REGULATION OF PP2Ac STABILITY – DISCOVERY OF A NOVEL α4 MONOUBIQUITINATION-DEPENDENT MECHANISM THAT IS ALTERED IN ALZHEIMER'S DISEASE

By

Guy R. Watkins

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Approved:

Professor Brian E. Wadzinski

Professor Benjamin Spiller

Professor Vsevolod Gurevich

Professor Randy Blakely

Professor Stephen R. Hann

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To my wife, Courtney, and family

for their unwavering support

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CHAPTER I

INTRODUCTION

Reversible phosphorylation of proteins

The reversible phosphorylation of proteins is an evolutionarily conserved post-translational modification that is responsible for the temporal and spatial regulation of most, if not all, cellular signal transduction cascades (Virshup, 2000). Proper maintenance of this modification is crucial, as dysfunctional regulation of phosphorylation has been linked to multiple pathologies including carcinogenesis, tumorogenesis, neurodegenerative disease, and diabetes (Eichhorn et al., 2009; Liu and Wang, 2009; Zolnierowicz, 2000). In general, protein phosphorylation is controlled by two major classes of enzymes: the protein kinases, which catalyze the addition of a phosphate to a serine, threonine, and/or tyrosine residue, and the protein phosphatases that catalyze the removal of the phosphate group (Fig. 1). In contrast to the substrates and regulatory mechanisms identified for protein kinases, much less is known about protein phosphatases, as they were initially thought to function as constitutively active enzymes that terminate signals. However, it has now become apparent that phosphatases are tightly regulated enzymes with a myriad of cellular roles.

Classification of phospho-serine/threonine phosphatases

The first protein phosphatase was identified by Carl and Gerty Cori in 1945. The Coris were interested in understanding the kinetic properties of phosphorylase, and found an enzyme, termed the PR enzyme, which converted phosphorylase *a* to phosphorylase *b*. While these studies identified the enzyme it was not until roughly 10 years later, in work performed by

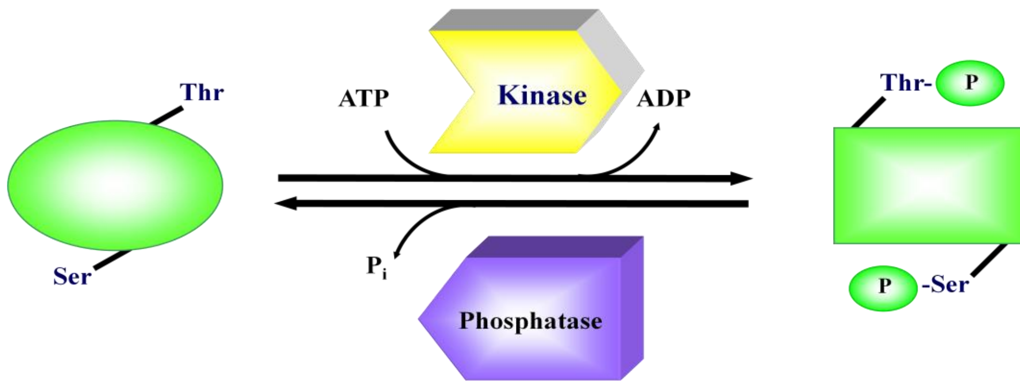


Figure 1. Reversible phosphorylation of proteins
Schematic representation of the role of kinases and phophatases in the reversible phosphorylation of proteins

Edmond Fischer and Edwin Krebs, that the PR enzyme was in fact determined to be a phosphatase. The seminal studies by Fischer and Krebs made two major discoveries. First, they identified a role for ATP in the conversion of phosphorylase *b* to phosphorylase *a*, and second they determined that phosphorylase *a* was a phospho-protein (Fischer and Krebs, 1955; Krebs and Fischer, 1956; Sutherland and Wosilait, 1955). Furthermore, Fischer and Krebs found that the conversion of phosphorylase *b* to phosphorylase *a* was catalyzed by phosphorylase *b* kinase. Together, these pioneering studies found that phosphorylation of phosphorylase altered its function, demonstrating for the first time the role of phosphorylation in the alteration of protein activity. Since the initial discovery of protein phosphorylation, it is now understood that in eukaryotic cells phosphorylation occurs primarily on serine, threonine or tyrosine residues. Closer examination of phosphorylation sites reveals that roughly 98% of total protein phosphorylation occurs on either serine or threonine (Olsen et al., 2006). Current studies of the mammalian proteome have identified 518 putative protein kinases that are classified as either tyrosine/dual-specificity kinases (~90) or serine/threonine kinases (~428) (Johnson and Hunter, 2005; Lander et al., 2001; Venter et al., 2001). Considering the large number of protein kinases and the tight regulation and specificity of protein phosphorylation, one would expect a similar number of phosphatases. Surprisingly, there have only been about 140 protein phosphatases identified, with 107 being protein tyrosine phosphatases and roughly 30 being protein serine/threonine phosphatases (Alonso et al., 2004). The relatively low number of serine/threonine phosphatases compared to serine/threonine kinases was unexpected and one of the reasons phosphatases were originally thought of as constitutively-active housekeeping proteins. However, based on recent discoveries, it is now appreciated that the stark difference in the total number of phosphatases and kinases can be explained in part by the relatively large

number of regulatory proteins that are able to bind serine/threonine phosphatase catalytic subunits to create multiple oligomeric forms of the same enzyme.

Similar to the protein kinases, phosphatases are classified as either tyrosine/dual-specificity phosphatases or serine/threonine phosphatases based on the amino acid they dephosphorylate, with the dual specificity phosphatase capable of dephosphorylating both tyrosine and serine/threonine residues. The serine/threonine phosphatases are divided into the following three major families based on their biochemical characteristics, sensitivity to inhibitors, and *in vitro* substrate specificity: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate based phosphatases (FCP/SCP) (Honkanen and Golden, 2002; Shi, 2009). Members of the PPM family include PP2C and are dependent on manganese/magnesium ions for activity, while the FCP/SCP family of phosphates is a relatively new family and the only known substrate for these enzymes is the C-terminal domain of RNA polymerase II (Shi, 2009). The PPP family is the largest family of serine/threonine phosphatases and includes five subfamilies: PP1, PP2A, PP2B (calcineurin), PP5, and PP7. The PP2A subfamily contains three distinct phosphatases: PP2A, the prototypical member of this subfamily, and the "PP2A like" phosphatases, PP4 and PP6, which share roughly 65% and 57% sequence identity with PP2A respectively (Fig. 2) (Honkanen and Golden, 2002; Shi, 2009). Members of the PPP family of serine/threonine phosphatases do not need manganese/magnesium ions for activity and rely on multiple regulatory subunits to control the enzymatic activity of the phosphatase. The remainder of this thesis will focus on the cellular functions and regulatory mechanisms for PP2A, which is the most abundant and best characterized member of the phospho-serine/threonine phosphatases.

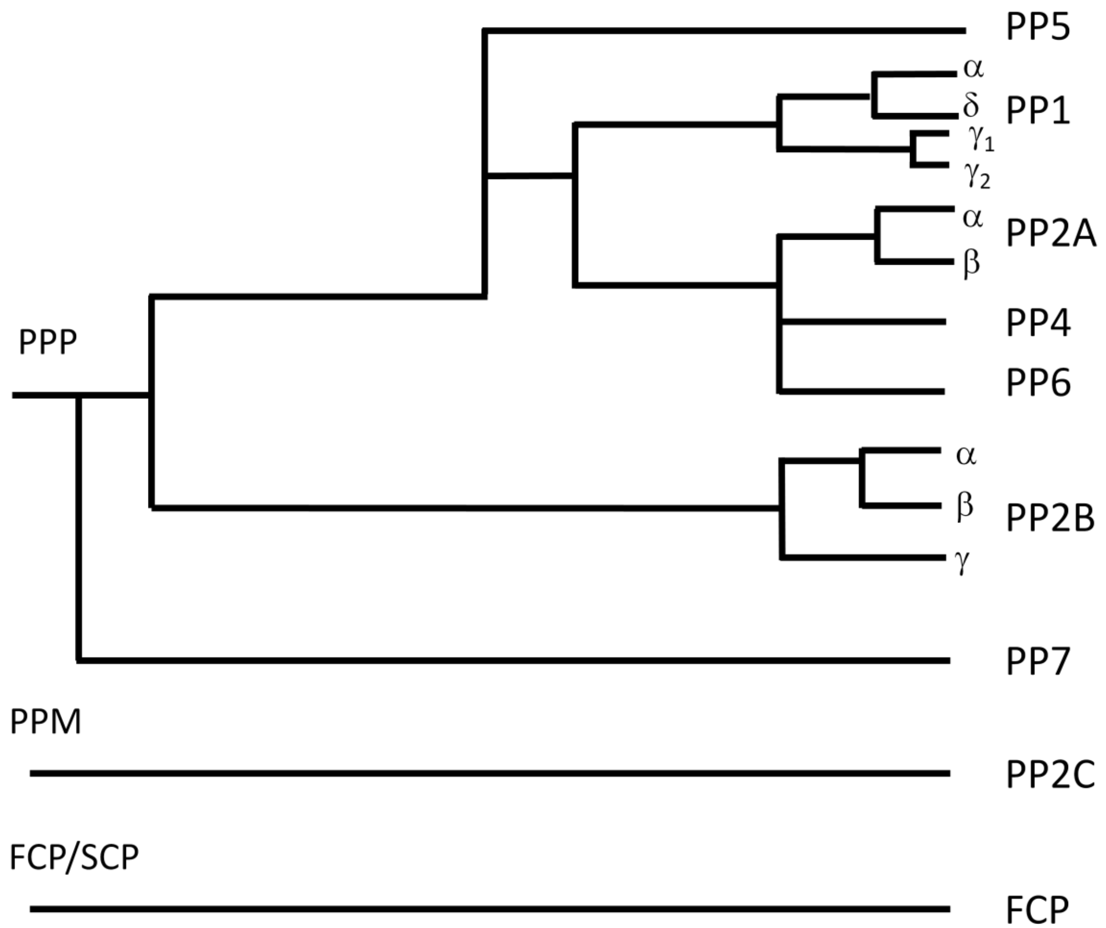


Figure 2. Phylogenetic tree of serine/threonine phosphatases
Classification of the three major families of serine/threonine phosphatases (PPP,PPM,FCP) based on amino acid sequence similarity. (Modified from Honkanen & Golden, 2002)

Protein phosphatase 2A (PP2A)

PP2A is an evolutionarily conserved member of the PPP family of phosphatases and is estimated to represent as much as 0.1% of total cellular protein (Cohen, 1997). As expected for a highly abundant cellular enzyme, a vast array of cellular functions are under the control of PP2A including cell growth (Adams et al., 2005), apoptosis (Van Kanegan et al., 2005), and development (Yang et al., 2003) (Fig. 3). Furthermore, PP2A activity must be tightly regulated, as its disregulation has been implicated in a wide variety of diseases including cancer, diabetes, and neurodegenerative disorders (Eichhorn et al., 2009; Liu and Wang, 2009).

The predominant form of PP2A in cells is a heterotrimeric holoenzyme consisting of a structural A subunit, a variable B subunit, and the catalytic C subunit (PP2Ac), which possesses the enzymatic activity of the phosphatase. Two major cellular isoforms for PP2Ac have been identified, α and β, which share over 97% sequence identity (Janssens and Goris, 2001). Studies of the PP2Ac isoforms have found that both are ubiquitously expressed, with the α isoform being much more abundant than the β isoform, although the role of each individual isoform in the control of phosphatase activity is unknown. Furthermore, the gene encoding the PP2Ac alpha isoform was found to be an essential gene, as knockout of this gene in mice causes embryonic lethality (Gotz et al., 1998). Structurally, the C subunit adopts the typical α/β fold found in PPP family members and contains two manganese ions in the active site (Xing et al., 2006). Furthermore, PP2Ac forms a pocket at the active site where the PP2A specific inhibitors okadaic acid (OA) and microcystin-LR (MCLR) bind (Xing et al., 2006).

In contrast to the C subunit, which has enzymatic activity, the A subunit functions as a scaffolding protein. Two isoforms of the A subunit (α and β) have been identified that share 86% sequence identity with the α isoform being much more abundant in mammalian cells;

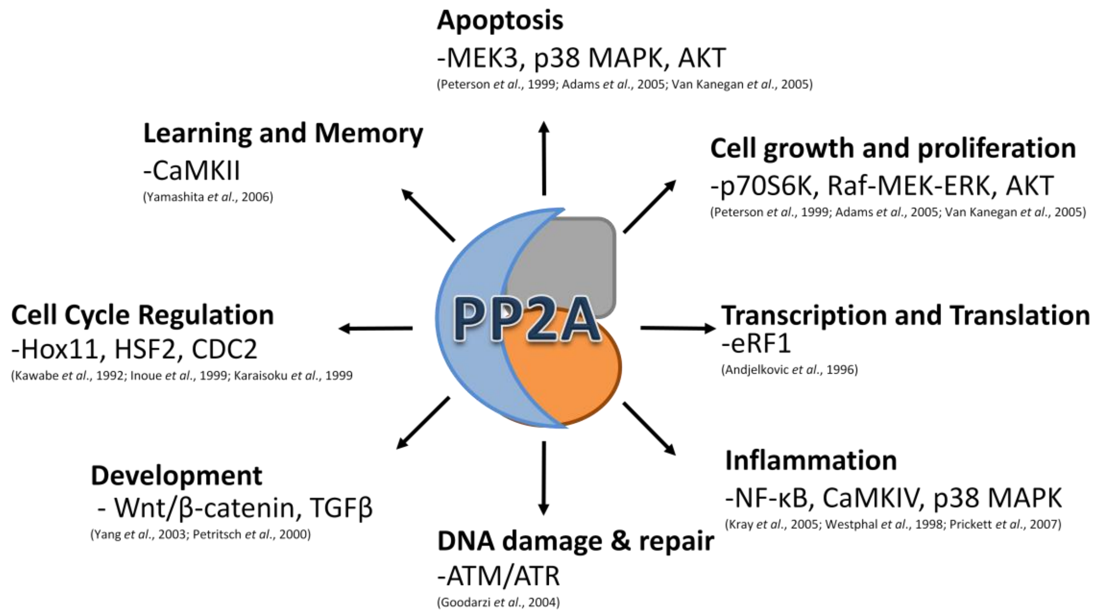


Figure 3. Cellular roles and substrates regulated by PP2A

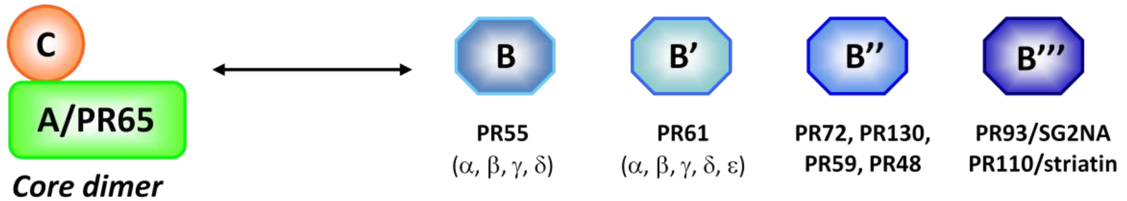
Schematic representation of select cellular processes and their corresponding protein substrates that are regulated by PP2A.

however, little is known about the different roles of the two isoforms (Janssens and Goris, 2001). The A subunit is a 65 kDa alpha helical protein containing 15 tandem HEAT repeat motifs, which gives the protein an elongated structure with a crescent shape, allowing the A subunit to function as the scaffolding subunit for the B and C subunits. In the cell, the A subunit tightly associates with either the C subunit alone to form the A/C core dimer or with both the B and C subunits to form the holoenzyme.

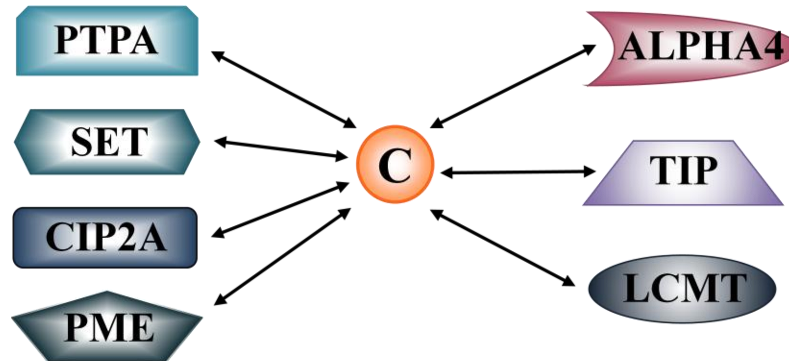
Substrate specificity for the PP2A holoenzyme is determined by the B subunit, which associates with the A/C core dimer to form the holoenzyme. Fifteen distinct B subunits have been identified and they are divided into four major families (B, B', B'', B''') with each family containing multiple members (Fig. 4A). Recent studies of the RAF·MEK·ERK signaling pathway highlight the specificity and importance of the B subunits. Bα-containing PP2A holoenzymes were shown to activate the RAF·MEK·ERK pathway by catalyzing the dephosphorylation of an inhibitory phospho-serine residue (p-Ser 259) in RAF, while B'-containing holoenzymes catalyze the dephosphorylation of ERK to inhibit its activity and inactivate the pathway (Adams et al., 2005; Van Kanegan et al., 2005).

In total, based on the multiple isoforms of each subunit of PP2A, there are over 70 distinct holoenzymes that can be formed, allowing this single enzyme to posses high substrate specificity (Fig. 4A) (Janssens et al., 2008). While the subunit composition alone adds diversity to PP2A, the function of PP2Ac is further regulated by other interacting proteins, known as atypical regulatory subunits, which associate with the catalytic subunit independent of the canonical A and B subunits.

A) Association with variable B regulatory subunits



B) Association with non-canonical regulatory subunits



C) Post-translational modifications of the catalytic subunit (C)

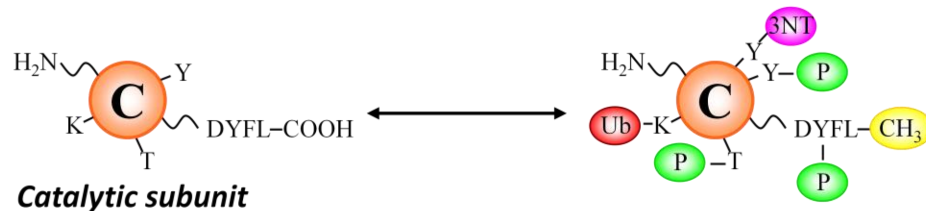


Figure 4. Multi-level regulation of PP2A

Schematic representation of mulit-level regulation of PP2A by (A) association with variable B regulatory subunits (B) association with non-canonical regulatory subunits and (C) post-translational modifications.

PP2Ac regulation by the atypical regulatory subunits

Recent studies have shown that the PP2Ac atypical regulatory subunits influence the assembly of the PP2A holoenzyme as well as the catalytic activity and stability of PP2Ac. The best characterized of these atypical subunits will be reviewed below and include LCMT, PME, SET, PTPA, CIP2A, TIP, and α4 (Fig 4B).

LCMT and PME

Leucine carboxyl methyltransferace (LCMT) is a 38 kDa protein that specifically catalyzes the methylation of the C-terminal leucine residue of PP2Ac (De Baere et al., 1999), and is crucial for proper PP2A function. LCMT is a critical protein, as knockdown of LCMT in cell culture leads to apoptosis (Lee and Pallas, 2007; Longin et al., 2007), and knockout of the LCMT gene in mice is embryonic lethal (Lee and Pallas, 2007). Much of what is known about the LCMT·PP2Ac complex was determined by structural analysis of LCMT bound PP2Ac; these studies identified a SAM-dependent methyltransfease domain and a lid domain on LCMT that is able to make multiple contacts and bind tightly to the PP2Ac active site (Stanevich et al., 2011). Furthermore, the active site of LCMT was shown to bind the highly conserved C-terminus of PP2Ac near the methylation site. Biochemical analysis of this complex revealed that PP2Ac must be in an active conformation to allow for LCMT-dependent methylation, suggesting that LCMT is only able to methylate an active form of PP2Ac (Stanevich et al., 2011).

The demethylation of PP2Ac occurs via a 42 kDa PP2A Methyl Esterase (PME) (Ogris et al., 1999). PME is conserved from yeast to humans and, similar to studies of LCMT, knockout of PME causes lethality (Ogris et al., 1999; Ortega-Gutierrez et al., 2008). The structure of the PP2Ac·PME complex reveals that PME binds directly to the phosphatase active site, and the conserved 6 amino acids at the C-terminus of PP2Ac occupy the active pocket of PME (Xing et

al., 2008) Engagement with PP2Ac causes a shift in PME from an inactive conformation to an active conformation allowing for the demethylation of the methylated C-terminal leucine residue of PP2Ac (Xing et al., 2008). Recent evidence suggests PME also shifts PP2Ac into an inactive conformation, most likely due to the loss of metal ions from its active site, and this inhibition of PP2Ac by PME is independent of methylation, as studies show LCMT is unable to re-activate PP2Ac inactivated by PME (Xing et al., 2008). Additionally, PP2Ac bound to a catalytically inactive mutant form of PME is still inhibited (Longin et al., 2008). In total, LCMT and PME not only play a major role in the regulation of PP2Ac methylation, but also in regulating the activity of the phosphatase.

PTPA, SET, and CIP2A

A second group of atypical regulatory subunits (PTPA, SET and CIP2A) do not appear to play a role in the regulation of post-translational modifications like LCMT and PME; rather they directly alter the activity of PP2Ac. Phosphatase Two A Phosphatase Activator (PTPA) enhances the activity of PP2Ac and is necessary for full activation of newly synthesized PP2Ac in yeast (Fellner et al., 2003). In addition, PTPA is unique among the PP2Ac atypical regulatory subunits in that activation of the phosphatase by PTPA requires a functionally bound A subunit (Hombauer et al., 2007). Consistent with a role for PTPA in the full activation of PP2Ac, knockdown of PTPA in cell culture shows phenotypes similar to those of PP2Ac-deficient cells (Fellner et al., 2003; Sablina et al., 2010). Although PTPA was initially shown to activate the tyrosyl phosphatase activity of PP2Ac *in vitro* using a synthetic tyrosine phosphorylated substrate (Cayla et al., 1994), recent studies indicate that PTPA reactivates a pool of PP2A that is inactive and bound to PME (Longin et al., 2004). The precise cellular mechanism for this activity

is unknown but may involve PTPA's peptidyl prolyl cis/trans-isomerase activity, which isomerizes Pro190 of PP2Ac (Jordens et al., 2006).

While PTPA is an activator of PP2Ac activity, SET and CIP2A are potent inhibitors of activity (Junttila et al., 2007; Li et al., 1996). SET, also known as TAF-I or I_2^{PP2A}, is a 39 kDa protein localized in the nucleus (Adachi et al., 1994). SET is cleaved into two fragments known as I_{2NTF} and I_{2CTF} allowing for translocation from the nucleus to the cytoplasm (Tanimukai et al., 2005). Both of the SET fragments are able to bind directly to PP2Ac leading to inhibition (Arnaud et al., 2011); however, the precise mechanism for the inhibition of PP2Ac by SET is currently unknown. Cellular studies have highlighted the importance of SET as they have identified a role for SET in a diverse set of biological functions including histone acetylation (Seo et al., 2001), cell growth (Canela et al., 2003), and transformation (Kumar et al., 2004). Furthermore, alterations in the levels of SET have been discovered in various cancers (Patel et al., 2008; ten Klooster et al., 2007) and Alzheimer's disease (Madeira et al., 2005).

A new inhibitor of PP2Ac activity has recently been discovered and named the Cancerous Inhibitor of Protein Phosphatase 2A or CIP2A. This protein is upregulated in multiple cancer types and via its inhibition of PP2Ac is thought to promote tumorogenesis (Bockelman et al., 2011; Come et al., 2009; Khanna et al., 2009; Niemela et al., 2012). Little is known about CIP2A in regards to its normal physiological function or mechanism of PP2Ac inhibition; however, studies have shown CIP2A inhibits the PP2Ac-dependent dephosphorylation of c-Myc pS62 leading to c-Myc stabilization (Junttila et al., 2007).

TIP and α4

The final group of atypical PP2A regulatory subunits, TIP and α4, are unique in the fact they bind to PP2Ac independent of both the A and B subunits of the holoenzyme, and there is

some evidence that they simultaneously interact with PP2Ac. TIP or Type 2A Interacting Protein is a 41 kDa protein that was initially identified in a yeast-2 hybrid screen using TAP42 (yeast α4) as bait, and has since been shown to bind the catalytic subunits of all three PP2A family members PP2Ac, PP4c, and PP6c (Jacinto et al., 2001; McConnell et al., 2007). Functional studies identified TIP as an inhibitor of PP2Ac activity *in vitro,* and uncovered a cellular role for TIP regulating the phosphorylation of a yet unidentified 32 kDa protein in the ATM/ATR pathway, which regulates the DNA damage and repair response (McConnell et al., 2007). In mammalian cells there is also a 20 kDa isoform of TIP which lacks the C-terminus and can no longer bind to PP2Ac, indicating the C-terminal tail of TIP is important for binding PP2Ac (McConnell et al., 2007). Recent studies have suggested TIP binds to TAK1 and promotes phosphorylation of the TAK1·TAB1 complex, which activates IKK signaling to NF-κB in cells (Prickett et al., 2008). Unpublished studies from our group also suggest that TIP is targeted for phosphorylation on S265, raising the intriguing possibility that TIP phosphorylation could influence its inhibitory potential and, hence, signaling pathways under the control of TIP·phosphatase complexes.

Probably the best studied PP2A atypical regulatory subunit is α4, which is the mammalian homologue of the yeast protein TAP42. Initially, α4 was characterized as a 52kDa phosphoprotein associated with the Ig receptor-associated protein MB-1 in B cells (Inui et al., 1995). Subsequent studies found α4 is ubiquitously expressed, with the highest levels found in the brain and thymus (Kloeker et al., 2003). Additional studies identified α4 as an atypical regulatory subunit of PP2Ac that is able to directly bind PP2Ac, PP4c, and PP6c independent of any other regulatory subunits for these phosphatases (Kloeker et al., 2003; Nanahoshi et al., 1999). In fact, the binding sites for both α4 and the A subunit on PP2Ac overlap and these proteins compete for binding to the catalytic subunit (Prickett and Brautigan, 2004). A closer

examination of the PP2Ac·α4 interaction revealed amino acids from 19-165 of PP2Ac are important in the binding of α4 whereas residues 94-202 of α4 are important for the binding to PP2Ac (Smetana et al., 2006; Yang et al., 2007a). Furthermore, mutation of the α4 amino acids arginine 155 to glutamic acid and lysine 158 to aspartic acid (α4 ED) generates a PP2Ac binding deficient mutant (McConnell et al., 2010; Yang et al., 2007a).

The effect of α4 on phosphatase activity has been extensively studied; however, the results have been controversial, as α4 has been shown to both enhance and inhibit phosphatase activity by multiple groups (Kong et al., 2009; Nanahoshi et al., 1999; Nien et al., 2007; Prickett and Brautigan, 2006). While the reason for the apparent contradictory nature of these findings is unclear, it does suggest that the activity dependent effects of α4 on PP2Ac is substrate dependent and direct changes in activity of PP2Ac is not a major role of α4. Structural studies of yeast TAP42, mouse α4, and human α4 have revealed it is a mainly alpha helical protein whose structure is most similar to the scaffolding proteins TPR and 14-3-3, with the C-terminus being highly flexible and unstructured (LeNoue-Newton et al., 2011; Smetana et al., 2006; Yang et al., 2007a). Further, biochemical analysis of the amino acid sequence of α4 identified a ubiquitin interacting motif (UIM) from amino acids 46-60 (McConnell et al., 2010). This amphipathic alpha helical region of α4 regulates the ubiquitination of PP2Ac in cells and will be discussed in detail in future sections.

