PP2Ac.

Overexpression of Alpha4 enhances expression and stability of ectopically expressed PP2Ac [191,215,217], but most studies have found no significant effect of overexpression of Alpha4 on endogenous PP2Ac levels [210,215,223]. On the other hand, knockout of Alpha4 appears to have a profound negative effect on expression levels of all three PP2A family members [215]. While knockout of Alpha4 has a pronounced effect on PP2Ac levels, knockdown of Alpha has only shown limited effects on PP2Ac expression and the effects of knockdown on the other PP2A family members has not been investigated [223]. It may be that complete knockout of Alpha4 versus knockdown of Alpha4 have profoundly different effects.

Regulation of Alpha4

It is established that Alpha4 interacts with the PP2A family of phosphatases, but it is unclear what factors regulate that association. In yeast, several studies show that binding of Alpha4 to the PP2A family is regulated by TOR and that Alpha4 plays a key role in regulation ofthe TOR pathway via suppression of PP2A family activity [11,131,200,201,208]. Alpha4 may serve a similar function in regulation of the mammalian Target of Rapamycin pathway, but the evidence for that is less convincing than in yeast [196,211,224]. The ability of rapamycin to lead to disassociation of the Alpha4-phosphatase complex appears to vary dependent upon cell type

and context [110,176,182,188,196,210,211,213,214]. Studies done using purified proteins have consistently found that the complex is insensitive to rapamycin [176,211], but many studies performed in cells have shown that the complex is sensitive to rapamycin [196,213,214]. For instance, the Alpha4-phosphatase complex dissociated in rapamycin-sensitive Jurkat cells when they treated with rapamycin, but not in rapamycin-insensitive Raji cells [213]. This implies that rapamycin induces a post-translational modification of either Alpha4, the phosphatase catalytic subunit, or another interacting protein that leads to disruption of the Alpha4-phosphatase complex.

A likely candidate for the rapamycin induced post-translational modification that leads to disruption of the Alpha4 phosphatase complex is phosphorylation, as in yeast it has been shown that TOR regulates the Tap42-phosphatase complex via phosphorylation of Tap42 [200]. Alpha4 has been shown to undergo multiple forms of post-translational modifications, including phosphorylation, glycosylation, acetylation and ubiquitination [192,214,216,217]. The effects of these various post-translational modification on Alpha4 expression, binding interactions and cellular functions need further research. Studies with Alpha4 and PP2Ac do not indicate that this association is dependent upon Alpha4 phosphorylation [214], though phosphorylation may still play a role in regulating the affinity of the binding interaction or serve to localize the proteins in close proximity allowing for more efficient binding. More investigation is needed to determine what role phosphorylation of Alpha4 plays in higher eukaryotes.

In addition to possible post-translational regulation of Alpha4 that modulates its interactions with the PP2A family of phosphatases, Alpha4 expression levels are also highly regulated both at the level of transcription and translation. Alpha4 is involved in regulation of translation through inhibiting PP2Ac activity towards 4E-BP1 and p70S6K and this leads to increased translation of eIF4E sensitive mRNAs which includes Alpha4 itself, thus initiating a positive-feedback loop [212]. In some cell types Alpha4 is downregulated by prolactin and overexpression of Alpha4 inhibits prolactin-inducible promoter activity [210,216]. Another

means of regulating Alpha4 expression levels is through microRNAs (miRNAs). miRNA34-b is downregulated in a number of cancers [223,225–229] and negatively regulates Alpha4 expression [223]. Interestingly, levels of miRNA-34b are regulated by p53 [226,228,230–233], while p53 is regulated by various PP2A family complexes[234–237], in particular it has been shown that expression of Alpha4 suppresses p53 mediated apoptosis [238,239]. Thus Alpha4, phosphatases, and p53 form a complex crosstalk network modulating each other's expression, stability, and activity; in doing so they regulate a diverse set of cellular functions.

Alpha4 and Cancer

Increased Alpha4 expression is found in a number of cancers and recent studies indicate that this increased expression may be sufficient to lead to transformation [223]. Alpha4 is overexpressed in over 80% of primary adenocarcinomas, breast cancers, hepatocellular carcinomas and bladder urothelial carcinomas compared to normal tissues [223,240]. High expression levels of Alpha4 are correlated higher rates of mortality, higher rates of recurrence, and a general poorer prognosis [240,241]. Several studies indicate that increased Alpha4 expression levels leads to increased ability of cells to form tumors *in vivo*, as well as increased cell proliferation, anchorage-independent growth, and migration, all steps in tumorigenesis and metastasis [223,240,242]. Knockdown or inducible knockout of Alpha4 has the opposite effect causing increased apoptosis, decreased cellular proliferation and decreased cellular migration [223,238,240,242]. These effects make Alpha4 a clear target for potential cancer therapies and a potential oncogenic protein.

Alpha4 regulates apoptosis, cell growth, cellular proliferation and DSB repair [215,223,238,239,243–245], all important aspects of tumorigenesis. Alpha4 knockout leads to an increased persistence of DNA damage markers following treatment with genotoxic agents or replication inhibitors [215]. Inactivation of p53 increased cell viability of these cells, but the cells still showed signs of defective DNA damage repair with higher basal γH2AX and a delay in

resolution of γH2AX foci [215]. Knockout of Alpha4 leads to apoptosis in a p53 dependent

fashion [238,245], as overexpression of Bcl-x$_L$, and an inhibitor of intrinsic apoptosis, protected

Alpha4 knockout MEFs from apoptosis [238]. In contrast, overexpression of Alpha4 leads to

increased cell viability and increased resistance to a number of stressors, including glutamine

deprivation and treatment with camptothecin, a replication inhibitor [215,223,239]. These

effects on apoptosis and DNA damage repair are in keeping with the idea of Alpha4 being a

putative oncogene and mirror many of the phenotypes seen with knockdown or overexpression

of PP2A family catalytic or regulatory subunits.

Interaction and regulation of PP2A family phosphatases is clearly a key component to

the any role of Alpha4 in cancer, but the C-terminal binding domain of Alpha4 may also have a

role. The C-terminus of Alpha4 binds to EDD [220], an E3 ubiquitin ligase that regulates p53

and is often upregulated in cancer [246–248]. In addition to the role of EDD in regulation of p53,

EDD also regulates cellular proliferation during development via regulation of polyubiquitination

and degradation of PP2Ac [220,249]. In another example of crosstalk networks, the canonical

PP2A heterotrimer containing the B55α subunit is known to dephosphorylate EDD and activate

p53. The assembly of this B55α heterotrimeric complex leads to increased cell viability in

response to glutamine deprivation [239,250],with Alpha4 playing a key role in promoting

assembly of this complex [239]. It is clear that Alpha4 plays a key role in promoting both cellular

proliferation and inhibiting apoptosis, two key steps in tumorigenesis and tumor growth, and that

it exerts this influence through multiple pathways. The goal of this thesis work is to elucidate

some of the pathways and mechanisms utilized by Alpha4 in regulating the expression levels

and stability of the PP2A family of phosphatases.

Chapter 2

Structural Analysis of the N-terminal PP2Ac Binding Domain of Alpha4

Introduction

Protein serine/threonine phosphatase 2A (PP2A) is regulated through a variety of mechanisms, including post-translational modifications and association with regulatory proteins. Alpha4 is one such regulatory protein that binds the PP2A catalytic subunit (PP2Ac) and modulates its polyubiquitination and degradation [215,217]. Alpha4, first discovered in murine B-cells as a 52 kDa phosphorylated protein [183], interacts with the entire PP2A family of serine/threonine phosphatases [176,188,211,214]. The cDNA was isolated from murine bone marrow and found to encode a 340 amino acid protein and that it is expressed in an array of diverse tissues [184]. The human homolog was identified on the X chromosome and found to share over 80% sequence identity with its murine counterpart [185]. A protein from yeast, involved in suppressing effects of mutations in Sit4 (a yeast PP2A family phosphatase), was identified and found to be homologous to murine Alpha4, sharing 24% sequence identity and 37% sequence similarity [200]. This protein, termed Tap42, was found to be essential in yeast and interacts with not only Sit4, but all the yeast PP2A family member homologs [11,200].

Studies in both yeast and mammals revealed that Alpha4/Tap42 interacts directly with the catalytic subunits of all the PP2A family phosphatases (PP2Ac, PP4c, PP6c) in the absence of the canonical regulatory/structural subunits of the phosphatases [176,188,200,213]. Its ability to interact with all members of the PP2A family makes it a rather uncommon phosphatase-interacting protein, as only a handful of proteins have been shown to interact with multiple members of the family [171,175]. Binding of Alpha4 to the PP2A family of phosphatases has been mapped to the N-terminal region of the phosphatase catalytic subunit [11,176,195]. There are three residues L35, E38 and N40 (in *S. cerevisiae* Sit4) that are strictly conserved across

species and between the PP2A family members, but are not found in other closely related serine/threonine protein phosphatases, such as PP1 [11]. Mutations in these residues reduce the ability of phosphatase catalytic subunits to bind to Tap42/Alpha4. Mutation of mammalian PP2Ac at E42A (corresponding to E38 in *S. cerevisiae*) both decreases binding to Alpha4 and increases PP2Ac binding to its canonical A subunit [11,194]. The reverse has also been found, in that mutations of key residues in PP2Ac can inhibit binding with A subunit and increase binding with Alpha4. This implies either that the binding sites overlap or that binding of one of these proteins leads to allosteric changes in PP2Ac that alters the binding site for the other [194].

Initial structural analysis of Alpha4, using limited proteolysis and circular dichroism, revealed a structured N-terminal domain composed of alpha helices and a highly unstructured C-terminal domain that is quickly degraded [197]. The alpha helical nature of the N-terminus was supported by the crystal structure of the Alpha4 yeast homolog, Tap42, which adopted an all alpha-helical structure with similarity to tetratricopeptide repeat (TPR) proteins [251]. The site of interaction between PP2Ac and Alpha4 was mapped within Tap42 and two key residues emerged, R163 and K166 (in *S. cerevisiae* Tap42), with mutations of these residues abrogating binding to PP2Ac [251]. Mammalian Alpha4 also contains a UIM, located between residues 46-60, that has been implicated in regulating the ability of Alpha4 to protect PP2Ac from ubiquitination and degradation [217]. This Chapter will focus on the structure of the N-terminal domain of mammalian Alpha4 determined by x-ray crystallography, as well double electron-electron resonance spectroscopy (DEER) data demonstrating that it is a flexible, TPR-like protein. Structurally, Alpha4 differs from its yeast homolog, Tap42, in two important ways: 1) the position of the helix containing the PP2Ac binding residues is in a more open conformation, and 2) Alpha4 contains a UIM.

Materials and Methods

Plasmids

The human 6xHis-Ubiquitin was expressed from a pet28a expression plasmid. Murine Alpha4ΔC (residues 1-222) was amplified by PCR from an Alpha4/pGEX4T2 vector and then inserted into the pET28a vector using the BamH1 and Nde1 restriction sites to create a N-terminal 6xHis-Alpha4ΔC construct. Murine 6xHis-Alpha4ΔC mutants were created using the QuikChange™ Site-Directed Mutagenesis method (Stratagene, La Jolla, CA) and the following primers: Alpha4ΔC_CF (C117S/C119S) forward 5'-CGTACATTTCTTAACTCAGAGTCATAGC TATCATGTGGCAGAG, reverse 5'-CTCTGCCACATGATGCTATGACTCTGAGTTAAGAAATG TACG; S44C forward 5'- CCAGGATAAGGTGTGCAAAGGACTAGAAC, reverse 5'-GTTCTAG TCCTTTGCACACCTTATCCTGG; M56C forward 5'-CTCCTTGAGAAGGCTGGATGTTTAT CGCAGCTTG ATTTG, reverse 5'-CAAATCAAGCTCGATAAACATCCAGCCTTCTCAAGGAG; K98C forward 5'-CAAGTCAACCCCAGCTGTCGTCTAGATCATTTGC, reverse 5'-GCAAATG ATCTAGACGACAGCTGGGGTTGACTTG; Y146C forward 5'-GCTCCTCCATGGCCTGT CCAAATCTCGTTGC, reverse 5'-GCAACGAGATTTGACAGGCCATGGAGGAGC; S154C forward 5'- CGTTGCTATGGCATGCCAAAGACAGGC, reverse 5'-GCCTGTCTTTGGCATGC CATAGCAACG; L206C forward 5'- GCATTGCTGTCAGCTGTGAAGAGCTTGAGAGC, reverse 5'- GCTCTCAAGCTCTTCACAGCTGACAGCAATGC. All constructs were verified by DNA sequencing.

Expression and purification of 6xHis-tagged proteins

Protein expression was performed at 20°C overnight in BL21 (DE3) competent *E. coli* following induction with 1mM isopropyl-beta-galactaside (IPTG). Proteins were purified using metal affinity chromatography, cleaved overnight with thrombin to remove the N-terminal

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hexahistidine tag, and dialyzed into gel-filtration (GF) buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM sodium azide, pH 7.5) (Figure 13A). The protein was further purified using size exclusion gel filtration on an S200 Amersham column in GF buffer (Figure 13B). Selenomethionine (SeMet) labeled protein was grown in minimal media [252] in BL834 (DE3) auxotrophic competent *E. coli* cells.

Crystallization and structure determination of Alpha4ΔC

Purified Alpha4ΔC was concentrated to approximately 17 mg/ml and crystallization trials were conducted. Crystals were obtained in two conditions: 1.6 M Ammonium sulfate, 2% PEG 400, 0.1 M Bis-Tris, pH 6.0; and 25% PEG 1500 (Figure 13C). Crystals were cooled in liquid nitrogen and diffraction data were collected to 2.35Å at the NECAT ID-C and SERCAT beamlines at Argonne National Lab. SeMet labeled crystals were produced in the ammonium sulfate conditions and an initial model was produced with data from these crystals. Data from these crystals was severely anisotropic (average diffraction intensities three times greater in one dimension than the other two) (Figure 13D), but SeMet positions were found using SHELX [253], refined using Sharp [254], and density modification was done by SOLOMON [255], as implemented in AutoSharp [254]. The structure was built using COOT [256] and refined using PHENIX [256] and CNS [257] (Figure 13E) . Data from crystals grown in 25% PEG 1500 were used in the final structure refinement (Figures 13F, G). Phasing and refinement statistics are given in Table 3. Coordinates have been deposited in the RCSB protein database with PDB code 3QC1.

Electron paramagnetic resonance (EPR) spectroscopy

Cysteine point mutants were created in the cysteine-free Alpha4ΔC (Alpha4ΔC_CF) background and then expressed and purified as described above. The protein concentration
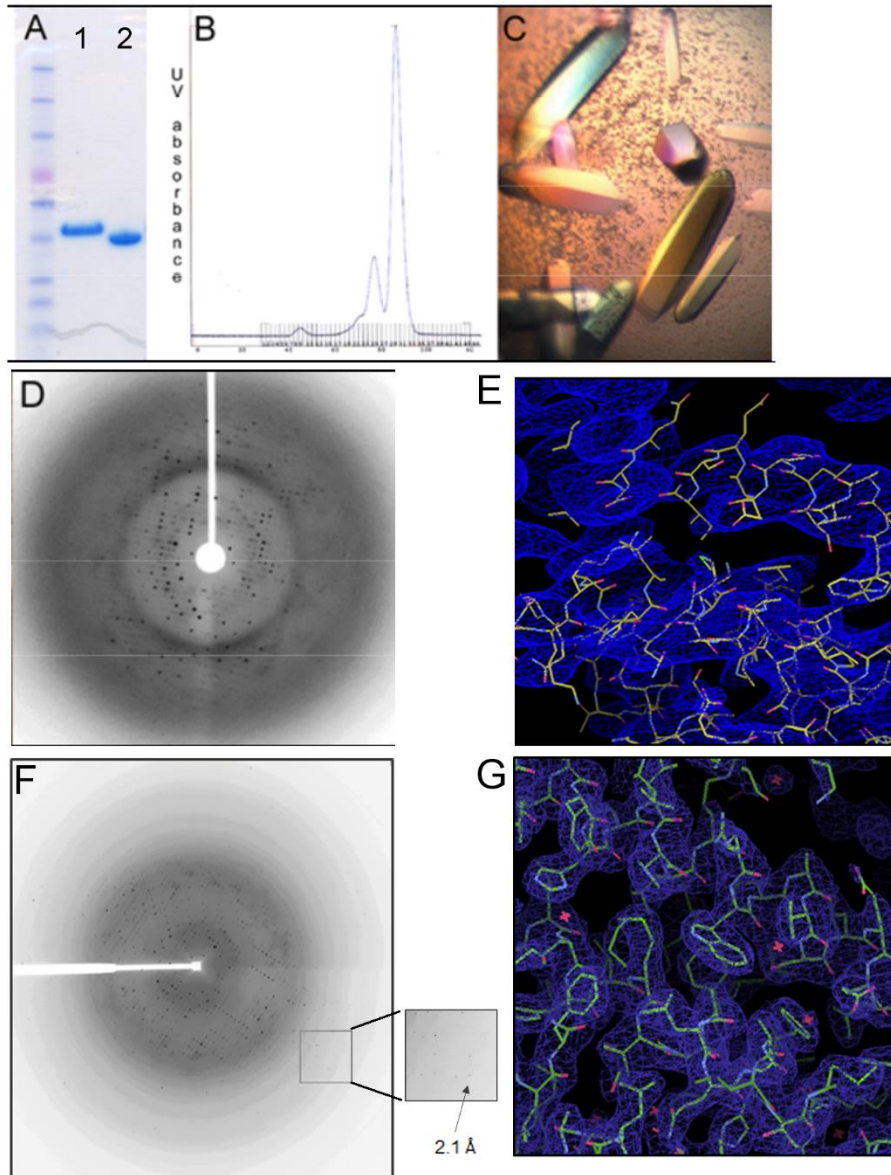
Figure 13: Alpha4ΔC protein expression, purification and crystallization. A) Coomassie stained SDS-PAGE gel of purified 6xHis-Alpha4ΔC before (lane 1) and after (lane 2) removal of the His tag. B) Chromatograph from a gel-filtration column showing a major peak of monodispersed, monomeric Alpha4ΔC. C) Image of crystals obtained in 1.6M ammonium sulfate, 2% Peg400, 01 M Bis-Tris pH 6.0. D) Diffraction from a crystal similar to those imaged in C. E) Electron density map derived from the diffraction of crystals grown in 1.6M ammonium sulfate, 2% Peg400, and 0.1 M Bis-Tris. Crystals were phased using selenomethionine. F) Diffraction from a crystal grown in 25% PEG1500, showing diffraction out to 2.35 Å. Enlargement of boxed area showing outer edges of diffraction data. G) Electron density map derived from diffraction of crystals grown in 25% PEG1500 and phased by molecular replacement.

Table 3: Crystallographic Data Collection and Refinement Statistics

Values in parentheses are for the highest resolution bin.

Parameters	Native Crystal	SeMet
Space Group	P3$_2$21	P3$_2$21
a =b (Å)	76.6	80.6
c (Å)	72.7	73.4
X-ray Source	APS 24 ID-C	APS 22-BM
Wavelength (Å)	0.97949	λ1: 0.97625
		λ2: 0.97949
		λ3: 0.9826
Resolution range (Å)	50-2.35	50-2.5
No. observed reflections	258,546	λ1: 96,531
		λ2: 108,359
		λ3: 9,5973
No. unique reflections	12,891	λ1: 9,876
		λ2: 9,843
		λ3 9,972
Completeness (%)	95.61 (91)	λ1: 99.9 (100)
		λ2: 99.9 (100)
		λ3: 99.9 (100)
Redundancy	6.9 (7.3)	λ1: 5.2 (3.9)
		λ2: 5.9 (5.9)
		λ3: 5.9 (3.8)
Rmerge	5.6 (28.0)	λ1: 5.5 (38.9)
		λ2: 5.3 (77.6)
		λ3: 4.3 (51.9)
FOM (50-2.8 Å)		0.44
FOM after dm[1] (50-2.5 Å)		0.86
I/σ	22 (3.8)	λ1: 23.5 (1.8)
		λ2: 23.6 (1.9)
		λ3: 28.9 (1.4)
No. reflections used in refinement (N)	10,142	
No. reflections used in Rfree	1,011	
No. water molecules	38	
Protein atoms	1,537	
Rcrystal (%)	20.7 (29.6)	
Rfree (%)	26.5 (36.9)	
Wilson B	54	
Average B	67	
Rmsd bond lengths (Å)	0.008	
Rmsd bond angles (deg)	1.044	
Rhamachandran (%)		
Favored	92.0	
Allowed	6.8	
Outliers	1.1	

[1] dm was performed using SOLOMON, as described in the material and methods.

after elution was measured by absorbance at 280 nm and 10X excess of methanethiosulfonate

spin label (MTSSL) (Toronto Research Chemicals) was added to ~10 mg of the protein. The

protein was incubated with MTSSL in the dark at room temperature for 2 h before being placed

overnight in the dark at 4°C. After overnight incubation, the protein was further purified using

size exclusion gel filtration on an S200 Amersham column in GF buffer. This purified protein

was concentrated to ~200 μM. Samples were analyzed using a described DEER protocol

[258,259]. MTSSL-labeled versions of all point mutants were analyzed using continuous wave

electron paramagnetic resonance spectroscopy (CW-EPR) to look at mobility at specific

residues. Binding between ubiquitin and Alpha4ΔC was analyzed by CW-EPR by using singly

MTSSL labeled samples of Alpha4ΔC and incubating with 500 µM of unlabeled ubiquitin.

Results

Crystallization and structural analysis

To gain insight into the molecular mechanism of Alpha4-mediated regulation of PP2Ac

polyubiquitination [217], we determined the structure of a mammalian version of Alpha4 that

contains the UIM consensus sequence, which is absent in Tap42. Given the intrinsically

disordered and proteolytically sensitive nature of the C-terminal 120 residues of Alpha4,

characterized as the Mid1 binding domain, we created an N-terminal hexahistadine-tagged

construct of murine Alpha4 spanning residues 1-222 for crystallization purposes, hereafter

referred to as Alpha4ΔC. We crystallized Alpha4ΔC and determined the structure to a

resolution of 2.35Å (Figure 14A). Statistics for data collection and structure refinement are

found in Table 3. Alpha4ΔC is an all alpha-helical protein with dimensions of 71 Å by 42 Å by

29 Å. This is similar to the dimensions found for TAP42ΔC of 65 Å by 35 Å by 25Å [251] and

the 72Å measured by scattering studies for the largest dimension [197]. A large flexible loop
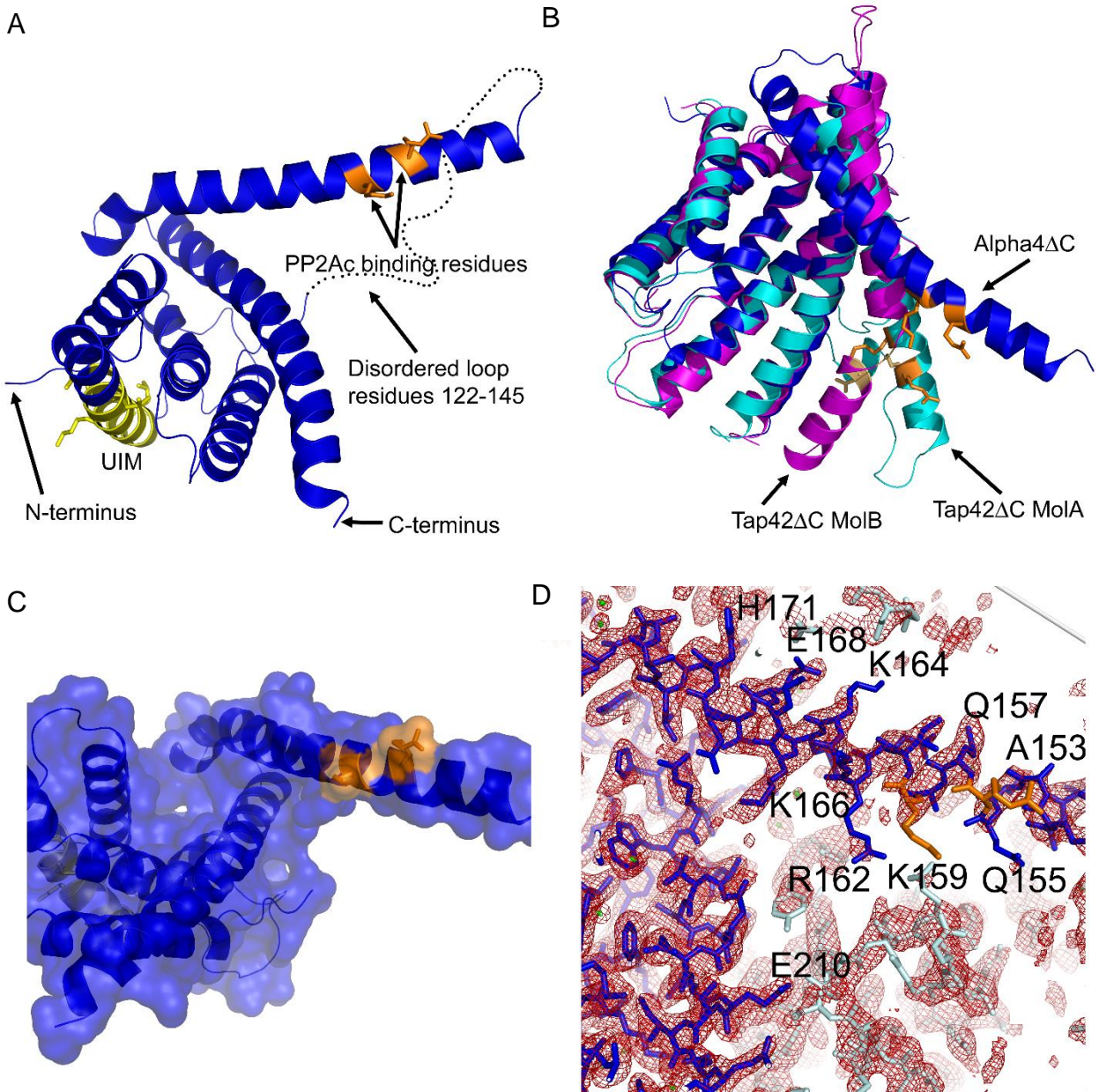
Figure 14: Structure of Alpha4ΔC. A) Ribbon diagram of Alpha4ΔC with residues important for PP2Ac binding shown in orange and the UIM consensus motif shown in yellow. B) Comparison of Alpha4ΔC (blue) to the Tap42ΔC structures (cyan and magenta)(PDB code 2V0P) that shows the variable positions of the extended helix (residues 147-182). C) Accessible surface rendering showing orientation of PP2Ac binding residues (orange) in Alpha4 (blue). D) 2mFo-DFc electron density map contoured at 1σ highlighting the PP2Ac binding residues (orange) within Alpha4 (blue). Symmetry molecules are shown in light blue and waters are shown in green. PYMOL was used to depict all molecular structures [260].

composed of residues 122-144 joins helix 4 to helix 5 and it is not observed in the crystal structure of Alpha4ΔC. A search in DALI [261] for structures similar to Alpha4ΔC revealed both tetratricopeptide repeat (TPR) proteins and 14-3-3 proteins, with the closest match being the yeast homolog of Alpha4, TAP42, and the next closest proteins being the TPR domain of prolyl 4-hydroxylase and 14-3-3 protein (Table 4).

