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Regulation and Function of PP2A Family Phosphatases

By

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Regulation and Function of PP2A Family Phosphatases

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B.S., Korea University, 2005
M.S., Korea University, 2007

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An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in Graduate Division of Biological and Biomedical Sciences
Biochemistry, Cell and Developmental Biology
2015

Abstract

Regulation and Function of PP2A Family Phosphatases

By Juyeon Hwang

PP2A is a multifunctional serine/threonine protein phosphatase that is critical for a variety of cellular processes and thus its dysfunction has been linked to human diseases including cancer and Alzheimer's disease (AD). PP2A is a heterotrimeric enzyme composed of a structural A subunit, a catalytic C (PP2Ac) subunit and one of many regulatory B-type subunits. Reversible carboxyl methylation of PP2Ac on its C-terminal leucine residue by leucine carboxyl methyltransferase 1 (LCMT-1) and protein phosphatase methylesterase 1 (PME-1) differentially regulates the binding of certain B-type subunits and thus PP2A heterotrimer formation and function. While the PP2A family phosphatases, PP4 and PP6, possess the conserved C-terminal leucine residue, only PP4c has been shown to be methylated before. In Chapter 2.1, I hypothesized that LCMT-1 is the major methyltransferase for both PP4 and PP6, and that methylation of PP4c and PP6c regulates holoenzyme assembly of PP4 and PP6. Results indicated that PP4c and PP6c are highly methylated on their C-termini and that LCMT-1 is the major methyltransferase responsible for methylating these phosphatases. In addition, blocking PP4c methylation by LCMT-1 knockout resulted in loss of methylation-dependent PP4 holoenzyme complexes. Consistent with this, phosphorylation of PP4R1 substrate, HDAC3, was increased by LCMT-1 loss. These results support the hypothesis that carboxyl methylation of PP2A family phosphatase C subunits is a general strategy for regulating holoenzyme assembly and function of these phosphatases. In Chapter 2.2, my objective was to determine the molecular organization of PP2A complexes containing the striatin family members as their B-type subunits. This novel, striatin-associated PP2A complex has been recently implicated in human diseases, especially cerebral cavernous malformations (CCM). Using a structure-function analysis, I found that striatin-associated PP2A regulates the phosphorylation and activation of Mst3 kinase. Finally, in Chapter 2.3, I found that inhibition of PP2A by okadaic acid increased recruitment of Mst3 and Mst4 kinases to striatin. Altogether, these studies provide insights into the regulation of PP2A family phosphatase holoenzyme assembly and function by methylation. Additionally, these studies also provide further insights into the molecular organization and function of striatin-associated PP2A complexes.

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Acknowledgements

First and foremost, I would like to express my sincerest gratitude to my dear God for giving me this opportunity to complete my Ph.D. research and for guiding me through all the ups and downs during my journey through grad school. I would like to give my greatest thanks to my advisor, Dr. David Pallas, for all of his support, excellent guidance, and patience throughout my stay at his lab. I would also like to recognize and thank my committee members, Dr. Andrew Kowalczyk, Dr. Carlos Moreno, Dr. Winfield Sale, and Dr. Keith Wilkinson, for their kindness, support, thoughtful advice, and encouragements. I am also very grateful to the past and current lab members for their help.

I would like to express my deepest gratitude to my loving parents and family, and dedicate this work to them. They have been the best supporters and the greatest motivation and inspiration to my life. I would never have been able to complete my Ph.D. without their love, prayers, and guidance. I would also like to remember and thank my dog, Julie. She was the most adorable furry friend of mine. I can't name them all here but I would like to thank all of my lovely friends too for their kind support and for all of our laughter. Last but not least, I am truly and deeply grateful to my lovely husband for his generous patience and support. I am truly blessed to have all of you in my life. Thank you.

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CHAPTER 1

General Introduction

Protein phosphatase 2A (PP2A)

Phosphorylation/dephosphorylation of proteins acts as a key switching mechanism for regulating a multitude of signal transduction pathways. The human genome encodes about 518 protein kinases, composing about 1.7% of all human genes [1]. By contrast, only about 147 protein phosphatases have been identified in humans to oppose the action of these kinases [2]. The unique strategy of some phosphatases to form heterotrimeric holoenzymes explains the smaller number of phosphatases as compared to that of kinases. Protein phosphatase 2A (PP2A) is one of the two most abundant serine/threonine phosphatases along with protein phosphatase 1 (PP1) and together they account for over 90% of all serine/threonine phosphatase activity in cells [3]. PP2A is a multifunctional serine/threonine protein phosphatase that is involved in numerous, critical cellular processes, including cell cycle regulation, signal transduction pathways, translation, apoptosis, and development, to name a few [4]. PP2A is ubiquitously expressed in various tissues and comprises up to 1% of total cellular protein.