The yeast homologue of α4, TAP42, binds to yeast PP2A catalytic subunits (Pph21/Pph22) and is an essential component of the target-of-rapamycin (TOR) kinase pathway controlling translation initiation and cell survival in yeast (Jacinto et al., 2001). However, studies linking α4 to the mammalian TOR pathway (mTOR) remains controversial and inconsistent (Bielinski and Mumby, 2007; Cygnar et al., 2005; Liu et al., 2001; Yoo et al., 2008). Despite the lack of convincing data linking α4 to the mTOR pathway, some insight into the physiological role

of mammalian α4 has come from studies of α4 knockout mice. Although knockout of α4 proved to be embryonic lethal, mouse embryonic fibroblasts derived from knockout animals exhibited increased apoptosis, which appeared to be due to increased phosphorylation of c-jun and p53 (Kong et al., 2004). Additional studies of these knockout MEFs revealed a role for α4 in the control of cell spreading and migration and in the regulation of the phosphorylation state of a multitude of proteins including RelA, AMPK, S6K, and PKA (Kong et al., 2007; Kong et al., 2009). Examination of α4 in cultured HEK cells revealed that α4 binds to the kinase MEK3, which regulates the activation of p38 MAPK (Prickett and Brautigan, 2007). Consistent with α4 null mice, recent studies in *Drosophila* found that total knockout of the α4 homologue TAP42 caused lethality; however, tissue-specific knockdown of TAP42 in wing imaginal discs led to a pleiotropic fly phenotype that included a thorax cleft and undeveloped wings (Wang et al., 2012). Silencing of *Tap42* also altered multiple signaling pathways (e.g., JNK, DPP, and HH) and triggered apoptosis in the wing discs. Furthermore, the $Tap42^{RNAi}$-induced defects were the direct result of disrupted regulation of *Drosophila* PP2A family members (Mts, PP4, and PPV) as enforced expression of wild type Tap42, but not a phosphatase binding-defective mutant of Tap42, completely rescued the phenotype of mutant flies. Studies are still ongoing to further characterize the role of α4 in the regulation of PP2Ac and Chapters IV and V in this thesis identify a novel role for the α4·PP2Ac complex.

The identification of novel regulatory subunits for PP2Ac has opened a new field in phosphatase research. Table 1 shows our current understanding of these atypical regulatory proteins. The cellular roles of the atypical regulatory proteins are just beginning to be identified, and there is a good possibility that many more atypical regulatory subunits exist to regulate PP2A in the cells.

Table 1. The role of the PP2Ac atypical regulatory subunits

Atypical PP2Ac Subunit	Regulatory Role	Reference
LMCT	Methylates L309 of PP2Ac	De Baere, I., *et al.* (1999) *Biochemistry*
PME	Demethylates L309 of PP2Ac	Ogris, E., *et al.* (1999) *J Biol Chem*
PTPA	Activator of PP2Ac activity	Longin, S., *et al.* (2004) *Biochem J*
SET	Inhibitor of PP2Ac activity	Li, M., *et al.* (1996) *J Biol Chem*
CIP2A	Inhibitor of PP2Ac activity	Junttila, M.R., *et al.* (2007) *Cell*
TIP	Inhibitor of PP2Ac activity in the ATM/ATR pathway	McConnell, J.L., *et al.* (2007) *Oncogene*
ALPHA4 (α4)	Stability of PP2Ac	LeNoue-Newton, M., *et al.* (2011) *J Biol Chem*

Regulation of PP2Ac by post-translational modifications

The activity of the catalytic subunit of PP2A is controlled not only by the holoenzyme composition and atypical regulatory subunits, but also by post-translational modifications. Studies to date have shown that PP2Ac is modified by methylation, phosphorylation, nitrosylation, and ubiquitination, and these post-translational modifications affect phosphatase activity, subcellular localization, and/or the ability of PP2Ac to bind the regulatory B subunits (Fig 4C). Table 2 summarizes the known post-translational modifications and lists the proteins involved in the regulation of each modification. PP2Ac methylation occurs on leucine residue 309 in the C-terminal tail, and is regulated by the atypical subunits LCMT and PME (Lee and Stock, 1993; Xie and Clarke, 1993, 1994), which were described earlier in this chapter. PP2Ac methylation plays an essential role in the formation of the PP2A holoenzyme and, more specifically, is important in the assembly of holoenzymes containing the Bα regulatory subunit (Bryant et al., 1999). Methylation can also regulate the phosphatase activity of PP2A in cells by altering the substrates for PP2Ac based upon holoenzyme formation (Bryant et al., 1999; Ikehara et al., 2007; Tolstykh et al., 2000). Methylation of PP2Ac appears to play a key role in the cell-cycle dependent regulation of PP2Ac (Turowski et al., 1995) as well as insulin secretion from pancreatic β-cells (Kowluru et al., 1996).

PP2Ac can also be phosphorylated on both a tyrosine residue and a threonine residue located in the C-terminal tail of the phosphatase. The best characterized of these phosphorylation events is phosphorylation of tyrosine 307 (Y307), which inhibits catalytic activity and may also affect holoenzyme assembly (Chen et al., 1994). Multiple tyrosine kinases, including Src, EGFR kinases, and insulin receptors, have been shown to mediate PP2Ac tyrosine phosphorylation (Chen et al., 1994). Moreover, misregulated tyrosine phosphorylation has been implicated in neurodegenerative diseases such as Alzheimer's disease (Liu et al., 2008). Y307

may not be the only site of phosphorylation, as unpublished results from our lab has detected phosphorylation of Y127 within the catalytic domain of PP2Ac. More studies are necessary to confirm the major site of cellular tyrosine phosphorylation. Threonine phosphorylation of PP2Ac has also been reported and the site is predicted to be T304 in the C-terminal tail (Guo et al., 1993). Similar to tyrosine phosphorylation, threonine phosphorylation inhibits the catalytic activity of the phosphatase (Damuni et al., 1994; Guo et al., 1993). Although the precise regulatory role that threonine phosphorylation plays in the control of phosphatase function remains unclear, there is some evidence that it can direct holoenzyme assembly with specific B subunits (Ogris et al., 1997).

In addition to phosphorylation, PP2Ac was shown to be nitrosylated at tyrosine 284, which increases the total level of cellular PP2Ac in a manner independent of the levels of either the A or B subunits (Ohama and Brautigan, 2010). The nitration of PP2Ac appears to be catalyzed by VCP/p97 but the precise mechanism and function of this novel post-translational modification has yet to be determined.

PP2Ac can also be modified by ubiquitin in cells. Ubiquitination is important for the regulation of PP2Ac stability and, more specifically, is thought to directly regulate the levels of a microtubule-associated pool of PP2Ac (Trockenbacher et al., 2001), as cells with decreased PP2Ac ubiquitination have decreased levels of microtubule-associated protein (MAP) phosphorylation (Trockenbacher et al., 2001). While the E3 ubiquitin ligase responsible for PP2Ac ubiquitination is unknown, a role for the PP2Ac atypical regulatory protein α4 in the control of PP2Ac ubiquitination has been identified (LeNoue-Newton et al., 2011; McConnell et al., 2010) and will be discussed in subsequent chapters.

Table 2. The regulatory role, site, and catalyzing enzymes of PP2Ac post-translational modifications

Post-Translational Modification	Site of PP2Ac Modifcation	Catalyzing Enzymes	Role of Modification	Reference
Tyrosine phosphorylation	Y307	Src, EGFR, InsulinR	Inhibits enzymatic activity	Chen, J., *et al.* (1992) *Science*
Threonine phosphorylation	T304	Unknown Kinase	Inhibits enzymatic activity	Guo, H., *et al.* (1993) *PNAS*
Carboxy-methylation	L309	LCMT	Facilitates Bα binding	Lee, J., *et al.* (1993) *J Biol Chem*
Nitrosylation	Y284	VCP/p97	Increases protein levels	Ohama, T., *et al.* (2010) *J Biol Chem*
Ubiquitination	K41	Unknown E3	Facilitates degradation	Trockenbacher, A., *et al.* (2001) *Nat Genet*

The ubiquitination cascade

The covalent attachment of ubiquitin, a 76 amino acid polypeptide, to target proteins occurs through a reversible multistep enzyme cascade, and is an evolutionarily conserved mode of protein regulation (Fig. 5). The ubiquitination cascade is initiated with the E1 ubiquitin-activating enzyme adenylating the C-terminal glycine residue of ubiquitin in an ATP-dependent reaction, allowing for a thioester bond to form between a cysteine residue on the catalytic site of the E1 enzyme and a glycine residue at the C-terminus of ubiquitin. The activated ubiquitin is then transferred to an E2 conjugating enzyme via a transthioesterification reaction to form a thioester bond between an active site cysteine residue of the E2 enzyme and the activated glycine residue of ubiquitin. The final step of the cascade is carried out by an E3 ubiquitin ligase that binds the target protein and facilitates the transfer of ubiquitin from the E2 enzyme to a lysine residue on the target protein. The transfer of ubiquitin from the E2 enzyme to the target protein is mediated via the amino group of the lysine residue on the target protein, which attacks the thioester bond to create an isopeptide bond between ubiquitin and the target protein lysine residue [reviewed in (Nandi et al., 2006)]. The attachment of ubiquitin to a lysine residue is the most studied and best characterized form of ubiquitination; however, ubiquitin can attach to serine, threonine, or cysteine residues, and in some instances to the amino terminal residue (Tait et al., 2007). Two major classes of E3 ubiquitin ligases have been characterized: the HECT (homologous to E6-AP carboxyl terminus) domain containing ligases, which form a thioester intermediate between the E3 ubiquitin ligase and ubiquitin leading to ubiquitination of the target protein, and the RING (really interesting new gene) finger domain containing ligases, which directly facilitate the transfer of ubiquitin from the E2 enzyme to the target protein (Weissman, 2001). Once a single ubiquitin moiety is adducted, the target protein can remain in a monoubiquitinated state or multiple ubiquitin moieties can be attached

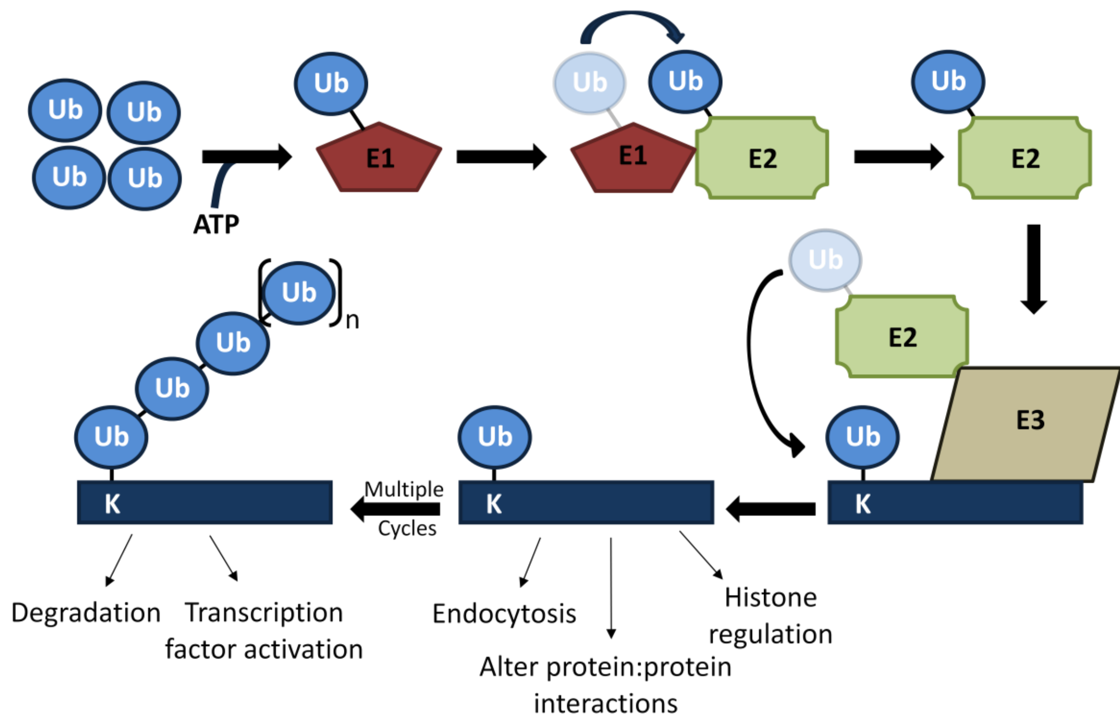


Figure 5. The ubiquitination cascade

Schematic representation of the ATP dependent multi-enzyme cascade responsible for the covalent attachment of ubiquitin to a lysine residue on a target substrate, and the known roles for both mono- and polyubiquitination of substrates.

to either a lysine residue on the bound ubiquitin to form an elongated ubiquitin chain termed polyubiquitination or to a second lysine residue on the target protein in what is known as multiubiquitination (Fig. 5) (Weissman, 2001). Initially it was hypothesized that a single E2/E3 complex was responsible for the polyubiquitination of a target protein, but recent studies have determined that the E2 enzyme is only able to adduct a single ubiquitin to a protein (Soss et al., 2011; Windheim et al., 2008). Furthermore, there is evidence that different E2 enzymes regulate protein monoubiquitination and the subsequent formation of polyubiquitin chains (Soss et al., 2011). The current hypothesis is that the E2 enzyme determines the type of ubiquitin chain formed; support for this stems from the fact that the mammalian genome contains only two E1 enzymes while there is greater diversity with the E2 and E3 enzymes, with 38 E2 enzymes and over 650 RING domain-containing E3 ligases discovered to date (Nandi et al., 2006).

Polyubiquitination of a target protein can play many roles depending on the type of polyubiquitin chain (reviewed in (Weissman, 2001)). Ubiquitin contains seven lysine residues and each lysine residue is a target residue for ubiquitin. The most common chain linkage occurs on lysine 48 of ubiquitin, which targets the polyubiquitinated protein for degradation via the 26S proteasome. On the other hand, ubiquitin chains linked via lysine 63 in ubiquitin can act as signals for DNA repair and/or activation of transcription factors. The other lysine residues on ubiquitin are still being characterized as to their role in protein regulation. The simplest form of protein ubiquitination, monoubiquitination, in which a single lysine in the target protein is bound to a single ubiquitin has been shown to influence the protein’s subcellular localization, lead to endocytosis, and play a role in histone regulation and virus budding (Weissman, 2001). Protein monoubiquitination and polyubiquitination is a rapidly evolving field of study as novel roles for these modifications are constantly being identified.

PP2Ac ubiquitination and the MID1·α4·PP2Ac complex

Studies of the rare genetic disorder Opitz syndrome led to the identification of PP2Ac as a target for polyubiquitination. Opitz syndrome is an X-linked genetic disorder characterized by mutations in the *MID1* gene, that encodes the microtubule associated protein MID1. This mutation causes defects in the development of the ventral midline with patients suffering from mental retardation, cleft lip and palate, and cardiac abnormalities (Gaudenz et al., 1998; Meroni, 1993; Quaderi et al., 1997). Trockenbacher and colleagues showed that human fibroblast cells treated with proteosome inhibitor exhibited an increase in the total level of PP2A, indicating PP2Ac is targeted for polyubiquitination (Trockenbacher et al., 2001). Furthermore, similar studies from fibroblasts of patients with Opitz syndrome did not detect any changes in PP2Ac levels between proteasome inhibitor treated and untreated cells (Trockenbacher et al., 2001). Together, data from these studies linked the polyubiquitination of PP2Ac to MID1.

Analysis of the domains within MID1 identified a RING finger domain (indicative of an E3 ubiquitin ligase), two B-box domains, a coiled-coil domain, a FNIII domain, and a C-terminal B30.2 domain. Further analysis of the link between PP2Ac and MID1 revealed that PP2Ac does not directly bind to MID1, but rather a ternary complex is formed in which the atypical regulatory subunit of PP2Ac, α4, binds to the B-box domain in MID1 and PP2Ac via domains in its C- and N-terminus, respectively (Fig. 6) (Liu et al., 2001; McConnell et al., 2010; Trockenbacher et al., 2001). These results highlight a key regulatory role for α4 in the modulation of PP2Ac ubiquitination, and forms the basis for the proposed model whereby α4 serves as a scaffolding protein to connect PP2Ac to MID1 allowing the polyubiquitination and degradation of a microtubule-associated pool of PP2Ac (Fig. 7) (Trockenbacher et al., 2001). While these studies supported a destructive role for α4 in the regulation of PP2Ac, several

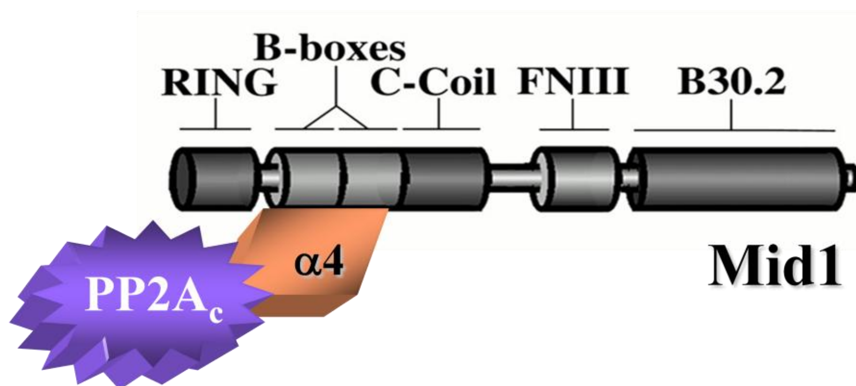


Figure 6. The Mid1·α4·PP2Ac ternary complex

Schematic representation of the Mid1·Alpah4·PP2Ac ternary complex showing the multiple domains found in Mid1 with α4 binding to the B-Box domain recruiting PP2Ac to the complex.

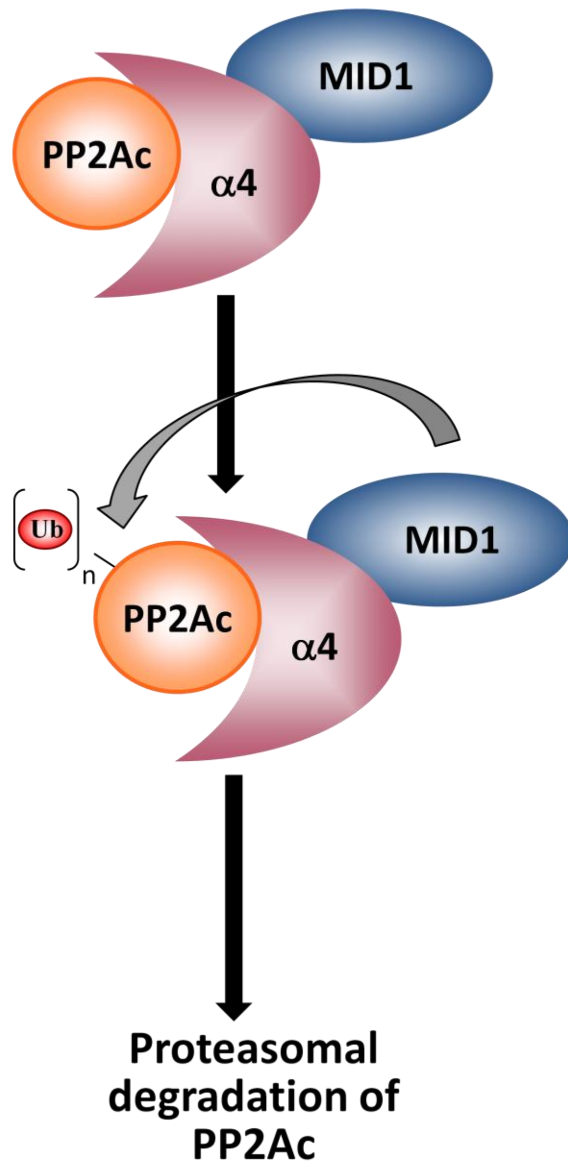


Figure 7. Initial model for the role of Mid regulation of PP2Ac
α4 was originally hypothesized to serve as a scaffolding protein responsible for bring PP2Ac to its proposed E3 ubiquitin ligase Mid1. Upon formation of the ternary complex Mid1 would polyubiquitinate a target lysine residue on PP2Ac leading to its proteasome mediated degredation.

recent reports have revealed α4 may actually protect PP2Ac from polyubiquitination and subsequent proteasomal degradation (Kong et al., 2009; LeNoue-Newton et al., 2011; McConnell et al., 2010). In fact, data from mammalian cell culture determined that α4 overexpression prevented PP2Ac polyubiquitination and increased the half-life of PP2Ac in cells (LeNoue-Newton et al., 2011; McConnell et al., 2010). In addition, analysis of α4 knockout mouse embryonic fibroblasts (MEFs) showed a loss of the catalytic subunit relative to WT MEFs (Kong et al., 2009). Furthermore, α4 was shown to contain a ubiquitin interacting motif (UIM) that inhibited PP2Ac from undergoing polyubiquitination both *in vitro* and in cells. Together these studies indicate a protective role for α4 in the regulation of PP2Ac from polyubiquitination and degradation, and highlight the importance of the 14 amino acid UIM region of α4 in this process. The apparent contradictory roles for α4 in the control of PP2Ac (protective vs. destructive) will be addressed in Chapter IV. The data we present in Chapter IV allowed us to formulate a new model that explains how α4 could possess both of these activities towards PP2Ac.

Summary

The multi-level regulation of PP2A is a rapidly evolving area of phosphatase research. PP2Ac is no longer thought to be regulated solely by the canonical B subunits, as our understanding of the roles of the atypical regulatory subunits and post-translational modifications of PP2Ac have become more appreciated. Recent advances in these areas have led to more questions since many of these regulatory proteins have been recently discovered, and the function that these proteins play in cellular physiology remain unclear.

The identification of ubiquitination as a novel mode of PP2Ac regulation and understanding the role of PP2Ac ubiquitination was a major theme of my graduate studies.

When I began these studies little was known about PP2Ac ubiquitination, as it was difficult to study this modification either *in vitro* or in cells. In chapter II of this thesis I will discuss the multiple techniques I adapted to study the ubiquitination state of PP2Ac both *in vitro* and in cells. The tools/protocols were used to identify the PP2Ac polyubiquitin chain linkage (lysine 48) and the site of modification on PP2Ac (lysine 41), which is discussed in chapter III.

The importance of the atypical regulatory protein α4 in the regulation of PP2Ac ubiquitination and the role of the MID1·α4·PP2Ac complex are the focus of chapter IV. This chapter identifies MID1 as the E3 ubiquitin ligase for α4, not PP2Ac, and elucidates a novel role for α4 ubiquitination. Studies presented in chapter IV also examine the relationship between α4 monoubiquitination and PP2Ac polyubiquitination, shedding light on the contradictory roles of α4 as protective and destructive towards PP2Ac. Furthermore, studies in this chapter allowed us to develop a new model for the role of α4 in the regulation of PP2Ac ubiquitination. A potential link between defective MID1 dependent monoubiquitination of α4 in both Opitz syndrome and Alzheimer's disease, as well as various cancers, are described in Chapter V. Finally, I end with studies aimed at identifying how α4 monoubiquitination is regulated, and highlight some interesting preliminary data pointing to a potential regulatory role for phosphorylation in the control of α4 function. Together, the studies presented in this thesis identify a novel mechanism for α4 in the regulation of PP2Ac, which may play an important role in the pathogenesis of MAP-dependent diseases such as OS and AD.

CHAPTER II

DETECTION OF UBIQUITINATED α4 AND PP2Ac SPECIES

Introduction

The regulation of proteins by the covalent attachment of ubiquitin occurs via an evolutionarily conserved multistep enzyme cascade (Fig. 5) (Nandi et al., 2006). The ubiquitination cascade is initiated by the E1 ubiquitin activating enzyme that adenylates the C-terminal glycine residue of ubiquitin in an ATP-dependent reaction. The activated ubiquitin is then transferred to an E2 conjugating enzyme and, ultimately, covalently attached to a lysine residue on a target protein via the actions of an E3 ubiquitin ligase. Ubiquitinated proteins can be modified by a single ubiquitin (monoubiquitination) or by multiple ubiquitins that form an elongated ubiquitin chain (polyubiquitination) (Weissman, 2001). Monoubiquitination can alter the protein's activity and subcellular localization, while protein polyubiquitination is most often a signal for proteasomal degradation of the targeted protein (Hicke, 2001).

PP2Ac and α4 are able to form a ternary complex with the E3 ubiquitin liagse MID1, thus suggesting that one or more of these proteins may be targeted for ubiquitination. Consistent with this idea, Trockenbacher and colleagues reported that the level of PP2Ac is increased in cells treated with proteasome inhibitor when compared to untreated cells (Trockenbacher et al., 2001). These findings indicate that PP2Ac is most likely a target for polyubiquitination, and raises the intriguing possibility that α4 is also targeted for ubiquitination. However, at the time I began my graduate studies, no reports had directly demonstrated that PP2Ac is targeted for polyubiquitination.

In this chapter I present both *in vitro* and cellular data showing that PP2Ac is indeed targeted for polyubiquitination. My studies also reveal that the PP2Ac regulatory protein α4 is targeted for monoubiquitination. This chapter reviews the development and utilization of both *in vitro* and cellular ubiquitination assays to study the ubiquitination state of both PP2Ac and α4 by Western and in live cells using ubiquitin bi-fluorescence complementation (UbFC).

Materials and Methods

Plasmids

The HA-ubiquitin plasmid was a gift from Dr. Hal Moses (Vanderbilt University, Nashville, TN) and the HA_3-PP2Ac and 6xHIS-ubiquitin constructs were gifts from Dr. David Brautigan (University of Virginia, Charlottesville, VA) and Dr. Dean Ballard (Vanderbilt University, Nashville, TN), respectively. Construction of the FLAG-tagged α4 wild type mammalian expression constructs was described previously (McConnell et al., 2010). The CFPc-HA_3-PP2Ac (amino acids 155-283 of cyan fluorescent protein attached to the N-terminus of HA_3-PP2Ac) construct was created using the following primers forward 5'-CCTGAAACAGAAAGTCATGAACCACCTGCAGCATGGCAATGGACAAGCA-3' and reverse 5'-CTCATCAATGTATCTTATCATCTGCAGGTACCGTGTCTGGATCCCCGCGGCCGCATACTT-3' to isolate nucleotides corresponding to amino acids 155-283 of CFP from the BiFC pJUN-CC155 plasmid (Dr. Tom Kerppola, University of Michigan, Ann Arbor, MI). The PCR product was ligated into the Pst1-digested HA_3-PP2Ac/pKH3 vector. The VN155-ubiquitin construct (amino acids 1-155 of venus fluorescent protein attached to the N-terminus of ubiquitin) was created by isolating amino acids 1-155 of venus fluorescent protein (a gift from Dr. Dave Piston Vanderbilt University, Nashville, TN) using the following PCR primers: forward 5'-

CCAAGCTTCATGGCAATGATGGTGAGCAAGGGCGAGGAGC-3’ and reverse 5’-GCTTGTATCGATGTACCGTTACCGGCGGTGATATAGACG-3’. The PCR product was ligated into the CLA1 and HindIII digested YN173-ubiquitin construct (Dr. Tom Kerppola, University of Michigan, Ann Arbor, MI) to create VN155-ubiquitin. The CFPc-HA_3-PP2Ac No K construct was created by mutating all codons encoding lysine residues to arginines using site-directed mutagenesis (Stratagene). Proper construction of all plasmids was verified by automated sequencing (Vanderbilt University DNA core facility).