Comparisons between Alpha4ΔC and Tap42ΔC indicate that helix 5 (residues 145-182) of Alpha4ΔC, adopts multiple conformations (Figure 14B). Helix 5 contains residues shown to be important for binding PP2Ac: R156 and K159 [251]. These residues face towards the main body of Alpha4 and are in an open conformation, allowing a high degree of accessibility to this interface for the globular PP2Ac subunit (Figures 14C, D). In the Alpha4ΔC structure, helix 5 protrudes away from the rest of the molecule. However, in the crystal lattice, helix 5 interacts with helix 2 of a neighboring molecule (Figure 14D), indicating that crystal lattice contacts might alter the position of helix 5. To determine the relative orientation of helix 5 in the absence of the crystal lattice, DEER spectroscopy was performed on Alpha4ΔC labeled with MTSSL at residues 98 and 146, 206 and 154, and 98 and 154. All three distance measurements support an open conformation (Figure 15), similar to that seen in the AlphaΔC crystal structure, as being the predominant conformation seen in solution, although the specific distance distribution is likely influenced by PP2Ac. The DEER data also support the idea that the protein is highly flexible within this region and adopts a range of conformational states, as the broad width of the peaks at half maximal height indicates flexibility in the position of the helix within Alpha4ΔC (Figure 15).

Analysis of the ubiquitin interacting motif (UIM)

A key difference between mammalian Alpha4 and non-mammalian homologs, such as Tap42, is the presence of an identifiable UIM consensus sequence, composed of residues 46-60, which has been shown to be functionally important in mammalian Alpha4 [217] (Figure 16A).

Table 4: Highest structural similarity matches to Alpha4ΔC defined by DALI

Protein	PDB ID	Z-score	rmsd (Å)[1]	No. of matched residues	% sequence ID
Tap42	2V0P	17.8	2.6	164	23
P4HA1	2V5F	10.9	2.0	93	14
14-3-3	3EFZ	10.1	4.1	109	10
APC7	3FF1	9.5	2.2	93	8
SycD	2VGY	9.1	2.3	90	10
PP5	1WAO	9.0	3.1	88	6
TOM20-3	1ZU2	9.0	4.3	109	14

[1]rmsd calculated using matched residue's C-alpha atoms
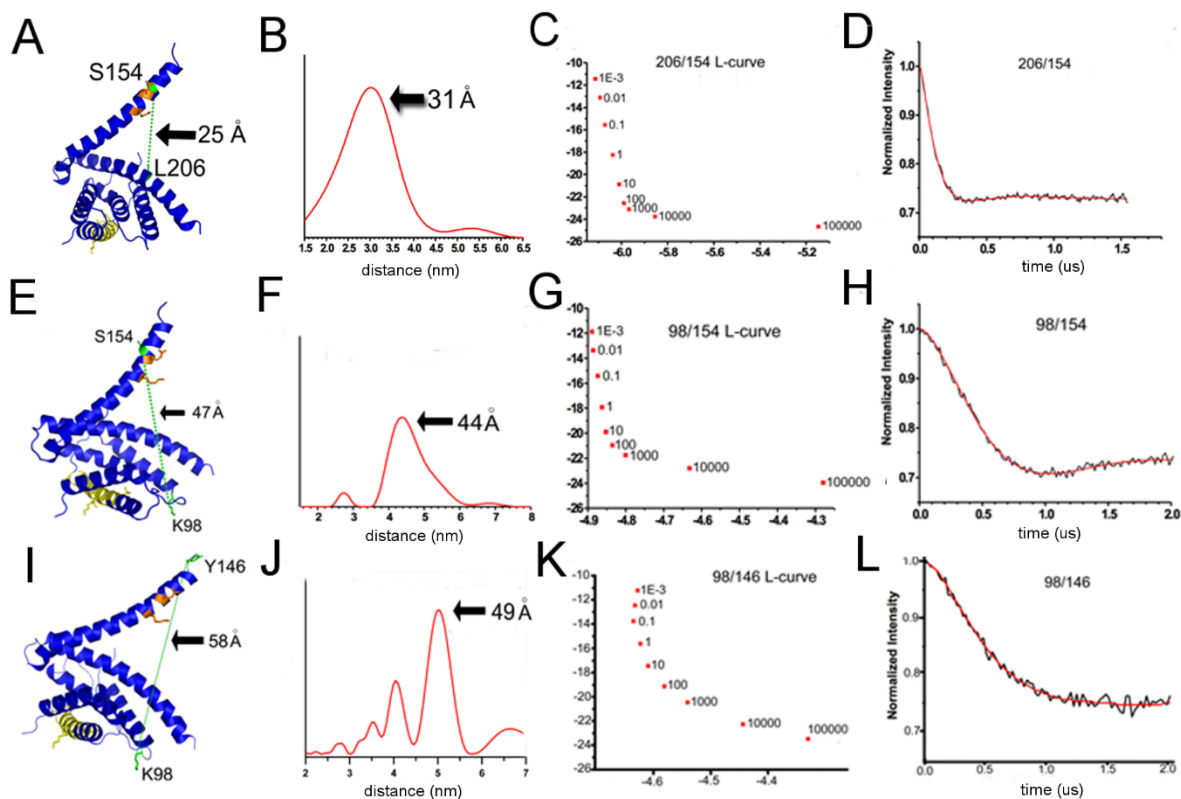
Figure 15: Distances between spin label pairs computed via DEER pulsed-EPR studies. A) Ribbon diagram showing location of spin labels L206C and S154C (green) and distance between β-carbons. UIM is shown in yellow and PP2Ac binding residues are shown in orange. (B) Distance distribution profiles corresponding to the best fit (shown in C, D), show a major distance distribution of ~31Å, compared to ~25Å in the crystal structure. C) Plot of the L-curve for L206C/S154C shows $\alpha = 100$ as the optimal value for computing the distance distribution. D) Background-subtracted dipolar modulation echo curves for spin-labeled L206C/S154C Alpha4ΔC. Red line shows the best solution using Tikhonov regularization analysis. E) Ribbon diagram showing location of spin labels K98C and S154C (green) and distance between β-carbons. UIM consensus motif is shown in yellow and PP2Ac binding residues are shown in orange. (F) Distance distribution profile for K98C/S154C, corresponding to the best fit (shown G, H), shows a major distance distribution of ~44Å, compared to ~47Å in the crystal structure. G) Plot of the L-curve for K98C/S154C shows $\alpha = 100$ as the optimal value for computing the distance distribution. H) Background-subtracted dipolar modulation echo curves for spin-labeled K98C/S154C Alpha4ΔC. Red line shows the best solution using Tikhonov regularization analysis. I) Ribbon diagram showing location of spin labels K98C and Y146C (green) and distance between β-carbons. UIM consensus motif is shown in yellow and PP2Ac binding residues are shown in orange. (J) Distance distribution profile for K98C/Y146C, corresponding to the best fit (shown K, L), shows a major distance distribution of ~49Å, compared to ~58Å in the crystal structure. K) Plot of the L-curve for K98C/Y146C shows $\alpha = 10$ as the optimal value for computing the distance distribution. L) Background-subtracted dipolar modulation echo curves for spin-labeled K98C/Y146C Alpha4ΔC. Red line shows the best solution using Tikhonov regularization analysis.

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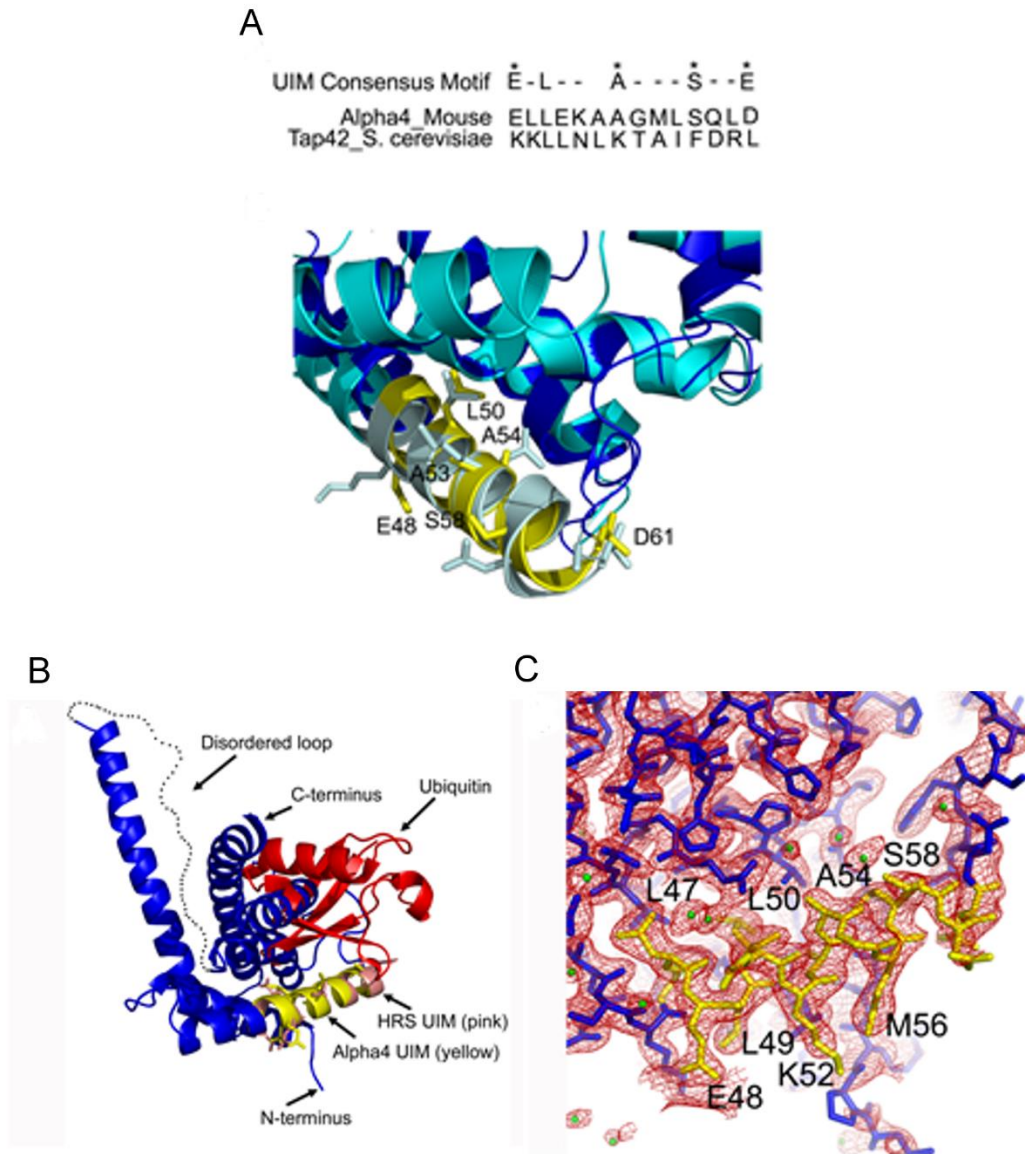
Figure 16: Structure of UIM consensus region not found in yeast homolog Tap42. A)
Sequence comparison of UIM consensus sequence, UIM motif in Alpha4, and aligned area of
TAP42 showing no UIM consensus motif in Tap42. Asterisks denote residues which when
mutated in hepatocyte growth factor-regulated tyrosine kinase substrate (HRS)-UIM have been
shown to have a detrimental effect on ubiquitin binding (top) [262]. Overlay of Alpha4 (blue) with
UIM of Alpha4 in yellow and corresponding region of Tap42 (cyan) (2V0P) with aligned
sequence residues (light cyan) showing differences in residue identity and position within the
UIM (bottom). B) Overlay of the Alpha4ΔC structure (blue) with its UIM colored in yellow and
the crystal structure of HRS-UIM bound to ubiquitin (2D3G) with the UIM in pink and ubiquitin in
red showing that the UIM of Alpha4ΔC would need to move in order for ubiquitin to bind in a
similar configuration. C) 2mFo-DFc electron density map contoured at 1σ showing residues
within the UIM consensus region in yellow, Alpha4 in blue, and water molecules in green.

The UIM is located within helix 2 of the structure and on the opposite face of Alpha4 relative to the PP2Ac binding site (Figure 14A). Overlaying the UIM consensus sequence with a known UIM-ubiquitin structure (2D3G) reveals that the UIM containing helix within Alpha4 must rotate if it is to bind ubiquitin (Figure 16B) and that this rotation would likely perturb the structure in this region of Alpha4 (Figure 16C). We utilized CW-EPR and DEER spectroscopy to assess whether this region was conformationally flexible. We double labeled the protein at residues M56C, located within helix 2, and K98C, located within the main body of the protein (Figure 17A). Interestingly, the DEER analysis showed two distinct, but overlapping peaks (Figure 17B). This indicates that this area may undergo a shift between two distinct conformations. We hypothesized that this conformational change was induced by interaction with ubiquitin. To test this, we incubated the doubly-labeled M56C/K98C construct with 500 µM unlabeled ubiquitin and tried to detect a shift in populations using DEER. Although the two curves are slightly different, the differences were not deemed significant enough to derive any conclusions on binding or conformational changes (Figure 17B). CW-EPR can be used to look for changes in mobility of a residue or a change in the overall tumbling time of the labeled protein upon binding to a ligand, as a measure of changes in the molecular mass and/or shape of the protein complex. We created several individually labeled mutants in Alpha4ΔC and incubated them with an excess of unlabeled ubiquitin and looked for changes in CW-EPR line spectra. We were not able to detect any significant changes in line spectra when Alpha4ΔC was incubated with unlabeled ubiquitin using any of the point mutants constructed (K98, S44, or M56) (Figure 17C). The multiple conformations observed for helix 5, and expected for helix 2, indicate that Alpha4 is a flexible molecule and that this flexibility may be functionally important. However, we were not able to induce any conformational changes in AlphaΔC using ubiquitin, nor were we able to demonstrate a ubiquitin-Alpha4 interaction via EPR.
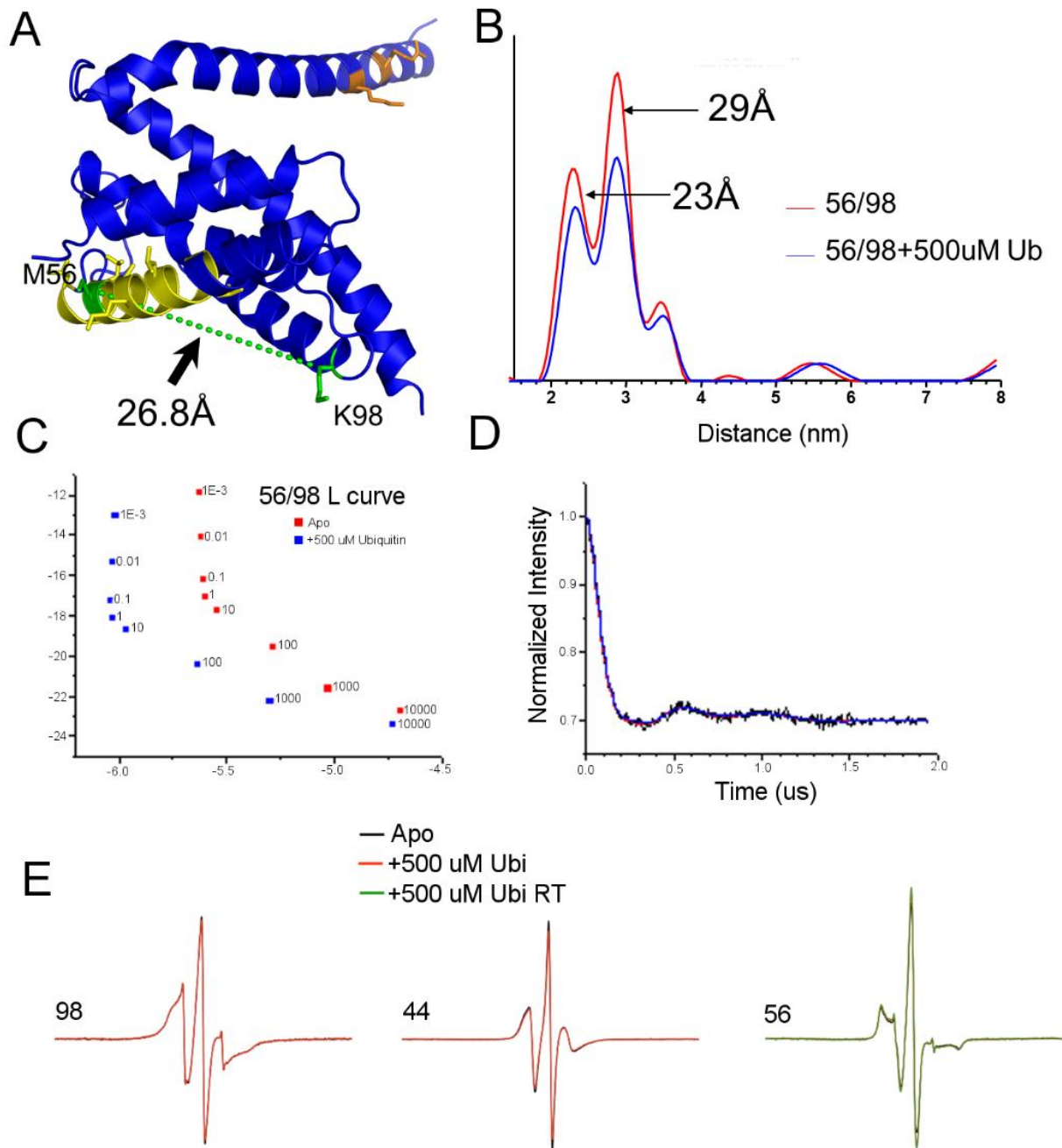
Figure 17: Measurement of conformational changes around the UIM in Alpha4ΔC in the presence and absence of ubiquitin. A) Ribbon diagram showing location of spin labels M56C and K98C (green) and distance between β-carbons. UIM is shown in yellow and PP2Ac binding residues are shown in orange. (B) Distance distribution profiles corresponding to the best fit (shown in C, D) show two major peaks within the distance distribution of ~23Å and ~29Å compared to ~26.8Å seen in the crystal structure. C) Plot of the L-curve for M56C/K98C shows $\alpha = 10$ as the optimal value for computing the distance distribution. D) Background-subtracted dipolar modulation echo curves for spin-labeled M56C/K98C Alpha4ΔC. Red line shows the best solution using Tikhonov regularization analysis. E) Overlaid CW-EPR line spectra of the three different MTSSL-labeled point mutants of Alpha4ΔC (K98C, S44C, M56C) incubated in the absence (Apo) (black line) or presence of 500 μM Ubiquitin (Ubi) at 4°C (red line) or at room temperature (Ubi RT) (green line).

Comparison of Alpha4 and standard tetratricopeptide repeat (TPR) proteins

Alpha4ΔC is a TPR-like protein with similarities to both TPR-containing and 14-3-3 proteins, but important topological differences create a possible binding site for PP2Ac. Both TPR proteins and 14-3-3 proteins are scaffolding proteins, which mediate protein-protein interactions [263–265]. TPR proteins are highly flexible molecules, with many TPR domains partially unstructured when not bound to their cognate ligands [266]. Structural analysis using PISA (Protein Interactions, Surfaces and Assemblies) [267] indicates that Alpha4ΔC has a high percentage of hydrophilic intramolecular interactions relative to alpha-helical proteins in general, but similar to that found in TPR motifs from other proteins (Figure 18). This high level of hydrophilic intramolecular interactions allow increased unfolding of a protein in solution due to lack of hydrophobic interactions, consistent with our findings that Alpha4 is a conformationally flexible molecule.

Although Alpha4ΔC adopts a TPR-like structure, it does not contain the TPR consensus residues [268], and the helices are longer and more irregular than a canonical TPR (Figure 19). In addition, the overall topology of Alpha4ΔC differs from a canonical three-repeat TPR in the arrangement of the final three helices, and these differences allow greater flexibility (Figure 19A). Both TPR proteins and 14-3-3 proteins are composed of pairs of anti-parallel helices stacked in parallel, with a twist, to create concave and convex faces (Figure 19B). In TPR proteins, these pairs of helices are labeled A and B, with the concave face of the motif lined by the A-helices (Figure 19B). Many TPR proteins also contain a final capping helix that extends the concave surface of the molecule [268]. In Alpha4ΔC, the first four helices are arranged as pairs of anti-parallel helices joined by a loop with the pairs stacking in parallel, as in a typical TPR or 14-3-3 protein (Figures 19A, B). Helix 6 occupies the position of the A-helix of a normal TPR motif and helix 5 extends away from the body of the protein analogous to the capping helix found in many TPR containing proteins, but in an opposite orientation (Figures. 19A-C). This
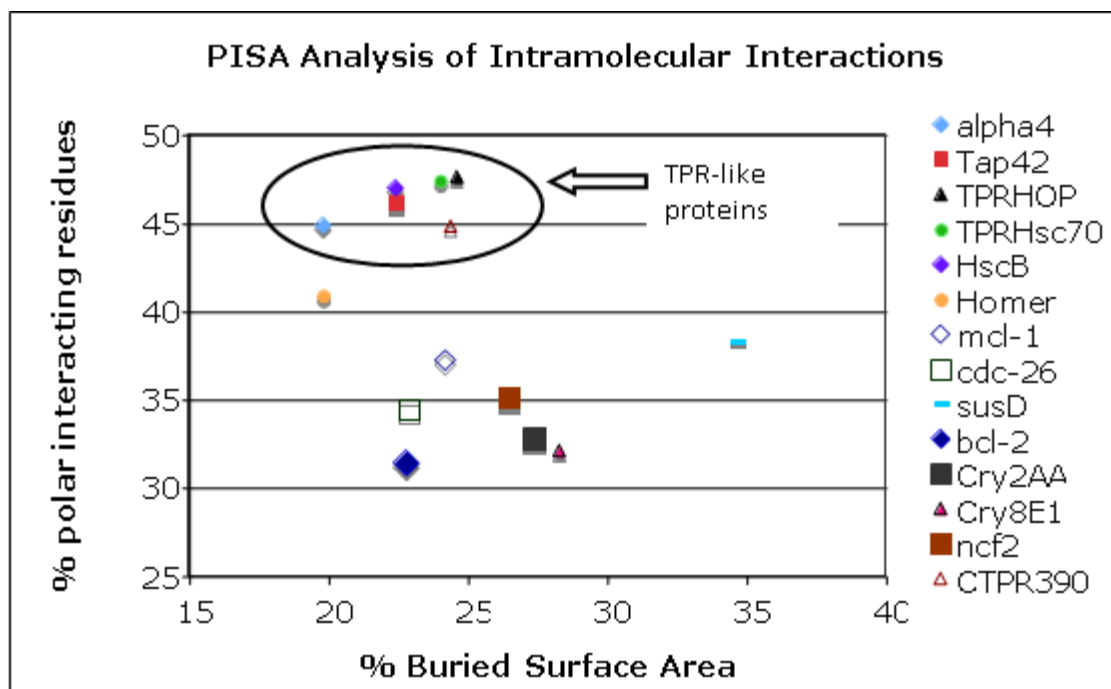
Figure 18: Structural analysis of buried surface area and polar interacting residues using PISA [267]. Proteins were analyzed by creating a separate chain for each helix for analysis by PISA. Total surface area of all helices was calculated and compared to the total buried surface area. Only interactions that were not part of secondary structures were counted as interacting residues. TPR-like proteins have a higher percentage of polar interacting residues than alpha-helical proteins in general and trend towards a lower percentage of buried surface area.
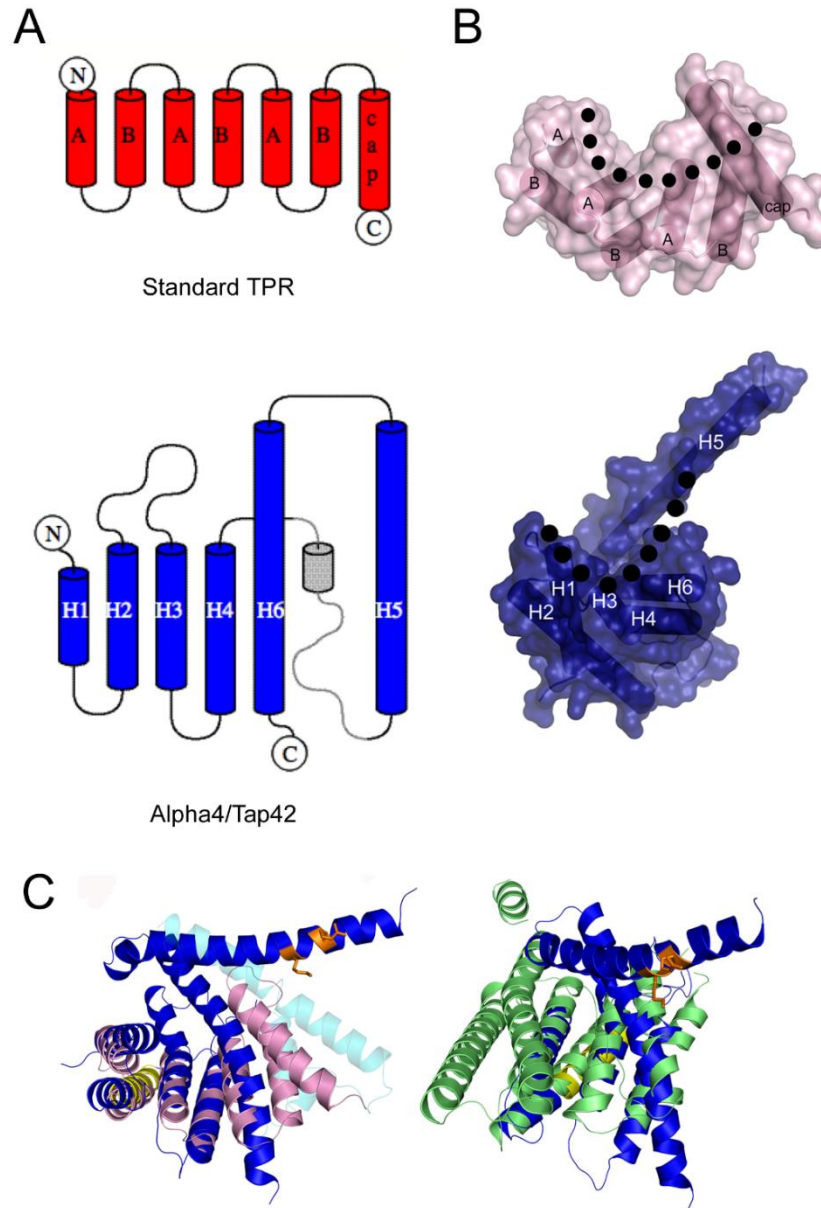
Figure 19: Comparison of Alpha4ΔC to TPR proteins. A) Topology diagrams of a standard TPR (top) and Alpha4ΔC (bottom) showing the altered topology of the final helices. The part represented in gray is based on crystal structure of Tap42ΔC, as these residues are not observed in the crystal structure of Alpha4ΔC. The diagrams were created in TOPDRAW [269]. B) Structures and surface representations of a standard TPR (top) and Alpha4ΔC (bottom) showing configuration of the helices and the formation of the concave and convex surfaces (outline of concavity denoted by dashed line). C) Superposition of Alpha4ΔC (colored blue, yellow, and orange, as in figure 14) with SycD TPR domain (pink) (2VGY) and 14-3-3 (green) (3EFZ) reveals similar tertiary structures, but indicate that the concave face of Alpha4 is more closed than a canonical TPR or 14-3-3 protein. The helices in cyan represent helices from Tap42ΔC that differ significantly in position from those in Alpha4ΔC.

extended helix is angled more steeply away from the rest of the protein than analogous capping helices, which pack against the concave surface. The distal portion of helix 5 is positioned above the concave binding surface, such that known binding residues (R156 and K159) point toward the concave surface. This extension of helix 5 and its positioning disrupts the typical TPR fold and creates a more closed concave face on the protein compared to standard TPR or 14-3-3 proteins (Figure 19B,C). In the crystal structure of Alpha4ΔC, the residues connecting helix 4 and 5 are not observed, and the third TPR-like motif lacks a B-helix. In the crystal structure of TAP42ΔC, the loop between helices 4 and 5 is observed and includes a small helix in a similar position to the B-helix of a third TPR motif [251] (Figure 19A), suggesting that Alpha4ΔC may contain a similar α helix in that position. Alpha4ΔC differs from a canonical TPR repeat in the topology of the final TPR motif and the capping helix with the inclusion of a large loop and an inversion in orientation. This altered topology in Alpha4ΔC allows for an opening and closing of the helix containing the PP2Ac binding residues creating a potential PP2Ac binding site (Figure 14C).