PP2A structure

PP2A is a heterotrimeric protein complex composed of a structural/scaffolding A subunit, a catalytic C subunit (PP2Ac), and a B-type regulatory subunit (Fig. 1-1). The PP2A A subunit provides a structural platform for binding of B and C subunits. The A

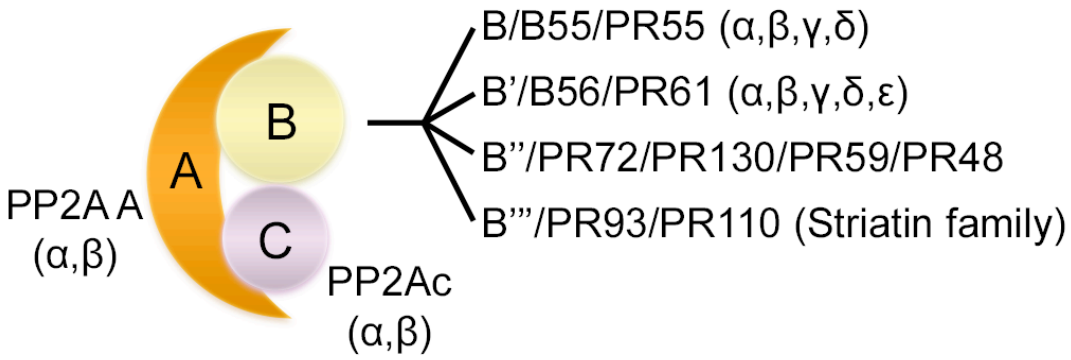

Figure 1-1. Heterotrimeric structure of the PP2A holoenzyme.

PP2A heterotrimers consist of a PP2A A subunit (α or β), a PP2A C subunit (α or β), and one of many B-type regulatory subunits (B, B', B'', or B'''). Each B-type subunit is encoded by several genes and some of them have multiple isoforms. In addition, some DNA tumor virus proteins, middle (MT) and small tumor (ST) antigens, act as viral B-type subunits and alter the function of PP2A (not shown).

subunits contain 15 HEAT (Huntingtin/Elongation factor/A subunit/TOR) repeats of about 39 amino acids [5, 6]. While the first 10 HEAT repeats bind B-type subunits, HEAT repeats 11-15 associate with PP2Ac [7, 8]. Two mammalian isoforms of the A (Aα and Aβ) and C (Cα and Cβ) subunits exist, with 86% and 97% amino acid sequence identity, respectively. There are 4 subfamilies of B-type regulatory subunits identified at present: B (B55/PR55; PPP2R2), B' (B56/PR61; PPP2R5), B'' (PR72/130/59 or 48; PPP2R3), and B''' (PR93/110; Striatin family) [4]. Each subfamily is encoded by several different genes and some of them have multiple splice variants or isoforms. PP2A A and C subunits form the core heterodimer, which then associates with one of B-type subunits, forming over 90 different PP2A heterotrimers. Association of the PP2A core heterodimer with various B-type subunits determines enzyme activity, substrate specificity, and subcellular localization of PP2A. Viral proteins including the polyoma virus small (PyST) and middle (PyMT) tumor antigens and simian virus 40 (SV40) small tumor antigen (SVST) can also bind PP2A A/C heterodimers by replacing certain PP2A B-type subunits, altering PP2A function [9]. Spatial and temporal expression of PP2A subunits also defines the enzyme characteristics. Moreover, post-translational modifications on PP2Ac play a role in regulation of PP2A activity and specificity. Additionally, modulation of PP2A activity by phosphorylation on the scaffolding A and regulatory B' family subunits has recently been reported [10].

The PP2Ac C-terminus and PP2A holoenzyme assembly

A complex and dynamic regulation exists to determine PP2A holoenzyme composition and activity of the enzyme. The PP2Ac C-terminus has a short stretch of

amino acid residues ($_{304}$TPDYFL$_{309}$) that are highly conserved among various species. These residues can be modified post-translationally. For example, T304 is a potential threonine phosphorylation site. Y307 is the site of tyrosine phosphorylation by receptor and nonreceptor tyrosine kinases, including v-Src [11]. PP2A activity is inhibited by phosphorylation on Y307 [11] or on unknown threonine residue(s) [12]. Lastly, L309 is the site of carboxyl methylation, which will be discussed in more detail in the following section.

Experiments using PP2Ac wildtype and mutant constructs that are modified either by truncation or substitution on the C-terminus showed that the PP2Ac C-terminus and post-translational modifications on it are crucial in PP2A holoenzyme construction. Truncation of the 9 PP2Ac C-terminal residues or substitution mutations on the phosphorylation sites T304 and Y307 abolished B subunit binding, without affecting the binding of a viral B-type subunit PyMT [13]. Similarly, corresponding substitution mutations of T304 and Y307 in yeast (T364 and Y367, respectively) showed decreased association with B (Cdc55) and A (Tpd3) subunits [14] (Table 1). Thus, post-translational modifications on the PP2Ac C-terminal residues influence PP2A holoenzyme assembly.

Carboxyl methylation of PP2Ac

PP2Ac is carboxyl methylated reversibly on its C-terminal leucine (Leu_{309}) residue by LCMT-1 (leucine carboxyl methyltransferase 1; 38kDa) [15-20] and demethylated by PME-1 (protein phosphatase methylesterase 1; 42kDa) [21-23] (Fig. 1-2). LCMT-1 is an AdoMet (S-adenosyl-methionine or SAM; methyl donor)-dependent methyltransferase and is inhibited by SAH (S-adenosylhomocysteine) [24, 25]. PP2Ac

	Human	*Saccharomyces Cerevisiae*
PP2A catalytic C subunit	PP2Ac	Pph21/Pph22
PP2A structural A subunit	PP2A A subunit	Tpd3
PP2A regulatory B-type subunit	PP2A B subunit	Cdc55
	PP2A B' subunit	Rts1
	PP2A B'' subunit	-[a]
	Striatin family (B''')	Far8
PP4 catalytic C subunit	PP4c	Pph3
PP4 regulatory subunit	PP4R1	-[b]
	PP4R2	YBL046w; Psy4
	PP4R3	YNL201c; Psy2
	PP4R4	-[b]
PP6 catalytic C subunit	PP6c	Sit4
PP6 regulatory subunit	PP6R1 (SAPS1)	SAP155
	PP6R2 (SAPS2)	SAP185
	PP6R2 (SAPS3)	SAP190
	-[c]	SAP4
	ARS-A	-[d]
	ARS-B	-[d]
	ARS-C	-[d]
Common binding protein of PP2A family phosphatases	Alpha4 (α4; also known as IGBP1)	Tap42
Leucine carboxyl methyltransferase	LCMT-1	Ppm1
Protein phosphatase methylesterase	PME-1	Ppe1

Table 1. Homologs of PP2A family phosphatases and their interacting proteins in human and yeast.