Antibodies and reagents

The mouse monoclonal PP2Ac antibody was from BD Biosciences (San Diego, CA). The rabbit polyclonal α4 antibody was from Bethyl Laboratories (Montegomery, TX), and the mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The purified PP2A catalytic subunit from bovine heart was a kind gift from Dr. Greg Moorhead (University of Calgary, Calgary, AB). MG132 was from BostonBiochem (Boston, MA). The HA-affinity agarose and FLAG-affinity agarose were from Roche Applied Science (Indianapolis, IN) and Sigma (St. Louis, MO), respectively. The TUBE2 agarose was from Lifesensors (Malvern, PA). FLAG peptide was from Sigma (St. Louis, MO).

In vitro ubiquitination reactions

Ubiquitination assays were performed using a ubiquitin−protein conjugation kit (BostonBiochem, Cambridge, MA), which contains a mixture of conjugation enzymes from rabbit reticulocyte fraction II. Fraction A contains E1 and E2 enzymes, and Fraction B contains E3 ligases and deubiquitinating enzymes. The kit also contains ubiquitin and an ATP-containing energy solution. Ubiquitination assays were conducted using 16 µg (20 µL) of combined

Fractions A and B, 34 µg of ubiquitin, 2.5 µL of energy solution, ~50 ng of purified bovine PP2Ac, or ~200 ng of immunopurified wild-type FLAG-α4. Reaction mixtures were incubated at 37 °C for 4 h, quenched with 10 mM EDTA, and then subjected to Western analysis.

Cell culture and transfection

HEK293FT cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. HEK293FT cells were transfected with mammalian expression constructs using Fugene6 (Roche, Indianapolis, IN) according to the manufacturer's protocol.

Immunoprecipitations

HEK293FT cells, seeded at 400,000 cells per well in 6 well plates, expressing FLAG- or HA-tagged proteins, were lysed in 200 µL 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 1µg/mL leupeptin] and centrifuged at 12,000 x g for 10 min. Clarified lysates (170 µL) were incubated with 20 µL of a 50% slurry of anti-HA agarose (Roche, Indianapolis, IN) overnight or anti-FLAG-agarose (Sigma, St. Louis, MO) for 4 hours. The immune complexes were washed three times in 1 mL IP buffer, and bound proteins were eluted in SDS sample buffer and subjected to Western analysis. In some cases, the FLAG-tagged proteins were eluted from the beads by incubation for 60 min at 4°C in Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl) containing 100 µg/ml FLAG peptide.

TUBE pulldowns

HEK293FT cells were lysed in 200 µl IP buffer [150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Igepal, 5 µg/mL aprotinin, 1 µg/mL pepstatin, 1 mM PMSF, and 1 µg/mL leupeptin] and centrifuged at 12,000 × *g* for 10 min. Clarified lysates (170 µl) were incubated with 20 µl of a 50% slurry of TUBE2-agarose (LifeSensors) overnight at 4 °C. TUBE2 complexes were washed three times with 1 ml of IP buffer, and bound proteins were eluted in SDS sample buffer and subjected to Western analysis.

IMAC

HEK293FT cells were lysed in IMAC wash buffer [50 mM NaH_2PO_4, pH 8.0, 1% Igepal, 40 mM imidazole, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, 1 mM PMSF, and 1 µg/ml leupeptin in PBS] and centrifuged at 12,000g for 10 min. Clarified lysates were incubated with 20 µl of a 50% slurry of His-select beads (Sigma) for 15 min at 4 °C. Bound proteins were washed twice in 1 mL IMAC wash buffer and eluted with 40 µl of IMAC elution buffer [50mM NaH_2PO_4, pH 8.0, 1% Igepal and 250mM imidazole in PBS] and subjected to Western analysis.

Western analysis

SDS-solubilized protein samples were separated on 10% SDS-polyacrylamide gels and transferred to 0.45 µm nylon-supported nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked in Odyssey Buffer (Li-COR, Lincoln, NE). All primary antibodies were used at a 1:1000 dilution in Tris-buffered saline containing 0.5% BSA and 0.1% Tween-20 (TTBS/BSA). For detection with the Odyssey Infrared Imaging system, the appropriate fluoraphore-conjugated secondary antibodies were used at a 1:10,000 dilution in TTBS/BSA.

Bound antibodies were visualized using the Odyssey Infrared Imaging system and Odyssey software (LI-COR, Lincoln, NE).

Ubiquitin fluorescence complementation (UbFC) assays

HEK293FT cells were plated at 50,000 cells per well in 35-mm glass bottom culture dishes (MatTek, Ashland, MA) and transfected with the UbFC plasmids, CFPc-HA_3-PP2Ac and VN155-Ub. At 24 h post-transfection, the live cells were imaged for the presence of fluorescence using a LSM meta confocal microscope.

Results

Detection of ubiquitinated α4 and PP2Ac species via in vitro ubiquitination assays.

PP2Ac has previously been recognized as a target for proteasomal degradation (Trockenbacher et al., 2001); however, no study has directly shown that PP2Ac is targeted for polyubiquitination. To address this issue we developed an *in vitro* ubiquitination assay to monitor the ubiquitination state of PP2Ac. Purified PP2Ac was incubated in a ubiquitin-protein conjugation solution containing a mixture of E1/E2 enzymes (Fraction A) and a mixture of E3 ligases (Fraction B), along with ubiquitin in the presence or absence of the Mg^{2+}/ATP activating solution. Western analysis of these reactions revealed a high molecular weight smear migrating above unmodified PP2Ac only in the lanes in which both Mg^{2+}/ATP and PP2Ac were present (Fig. 8A), indicating that PP2Ac is indeed targeted for polyubiquitination under these *in vitro* assay conditions.

We also utilized the *in vitro* ubiquitination assay to test whether the PP2Ac interacting protein α4 is targeted for ubiquitination. Immunopurified FLAG-α4 was incubated in the

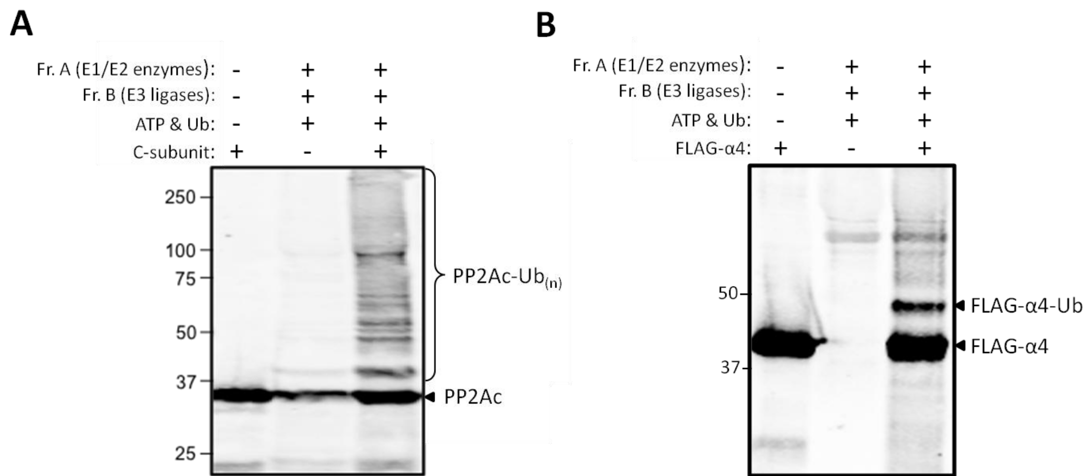


Figure 8. PP2Ac is targeted for polyubiquitination and α4 is targeted for monoubiquitination *in vitro*

A.) *In vitro* ubiquitination assays were carried out in the absence (-) or presence (+) of purified catalytic subunit and/or ATP, ubiquitin (Ub), Fraction A (HeLa cell lysate containing E1/E2 enzymes) and Fraction B (HeLa cell lysate containing E3 ligases). Samples were analyzed by SDS-PAGE and immunoblotting using a PP2Ac-specific antibody. B) *In vitro* ubiquitination assays were carried out in the absence (-) or presence (+) of FLAG purified α4 and/or ATP, Ub, Fraction A, and Fraction B. Samples were analyzed by SDS-PAGE and immunoblotting using an α4 specific antibody.

presence or absence of the ubiquitin-protein conjugation mixture containing Fraction A, Fraction B, Mg^{2+}/ATP and ubiquitin. Western analysis of these reactions revealed the presence of a predominant band shifted roughly 8 kDa above unmodified α4 but no high molecular weight α4 species (Fig 8B), thus supporting the idea that α4 is targeted for monoubiquitination *in vitro*. Taken together these data demonstrate that the *in vitro* ubiquitination assay is a viable approach to monitor PP2Ac and α4 ubiquitination; furthermore, this assay readily detects both poly- and monoubiquitinated proteins.

α4 is monoubiquitinated in cells.

Given that α4 is monoubiquitinated in our *in vitro* ubiquitination assay, we next sought to determine if α4 is targeted for monoubiquitination in cells. FLAG-α4 and HA-ubiquitin were co-transfected into HEK293FT cells, and immunoprecipitations were performed using anti-HA-agarose beads. Western analysis of the ubiquitinated proteins revealed the presence of an α4 immunoreactive band that corresponds with the molecular weight of monoubiquitinated α4 (~50 kDa) from lysates of cells co-expressing FLAG-α4 and HA-ubiquitin (Fig 9). α4 did not appear to be polyubiquitinated under these experimental conditions, as no high molecular weight proteins were detected on the immunoblot. Moreover, treatment of cells with a proteasome inhibitor (MG132) did not alter the levels of ubiquitinated FLAG-α4. These data indicate that alpha4 is monoubiquitinated in mammalian cells.

Detection of PP2Ac ubiquitination in cells using tagged ubiquitin.

HEK293FT cells expressing HA-ubiquitin or an empty vector control plasmid were untreated or treated with a proteasome inhibitor (MG132) to enhance the detection of polyubiquitinated proteins. Western analysis of the ubiquitinated proteins isolated from the cell

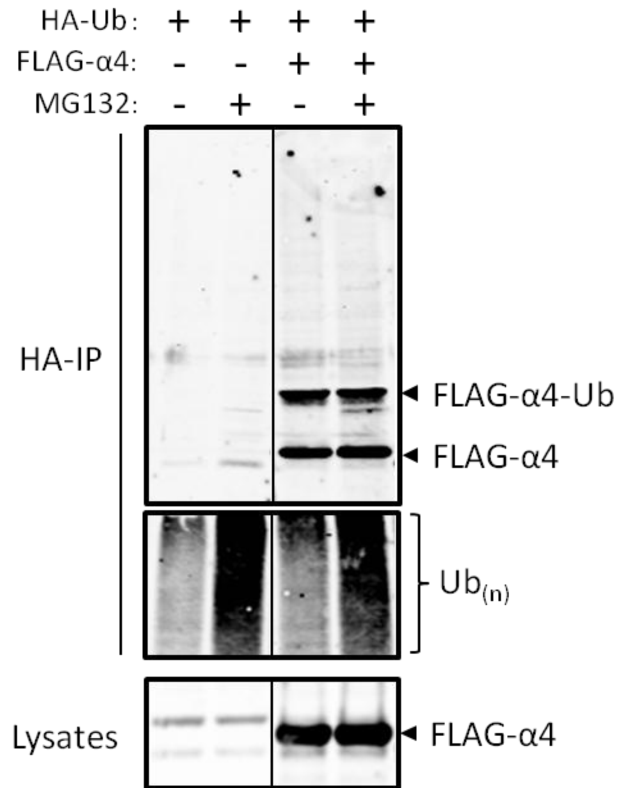


Figure 9. α4 is targeted for monoubiquitination in cells
HEK 293FT cells co-expressing FLAG-α4 and/or HA-Ub expression plasmids were treated plus (+) or minus (-) proteasome inhibitor MG132 for 4 hours prior to lysis. HA-tagged proteins were immunoprecipitated from cell lysates using anti-HA agarose beads. Ubiqutinated proteins were anyalzyed via SDS-PAGE and Western analysis using an α4 and ubiquitin specific antibody.

lysates, via the anti-HA resin, revealed the presence of both monoubiquitinated PP2Ac and di-ubiquitinated PP2Ac. However, unlike what we previously found *in vitro,* no polyubiquitinated PP2Ac was identified, even in the presence of proteasome inhibitor (Fig 10A). Since our previous *in vitro* ubiquitination data identified PP2Ac as a target for polyubiquitination (Fig. 8) and a previous study showed PP2Ac is degraded by the proteasome (Trockenbacher et al., 2001), we hypothesize that the HA tag in ubiquitin interferes with our ability to detect polyubiquitinated PP2Ac species in these cellular assays.

To address this question we utilized a different tagged form of ubiquitin, 6xHis-ubiquitin, and monitored PP2Ac polyubiquitination in the absence or presence of proteasome inhibitor. Ubiquitinated proteins were isolated from lysates of HEK293FT cells treated expressing both 6xHis-ubiquitin and HA_3-PP2Ac using immobilized metal affinity chromatography (IMAC). Western analysis of polyubiquitinated proteins revealed PP2Ac polyubiquitination, which is increased in the presence of MG132; no polyubiquitinated PP2Ac species were observed in the control reactions (i.e., cells expressing 6xHis-Ub or HA_3-PP2Ac alone) (Fig. 10B). Taken together, these data show that we are able to detect PP2Ac ubiquitination in cells.

Detection of PP2Ac polyubiquitination using a Tandem Ubiquitin Binding Element (TUBE) pulldown.

The cellular ubiquitination assays presented above rely on overexpression of either ubiquitin or PP2Ac. To test whether we could detect PP2Ac ubiquitination without having to use protein overexpression we utilized TUBE2 agarose. The TUBE2 resin binds polyubiquitin chains with high affinity, thereby circumventing the use of epitope tagged ubiquitin or ubiquitin

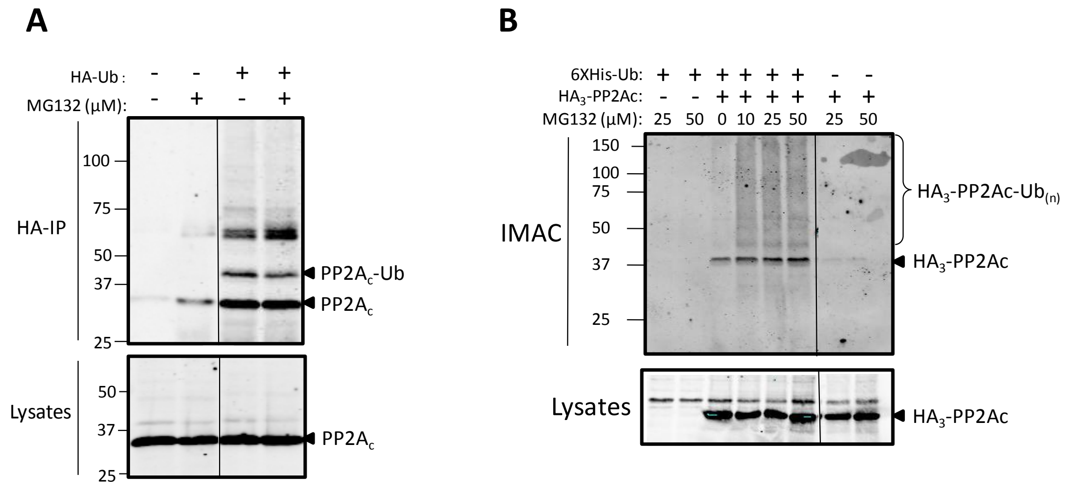


Figure 10. PP2Ac is targeted for polyubiquitination in cells.

A.) Ubiquitinated proteins were isolated using HA affinity agarose from lysates of HEK 293FT cells expressing HA-Ub treated plus (+) or minus (-) proteasome inhibitor MG132. PP2Ac ubiquitination was determined via SDS-PAGE and Western analysis using a PP2Ac specific antibody. B.) Ubiquitinated proteins were isolated by IMAC from lystates of HEK 293FT cells expressing 6xHis-Ub and HA_3-PP2Ac treated with increasing concentrations of MG132. PP2Ac ubiquitination was analyzed via SDS-PAGE and Western using a PP2Ac specific antibody.

antibodies, which are notoriously inefficient for isolating polyubiquitinated proteins from cell extracts. Moreover, TUBE2 agarose allows for the detection of endogenous protein ubiquitination. The TUBE2 resin was utilized to isolate ubiquitinated proteins from lysates of non-transfected cells or cells expressing HA_3-PP2Ac treated +/- MG132. Western analysis of TUBE2 pulldowns revealed the presence of endogenous ubiquitinated PP2Ac (Fig. 11A lanes 1 and 2), which was more easily visualized on a longer exposure (Fig. 11B). To our knowledge, this is the first direct evidence indicating that endogenous PP2Ac is targeted for polyubiquitination by endogenous ubiquitin. Furthermore, we detected the polyubiquitination of HA_3-PP2Ac using the TUBE2 resin (Fig 11A).

Detection of PP2Ac ubiquitination by Ubiquitin Fluorescence Complementation (UbFC) imaging.

To begin to explore the subcellular distribution of ubiquitinated PP2Ac we utilized a ubiquitin fluorescence complementation assay (UbFC) (Fig 12A). This approach is based upon the complementation between two halves of a single fluorescent protein that associate when proximal to one another. This system allows for the visualization of only ubiquitinated proteins since neither half of the fluorescent protein alone is able to be detected. HEK293FT cells were co-transfected with a fusion construct encoding amino acids 155-283 of the cyan fluorescent protein, attached to the N-terminus of PP2Ac (CC155-PP2Ac), and a fusion construct encoding amino acids 1-155 of the Venus fluorescent protein, attached to the N-terminus of ubiquitin (VN155-Ub). Live cell confocal imaging of cells expressing either CC155-PP2Ac WT or CC155-PP2Ac NoK (all lysines mutated to arginine) alone produced no fluorescence (Fig. 12 B1-2); however, when CC155-PP2Ac WT was co-expressed with VN155-ubiquitin a strong fluorescent signal was detected with most of the signal residing in the cytoplasm (Fig. 12 B3). The

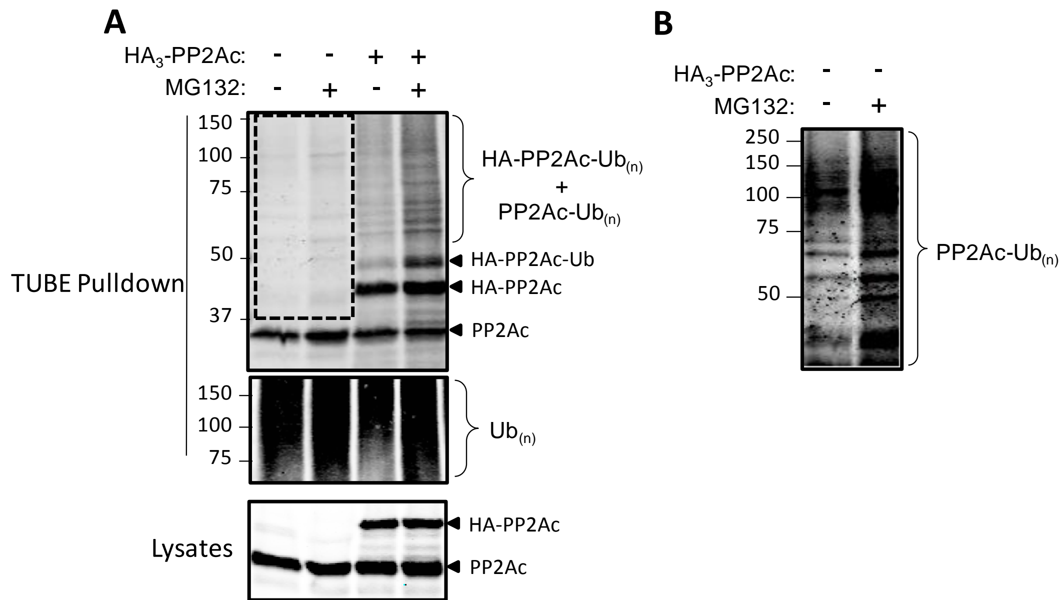


Figure 11. Isolation of both endogenous and ectopic polyubiquitinated PP2Ac via TUBE pulldown

A.) HEK 293FT cells transfected plus or minus HA_3-PP2Ac were treated with (+) or without (-) MG132. Ubiquitinated proteins were isolated from cell lysates using TUBE-2 agarose beads and analyzed via SDS-PAGE and Western using PP2Ac and ubiquitin specific antibodies. B.) Longer exposure of the highlighted region from (A) to better show polyubiquitination of endogenous PP2Ac.

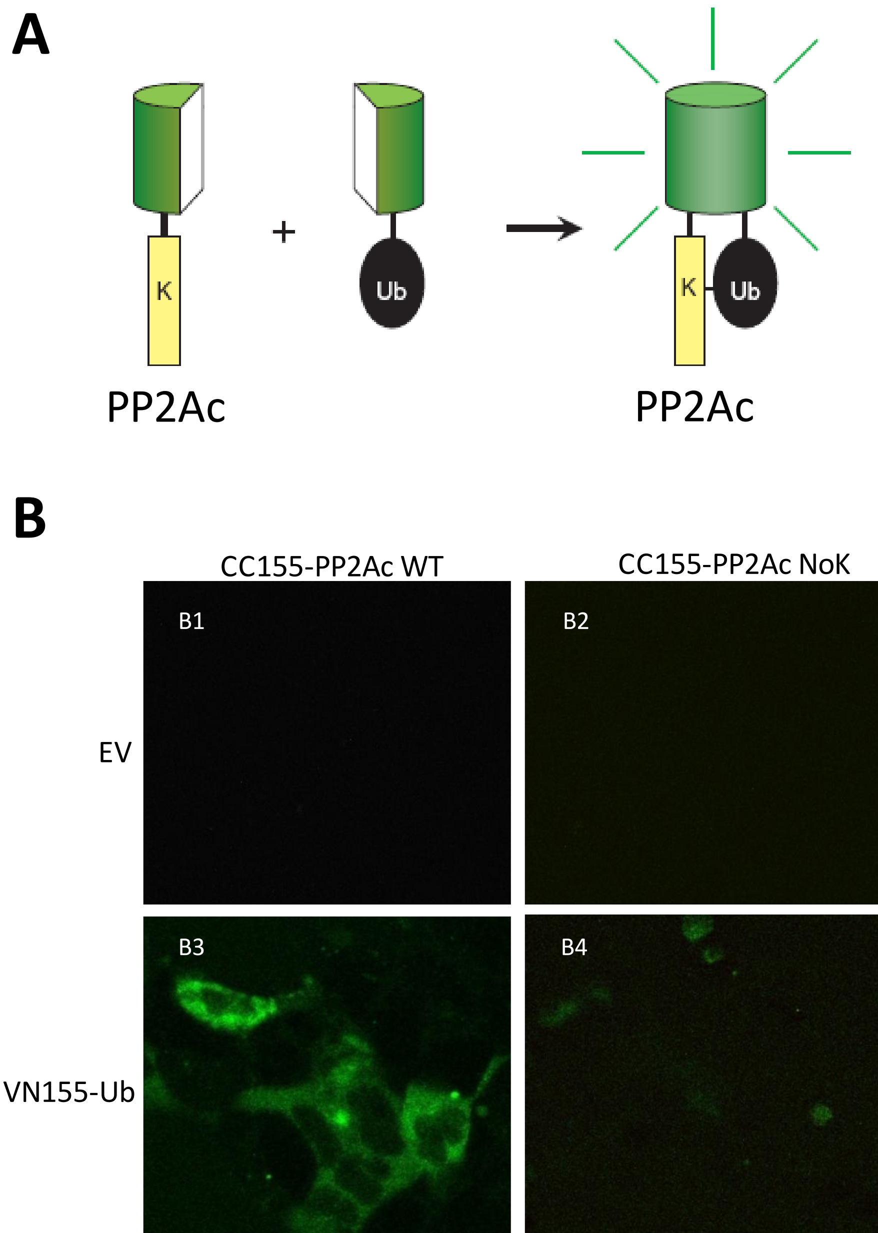


Figure 12. Detection of PP2Ac ubiquitination via ubiquitin fluorescence Complementation (UbFC)

A) Schematic representation of UbFC assay (Modified from Kerppola TK 2008). B.) HEK 293FT cells were transfected with either Empty vector or VN155-Ub and CC155-PP2Ac WT or a mutant in which all lysine residues were mutated to arginine (NoK). Fluorescence was detected in live cells using confocal microscopy with fluorescent signal indicating ubiquitinated PP2Ac.

fluorescent signal represents ubiquitinated PP2Ac species since very little fluorescence was detected in cells expressing VN155-Ub and the CC155-PP2Ac NoK control (Fig. 12 B4). These imaging assays confirm PP2Ac is undergoing ubiquitination in cells and further suggest that ubiquitinated PP2Ac is primarily localized in the cytoplasm. To determine if the fusion proteins function similar to their WT counterparts, ubiquitinated proteins were isolated from HEK293FT cells co-expressing CC155-PP2Ac and 6xHis-Ub treated +/- MG132. Western analysis of ubiquitinated proteins isolated by IMAC revealed polyubiquitinated CC155-PP2Ac species (Fig. 13A). Conversely, purification of ubiquitinated proteins via anti-HA agarose from HEK293FT cells expressing VN155-Ub also revealed ubiquitinated endogenous PP2Ac species (Fig. 13B), indicating the fusion constructs function similarly to their wild-type counterparts in cells. In addition, analysis of the CC155-PP2Ac protein revealed similar binding to the regulatory subunit α4 (Fig. 14). Together these data indicate that based on the assays we tested; the fusion of the fluorescent fragment is not significantly altering the function of either PP2Ac or ubiquitin.

Discussion

Previous studies have suggested PP2Ac is targeted for polyubiquitination in cells; however, the ubiquitination of PP2Ac has not been shown directly. In this chapter we demonstrate that PP2Ac is indeed targeted for ubiquitination both *in vitro* and in cells. Furthermore, the data presented demonstrate for the first time that the PP2Ac regulatory protein α4 is targeted for monoubiquitination.

In vitro ubiquitination reactions employing a mixture of E1/E2 enzymes (Fraction A) and E3 ligases (Fraction B) allowed direct visualization of PP2Ac polyubiquitination. The development of this assay also allowed us to test the ubiquitination state of other potential ubiquitinated proteins of interest, leading to the identification of α4 as a target for

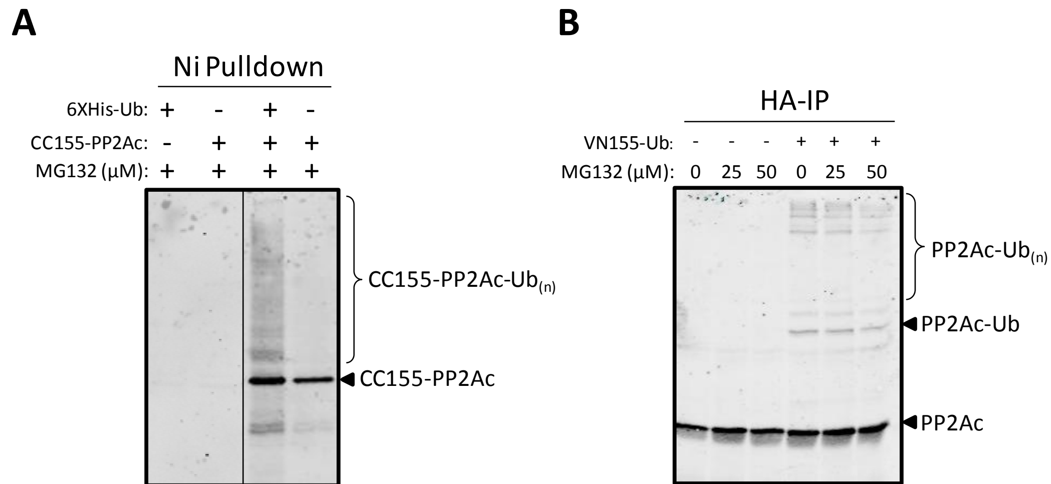


Figure 13. Detection of PP2Ac polyubiquitination via Western using the UbFC constructs. A.) HEK293FT cells co-transfected with CC155-PP2Ac and/or 6xHis-Ub were treated with MG132. Ubiquitinated proteins were isolated from cell lysates using IMAC and analyzed via SDS-PAGE and Western using a PP2Ac specific antibody. B.) Ubiquitinated proteins were isolated using HA affinity agarose from lysates of HEK293FT cells transfected with (+) or without (-) VN155-Ub treated with increasing concentrations (μM) proteasome inhibitor MG132. PP2Ac ubiquitination was determined via SDS-PAGE and Western analysis using a PP2Ac specific antibody.