Discussion

Our final structure was based on diffraction data from two different crystal conditions. The first crystals that we obtained in 1.6M ammonium sulfate, 2% PEG400, 0.1M Bis-Tris pH 6.0 diffracted out to a resolution of 2.35 Å. Selenomethioine labeled protein was crystallized under identical conditions and the crystals obtained diffracted out to 2.5.Å resolution. Unfortunately, the diffraction data from these crystals were highly anisotropic with diffraction intensities in one dimension approximately 3 times that than in other two dimensions. Even with this high level of anisotropy the data was able to be phased and the selenomethionines located within the structure. An initial structure model was developed using this electron density map, but the model was never able to be refined to a good resolution. Although, the crystals diffracted out to 2.5 Å, the electron density maps looked more like 3.5Å resolution and the refinement

statistics could not improve even after repeated rounds of refinement. At this point, we decided that a new crystal form needed to be identified. Initial crystal screens were conducted and crystals were obtained and optimized in a 25% PEG1500 condition. These crystals diffracted out to 2.35 Å and we were able to use the initial structure determined using the anisotropic diffraction data to phase the new diffraction data by molecular replacement. This data produced much higher quality electron density maps and allowed us to refine the structure with appropriate refinement statistics for its resolution.

We determined that Alpha4ΔC adopts an alpha-helical TPR-like structure that differs from canonical TPR proteins in length, irregularity, and topology of their helices. Comparison of the structure of Alpha4ΔC to those of its yeast homolog, Tap42, shows the extended helix containing the PP2Ac binding determinants existing in multiple conformations, indicating that the PP2Ac binding region is flexible. The structure of Tap42 was determined in two conformations and Alpha4 adopts yet a third conformation with most of the variation between the three structures occurring in the relative position of the extended helix containing the PP2Ac binding residues (Figure 14B). The Alpha4 structure contains the most open conformation of this helix with the residues important for binding to PP2Ac in an open and accessible conformation. In the two structures of Tap42ΔC, these resides are less accessible, indicating that binding of a globular protein, like PP2Ac, would require an opening of this helix in Tap42 to allow binding.

DEER studies interrogating the extended helix of Alpha4 indicate that an open conformation is the predominant conformation found in solution, and that this helix is highly flexible with a continuum of conformations existing based on the broad width of the peaks at half maximum height in the DEER distance measurements (Figure 15). The Alpha4 structure also has the critical PP2Ac binding residues oriented such that they point towards the concave face of the molecule (Figure 14). This is of note because structures of other TPR and 14-3-3 proteins with their cognate interacting proteins show that the interactions are mediated either by

the concave face or via the interhelix loops [263,270–273]. Thus, it is likely that Alpha4 interacts with PP2Ac in a similar fashion.

We also interrogated the position and structure of the UIM within helix 2 of Alpha4. It has been reported that this region of the protein is important for Alpha4 regulation of PP2Ac polyubiquitination. We hypothesized that the most likely way in which this region was involved was that the UIM was binding to the ubiquitin moiety and blocking the K48 linkage site, in a manner similar to the HRS-UIM. Comparing the UIM in Alpha4 with that of an HRS-UIM bound to ubiquitin made it clear that the orientation of the UIM within our structure would preclude binding to ubiquitin in a similar manner (Figure 16B). DEER studies conducted to look at flexibility within this region revealed two distinct distance measurement peaks indicative of two distinct conformations of the protein (Figure 17B). We attempted to use an excess of ubiquitin to shift the protein into one conformation, but were unable to do so (Figure 17B). Using EPR based approaches, we were not able to detect any changes in the conformation of Alpha4ΔC induced by incubation with ubiquitin nor were we able to detect any level of interaction between ubiquitin and Alpha4 (Figure 17C). The data clearly show that Alpha4ΔC, while structured, is capable of a great degree of flexibility, the role and importance of this flexibility in its function has yet to be determined.

Chapter 3

Role of the PP2Ac- and E3 Ubiquitin-Ligase Binding Domains of Alpha4 in the Stability of Ectopically Expressed PP2A Catalytic Subunit

Introduction

Protein phosphatase 2A (PP2A) is a ubiquitous serine/threonine phosphatase involved in the regulation of numerous cell signaling pathways and cellular functions, including proliferation, cytoskeletal rearrangement, apoptosis and cell migration [4,238,242]. Several pathologies have been linked to dysregulation of PP2A, including Alzheimer's disease, cancer, and diabetes [5,20,23,26,274]. The activity of PP2A is tightly controlled *in vivo* via association with regulatory subunits, interactions with other cellular proteins, and various post-translational modifications [7,47,218,275]. PP2A regulatory subunits play a critical role in determining phosphatase activity and substrate selectivity, as well as directing the subcellular localization of the PP2A holoenzyme [4]. PP2A exists primarily as a heterotrimeric holoenzyme consisting of a structural A subunit, a variable B regulatory subunit, and the catalytic subunit (PP2Ac). However, a pool of PP2Ac exists in complex with an atypical regulatory subunit, termed Alpha4, that binds directly to PP2Ac in the absence of the A and B subunits [176,188,194,214]. Recent studies have shown that Alpha4 plays a crucial role in the control of PP2A's ubiquitination and stability [215,217,218].

Alpha4, a multidomain protein with similarity to Tap42 from yeast, was initially discovered as a 52 kDa phosphoprotein in B-cell receptor complexes [213,214]. Both Alpha4 and Tap42 consist of an N-terminal domain that contains the residues important for PP2Ac binding [251] and a C-terminal domain that is protease sensitive and intrinsically disordered [197]. Alpha4 regulates all three type 2A protein phosphatase catalytic subunits (PP2Ac, PP4c, and PP6c), modulating both enzymatic activity and expression levels [176,188,215,276].

Charge reversal mutations of R156 and K159 within Alpha4 were shown to abolish binding to PP2Ac, indicating that these residues are critical for the Alpha4-PP2Ac interaction [251]. In addition to its association with PP2A family members, Alpha4 has been shown to interact via its C-terminal domain with two known E3 ubiquitin ligases, EDD and Mid1. Both of these proteins have been implicated in regulating PP2Ac polyubiquitination and degradation [217–220,249,277]. Alpha4 co-localizes with Mid1 in cells, with the C-terminus of Alpha4 and the B-Box1 domain of the Mid1 protein mediating the association [218,219,278]. Mutations in Mid1, including ones that disrupt binding to Alpha4, have been linked to Opitz Syndrome, a developmental disorder characterized by incomplete closure of midline structures [279–282]. At the cellular level, mutations in Mid1 lead to decreases in ubiquitination and degradation of PP2Ac, especially microtubule-associated PP2Ac, and hypophosphorylation of microtubule associated proteins [218,283].

Although it was originally postulated that Alpha4 increased PP2Ac polyubiquitination and degradation by promoting interaction between PP2Ac and its putative E3 ubiquitin ligase Mid1 [218], several studies have been published showing a protective role of Alpha4 on PP2Ac polyubiquitination [215,217]. Immunoprecipitation experiments have shown that Alpha4 is serves as a scaffold for PP2Ac and Mid1 and that these proteins do not interact in the absence of Alpha4 [217]. Mammalian orthologs of Alpha4 also contain an ubiquitin-interacting motif (UIM) between residues 46-60, deletion of which abolishes the protective effect of Alpha4 on PP2Ac polyubiquitination [217]. This led to a model in which Alpha4 protected PP2Ac from polyubiquitination via a capping mechanism in which the UIM in Alpha4 interacted with monoubiquitinated PP2Ac to prevent further addition of ubiquitin [217].

In this Chapter, we examine the role of the E3 ubiquitin ligase and PP2Ac binding domains of Alpha4 in regulation of PP2Ac ubiquitination and degradation. Given the capping model described above, we hypothesized that the N-terminal PP2Ac binding domain would be sufficient to provide protection of PP2Ac from polyubiquitination and degradation. The effects of

wild type and mutated Alpha4 on PP2Ac ubiquitination and stability were examined *in vitro* by

performing ubiquitination assays with purified proteins and in mammalian cells by performing

precipitations with tandem ubiquitin binding entities (TUBEs) and cycloheximide chase

experiments. Contrary to our initial hypothesis, our results revealed that both the C-terminal E3

ubiquitin ligase binding domain and the PP2Ac binding determinants were required for Alpha4-

mediated protection of PP2Ac from polyubiquitination and degradation.

Materials and Methods

Work in this chapter was done in collaboration with Guy Watkins, Ph.D. in the laboratory

of Dr. Brian Wadzinski. All work with recombinant and purified proteins, as well as *in vitro*

assays were performed by me. Cell based assays were performed by Guy Watkins. Data

generated by Guy Watkins is credited beneath the corresponding figure.

Plasmids

The HA-ubiquitin plasmid was a gift from H. Moses (Vanderbilt University, Nashville,

TN), the myc-Mid1/pCMV tag3A construct was a gift from S. Schweiger (University of Dundee,

Dundee, U.K.), and the HA_3-PP2Ac construct was a gift from D. Brautigan (University of

Virginia, Charlottesville, VA). Construction of the FLAG-Alpha4/pcDNA5TO, FLAG-Alpha4∆C/

pcDNA5TO, and FLAG-Alpha4_ED/pcDNA5TO constructs were described previously [175,217].

Murine Alpha4∆C mutants were created using the QuikChange™ Site-Directed Mutagenesis

method (Stratagene, La Jolla, CA) and the following primers: Alpha4∆C_AA (R156A/K159A)

forward 5'-GCTATGGCATCTCAAGCACAGGCT GCAATGAGAGATACAAGC, reverse 5'-

GCTTGTATCTCTCTATTGCAGCCTGTGCTTGAGATGCCATAGC.

Antibodies

The mouse monoclonal PP2Ac antibody was from BD Transduction Laboratories (San Jose, CA). The rabbit monoclonal myc-tag antibody was from Cell Signaling Technology, Inc. (Danvers, MA). The rabbit polyclonal FLAG antibody was from Sigma (St Louis, MO). The rabbit polyclonal Alpha4 antibody was from Bethyl Laboratories (Montgomery, TX). The rabbit polyclonal ubiquitin antibody was from Dharmacon (Lafeyette, CO). The rabbit polyclonal 6xHis and mouse monoclonal HSP90 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and transfection

HEK293FT cells were grown at 37ºC in a humidified atmosphere with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cell were transfected using FuGene 6 transfection reagent (Roche) according to the manufacturer's directions.

Protein expression and purification

Recombinant 6xHis-tagged murine Alpha4, Alpha4ΔC and Alpha4ΔC_RK_AA proteins were expressed in BL21 (DE3) *E. coli* cells at 20°C overnight. The proteins were purified using metal affinity chromatography followed by size exclusion gel filtration on an S200 Amersham column in gel-filtration buffer (10 mM Tris-HCl, 150 mM NaCl, 1 mM sodium azide, pH 7.5). Proteins concentrations were determined using absorbance spectroscopy at 280 nm.

Human Flag-Alpha4 and Flag-AlphaΔC proteins were expressed and immunopurified from transfected HEK293T cells. At 48 hours post-transfection, cells were harvested in 500 µl cold immunopurification buffer (20 mM sodium phosphate pH 8.0, 150 mM NaCl, 1% Igepal) with fresh protease inhibitors (5 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 1mM PMSF). Clarified lysate was added to 40 µl of a 50% slurry of pre-washed Flag M2 agarose and incubated for 4 hours at 4°C. The resin was washed three times with cold immunopurification

buffer and proteins were eluted using 100 μl of 100 μg/ml Flag peptide. Protein concentrations were determined via densitometry using a BSA standard curve.

Binding assays

Binding assays were conducted using 40 μg of purified recombinant 6xHis-Alpha4ΔC or 6xHis-Alpha4ΔC_AA protein and 100 μl whole cell lysate from HEK293FT cells lysed with 500 μl RIPA buffer (20 mM sodium phosphate pH 8.0, 150 mM NaCl, 1% Igepal, 0.5% sodium deoxycholate, 0.1% SDS) per 10 cm plate. The lysate was incubated with the purified recombinant protein for 30 min at 4°C before adding 40 μl of a 50% slurry of Co-NTA resin and incubating for an additional 30 min at 4°C. The resin was washed three times with 1 ml of 20 mM sodium phosphate pH 8.0, 150 mM NaCl, 20 mM imidazole. Bound proteins were eluted with 200 mM imidazole and analyzed with SDS-PAGE and immunoblotting for PP2Ac and the hexahistidine tag.

In vitro *ubiquitination ass*ays

In vitro ubiquitination assays were conducted using immunopurifed wild-type human Flag-Alpha4 or Flag-Alpha4ΔC and purified bovine PP2Ac (a gift from Dr. Greg Moorhead, University of Calgary). The assays were carried out using a ubiquitin-protein conjugation kit (Boston Biochem, Cambridge, MA), following the protocol outlined in [217]. In brief, 4 μg purified ubiquitin (2 mg/ml), 150 ng purified Alpha4, and 50 ng purified PP2Ac were added to 10 μl of Fraction B (E1/E2 containing fraction) ± 10 μl Fraction A (E3 containing fraction) and total volume was brought to 80 μl with 1X ubiquitin assay buffer. For experiments involving deubiquitinase inhibitors, 2 μl of 50 μM PR-619 in 10% DMSO or 10% DMSO alone (vehicle control) were added to the reaction mixture and reactions were carried out as outlined above.

Immunoprecipitations

Cells were lysed in immunoprecipitation (IP) buffer [20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 5 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM PMSF, and 1 µg/ml leupeptin] and centrifuged at 12,000 x g for 10 min. Clarified lysates were incubated with 20 µl of a 50% slurry of anti-FLAG M2 agarose (Sigma) or 20 µl of a 50% slurry of anti-HA agarose (Roche) overnight at 4°C with rotation. Immunoprecipitations were washed three times in 1 ml IP buffer and bound proteins were eluted in SDS sample buffer and subjected to Western analysis.

Cycloheximide chase experiments

HEK293FT cells, seeded in six-well tissue culture plates at 300,000 cells per well, were transfected with either HA$_3$-PP2Ac alone or in combination with FLAG-Alpha4, FLAG-Alpha4ΔC, or FLAG-Alpha4_ED. At 48 hours post-transfection, cells were treated with 100 µg/ml cycloheximide (Sigma) for the indicated times and then lysed in IP buffer. Cell lysates were prepared and subjected to Western analysis using antibodies recognizing PP2Ac, Alpha4, and HSP90 (as a loading control).

Tandem Ubiquitin Binding Entities (TUBE) isolations

HEK293FT cells were lysed in IP buffer and centrifuged at 12,000 x g for 10 min. Clarified lysates were incubated with 20 µl of a 50% slurry of Agarose-TUBE2 (LifeSensors) overnight at 4°C with rotation. TUBEs, are Tandem Ubiquitin Binding Entities, linked to beads as a matrix to isolate and pulldown ubiquitinated proteins from cells. TUBE2 complexes were washed three times in 1 ml IP buffer and bound proteins were eluted in SDS sample buffer and subjected to Western analysis.

Western analysis

SDS-solubilized protein samples were separated by SDS-PAGE (4-12% Bis-Tris gradient acrylamide gels or 10% acrylamide gels) and transferred to 0.45 µm nylon-supported nitrocellulose membranes. Membranes were blocked in Odyssey Blocking Buffer (Li-COR, Lincoln, NE). All primary antibodies were used at 1:1000 diluted in a 1:1 mixture of Odyssey Blocking Buffer and Tris-Tween buffered saline (TTBS) (0.1% Tween-20, 50 mM Tris, 150 mM NaCl). For detection with the Odyssey Infrared Imaging system, appropriate secondary flouraphore-conjugated antibodies were used at 1:20,000 diluted in a 1:1 mixture of Odyssey Blocking Buffer and TTBS. Bound antibodies were visualized using the Odyssey Infrared Imaging system and Odyssey Classic Software Version 3.0 (Li-COR).

Results

The Alpha4ΔC mutant is capable of binding to PP2Ac, but not to Mid1

To determine whether the recombinant murine Alpha4ΔC used in our structural studies was capable of binding to PP2Ac and if this interaction could be disrupted by the mutations R156A and K159A (Alpha4ΔC_AA), we conducted *in vitro* binding assays using purified 6xHis-Alpha4ΔC, 6xHis-Alpha4ΔC_AA, and whole cell HEK293FT cell lysate as a source of PP2Ac. Alpha4ΔC, but not Alpha4ΔC_AA, bound to endogenous PP2Ac indicating that murine Alpha4ΔC is capable of binding to human PP2Ac, and that the residues R156 and K159 mediate this interaction (Figure 20A). To investigate the ability of Alpha and Alpha4ΔC to interact with PP2Ac and Mid1 in cells, HEK293FT cells were transfected with full-length FLAG-Alpha4 or FLAG-Alpha4ΔC and either HA_3-PP2Ac, myc-Mid1, or both HA_3-PP2Ac and myc-Mid1. Western analysis of FLAG immunoprecipitations revealed that while full-length Alpha4 bound both PP2Ac and Mid1, Alpha4ΔC only bound HA_3-PP2Ac (Figure 20B).

A

	Lysate	Co²⁺ affinity resin eluate		
6xHis-Alpha4ΔC		-	+	-
6xHis-Alpha4ΔC_RK_AA		-	-	+

MW kDa

WB: PP2Ac   37—

WB: 6xHis   25—

B

Flag-Alpha4	+	-	-	-	-	-	-	+	+	+
Flag-Alpha4ΔC	-	+	-	-	+	+	+	-	-	-
Myc-Mid1	-	-	-	+	-	+	+	-	+	+
HA₃-PP2Ac	-	-	+	-	+	-	+	+	-	+

Flag IPs — Myc-Mid1 / HA₃-PP2Ac / Flag-Alpha4

Lysates — Myc-Mid1 / HA₃-PP2Ac / Flag-Alpha4
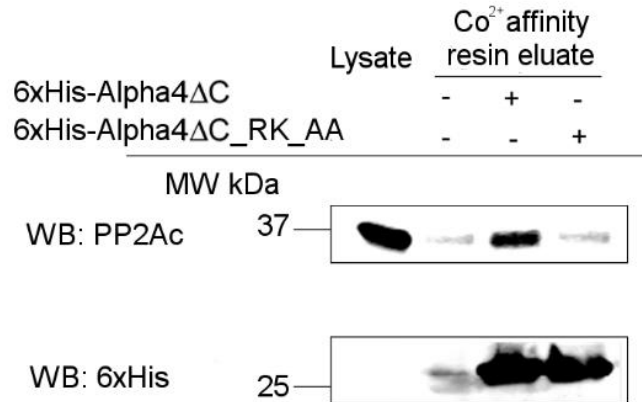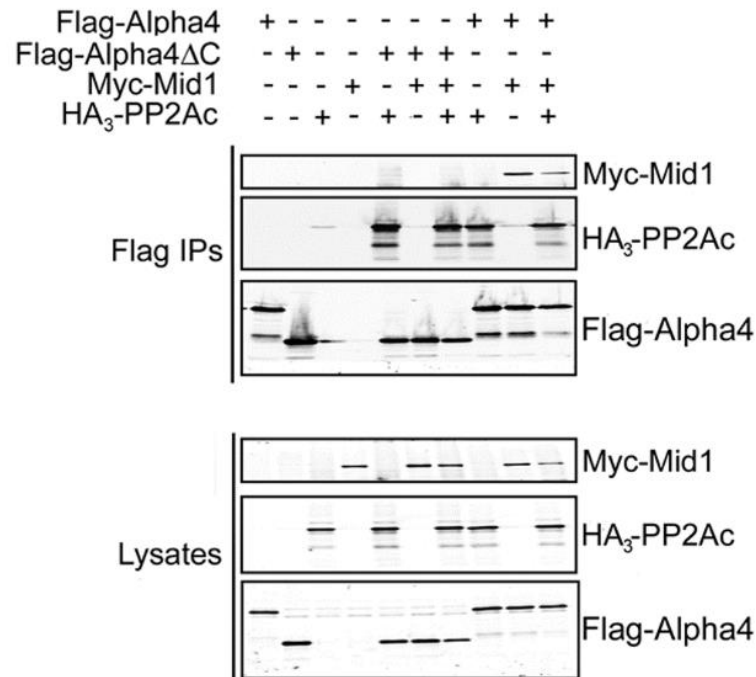
Data for panel B generated by Guy Watkins

Figure 20: Alpha4ΔC binds to PP2A, but fails to bind Mid1. A) HEK293FT whole cell lysate was incubated with Co-NTA resin in the absence (-) or presence (+) of either 6xHis-Alpha4ΔC or Alpha4ΔC_AA (mutation of the PP2Ac binding residues). Bound proteins were eluted with 200 mM imidazole, separated by SDS-PAGE, and analyzed by Western using antibodies recognizing PP2Ac (top panel) and the 6xHis tag (bottom panel). Data are representative of 3 independent experiments. B) HEK293FT cells were transfected with HA₃-PP2Ac, FLAG-Alpha4, FLAG-Alpha4ΔC, myc-Mid1 or a combination of the constructs. An aliquot of the cell lysates and FLAG immune complexes (FLAG-IPs) isolated from cell lysates were subjected to SDS-PAGE and Western analysis using antibodies recognizing Myc, PP2Ac, and Alpha4.

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Both the Mid1 binding domain and the PP2Ac binding residues of Alpha4 are essential for regulation of PP2Ac polyubiquitination

To investigate the role of the Mid1 binding domain and PP2Ac binding residues of Alpha4 in protecting PP2Ac from polyubiquitination, we performed TUBE isolation experiments on cell lysates of cells transfected with HA_3-PP2Ac and either empty vector, full-length FLAG-Alpha4, FLAG- Alpha4ΔC, or FLAG-Alpha4_ED. We found that when HA_3-PP2Ac was expressed alone polyubiquitinated PP2Ac could be observed and treatment with MG132, a proteasome inhibitor, greatly increased levels of polyubiquitinated PP2Ac. Expression of full-length FLAG-Alpha4, but neither FLAG-Alpha4ΔC nor FLAG-Alpha4_ED, decreased levels of PP2Ac polyubiquitination both in the presence and absence of MG132. All Alpha4 constructs were expressed at equal levels, as was HA_3-PP2Ac. Cells that were pre-treated with MG132, all showed increased levels of polyubiquitinated proteins, and expression of the various Alpha4 constructs did not affect levels of total poly-ubiquitinated proteins (Figure 21A). These findings demonstrate that both the Mid1 binding domain and the PP2Ac binding residues of Alpha4 are essential for the Alpha4-mediated protection of PP2Ac from polyubiquitination.

Both the Mid1 binding domain and PP2Ac binding residues of Alpha4 are required for Alpha4 to protect PP2Ac from degradation

In order to examine the role of Mid1 and PP2Ac binding on the ability of Alpha4 to decrease PP2Ac degradation, we performed cycloheximide chase experiments to evaluate the half-life of HA_3-PP2Ac when co-expressed with or without various Alpha4 constructs. Cells were treated with cycloheximide to inhibit new protein translation, and the levels of HA_3-PP2Ac were monitored at various time points after initiating cycloheximide treatment. This protocol allows us to monitor levels of PP2Ac and their degradation over time, as new PP2Ac is not produced. Cells that expressed HA_3-PP2Ac alone showed a progressive decline in the level of ectopic
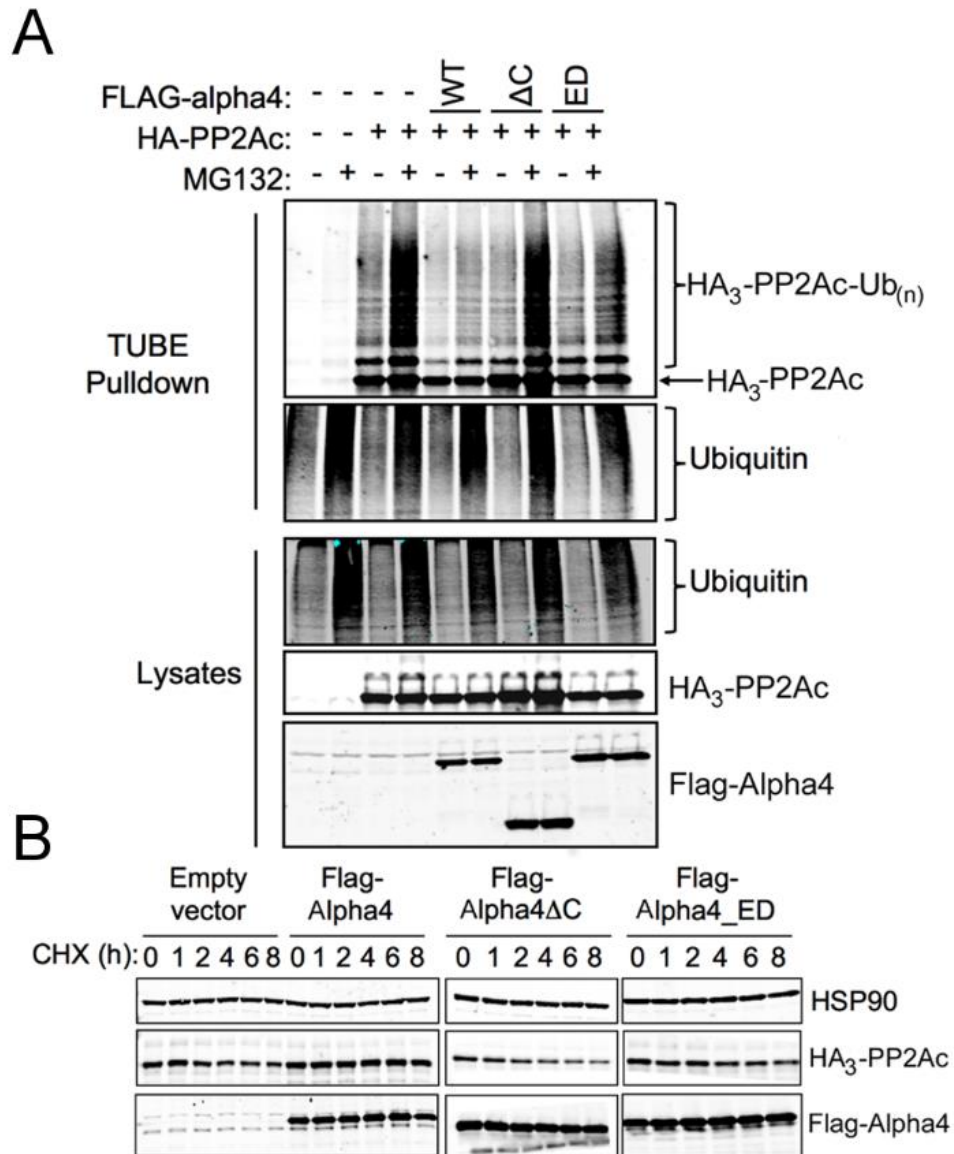
Figure 21: Both the Mid1 binding domain and PP2Ac binding are essential for Alpha4's inhibition of PP2Ac polyubiquitination and degradation. A) HEK 293FT cells were transfected with HA_3-PP2Ac and either empty vector, full-length FLAG-Alpha4 WT, FLAG-Alpha4ΔC, or FLAG-Alpha4_ED. At 48 h post-transfection cells were treated with(+) or without (-) 25 µM proteasome inhibitor (MG132) for 4 h at 37°C prior to lysis. Total polyubiquitinated proteins were isolated from the cell lysates using agarose TUBE2 beads. Protein expression and the polyubiquitination state of ectopic PP2Ac were analyzed via immunoblotting using PP2Ac, Alpha4, and ubiquitin specific antibodies. B) HEK293FT cells were transfected with HA_3-PP2Ac alone or with HA_3-PP2Ac and either FLAG-Alpha4, FLAG-Alpha4ΔC or FLAG-Alpha4_ED. Cells were treated with 50 µM cycloheximide (CHX) 48 hours post-transfection and then lysed at the indicated time points post-treatment. The lysates were subjected to Western analysis using antibodies recognizing Alpha4, PP2Ac and HSP90.