[a], *S. cerevisiae* homolog has not been identified.

[b], no *S. cerevisiae* homologs exist [26].

[c], human homolog has not been identified.

[d], no *S. cerevisiae* homologs exist [27].

carboxyl methylation on a leucine residue is distinct from other types of carboxyl methylation on a prenylcysteine residue found in proteins such as the small GTP-binding protein (G protein), Ras, γ-subunits of the heterotrimeric G proteins, and nuclear lamins, in that PP2Ac and its methyltransferase are cytosolic and not membrane-associated [16]. PME-1 is a lipase with a serine residue (S156) in its active site and is inhibited by okadaic acid (OA) but not phenylmethylsulfonyl fluoride (PMSF) [23]. Both LCMT-1 and PME-1 interact with PP2Ac. LCMT-1, via its active site pocket, recognizes PP2Ac C-terminal residues and regulates PP2A methylation and activation [28]. PME-1 directly binds to the active site of PP2Ac and the C-terminal region of PP2Ac is placed in the active site of PME-1, resulting in demethylation and inactivation of PP2A [23, 29]. PME-1 stably associates with catalytically inactive mutants of PP2Ac (H59Q and H118Q) and this interaction is decreased by PP2A inhibitors including OA, sodium fluoride, and sodium pyrophosphate [23]. An inactive population of wildtype PP2Ac bound to PME-1 has also been described and this inactive PP2A population bound by PME-1 can be reactivated by PP2A activator (PTPA, phosphotyrosyl phosphatase activator) [4]. The methylation state of PP2Ac is cell cycle-dependent. PP2Ac in the cytoplasm is demethylated at the G_0/G_1 boundary and remethylated as cells enter S phase, while PP2Ac in the nucleus is demethylated at the G_1/S boundary [30].

As previously mentioned, the PP2Ac C-terminus and the post-translational modifications on it have a profound effect on which PP2A holoenzymes are constructed. PP2Ac methylation especially plays an important role in PP2A holoenzyme assembly. One major function of PP2Ac carboxyl methylation is that it dictates the relative amount of formation of methylation-dependent (methylation-sensitive) and methylation-

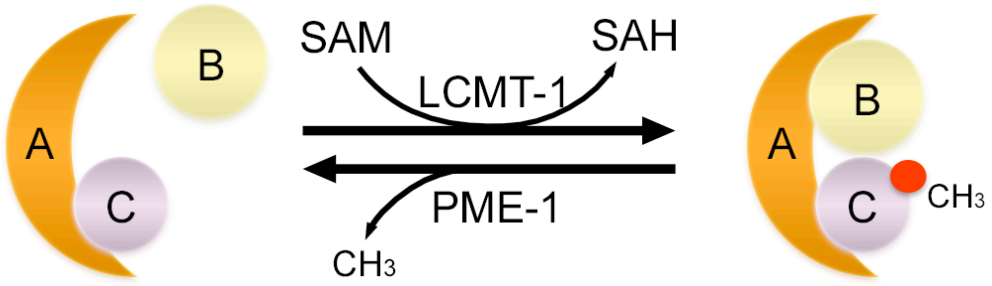

Figure 1-2. Reversible carboxyl methylation of PP2Ac.

PP2Ac is reversibly methylated on its C-terminal leucine residue by leucine carboxyl methyltransferase 1 (LCMT-1) and protein phosphatase methylesterase 1 (PME-1). The action of LCMT-1 is dependent on S-adenosyl-methionine (SAM), the methyl donor, and is inhibited by S-adenosylhomocysteine (SAH). Methylation of PP2Ac promotes binding of a number of methylation-dependent B-type subunits to PP2A A/C heterodimer. Thus, PP2Ac methylation determines enzyme activity, substrate specificity, and function of PP2A.

independent (methylation-insensitive) PP2A holoenzymes by determining which B-type subunits bind to the PP2A A/C heterodimer. For example, in yeast deletion of Ppm1, the major Pph21/Pph22 (PP2Ac) methyltransferase, led to a decrease in PP2Ac methylation as well as PP2Ac association with Tpd3 (A) and Cdc55/Rts1 (B/B') subunits [14, 31] (Table 1). However, loss of Ppm1 had a more severe impact on formation of PP2A heterotrimer containing Cdc55 ($PP2A_{Cdc55}$) than $PP2A_{Rts1}$, suggesting that assembly of $PP2A_{Cdc55}$ is more methylation-dependent [32]. In mammalian cells, PP2Ac methylation is an absolute requirement for assembly of PP2A heterotrimers containing Bα but not B', B'', striatin family (B''') or MT [33-35]. A PP2Ac mutant, PP2Ac L309A lost most of its methylation and association with Bα, indicating that modification on the L309 residue is critical for Bα binding [36]. Modifications at other residues on the PP2Ac C-terminus, including T301/T304/Y307, also seem to affect methylation state of PP2Ac on L309 and/or binding to B-type regulatory subunits. Substitution mutations of these residues decreased the methylation level of PP2Ac to different degrees and thus binding of methylation-dependent Bα to PP2Ac while others directly affecting Bα binding without affecting methylation [34].