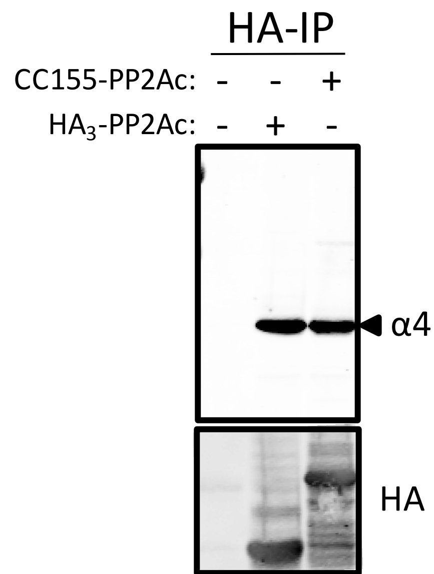


Figure 14. CC155-PP2Ac retains binding to its regulatory subunit α4
HEK293FT cells were transfected with (+) or without (-) CC155-PP2Ac. HA immune complexes (*HA IPs*) were isolated from cell lysates and analyzed by SDS-PAGE and immunoblotting using α4 and HA specific antibodies

monoubiquitination. Since this *in vitro* ubiquitination assay uses pools of enzymes we were unable to identify the exact enzyme cascade that facilitates PP2Ac polyubiquitination and α4 monoubiquitination. However, subsequent chapters will discuss other *in vitro* ubiquitination methodologies that I developed to identify the relevant ubiquitination machinery for α4.

Once we determined that both PP2Ac and α4 are subject to ubiquitination *in vitro*, we sought to develop assays to monitor their ubiquitination in cells. Our studies demonstrate that purifying ubiquitinated proteins from MG132-treated cells, expressing ectopic ubiquitin alone or co-expressing ubiquitin and PP2Ac, followed by Western analysis, allowed us to detect the polyubiquitination of PP2Ac. Furthermore, the co-expression of HA-ubiquitin and FLAG-α4, and subsequent Western analysis of the HA immune complexes, allowed for the detection of monoubiquitinated α4 species in cells. Together, these cellular assays allow for the identification of both PP2Ac and α4 ubiquitination in cells; however, these assays are limited by the fact that the proteins need to be overexpressed.

To overcome the complications associated with protein overexpression, we adapted a new method to detect the polyubiquitination of PP2Ac in cells. The TUBE2 resin binds polyubiquitinated proteins with high affinity and allows for the purification of ubiquitinated proteins from cell lysates. This methodology gave us the ability to visualize for the first time the polyubiquitination of endogenous PP2Ac in cells. This assay was also used to detect polyubiquitinated HA_3-PP2Ac species allowing for the expression of PP2Ac alone to assay for polyubiquitination instead of co-overexpression of ubiquitin and PP2Ac. While TUBE2 agarose has been valuable for the study of PP2Ac polyubiquitination, it preferentially binds polyubiquitinated proteins, making them unsuitable for monitoring α4 monoubiquitination.

Since the detection of polyubiquitinated PP2Ac by Western requires cells to be lysed, we were unable to determine the subcellular distribution of ubiquitinated PP2Ac. To begin to

explore its subcellular distribution we developed a ubiquitin fluorescence complementation assay for PP2Ac to allow us to image ubiquitinated PP2Ac in live cells. These studies revealed PP2Ac was polyubiquitinated in the cytoplasm of cells; however, we are currently limited by both the level of protein overexpression and resolution to allow us to identify the specific subcellular localization of ubiquitinated PP2Ac.

The data presented in this chapter demonstrate that PP2Ac and α4 are targeted for polyubiquitination and monoubiquitination, respectively. Furthermore, the methodologies discussed in this chapter paved the way for additional studies aimed at elucidating the regulation and function of these post-translation modifications in target cells.

CHAPTER III

IDENTIFICATION OF THE UBIQUITIN ACCEPTOR SITE IN PP2Ac

Introduction

Polyubiquitination has been implicated in a wide variety of protein responses including activation, degradation by the proteasome, and changes in subcellular localization (Weissman, 2001). The type of response is encoded by the ubiquitin chain topology used to generate the polyubiquitin chains. Ubiquitin is a 76 amino acid polypeptide containing seven lysine residues, each of which can serve as a ubiquitin acceptor site(s) for polyubiquitin chain formation. Lysine 48 (K48) and lysine 29 (K29) linked polyubiquitin chains target the protein for proteasomal degradation; whereas, polyubiquitin chains linked via lysine 63 (K63) can alter both protein activity and subcellular localization. The four remaining lysine residues on ubiquitin (residues 6, 11, 27, and 33) are also able to form polyubiquitin linkages; however, relatively little is known about the functional roles of these polyubiquitin chains (Nandi et al., 2006).

My studies presented in Chapter II, together with previous findings (Trockenbacher et al., 2001), indicate that PP2Ac is targeted for polyubiquitin-mediated proteasomal degradation; however, the ubiquitin acceptor site on PP2Ac and the polyubiquitin chain topography have not yet been reported. In this chapter, we demonstrate that polyubiquitination of PP2Ac occurs via K48 linkage on ubiquitin, consistent with its role in proteasomal degradation. Furthermore, mutagenesis studies coupled with cycloheximide chase experiments identified lysine 41 (K41) in PP2Ac as the major site of ubiquitin modification.

Materials and Methods

Plasmids

The HA_3-PP2Ac construct was a gift from Dr. David Brautigan (University of Virginia, Charlottesville, VA). The HA_3-PP2Ac K41R, K21R, K29R, and K34R single point mutant constructs were created by mutating the respective lysine residue to arginine using site-directed mutagenesis (Stratagene). Proper construction of all plasmids was verified by automated sequencing (Vanderbilt University DNA core facility).

Antibodies and reagents

The mouse monoclonal PP2Ac antibody was from BD Biosciences (San Diego, CA). The mouse monoclonal ubiquitin antibody and mouse monoclonal HSP90 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Purified K48R, K63R, and NoK (all lysines mutated to arginine) ubiquitin were from Boston Biochem (Boston, MA). The purified PP2A catalytic subunit from bovine heart was a kind gift from Dr. Greg Moorhead (University of Calgary, Calgary, AB).

In vitro ubiquitination reactions

See "Materials and Methods" for details of this methodology in Chapter II

Cell culture and transfection

HEK293FT cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2

mM L-glutamine. HEK293FT cells were transfected with mammalian expression constructs using Fugene6 (Roche; Indianapolis, IN) according to the manufacturer's protocol.

TUBE pulldowns

HEK293FT cells were lysed in 200 µl immunoprecipitation buffer [150 mM NaCl, 20 mM Tris-HCl pH 7.4, 1% Igepal, 5 µg/mL aprotinin, 1 µg/mL pepstatin, 1 mM PMSF, and 1 µg/mL leupeptin] and centrifuged at 12,000 × *g* for 10 min. Clarified lysates (170 µl) were incubated with 20 µl of a 50% slurry of TUBE2-agarose (LifeSensors) overnight at 4 °C. TUBE2 complexes were washed three times with 1 ml of immunoprecipitation buffer, and bound proteins were eluted in SDS sample buffer and subjected to Western analysis.

Cycloheximide chase experiments

HEK293FT cells were seeded in six-well tissue culture plates (350,000 cells per well) and transfected with either wild type (WT) HA_3-PP2Ac or various Lys to Arg point mutants of HA_3-PP2Ac (K41R, K21R, K29R, K34R). At 48 h post-transfection, cells were treated with 100 µg/mL cyclohexamide (Sigma, St. Louis, MO) for the indicated times. Cell lysates were then prepared and subjected to Western analysis.

Western analysis

See "Materials and Methods" for details of this methodology in Chapter II

Results

The polyubiquitin chain on PP2Ac is linked via K48 on ubiquitin.

To better understand the role of PP2Ac ubiquitination, we performed experiments to determine the ubiquitin chain topology of polyubiquitinated PP2Ac. *In vitro* ubiquitination assays were performed using wild type (WT) ubiquitin or a mutant form of ubiquitin (e.g., lysine 48 mutated to arginine (K48R), lysine 63 mutated to arginine (K63R), or all lysine residues mutated to arginine (NoK)). Western analysis of these reactions showed appreciable PP2Ac polyubiquitination in the presence of WT or K63R ubiquitin, but very little polyubiquitination in the presence of K48R or NoK ubiquitin (Fig 15). These findings demonstrate that the PP2Ac polyubiquitin chain is formed via K48 in ubiquitin. Furthermore, the detection of only monoubiquitinated PP2Ac species in reactions containing a ubiquitin that cannot undergo polyubiquitination (NoK) indicates that PP2Ac ubiquitination likely occurs at a single residue. If multiple residues were targeted for ubiquitination we would have expected to se di- or tri-ubiquitinated species, but we did not detect these higher molecular weight forms of PP2Ac.

Identification of the PP2Ac ubiquitination site.

Since our *in vitro* ubiquitination studies of PP2Ac support a single site of modification, we next sought to identify the ubiquitin acceptor residue within PP2Ac. Ubiquitinated proteins were isolated via TUBE2 pulldowns from lysates of HEK293FT cells expressing HA_3-PP2Ac WT or a panel of HA_3-PP2Ac point mutants in which lysine 29, 34, or 41 of PP2Ac were individually mutated to arginine. Western analysis of the TUBE2 pulldowns reveals the polyubiquitination of PP2Ac was unchanged by any of the single point mutations (Fig 16). A second series of PP2Ac lysine mutants were created in which multiple lysine residues of the same construct were

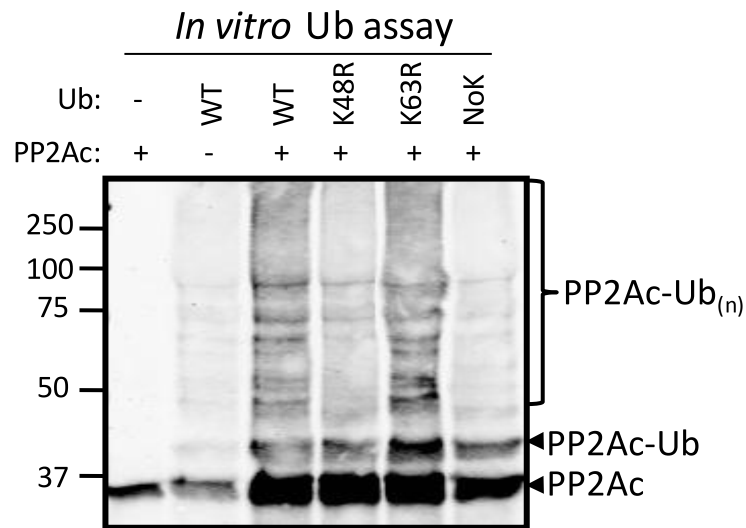


Figure 15. The PP2Ac polyubiquitin chain is linked via K48 in ubiquitin
In vitro ubiquitination assays were carried out in the absence (-) or presence (+) of purified PP2Ac and wild-type ubiquitin (*WT*) or a ubiquitin mutant containing arginine substitutions for either lysine-48 (*K48R*), lysine-63 (*K63R*), or all lysines (*No K*). Samples were analyzed by SDS-PAGE and immunoblotting using a PP2Ac-specific antibody.

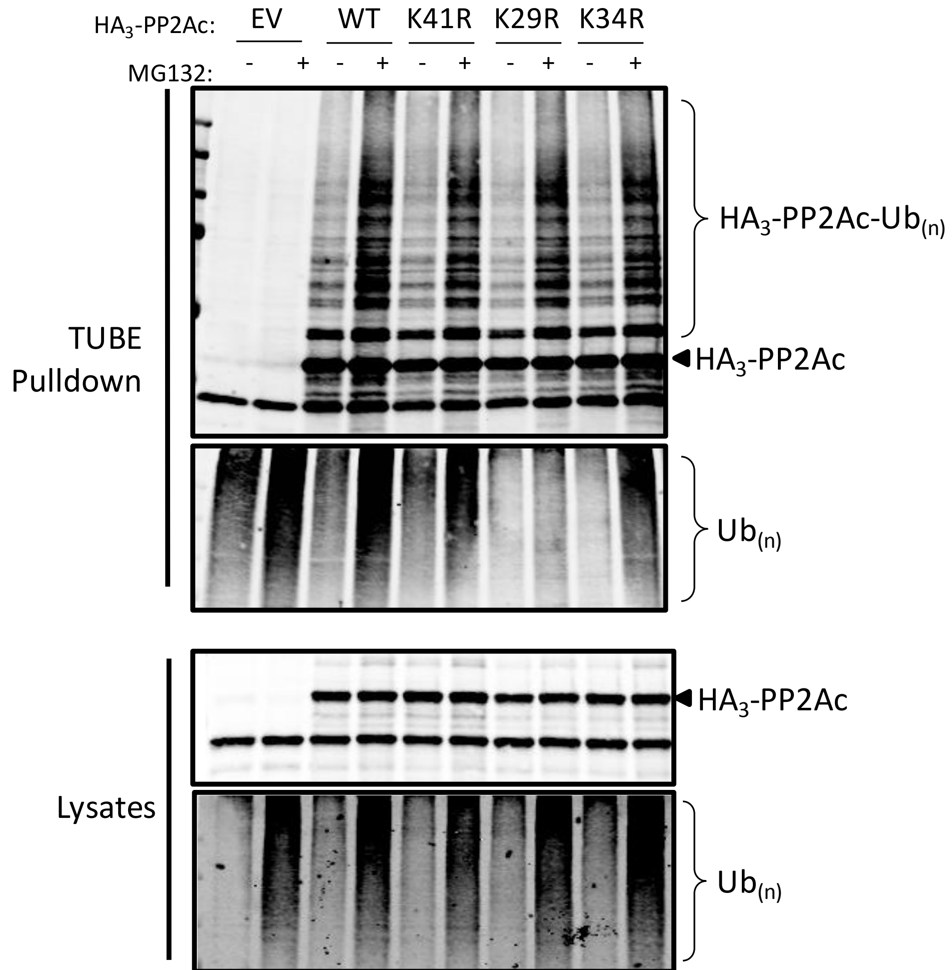


Figure 16. PP2Ac lysine point mutants show no changes in polyubiquitination
Ubuquitinated proteins were isolated using TUBE2 agarose from HEK 293FT cell lysates expressing either HA_3-PP2Ac wild type (WT) or a point mutant of PP2Ac in which lysine 41 (K41R), lysine 29 (K29R), or lysine 34 (K34R) was mutated to an arginine residue. Ubiquitinated proteins were analyzed via SDS-PAGE and Western analysis using PP2Ac and ubiquitin specific antibodies.

mutated to arginine. Analysis of these mutants revealed near wild type ubiquitination of all the multi-site mutations, with the exception of a set of PP2Ac mutants with eight or more lysine to arginine mutations, which exhibited a significant decrease in polyubiquitination (data not shown); however, none of the ubiquitin deficient mutants retained enzymatic activity (Table 3). To test if the Lys to Arg mutants have similar biochemical properties as WT HA_3-PP2Ac we assayed their ability to bind both the PP2A A subunit and α4 as well as analyzed their phosphatase activity using phosphorylated-histone radiolabeled by PKC. The results from these analyses are summarized in Table 3, and reveal near WT properties for all mutants tested, except for a set of mulit-site mutants which no longer possess phosphatase activity. Although we were unable to identify the PP2Ac ubiquitination site by this approach, the data are consistent with the idea that ubiquitin is able to switch from the major site of modification to a secondary site(s) when the primary site is mutated.

Identification of K41 as the "functional" ubiquitin acceptor site of PP2Ac.

Given that we were unable to detect the ubiquitin acceptor site via TUBE2 pulldown and polyubiquitinated PP2Ac is degraded by the proteasome, we sought to identify the functional PP2Ac ubiquitination site by assaying changes in turnover rates of our PP2Ac mutants. HEK293FT cells expressing WT HA_3-PP2Ac or a panel of lysine to arginine HA_3-PP2Ac single point mutants were treated with cycloheximide, to inhibit protein synthesis, and lysed at the indicated times after treatment. Western analysis of the cell lysates revealed a time-dependent decline in the levels of WT HA_3-PP2Ac along with the K21R, K29R, and K34R point mutants of HA_3-PP2Ac; however, the levels of the HA_3-PP2Ac K41R mutant remained stable over the eight hour time course (Fig. 17). These data indicated that K41 is the functional site of polyubiquitination.

Table 3. Biochemical characteristics of the HA_3-PP2Ac lysine to arginine point mutants
Cumulative data of the HA_3-PP2Ac lysine to arginine point mutant polyubiquitination state, binding to α4 and the A-subunit, and phosphatase activity compared to WT HA_3-PP2Ac. Differences are depicted by + (increase), - (decrease), = (no change), and none (could not detect). The degree of differences is shown by the number of + or – signs.

HA_3-PP2Ac point mutant	Mutant ubiquitination relative to WT	Mutant binding to α4 relative to WT	Mutant binding to the A subunit relative to WT	Mutant phosphatase activity relative to WT
T40A	=	+	Not tested	++
T40E	=	-	Not tested	=
K41R	=	=	Not tested	=
K34R	=	=	Not tested	Not tested
K144R	=	=	Not tested	=
K21R	=	Not tested	Not tested	Not tested
K29,41R	=	=	Not tested	Not tested
K34,36R	=	Not tested	Not tested	=
K8,29,41R	=	Not tested	Not tested	Not tested
K8,34,36R	=	Not tested	Not tested	Not tested
K8,34,36,283R	=	Not tested	Not tested	=
K8,41,34,36R	-	Not tested	Not tested	Not tested
K8,34,36,41,283R	-	Not tested	Not tested	=
K8,29,41,34,36,283R	=	Not tested	Not tested	=
K8,21,34,36,41,283R	=	=	=	Not tested
K8,29,34,36,41,136,283R	-	=	=	Not tested
K8,21,29,34,36,41,283R	Not tested	=	=	=
K8,29,34,36,41,144,283R	=	=	=	Not tested
K8,21,29,34,36,41,104,144,283R	-	----	None	None
K8,21,29,34,36,41,74,104,144,283R	--	Not tested	Not tested	Not tested
K8,34,36,41,104,136,283R	Not tested	----	None	None
K8,21,29,34,36,41,104,136,283R	----	Not tested	Not tested	Not tested
K8,29,34,36,41,136,104,283R	----	----	None	None
K8,21,29,34,36,41,136,144,283R	Not tested	=	None	Not tested
K4,8,21,29,34,36,41,136,144,283R	Not tested	=	None	Not tested
K4,8,21,29,34,36,41,74,104,144,283R	----	----	None	Not tested
K8,21,29,34,36,41,74,136,144,283R	Not tested	--	----	----
K4,8,21,29,34,36,41,74,136,144,283R	Not tested	--	----	----
K8,21,29,34,36,41,74,104,136,144,283R	Not tested	----	None	None
K4,8,21,29,34,36,41,104,136,144,283R	Not tested	None	None	None
NoK	----	None	None	None

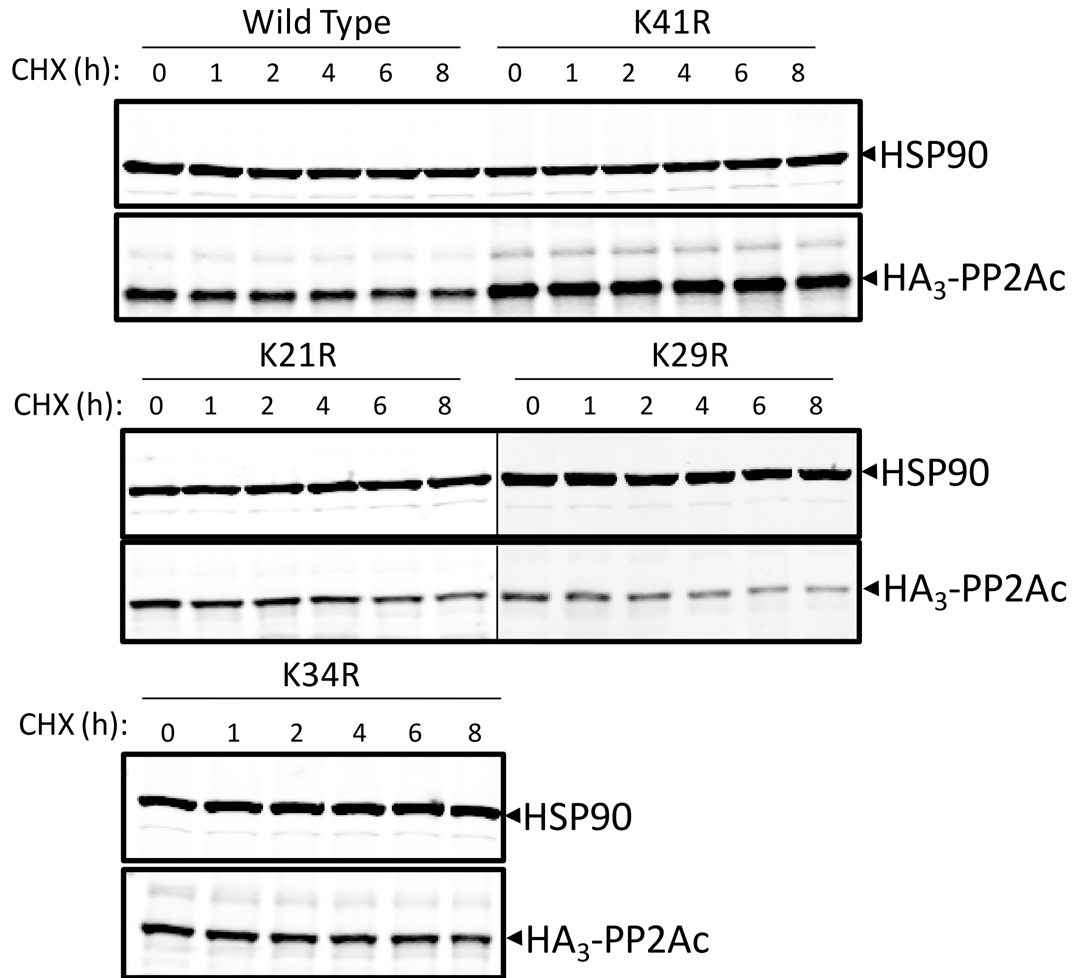


Figure 17. Lysine 41 in PP2Ac regulates its polyubiquitin mediated turnover

HEK 293 FT cells were transfected with HA-PP2Ac wild type (WT) or a point mutant of PP2Ac in which lysine 41 (K41R), lysine 29 (K29R), or lysine 34 (K34R) was mutated to an arginine residue. Forty-eight hours post-transfection cells were treated with 100 µg/mL cycloheximide for the indicated time (h). Cell lysates were subjected to SDS-PAGE and immunoblotting using PP2Ac- and HSP90- specific antibodies.

Discussion

In this chapter, we show that the chain topology for PP2Ac polyubiquitination occurs via lysine 48 on ubiquitin. These data are consistent with prior reports indicating that PP2Ac is subject to proteasomal degradation (Trockenbacher et al., 2001), since K48 ubiquitin chain topology signals for ubiquitin-mediated degradation.

PP2Ac ubiquitination appears to occur at a single site, as only monoubiquitinated PP2Ac is detected with the NoK ubiquitin mutant; however, we cannot rule out the possibility of a mixed population of ubiquitinated PP2Ac. Based on this result, we sought to identify the ubiquitin acceptor site within PP2Ac; however, the identification of ubiquitination sites is a notoriously difficult task as ubiquitin is known to adduct to secondary lysine residues if the primary site is mutated. Our lysine mutagenesis studies did not reveal a ubiquitin deficient mutant that still retained catalytic activity, most likely due to the redundancy of ubiquitination. As a secondary approach we used a functional assay to identify the site of modification. Using cycloheximide chase experiments we showed that the K41R mutation significantly increased the half-life of PP2Ac. Based on these studies we believe K41 is the major site of PP2Ac polyubiquitin mediated turnover of PP2Ac; however, we cannot rule out the possibility the mutation could be stabilizing PP2Ac by an unknown mechanism. Consistent with our results identifying K41 as the ubiquitin acceptor site, a recent report by Meierhofer and colleagues identified ubiquitinated peptides by mass spectrometry which corresponded to 669 ubiquitinated proteins including PP2Ac (Meierhofer et al., 2008). In unpublished correspondence with the author of this study, two isolated peptides that corresponded to PP2Ac showed the ubiquitin adducted to K41. The identification of K41 as the functional ubiquitination site gives us a valuable tool to study PP2Ac in cells. Utilizing the K41R mutant

construct will potentially allow us to manipulate the level of PP2Ac present in cells and help identify substrates that are regulated by PP2Ac ubiquitination.

Taken together, data presented in this chapter identify the K48 ubiquitin chain topology for PP2Ac, which further confirms the role of PP2Ac polyubiquitination in the degradation of PP2Ac. In addition, we demonstrate K41 is the functional ubiquitin acceptor site for PP2Ac.

CHAPTER IV

MID1-DEPENDENT MONOUBIQUITINATION PROMOTES CALPAIN CLEAVAGE OF α4 ALTERING PP2A STABILITY

Introduction

Several regulatory mechanisms have been described for PP2A including post-translational modifications of the PP2A catalytic subunit (PP2Ac) and the association of PP2Ac with regulatory subunits (Janssens and Goris, 2001; Virshup and Shenolikar, 2009). Although the most studied forms of PP2A are the heterotrimeric holoenzymes, which consist of PP2Ac, a structural A subunit, and a variable B subunit that dictates substrate selectivity and subcellular localization of the phosphatase holoenzyme, PP2Ac also interacts with a number of atypical regulatory subunits independent of the canonical A and B subunits. Within this group is α4, a direct interacting partner of PP2Ac that also binds the microtubule-associated E3 ubiquitin ligase MID1 and plays a crucial role in modulating PP2Ac polyubiquitination and stability (Kong et al., 2009; LeNoue-Newton et al., 2011; Liu et al., 2001; McConnell et al., 2010). Loss of function mutations in the *MID1* gene are the underlying cause of OS (Quaderi et al., 1997), a congenital disorder characterized by defects in midline development and significant increases in microtubule-associated PP2A activity (Trockenbacher et al., 2001). α4 was initially shown to tether PP2Ac to MID1 and promote polyubiquitination and degradation of microtubule-associated PP2Ac (Trockenbacher et al., 2001); however, subsequent studies revealed that α4 can also protect PP2Ac from polyubiquitination and proteasomal degradation (Kong et al., 2009; LeNoue-Newton et al., 2011; McConnell et al., 2010). These findings indicate that α4 may exhibit both protective and destructive actions in the control of PP2Ac cellular levels.