PP2Ac over the 8 h time course, while the samples co-expressing wild-type FLAG- Alpha4 had stable PP2Ac levels over this same period (Figure 21B). Cells co-expressing FLAG-Alpha4ΔC or FLAG-Alpha4_ED failed to promote this stabilization, but rather showed a progressive decline in HA$_3$-PP2Ac levels similar what was observed in cells expressing HA$_3$-PP2Ac alone (Figure 21B). In fact, the cells expressing Flag-Alpha4ΔC appeared to show an increased decline in HA$_3$-PP2Ac levels as compared to cells expressing HA$_3$-PP2Ac alone. These results indicate that both the Mid1 binding domain and the PP2Ac binding residues are essential for the PP2Ac stabilizing effect of Alpha4.

In vitro *ubiquitination assay supports that the Mid1 binding domain is required for Alpha4 reduction of PP2Ac ubiquitination*

In addition to investigating the effects Alpha4 and Alpha4 mutations when overexpressed in cells, we also wanted to look at the effects of Alpha4 and Alpha4ΔC on PP2Ac polyubiquitination using purified proteins in an *in vitro* assay to clarify that any effects seen were not an indirect effect of Alpha4 overexpression. To investigate the role of the C-terminus in protecting PP2Ac from polyubiquitination and degradation, we performed *in vitro* ubiquitination assays using a ubiquitin-protein conjugation kit (Boston Biochem), purified bovine PP2Ac, and either Flag-Alpha4 or Flag-Alpha4ΔC purified from HEK293FT cell lysate. Full-length Flag-Alpha4 (lane 4) consistently showed a protective effect towards PP2Ac polyubiquitination as evidenced by a decreased high molecular weight smear and reduced intensity of lower molecular weight ladders compared to the control lane (lane 3) with no ectopic Alpha4 present. This is more evident in the higher contrast image to the right. In contrast, addition of Flag-Alpha4ΔC (lane 5) showed no change in polyubiquitination or laddering compared to control (lane 3), indicating that the C-terminal domain of Alpha4 is required for Alpha4 to reduce levels of PP2Ac polubiquitination (Figure 22). This is consistent with the results seen in the cell-based assays described above.
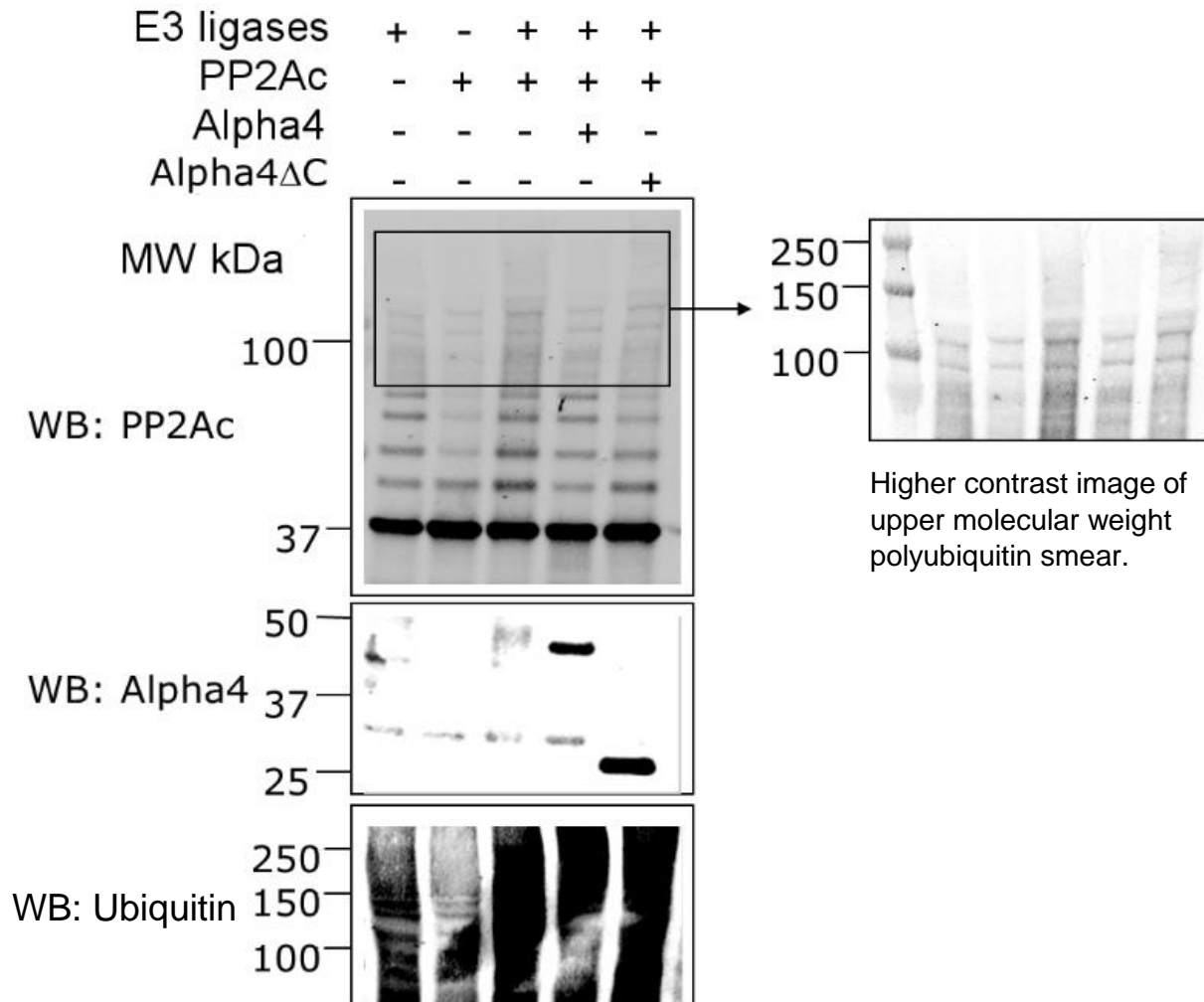
Figure 22: I*n vitro* ubiquitination assay supports that the Mid1 binding domain is required for Alpha4 reduction of PP2Ac ubiquitination. Immunopurified full length FLAG-Alpha4 (WT) or FLAG-Alpha4ΔC were used in an *in vitro* ubiquitin conjugation assay (Boston Biochem), along with purified PP2Ac, following manufacturer's protocols. Reaction mixtures were analyzed via immunoblotting using PP2Ac, Alpha4, and ubiquitin specific antibodies.

Inhibition of deubiquitination by PR-619 leads to increased polyubiquitination in both the

presence and absence of Alpha4

       Alpha4 could be acting in at least two ways to regulate PP2Ac polyubiquitination, it could

either prevent ubiquitination or promote deubiquitination. To investigate the mechanism of

action of Alpha4, we tested the effects of PR619, a deubiquitinase inhibitor, on polyubiquination

of PP2Ac in the *in vitro* ubiquitination assay. Fraction B of the ubiquitin- protein conjugation kit

contains both E3 ubiquitin ligases and deubiquitinating enzymes, as well as ubiquitin C-terminal

hydrolases, according to the manufacturer. We hypothesized that if Alpha4 prevented

polyubiquitination of PP2Ac from taking place, then treatment with PR-619 would have no effect

on the ability of Alpha4 to reduce polyubiquitination levels. On the other hand, if Alpha4

enhances deubiquitination then treatment with PR-619 should abrogate any effects of Alpha4 on

PP2Ac polyubiquitination and levels of polyubiquitinated PP2Ac should be the same in the

presence and absence of Alpha4 with PR-619 treatment.

       Treatment with PR-619 (lanes 6-8) caused an increase in ubiquitination in all conditions

compared to untreated control lanes, as evidenced by complete loss of lower molecular weight

laddering and an increase in higher molecular weight bands as compared to controls treated

with DMSO (lanes 3-5) (Figure 23). The appearance of the higher molecular weight bands (at

~200 kDa) is shown more clearly in the higher contrast image to the right. This change in

banding pattern of PP2Ac occurred both in the presence (lane 7) and absence (lane 6) of full

length Flag-Alpha4, as well as Flag-Alpha4ΔC (lane 8). This indicates that PP2Ac does

undergo deubiquitination within the *in vitro* assay and that inhibition of this activity increases

PP2Ac polyubiquitination, but we cannot ascertain the mechanism by which Alpha4 provides

protection. Very little (if any) protection of PP2Ac was seen in the control Alpha4 samples

treated with DMSO, thus confounding our ability to interpret the results of this experiment. This

decrease in protection in the assay could be due to the presence of DMSO or given our newly

gained insight into the relevance of Alpha4 cleavage, it could be due to the increased amount of
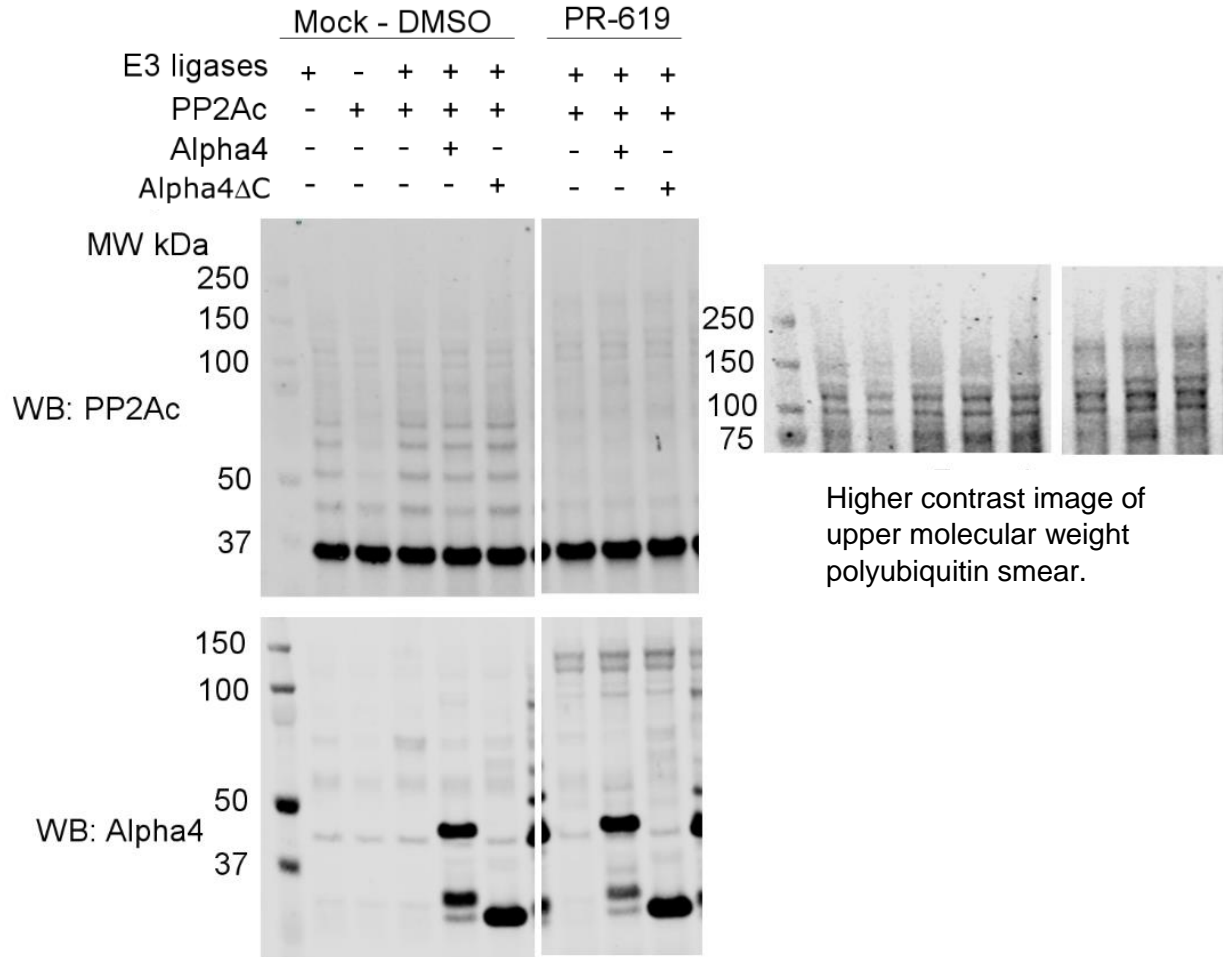
Figure 23: Inhibition of deubiquitination by PR-619 leads to increased polyubiquitination in both the presence and absence of Alpha4. Effects of the deubiquitinase inhibitior, PR-619, on the ability of Alpha4 to reduce levels of ubiquitinated PP2Ac in the in vitro ubiquitination assay were investigated by treating the reaction mixtures with either DMSO (vehicle control) or 50 µM PR-619. Reaction mixtures were analyzed via immunoblotting using PP2Ac and Alpha4 specific antibodies. Representative blots from 3 independent experiments.

Alpha4 cleavage product seen in these experiments as compared with the experiments conducted for Figure 22. The results indicate that full-length Flag-Alpha4 is not able to provide any protection from the increase in polyubiquitination caused by treatment with PR-619, and as such we cannot conclude that Alpha4 is acting via a mechanism independent of deubiquitination.

Treatment with PR-619 also appeared to increase polyubiquitination of Alpha4, indicating that Alpha4 itself may be a substrate for ubiquitination and deubiquitination within this assay (Figure 23 – bottom panel). As treatment with PR-619 exerts an effect on PP2Ac leading to increased ubiquitination in the both the presence and absence of Alpha4, this still leaves two possible explanations for decreases in PP2Ac polyubiquitination in the presence of full-length Alpha4 when cells are not treated with PR-619. Alpha4 could enhance deubiquitination and this effect is inhibited by PR-619 or it is acting in a manner independent of deubiquitination, but the effects of PR-619 override any protective effects that may be induced by Alpha4. More experiments need to be conducted to ascertain the mechanism by which Alpha4 is exhibiting the protective effect on Alpha4 in the absence of PR-619.

Discussion

Our initial hypothesis was that Alpha4 protected PP2Ac from being ubiquitinated via a yet to be determined mechanism involving the UIM site within Alpha4 that involved blocking the site the ubiquitination. We found that the N-terminal domain that contains the UIM and is responsible for binding to PP2Ac is necessary, but not sufficient for protection of PP2Ac from polyubiquitination. In fact, overexpression of Flag-Alpha4ΔC seems to promote degradation as compared to controls. This indicates that the C-terminus is a key player in protection of PP2Ac from polyubiquitination and degradation. The mechanism by which Alpha4 protects PP2Ac from degradation has yet to be determined. It could be that the C-terminus serves to block the interaction between PP2Ac and its E3 ubiquitin ligase via steric hindrance.

The C-terminus could also interact with an unknown protein that serves as a chaperone to PP2Ac and prevents its ubiquitination and degradation. Although, Alpha4 does reduce the levels of PP2Ac ubiquitination in *in vitro* assays, it appears to be considerably more effective at reducing polyubiquitination in cell-based assays. This suggests that Alpha4 may be recruiting an additional factor that is providing this protective effect and this factor is in limited supply in the *in vitro* reaction mixture. This other factor could be a chaperone molecule that assists in stabilizing and/or refolding PP2Ac making it a less viable target for ubiquitination. Another possible option is that Alpha4 recruits a deubiquitinase (DUB) that serves to deubiquitinate either PP2Ac, Alpha4 itself, or both; thus increasing the ability of Alpha4 to protect PP2Ac from degradation. Our studies with PR-619 showed that inhibition of deubiquitination by treatment with PR-619 increases polyubiquitination of both PP2Ac and Alpha4, but that treatment with PR-619 leads to increased polyubiquitination of PP2Ac in both the presence and absence of Alpha4. Therefore, the data do not support a conclusion that the protective effect is mediated by a process other than enhancement of deubiquitination, but it also does not exclude this possibility. The real insights gained from this experiment is that deubuitination is playing a significant role in regulating ubiquitination of proteins, even within *in vitro* ubiquitination assays and that their contribution should not be overlooked.

Prior studies have revealed that Alpha4 acts to both inhibit and promote PP2Ac degradation [215,217,218]. The initial model posited that Alpha4 was a scaffolding molecule that promoted polyubiquitination of PP2Ac by scaffolding PP2Ac to Mid1 [218]. Subsequent studies showed Alpha4 having a protective effect on PP2Ac degradation and polyubiquitination [215], and that Alpha4 contained a UIM which played a crucial role in protection of PP2Ac from polyubiquitination [217]. The present studies investigated the role of both PP2Ac binding and the Mid1 binding domain on Alpha4 regulation of PP2Ac ubiquitination and degradation. Our data demonstrate that both of these domains are required for Alpha4 to protect PP2Ac from degradation. These findings indicate that the protective effects of Alpha4 cannot be entirely

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accounted for by a hypothesis that Alpha4 inhibits Mid1 function or sequesters Mid1 from PP2Ac, and also raise questions about Mid1's role in PP2Ac polyubiquitination and degradation.

Interestingly, an Alpha4 reactive band that runs at a slightly higher molecular weight than our truncated Alpha4 construct, is consistently found in both cell lysates and purified proteins. This band has since been identified as an N-terminal cleavage product of Alpha4 produced by calpain cleavage that is induced by Mid1 catalyzed monoubiquitination of Alpha4 [191]. The findings that both the PP2Ac binding domain and the Mid1 binding domain are required for Alpha4 to exert its protective effects on PP2Ac, along with results indicating the importance of the UIM consensus motif in regulating both protection of PP2Ac from degradation and Alpha4 cleavage [191,217], imply a more complex mechanism of Alpha4's inhibition of PP2Ac degradation that involves contributions from all of these domains.

Chapter 4

Differential Effects of Knockdown and Expression of Alpha4 on the Expression of Protein Phosphatase 2A Family Members

Introduction

Protein phosphatase 2A (PP2A), and its closely related Type 2A family members PP4 and PP6, are abundant phospho-serine/threonine phosphatases that modulate a wide variety of cellular processes. Several regulatory mechanisms have been described for these enzymes, including post-translational modifications and association with regulatory subunits [4,8,9]. PP2A primarily exists as heterotrimeric holoenzymes consisting of a catalytic subunit (C subunit), a structural subunit (A subunit), and a regulatory subunit (B subunit). The canonical regulatory subunits are generally specific for a particular Type 2A family member and modulate phosphatase activity, substrate selectivity, and subcellular localization [3,4]. However, a number of non-canonical regulatory subunits exist that bind to the phosphatase catalytic subunit in the absence of the structural/canonical regulatory subunit and are capable of binding multiple Type 2A family members [175,176]. Our studies focus on one of these non-canonical regulatory subunits, IGBP1 or Alpha4.

IGBP1, or Alpha4, is a multidomain protein with similarity to Tap42 from *Saccharomyces cervisiae* [185,200]. It was initially discovered as a 52-kDa phosphoprotein associated with B-cell receptors[183,184], but has since been shown to be present in a wide range of tissues and cell types [110,184,187,243]. The N-terminus of Alpha4 consists of a tetratricopeptide repeat (TPR) domain and binds to the catalytic subunit Type 2A phosphatases [110,188,214]. Recent crystal structures of a partial fragment of PP2Ac complexed with the PP2Ac binding domain of Alpha4 show the N-terminal domain of Alpha4 bound to a partially unfolded and catalytically inactive fragment of PP2Ac [152], consistent with observations that PP2Ac associated with

Alpha4 has greatly diminished activity [211,212,214,215]. Although the fragment of PP2Ac used for crystallization is incomplete and does not contain the full catalytic domain, both of the residues shown to be essential for Alpha4-PP2Ac binding (R155, K158) make internal packing contacts within the n-PP2Ac/N-Alpha4 structure [152]. Although the PP2Ac-Alpha4 complex is inactive, several studies show increased PP2Ac activity upon Alpha4 overexpression [213,215,242] and reduced PP2Ac activity upon Alpha4 knockdown [215,242]. The mechanism of these changes in activity is not well understood.

One explanation supported by the crystal structure of Alpha4 bound to a fragment of PP2Ac is that Alpha4 stabilizes partially unfolded PP2Ac, preventing aggregation, and promoting proper folding and activation of PP2Ac [152], thus allowing for greater expression of functional PP2Ac. Consistent with this, overexpression of Alpha4 allows for increased expression of ectopic PP2Ac and protects exogenously-expressed PP2Ac from polyubiquitination and degradation by the proteasome [215,217,284]. Our previous studies have shown that the Alpha4-mediated protection of PP2Ac from polyubiquitination and degradation is dependent not only the N-terminus of Alpha4 that is involved in binding to PP2Ac, but also on the C-terminal portion of Alpha4 [284]. The C-terminal portion of Alpha4 has been shown to be important for binding to a number of E3 ubiquitin ligases, such as MID1 and EDD, which have been implicated in PP2Ac polyubiquitination [218,220,249,277]. MID1 has also been shown to be involved in the monoubiquitination of Alpha4, which in turn has been linked to cleavage of the C-terminal portion of Alpha4, providing a possible means of switching Alpha4's phosphates regulatory function from protective to degradative [191,193].

Studies of the effects of Alpha4 overexpression or knockdown on endogenous levels of PP2Ac have been inconclusive. The majority of studies show no significant changes in PP2Ac expression levels when Alpha4 levels are altered, either by knockdown or overexpression [210,223], but some studies have shown a decrease in PP2Ac and $PP2A_A$ levels when Alpha4 is deleted [215]. These data support the idea that Alpha4 effects on PP2Ac are dependent on a

direct interaction between Alpha4 and the catalytic subunit and act to stabilize an otherwise unstable pool of PP2Ac. The amount of PP2Ac, or any of the PP2A-like phosphatases, that are in complex with Alpha4 is not known, although studies done in yeast suggest a relatively small pool of the PP2Ac homologs interact with the Alpha4 homolog [200]. If true in mammalian cells, this might support a chaperone role in which proper phosphatase folding is enhanced by Alpha4, as this would explain its ability to affect expression levels and stability at a much greater level than amount of phosphatase bound to Alpha4 at any given time.

The Type 2A family of phospho-serine/threonine phosphatases in both humans (PP2A, PP4, and PP6) and yeast (Pph21, Pph22, Pph3, Sit4, and Ppg1) share approximately 60% sequence identity and all contain a conserved N-terminal region that binds Alpha4 (human) and Tap42 (yeast/Drosophila) [11,176,213,285]. This ability to bind to Tap42 is essential to the functionality of pph21 and Sit4 [11] and mutations in Alpha4/Tap42 that abrogate binding to PP2Ac fail to rescue the effects of Alpha4 knockdown or protect ectopic PP2Ac from degradation [284,285]. The regulatory effects of Alpha4 on PP6c and PP4c are somewhat controversial although it has been demonstrated that deletion of Alpha4 leads to decreased expression levels of all three mammalian Type 2A phosphatases [215]. This implies that Alpha4 may play a similar role in stabilizing PP4c and PP6c, as it does for PP2Ac.

Lentiviral-based transfection is a popular method to create stable cell lines expressing transgenes or lacking a target gene, especially in cells that are difficult to transfect. Typically, this method is used to knockdown or express a single gene, but recent protocols have been developed to allow for expression of multiple shRNAs within a single plasmid to either more efficiently knockdown a single gene or knockdown multiple genes of interest [286–290]. Very recently protocols have been published looking at simultaneous knockdown and expression of a target gene or genes [291]. Here we establish a relatively simple protocol, using a single lentiviral expression plasmid and readily available resources, to create stable cell lines that

simultaneously knock down an endogenous protein (Alpha4) and express an ectopic copy of the gene (FLAG-Alpha4).

Materials and Methods

Plasmids

We used a second generation lentiviral transfection system consisting of three plasmids: a packaging plasmid (psPAX2; gift from Didier Trono, Addgene #12260), an envelope plasmid (pMD2.G; gift from Didier Trono, Addgene #12259), and a transfer plasmid (pLKO.1-TRC; gift from David Root, AddGene #10878)[292]. The scrambled shRNA in pLKO.1 was a gift from David Sabatini (Addgene #1864)[293], shRNA directed to the 3'UTR (NM_001551.x-1110s1c1) or coding regions of Alpha4 (NM_001551.2-752s21c1) were from Sigma-Aldrich. The pcDNA5TO expression vector containing Flag-tagged human Alpha4 has been described[175].

Antibodies and Reagents

The rabbit polyclonal Alpha4 antibody was from Bethyl Laboratories (Cat# A300-471A). The mouse monoclonal PP2Ac antibody was from BD Biosciences. The sheep PP4c and PP6c antibodies have been described [110]. The mouse monoclonal HSP90 antibody was from Santa Cruz Biotechnology. The mouse tubulin antibody was from Sigma-Aldrich. The siRNA targeting Alpha4 and the control Block-iT siRNA were from Invitrogen. Puromycin was from Mediatech, Inc (Manassas, VA). Protein A agarose was obtained from Genscript. FuGENE 6 was from Promega (Madison, WI). The PCR primers used for subcloning human Flag-Alpha4 from pcDN5/TO were F: GGCAAGGCTTGACCGACAATTGCATGAAGAATCTGC and R: GTGGTGCAATTGGAGCCCCAGCTGGTTCTTTCCGC (Sigma).

Cell culture and transfection

HEK293T and HeLa cell stocks were obtained from the ATCC. A549 cell stocks were a gift from Dr. John V. Williams (University of Pittsburg Medical Center). All cell lines were grown in DMEM supplemented with 10% FBS and incubated at 37 °C in 5% CO_2. HEK293FT cells were transfected with mammalian expression constructs using LTX transfection reagent (Invitrogen) following manufacturer's protocols. HEK293FT cells were transfected with siRNA using RNAi Max (Invitrogen) following manufacturer's protocols.

Lysis of transfected cells

HEK293FT cells were seeded in 10 cm tissue-culture treated plates at a density of $2x10^6$ cells/plate and allowed to grow overnight. The next day they were transfected with either empty vector or wild-type Flag-Alpha4 for expression experiments or with control Block-iT siRNA or Alpha4 targeted siRNA for knockdown experiments. Forty-eight hours post-transfection, cells were gently dislodged from the plate by pipetting with 6 ml Earle's Balanced Salt Solution (EBSS) and the cell suspension was then centrifuged at 500 xg. The pelleted cells were lysed in 200 μl of ice cold lysis buffer (85 mM PIPES, pH 6.93, 1 mM EGTA, 1 mM $MgCl_2$, 2 M glycerol, 0.5% Triton) containing fresh protease inhibitors (1 μM PMSF, 1 μg/ml leupeptin, 0.7μg/ml pepstatin, 2μg/ml aprotinin). Cell lysates were clarified by centrifugation at 17,000 x g for 20 minutes and protein concentrations of the cell supernatants were determined by Bradford Assay (Bio Rad Protein Assay Kit). Laemmli loading buffer was added and cell lysates were stored frozen at -20°C for further analysis by Western.

Immunodepletion

Wild-type HEK293T cells were seeded at a density of $3x10^6$ cells in 10 cm tissue culture plates and allowed to grow to near confluency over 48 hours. Subsequent manipulations were

performed in parallel at room temperature and 4 °C (either in a cold room or on ice). The cells

were lysed with 400 μl lysis buffer (10 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1% Igepal)

containing freshly added protease and phosphatase inhibitors (1 μM PMSF,1 ug/ml leupeptin,

0.7 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM Na_3VO_4, 30 mM NaF, 20 mM $Na_4O_7P_2$, 50 mM β-

glycerophosphate disodium, pH 7.2. Cell lysates were clarified by centrifugation at 17,000 x g

for 15 minutes. Protein A resin was washed three times in a PBS buffer containing 1% BSA and

resuspended in a 50% slurry with PBS buffer containing 1% BSA. Immunodepletions were

conducted using 300 μl of clarified cell lysate and 20 μl of the pre-washed Protein A resin slurry

in the presence or absence of 3 μl Alpha antibodies (1:100 dilution). The first round of

immunodepletions was conducted for 4 hours and then lysates were centrifuged at 1,400 x g for

5 minutes. The supernatants were collected and 40 μl aliquots were taken for analysis. Alpha4

antibodies or an equal volume of buffer were added to the remaining lysates at a 1:100 dilution

and incubated overnight. The next morning 20 μl of the pre-washed Protein A resin slurry was

added and incubated for 1 hour and then lysates were centrifuged at 1,400 x g for 5 minutes.