In summary, changes in PP2Ac methylation alter affinity of the PP2A A/C heterodimers for certain B-type subunits, leading to changes in enzyme substrate specificity and function due to switching of B-type subunits. Thus, precise control of PP2Ac methylation is critical for proper functioning of cells and deregulation of this process can result in diseases. As an example, downregulation of LCMT-1 causes significant reduction of PP2Ac methylation, which then results in loss of Bα-associated

PP2A heterotrimers and accumulation of hyperphosphorylated Tau, which has been reported to be a major cause of Alzheimer's disease [37].

PP2A and human diseases

Several lines of evidence suggest that PP2A is a tumor suppressor (for reviews, see [3, 38]). Mutations in genes encoding both the α and β isoforms of the PP2A A subunit have been identified in multiple cancers and cancer cell lines [39-42]. Some of these mutations disrupted the interaction of the A subunit with B or that of the A subunit with B and C subunits. PP2A B-type subunits are clearly involved in cancer as well. First of all, overexpression of B'γ was found in malignant melanoma cells [43]. Secondly, N-terminal truncation mutant of B'γ has been linked to metastasis. This mutant was shown to target PP2A to paxillin normally but failed to dephosphorylate paxillin, leading to enhanced cell spreading [44].

Several naturally occurring toxins such as okadaic acid and calyculin A are potent PP2A inhibitors and they promote tumors [45-47]. In addition, there is a cellular inhibitor of PP2A called SET (Suvar 3-9/Enhancer of zeste/Trithorax) [48], which forms a fusion protein with CAN (nucleoporin Nup214) and impairs the function of PP2A in acute lymphocytic myeloid leukemia [49]. Interfering with the normal functioning of PP2A in cell cycle regulation, transcription, translation, and replication could also contribute to genomic instability. Lastly, DNA tumor viruses transform cells in part by replacing the B-type subunits and hampering PP2A function. The MT and ST oncoproteins substitute for a number of B-type subunits and function as viral B subunits themselves [9, 13, 50].

Suppression of B'γ had a very similar effect with ST-mediated tumorigenesis, suggesting B'γ as a major mediator of ST-induced cell transformation [51].

PME-1 overexpression has been observed in some malignant glioblastomas [52] where increased PME-1 expression level may result in enhanced association of PME-1 with PP2Ac, increasing PP2Ac demethylation. PME-1 was also overexpressed in endometrial adenocarcinomas and correlated with malignant, invasive phenotypes of the cancer [53]. A recent discovery of aza-β-lactam (ABL) and sulfonyl acrylonitrile (AMZ30) compounds as selective inhibitors of PME-1 promises a potential therapeutic intervention against cancer [54, 55].

PP2A has a proapoptotic role by inhibiting anti-apoptotic protein signaling and promoting proapoptotic signaling. Thus, misregulation of its proapoptotic function could result in uncontrolled cell growth and survival, leading to tumorigenesis. For example, PP2A B'α is implicated in dephosphorylation and inhibition of anti-apoptotic Bcl-2 protein, inducing programmed cell death [56, 57]. PP2A also dephosphorylates a proapoptotic protein called Bad, another member of the Bcl-2 family of proteins, which when dephosphorylated inhibits the anti-apoptotic Bcl-2 protein, promoting apoptosis [58]. Moreover, it is believed that viruses make use of apoptosis as a means to enhance virus spread. For instance, the viral oncoprotein E4orf4 of human adenovirus type 2 (Ad2) induces p53-independent apoptosis and this process requires its interaction with PP2A Bα [59, 60]. In addition to the tumor suppressor PP2A that could be utilized as a target for therapeutic strategies, the PP2A-interacting viral oncoprotein, E4orf4 is also a potential therapeutic approach since it only induces apoptosis in transformed cells without disturbing normal cells [59].

As briefly stated before, PP2A has been implicated in Alzheimer's disease (AD). PP2A ABαC heterotrimer binds and dephosphorylates the microtubule-associated protein Tau, whose hyperphosphorylation is thought to be central in AD pathogenesis [61]. In agreement with this idea, AD-affected brain regions showed downregulation of LCMT-1 and PP2Ac methylation, which resulted in loss of ABαC heterotrimer assembly, suggesting that PP2A dysfunction leads to accumulation of hyperphosphorylated Tau, formation of neurofibrillar tangles in brain, neuronal cell death, and onset of AD [37]. In addition, other events potentially leading to PP2A inactivation have been reported in AD: increased PP2Ac-inactivating phosphorylation at Y307, increased expression of cellular inhibitor of PP2A, SET, and decreased expression of the PP2A activator, PTPA in AD-affected brain regions [10].

PP2A-related protein phosphatases

PP2A family phosphatases include PP2A and two additional PP2A-related protein phosphatases called protein phosphatase 4 (PP4) and protein phosphatase 6 (PP6). C subunits of these PP2A-related phosphatases share about 60% amino acid identity with PP2Ac including the 3 conserved amino acid residues at C-terminus ($_{307}$YFL$_{309}$ as in PP2Ac). Therefore, PP4c and PP6c have the potential to be modified by post-translational modification mechanisms involved in PP2Ac regulation. Carboxyl methylation of PP4c has been reported [62], but whether PP6c is also methylated is not known.