In this chapter I demonstrate that MID1 functions as the E3 ubiquitin ligase for the monoubiquitination of α4, which triggers calpain-mediated cleavage of the C-terminal MID1-binding domain of α4. I also demonstrate that PP2Ac stability is influenced by the monoubiquitination state and cleavage of α4. Together these findings reveal that monoubiquitination of α4 promotes calpain-mediated inter-domain cleavage of α4 that switches its activity towards PP2Ac from protective to destructive. Moreover, these data reconcile the apparent contradictory protective and destructive roles of α4 in the control of PP2Ac and support a new model for the regulation of PP2Ac by MID1 and α4.

Materials and Methods

Plasmids

The HA-ubiquitin plasmid was a gift from Dr. Hal Moses (Vanderbilt University, Nashville, TN) and the HA_3-PP2Ac construct was a gift from Dr. David Brautigan (University of Virginia, Charlottesville, VA). The mammalian expression constructs encoding FLAG-MID1 and Myc-MID1 were described previously (Aranda-Orgilles et al., 2008b; Cainarca et al., 1999). Construction of the FLAG-tagged wild type, ED, and ΔUIM α4 mammalian expression constructs were also described previously (McConnell et al., 2007; McConnell et al., 2010). The N-terminal fragment of FLAG-α4 containing a stop codon at G256 (G256*) and the double point mutant of FLAG-α4 containing Leu residues in place of Ala52 and Ala53 (A52L/A53L) were generated using FLAG-α4/pcDNA5TO as a template and the Quick Change site-directed mutagenesis kit (Stratagene, LaJolla, CA) with the following primers: G256* forward 5'-AAC ATG GCT CAA GCC AAA GTA TTT TGA GCT GGT TAT CC-3'; G256* reverse 5'-GGA TAA CCA GCT CAA AAT ACT TTG GCT TGA GCC ATG TT-3'; A52L/A53L forward 5'-GCT TGG ACC TCC TTG AGA AGC TGC TGG AAA

TGT TAT CGC AGC TCG ACT TGT TCA GCCG-3'; A52L/A53L reverse 5'-CGG CTG AAC AAG TCG AGC TGC GAT AAC ATT TCC AGC AGC TTC TCA AGG AGG TCC AAG C-3'. Proper construction of all plasmids was verified by automated sequencing (Vanderbilt University DNA core facility).

Antibodies and reagents

The mouse monoclonal PP2Ac antibody was from BD Biosciences (San Diego, CA). The rabbit polyclonal α4 antibody and the rabbit polyclonal MID1 antibody were from Bethyl Laboratories (Montgomery, TX). The mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse monoclonal Myc antibody and the mouse monoclonal FLAG antibody were from Cell Signaling (Billerica, MA) and Sigma-Aldrich (St. Louis, MO), respectively. The rabbit polyclonal phospho-tau and total tau antibodies were from Invitrogen (Carlsbad, CA). The E1 ubiquitin ligase inhibitor, PYR-41, was from EMD Chemicals USA (Gibbstown, NJ). The calpain inhibitors Z-Leu-Leu-CHO and calpeptin were from Enzo Life Sciences (Plymouth Meeting, PA) and EMD chemicals (Gibbstown NJ), respectively. The siGENOME SMART pool human MID1-targeted siRNA was from Thermo Scientific (Lafayette, CO).

Cell culture and transfection

HEK293FT cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. HEK293FT cells were transfected with mammalian expression constructs using Fugene6 (Roche; Indianapolis, IN) according to the manufacturer's protocol. MID1-targeted siRNA was introduced into HEK293FT cells using Dharmafect (Thermo Scientific; Rockford, IL) according to the manufacturer's protocol.

MID1/E2 activity assay

The ability of MID1·E2 pairs to form polyubiquitin chains was determined using the E3LITE Customizable Ubiquitin Ligase Kit from LifeSensors (Malvern, PA). Briefly, increasing concentrations of immunopurified FLAG-MID1 were incubated with 20 nM E1, 400 nM of the indicated E2 (LifeSensors, Malvern, PA), 4 μM ubiquitin, and 0.4 mM ATP for 60 min at room temperature in separate wells of a 96-well microtiter plate. The cells were subsequently washed and incubated for 60 min with a 1:1000 dilution of the detection reagent followed by 60 min incubation with a 1:10,000 dilution of streptavadin-HRP. Polyubiquitin chain formation was measured using SuperSignal West Dura Extended Duration Substrate from Pierce (Rockford, IL) and a 96-well plate reader.

In vitro ubiquitination assays

Some *in vitro* ubiquitination assays were performed using a ubiquitin-protein conjugation kit (BostonBiochem, Cambridge, MA) as described previously (McConnell et al., 2010). For these experiments, purified FLAG-α4 or PP2Ac was incubated in ubiquitination assay buffer containing a mixture of E1/E2 enzymes (Fraction A) and either a mixture of E3 ligases (Fraction B) or an immunopurified preparation of FLAG-MID1. Ubiquitination assays were also performed using purified conjugation enzymes. These reactions consisted of 2.5 μM ubiquitin (BostonBiochem, Cambridge, MA), 100 nM E1 (Enzo Life Sciences, Plymouth Meeting, PA), 2.5 μM of the indicated E2 enzyme (LifeSensors, Malvern, MA), 100 nM FLAG-MID1, Mg-ATP solution (Enzo Life Sciences; Plymouth Meeting, PA), 50 mM DTT, 100 U/mL inorganic pyrophosphatase (Sigma, St. Louis, MO), and 1 μM FLAG-α4 and/or 50 ng PP2Ac. All reactions were incubated at 37°C for 1 h and terminated by the addition of SDS sample buffer.

Mass spectrometry (MS)

FLAG-α4 was immunopurified from HEK293FT cells and subjected to SDS-PAGE. Full-length FLAG-α4 and the proteolytic fragment of FLAG-α4 were visualized by Colloidal Blue staining and excised from the gel. The gel pieces were cut into 1 mm cubes and equilibrated in 100 mM NH_4HCO_3. Proteins in the gel pieces were reduced with DTT (1/10 volume of 45 mM DTT for 20 min at 50°C) followed by alkylation with iodoacetamide (1/10 volume of 100 mM iodoacetamide for 20 min in the dark at room temperature). The gel pieces were then dehydrated with acetonitrile and rehydrated with 15 μL of 12.5 mM NH_4HCO_3 containing 0.01 μg/μL trypsin (Trypsin Gold from Promega; Madison, WI); the trypsin digestions were carried out for >2 h at 37°C. Peptides were extracted with 60% acetonitrile and 0.1% formic acid, dried by vacuum centrifugation, and reconstituted in 15 μL 0.1% formic acid. Five μL of peptide hydrosylate were analyzed by C18 reverse-phase LC-MS/MS using a Thermo LTQ-XL Orbitrap ion trap tandem mass spectrometer equipped with an Eksigent autosampler and nanoLC ultra 1D-plus HPLC pump system, nanospray source, and Xcalibur 2.0 instrument control using standard data-dependent methods. Tandem MS data were analyzed with the Sequest algorithm.

Cycloheximide chase experiments

HEK293FT cells were seeded in six-well tissue culture plates (350,000 cells per well) and transfected with either HA_3-PP2Ac alone or together with FLAG-α4 WT, FLAG-α4 ΔUIM, FLAG-α4 A52L/A53L, or FLAG-α4 G256*. At 48 h post-transfection, cells were treated with 100 ug/mL cyclohexamide (Sigma; St. Louis, MO) for the indicated times. Cell lysates were then prepared and subjected to Western analysis.

Immunoprecipitations

HEK293FT cells expressing FLAG- or HA-tagged proteins were lysed in 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-HA agarose (Roche, Indianapolis, IN) overnight or anti-FLAG-agarose (Sigma, St. Louis, MO) for 4 hours. The immune complexes were washed three times in 1 mL IP buffer, and bound proteins were eluted in SDS sample buffer and subjected to Western analysis. In some cases, the FLAG-tagged proteins were eluted from the beads by incubation for 60 min at 4°C in Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 3 mM KCl) containing 100 μg/ml FLAG peptide to create immunopurified preparations for *in vitro* ubiquitination assays and mass spectrometry.

Western analysis

SDS-solubilized protein samples were separated on 10% SDS-polyacrylamide gels (unless otherwise indicated) and transferred to 0.45 μm nylon-supported nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked in Odyssey Buffer (Li-COR; Lincoln, NE). All primary antibodies were used at a 1:1000 dilution in Tris-buffered saline containing 0.5% BSA and 0.1% Tween-20 (TTBS/BSA). For detection with the Odyssey Infrared Imaging system, the appropriate fluoraphore-conjugated secondary antibodies were used at a 1:10,000 dilution in TTBS/BSA. Bound antibodies were visualized using the Odyssey Infrared Imaging system and Odyssey software (LI-COR; Lincoln, NE).

Results

α4 suppresses PP2Ac polyubiquitination

α4 has previously been shown to regulate the ubiquitination of PP2Ac (Kong et al., 2009; LeNoue-Newton et al., 2011; McConnell et al., 2010; Trockenbacher et al., 2001); however, the precise role for α4 in this process is unclear. To understand the effect of α4 on the ubiquitination state of PP2Ac, HEK293FT cells expressing HA_3-PP2Ac alone or together with FLAG-α4 were treated +/- MG132. Ubiquitinated proteins were purified from lysates via TUBE2 pulldown assays. Western blots of bound proteins revealed a decrease in the amount of polyubiquitinated PP2Ac when α4 is co-overexpressed, as compared to cells expressing PP2Ac alone (Fig. 18A). To confirm our results from the overexpression studies (ie., α4 suppresses PP2Ac ubiquitination), we evaluated the effect of α4 siRNA on PP2Ac ubiquitination. Western analysis of ubiquitinated proteins isolated from these cell lysates using TUBE2 agarose showed an increase in the amount of ubiquitinated PP2Ac when α4 siRNA was present compared to control siRNA (Fig 18B). Taken together, these data indicate α4 is able to suppress the polyubiquitination of PP2Ac and calls into question the previously proposed role of α4 as a facilitator of PP2Ac ubiquitination (Trockenbacher et al., 2001).

α4 serves as an adaptor protein linking Mid1 and PP2Ac

Since the Mid1- and PP2Ac-binding domains within α4 are nonoverlapping, it is possible that a Mid1·α4·PP2Ac ternary complex exists in cells. Although prior immunoprecipitation experiments have revealed that α4 associates with PP2Ac and MID1, it is unclear whether this is representative of one complex (MID1·α4·PP2Ac) or two distinct complexes (MID1·α4 and α4·PP2Ac). To investigate whether α4 acts as a scaffold protein to tether PP2Ac and MID1, we

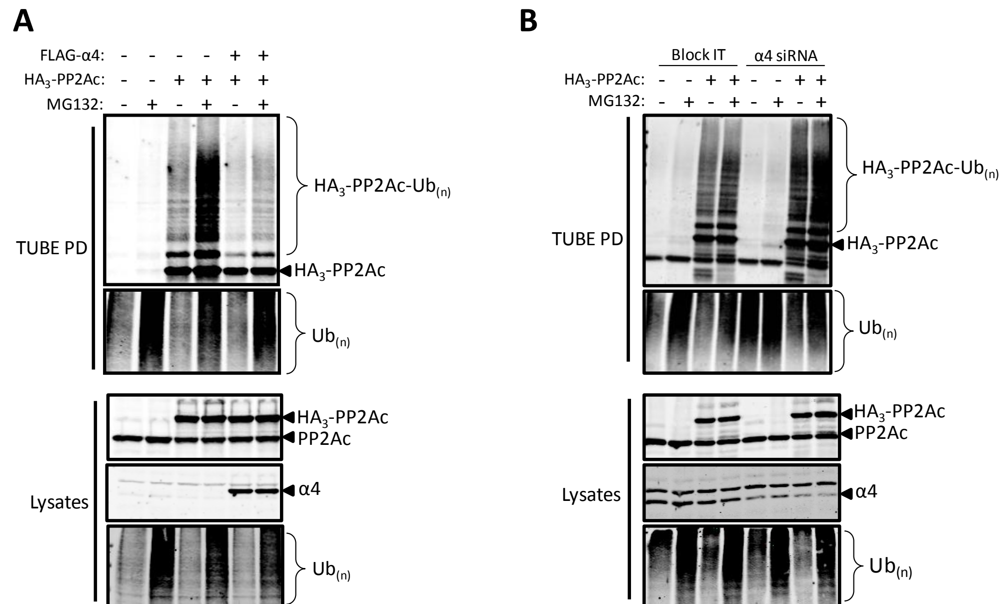


Figure 18. α4 regulates the polyubiquitination of PP2Ac

A.) HEK 293FT cells transfected with HA-PP2Ac alone or with the combination of HA_3-PP2Ac and Flag-α4 wild type (WT) were treated plus (+) or minus (-) proteasome inhibitor MG132. Cell lysates were incubated with agarose-TUBE2 beads and bound proteins (TUBE pulldown) were analyzed by SDS-PAGE and immunoblotting using antibodies recognizing PP2Ac, α4, and ubiquitin. Aliquots of the cell lysates were analyzed in the same manner. B.) HEK 293FT cells expressing HA_3-PP2Ac and/or FLAG-α4 were transfected with either control (Block IT) or α4 specific siRNA. Cell lysates were incubated with agarose-TUBE2 beads and bound proteins (TUBE pulldown) were analyzed by SDS-PAGE and immunoblotting using antibodies recognizing PP2Ac, α4, and ubiquitin. Aliquots of the cell lysates were analyzed in the same manner.

examined the protein composition of myc-MID1 immune complexes isolated from lysates of HEK293FT cells lacking or coexpressing FLAG-α4 and HA_3-PP2Ac. As shown in Fig. 19A, we were able to detect an interaction of MID1 with PP2Ac in myc-MID1 immune complexes isolated from cells coexpressing FLAG-α4 and HA_3-PP2Ac; however, we were unable to detect HA_3-PP2Ac in myc-MID1 immune complexes from lysates of cells lacking FLAG-α4. The finding that PP2Ac does not associate with MID1 in the absence of coexpressed α4 supports the conclusion that α4 is an adaptor protein necessary for the formation of a MID1·α4·PP2Ac complex. To determine if an association between α4 and PP2Ac is necessary for their interaction with MID1, we exploited an α4 mutant protein lacking the PP2Ac binding determinants (FLAG-α4 R155E/K158D), which was characterized in a previous report (Yang et al., 2007a). MID1, but not PP2Ac, co-immunoprecipitated with FLAG-α4 R155E/K158D (Figure 19B), indicating that α4 can interact with MID1 independently of PP2Ac. Together, these data demonstrate that α4 serves as a scaffolding protein to promote formation of a MID1·α4·PP2Ac complex.

MID1 is the E3 ubiquitin ligase that facilitates α4 monoubiquitination.

MID1, α4, and PP2Ac form a ternary complex in cells (Fig. 19A). Both α4 and PP2Ac are targeted for ubiquitination (monoubiquitination of α4 and polyubiquitination of PP2Ac) (Han et al., 2011; McConnell et al., 2010; Trockenbacher et al., 2001), but the target of MID1 remains unclear. Although MID1 was initially postulated to function as the E3 ubiquitin ligase for PP2Ac polyubiquitination (Trockenbacher et al., 2001), a recent *in vitro* study demonstrated that the RING and B-box domains of MID1 possess E3 ligase activity and can monoubiquitinate a 45 amino-acid polypeptide derived from the C-terminus of α4 (Han et al., 2011). However, no reports have examined whether full-length MID1 directly promotes PP2Ac and/or α4 ubiquitination. To address this question, we incubated purified PP2Ac and full-length FLAG-α4 in

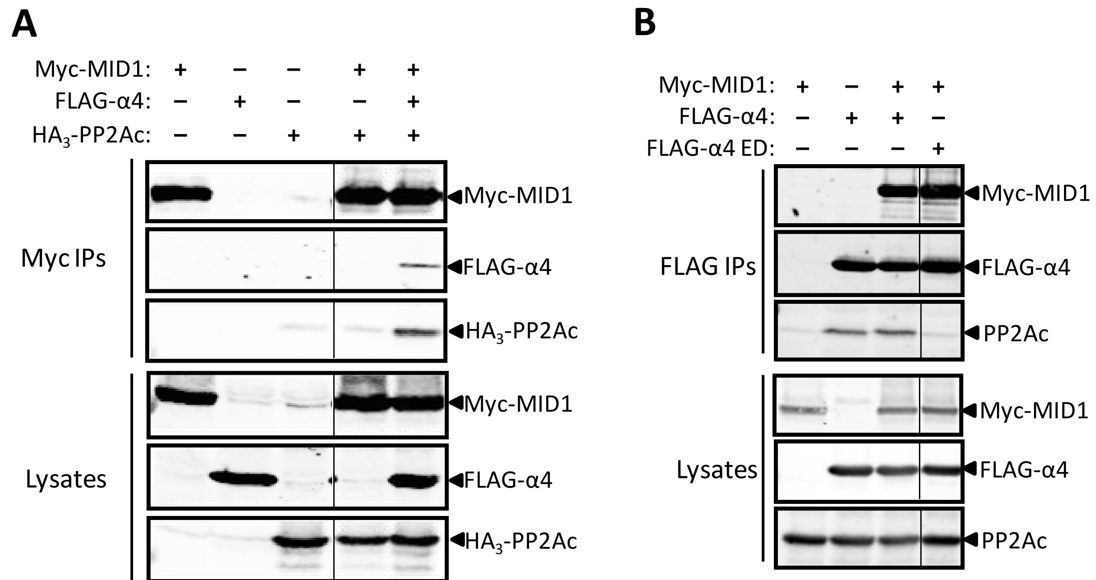


Figure 19. α4 is an adaptor protein linking Mid1 and PP2Ac

A) HEK293FT cells were transfected with myc-MID1, HA-PP2Ac, FLAG-α4, or the indicated combinations of these constructs. Myc immune complexes (*Myc IPs*) were isolated from cell lysates and analyzed by SDS-PAGE and immunoblotting using myc, FLAG, and HA antibodies to detect the epitope-tagged forms of MID1, α4, and PP2Ac, respectively. Aliquots of the cell lysates were analyzed in the same manner. B.) HEK293FT cells were co-transfected with HA-ubiquitin and the indicated construct(s). FLAG immune complexes (*FLAG IPs*) were isolated from cell lysates and analyzed by SDS-PAGE and immunoblotting using myc, FLAG, and PP2Ac antibodies to detect the epitope-tagged forms of MID1 and α4, as well as endogenous PP2Ac.

a ubiquitin-protein conjugation solution containing a mixture of E1/E2 enzymes and either a mixture of E3 ligases (Fraction B) or immunopurified FLAG-MID1. Western analysis of the reactions containing the E3 ligase mixture revealed both polyubiquitinated PP2Ac species and monoubiquitinated α4, whereas analysis of reactions containing FLAG-MID1 revealed only α4 monoubiquitination and no detectable polyubiquitinated PP2Ac species (Fig. 20A). To determine whether MID1 is capable of promoting polyubiquitin chain formation and to identify the E2 ubiquitin conjugation enzymes that pair with MID1, we assayed immunopurified full-length MID1 for E3 activity using a panel of purified E2 ligases. As shown in Fig. 20B, a MID1 dose-dependent increase in polyubiquitin chain formation was observed in presence of the E2 ligases UBE2D3 and UBE2D2, but no detectable polyubiquitin chain formation was seen in reactions containing the other E2 ligases. To further characterize the ligase activity of MID1, we incubated FLAG-MID1 with α4 and/or PP2Ac in ubiquitination assay mixtures containing purified E1 and various E2 ligases. The MID1-UBE2D3 and MID1-UBE2D2 pairs, but not the MID1-UBE2A pair, facilitated α4 monoubiquitination and MID1 auto-polyubiquitination in the presence or absence of PP2Ac; however, no appreciable PP2Ac polyubiquitination was observed in any of the experimental conditions (Fig. 20C).

To determine whether MID1 facilitates α4 monoubiquitination in mammalian cells, we co-transfected HEK293FT cells with HA-ubiquitin and FLAG-α4, together with control siRNA or MID1-targeted siRNA. The expression MID1 was dramatically reduced in cells transfected with MID1 siRNA relative to cells transfected with control siRNA (Fig. 21A). Western analysis of the ubiquitinated proteins isolated from the cell lysates revealed significantly decreased α4 monoubiquitination but not total protein ubiquitination in cells harboring MID1 siRNA compared to the control cells (Figs. 21B). These cellular findings further establish MID1 as the E3 ubiquitin ligase for α4.

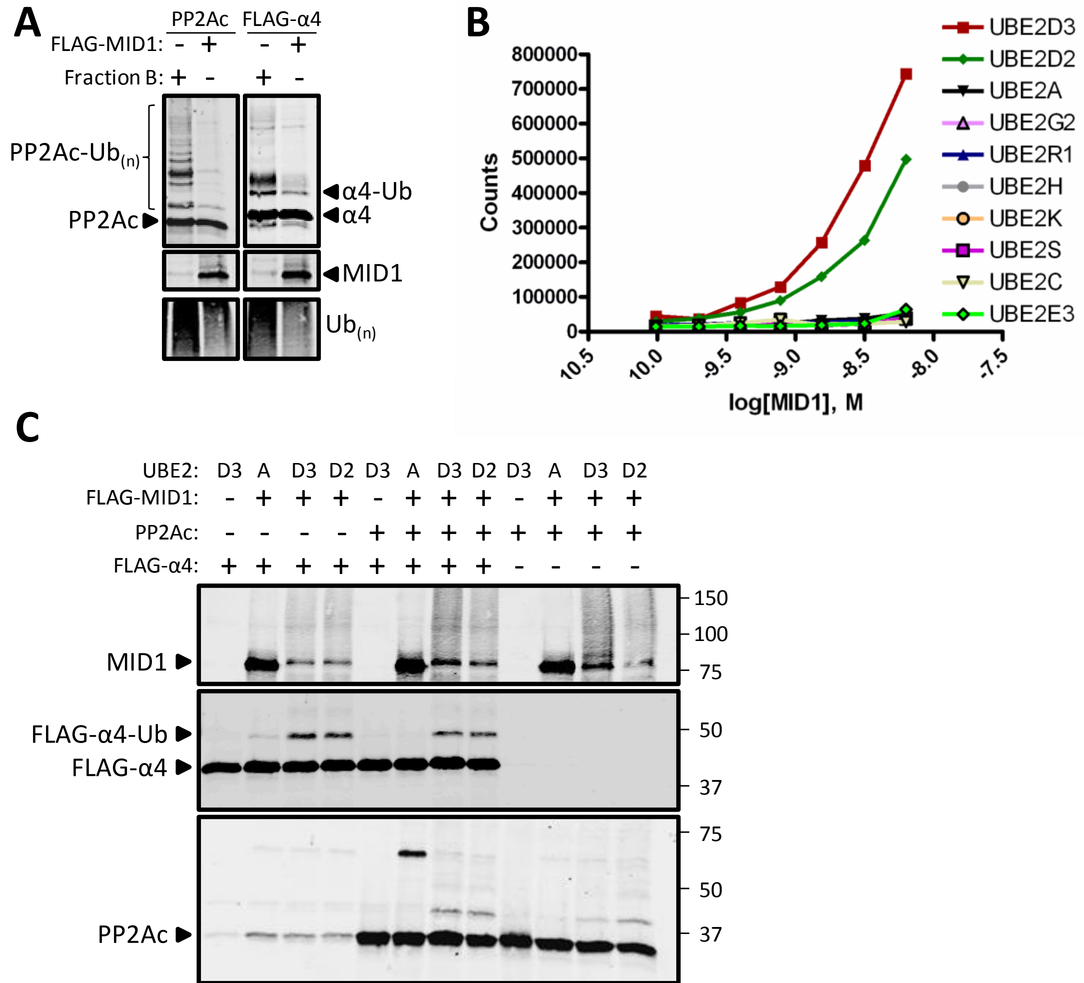


Figure 20. Mid1 functions as an E3 ubiquitin ligase for α4 monoubiquitination
A.) Purified FLAG-α4 or PP2Ac was incubated in a ubiquitination assay solution containing a mixture of E1 and E2 enzymes and either a mixture of E3 ligases (*Fraction B*) or FLAG-MID1. The reactions were analyzed via Western using antibodies recognizing PP2Ac, α4, MID1, and ubiquitin. B.) The indicated E2 ligase was incubated with increasing concentrations of purified FLAG-MID1, and the ability of each MID1/E2 pair to form polyubiquitin chains was measured using the customizable E3LITE ubiquitin ligase kit. C.) Ubiquitination assays were performed in the presence (*+*) or absence (-) of purified FLAG-MID1, PP2Ac, FLAG-α4, and the indicated E2 ligase (*UBE2*). Ubiquitinated MID1, α4, and PP2Ac species were visualized by Western analysis using the corresponding antibodies.

Monoubiquitination of α4 promotes its cleavage.

α4 is a multi-domain protein with an unstructured C-terminus (LeNoue-Newton et al., 2011; Smetana et al., 2006; Yang et al., 2007a). We detected a proteolytic fragment of α4 (27 kDa) in lysates of cells expressing an N-terminal FLAG-tagged form of this protein (Fig. 21B). Both full-length and truncated α4 were immunoreactive with an anti-FLAG antibody and an antibody directed against the N-terminus of α4, but only the full-length protein was recognized with an antibody directed against the C-terminus of α4 (Figs. 21B and 22), thus demonstrating that in cells α4 is subject to proteolytic cleavage resulting in a truncated protein that lacks the C-terminus (α4ΔC). Unexpectedly, we also observed that the amount of α4 cleavage product appeared to parallel the monoubiquitination state of α4, as cells harboring MID1 siRNA exhibited decreased cleavage and monoubiquitination in comparison to control cells (Fig. 21B). These findings point to an unprecedented monoubiquitination-regulated proteolysis event.

To better understand the relationship between α4 monoubiquitination and cleavage, and to rule out any MID1-independent effects of the MID1 siRNA on α4 cleavage, we treated target cells with increasing concentrations of the E1 ubiquitin ligase inhibitor PYR41. As shown in Fig. 23, the monoubiquitination and cleavage of α4 both decreased in a strikingly similar PYR41 dose-dependent manner. In agreement with previous reports (Yang et al., 2007b), we did not observe significant changes in total ubiquitin conjugates with PYR41 (Fig. 23). These data further illustrate that alterations in α4 monoubiquitination influence its own cleavage.