Supernatants were collected and 40 μl aliquots were taken for analysis. Alpha4 antibodies or

equal volume of buffer were added to the remaining lysates at 1:100 dilution along with 20 μl of

the Protein A resin slurry and incubated for 3 hours. Lysates were centrifuged at 1,400 x g for 5

minutes and supernatants collected for analysis. All samples for analysis were placed

immediately on ice, 2X Laemmli Sample buffer was added and samples were heated to 95 °C

for 10 minutes.

Lentiviral Production

HEK293T cells were seeded at a density of $7x10^5$ cells/well in 6-cm tissue culture plates.

Lentiviral plasmids (250 ng pMD2.G, 750 ng psPAX2, 1 μg PLKO.1 vector plasmid) were

transfected into HEK293T cells using FuGENE 6, following the manufacturer's protocol for

packaging into viral particles. Media was exchanged after 15 hours and virus-containing

supernatant was harvested and pooled at 24 hours and 48 hours. Supernatant was clarified by

centrifugation at 1,000 x g for 5 minutes and stored at -20°C.

Creation of stable cell lines

Cells were seeded at a density of $5x10^5$ cells/well in 6-well tissue culture plates and

allowed to grow overnight before infection with lentivirus using 0.5 ml of viral supernatant.

Media was replaced after 24 hours and cells were treated with puromycin for selection of stably

infected cells. Puromycin concentrations used for selection were 7 μg/ml (A549), 3μg/ml

(HEK293T), and 1 μg/ml (HeLa).

Cell lysis of stable cell lines

Cells were seeded at $4x10^5$ cells/well in 6-well plates in DMEM supplemented with 10%

FBS and incubated at 37°C in 5% CO2 for 72 hours. Plates were placed on ice, rinsed 2x with

1 ml of cold PBS, and then lysed with 200 µl of cold lysis buffer (20 mM MOPS pH 7.0, 5 mM

EDTA, 2 mM EGTA, 1 mM DTT) containing freshly added protease and phosphatase inhibitors

(1 μM PMSF, 1 μg/ml leupeptin, 0.7 μg/ml pepstatin, 2 μg/ml aprotinin, 1 mM Na_3VO_4, 30 mM

NaF, 20 mM $Na_4O_7P_2$, 60 mM β-glycerophosphate disodium, pH 7.2). Cell lysates were clarified

by centrifugation at 16,000 x g for 20 minutes at 4°C. Protein concentrations of supernatants

were determined using a Bradford assay (BioRad Protein Concentration reagent).

Supernatants were diluted, to 1 mg/mL, aliquoted and stored at –20 ˚C.

Western Analysis

Approximately 15-20 μg of protein were separated by SDS-PAGE using 4-12% Bis-Tris

NOVEX NU_PAGE gels (Lifetech). Proteins were transferred to 0.45 μm nylon-supported

nitrocellulose membranes (GE Life Science, Amersham) and membranes were stained with

PonceauS to verify transfer and protein loading. Membranes were blocked overnight in

Odyssey Buffer (Li-COR; Lincoln, NE) and then probed with primary antibodies to the proteins

of interest overnight at 4°C. All antibodies were diluted in a 1:1 solution of Tris buffered saline

with Tween (TTBS: 50 mM Tris, 150 mM NaCl, 0.05 % Tween 20, pH 7.6) and Odyssey

blocking buffer (Li-COR). Antibody dilutions were as follows: anti-Alpha4 (1:1000), anti-PP2Ac

(1:1000), anti-PP4c (1:500), anti-PP6c (1:500), anti-HSP90 (1:1000), and anti-tubulin (1:1000).

Membranes were washed 3 times with TTBS then probed with appropriate fluorescently labeled

secondary antibodies (LiCOR; diluted 1:20,000 in TTBS) for 30 minutes. Membranes were

washed 3 times then images were obtained using the Odyssey Imaging platform.

Analysis

All statistical analysis and graphing was performed in Graph Pad Prism version 6.0 for

Windows (GraphPad Software, La Jolla, CA USA). The specific tests used are described in the

figure legends.

Results and Discussion

Association of Alpha4 with Type 2A phosphatases

To determine the fraction of each of the phosphatase catalytic subunits (PP2Ac, PP4c,

and PP6c) bound to Alpha4, we conducted immunodepletion experiments. Alpha4 was depleted

from whole cell lysates using an Alpha4-specific antibody and the resulting supernatants were

probed for PP2Ac, PP4c, and PP6c (Figure 24A). Experiments were conducted in parallel at

4°C and at room temperature as temperature has previously been shown to affect the

association of Alpha4 with PP2Ac [215]. Alpha4 immunodepletion did not significantly reduce

PP2Ac levels under either condition (Figure 24A), indicating that a relatively small cellular pool
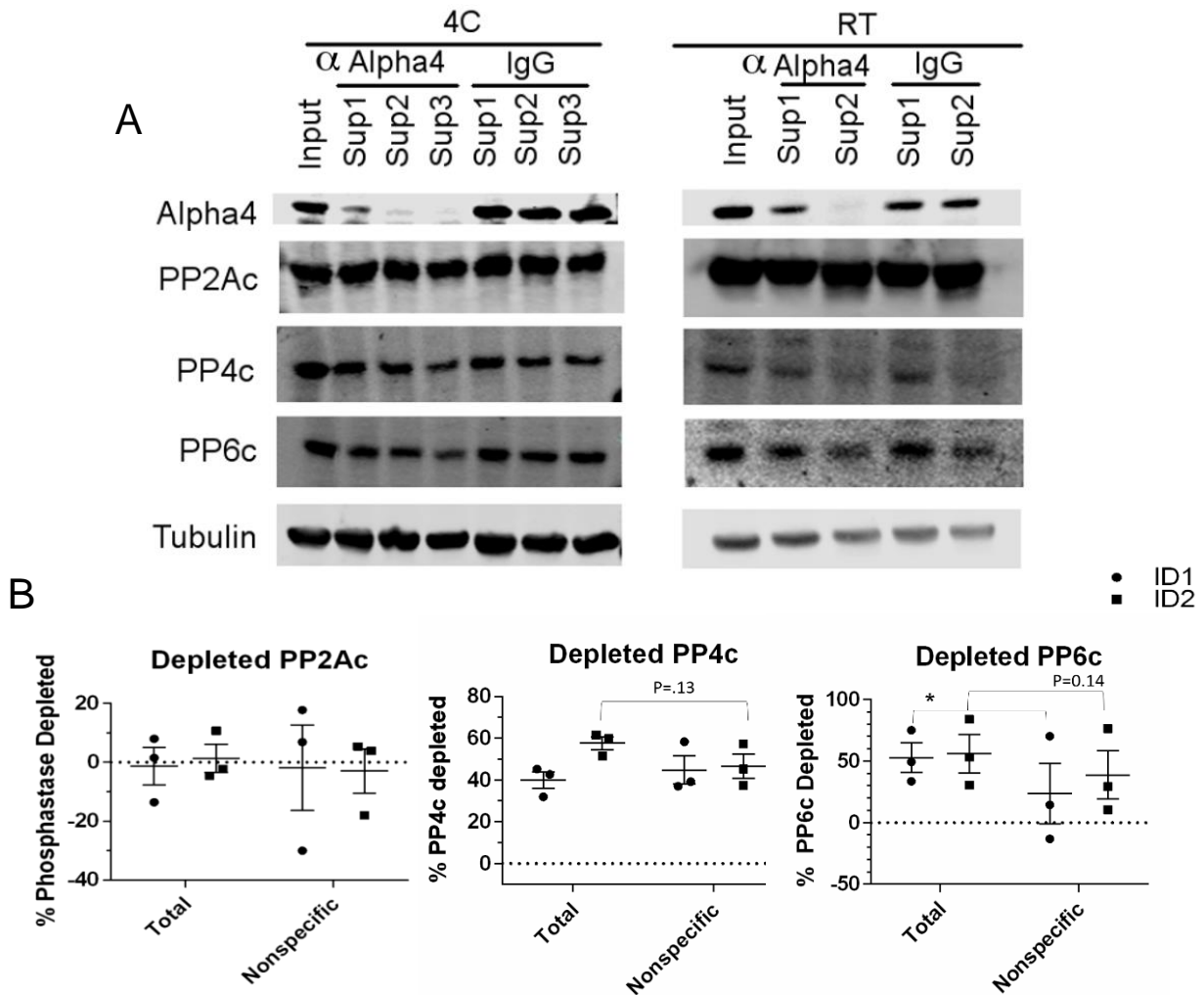
Figure 24: Association of phosphatase catalytic subunits with Alpha4. Cell lysis and immunodepletion were conducted at either 4°C or room temperature. Cell lysates were cleared by centrifugation and the cleared supernatants were split into equal aliquots for immunodepletion experiments. Supernatants were incubated with either 20 μl Protein A resin alone or with Alpha4 antibody (1:100 dilution) and 20 μl Protein A resin. Three successive rounds of immunodepletion were conducted and aliquots were removed after centrifugation for analysis. Samples were separated via SDS-PAGE gel electrophoresis and transferred to nitrocellulose. A) Western blot of input and supernatants from successive rounds of immunodepletion (at both 4°C and room temperature) probed for Alpha4, tubulin, PP2Ac, PP4c, and PP6c. B) Membranes were probed for Alpha4, tubulin, PP2Ac, PP4c, and PP6c and quantified using Odyssey Imaging software. Graphs shows % of phosphatase catalytic subunit depleted after each round of immunodepletion. A total of 3 independent experiments were conducted. Statistical significance was calculated using two-way ANOVA analysis. Graph shows mean ± SEM. *, $p < 0.05$

of PP2Ac is associated with Alpha4. In contrast, both PP4c and PP6c showed signs of depletion when Alpha4 was immunodepleted at 4°C (Figure 24A, B). Overall, these results indicate that larger fractions of PP4c and PP6c associate with Alpha4 than PP2Ac, with PP6c being the most highly associated with Alpha4.

Alpha 3'UTR targeted shRNA is effective in achieving Alpha4 knockdown

Given these results, we sought to determine the effects of Alpha4 knockdown and overexpression on PP2Ac, PP4c, and PP6c expression levels. Specifically, to determine if transient versus stable knockdown or overexpression have similar effects on phosphatase catalytic subunit expression and if these effects are similar for all the Type 2A phosphatases. To test the effects of both transient and stable knockdown and expression, we developed a lentiviral-based panel of Alpha4 knockdown cell lines. Lentiviral particles expressing either scrambled shRNA, shRNA targeted to 3'UTR of Alpha4, or shRNA targeted to the coding region of Alpha4 (Figure 25A) were used to infect HEK293T cells. Effective knockdown of Alpha4 was accomplished using both of the shRNAs (Figure 25A, top left). With the efficacy of the 3'UTR construct confirmed, we infected three different adherent cell lines (HEK293T, HeLa, and A549) with either scrambled shRNA-expressing lentivirus or 3'UTR shRNA-expressing lentivirus and selected for stable incorporation using puromycin. We determined that the knockdown efficiency of Alpha4 was approximately 85% in the HEK293T and HeLa cell lines and approximately 80% in the A549 cell line (Figure 25B, C, D).

A dual promoter plasmid for simultaneous knock down and expression of Alpha4 in cells

To create the dual promoter plasmid allowing for simultaneous knockdown and expression, we modified a PLKO.1-TRC cloning vector containing the 3'UTR targeted Alpha4 shRNA by inserting a fragment containing Flag-Alpha4 driven by a pCMV promoter. The fragment was created using PCR primers containing Mfe1 restriction sites and the
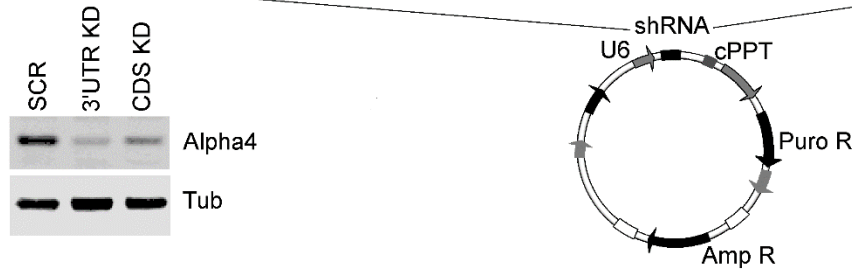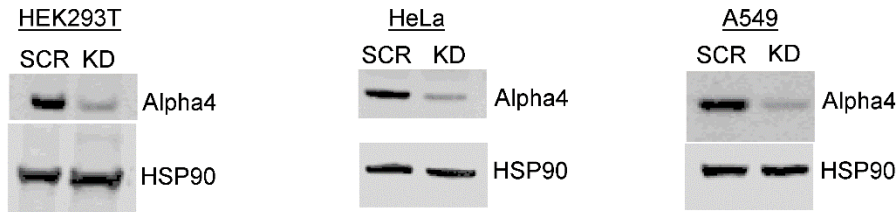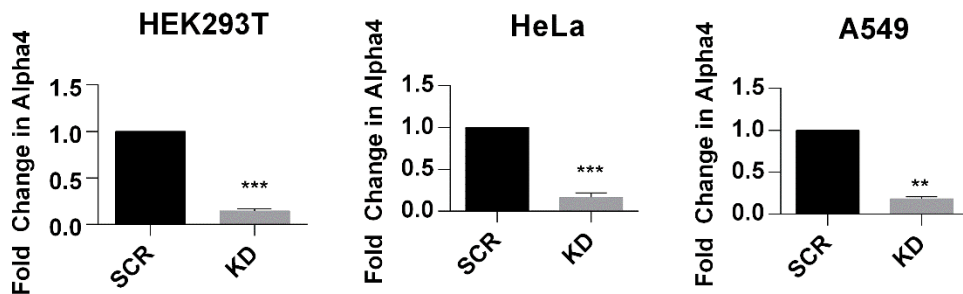
Figure 25: Creation of stable knockdown cell lines. A) Sequences of shRNAs: scr shRNA, coding region (CDS) shRNA, 3'UTR shRNA (top). Schematic of PLKO.1-TRC vector showing location of shRNA insert (bottom,right).U6: RNA promoter; cPPT: central polypurine tract; Puro R: Puromycin resistance gene; Amp R: Ampicillin resistance gene. Representative western blot of cell lysates from HEK293T stable cells, showing knockdown of Alpha4 with both the 3'UTR shRNA (3'UTR KD) and coding region shRNA (CDS KD) (bottom, left) B) Representative western blots of cell lysates from HEK293T (left), HeLa (middle), and A549 (right) stable cells expressing either scrambled shRNA or 3'UTR targeted shRNA probed for Alpha4 and HSP90 (loading control). C) Graphs showing Alpha4 expression of KD cell lines relative to scrambled control shRNA in HEK293T (left), HeLa (middle), and A549 (right). At least 3 independent experiments were used for quantifications. Graphs show mean ± SD. Statistical significance was calculated using one-sample t-test against a hypothetical mean of 1. **, p<.001; ***, p<0.0001

Alpha4/pcDNA5TO expression vector as a template (Figure 26A). The PCR product was then digested with MfeI and ligated into an EcoRI digested PLKO.1-TRC vector. This resulted in the plasmid shown in Figure 26A, in which a U6 promoter drives shRNA expression and a pCMV promoter drives Flag-Alpha4 expression. It should be noted that the cDNA construct of Flag-Alpha4 does not contain the native 3'UTR, this allows the 3'UTR directed shRNA to knockdown endogenous Alpha4, but leave the Flag-Alpha4 unaffected. To create the stable cell lines, we transfected our modified PLKO.1-TRC constructs, psPAX2 and pMD2.G into HEK293T cells to produce lentivirus particles. The lentivirus-containing cell supernatant was then used to infect multiple cell lines (HEK293T, HeLa, and A549).

One of the goals of this investigation was to efficiently and stably knockdown Alpha4 to allow studies of long-term effects of Alpha4 repression. As well as, investigate the ability of Alpha4 and Alpha4 mutants to rescue the knockdown. As a first step toward this goal, stable cell lines expressing scrambled shRNA, 3'UTR Alpha4 shRNA, 3'UTR Alpha4 shRNA plus Flag-Alpha4 cDNA, and scrambled shRNA plus human Flag-Alpha4 cDNA were created in HEK293T cells, and expression levels of Alpha4 were compared by Western analysis of cell lysates (Figure 26B). Knockdown efficiency was approximately 85% in both 3'UTR Alpha4 shRNA alone and 3'UTR Alpha4 shRNA plus Flag-Alpha4 cDNA cell lines. Expression of the Flag-Alpha4 was approximately equal in the cell lines expressing both scrambled shRNA and 3'UTR shRNA and was approximately 3-fold more than endogenous Alpha4 expression.

Effects of transient Alpha4 knockdown and overexpression on phosphatase catalytic subunit expression

Previous experiments have shown limited effects of transient Alpha4 knockdown and overexpression on endogenous PP2Ac levels [210,242], and the effects of knockdown and overexpression on PP4c and PP6c have not been extensively studied. Given the results from
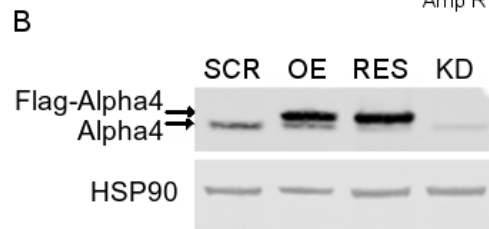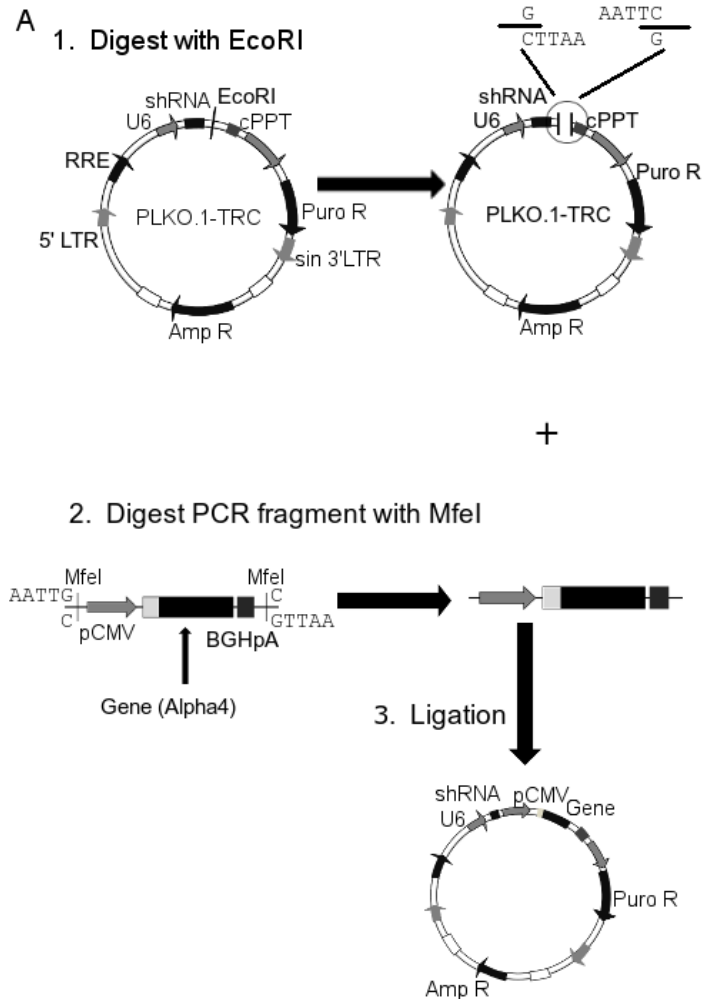
Figure 26: Creation of simultaneous knockdown and expression vector. A) Schematic representation of creation of simultaneous knockdown and expression vector using PLKO.1-TRC as a backbone. U6: RNA promoter; cPPT: central polypurine tract; Puro R: Puromycin resistance gene; Amp R: Ampicillin resistance gene; EcoR1: EcoR1 restriction site; 5'LTR: 5' long terminal repeat promoter sequence; sin 3' LTR: Self-Inactivating 3' long terminal repeat; RRE: Rev response element. In step 1, PLKO.1-TRC vector with inserted shRNA is digested with EcoRI. In step 2, PCR primers are used to amplify the pCMV promoter and gene from the pcDNA5/TO expression vector. pCMV: constitutive CMV promoter; pBGHrev: reverse primer site; MfeI: MfeI restriction site. The PCR fragment is then digested with MfeI. In step 3, the fragment is ligated into the PLKO.1-TRC backbone at the EcoRI restriction site. B) Representative western blot showing Alpha4 expression profiles in stable cell lines expressing scrambled shRNA (SCR), scr shRNA + Flag-Alpha4 (OE),3'UTR shRNA + Flag-Alpha4 (RES), 3'UTR shRNA (KD).

the immunodepletion experiments, we hypothesized that knockdown and overexpression of Alpha4 would have greater effects on PP4c and PP6c levels than on PP2Ac levels. To assess the effects of transient Alpha4 knockdown on phosphatase catalytic subunit expression, we determined PP2Ac, PP4c, and PP6c levels in HEK293T cells that were transfected with either control siRNA or Alpha4 siRNA. Only PP6c showed a significant decrease in expression upon Alpha4 knockdown (Figure 27A). We also determined PP2Ac, PP4c, and PP6c expression levels in HEK293T cells overexpressing a Flag-Alpha4 construct, and found no significant differences in expression of any of the phosphatase catalytic subunits (Figure 27B).

Stable knockdown and expression of Alpha4 differentially effects phosphatase expression levels

Chronic, rather than transient, changes in Alpha4 expression levels likely better recapitulate the perturbations seen in diseases where Alpha4 is mutated or misregulated, as is the case in the many cancers [223,240,241,294]. Using our simultaneous knockdown and expression cell lines, we investigated the effect of stable knockdown and expression of Alpha4 on the levels of endogenous PP2Ac, PP6c, and PP4c in HEK293T cells under both 10% FBS conditions and after overnight serum starvation. Knockdown of Alpha4 does not significantly impact PP2Ac expression, but there was an increase in PP2Ac expression in Flag-Alpha4 expressing cells compared to knockdown cells (Figure 28A, B). This increase in PP2Ac levels with Flag-Alpha4 expression was more pronounced under low serum conditions (Figure 28A, B). Interestingly, PP6c expression was decreased upon Alpha4 knockdown and expression of wild-type Flag-Alpha4 cDNA rescued this effect (Figure 28E, F). PP4c also showed pronounced decreases in expression levels with Alpha4 knockdown under both normal and serum-starved conditions; however, this decrease in expression was unable to be rescued with expression of wild-type Alpha4 cDNA (Fig 28C, D). These results are consistent with findings shown in Figure 21, in which immunodepletion experiments revealed that a greater proportion of PP4c and PP6c are associated with Alpha4. The variability in results of Alpha4 knockdown and expression on

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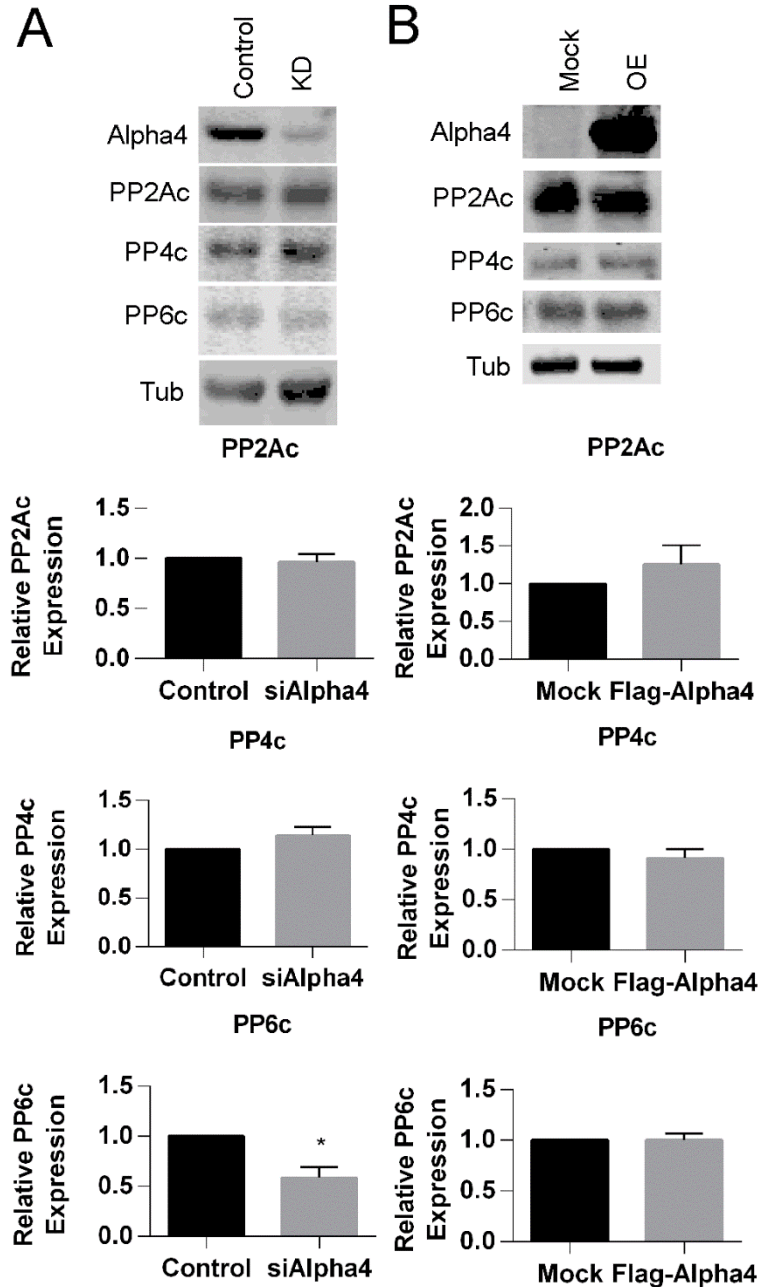
Figure 27: Effects of transient Alpha4 knockdown and overexpression on PP2A family phosphatase expression. HEK293T cells were grown in 10% FBS DMEM. A) Cells were transfected with either Control BlockIT siRNA or Alpha4 targeted siRNA. Cells were harvested 48 hours post-transfection. (top) Representative Western blots probed for PP2Ac, PP4c, PP6c, Alpha4 and tubulin (loading control). Quantification of PP2Ac, PP4c, and PP6c levels relative to control. B) Cells were transfected with either empty pcDNA5/TO vector or Flag-Alpha4 pcDNA5/TO vector. Cells were harvested 48 hours post-transfection. (top) Representative western blots probed for PP2Ac, PP4c, PP6c, Alpha4 and tubulin (loading control). Quantification of PP2Ac, PP4c, and PP6c levels relative to control. At least 3 independent experiments were performed for all analyses. Graphs shows mean ± SEM. Statistical significance was calculated using one-sample t-test against a hypothetical mean of 1. *, $p < 0.05$
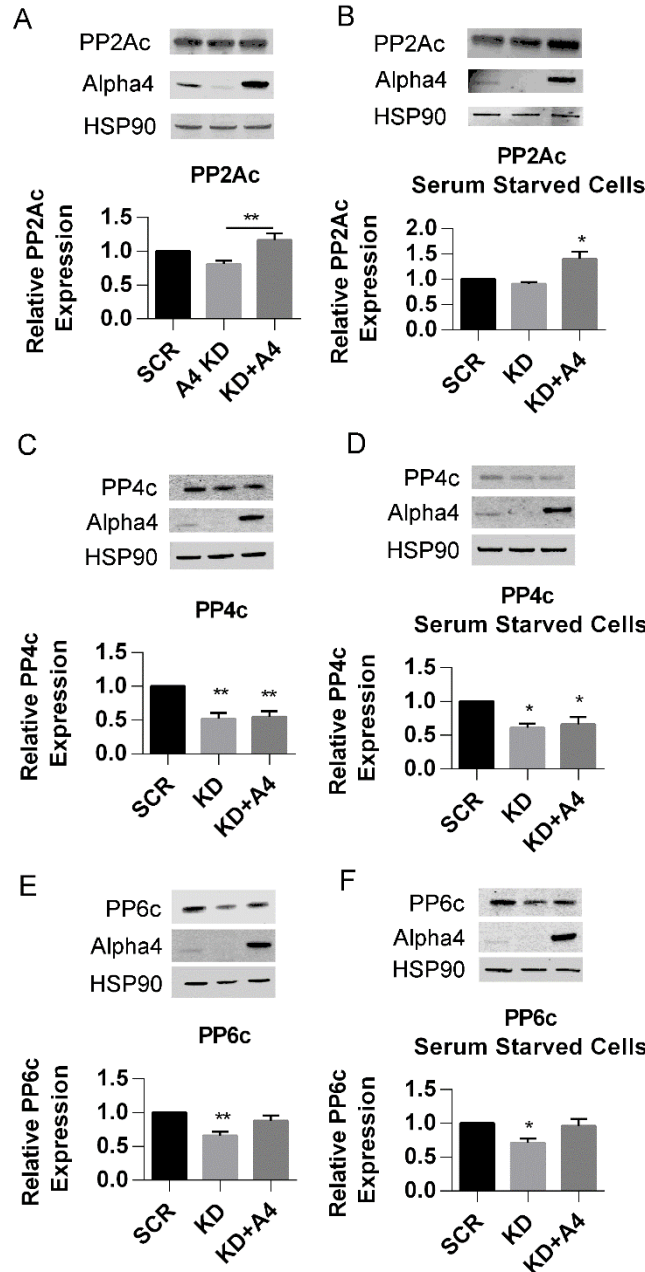
Figure 28: Effects of stable Alpha4 knockdown and expression on PP2A family phosphatase expression. HEK293T cell were grown either in 10% FBS DMEM (A, C, E) or serum starved overnight prior to harvesting (B, D, F). A, B) (top) Representative Western blots probed for PP2Ac, Alpha4, and HSP90 (loading control). (bottom) Quantification of PP2Ac expression levels relative to scrambled control cells. C ,D) (top) Representative western blots probed for PP4c, Alpha4, and HSP90 (loading control). (bottom) Quantification of PP4c. E, F) (top) Representative western blots probed for PP6c, Alpha4, and HSP90 (loading control). (bottom) Quantification of PP6c expression levels relative to scrambled control cells. At least 3 independent experiments were performed for all analyses. Graphs shows mean ± SEM. Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparison analysis. *, $p < 0.05$; **, $p < 0.01$

Type 2A phosphatase levels implies differential regulation of these three very similar

phosphatases and possibly a more complex role of Alpha4 in regulation of phosphatase levels

in mammalian cells.