Protein phosphatase 4 (PP4)

PP4 (35kDa) is a PP2A-like protein phosphatase whose C subunit has a conserved C-terminal sequence, $_{304}$DYFL$_{307}$, with PP2Ac. Like PP2Ac, PP4c is carboxyl methylated [62]. PP4c shares about 65% amino acid identity with PP2Ac [63]. Despite the high amino acid sequence identity, PP4c does not bind to the PP2A A subunit (PR65) [63]. Instead, PP4c forms holoenzyme with its own set of regulatory subunits. PP2Ac and PP4c show distinct tissue and cellular distribution, suggesting that despite their similarities each phosphatase possesses distinct functions. While both phosphatases are expressed in various tissues and show both nuclear and cytosolic localizations, PP2Ac is more cytosolic while PP4c is more nuclear [62]. Moreover, PP2Ac is more abundant in brain while PP4c is more abundant in testes [62].

Like PP2A, PP4 is inhibited by a number of drugs such as OA, fostriecin, microcystin, calyculin A, cantharidin, and tautomycin. The extent of inhibition by these molecules is very similar between PP2A and PP4. For example, purified PP4 catalytic subunit was inhibited by antitumor antibiotic called fostriecin with an IC_{50} of 3nM while IC_{50} of PP2A catalytic subunit was 1.5nM [64]. Therefore, one needs to be extremely cautious when interpreting results from experiments using these inhibitors that would inhibit both PP2A and PP4 at similar IC_{50}.

The essential function of PP4 in the centrosome is conserved in human, *Drosophila melanogaster*, and *Caenorhabditis elegans* [63, 65, 66]. Intense staining of human PP4c in centrosomes has been observed, suggesting that PP4 may play a role in centrosome function [63]. The *Drosophila* PP4c homolog is about 91% identical and 94% similar to human PP4c at the amino acid level and shows similar intense localization at the centrosomes of *Drosophila* cells and embryos [65]. *Drosophila* embryos show a

number of phenotypes due to loss of PP4c expression: 1) asynchronous nuclear divisions; 2) presence of free centrosomes not associated with microtubules; 3) presence of aberrant polar microtubules not properly connected to chromosomes; and 4) reduced γ-tubulin staining without changing the total γ-tubulin protein level [65]. These phenotypes implicate PP4 function in cell cycle regulation as well as microtubule nucleation, growth, or stabilization [65]. Of the two *C. elegans* homologs of PP4c, PPH-4.1 and PPH-4.2, PPH-4.1 shows a higher amino acid identity to human PP4c than PPH-4.2 and seems to carry out a more significant role in embryonic development since PPH-4.1 depletion led to embryonic lethality while PPH-4.2 depletion did not [66]. As in human and fly, *C. elegans* PP4c homolog PPH-4.1 also localizes to centrosomes during mitosis [66]. Depletion of PPH-4.1 revealed that PPH-4.1 is not required for oocyte meiosis but for spindle formation in mitosis and sperm meiosis [66]. Moreover, PPH-4.1 is also needed for proper localization of γ-tubulin and Polo-like kinase, PLK-1, to centrosomes [66]. It would be interesting to see whether the essential function of PP4c in the centrosome is also conserved in the yeast homolog of PP4c, Pph3. However, it has been predicted that yeast Pph3 would not have an essential function at the centrosome since yeast with loss of Pph3 are still viable [67, 68].

PP4 regulatory subunits (PP4Rs) and functions

Four different types of PP4 regulatory subunits have been identified: PP4R1, PP4R2, PP4R3 (PP4R3α and PP4R3β), and PP4R4 (Fig. 1-3)[69-72]. These regulatory subunits specifically interact with PP4c forming a number of independent PP4 holoenzymes. Similar to PP2A regulatory subunits, PP4 regulatory subunits are believed

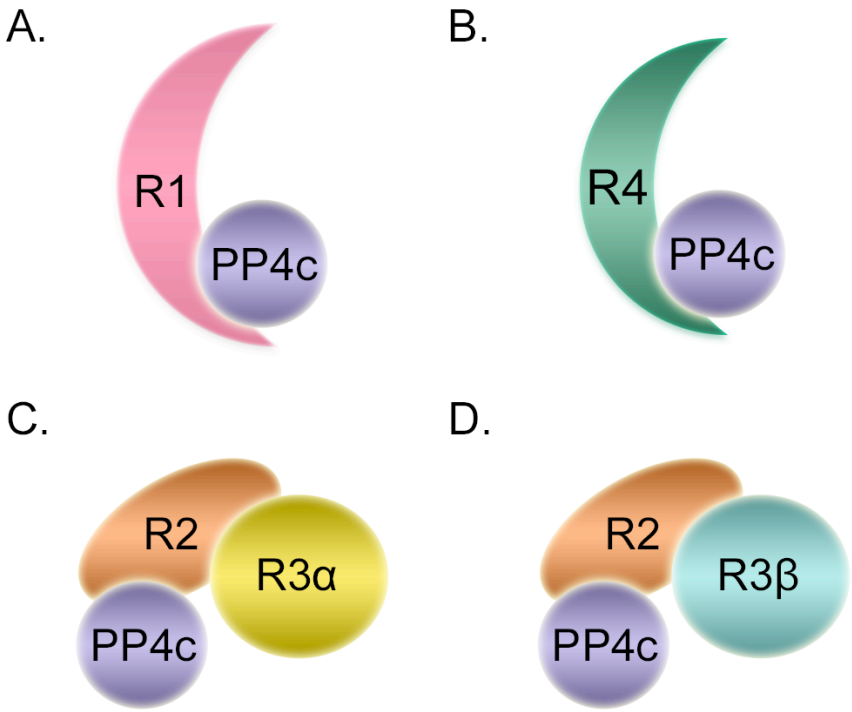

Figure 1-3. Structure of PP4 holoenzymes.