Since our previous study demonstrated that human α4 contains a ubiquitin interacting motif (UIM; residues 46-60) (McConnell et al., 2010), we asked whether mutation or deletion of this motif influenced α4 monoubiquitination and cleavage product formation. Like other UIM-containing proteins (Hicke et al., 2005), we found that deletion of the UIM within α4 (ΔUIM)

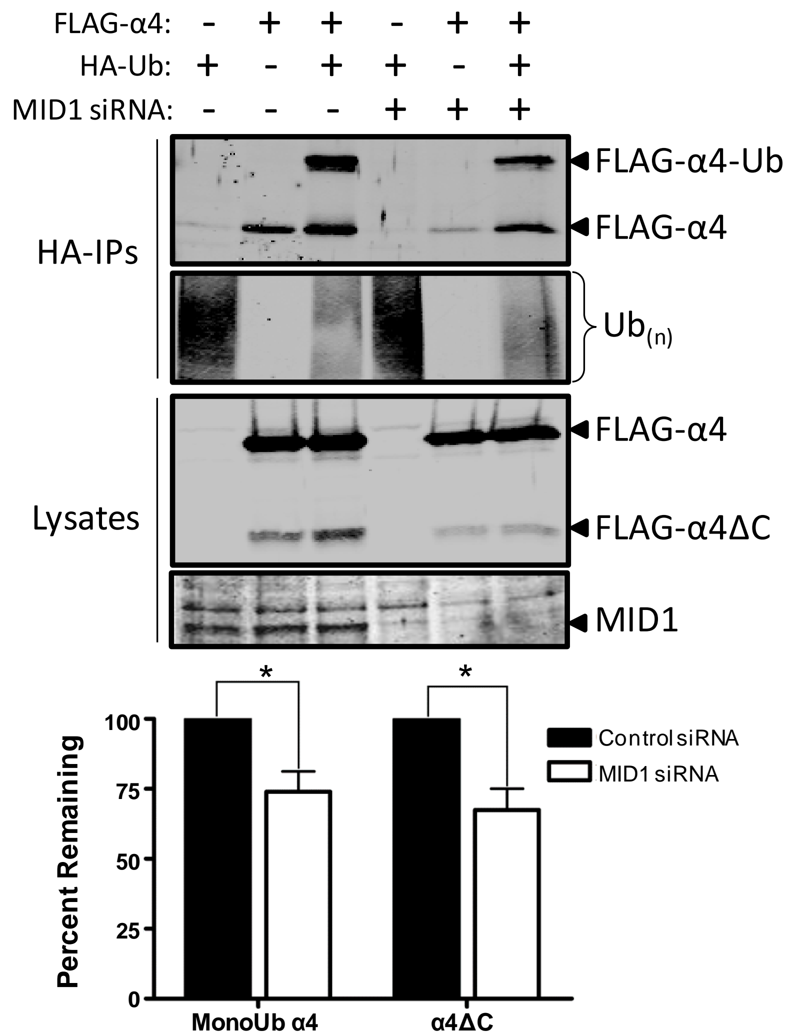


Figure 21. Knockdown of Mid1 decreases both α4 monoubiquitination and C-terminal α4 cleavage

HEK293FT cells were transfected with HA-ubiquitin (*HA-Ub*) and FLAG-α4 plasmids alone or together with MID1 siRNA (+) or Control siRNA (-). Ubiquitinated proteins were isolated from the cell extracts using a HA-affinity matrix (*HA-IPs*). The HA-IPs and cell lysates were analyzed by Western using antibodies recognizing α4 and ubiquitin. The cell lysates were also analyzed by Western using a MID1 specific antibody to confirm protein knockdown. The monoubiquitinated α4 and cleaved α4 signals were normalized to the total α4 signal in the corresponding cell lysate, and the normalized monoubiquitinated and cleaved α4 values from the MID1 knockdown conditions were compared with the corresponding values in the control siRNA conditions, which were set at 100. Values represent means +/- SEM; *, $p < 0.001$. Some unmodified FLAG-α4 (non-specific) was detected in the HA-IPs, but the levels of unmodified FLAG-α4 were not statistically different between the control and experimental condition from multiple experiments.

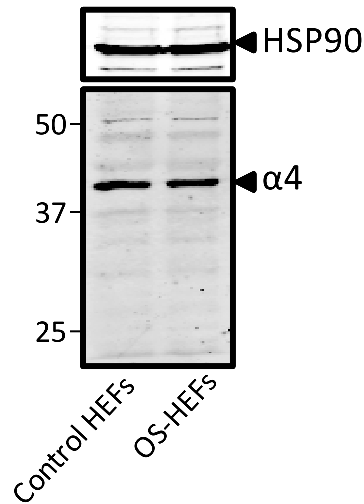


Figure 22. The α4 cleavage product is not recognized by a C-terminal α4 antibody.

Lysates from Opitz syndrome-derived HEFs (*OS-HEFs*) and aged-matched control HEFs were analyzed by Western using antibodies recognizing HSP90 and the C-terminus of α4.

prevented the protein from undergoing monoubiquitination (Fig. 24A). Our analyses of multiple α4 UIM point mutants also identified a double point mutant of α4 (A52L/A53L) that exhibited increased monoubiquitination (Fig. 24A). Importantly, notable differences in the amount of cleavage product (α4ΔC) were observed in these cells; A52L/A53L-expressing cells exhibited higher levels of α4ΔC relative to wild type α4-expressing cells, but very little α4ΔC was detected in ΔUIM-expressing cells (Fig. 24A). Thus, α4 cleavage is dependent on its monoubiquitination: A52L/A53L>wild type α4>>ΔUIM. These findings, together with the results of the MID1 knockdown and PYR41 experiments, support our hypothesis that MID1-dependent monoubiquitination of α4 triggers its proteolytic cleavage.

α4 monoubiquitination and cleavage are important in the regulation of PP2Ac stability.

The gene encoding α4 (*IGBP1*) is an essential gene since its deletion causes lethality of the host and cellular apoptosis (Kong et al., 2004). Furthermore, studies of conditional α4-null mouse embryonic fibroblasts have revealed that α4 plays a crucial role in the maintenance of PP2Ac stability (Kong et al., 2009). To explore a potential role of α4 monoubiquitination and cleavage in the regulation of PP2Ac stability, we performed cycloheximide chase studies of cells expressing HA_3-PP2Ac alone or together with various FLAG-tagged α4 constructs exhibiting differing degrees of monoubiquitination and cleavage. The cells were treated with cycloheximide 48 h post-transfection to inhibit new protein synthesis, and the levels of HA_3-PP2Ac were monitored at various time points after cycloheximide treatment (Fig. 24B). Consistent with our previous report (LeNoue-Newton et al., 2011), we observed a progressive decline in HA_3-PP2Ac levels over the 8 h cycloheximide time course in cells expressing HA_3-PP2Ac alone but the levels of HA_3-PP2Ac remained stable during this period in cells coexpressing wild type α4. HA_3-PP2Ac levels also remained stable in cells coexpressing ΔUIM after

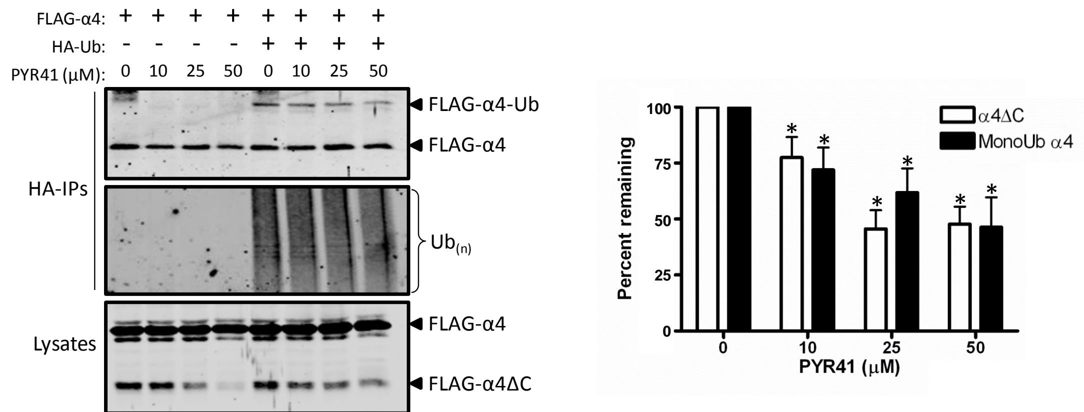


Figure 23. α4 monoubiquitination promotes cleavage of the C-terminus
HEK293FT cell expressing HA-Ub and/or FLAG-α4 were treated with increasing concentrations of PYR41 for 4 h prior to lysis. Monoubiquitinated FLAG-α4 and total ubiquitinated proteins were visualized by Western analysis of the HA-IPs using α4- and ubiquitin-specific antibodies. Cell lysates were also subjected to Western analysis using an α4 antibody. The α4 monoubiquitin signals from PYR41-treated cells were normalized to the signal from untreated cells. Values represent means +/- SEM. Statistical significance between untreated and treated samples; *, $p < 0.01$.

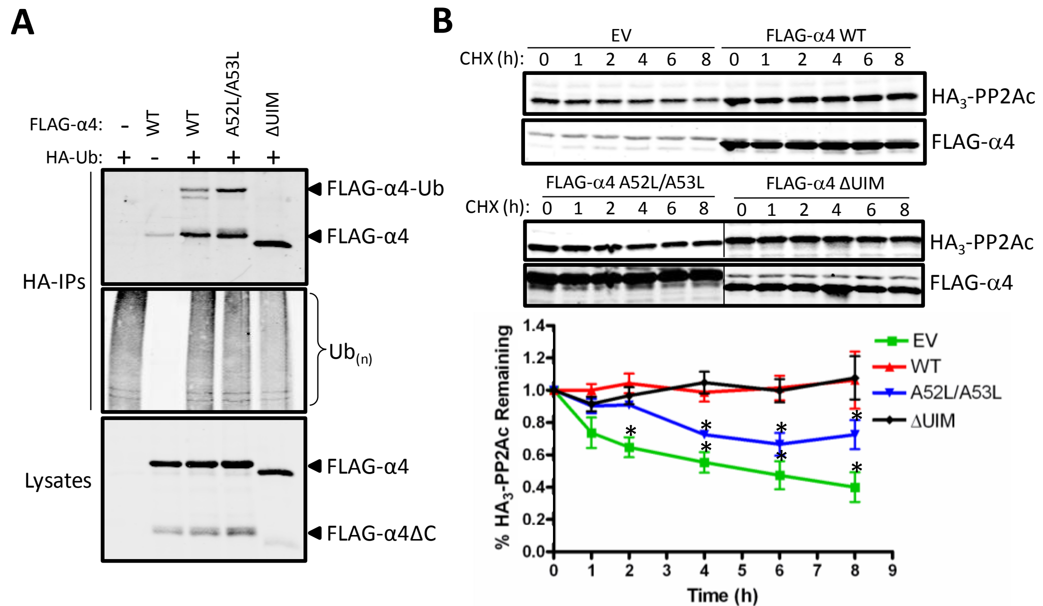


Figure 24. α4 monoubiquitination and cleavage are essential for PP2Ac turnover. (A) HEK293FT cells were transfected with HA-Ub and either empty vector (-), wild type FLAG-α4 (*WT*), a UIM deletion mutant of FLAG-α4 (*ΔUIM*), or a FLAG-α4 double point mutant (*A52L/A53L*). Western analysis of the HA-IPs was performed using α4 and ubiquitin antibodies. Cell lysates were similarly probed using an α4-specific antibody. (B) HEK293FT cells expressing HA_3-PP2Ac alone or together with empty vector (*EV*), FLAG-α4 WT, A52L/A53L, or ΔUIM were treated with 100 µg/ml cycloheximide (*CHX*) at 48 h post-transfection and then lysed at the indicated time points after treatment. The lysates were analyzed by Western using antibodies recognizing HA_3-PP2Ac and α4. Samples were analyzed for statistically significant changes relative to the corresponding HA_3-PP2Ac + FLAG-α4 WT samples; *, $p < 0.05$ (ANOVA).

cycloheximide treatment; however, the levels of HA_3-PP2Ac progressively declined in cells coexpressing A52L/A553L. These results indicate that the monoubiquitination- and cleavage-resistant ΔUIM construct stabilizes HA_3-PP2Ac, whereas the A52L/A53L mutant, which exhibits increased monoubiquitination and cleavage, is relatively ineffective in stabilizing HA_3-PP2Ac. Furthermore, these data highlight a role for α4 monoubiquitination and cleavage in the control of PP2Ac turnover.

Calpain-mediated cleavage of α4 occurs at the F255-G256 bond.

We next sought to determine the cleavage site in α4. Electrospray ionization high-pressure liquid chromatography tandem mass spectrometry (ESI-HPLC MS/MS) of α4 and α4ΔC identified multiple overlapping peptides that corresponded to the N-terminal portion of α4. However, one peptide was found to be unique to α4ΔC – NMAQAKVF (Fig. 25); no α4 peptides beyond this region were identified in the cleaved protein but peptides covering the entire sequence were identified in the full-length α4 sample (Fig. 26A). Western analysis of cells expressing FLAG-tagged wild type α4 or an α4 construct encompassing residues 1-255 (G256*) revealed that G256* comigrated exactly with the authentic cleavage fragment derived from full-length α4 (Fig. 26B). Together, these data demonstrate that the 27 kDa fragment of α4 (α4ΔC) is the result of proteolytic cleavage of the full-length protein between residues F255 and G256.

Analysis of the amino acid residues flanking the α4 cleavage site identified a potential calpain consensus sequence (Fig. 27A). To test whether calpains are responsible for the cleavage of α4, we treated cells expressing FLAG-α4 with increasing concentrations of the calpain inhibitors calpeptin or Z-Leu-Leu-CHO and monitored cleavage product formation by Western analysis. The calpain inhibitors potently protected FLAG-α4 from cleavage (Fig. 27B). Since the

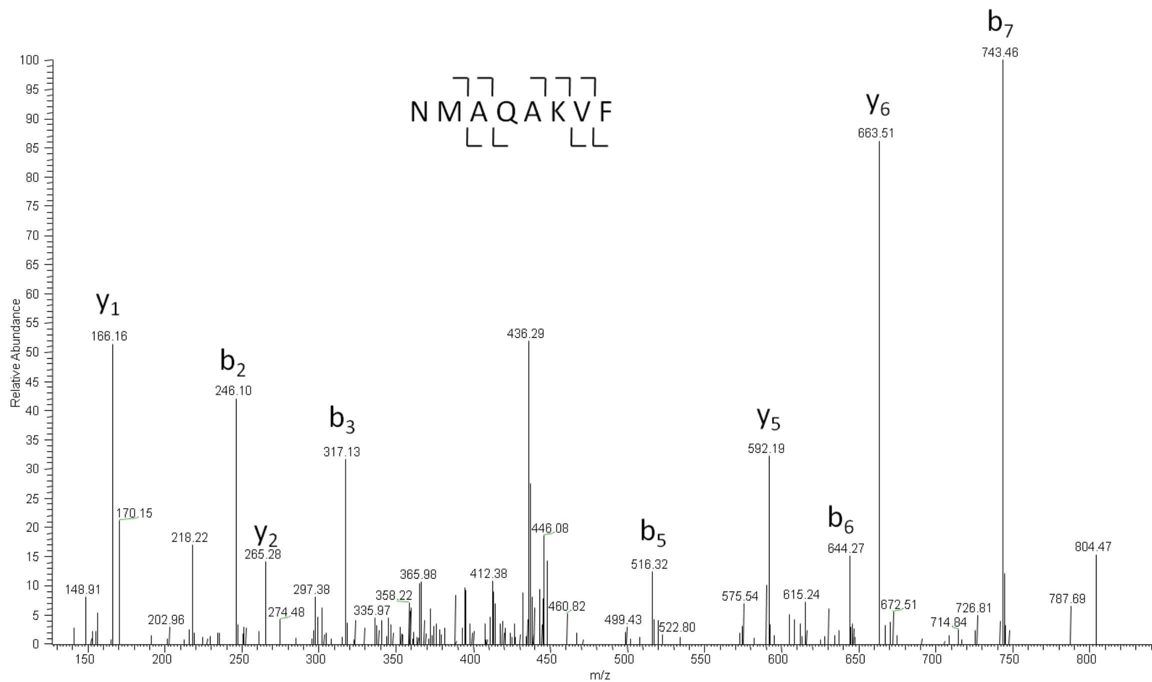


Figure 25. Identification of the α4 cleavage site via mass spectrometry

Tandem mass spectrum of the doubly-charged NMAQAKVF peptide (m/z 454.73). Labeled b-ions and y-ions are denoted by cleavage brackets above and below the sequence, respectively.

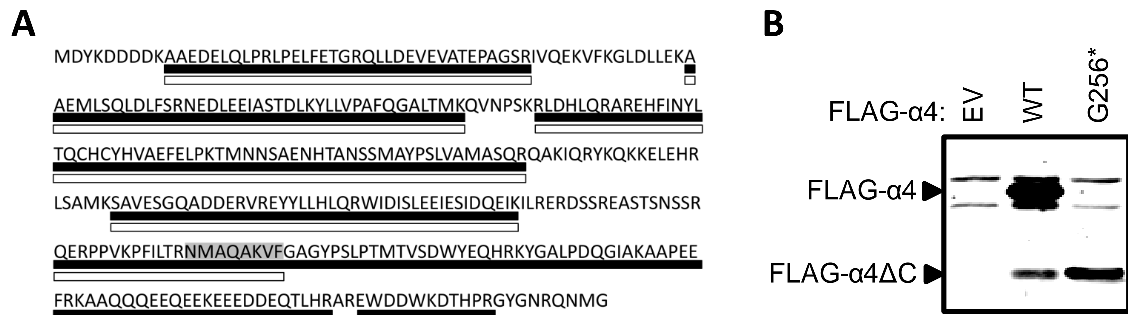


Figure 26. α4 cleavage occurs at the F255-G256 bond.

(A) Coverage map of the full-length α4 and cleaved α4 peptides determined by mass spectrometry. Closed boxes mark peptide regions identified in full-length FLAG-α4; open boxes mark peptide regions identified in the FLAG-α4 cleavage product. The residues highlighted in grey correspond to the unique peptide found only in the α4 cleavage product. The MS spectrum for this peptide can be found in Fig. S2 (B) HEK293FT cells expressing empty vector (*EV*), wild type FLAG-α4 (*WT*), or FLAG-α4 1-255 (*G256**) were analyzed by Western using an α4-specific antibody.

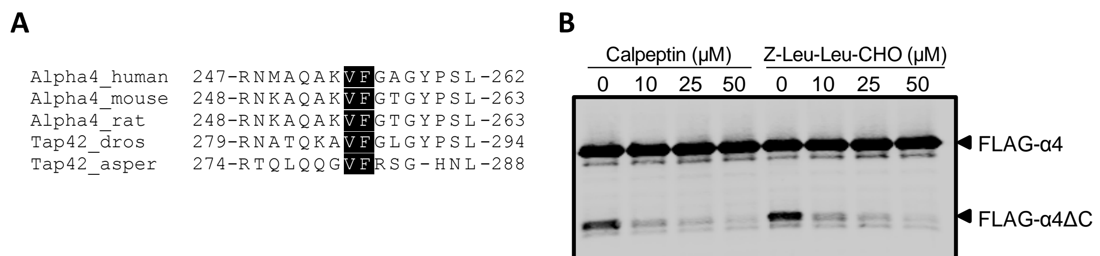


Figure 27. Calpain-mediated cleavage of α4 results in an N-terminal fragment that binds PP2Ac but not MID1. (A) Sequence alignment of the region within multiple species of α4 that encompasses the calpain cleavage site; the conserved VF residues are highlighted. (B) HEK293FT cells expressing wild type FLAG-α4 were treated with increasing concentrations of calpeptin or Z-leu-leu-CHO for 4 h prior to lysis. Cell lysates were subjected to Western analysis using an α4 antibody.

VF residues are conserved amongst many different species of α4 (Fig. 27A), calpain-mediated cleavage at F255 may represent an evolutionarily conserved mode of regulation for α4. Additional support for this proposal comes from our observations showing that the *Drosophila* homolog of α4, Tap42, is also targeted for both monoubiquitination and cleavage (Fig. 28).

Cleavage of α4 regulates its interaction with MID1.

Previous studies have shown that PP2Ac binds to the N-terminal domain of α4 (Yang et al., 2007a), whereas MID1 binds to the C-terminal domain of α4 (Liu et al., 2001; Trockenbacher et al., 2001), yet both domains are required for α4-mediated protection of PP2Ac from polyubiquitination and degradation (LeNoue-Newton et al., 2011). Since α4 cleavage (at the F255-G256 bond) occurs within the previously identified MID1 binding region, we performed experiments to determine if the cleavage product of α4 still retains the ability to bind MID1. Western analysis of FLAG immune complexes from HEK293FT cells co-expressing Myc-MID1 and either wild type FLAG-α4 or FLAG-α4 G256* revealed that both forms of α4 bound to PP2Ac, but only full-length α4 interacted with MID1 (Fig. 29). These results demonstrate that the C-terminal 84 amino acids of human α4 (aa 256-340) are necessary for MID1 binding. Furthermore, these findings indicate that α4 cleavage likely leads to the disruption of MID1·α4·PP2Ac complexes.

Discussion

In this chapter, we show that MID1 possesses E3 ligase activity and directly facilitates α4 monoubiquitination, which triggers calpain-mediated cleavage of the PP2A regulatory subunit. Monoubiquitination has previously been shown to impact the activity and subcellular localization of many proteins (Hicke, 2001); however, to our knowledge, monoubiquitination-

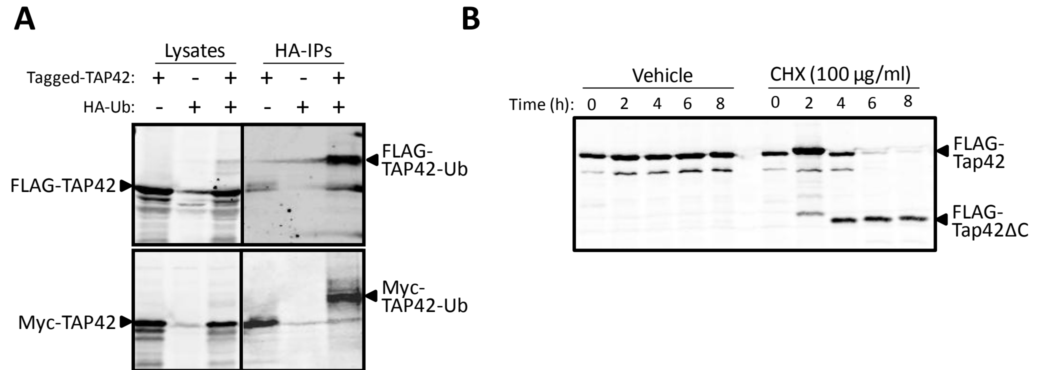


Figure 28. *Drosophila*TAP42 is subject to both monoubiquitination and cleavage.
A.) *Drosophila* TAP42 is subject to monoubiquitination. Ubiquitinated proteins (*HA-IPs*) were isolated from extracts of *Drosophila* S2 cells expressing either Myc-TAP42 or FLAG-TAP42 alone or together with HA-Ub. The HA-IPs and cell lysates were analyzed by Western using antibodies recognizing the FLAG and Myc epitopes. B.) *Drosophila* TAP42 undergoes C-terminal cleavage. *Drosophila* S2 cells expressing FLAG-TAP42 were treated with vehicle or 100 µg/ml cycloheximide and lysed at the indicated time points. Lysates were analyzed by Western using antibodies recognizing FLAG-TAP42.

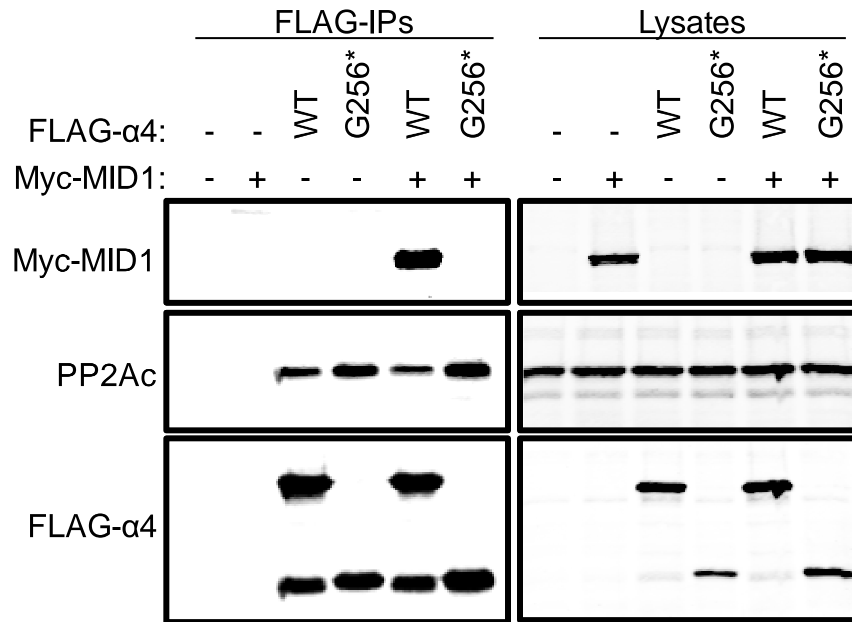


Figure 29. α4 cleavage results in a N-terminal fragment that binds PP2Ac but not MID1.

HEK293FT cells were transfected with wild type FLAG-α4 WT and G256* alone or together with (*+*) or without (-) Myc-MID1. FLAG-tagged proteins were immunoprecipitated (*Flag IPs*) from the cell lysates and analyzed by Western using the Myc, PP2Ac, and α4 antibodies. The cell lysates were similarly analyzed.

induced cleavage represents a hitherto undescribed activity for ubiquitin. Considering the relatively large number of cellular proteins that undergo both monoubiquitination and cleavage (e.g., IL-1R1 (Twomey et al., 2009) and Notch (Gupta-Rossi et al., 2004)), it will be interesting to determine whether monoubiquitination-induced cleavage represent a more general phenomenon for the control of protein function.

How is the monoubiquitinated form of α4 targeted for cleavage? Deletion of the UIM within α4 prevents its monoubiquitination and cleavage, thus supporting the idea that a functional UIM is necessary for these events. We propose that the UIM within α4, which is known to form non-covalent interactions with ubiquitin (McConnell et al., 2010), binds in *cis* to the ubiquitin moiety on α4 leading to a conformational change in α4 that unmasks a calpain cleavage site. Studies of calpain substrates have revealed that the tertiary structure of the protein is important for cleavage (Stabach et al., 1997); therefore, a monoubiquitin-induced conformational change in α4 could explain why only a fraction of α4 (i.e., monoubiquitinated α4) is targeted for cleavage.