Conclusions

Herein we describe a simple, fast, and efficient protocol to create lentiviral vectors

capable of simultaneous knockdown and expression of an unmodified target gene. This method

allows for creation of stable cell lines within 3-4 weeks with a single round of

transfection/infection. These cell lines remain stable over time with levels of knockdown and

expression remaining constant within each cell line, thus reducing variability between

experiments. In addition, the ability to express a cDNA simultaneous with knockdown of the

corresponding endogenous protein allows more efficient study of the effects of mutations on the

activity of proteins involved in cell growth, proliferation and apoptosis. Our results indicate that

we were successfully able to stably knockdown Alpha4 in a variety of cell lines. Stable

knockdown had more pronounced effects upon PP4c expression than transient knockdown

(Figure 27A; Figure 28B, C), but transient and stable knockdown of Alpha4 had similar effects

upon PP6c (Figure 27A; Figure 28E, F) and PP2Ac (Figure 27; Figure 28A, B) expression

levels. Transient overexpression of Alpha4 in the presence of endogenous Alpha4 had very little

effect upon the expression levels of any of the Type 2A phosphatases (Figure 27), indicating

that the effects of Alpha4 mutations are better studied in the absence of endogenous Alpha4.

Stable expression of Flag-Alpha4 in the absence of endogenous Alpha4 partially rescued the

depletion of PP6c levels caused by Alpha4 knockdown (Figure 28E, F) and increased

expression of PP2Ac (Figure 28A, B), but had no effect upon levels of PP4c (Figure 28B,C).

The inability to rescue PP4c expression levels with a Flag-Alpha4 cDNA construct is intriguing

as it implies that PP4c is regulated by Alpha4 in a different fashion then PP2Ac and PP6c.

Most studies on Alpha4 have focused on its interactions with PP2Ac and have attributed alterations in cellular functions to changes in PP2Ac expression or activity. This study indicates that the role of Alpha4 in regulating the closely related Type 2A phosphatases PP4c and PP6c should also be considered when investigating the effects of Alpha4 on cell functions. Moreover this study also shows that while these three phosphatases are closely related, Alpha4 has differential effects upon their expression levels.

Chapter 5

Alpha4 Effects on Cell Adhesion, Growth and Viability

Introduction

Alpha4 is overexpressed in a number of cancer cell lines (including breast, lung, liver and bladder tumors) [223,241], with overexpression of Alpha4 linked to poor prognosis and increased recurrence of tumors [240,241]. Ectopic overexpression of Alpha4 in non-tumorigenic cell lines allows tumor formation *in vivo* and increases cellular proliferation and colony forming capacity; whereas knockdown of Alpha4 in tumorigenic cell lines reduces their tumor forming capacity, rate of growth and colony formation [223]. Knockout of Alpha4 in mouse models is embryonic lethal, while induced knock out leads to apoptosis in cell lines expressing wild-type p53 [238]. This apoptosis appears to be via the intrinsic mitochondrial apoptosis pathway, as overexpression of Bcl-x_L can abrogate the effects of Alpha4 knockout [238]. These roles of Alpha as a tumor promoter and possible driver of carcinogenesis make it a potential target of interest for anti-cancer therapies.

Alpha4 is most well-characterized as a regulator of the PP2A family of serine/threonine phosphatases, though its exact function in regulating these proteins is not fully understood [110,188,211,213,217]. Alpha4 plays a role in protecting PP2Ac, the most well studied of the PP2A family phosphatases, from polyubiquitination and degradation [191,215,217,284]. Studies presented in this thesis and previously published show that this protection is dependent on both the PP2Ac binding domain and the C-terminal domains of Alpha4 [284]. Subsequent studies show that Alpha4 is subject to ubiquitination and that this ubiquitination leads to a calpain dependent cleavage event that cleaves the C-terminal domain and that this cleavage event abrogates the ability of Alpha4 to protect PP2Ac from degradation [191,222].

The effects of Alpha4 on the other members of the PP2 family have not been extensively studied. In yeast, the interaction between Alpha4 and the phosphatase catalytic subunits is critical to their protein function [110,188], and in murine cells knockout of Alpha4 leads to dramatic decreases in expression levels of PP2Ac, PP4c, and PP6c [215]. Few studies have been conducted looking at the effect of knockdown or overexpression on the expression levels of endogenous PP2Ac, PP4c, or PP6c. Previous studies presented in this work (Chapter 4) explore the effects of Alpha4 knockdown and expression on PP2Ac, PP4c and PP6c and we found that Alpha4 has differential effects on the PP2A family members. Stable knockdown of Alpha4 leads to significant decreases in PP4c and PP6c expression levels, while having minimal impact upon expression levels of PP2Ac. Only PP6c was significantly decreased by transient knockdown of Alpha4 and immunodepletion experiments indicated that PP6c was also the phosphatase most highly associated with Alpha4. In this section, we explore the effects of altered Alpha4 expression levels and Alpha4 mutations on some selected cell functions modulated by these phosphatases that have been previously linked to Alpha4, such as apoptosis, cell adhesion, and cellular proliferation [223,238,240,242]. As the apoptotic effects of Alpha4 have been linked to p53 dependent apoptosis, our studies are conducted primarily using either HEK293T cells (a transformed, but non-tumorigenic cell line that is p53 transcriptionally incompetent) and A549 (a lung cancer cell line that expresses wild-type p53) in order to assess possible effects of Alpha4 manipulation in cancers that express both mutated and wild-type versions of p53.

Materials and Methods

Plasmids and Stable Cells

Construction of the FLAG-Alpha4/pcDNA5TO, FLAG-Alpha4ΔC/ pcDNA5TO, and FLAG-Alpha4_ED/pcDNA5TO constructs were described previously [175,217]. A complete description

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of the protocol for creation of the stable cell lines can be found in Chapter 4. The PP2Ac binding deficient mutant (Flag-Alpha4 RKED) stable knockdown and expression cell line was created using the same protocol found in Chapter 4. The Flag-Alpha4_ED/pcDNA5TO construct was used as a template for the PCR fragment, using the same primers used for full-length Flag-Alpha4 as previously described. HEK293T and A549 cells were infected and stable cells were selected with puromycin.

Cell culture and transfection

HEK293FT, HeLa and A549 cells were grown at 37ºC in a humidified atmosphere with 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (FBS). Cell were transfected using Lipofectamine RNAiMAx (siRNAs) or Lipofectamine LTX transfection reagent (cDNA plasmids) (Invitrogen) according to manufacturer's directions.

Cell Lysis and Western analysis

For a description of these protocols, see Chapter 4, Material and Methods section.

Cell spreading Assay

HeLa cells were plated in 10 cm plates at a density of $1.2x10^6$ cells/plate in DMEM supplemented with 10% FBS. For tests of Alpha4 knockdown, the cells were grown for 48 hours and then transfected with either Alpha siRNA or a Control Block-iT siRNA (described in Materials and Methods in Chapter 4), using Lipofectamine RNAiMax according to manufacturer's protocols. For tests of Alpha4 overexpression, cells were grown for 48 hours and then transfected with empty pcDNA5/TO vector, Flag-Alpha4, or Flag-Alpha4_RKED, using Lipofectamine LTX with PLUS reagent according to manufacturer's protocols. At 48 hours post-transfection, cells were rinsed gently with pre-warmed PBS and then dislodged by incubation with 0.25% trypsin/EDTA for 2 minutes at 37°C. Cells were harvested in 9 mls of DMEM

supplemented with 0.1% FBS and then centrifuged at 1200 rpm to remove trypsin. Supernatant

was removed and cells were resuspended in 9 mls of DMEM supplemented with 0.1% FBS and

pipetted gently to create a single cell suspension. Cells were then plated in 6-well tissue culture

plates coated with 5 µg/ml fibronectin at a density of 200,000 cells/well and incubated for 40

minutes at 37°C. The cells were then imaged at 10x on a Zeiss Axiovert 135 microscope using

Q-capture software and a CCD camera. The imaged cells were then assessed for attachment

and spreading. Cells were considered unattached if round and phase bright.

ATP-based cell growth assay and viability assay

HEK293T cells stably expressing either scrambled shRNA, Alpha4 targeted shRNA,

Alpha4 shRNA+Flag-Alpha4 cDNA, or Alpha4 shRNA + Flag-Alpha4_ED cDNA grown in 10-cm

tissue culture plates were trypsinized, washed to remove trypsin, then brought up in fresh

DMEM supplemented with 10% FBS. Viable cells were counted with a hemocytometer after

staining with 0.4% trypan blue and cells were plated at 10,000 viable cells/well in 96-well plates.

Cell proliferation/ATP production was measured using Cell Titer Glo (Promega) at 0, 24, 48 and

72 hour time points, following manufacturer's protocols. Standard curves using serially diluted

cells at 80,000 cells/well; 40,000 cells/well; 20,000 cells/well; 10,000 cells/well and 5,000

cell/well were also plated and linear regression was performed to calculate a regression line

correlating cell number and ATP production for each cell type.

Trypan blue viability assay and cell counting

Cell were seeded at a density of 500,000 cells/plate in 10 cm plates and allowed to grow

for either 24, 48 or 72 hours before harvesting and counting. Cells were gently washed two

times with pre-warmed Dulbecco's phosphate buffered saline (DPBS) and then detached with

0.25% tryspin/EDTA. Cells were allowed to incubate at 37°C for 2 and 5 minutes depending

upon cell type and then 9 mls of DMEM supplemented with 10% FBS was added to both stop

the reaction and resuspend cells. Cells were harvested by gentle pipetting and transferred to 15 ml conical tubes. Cells were centrifuged at low speed (1200 rpm) to pellet cells and supernatant was removed. Cells were resuspended in 9 mls of DMEM supplemented with 10% FBS and cells were pipetted gently to create a single cell suspension. In order to count the number of viable cells, equal amounts of cells suspension and 0.4% trypan blue were mixed in a 1.5 ml Eppendorf tube and 10 µl of this suspension was placed on a hemocytometer. Both viable and non-viable cells were counted to assess cell viability and proliferation.

Caspase 3/7 Activation, LDH Release Assays, Cell Viability assays

For these assays cell were plated in clear, flat bottomed black walled 384-well plates. Cells were plated at 5,000 cells/well and allowed to adhere overnight. Cells were assessed the next day for viability (Cell Titer Glo, Promega), LDH release assay (Promega), and Caspase 3/7 activation assay (Promega) using a Biotek plate reader.

Fluorescent based cell viability assay

We measured cell viability and cell death by counting live and dead cells before and after treatment with various stressors. We used a fluorescent-based live/dead assay containing calcein AM to stain live cells and ethidium homodimer to stain dead cells (Thermo Fischer). For initial assays, we plated cells in triplicate for each condition at a density of 20,000 cells/well and incubated them overnight at 37°C before treatment with either mock (sterilized ddH$_2$O), H$_2$O$_2$, or cycloheximide (CHX) at the specified concentrations. After either 24 hours or 48 hours of treatment, calcein AM and ethidium homodimer were added to the wells and allowed to penetrate the cells. Cells were then imaged with the Zeiss Axiovert 135 at 10x using either the FITC or TRITC filters.

Analysis

All statistical analysis and graphing was performed in Graph Pad Prism version 6.0 for Windows (GraphPad Software, La Jolla, CA USA). The specific tests used are described in the figure legends. All microscope image analysis was performed using FiJI image analysis software (NIH).

Results

The ability of Alpha4 to interact PP2A family phosphatases is required for Alpha4 effects on cell adhesion

Cre-inducible overexpression and knockout of Alpha4 in MEF/p53$^{-/-}$ cells leads to alterations in cell attachment and spreading with increased expression of Alpha4 increasing rates and times of cell adhesion and spreading [242]. We wanted to investigate not only the effects of Alpha4 knockdown and overexpression, but the overexpression of an Alpha4 mutant that is defective in binding to the PP2A family of phosphatases (RKED), on the cell spreading and attachment in HeLa cells. We found that knockdown of Alpha4 has a significant negative effect upon rates of cell adhesion and spreading of HeLa cells when plated on fibronectin-coated plates under low serum conditions (Figure 29A, B). We also found that overexpression of WT Alpha4, but not a PP2A family binding deficient mutant (RKED), enhanced rates of cell adhesion and spreading (Figure 29C, D). This would indicate that the ability to bind to the PP2A family of phosphatases is essential to Alpha4 effects upon cell adhesion and spreading.

Effects of expression of RKED binding deficient mutant on expression of PP2A family members

Previous studies attributed the increases in cell spreading and adhesion to increases in PP2Ac activity and Rac activation, but found no significant changes in PP2Ac expression levels [242]. Our studies showing that knockdown of Alpha4 has limited effects upon PP2Ac but
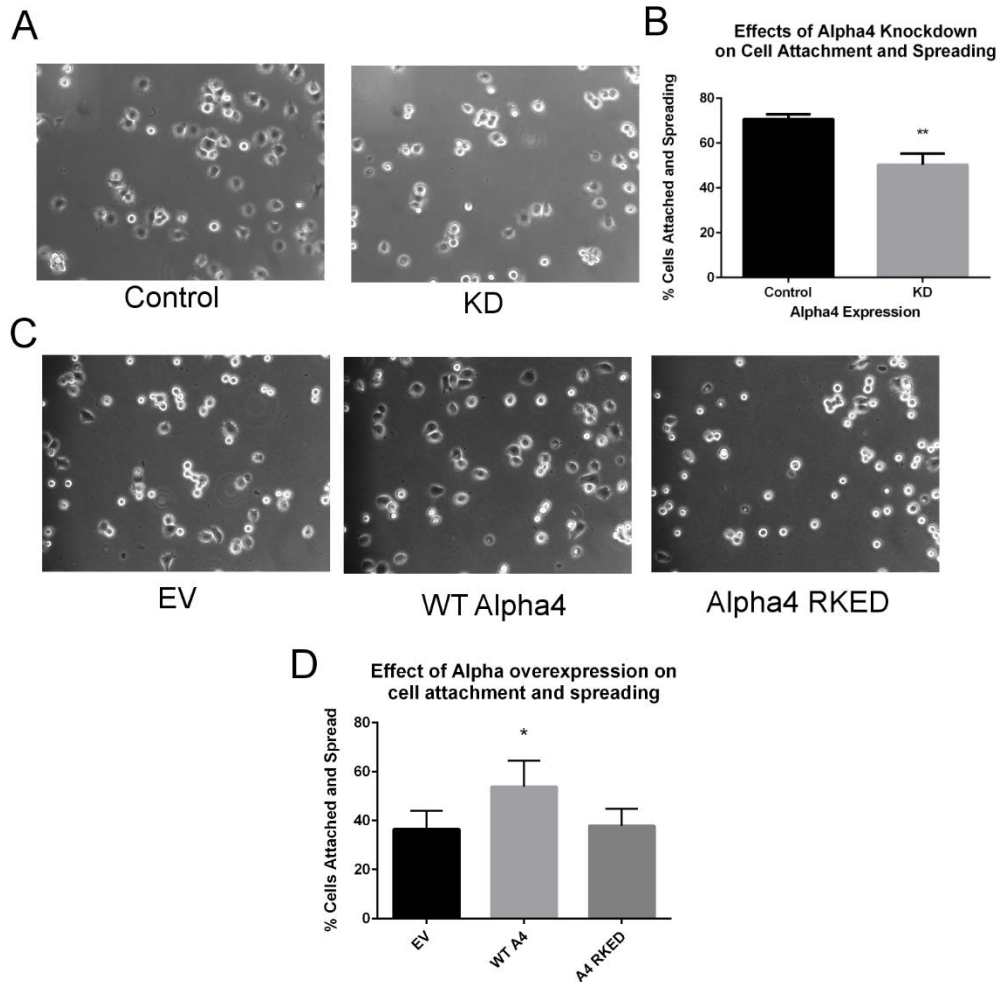
Figure 29: Alpha4 regulates cell attachment and spreading. HeLa cells transfected with either non-targeting control siRNA or Alpha4 targeted siRNA (A,B) or transfected with pcDNA/5TO control vector, WT Flag-Alpha4, or Flag-Alpha4_RKED (C,D) were plated on fibronectin coated plates under serum deprivation and allowed to adhere for 40 minutes. A) Representative images of cells showing attachment and spreading in control versus knockdown cells. B) Quantification of cell attachment and spreading in control versus knockdown cells. Statistical significance was calculated using two-tailed Student's t-test. Average of at least three independent experiments. **, $p < 0.01$. C) Representative images of cells showing attachment and spreading in control versus WT overexpressing and RKED mutant overexpressing cells. D) Quantification of cell attachment and spreading in control, WT, RKED cells. Statistical significance was calculated using one-way ANOVA analysis. Average of at least three independent experiments.*, $p < 0.05$.

significant effects on PP4c and PP6c indicate that Alpha4 affects originally attributed wholly to PP2Ac activity, may in fact be mediated by one of these two closely related phosphatases.

Although, it is possible that Alpha modulates activity of PP2Ac without changing expression levels through shifting of heterotrimer compositions or directly altering PP2Ac activity. Given that our previous experiments indicated that Alpha4 differentially regulates the members of the PP2A family, we decided to determine if the RKED mutant also differentially regulates the various members of the PP2A family. We investigated PP2Ac, PP4c, and PP6c expression levels with both transient overexpression of wild-type and RKED mutant in the presence of endogenous Alpha4 and with stable expression of wild-type and the RKED mutant in cells expressing the 3'UTR Alpha4 targeted knockdown plasmid (Figure 30).

Flag-Alpha4 WT or theFlag-Alpha4 RKED mutant were transiently overexpressed in the presence of endogenous Alpha4 in HEK293T cells and expression levels of the PP2Ac, PP4c and PP6c were assessed by Western (Figure 30A, B, C,D). Similar to previous results that assessed the effects of transient overexpression, no statistically significant differences between the SCR, WT and RKED expressing cells was observed. Although the differences do not rise to the level of statistical significance with the sample sizes used in these studies, it should be noted that in cells expressing both Flag-Alpha4 and the Flag-Alpha4 RKED mutant, expression levels of PP2Ac showed an uptick of approximately 25-30% in all experiments conducted using both transient and stably expressing cell lines in four sets of independent experiments (Figures 27B, 28A, and 30B, D). Given the consistency and repeated nature of this uptick in expression with increased Alph4 expression, further research is warranted to determine if an actual effect of Alpha4 on PP2Ac expression levels can be shown.

In SCR, KD, RES, or RKED stable HEK293T cells, expression levels of PP2Ac, PP4c and PP6c were assessed by Western blot. In this set of experiments, none of the differences rose to the level of statistical significance, even though previous experiments have shown differences in PP4c and PP6c expression levels upon stable KD and RES (Figure 30E, G, H).

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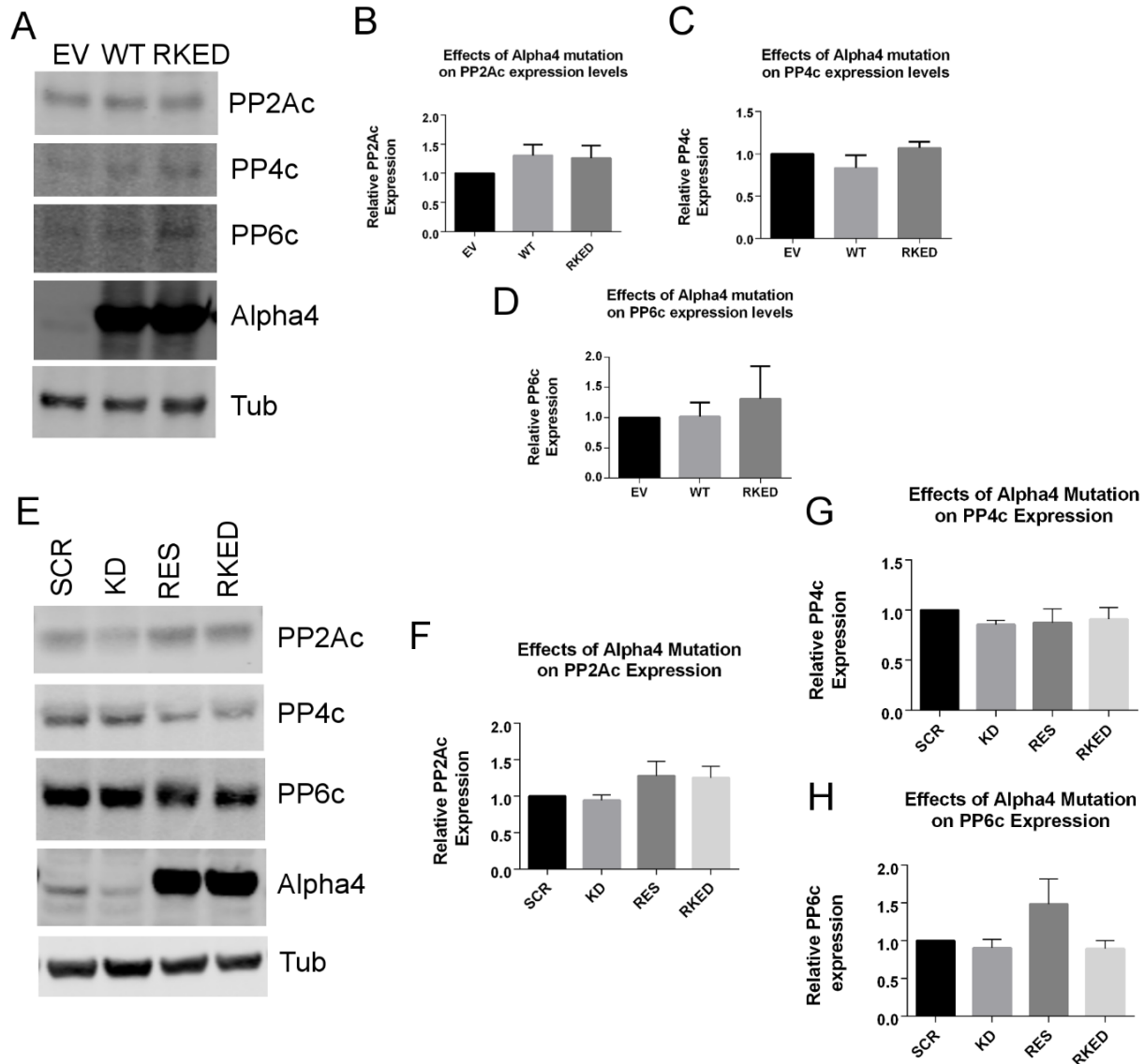
Figure 30: Differences in functional effects of RKED mutant compared to WT Alpha4 cannot be explained by differences in phosphatase expression. HEK293T cells were grown in 10% FBS DMEM. A) Cells were transfected with either empty pcDNA5/TO vector or Flag-Alpha4 pcDNA5/TO vector. Cells were harvested 48 hours post-transfection. Representative western blots probed for PP2Ac, PP4c, PP6c, Alpha4 and tubulin (loading control). B, C, D) Quantification of PP2Ac, PP4c, and PP6c levels relative to control. At least 3 independent experiments were performed for all analyses. Graphs shows mean ± SEM. Statistical significance was calculated using one-way ANOVA analysis. E) Representative western blots probed for PP2Ac, PP4c, PP6c Alpha4, and tubulin (loading control). F, G, H) Quantification of PP2Ac, PP4c, and PP6c expression levels relative to scrambled control cells. At least 3 independent experiments were performed for all analyses. Graphs shows mean ± SEM. Statistical significance was calculated by one-way ANOVA and Tukey's multiple comparison analysis.

The trends remained the same as in previous experiments with decreases in expression with Alpha4 KD and increased expression back to SCR levels for PP6c, but not PP4c with Flag-Alpha4 expression. The pattern also indicates that the Flag-Alpha4 RKED mutant may behave more like KD than Flag-Alpha in regulating PP6c expression, whereas it appears to behave more like Flag-Alpha4 in regulating PP2Ac expression. Although the differences do not rise to the level of statistical significance in this set of experiments, they may warrant further study. The lack of statistical significance could be due to the limited sample size (N=4) or due to large differences in growth morphology observed between the stable knockdown cells lines.