PP4c associates with PP4 regulatory subunits (PP4Rs) to form either a heterodimeric or heterotrimeric complexes. Four different PP4Rs have been identified so far: PP4R1, PP4R2, PP4R3 (PP4R3α and PP4R3β), and PP4R4. PP4c associates with either PP4R1 (A) or PP4R4 (B) forming a heterodimeric PP4 holoenzyme complex. Pre-assembly of PP4c and PP4R2 is required for binding of either PP4R3α (C) or PP4R3β (D) to form heterotrimeric holoenzyme complexes. PP4c can also associate with α4 independently (not shown).

to target PP4c to different substrates and subcellular localizations as well as to regulate PP4c enzyme activity. Thus, understanding PP4 holoenzyme assemblies and identifying their interacting proteins would help us better understand biological functions of PP4.

Interestingly, PP4 has been shown to form a number of different, high-molecular weight protein complexes. Purification of PP4 holoenzyme complexes from bovine testis extracts showed that two major PP4 complexes are found at about 270-300kDa and 400-450kDa [69]. Mass spectrometry (MS) analysis of the smaller PP4 complex (270-300kDa) identified PP4R1 in complex with PP4c [69]. Human PP4R1 is a protein of 950 amino acids (migrates at about 120-125kDa on SDS-PAGE) and possesses 13 HEAT repeats that are similarly found in PP2A A subunit [69]. In an independent study, purification of PP4 holoenzyme complexes from rat tissues detected three separate fractions of PP4 complexes, of which two were found at about 450kDa and 600kDa containing PP4c and PP4R2 [70]. Human PP4R2, a protein of 453 amino acids (50kDa protein but migrates at about 65kDa on SDS-PAGE), is thought to be a highly asymmetric dimer that binds to two PP4c molecules, forming a high-molecular weight complex [70]. Human PP4R2 exhibits a weaker staining in the nucleus and cytoplasm but a very intense localization at centrosome in human cells, suggesting that similar to PP4c, PP4R2 might be involved in regulation of centrosome function [70]. Novel PP4 regulatory subunits, PP4R3α (820 amino acids; migrates at about 110kDa on SDS-PAGE) and PP4R3β (764 amino acids; 97kDa), were identified by combined analyses of affinity purification and mass spectrometry (AP-MS) [71]. Chaperonin containing TCP-1 (CCT) complex subunits were also identified as new interacting proteins of PP4c and α4 in the same study [71]. Iterative AP-MS analysis not only confirmed the presence of PP4

complexes containing the previously identified PP4Rs but also established that at least three independent, mutually exclusive mammalian PP4 holoenzymes exist: 1) one containing PP4c with PP4R1; 2) a second containing PP4c in complex with PP4R2 and PP4R3 (PP4R3α or PP4R3β) where pre-assembly of PP4c and PP4R2 is necessary for PP4R3 binding; 3) the last containing PP4c with α4 and CCT complex [71]. Lastly, using a similar AP-MS approach, the newest member of the PP4Rs, PP4R4 (873 amino acids; about 100kDa) was identified, forming an independent PP4 holoenzyme without containing other known binding partners [72]. PP4R4 comprises three canonical HEAT repeats in addition to lower confidence repeat sequences and exhibits high sequence homology to other HEAT repeat-containing proteins PP2A A and PP4R1 subunits [72]. PP4R4 protein level was severely affected by depletion of PP4c, suggesting that PP4c may regulate PP4R4 expression or PP4c-PP4R4 interaction is needed for PP4R4 protein stability [72]. Intriguingly, a small amount of PP4c was found to interact with PP2A Aα subunit and PP2A B'δ, although the functional significance of this interaction is not fully understood [72]. Taken together, PP4 holoenzymes seem to include at least 5 independent complexes having PP4c together with: 1) PP4R1; 2) PP4R2-PP4R3 (PP4R3α or PP4R3β); 3) PP4R4; 4) α4 and CCT complex; and 5) PP2A Aα subunt-PP2A B'δ [72]. A few other studies detected minor interactions between PP4c and PP2A subunits as well. For example, interactions between PP4c and Aα, Bδ, or B'δ was observed by AP-MS [73]. Additionally, a very minor fraction (1-2%) of a PP4c complex containing PP2A Aα and PP2A Bα was detected, even though its biological function remains unexplored [74].

PP4c-PP4R1 was found to interact with Histone deacetylase 3 (HDAC3) and downregulates deacetylase activity of HDAC3 by dephosphorylation at S424, implicating

the PP4c-PP4R1 complex in transcriptional regulation through its substrate HDAC3 [75]. Protein kinase CK2 phosphorylates HDAC3 at S424, a site whose phosphorylation is critical for HDAC3 activity [75]. HDAC3 belongs to class I histone deacetylases along with HDAC1, 2, and 8. Similar to HDAC1 and 2, HDAC3 is involved in regulation of mammalian gene expression by deacetylation of histone proteins to transcriptionally repress target genes. HDAC3 is recruited to specific promoters via association with N-CoR (nuclear receptor corepressor)/SMRT (silencing mediator of retinoic and thyroid receptors) and mediates transcriptional repression [76]. In addition to the histone proteins, accumulating evidence indicates that HDAC3 targets nonhistone substrates as well, implicating HDAC3 in other cellular functions. Like HDAC1 and 2, HDAC3 is ubiquitously expressed. However, unlike HDAC1 and 2 which are predominantly nuclear, HDAC3 localizes to the nucleus, the cytoplasm and also at the plasma membrane. Interestingly, HDAC3 has been implicated in cancer since its overexpression has been reported in several tumors [77-79]. It is believed that specific targeting of the dysregulated HDAC activity in cancer using HDAC inhibitors is a promising therapeutics strategy against the disease.