Our studies demonstrate that α4 monoubiquitination and cleavage are important for facilitating PP2Ac degradation. Since the cleaved form of α4 (α4ΔC) retains its ability to bind PP2Ac but fails to bind MID1, α4 cleavage could be important for redirecting the localization of PP2Ac and promoting the polyubiquitination of this phosphatase by a yet unknown E3 ubiquitin ligase. Although the ubiquitination machinery necessary for PP2Ac ubiquitination remains to be identified, our studies provide compelling support for a new model of MID1/α4 regulation of PP2Ac in which MID1-mediated monoubiquitination of α4 triggers a conformational change in α4 leading to calpain-cleavage of its MID1 binding domain and, ultimately, PP2Ac polyubiquitination and proteasomal degradation (Fig. 30). Our studies also reconcile the apparent contradictory protective and destructive roles of α4 in the control of PP2Ac levels

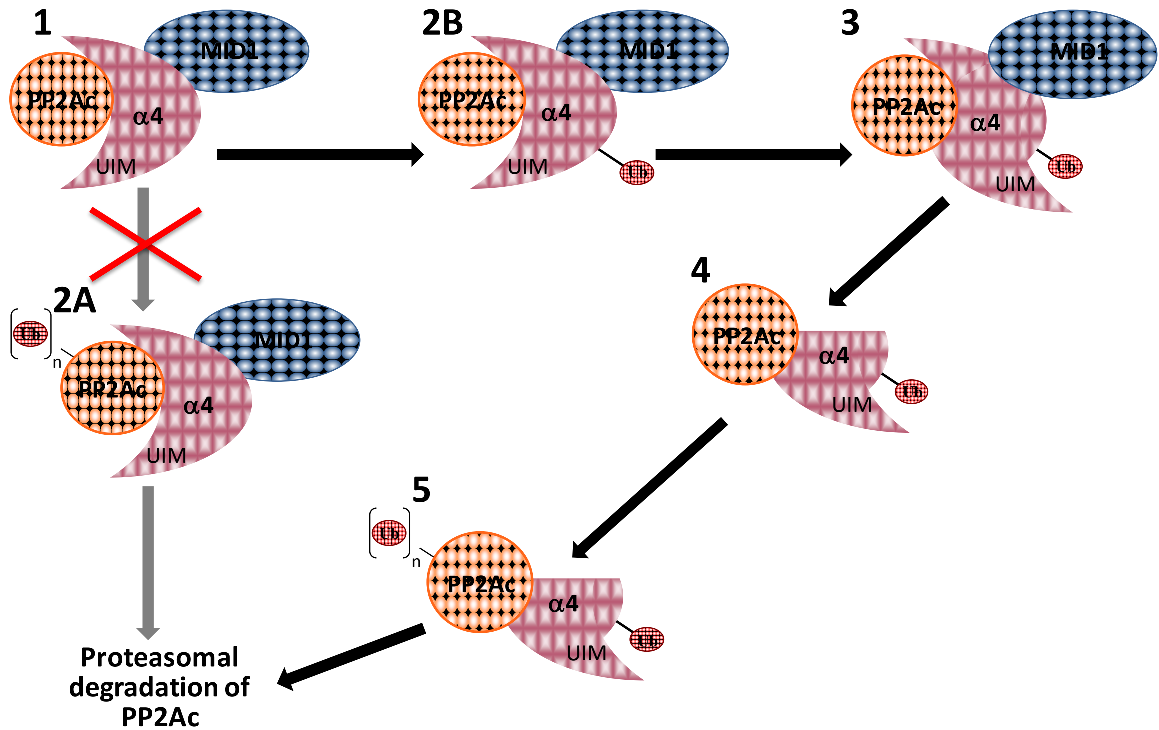


Figure 30. Working model for MID1/α4 regulation of PP2Ac.
MID1, α4, and PP2Ac form a ternary complex (***1***) in cells. Previous studies have suggested that α4 facilitates MID1-dependent polyubiquitination of PP2Ac (***2A***) and subsequent proteasomal degradation; however, our findings challenge this paradigm and support a model in which MID1 serves as the E3 ligase for α4 (***2B***) leading to a conformational change in α4 whereby the UIM of α4 binds in *cis* to the covalently attached ubiquitin (***3***). This structural rearrangement then leads to calpain-mediated cleavage of the C-terminus of α4 (***4***) allowing for polyubiquitination of PP2Ac by a currently unknown E3 ligase (***5***) and subsequent degradation by the proteasome.

(Kong et al., 2009; LeNoue-Newton et al., 2011; McConnell et al., 2010; Trockenbacher et al., 2001), and place α4 in a critical position where it can signal either protection or degradation of PP2Ac depending on the ubiquitination state of α4. The cytosolic form of α4 that is not associated with MID1 likely stabilizes PP2Ac in an inactive form until it can be incorporated into active PP2A holoenzymes (Kong et al., 2009), whereas the microtubule-associated MID1·α4-bound PP2Ac is subject to proteasomal degradation as a result of MID1-dependent monoubiquitination and cleavage of α4. In summary, our studies presented in this chapter have uncovered a novel regulatory process for PP2A involving ubiquitination-induced cleavage of α4, which plays a crucial role in modulating PP2Ac levels.

CHAPTER V

α4 CLEAVAGE IS ALTERED IN HUMAN DISEASE

Introduction

Deregulation of protein phosphatase 2A (PP2A) has been implicated in a variety of microtubule-associated protein (MAP)-dependent pathologies such as cancer, Alzheimer's disease (AD), and Opitz syndrome (OS) (Liu and Wang, 2009; Sontag and Sontag, 2006; Trockenbacher et al., 2001). PP2Ac levels at the microtubules are controlled, in part, by the MID1·α4·PP2Ac complex (Trockenbacher et al., 2001). In Chapter IV, we identified a novel monoubiquitin-dependent cleavage of α4, which is regulated by the E3 ligase activity of the MAP MID1. Inter-domain cleavage of α4, by calpain, seperates the MID1- and PP2Ac- binding domains leading to the proteasomal degradation of PP2Ac. Since MID1 is a MAP and the MID1·α4·PP2Ac complex regulates microtubule-associated PP2Ac (Trockenbacher et al., 2001), we hypothesized that α4 cleavage may control MAP phosphorylation. Consistent with this hypothesis, studies of OS-derived fibroblast cells, a disease characterized by mutations in MID1, revealed an increase in the microtubule-associated pool of PP2Ac and a hypophosphorylation of MAPs (Schweiger and Schneider, 2003; Trockenbacher et al., 2001; Winter et al., 2003). These data raise the intriguing possibility that misregulation of α4 cleavage may contribute to the pathogenesis of Opitz syndrome, as well as other MAP pathologies such as AD and various cancers.

We observe marked alterations in α4 cleavage from both post-mortem AD tissue and fibroblast cells derived from an OS fetus. Examination of human melanocytes revealed changes in α4 cleavage, thus indicating that defective α4 monoubiquitination/cleavage and

consequential deregulation of PP2A function may be involved in the pathogenesis of OS, AD, and cancer. Finally, we detail the development and characterization of an α4 cleavage product-specific antibody, which undoubtedly will be valuable for future characterization of α4 cleavage in disease.

Materials and Methods

Plasmids

The HA_3-PP2Ac/pKH3 plasmid was a gift from Dr. David Brautigan (University of Virginia, Charlottesville, VA). Construction of the mammalian expression constructs for FLAG-tagged wild type α4 and 1-256 was described previously (Ref). Proper construction of all plasmids was verified by automated sequencing (Vanderbilt University DNA core facility)

Antibodies and reagents

The rabbit polyclonal α4 antibody was from Bethyl Laboratories (Montegomery, TX). The mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell culture and transfection

HEK293/tau stable cells were from Dr. Brian Kraemer (University of Washington, Seattle, WA) and grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine and 100 µg/ml zeocin to maintain tau selection (Guthrie and Kraemer, 2011). The SK-MEL2 and SK-MEL5 cells were from Dr. Ann Richmond (Vanderbilt University, Nashville, TN) and grown at 37°C in a humidified atmosphere with 5% CO_2 in DMEM/F12 with 10% fetal calf serum and 2 mM L-

glutamine. The MEL-ST cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in DMEM supplemented with 5% fetal calf serum and 2 mM L-glutamine. HEK293/tau cells were transfected with mammalian expression constructs using Fugene6 (Roche; Indianapolis, IN) according to the manufacturer's protocol.

Human tissue

After receiving human subjects approval from the University of Washington human subjects division (HSD# 06-0492-E/A 01: Molecular Regulators of Tauopathy; Dr. Brian Kraemer, Principal Investigator), de-identified samples of post-mortem temporal cortex tissue from AD and age-matched control cases were obtained from the University of Washington Alzheimer's Disease Research Center (ADRC) Neuropathology Core (Core Leader, Dr. Thomas Montine). The tissues were lysed by sonication in a high salt buffer and the clarified lysates were analyzed via Western.

Western analysis

SDS-solubilized protein samples were separated on 10% SDS-polyacrylamide gels and transferred to 0.45 µm nylon-supported nitrocellulose membranes (Whatman, Dassel, Germany). Membranes were blocked in Odyssey Buffer (Li-COR; Lincoln, NE). All primary antibodies were used at a 1:1000 dilution in Tris-buffered saline containing 0.5% BSA and 0.1% Tween-20 (TTBS/BSA). For detection with the Odyssey Infrared Imaging system, the appropriate fluoraphore-conjugated secondary antibodies were used at a 1:10,000 dilution TTBS/BSA. Bound antibodies were visualized using the Odyssey Infrared Imaging system and Odyssey software (LI-COR; Lincoln, NE).

In cell Western analysis

HEK293/tau cells expressing FLAG-α4 wild type or FLAG-α4 G256* were fixed in a 96-well plate with 4% paraformaldehyde and then permeabilized in 1X PBS containing 0.1% Triton X-100. The permeabilized cells were incubated with primary antibodies (diluted 1:1000 in Odyssey Buffer) overnight at 4°C and incubated with secondary antibodies (diluted 1:500 in Odyssey Buffer containing 0.2% Tween-20) for 60 min at room temperature. Bound antibodies were visualized and quantified using the Odyssey Infrared Imaging system and Odyssey software.

Results

Regulation of tau phosphorylation by α4 cleavage.

The MID1·α4·PP2A complex is localized to microtubules via the interaction of MID1 with microtubule structures, and is thought to be involved in the maintenance of microtubule stability (Aranda-Orgilles et al., 2008a; Liu et al., 2001; Schweiger et al., 1999). Although the precise function of this complex in microtubule stabilization remains unclear, it likely involves PP2A-mediated dephosphorylation of various MAPs. Tau is one such MAP that is targeted for PP2A dephosphorylation at multiple epitopes (Goedert et al., 1995). To test whether α4 cleavage plays a role in the control of PP2Ac-dependent tau dephosphorylation, we transfected HEK293 cells stably expressing the tau protein (Guthrie and Kraemer, 2011) with HA_3-PP2Ac and either wild type FLAG-α4 or FLAG-α4 G256*. The levels of tau S262 and T205 phosphorylation were significantly elevated in FLAG-α4 G256*-expressing cells relative to wild type FLAG-α4 expressing cells, as monitored by In-cell Western (Fig. 31). Since α4 cleavage disrupts PP2Ac binding to MID1 (Fig. 29) these data support the hypothesis that α4 cleavage leads to a loss of

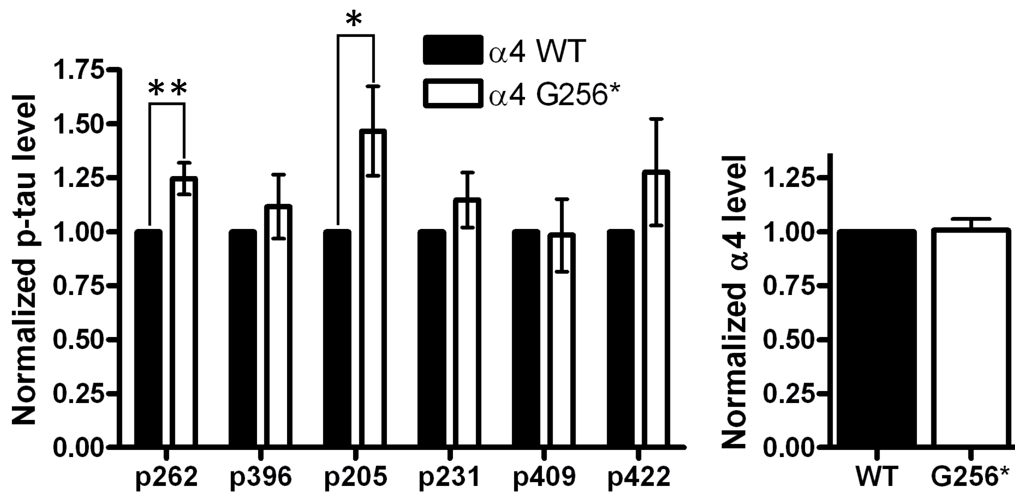


Figure 31. Cleaved α4 increases tau phosphorylation

A.) HEK/tau cells expressing HA_3-PP2Ac and either wild type FLAG-α4 (*α4 WT*) or FLAG-α4 G256* (*α4-G256**) were fixed in a 96-well plate and subjected to in-cell Western using antibodies recognizing the FLAG epitope, HSP90, total tau, or the indicated phospho-tau epitope. The phospho-tau signal was normalized to the total tau signal in each well, and the normalized phospho-tau values in α4 G256*-expressing cells were compared with the corresponding values in α4 WT-expressing cells, which were set at 1. Likewise, the FLAG-α4 signal was normalized to the HSP90, and the normalized α4 values in G256*-expressing cells were compared with corresponding values in α4 WT-expressing cells, which were set a 1. Values represent means +/- SEM; *, $p < 0.05$, **, $p < 0.01$.

PP2Ac at microtubules and consequential increased phosphorylation of PP2A-sensitive sites within tau.

α4 cleavage is decreased in OS.

Loss of function mutations in the MID1 gene are the underlying cause of OS (Quaderi et al., 1997), a congenital disorder characterized by defects in midline development, increases in microtubule-associated PP2A activity, and hypophosphorylation of MAPs (Trockenbacher et al., 2001). To determine if α4 cleavage is altered in OS, we probed lysates of human embryonic fibroblasts derived from a fetus with OS (OS-HEFs) and control HEFs for the presence of the α4 cleavage product (α4ΔC). The amount of cleaved α4 detected in the OS-HEFs was significantly reduced compared to control HEFs (Fig. 32). Furthermore, like the HEK293FT cells expressing FLAG-α4 (Fig. 27B), treatment of the HEFs with calpain inhibitors also protected endogenous α4 from cleavage (Fig. 33). These findings support the notion that defects in MID1 function, as is the case in OS cells, may lead to reduced α4 monoubiquitination/cleavage and increased PP2A levels at microtubules, which would explain the hypophosphorylation of MAPs seen in OS (Trockenbacher et al., 2001).

α4 cleavage is increased in AD.

Amyloid-β-containing plaques and neurofibrillary tangles (NFTs), composed of hyperphosphorylated forms of tau, are hallmark features of AD (Selkoe, 2001). Although the precise role of amyloid-β plaques and NFTs in disease progression remains unknown, recent evidence indicates that these lesions are closely linked and points to a role for abnormally hyperphosphorylated tau in amyloid-β toxicity (Haass and Mandelkow, 2010; Ittner and Gotz, 2011). To determine if deregulation of tau during disease coincides with alterations in α4

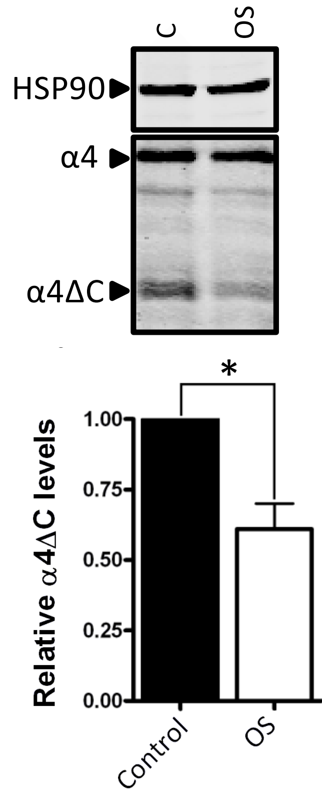


Figure 32. α4 cleavage is altered in Opitz syndrome
Lysates from Opitz syndrome-derived HEFs (*OS-HEFs*) and aged-matched control HEFs were analyzed by Western blotting using antibodies recognizing HSP90, α4, and PP2Ac. The graph depicts the relative $\alpha 4\Delta C$ levels [ratio of $\alpha 4\Delta C$ signal to total α4 signal ($\alpha 4\Delta C$ + full-length α4)] in the two cell types with the relative $\alpha 4\Delta C$ levels in control HEFs set at 1. Values represent means +/- SEM; *, $p < 0.001$.

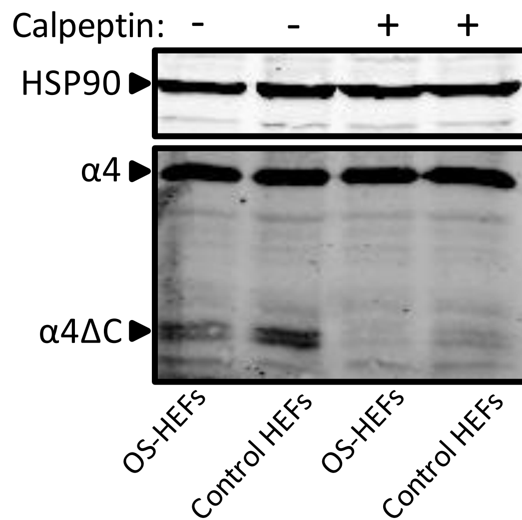


Figure 33. Calpeptin inhibits α4 cleavage in OS HEFs
Opitz syndrome-derived HEFs (*OS-HEFs*) and aged-matched control HEFs were treated with 10 µM calpeptin for 4 hours prior to lysis. Cell lysates were analyzed by Western blotting using antibodies recognizing HSP90 and α4.

cleavage, and hence PP2Ac stability, we analyzed α4 cleavage in temporal cortex tissue samples from 10 Alzheimer's cases and 5 control cases. As shown in Fig. 34, α4 cleavage was significantly enhanced in AD brains.

α4 cleavage is increased in oncogenic melanocytes.

Given that α4 is highly expressed in a variety of primary human cancers (Chen et al., 2011), we examined α4 cleavage in multiple melanoma cell lines (SK-MEL2 and SK-MEL5) and control melanocytes (MEL-ST). Western analyses of these lysates showed an increase in α4 cleavage in the melanoma cell lines, as compared to control cells (Fig. 35). These data indicate misregulation of α4 cleavage could play a role in the development of oncogenic cells.

Development of an α4 cleavage product-specific antibody.

Since we are only able to identify the cleavage of α4 using an antibody that also detects full-length α4, we sought to create an α4 cleavage product-specific antibody. Four mice were inoculated with a KLH-conjugated peptide corresponding to the final seven amino acids for the α4 cleavage product (CAQAKVF). Western analysis of HEK293FT cells expressing empty vector, α4 WT, α4 1-256, or α4 1-222 showed that the mouse antisera only detected the cleaved form of α4 and not the full-length version of the protein or a mutant form of α4 expressing amino acids 1-222 (Fig. 36), while the control α4 N-terminal specific antibody detected both the full-length and cleaved forms of α4. Hybridoma production has begun in order to create a monoclonal mouse antibody; however, based on these results currently we have a polycolonal mouse antibody that successfully detects only the cleaved form of α4.

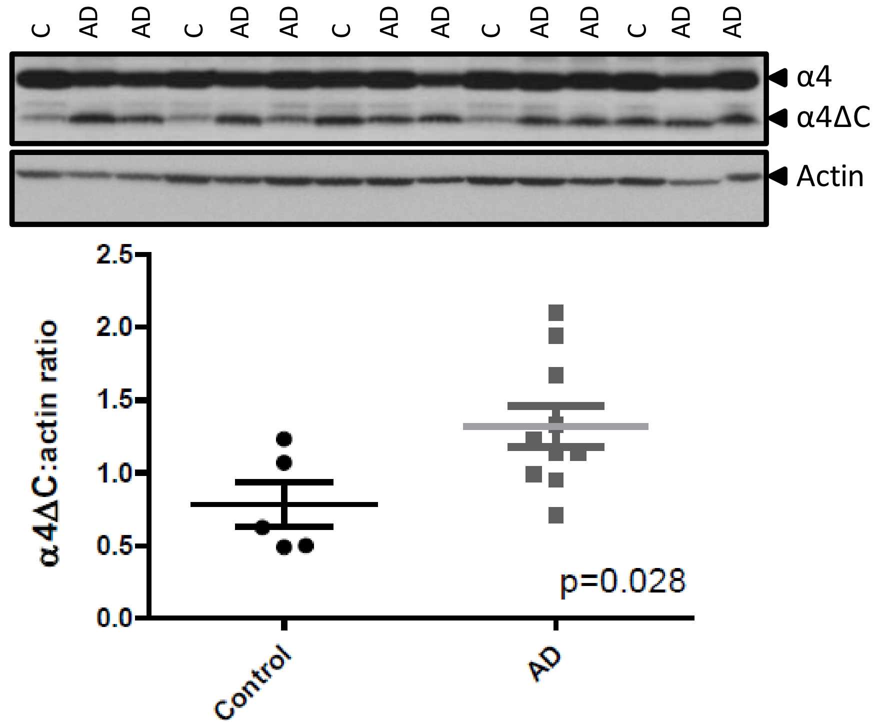


Figure 34. α4 cleavage is altered in Alzheimer's disease
Trios of age-matched AD cases (N=10) and control patients (N=5) post mortem temporal cortex tissue samples were analyzed by Western for full-length α4 and α4ΔC levels as described above. Samples are loaded in order of descending age. AD is Alzheimer's disease case, C is normal control case. Quantification of α4ΔC levels were carried out using Adobe Photoshop analysis functions and normalized to actin levels. Mean α4ΔC/actin ratios are 1.3 +/- 0.14 for AD cases and 0.78 +/- 0.15 for controls. Depicted error represents SEM. The difference between AD cases and controls is statistically significant; $p=0.028$.

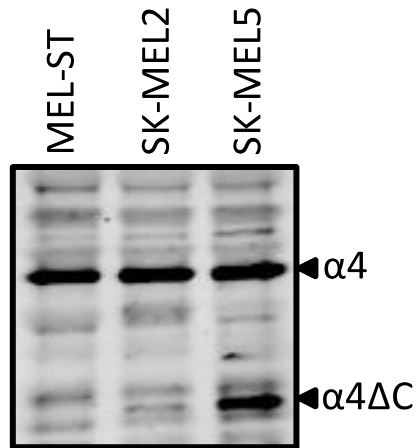


Figure 35. α4 cleavage is altered in melanoma cells
Cell lysates from control melanoma cells (MEL-ST) and a panel of melanocyte cell lines (SK-MEL2 and SK-MEL5) were analyzed via SDS-PAGE and Western for changes in α4 cleavage using an α4 specific antibody.

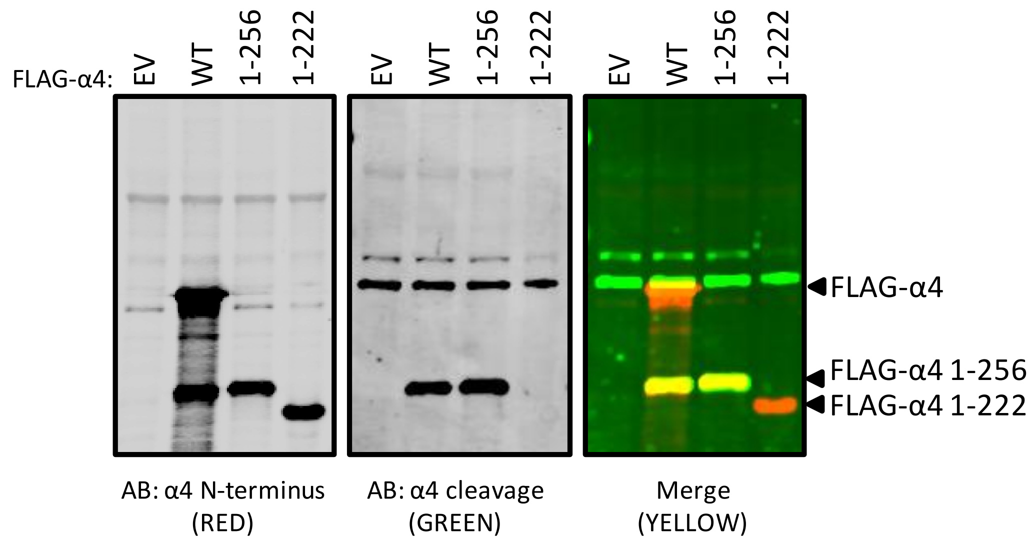


Figure 36. α4 cleavage specific antibody detects only the α4 cleavage fragment
HEK 293FT cell lysates expressing either α4 wild type (WT), cleavage memetic (1-256), or short form (1-222) were analyzed via SDS-PAGE and Western using an α4 N-terminal or cleavage specific antibody.

Discussion

Our studies indicate that MID1-dependent regulation of PP2Ac levels at microtubules (via α4 monoubiquitination/cleavage-induced PP2Ac degradation) plays a crucial role in maintaining the proper phosphorylation state of MAPs. Defects in this phosphatase regulatory process, such as those that occur in OS (mutated MID1, decreased α4 cleavage, stabilization of microtubule-associated PP2Ac) and AD (increased α4 cleavage, destabilization of microtubule-associated PP2Ac), may explain the MAP hypophosphorylation and tau hyperphosphorylation seen in OS and AD, respectively. At the molecular level, increases in intracellular calcium have been shown to promote tau hyperphosphorylation (Zempel et al., 2010). Since α4 cleavage is mediated by calpains (Fig. 27), which can be hyperactivated in response to elevated calcium flux (Kambe et al., 2005), it is enticing to speculate that the hyperphosphorylation of tau could result from upregulated α4 cleavage and a loss of PP2Ac at microtubules. Pharmacological agents that increase microtubule-associated PP2A levels, by interfering with α4 monoubiquitination or cleavage, are an attractive avenue for the treatment of AD and other tauopathies. Two drugs that should be considered in this regard are metformin and sodium selenate, as they have been shown to stabilize tau-associated PP2A and reduce tau phosphorylation in cellular and animal models of AD (Corcoran et al., 2010; Kickstein et al., 2010; van Eersel et al., 2010).

The creation of an α4 cleavage specific antibody is an important tool that will aide in our studies on the biological role of α4 cleavage. Specifically, this antibody will allow us to exploit high throughput assays to screen for compounds that can alter α4 cleavage. Furthermore, this new antibody could potentially be used as a diagnostic tool as it will allow us to perform immunohistochemical experiments as well as detect cleavage in human tissue, and will give insight into the role of cleavage in disease.

In summary, our studies have uncovered a novel regulatory process for PP2A involving ubiquitination-induced cleavage of α4, which plays a crucial role in modulating PP2Ac levels in both normal and pathophysiological conditions.

CHAPTER VI

POTENTIAL REGULATION OF α4 MONOUBIQUITINATION BY PHOSPHORYLATION

Introduction

α4 is targeted for monoubiquitination by the E3 ubiquitin ligase MID1 leading to calpain mediated cleavage of α4 between residues 255 and 256 in the C-terminus (Chapter IV). This novel monoubiquitination-dependent cleavage was found to be an important regulator of PP2Ac stability and misregulation of α4 cleavage was identified in multiple MAP-dependent pathologies (Chapter V). α4 was initially discovered as a 52 kDa phosphoprotein associated with the Ig receptor associated protein MB-1 in B cells (Inui et al., 1995). Furthermore, the phosphorylation of α4 was shown to occur on a serine residue (Murata et al., 1997); however, little is known about the role of α4 phosphorylation. Interestingly, phosphorylation can regulate protein ubiquitination (Hunter, 2007) raising the intriguing possibility that the phosphorylation state of α4 influences its monoubiquitination.

While both PKC and $p56^{lck}$ phosphorylate α4 *in vitro*, the kinase responsible for α4 phosphorylation in cells is unknown. Recent studies have shown knockdown of the mammalian sterile-20 like kinsas 2 (MST2) leads to a proteasome-dependent decrease in the level of PP2Ac in cells, making MST2 a potential kinase for α4 phosphorylation (Kilili and Kyriakis, 2010).

In this chapter, we demonstrate that MST2 phosphorylates α4 *in vitro* and identified two amino acids in α4 that are phosphorylated. We also show that phosphorylated α4 fails to undergo monoubiquitination.

Materials and Methods

Plasmids

Construction of the mammalian expression constructs for FLAG-tagged wild type α4 and 1-256 was described previously (Watkins et al., 2012). Proper construction of both plasmids was verified by automated sequencing (Vanderbilt University DNA core facility).

Antibodies and reagents

The rabbit polyclonal α4 antibody was from Bethyl Laboratories (Montegomery, TX). The mouse monoclonal ubiquitin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Purified MST2 was from ProQuinase (Freiburg, Germany)

Cell culture and transfection

HEK293FT cells were grown at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 2 mM L-glutamine. Cells were transfected with the indicated mammalian expression constructs using Fugene6 (Roche; Indianapolis, IN) according to the manufacturer's protocol.

In vitro kinase assay

A reaction mixture containing 100 ng FLAG purified FLAG-α4, 100 ng MST2 kinase, 40 μM ATP, and 10 μCi [γ-^{32}P]ATP was incubated in the presence or absence of purified PP2Ac and okadaic acid for 30 min at 30° C and then terminated by the addition of 5x SDS sample buffer. The reaction mixtures were subjected to SDS-PAGE and analyzed by Western and autoradiography for the presence of phosphorylated α4. *In vitro* phosphorylated α4 for use in

mass spectrometry was created using the same reactions as indicated above using non-radiolabeled ATP in place of 10 µCi [γ-^{32}P]ATP.