Expression of PP2Ac-binding deficient mutant of Alpha4 leads to increased cell death in HEK293T cells

The known relationship between cell growth, apoptosis and Alpha4 led us to explore the possibility that the differences in cell growth between the HEK293T stable cells were due to baseline differences in rates of cell death or cell proliferation. Cells stably expressing scrambled shRNA (SCR), Alpha4 targeted shRNA (KD), Alpha4 targeted shRNA + Flag-Alpha4 cDNA (KD+A4), and Alpha4 targeted shRNA + Flag-Alpha4_RK_ED mutant (KD+RKED) (Figure 31A) were assessed for cell viability using a live/dead cell fluorescent staining kit (Promega). Assessment of cell death in 293T cells showed that expression of the PP2Ac binding deficient mutant leads to increased cell death compared to the other cell lines (Figure 31B). It should be noted that neither Alpha4 knockdown or overexpression of Alpha4 lead to increases in cell death, indicating that overexpression of a PP2Ac binding deficient mutant is having a unique effect on cell viability that does not recapitulate either knockdown or overexpression phenotypes.
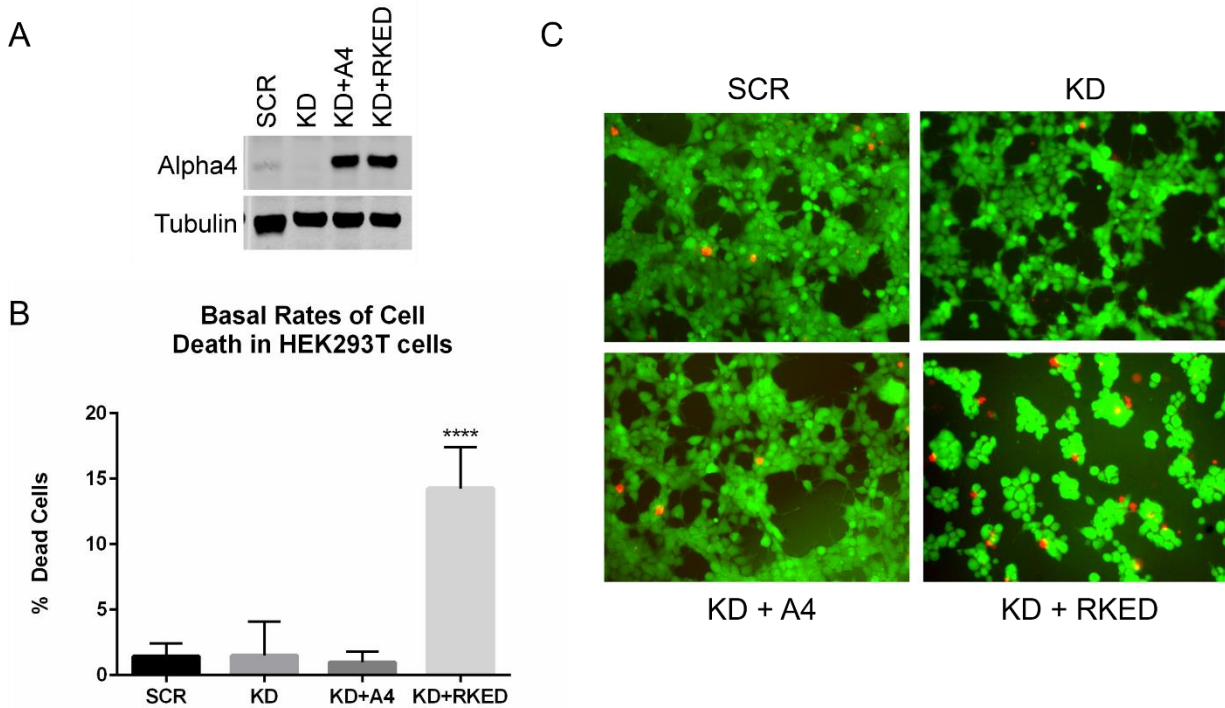
Figure 31: Expression of PP2Ac binding deficient mutant has negative effect on cell viability. HEK293T cell lines stably expressing either scr shRNA (SCR), 3'UTR Alpha4 shRNA (KD), 3'UTR Alpha4 shRNA+Flag-Alpha4 (KD+A4), or 3'UTR Alpha4 shRNA + Flag-Alpha4 RKED (KD+RKED). A) Western blot of stable cell line lysates probed for Alpha4 and Tubulin. B) HEK293T cells were plated in 96 well plates and incubated overnight. Cells were treated with fluorescent live/dead cell staining reagents and imaged. The number of live and dead cells were counted in a representative section of each image and the percent of dead cells was calculated. Graphed is the average of two independent experiment, showing increased rates of cell death in RKED expressing cells. Statistical significance was assessed using one-way ANOVA and Dunnett's multiple comparison correction.****, $p<0.0001$ C) Representative images of fluorescently stained cells with live cells in green and dead cells in red.

Alpha4 expression has a negative effect on cell proliferation in HEK293T cells

As noted above, differences in the HEK293T stable cell lines in both rates of cell growth and cell morphology were noted. Images of cells stably expressing scrambled shRNA (SCR), Alpha4 targeted shRNA (KD), Alpha4 targeted shRNA + Flag-Alpha4 cDNA (KD+A4), and Alpha4 targeted shRNA + Flag-Alpha4_RK_ED mutant (KD+RKED) show differences in cell morphology and ability to form a cohesive monolayer (Figures 31C (fluorescent) and Figure 32A (phase contrast)). The images show that overexpression of both Flag-Alpha4 and of the PP2Ac binding deficient mutant Flag-Alpha4 RKED have an apparent detrimental effect on formation of a cohesive cell monolayer with cells forming overgrown patches and failing to form a cohesive monolayer (Figures 31C, 32A). This is very different than the cell growth phenotype of the Alpha4 knockdown cells which showed very little signs of overgrowth and formed an even and cohesive monolayer (Figure 31C, 32A). We wanted to further characterize these differences in growth by measuring rates of cell proliferation.

To investigate cell proliferation, we used a luminescence based ATP production assay as a measure of the number of viable cells. Since we were using stable cell lines, one of our concerns was that the different cell lines would produce different amounts of ATP per cell, so we set up standard curves using serially diluted cells from each cell line and measured ATP production using Cell Titer Glo. Interestingly, we found significant differences between cell lines in ATP production with stable cells expressing the PP2A binding mutant RKED showing much lower rates of ATP production per cell and cell expressing WT Alpha4 showing slightly higher rates of ATP production per cell (Figure 32B). We did not see any significant difference in ATP production between HEK293T cells expressing the scrambled shRNA versus Alpha4 knockdown HEK293T cells (Figure 32B).

We then assessed cellular proliferation using the ATP-based luminescence assay and measured differences between cell lines in two different manners. Changes in ATP production over time for each of the cell lines were assessed by normalizing each cell line to ATP
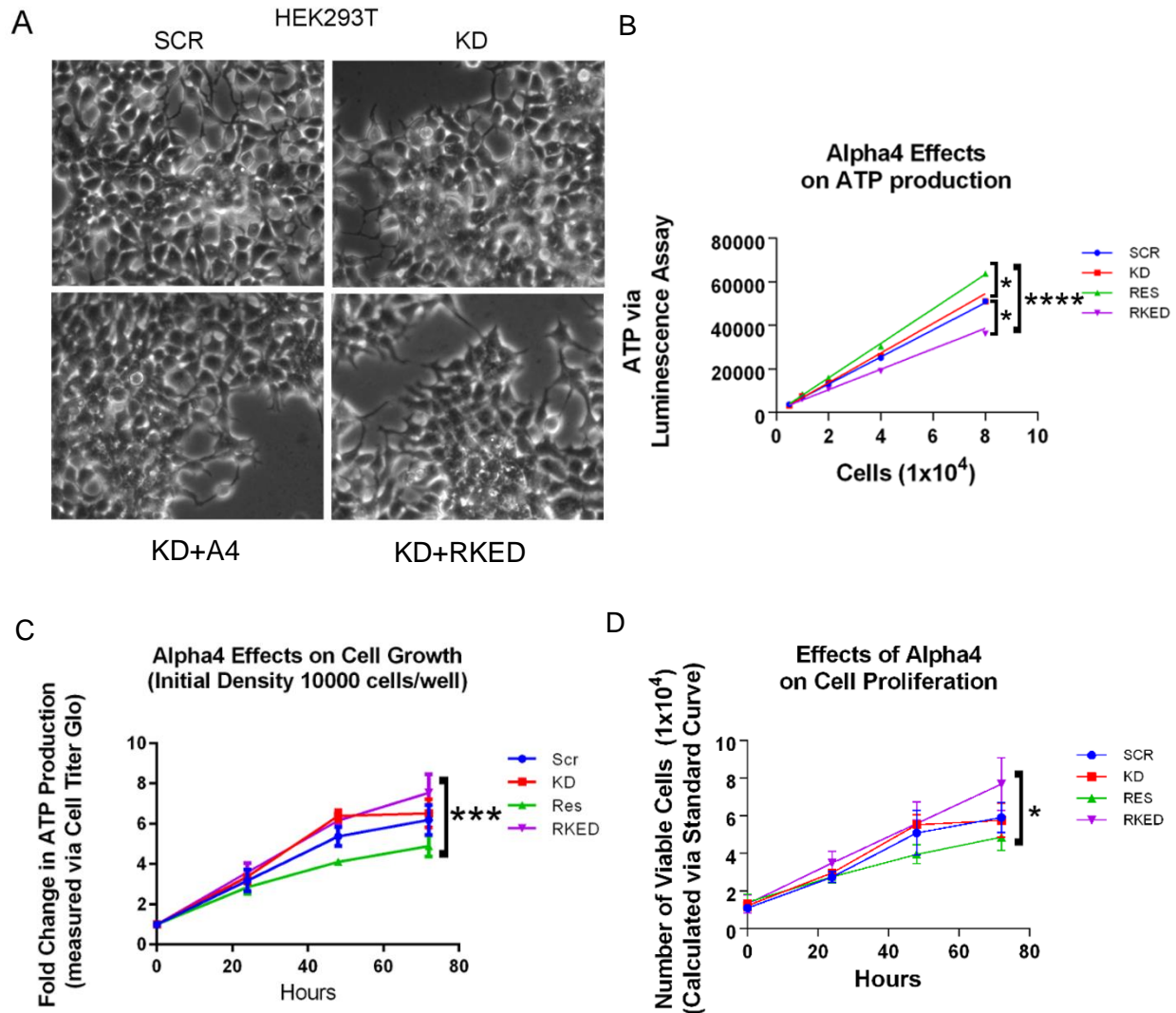
Figure 32: Alpha4 knockdown, overexpression and mutation have different effects on cell growth in HEK293T cells. A) HEK293T cells plated at 10,000 cells/well were imaged by phase at 10X resolution 72 hours after plating showing differences in growth morphology and confluency between cell lines. B) Standard curve of cell number versus ATP production for each cell line. Cells were plated at 5000, 10000, 20000, 40000, and 80000 cells/well in triplicate and ATP production was measured using Cell Titer Glo, after allowing the cells to incubate for 1 hour to recover from plating. Nonlinear regression analysis was performed at concluded that standard curves for each cell line were significantly different from one another with a $p=0.0004$. The regression lines calculated were used to calculate the number of viable cells for each cell line from the raw luminescence data. Two-way ANOVA comparisons with Holm-Sidak multiple comparison correction was performed. *, $p<0.05$; ****, $p<0.0001$ C) Cells were plated at 10,000 cells/well in 96 well plates in triplicate for each time point and cell viability was assessed using the Cell Titer Glo luminescent ATP assay. Fold change in ATP production as readout by Cell Titer Glo normalized to T=0 for each cell line. Significance was calculated using two-way ANOVA with Tukey's multiple comparison correction. ***, $p<0.001$. D) Comparison of cell proliferation rates after correction using the standard curves calculated in C for each cell line showing differences in cell proliferation rates. Significance was calculated using two-way ANOVA analysis and Tukey's multiple comparison analysis. *, $p<0.05$.

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production at T=0 and looking at fold change in ATP production. Using this assessment, the

cell expressing WT Alpha4 had the lowest rates of cell growth and cells expressing the RKED

mutant the highest rates of cell growth (Figure 32C).

Next the number of cells for each cell line were calculated at each time point, by using

the standard curve previously calculated (Figure 32C). This assessment gave a similar result

with expression of WT Alpha4 having the lowest cell proliferation and the RKED mutant having

the highest cell proliferation (Figure 32D). The differences in growth between the cells

expressing WT and RKED Flag-Alpha4 was significantly different from each other using both

methods. The effects of Alpha4 expression and knockdown in HEK293T cells are at odds with

what has been seen in other cell lines where Alpha4 promotes cell proliferation.

Knockdown of Alpha4 led to decreased cell proliferation in A549 cells

Stable cell lines expressing either scrambled shRNA (SCR), scrambled shRNA plus

Flag-Alpha4 (OE), 3'UTR shRNA(KD), or 3'UTR shRNA plus Flag-Alpha4_RKED (RKED) were

created in the A549 lung cancer cell line (Figure 33A,B). Characterization of this cell line

revealed that unlike in the HEK293T stable cell lines, knockdown of Alpha4 had a significant

negative effect on cell growth (Figure 33B, C). Neither OE nor expression of RKED had any

significant effects on cell proliferation rates in A549 cells.

Overexpression of Alpha4 increases cell viability in A549 cells

Cell viability between SCR A549 cells and OE A549 cells was assessed using a variety

of measures and OE A549 cells consistently had higher measures of cell viability. Assessments

of SCR A549 and OE A549 cells showed significantly decreased percentage of dead cells

(Figure 34A). The OE A549 cells also exhibited slightly increased cell viability as measured by

ATP production using Cell Titer Glo and decreased levels of cell death either via apoptosis, as
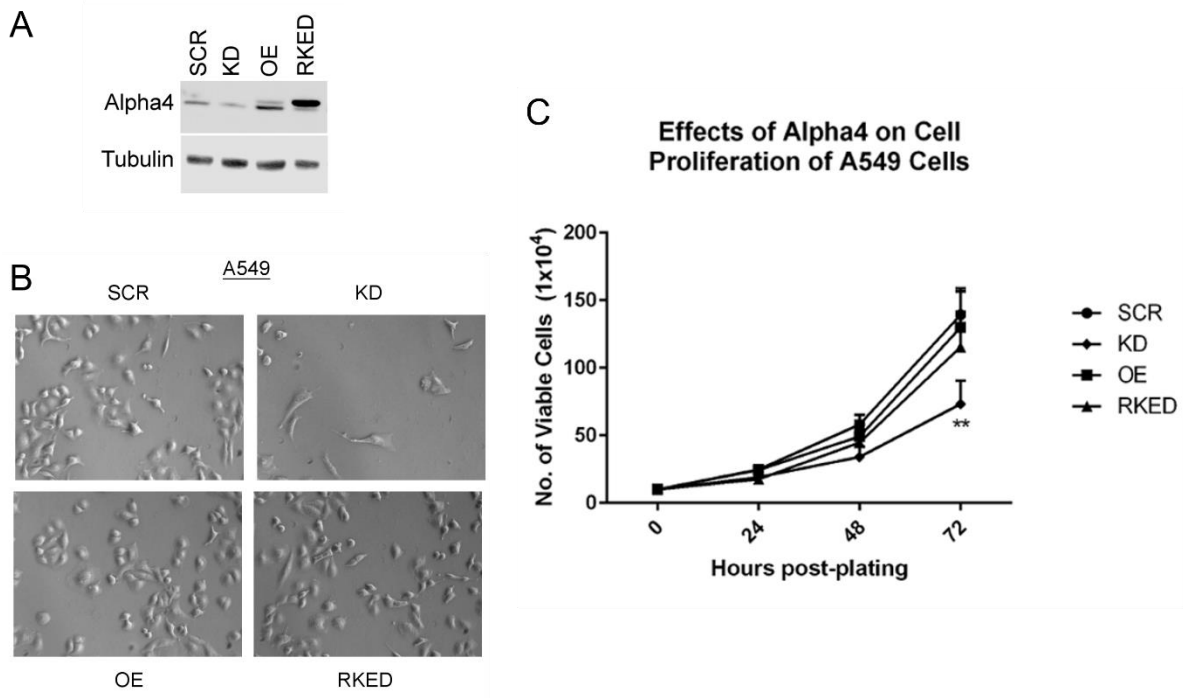
Figure 33: Knockdown of Alpha4 has a negative impact on cell growth in A549 cells. A) Western blot of stable scr shRNA (SCR), scr shRNA+WT Flag-Alpha4 (OE), 3'UTR shrNA + Flag-Alpha4-RK_ED mutant (RKED) SCR, KD, OE and RKED A549 cell lysates probed for Alpha4 and Tubulin. B) Images of A549 cells stable cells plated at equal density (500,000 cell/well) after 48 hours of cell growth using phase-contrast microscopy and imaged at 10X resolution. C) Cells were plated at 500,000 cells/well in 6-well tissue culture plates and harvested at the specified time points (0, 24, 48, and 72 hours). Cells were stained with 0.4% trypan blue and the number of viable cells were counted. Results graphed are the average of 4 independent experiments. Statistical significance was calculated using two-way ANOVA analysis and Dunnett's multiple comparison correction. Comparisons were made between SCR cells and each of the other cell lines at each time point. **, $p<0.01$.
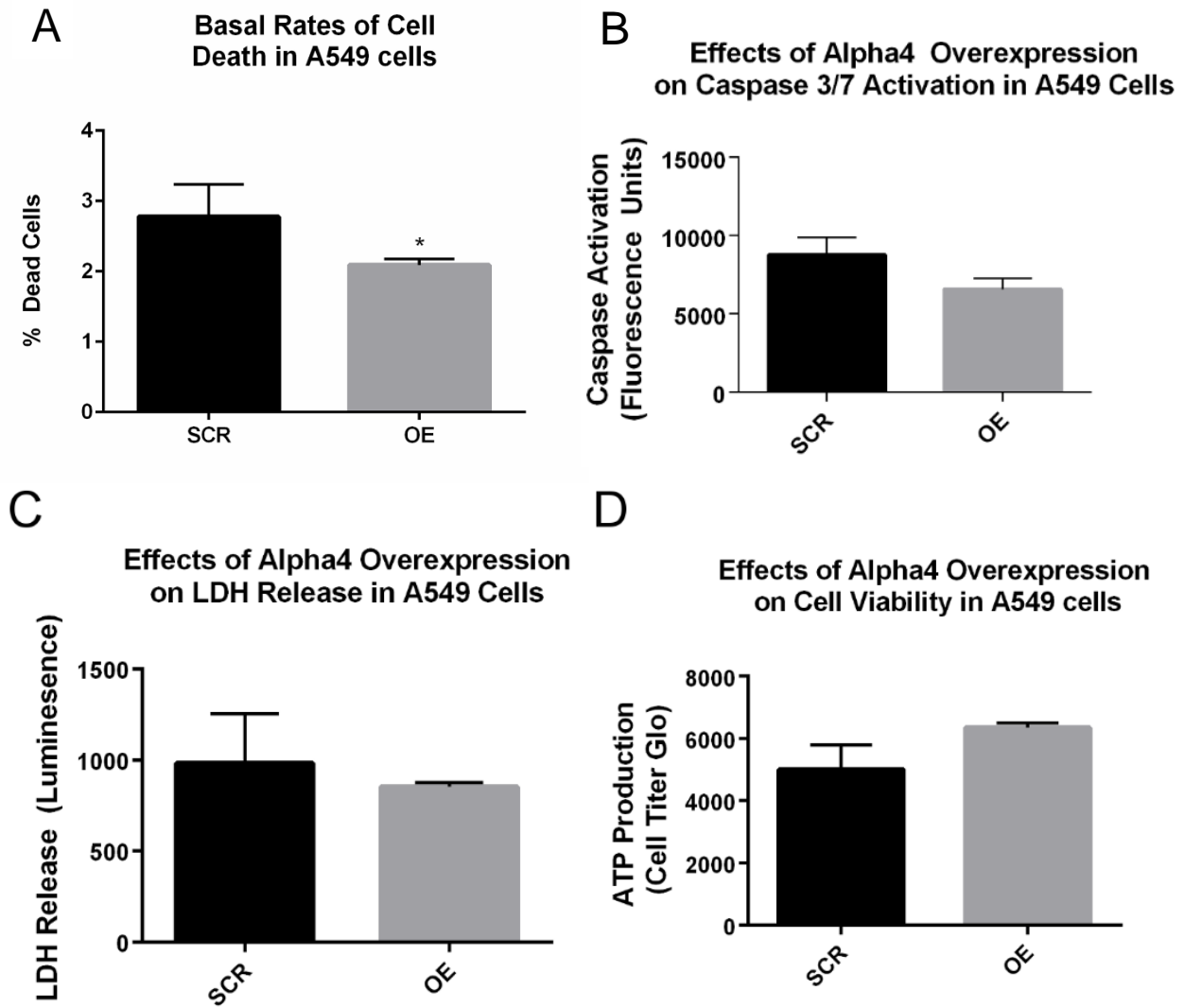
Figure 34: Overexpression of Alpha4 decreases cell death in A549 cells. A) (scr shRNA) SCR and (scr shRNA +Flag-Alpha4) OE A549 cells were plated at equal densities and harvested 48 hours after plating, stained with 0.4% trypan blue, and viable and non-viable cells were counted using a hemocytometer. The percent of dead cells was determined and graphed. Results shown are the average of four independent experiments. Student's t-test was used to calculate significance. *, $p < 0.05$. B, C, D) SCR and OE A549 cells were plated in 384-well black-walled, clear-bottomed plates at 4000 cells/well and allowed to incubate overnight. Cells were then assessed for (B) apoptosis via caspase 3/7 activation, (C) necrosis via LDH release, and (D) cell viability via ATP production using luminescent and fluorescent based reagents and signal was read out using a BioTek plate reader. Results reported are the average of two independent experiments.

measured by caspase 3/7 activation, or necrosis, as measured by LDH release, though these effects did not reach the levels of statistical significance (Figures 34B, C, D).

Increased Alpha4 expression A549 cells leads to greater sensitivity to cell death induced by ROS in A549 cells, but decreased sensitivity to cycloheximide

Alpha4 has been shown to be an essential inhibitor of apoptosis under normal conditions [238] and to decrease apoptosis in response to some types of cell stress [239]. The effects of Alpha4 overexpression on the response of the lung cancer cell line A549 to two different cell stressors, hydrogen peroxide (H_2O_2) and cycloheximide (CHX), were investigated. A549 cells were treated with increasing concentrations of H_2O_2, to induce oxidative stress and assessed for viability after 24 hours of treatment. Contrary to our hypothesis, overexpression of Alpha4 led to a dramatic increase in cell death of A549 cells exposed to H_2O_2 (Figure 35). It remains to be determined whether this cell death is apoptotic or necrotic based cell death, as well as the mechanism for this increased sensitivity to reactive oxygen species (ROS). A549 cells were also treated with increasing levels of CHX, which blocks protein translation, and assessed for viability after 24 hours. Overexpression of Alpha4 led to decreased cell death in comparison to WT A549 cells when cells were treated with CHX, which is consistent with the idea that Alpha4 plays a role in protecting cells from apoptosis (Figure 36).

Discussion

Alpha4 has been shown to positively regulate rates of cell spreading and attachment in MEF cells [242]. We investigated whether Alpha4 had similar effects in HeLa cells and if this effect was dependent upon the ability of Alpha4 to bind to the PP2A family of phosphatases. We found that rates of cell attachment were positively correlated with Alpha4 expression and that this effect was dependent on the ability to bind to PP2A family phosphatases, as the RKED binding deficient mutant failed to have this positive effect (Figure 29). As we previously found
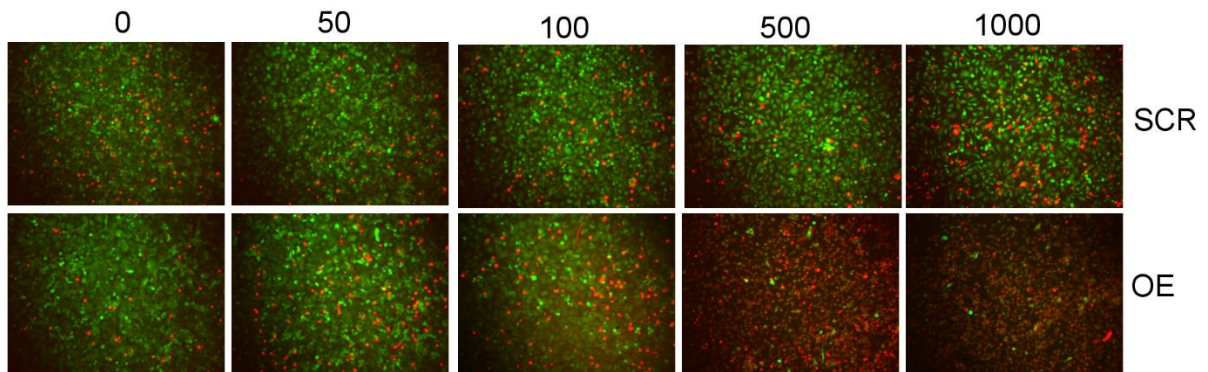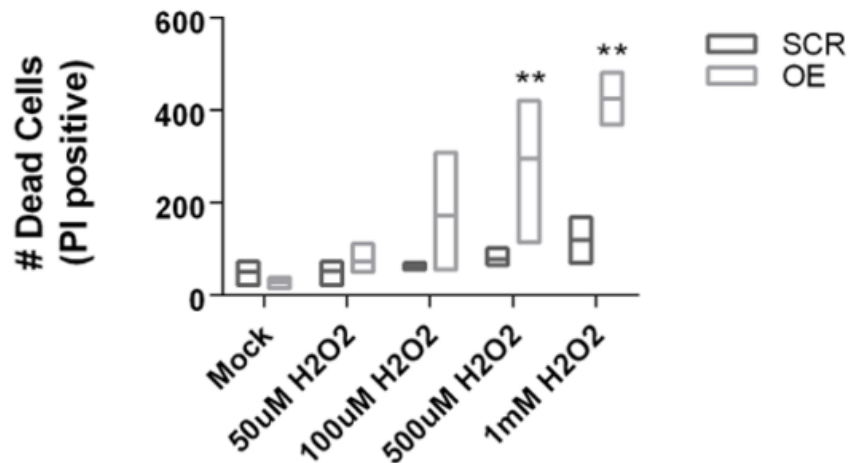
Figure 35: Overexpression of Alpha4 increases A549 sensitivity to H_2O_2 . A549 cells were treated with increasing concentrations of H_2O_2 for 24 hours, stained with live/dead cell fluorescent dyes and imaged. Results were analyzed using two-way ANOVA comparing SCR to OE at each concentration with Sidak's multiple comparison correction. Three independent experiments were conducted. p, **<0.01. Plots of # of dead cells at each concentration for H_2O_2 (top). Representative images of stained cells (bottom) with live cells (green) and dead cells (red)
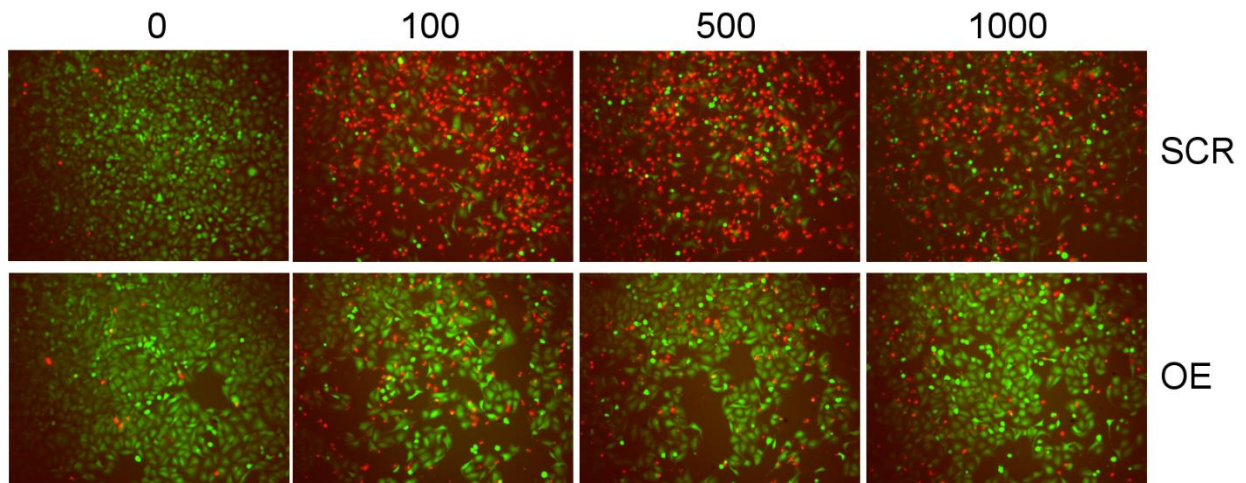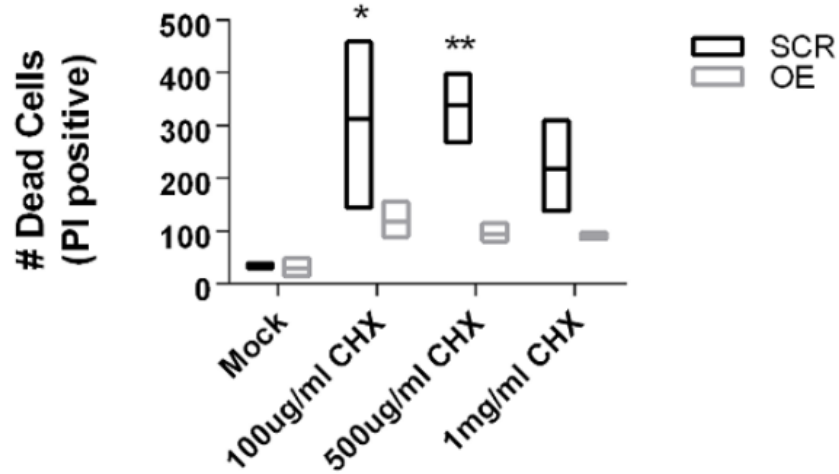
Figure 36: Overexpression of Alpha4 decreases A549 sensitivity to Cycloheximide (CHX)
A549 cells were treated with increasing concentrations of CHX for 24 hours, stained with live/dead cell fluorescent dyes and imaged. Results were analyzed using two-way ANOVA comparing SCR to OE at each concentration with Sidak's multiple comparison correction. Three independent experiments were conducted. p, **<0.01. Plots of # of dead cells at each concentration for CHX (top). Representative images of stained cells (bottom) with live cells (green) and dead cells (red).

that Alpha4 knockdown had minimal effects on PP2Ac expression (Chapter 4, Figures 27, 28), we hypothesized that the effects on cell spreading and attachment may be due to effects on other members of the PP2Ac family. Given this, we investigated whether expression of the RKED mutant had differential effects on PP2Ac, PP4c and PP6c expression to elucidate which of the PP2A family members were involved in the cell spreading effects of Alpha4. However, the experiments were inconclusive as none of our findings met the threshold for statistical significance, even with Alpha4 knockdown (Figure 30). This is in contrast to previous experiments that did show a significant effect of stable knockdown Alpha4 upon PP4c and PP6c (see Chapter 4, Figure 28). Although we did not see a significant difference in this set of experiments the overall trends for the repeated conditions (KD and WT) were the same as in our previous experiments and with an increased sample size may reach the level of significance. Given that, it is of interest to note that the expression of the RKED mutant appeared to have differential effects upon the various PP2A family members. The data though not reaching the level of significance when quantified indicate that the RKED mutant may have the same positive impacts as WT on PP2Ac expression, but not on PP6 expression (Figure 30B, F). As in previous experiments (Chapter 4, Figure 28), both knockdown and expression of Flag-Alpha4 have a negative effects on PP4c expression though the effect in this set of experiments was very minimal. One of the possible causes for the decreased differences in expression levels in these experiments is the differences in growth morphology observed between cell lines. The cells in these experiments were plated at a slightly lower initial density and this allowed for differences in growth kinetics to become more observable. The lack of significance in PP6 expression especially may be due to this effect, as expression of PP6 has shown cell density dependence in other experiments [146].