PP4c-PP4R2 has been implicated in Survival of Motor Neurons (SMN) complex function in the maturation process of small nuclear ribonucleoproteins (snRNPs) since PP4c-PP4R2 interacts with SMN complex proteins and, when overexpressed, enhances movements of the newly formed snRNPs during maturation processes [80]. In addition, a recent study found that PP4R2, potentially acting upstream of SMN, is needed for proper differentiation of neuronal cells [81]. Additional functions of PP4R2 and PP4R3 (PP4R3α or PP4R3β) in DNA repair, including dephosphorylation of γ–H2AX during

DSBs (double-stranded DNA breaks), will be discussed in the section below entitled '*One common function of PP2A family phosphatases as a γ-H2AX phosphatase'*.

Finally, the function of the novel PP4R4 remains largely unknown. Knockdown (KD) of PP4R4 did not accumulate cells at S-G_2/M, indicating that PP4R4 is not involved in cell cycle regulation [72]. Unlike PP4c and PP4R2, PP4R4 was found to be a cytoplasmic protein and not localized to centrosome [72], suggesting that it is probably not linked to regulation of centrosomal function. Knockdown of PP4R4 also did not lead to elevated level of γ–H2AX [72], suggesting that PP4R4 function is not linked to dephosphorylation of γ–H2AX during DNA damage repair signaling. However, a recent human genome-wide siRNA screen for novel NF-κB activation pathway components identified PP4R4, implicating PP4R4 in NF-κB signaling pathway [82].

Protein phosphatase 6 (PP6)

Mammalian PP6 is a heterotrimeric holoenzyme complex that consists of the PP6 catalytic subunit (PP6c), a Sit4-associated proteins (SAPS) domain-containing regulatory subunit (PP6R1, PP6R2, and PP6R3), and an ankyrin repeat domain-containing subunit (ARS-A, ARS-B, and ARS-C). Similar to other PP2A family phosphatases, specificity of PP6 is determined by binding of different SAPS and ankyrin repeat domain subunits. SAPS domain subunits serve as scaffolds by binding the catalytic subunit via their SAPS domain on the N-terminus and ARS proteins via their C-terminal region [27, 83]. Of the three SAPS domain regulatory subunits, PP6R1 along with PP6c was found to dephosphorylate IκBε and protects it from degradation in response to tumor necrosis factor-α (TNF-α), implicating PP6c-PP6R1 in NFκB signaling pathway [83]. Later, it

was also demonstrated that depletion of PP6R1 or ARS-A, but not PP6R3, leads to a reduction of IκBε levels upon TNF-α treatment, implicating PP6 again in NFκB signaling [27]. PP6 also plays a critical role in mitotic spindle formation. PP6 holoenzyme dephosphorylates a threonine residue within the activation or T loop of Aurora A, an essential mitotic kinase involved in mitotic spindle formation [84]. Depletion of PP6c or its regulatory subunits led to increased Aurora A activity which then interfered with spindle formation and chromosome alignment [84].

Sit4, the yeast homolog of mammalian PP6c (Table 1), is required in late G_1 for progression into S phase during cell cycle [85]. Sit4 is a cytoplasmic protein whose localization does not change during cell cycle [85]. However, the interaction of Sit4 with its regulatory, Sit4-associated proteins, SAP155 and SAP190 (Table 1), changes during cell cycle. While Sit4 exists as a monomer during G_1 phase, it associates with SAP155 or SAP190 during the G_1/S transition, suggesting that formation of these Sit4 complexes is required for progression into S phase [85]. SAP155, SAP190, and an additional protein called SAP185, get hyperphosphorylated in the absence of Sit4 (Table 1), suggesting that function of the SAPS domain proteins could be regulated by phosphorylation [86].

One common function of PP2A family phosphatases as a γ-H2AX phosphatase

One major common function of the PP2A family phosphatases is that they dephosphorylate γ-H2AX formed during DSBs (double-stranded DNA breaks). DSBs trigger formation of γ-H2AX by phosphorylation of histone H2A variant protein H2AX on Serine 139, which then recruits DNA repair proteins to DSBs for repair [87]. During or after repair is completed, γ-H2AX is cleared by dephosphorylation. Three PI3K-like

kinases, ATM (ataxia telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-PK (DNA-dependent protein kinase), phosphorylate H2AX in response to DSBs [88]. ATM and DNA-PK function in response to IR (ionizing radiation) and radiomimetic drugs while ATR is activated in DNA replication-interfering conditions [88]. PP2A was first recognized as a γ-H2AX phosphatase during DSB repair [89]. Once DSBs form, PP2A gets recruited to DSBs forming discrete foci in the nucleus that co-localize with γ-H2AX [89]. In the absence of functional PP2A, either by PP2A inhibitor treatment or depletion, elevated levels of γ-H2AX after DSB induction persisted longer as compared to control cells [89]. In addition, PP2A interacts with γ-H2AX only after DSB induction [89]. However, in the absence of functional PP2A, the γ-H2AX level seemed to decrease eventually, suggesting that other phosphatases may be involved as well. Consistent with this, PP4 and PP6 have also been recognized as γ-H2AX phosphatases.