Western analysis

See “Materials and Methods” for details of this methodology in Chapter II.

In vitro ubiquitination assay

A reaction mixture was made containing 150 ng purified FLAG-MID1, 2.5 µM E2 Ube2D3, 100 nM E1, 2.5 µM Ub, ATP/Mg^{2+} solution, 1 µM okadaic acid and either 50 µg *in vivo* phosphorylated or non-phosphorylated control purified FLAG-α4 WT and incubated at 37°C for 1 hour. Reactions were quenched by the addition of 5x SDS sample buffer and analyzed by Western.

Mass spectrometry

FLAG-α4 was immunopurified from HEK293FTs cell treated plus or minus 1 µM okadaic acid and subjected to SDS-PAGE. Phosphorylated FLAG-α4 was visualized by Colloidal Blue staining and excised from the gel. Excised gel fragments were subjected to mass spectrometry (see Chapter IV methods).

Results

MST2 phosphorylated α4 *in vitro*

α4 was initially characterized as a phosphorylated protein (Inui et al., 1995); however, the kinase responsible for this post-translational modification has not yet been reported. Since

MST2 can alter the levels of PP2Ac in a proteasome-dependent manner (Kilili and Kyriakis, 2010), I tested whether MST2 plays a role in the phosphorylation of α4. Purified FLAG-α4, in the presence or absence of PP2Ac, was used as a substrate in a *in vitro* kinase reaction mixture containing purified MST2 kinase and [γ-^{32}P]ATP plus or minus okadaic acid. Western analysis and autoradiography revealed that MST2 phosphorylates α4 and that PP2Ac dephosphorylates the phospho-α4 species (Fig. 37). These data indicate that MST2 may function as the kinase for α4 in cells and also indicates that PP2Ac dephosphorylates the amino acid(s) targeted by MST2 for phosphorylation on α4.

MST2 phosphorylates α4 on T89 and T314

To further understand the biological consequence of α4 phosphorylation we first sought to determine the site(s) of modification on α4. Purified FLAG-α4 was phosphorylated using purified MST2 in an *in vitro* kinase assay and then subjected to SDS-PAGE. Phosphorylated FLAG-α4 was visualized by Colloidal Blue Coomassie staining, and excised from the gel for analysis by mass spectrometry, which identified both T89 and T314 as phospho-sites. To determine if these residues are phosphorylated in cells, I immune-purified FLAG-α4 from lysates of HEK293FT cells treated with okadaic acid and then fractionated the sample by SDS-PAGE (Fig. 38). Mass spectrometry analysis of the slower migrating FLAG-α4 species identified T314 as a site of phosphorylation on cellular α4, consistent with what was found with *in vitro* MST2 phosphorylated α4. Together, these data indicate that T314 is a novel α4 phosphorylation site and MST2 could be the cellular kinase of α4.

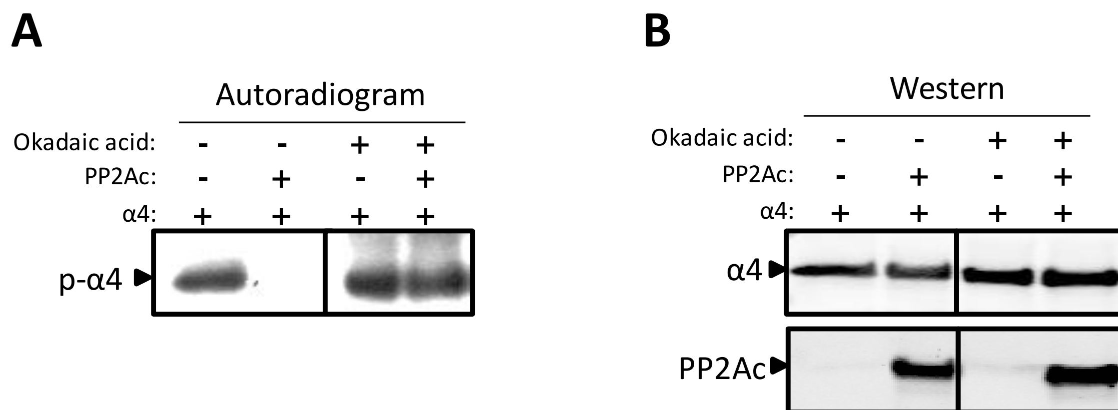


Figure 37. α4 is phosphorylated *in vitro* by MST2
MST2 (100 ng) and FLAG-α4 (F-α4; 100 ng) were incubated with (+) or without (-) PP2Ac (50 ng) in the presence of 1 mM okadaic acid or DMSO (vehicle) for 30 min at 30°C in kinase assay buffer containing ^{32}P-ATP. Reaction mixtures were terminated by adding SDS sample buffer and subjected to SDS-PAGE followed by autoradiography (ARG) and Western blotting (WB) with antibodies recognizing FLAG-α4 and PP2Ac.

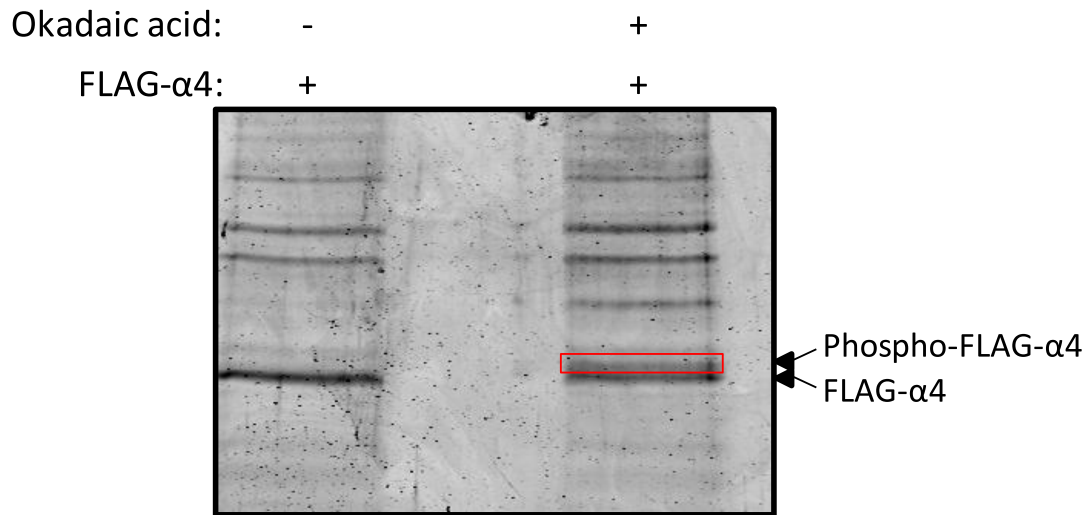


Figure 38. Okadaic acid induces a phosphorylated shift for α4
HEK293FT cells expressing FLAG-α4 were treated in the presence (+) or absence (-) of okadaic acid prior to lysis. FLAG-α4 was purified from lysate by FLAG immunoprecipitation and samples were analyzed by comassie for an okadaic acid induced shift. The area highlighted in red was excised from the gel and used for mass spectrometry analysis of endogenous phosphorylation sites

Phosphorylation of α4 inhibits its monoubiquitination

Protein phosphorylation and ubiquitination are closely linked, and phosphorylation can both inhibit or promote protein ubiquitination (Hunter, 2007). In light of prior studies implicating a role for MST2 in the control of PP2Ac levels (Kilili and Kyriakis, 2010) and our data showing MST2 phosphorylation of α4 (Fig. 37), we hypothesized that MST2 phosphorylation of α4 may influence its ability to undergo monoubiquitination. To test this hypothesis, I performed *in vitro* ubiquitination reactions using MID1 as the E3 ubiquitin ligase and either MST2-phosphorylated α4 or non-phosphorylated α4 as the substrate. Western analysis of these reactions showed a marked decrease in the level of monoubiquitinated α4 for the MST2-phosphorylated α4, as compared to non-phosphorylated α4 (Fig. 39). These data indicate that α4 phosphorylation by MST2 may inhibit the ubiquitination of α4, and suggest a possible mechanism of regulation for α4 monoubiquitination.

Discussion

In this chapter, I show that MST2 is able to phosphorylate α4 *in vitro* and that the phospho- form of α4 exhibits decreased monoubiquitination compared to non-phosphorylated α4. Furthermore, I identified two phosphorylation sites in α4 (T89 and T314), with T314 occurring both *in vitro* and in cells. Interestingly, the identification of two threonine residues as phosphorylation site(s) contrasts with previous work showing that α4 phosphorylation occurs on a serine residue (Murata et al., 1997). This data raises the intriguing possibility that α4 is phosphorylated at multiple sites by different kinases, and these distinct phosphorylation events may differentially alter the function of α4. Although further studies are needed to determine the precise role of α4 phosphorylation, my studies indicate threonine phosphorylation may be a key regulator for α4 monoubiquitination and its subsequent cleavage.

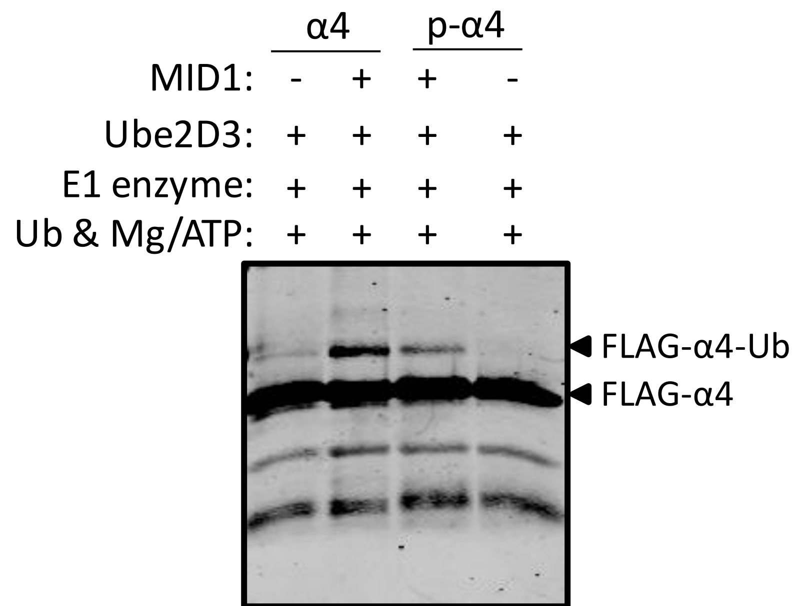


Figure 39. Phosphorylation of α4 inhibits its monoubiquitination
Purified FLAG-α4 or *in vitro* MST2 phosphorylated α4 was incubated in a ubiquitination assay solution containing the E1 and E2 enzymes plus (+) or minus (-) the FLAG purified E3 ligase MID1. The reactions were analyzed via Western using an α4 specific antibody.

Studies in Chapter IV identified a novel role for α4 monoubiquitination and cleavage in the control of PP2Ac levels. One major question that stems from these studies is "how is monoubiquitination of α4 controlled?" Our data indicate a potential regulatory mechanism in which α4 phosphorylation by MST2 inhibits MID1-dependent monoubiquitination of α4. Based on our model (Fig. 29), this would lead to decreased α4 cleavage thereby stabilizing the cellular levels of PP2Ac. Consistent with this hypothesis, when MST2 is knocked down in cells, which would lead to decreased α4 phosphorylation and increased α4 monoubiquitination, there is a proteasome dependent decrease in the level of PP2Ac (Kilili and Kyriakis, 2010). More studies will be needed to determine if MST2 phosphorylates α4 in cells and to verify the inhibitory role of α4 phosphorylation in the regulation of its monoubiquitination.

CHAPTER VII

SUMMARY AND FUTURE DIRECTIONS

Summary and Significance

Studies described in this thesis identify a novel regulatory mechanism for α4 in which monoubiquitination induces calpain-mediated cleavage of α4, changing the activity of this atypical phosphatase regulator from protective to destructive. The role of α4's regulation of PP2Ac has been controversial, as α4 has been shown to both protect PP2Ac from polyubiquitination (Kong et al., 2009; LeNoue-Newton et al., 2011; McConnell et al., 2010) and promote PP2Ac polyubiquitination (Trockenbacher et al., 2001). Our studies provide a mechanism whereby α4 can possess both activities based on its ubiquitination state. A series of *in vitro* ubiquitination assays and MID1 knockdown experiments revealed that α4 is targeted for monoubiquitination by the E3 ubiquitin ligase MID1, and that MID1 fails to facilitate the polyubiquitination of PP2Ac (Figs. 18 & 19). These data called into question the initial model for α4 regulation of PP2Ac, whereby α4 serves as a scaffold to facilitate the polyubiquitination of PP2Ac by MID1 (Trockenbacher et al., 2001). Interestingly, analysis of α4 expression from cells that harbored MID1 siRNA or treated with an E1 inhibitor revealed that the C-terminus of α4 was targeted for cleavage and that the cleavage pattern mirrored the changes in α4 monoubiquitination (Figs. 19, 20, & 21). Further study of α4 showed that the cleavage occured between F255 and G256 in α4 and was mediated by calpain (Figs. 23 & 24). The calpain cleavage motif in α4 is conserved among many species and studies of *Drosophila* Tap42, the homologue of mammalian α4, showed that it too is subject to both monoubiquitination and cleavage, thus indicating this may be an evolutionarily conserved mechanism of α4 regulation

(Figs. 24 & 25). To identify a cellular role for α4 cleavage, we first tested the ability of α4 to protect PP2Ac from degradation. Studies of PP2Ac turnover revealed that α4 cleavage switches α4's function from protective to destructive towards PP2Ac (Fig. 22). These data, coupled with the fact the α4 cleavage site lies in the MID1 binding domain of α4 and cleaved α4 no longer binds to MID1 (Fig. 26), led us to formulate a new model for the role of α4 in the regulation of PP2Ac. In this new model (Fig. 27), MID1 facilitates α4 monoubiquitination leading to a conformational change in which the UIM of α4 binds in a *cis* fashion to the attached monoubiquitin. This conformational change exposes a calpain cleavage site allowing cleavage of the C-terminus of α4, which liberates the α4·PP2Ac complex from MID1 and ultimately causes both the loss of PP2Ac localization at the microtubules and increased PP2Ac turnover. Further exploration of this model, as well as potential mechanisms of α4 monoubiquitin regulation, is discussed under future directions.

Initial studies on the role of α4 regulation of PP2Ac revealed changes in the phosphorylation of microtubule-associated proteins (MAPs) in cells derived from patients with Opitz syndrome, which is characterized by mutations in MID1 (Trockenbacher et al., 2001). Since our data indicate MID1 regulates PP2Ac via α4 monoubiquitination and cleavage, we tested whether α4 cleavage is involved in regulating MAP phosphorylation and whether it is altered in various MAP-dependent diseases. Consistent with an α4 cleavage-dependent decrease in PP2Ac levels at the microtubules, we observed a significant increase in tau phosphoryation in cells expressing the cleavage mimetic form of α4 relative to wild type α4-expressing cells, thus cleavage is important for proper MAP phosphorylation (Fig. 28). We also examined α4 cleavage in two diseases characterized by aberrant MAP phosphorylation. In Optiz syndrome, a disease characterized by decreased MAP hypophosphorylation (Trockenbacher et al., 2001), we saw a significant decrease in cleaved α4 (Fig. 29), whereas in Alzheimer's disease, which is

characterized by tau hyperphosphrylation (Selkoe, 2001), we saw a significant increase in the amount of cleaved α4 (Fig. 30). Taken together, these data indicate that α4 cleavage is important for the maintenance of proper MAP phosphorylation, and indicate that misregulated cleavage may be involved in the pathogenesis of these diseases. Future experiments aimed at elucidating whether α4 cleavage is either a cause or an effect of these diseases are discussed below ("Future Directions").

Studying the biology of PP2Ac polyubiquitination proved to be difficult from the start due to the inherent limitations in technologies available to study polyubiquitinated proteins. However, I was able to develop multiple experimental platforms that allowed me to visualize PP2Ac polyubiquitination both *in vitro* and in cells (Figs. 8-12), and was able to show for the first time the presence of polyubiquitinated endogenous PP2Ac in target cells (Fig. 11). Furthermore, these studies allowed me to determine the polyubiquitin chain topology for PP2Ac, which occurs via K48 ubiquitin linkage, consistent with PP2Ac polyubiquitination targeting its degradation (Fig. 13). Another goal of these studies was to identify the ubiquitin acceptor site of PP2Ac. These experiments proved challenging as mutatgeneis of multiple lysine residues did not show any changes in PP2Ac polyubiquitination (Fig. 14). As a secondary means to identify the site, we employed a functional assay that measured the turnover of PP2Ac mutants, these experiments identified K41 as the ubiquitin acceptor site within PP2Ac (Fig. 17). These data fit in well with the crystal structure of the α4·PP2Ac complex solved by our collaborator (Dr. Yongna Xing, University of Wisconsin), and are currently being incorporated into a manuscript.

Future Directions

My studies identified a novel phosphatase regulatory mechanism in which MID1 monoubiquitinates α4, leading to calpain-mediated cleavage of α4 and subsequent proteasomal

degradation of PP2Ac. Although these studies have resulted in a reformulation of the mechanism of MID1/α4-dependent ubiquitination of PP2Ac, questions still remain on how this novel mechanism is regulated and its involvement in human pathologies.

Define the molecular mechanisms that regulate the MID1·α4·PP2Ac complex

Mapping the phosphorylation site(s) within α4.

Our preliminary data indicate that MST2 phosphorylates α4 at T89 and T314, which attenuates subsequent monoubiquitination of α4. This is an intriguing finding and could be a potential mechanism for regulating α4 monoubiquitination. To better understand the role of α4 phosphorylation we will need to perform assays to confirm the sites of phosphorylation that were detected *in vitro* and in cells by mass spectrometry. We have generated both phospho-mimetic (T to D or E) and phospho-null (T→A) mutants, which can be tested in both *in vitro* phosphorylation assays and cellular assays to verify their inability to undergo phosphorylation.

Does α4 phosphorylation affect its monoubiquitination and cleavage?

We have data from our *in vitro* ubiquitination assays showing that phosphorylated α4 exhibits decreased monoubiquitination, when compared to non-phosphorylated α4. To determine if this also occurs in cells, we can test the α4 phospho-site mutants (e.g., phospho-mimetic and phospho-null) for their ability to undergo both monoubiquitination and cleavage in our standard cellular ubiquitination assay. If α4 phosphorylation prevents its monoubiquitination in cells in a manner similar to what we previously identified *in vitro*, then we would expect to see much less monoubiquitination and cleavage of the phospho-mimetic mutants compared to the phospho-null mutant. The major caveat to this approach is that

phospho-mimetic mutants do not always act the same as phosphorylated proteins. If this is the case, we would expect similar results for both the phospho-null and phospho-mimetic mutants. As an alternative approach to examine the effects of α4 phosphorylation on ubiquitination, we could monitor α4 monoubiquitination and cleavage in cells overexpressing or lacking candidate kinases, like MST2.

Is MST2 the cellular kinase for α4?

Our *in vitro* kinase assays demonstrate that MST2 is able to phosphorylate α4; however, it remains to be determined if this kinase is responsible for phosphorylating α4 in cells. MST2 was chosen as a candidate kinase for the regulation of α4 function based on prior studies that revealed that knockdown of MST2 led to decreased PP2Ac levels (Kilili and Kyriakis, 2010). To test if MST2 also phosphorylates α4 in cells, we can exploit MST2 specific knockdown in target cells. If MST2 phosphorylates α4 in cells, we should see a decrease in the phospho-shift of α4 via SDS-PAGE in cells harboring MST2 siRNA. Alternatively, we could overexpress MST2 and test for enhanced α4 phosphorylation. A caveat to these studies is the possibility that multiple kinases are able to phosphorylate α4. If this is the case, then we would still expect some change in α4 phosphorylation; however, these changes may be relatively small.

Does TIP, an atypical PP2Ac regulatory protein, also regulate α4 phosphorylation?

The atypical PP2Ac regulatory protein TIP has previously been found to inhibit the catalytic activity of the phosphatase (McConnell et al., 2007). Additional studies from our lab have provided genetic and biochemical evidence for a link between α4 and TIP in the control of PP2Ac function (unpublished data). Our studies of α4 phosphorylation indicate that PP2Ac is able to dephosphorylate α4, which would lead to its monoubiquitination and subsequent

cleavage. We hypothesize that TIP-mediated inhibition of PP2Ac in this complex will block the ability of PP2Ac to dephosphorylate α4. To test this hypothesis, we can utilize our *in vitro* kinase assay +/- TIP to determine if we see changes in α4 phosphorylation in the presence of PP2Ac. Furthermore, since dephosphorylation of α4 promotes ubiquitination cellular ubiquitination assays can be performed either in the presence of overexpressed TIP or in TIP knockdown cells to test if α4 monoubiquitination is altered. Together these studies would give a good indication as to whether TIP was able to modulate the monoubiquitination and cleavage of α4 in a phosphorylation dependent manner.

Additional studies to firmly establish the validity of our model.

The data presented in this thesis have allowed us to develop a new model for the functional role of the MID1·α4·PP2Ac complex; however, some elements of the mechanism still need to be verified experimentally. First, we believe that the monoubiquitination of α4 causes a conformational change in which the UIM of α4 binds in *cis* to the attached ubiquitin, thereby unmasking a calpain cleavage site within α4. To test this idea, we can exploit an α4 fusion protein containing ubiquitin at the N-terminus of α4, which should engage the UIM within α4. If our hypothesis is correct, we can expect to see nearly all of this protein cleaved in cells since its calpain cleavage site would be persistently exposed. A second strategy would be to directly identify the α4 ubiquitination site using mass spectrometry or site-directed mutagenesis. Once we identify the site of modification we can exploit a novel approach pioneered by Tom Muir (McGinty et al., 2009), in which we could fuse ubiquitin to its target lysine within α4 and monitor its susceptibility to calpain cleavage. We could also attempt to elucidate the crystal structure of ubiquitin bound to α4 and compare this structure to the previously determined structure of unmodified α4 (LeNoue-Newton et al., 2011).

A second feature of our model where further experimentation is necessary is identification of the E3 ubiquitin ligase for PP2Ac. Our data show that MID1 is the E3 ligase for α4 but we do not have any data indicating if it is able to facilitate PP2Ac polyubiquitination. Although the identification of E3 ligases for a target protein is very challenging, one method would be to identify potential candidates based on known interacting proteins and test each potential E3 ligase individually. EDD and MID2 are candidate E3 ubiquitin ligases that have been implicated in the regulation of PP2A. These two candidates could be tested for their ability to polyubiquitinate PP2Ac using our standard ubiquitination assays. As an alternative, a shotgun proteomics approach could be used to identify novel binding partners for PP2Ac that may include potential E3 ligases, which could then be tested for their ability to polyubiquitinate PP2Ac.

Studies of α4 cleavage in Alzheimer's disease and cancer

Alzheimer's disease (AD)

Amyloid-β-containing plaques and neurofibrillary tangles (NFTs), composed of hyperphosphorylated forms of tau, are hallmark features of AD (Selkoe, 2001). Although the precise role of amyloid-β plaques and NFTs in disease progression remains unknown, recent evidence indicates that these lesions are closely linked and points to a role for abnormally hyperphosphorylated tau in amyloid-β toxicity (Haass and Mandelkow, 2010; Ittner and Gotz, 2011). Based on our data presented in Chapter V, it is enticing to speculate that the hyperphosphorylation of tau could result from upregulated α4 cleavage and a loss of PP2Ac at microtubules. In-cell Westerns allowed us to identify changes in tau phosphorylation in cells expressing WT α4 as compared to a cleavage mimetic form of α4; however, these studies relied

on the overexpression of α4 and PP2Ac. To further this work, we could examine the effects of α4 knockdown on tau phosphorylation. To look more directly at the effect of α4 cleavage on tau phosphorylation, we can overexpress either WT α4 or cleaved α4 in cells lacking endogenous α4 to try and rescue the knockdown phenotype. Based on our studies to date, knockdown of α4 should increase tau phosphorylation, which we hypothesize would be rescued by the reintroduction of WT α4 but not cleaved α4. To further understand the involvement of α4 cleavage in the regulation of tau, we could examine tau aggregation using immunofluorescence in cells overexpressing WT or cleaved α4. In addition, since MID1 influences the monoubiquitination and cleavage of α4, it would be interesting to examine the levels of MID1 in AD tissue compared to control tissue.

Cancer

Data presented in Chapter V of this thesis show that α4 cleavage is increased in melanoma cell lines, as compared to control cell lines. These findings raise the possibility that misregulation of α4 cleavage could play an important role in tumor development. To explore a role for α4 cleavage in oncogenesis we can utilize cellular assays that examine cell growth, survival, transformation, and migration. In each assay, the effect of expressing either WT α4 or cleaved α4 would be compared to elucidate a role for α4 cleavage in oncogenesis/tumor progression. We would expect to see increased "cancer-like" characteristics, such as increased growth and anchorage-independent growth, of cells expressing the cleaved form of α4 compared to the WT form. To further these studies, cells expressing WT or cleaved α4 could be introduced into nude mice to allow us to test for tumor formation, tumor size, and monitor for metastases. These studies will allow us to explore whether cleavage contributes to the

formation of tumors, and will lay the foundation for future studies aimed at determining whether α4 cleavage is a cause or an effect of the disease.

Monitoring cleavage in oncogenic cell lines and human pathologies

We are in the process of developing an α4 cleavage product-specific monoclonal antibody. The availability of such an antibody will allow us to screen a myriad of cell lines and tissue slices from disease and control tissues to determine if α4 cleavage is a hallmark of multiple tumors. We anticipate these studies will allow us to determine if α4 cleavage is a biomarker for different disease states. Furthermore, we can use this antibody for immunohistochemical experiments to examine the expression and localization of cleaved α4 in diseased tissue.

Identification of antagonists for α4 monoubiquitination and cleavage

Recent literature has identified sodium selenate and Metformin as two drugs that alter tau phosphorylation in a PP2A-dependent manner (Corcoran et al., 2010; Kickstein et al., 2010; van Eersel et al., 2010). Therefore, it would be interesting to test whether these compounds influence α4 monoubiquitination and cleavage. Furthermore, we believe that an α4 cleavage product-specific antibody would be valuable for high-throughput screens to identify a specific antagonist for α4 cleavage. Based on the studies presented in this thesis (Fig. 29), we predict that an antagonist of α4 cleavage could be of therapeutic value for diseases characterized by deregulated microtubule-associated PP2A and MAP hyperphosphorylation including AD.

Studying α4 cleavage in an in vivo model system

Our studies suggest that changes in α4 cleavage may be linked to diseases associated with changes in MAP phosphorylation; however, we currently do not know if altered α4 cleavage is the cause or the result of the disease. To better understand the role of cleavage in disease, we could utilize *in vivo* model systems. For these studies, we first would need to identify an α4 mutation that prevents its cleavage either by direct mutatation of the cleavage site, phospho-site, or ubiquitin acceptor site. Transgenic mice harboring a ubiquitination/cleavage-resistant form or a cleavage mimetic form of α4 could be generated and subsequently analyzed for the pathological hallmarks of AD or OS.

Concluding Remark

In conclusion, these studies have uncovered a novel PP2A regulatory mechanism involving monoubiquitin-dependent cleavage of α4. Moreover, these studies identified defects in this phosphatase regulatory mechanism (i.e., α4 cleavage), which could potentially cause various human diseases, including AD and cancer.

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