These findings do not support the idea that Alpha4 alters cell spreading via changes in PP2Ac expression, as we saw no differences in expression profile between wild-type and the RKED mutant, where clear differences in cell adhesion do exist. If further experiments conclude

that expression Flag-Alpha4 and Flag-Alpha4 RKED do have similar effects on PP2Ac expression that differ from empty vector, this may current model of Alpha4 effects on PP2Ac, as it would mean that some effects of Alpha4 on PP2Ac do not require binding of Alpha4 to PP2Ac.

Differences in cell growth as measured by ATP production and a calculation of the number of viable cells over a 4 days growth curve indicated that xpression of Flag-Alpha in HEK293T cells causes a decrease in cell proliferation rates compared to control cells or cells expressing either the RKED mutant or Alpha4 targeted shRNA (Figure 31). This contrasts with numerous studies conducted in a variety of cell lines showing that increased expression of Alpha4 increased rates of cellular proliferation and decreased cellular apoptosis [223,238]. The previous studies investigating cellular proliferation and apoptosis have generally been conducted in cells that express wild-type p53, our choice of HEK293T cells for our initial studies may have highlighted a difference in the effects of Alpha4 in cells that express wild-type transcriptionally competent p53 compared to cells that express p53 mutants that cannot induce regulate transcription, as expression of the SV40 virus renders the p53 in HEK293T cells transcriptionally incompetent.

The increase in apoptosis caused by Alpha4 knockdown/knockout is mediated by p53 and this effect can be blocked by either knocking out p53 or overexpressing anti-apoptotic proteins, such as Bcl-X_L [238]. The idea that overexpression of Alpha4 may have negative impacts in cell lines with mutated or inactive p53 should be further explored, as mutations that render p53 inactive are commonly seen in cancer [149,295,296]. The idea that Alpha4 may play a proapoptotic role due to its positive effects on phosphatase activity, should be investigated since both PP2A and PP6c are classified primarily as tumor suppressors [21,124,142,149].

Another interesting finding was that although RKED mutant expressing cells did not proliferate more slowly than control or knockdown cells, they did produce less ATP and experienced higher rates of cell death (Figures 31, 32). The increased growth rates coupled

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with increased apoptosis points to a possible dysregulation of cell cycle checkpoints allowing cells to grow and divide without regard to resources, DNA damage or appropriate mitotic spindle formation. This would lead to an increased likelihood of mutations, as well as chromosomal and nuclear abnormalities, eventually leading to increased rates of cell death [297]. All three of the of PP2A phosphatases play roles in regulating these facets of cell division [96,109,136,138,298], as well as regulating p53 which initiates cell cycle checkpoints that arrest cell division until a damaged cell can initiate repair [295,299]. Expression of a PP2A binding mutant may disrupt the formation of necessary complexes to appropriately repair DSBs and coupled with the deficiencies in p53 lead to increased accumulation of DNA damage and chromosomal instability.

We also assessed the effect of Alpha4 overexpression, knockdown and mutation on A549 cells, a lung cancer cell line that overexpresses Alpha4 compared to normal lung epithelial cells [223]. Knockdown of Alpha4 expression in A549 cells, which has competent p53, leads to a significant decrease in cell proliferation (Figure 33), as to be expected. Overexpression of Flag-Alpha4 or expression of the RKED mutant does not have profound effects on A549 growth or viability, though overexpression does slightly though significantly, decrease rates of cell death. Investigation of the effects of overexpression of Flag-Alpha4 on the responses of A549 cells to two different cell stressors led to some unexpected findings, in that overexpression of Alpha4 greatly enhanced sensitivity of A549 cells to ROS, in the form of H_2O_2 (Figure 35). This increased sensitivity of A549 cells to ROS imparted by further increases in Alpha4 expression is intriguing as many tumors show increased levels of ROS, which is generally considered to have a slight protective effect on cell survival due in part to inhibition of phosphatase activity [82,300,301]. The role of p53 in regulating cellular redox states [302–305] implies that Alpah4 suppression of p53 activity [215,238] could be a double-edged sword having both pro-survival and pro-apoptotic effects depending upon conditions. This could have implications for both

cancer and neurodegenerative diseases, as ROS induced inhibition of PP2A plays a role in both

of these diseases [82,300,306].

Chapter 6

Summary and Future Directions

Summary

Alpha4 interacts with the PP2A family of serine/threonine phosphatases but the role and consequences of these interactions are still up for debate and contention [110,176,188]. It is known that Alpha4 is essential for cell survival and development, as knockout of Alpha4 at the organismal level is generally lethal [238]. Initial studies of Alpha4 focused on its role as either an activator or an inhibitor of PP2A activity with variable conclusions dependent upon cell type and treatment, as well as the assay used to measure activity [188,210–215]. Since then, it has been found that Alpha4 plays a role in regulating PP2A family expression levels and stability, but again whether it acts to increase or decrease PP2A expression has been debated [215,217,218]. Appropriate function of the PP2A family of phosphatases, especially Sit4, is dependent upon their ability to interact with Tap42, as abrogation of this interaction impairs function [11,200–203]. In yeast, Tap42 plays a critical role in regulation of the nutrient sensing via interactions within the TOR pathway [200,201,203,206]. The exact role and pathways that Alpha4 is involved in are less understood, in higher eukaryotes, and the nature of its interactions with the PP2A family of phosphatases appears to be more complicated than straightforward activation or inhibition.

Insights gained from structural analysis of the N-terminal PP2Ac binding domain of Alpha4

We determined the crystal structure of the N-terminal PP2Ac binding domain of Alpha4 to a resolution of 2.35Å and found that similar to its yeast homolog, Tap42, it is an all alpha-helical protein similar in structure to tetratricopeptide repeat (TPR) proteins (Figure 14). These proteins generally facilitate protein-protein interactions [263]. Alpha4 does contain some key

differences from a traditional TPR motif. One of the most striking differences is the reversed topology in the third TPR motif and the insertion of a very large loop connecting helices 5 and 6 (Figure 19). This structure allows for a very high degree of conformational flexibility in this region of the protein. In fact, in our crystal structure, helix 5 was in an open position pointing out into solution, whereas structures of Tap42 had this helix in a more closed conformation (Figure 13). We confirmed that this open conformation seen in our structure was not due to a crystal packing by performing double-electron-electron resonance (DEER) spectroscopy to measure the distance between residues in protein in solution. Using several sets of residues, we found that this open conformation did exist in solution, as the average distance measurements found by DEER were very close to the measurements found in our crystal structure. The DEER data also confirmed that this area of the protein undergoes a large degree of conformational changes as the peaks in the distance spectrum were quite wide indicative of a wide range of conformational space sampled (Figure 15). We hypothesized that this large degree of flexibility would allow Alpha4 to more easily bind the large PP2A catalytic subunit. A more recent structure of the N-terminal PP2Ac binding domain of Alpha4 (residues 2-233) and a N-terminal fragment of PP2Ac containing residues (1-153) have shown that this extended helix forms part of a 'helix tweezer' that closes upon binding to a helix motif within a partially unfolded structure of PP2Ac [152]. This new structure elucidates a possible mechanism by which Alpha4 exerts its protective effects upon PP2Ac as binding of Alpha4 to the partially unfolded PP2Ac fragment prevents aggregation and blocks the K41 ubiquitination site of PP2Ac, though it does not explain the necessity of the C-terminal domain for this protective effect [152].

Previous studies indicated that Alpha4 contained an ubiquitin binding motif (UIM) that is important to its function in protecting PP2Ac from polyubiquitination and degradation. Our hypothesis was that it did so by binding to monoubiquitinated PP2Ac and blocked the ubiquitination reaction. Looking at that region of the protein within our crystal structure, it is clear that in the conformation seen in our structure that ubiquitin would be unable to bind to this

UIM (Figure 16). We investigated this region using DEER spectroscopy and CW-EPR. We found that there was an indication that this region was capable of adopting more than one conformation indicated by the two peaks found in the DEER distance analysis (Figure 17). We hypothesized that we could induce a shift in this spectrum by incubating with excess quantities of ubiquitin, but we were not able to see any significant changes in the spectrum upon exposure to ubiquitin (Figure 17).

Role of the PP2Ac-Alpha4-Mid1 heterotrimeric complex

One of the areas of debate has been the function of the heterotrimeric complex formed between Alpha4, PP2Ac, and Mid1. When this complex was originally discovered it was concluded that formation of this complex led to increased degradation of PP2Ac, specifically the microtubule-associate pool of PP2Ac, by allowing a complex to form between PP2Ac and its E3 ubiquitin ligase, Mid1 [217,218]. Later studies have disputed this hypothesis, showing that Alpha4 actually protects PP2Ac from polyubiquitination and degradation [215,217]. A hypothesis was put forth that this was due to an ubiquitin-interaction motif within the N-terminus of Alpha4 that acted to block ubiquitination of PP2Ac [217]. Our studies investigating the domains of Alpha4 that are required for the protective effect of Alpha4 expression found that the N-terminal domain, while essential for full protection of PP2Ac was not sufficient to provide protection (Figures 21, 22). The C-terminal domain of Alpha4 that binds to the E3 ubiquitin ligase is also essential to this protective effect, but its mechanism of action is still unknown. Further studies of the PP2Ac-Alpha4-Mid1 heterotrimer have revealed that Mid1 serves as an E3 ubiquitin ligase for Alpha4 and its role as an E3 ligase for PP2Ac has been brought into question [191,193]. Studies found that Mid1 monoubiquitinated Alpha4 leading to a subsequent calpain-mediated cleavage event that removes the C-terminal Mid1 binding region [191]. Our data indicate that this cleavage would render Alpha4 incapable of providing a protective effect towards PP2Ac and in fact may even promote its eventual degradation, this was confirmed in

further experimental studies exploring the role of this cleavage product [191,222]. The events that trigger this monoubiquitination event have not been elucidated though some initial studies indicate that it may be regulated by the phosphorylation status of Alpha4 [222]. The current model takes into account these new discoveries and the possible dual roles of Alpha4 as both an inhibitor and promotor of PP2Ac degradation dependent upon Alpha4 post-translational modifications (Figure 37).

Consequences of differential regulation of the PP2A family of phosphatases by Alpha4

Our results demonstrate that Alpha4 has differential effects on the expression levels of the various family members of the PP2A serine/threonine phosphatase family and theoretically upon their activity levels in the cell (Figures 27 and 28). Our results also indicate that interaction of Alpha4 with the phosphatase catalytic subunit may not be necessary for some of the effects of Alpha4 in the cell (Figures 31 and 32). The expression of the PP2A binding deficient mutant Flag-Alpha4 RKED in p53-incompetent HEK293T cells had a profoundly different impact upon cell proliferation, cell growth morphology and cell death than expression of either Flag-Alpha4 or knockdown of Alpha4 (Figures 31,32). Mutants of Alpha4 that interfere with complex formation, like the RKED mutant tested, may act as a dominant negative, but the idea that Alpha4 may have a function that does not require interaction with phosphatases has not been ruled out. The differential regulation of members of the PP2A family by Alpha4 may allow for elucidation of which phosphatases are involved in particular cellular functions by assessing effects on function when Alpha4 levels are altered or mutations introduced and comparing these to effects on phosphatase expression.

The differences in cell growth exhibited in response to Alpha4 knockdown, overexpression and mutation in both 293T cells and A549 cells has impacts on examining the role of Alpha4 as a potential oncogene and to examine the role of various phosphatases in cancer cell biology (Figures 31-33). The results that Alpha4 overexpression can have a

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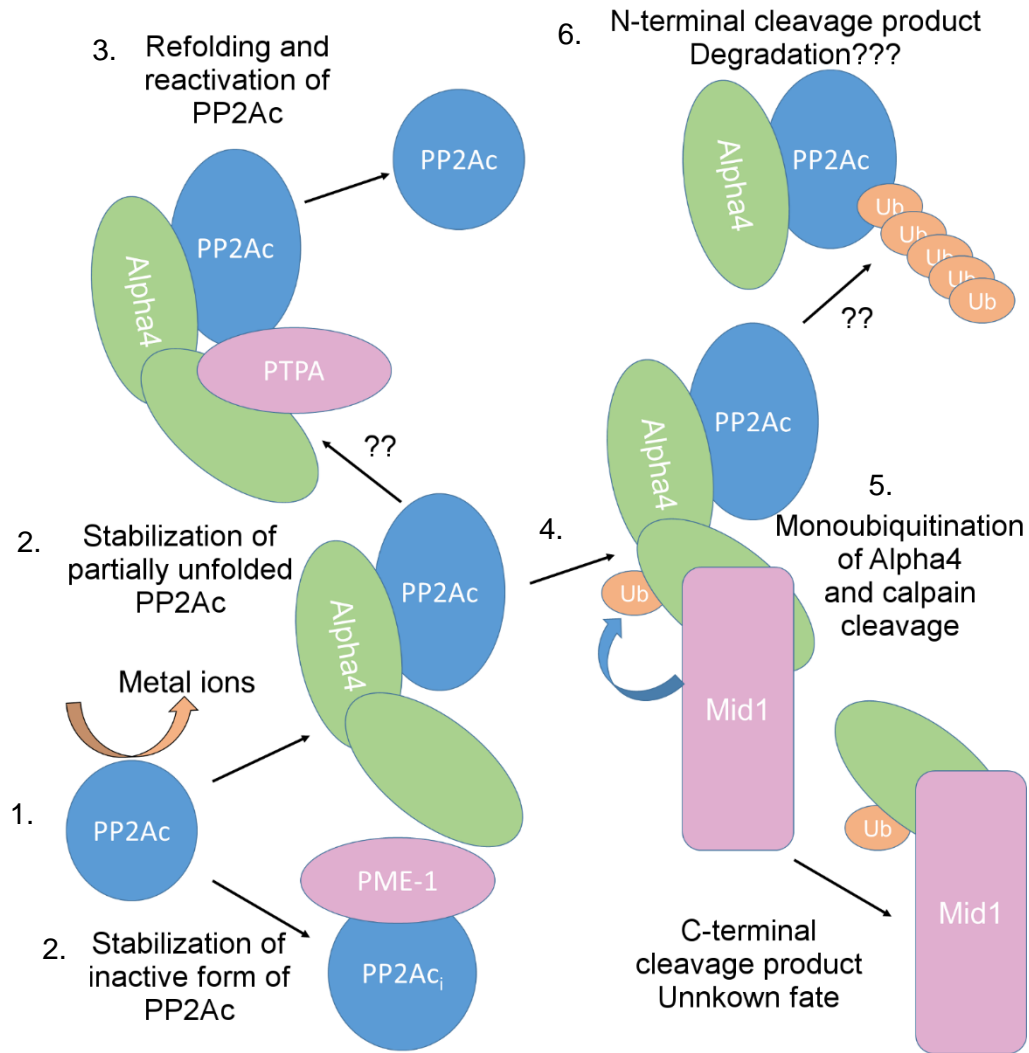
Figure 37: Model of Alpha4 role in PP2Ac stabilization, reactivation and degradation

1) Nascent Alpha4 is synthesized or Alpha4 destabilizes and loses its catalytic metal ions.
2) This leads to either binding by PME-1 to stabilize an inactive, but folded form of PP2Ac or to partial unfolding of PP2Ac and binding by Alpha4 to stabilizing a partially folded state.
3) PP2c bound to Alpha4 can be chaperoned to be refolded by an as yet to be determined mechanism, possibly involving PTPA. 4) PP2Ac bound to Alpha4 can form a heterotrimer with Mid1. 5) Mid1 monoubiquitinates Alpha4 leading to calpain induced cleavage of Alpah4 at F255-G256. 6) Cleaved Alpha4 fails to protect PP2Ac from polyubiquitination from an as yet to be determined E3 ubiquitin ligase and PP2c is degraded.

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negative impact on cell growth in the absence of p53 or when cells are exposed to certain types of stress has not previously been observed and gives us a better understanding of the complexity of the Alpha4 functions in the cell (Figures 31, 32, 35). These results show that although Alpha4 is generally considered anti-apoptotic its role may be shifted to a pro-apoptotic one under certain cellular conditions. This idea is important to understanding the possible roles and therapeutic value of Alpha4 as a target in both cancer and neurodegenerative diseases.

Future Directions

Role of the UIM and ubiqutin

Studies have shown that the UIM plays an important role in regulating Alpha4 protection of PP2Ac from degradation [191,217], but the mechanism by which it does so is still not understood. The composition of the UIM regulates the monoubiquitination and subsequent cleavage of Alpha4 by calpains with deletion of the UIM blocking monoubiquitination and mutations within the UIM enhancing monoubiquitination [191]. Our attempts to interrogate the nature of the interaction between UIM and ubiquitin via EPR and through crystallography have not been successful. We have also not been successful in our attempts to crystallize the Alpha4 UIM mutant that enhances monoubiquitination. The initial experiments that elucidated the interaction between the Alpah4 UIM and ubiquitin were conducted by NMR. Investigations of the UIM deletion mutant and the mutant that enhances monoubiquitination using this same methodology could prove enlightening. In this experiment, labelled ubiquitin would be titrated with increasing quantities of Alpha and a shift in the peaks of ubiquitin measured as an indication of binding. A second, but more involved approach, would be to label Alpha4 and assign the spectra. This would allow one to map the binding residues of ubiquitin on Alpha4 by subsequently titrating in unlabeled ubiquitin and looking at peak shifts and line broadening within the spectra.

Investigations into the roles of specific Alpha4 domains in Alpha4 functions

Our initial experiments concluded that both the PP2Ac binding domain and the C-terminal E3-ligase binding domains were essential for Alpha4 protective effects towards ectopically expressed PP2c [284]. Using the lentiviral methodology described in this work, the role of these domains and others in regulating phosphatase expression levels and cellular functions in a variety of cell lines can be more easily tested. Creation of a stable cell line expressing the C-terminally cleaved mutant Alpha4 G256* would allow investigation of the effects not only on PP2A, PP4 and PP6c expression levels, but also on cell functions such as cell adhesion, proliferation and cell death. Other mutants that effected various known post-translational modifications could also be tested in this fashion, such as mutation of K241, which would block acetylation of this residue [192], or mutation of K287 which would block the known Mid1 ubiquitination site [193].

Alpha4 effects on mitosis

Dysregulation of PP4 and PP6 expression have been found to lead to defects in chromosome segregation and mitosis evidenced by increased multinucleation and micronucleation. Observations of DAPI stained cells revealed some possible effects of Alpha4 knockdown and overexpression on the prevalence of both of these abnormalities in both HEK293T and A549 cells. As our experiments concluded that Alpha4 has a greater impact on PP4 and PP6, Alpha4 effects on pathways controlled by these two phosphatases should be more fully explored. This could help elucidate which phosphatase Alpha4 is regulating in these pathways as overexpression of PP4 has been linked to increased micronucleation [97], whereas knockdown of PP6 has the effect of increased micronucleation [137,138].

Role of Alpha4 in DNA damage repair: γH2AX dephosphorylation

An enlightening experiment to look at the role of Alpha4 in regulating the PP2A family of phosphatases would be to investigate the effects of Alpha4 knockdown and expression on γH2AX. Knockout of Alpha4 causes a delay in dephosphorylation of γH2AX after treatment with doxorubicin, as well as increased basal γH2AX [215], but the effects of Alpha4 knockdown, overexpression or mutations on basal γH2AX or foci resolution have not been studied. All three PP2A family members are involved in regulating γH2AX, but the routes of activation and the pool of H2AX that each of the PP2A family members act upon is slightly different. For instance, knockdown of PP4c leads to increases in basal levels of γH2AX, but does not increase the levels of γH2AX when cells are exposed to ionizing radiation (IR) [96]. In contrast, knockdown of PP2Ac has no effect upon levels of basal γH2Ax, but significantly increases the peak levels of γH2AX that accumulate in cells after exposure to IR [96]. By staining cells that stably knockdown or express Alpha4 for γH2Ax before and after exposure to IR over a time course, one could determine if Alpha4 effects basal levels, peak levels after IR damage, or time to resolution of foci. This could shed light on which phosphatase is the primary target of Alpha4 in this situation.

Effects of Alpha4 overexpression, knockdown and mutation on cell death in response to stressors

We discovered that overexpression of Alpha4 in A549 cells leads to increased cell death in response to oxidative stress induced by H_2O_2. Further exploration of the effects of Alpha4 knockdown and expression of the RKED mutant should be investigated. It would be interesting to determine if knockdown of Alpha4 has a protective effect under these circumstances or if any perturbations of Alpha4 levels in A549 cells increases sensitivity to H_2O_2. These experiments could be followed up with a series of experiments using the stable SCR, OE, KD and RKED A549 cell lines to look at effects on sensitivities to other stressors, especially agents used for

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chemotherapies. Some preliminary work has been done with staurosporine, a known inducer of apoptosis and a similar increased sensitivity with Alpha4 overexpression was seen (data not shown), but more work is need before conclusions can be made. A few other strong candidates to investigate would be camptothecin, a DNA topoisomerase inhibitor; doxorubicin, a DNA replication inhibitor; and cisplatin, also a DNA replication inhibitor that is a common therapeutic in chemotherapies and to which many cancers are resistant. Cisplatin resistance is driven by PP4 phosphatase complexes [112,118,119] and given that Alpha4 has a significant impact on PP4c levels (Figure 25), this agent is of particular interest.

These experiments should also be conducted in the HEK293T cell lines, as a preliminary work to investigate the role of Alpha4 in p53 incompetent cell lines. It would be beneficial to create various Alpha4 stable cell lines in other p53 deficient cell lines to determine if the effects seen in HEK293T cells are due to the p53 deficiency or another aberration present within these cells. As p53 is mutated in a number of cancers, it would be of import to understand the effects of Alpha4 manipulation on p53 mutated cells, if Alpha4 is to be studied as a possible therapeutic target.

Use of stable cell lines in in vivo *and* in vitro *models of tumorigenesis and metastasis*

The stable cell lines created using the lentiviral based approach can also be used in *in vivo* animal models with nude mice. Cells would be injected under the skin or in the fat pads of mice and tumor formation and growth would be assessed over time. Use of the HEK293T cell line would allow one to assess the tumorigenic potential of various Alpha4 constructs, as WT HEK293T cells doo not readily form tumors. Use of the A549 cell line could detect mutations that lead to a reduction in tumorigenesis or tumor growth, as this cell line does readily form tumors. Similar assays could be conducted using tail-vein injection and assessing the formation of metastasis in the lung or other organs.

These in vivo models can then be used to test Alpha4 mutant cells for sensitivity tvarious genotoxic agents and cancer therapeutics in a relevant animal model. One agent of particular interest is cisplatin. Cisplatin is a commonly used cancer therapeutic, but may cancers show cisplatin resistance. PP4c complexes are a known player in conveying cisplatin resistance and given that alterations in Alpha4 expression have a significant negative impact on PP4c expression levels, the hypothesis would be that alterations in Alpah4 would decrease cisplatin resistance thus making cancer cells more susceptible to cisplatin based therapies. This hypothesis could be tested in both *in vitro* and *in vivo* with the stable cell lines described within this work (or others created using a similar protocol) by assessing changes in cell proliferation, ATP production, cell death using some of the same methodologies described in Chapter 5 (Cell Titer Glo, Live/Dead cell staining, LDH release, Caspase 3/7 activation) when cells were treated with cisplatin or other genotoxic/chemotherapeutic agents.. These Alpha stable cell lines could also be tested *in vivo* using the tail vein injection or subcutaneous injection methods to create tumors in nude mice. The mice could then be treated with cisplatin and changes in tumor growth or formation could be evaluated in a live animal model.

Concluding Remarks

The complexities of Alpha4 regulation of the PP2A family of phosphatases is only beginning to be understood. The work presented here has elucidated the importance of both the N-terminal and C-terminal domains of Alpha4 in regulation of PP2Ac degradation and led to a change in the working model of Alpha4 protection of PP2Ac. Subsequent work has shown that cleavage of Alpha4 is a regulated process within the cell that is dysregulated in a number of diseases, including Optiz syndrome, melanoma, and Alzheimer's disease [191]. Further investigations have uncovered that Alpha4 does not associate with each of the PP2A family member equally or regulate them in the same manner. This indicates that, although a very high degree of homology exists between these phosphatases, the interactions differ enough that

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Alpha4 has differential effects upon the catalytic subunits. The causes and consequences of these differential effects have yet to be studied. In addition, Alpha4 is known to be a highly post-translationally modified protein [192,214,216,217] and the effects of these post-translational modifications are not understood. Our results show that in addition to Alpha4 playing an essential role in the cell in inhibiting apoptosis, it also increases cellular sensitivity to certain agents (e.g. ROS). This finding has implications for both understanding the role of Alpha in diseases such as cancer and Alzheimer's disease, but also in the development of potential therapeutics.

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