The yeast PP4c counterpart, Pph3, was found to dephosphorylate γ-H2AX in *Saccharomyces Cerevisiae* (Table 1)[90]. Interestingly, while DSB repair was as efficient in Δpph3 cells as in wildtype cells, persistent γ-H2AX induced by Pph3 depletion led to checkpoint recovery defects and cells accumulated at G_2/M. These results suggest that γ-H2AX formation keeps cells arrested at G_2/M until the repair is completed and that dephosphorylation of γ-H2AX by Pph3 is necessary for timely recovery from the DNA damage checkpoint [90]. As in yeast, human PP4, more specifically the PP4c-PP4R2 complex, has been shown to dephosphorylate γ-H2AX in both damaged and undamaged cells, promoting recovery from the DNA damage checkpoint at G_2/M [91]. PP2A and PP4 seem to exhibit distinct phosphatase activity toward γ-H2AX depending on the types of damage, for example, PP2A only showed minimal activity toward γ-H2AX at IR-

induced DSBs as compared to PP4 [91]. The heterotrimeric PP4 complex, PP4c-PP4R2-PP4R3β, was also found to dephosphorylate γ-H2AX generated by ATR during DNA replication [92]. Depletion of other PP4 regulatory subunits, PP4R1 or PP4R3α, did not induce γ-H2AX formation in undamaged cells and also did not interfere with γ-H2AX dissolution after CPT (camptothecin)-induced DSBs [92]. Additionally, the heterodimeric PP4 complex PP4c-PP4R2, independently of PP4R3α or PP4R3β, dephosphorylates RPA2, a single-stranded DNA binding protein involved in DNA repair, and mediates efficient homologous recombination (HR)-mediated repair of DSBs [93]. Besides HR-mediated repair, DSBs are also repaired through an error-prone nonhomologous end joining (NHEJ)-mediated repair process. The PP4 holoenzyme complex containing PP4c-PP4R2 was implicated in NHEJ-mediated repair of DSBs, potentially through regulating phosphorylation state of its novel substrate called KAP-1 [94]. Another group has also proposed that the PP4 complex containing PP4c and PP4R3β functions in DNA damage response by regulating the G_2/M checkpoint and chromatin structure through KAP-1 [95].

Another member of the PP2A family phosphatases, PP6, has recently been found to regulate dephosphorylation of γ-H2AX, removal of γ-H2AX foci, and recovery from the G_2/M checkpoint after IR-induced DNA damage [96]. Depletion of PP6c or PP6R1, but not depletion of PP6R2 or PP6R3, led to persistent γ-H2AX in response to IR, implicating the PP6 holoenzyme containing the PP6R1 regulatory subunit in γ-H2AX dephosphorylation during the IR-induced DNA damage response [96]. Interestingly, another form of PP6 holoenzyme containing PP6c and PP6R2 was found to be responsible for dephosphorylation of γ-H2AX after CPT treatment and homology-directed repair of DSBs [97].

PP4 and PP6 in human diseases

Several lines of evidence implicate PP4 and PP6 in a number of human diseases, especially cancer. Overexpression of PP4c and its regulatory subunits was detected in human breast and lung tumors, suggesting a correlation between PP4 expression levels and tumorigenesis [98]. In support of this idea, knockdown of PP4c sensitized breast and lung cancer cells to cisplatin treatment [98]. PP4c was also overexpressed in stage II pancreatic ductal adenocarcinoma (PDAC) and PP4c overexpression was associated with poor prognosis in stage II PDAC patients [99]. These data suggest that targeting PP4 may be a potential therapeutic strategy against cancer.

As for PP6, both oncogenic and tumor suppressive properties of PP6 have been reported in cancer. In an attempt to identify genes differentially regulated in malignant mesothelioma (MM), miR-31 was found to be lacking [100]. As a result of miR-31 downregulation, one of its targets, PP6c, was found upregulated profoundly in MM samples, suggesting a role of PP6c in tumorigenesis [100]. In contrast, PP6c was downregulated in human hepatocellular carcinoma (HCC) tissues due to upregulation of its negative regulator miR-373 [101]. Upregulation of PP6c in glioblastoma multiforme (GBM) tissues has also been reported [102]. In that study, knockdown of PP6c in GBM cell lines as well as in a GBM mouse model increased sensitivity to radiation, suggesting that targeting of PP6c in GBM is a promising method of improving the effect of radiation treatment and patient survival [102]. Several PP6c mutations were identified in melanoma. These mutants exhibited compromised holoenzyme assembly and enzyme activity and promoted chromosome instability and DNA damage due to elevated Aurora A kinase activity [103]. Loss of PP6c in a skin keratinocyte-specific PP6c conditional

knockout mouse model predisposed the animals to skin carcinogenesis, suggesting a tumor suppressor function of PP6c [104]. Another study also performed a detailed analysis of a number of PP6c mutations observed in melanoma and found that they were of a heterogeneous nature, some showing oncogenic effect while others displayed tumor suppressive function [105].

Besides cancer, PP6 has also been implicated in Crohn's disease (CD) since its overexpression was detected in the inflamed colonic CD tissues [106].

Alpha4 (α4): a common binding partner of PP2A family phosphatases

Alpha4 (α4; homolog of the yeast Tap42 protein; also known as IGBP1, immunoglobulin-α-binding protein 1) is a common binding partner of PP2A, PP4, and PP6 catalytic subunits (Table 1)[107-112]. Tap42 was first identified as a high copy suppressor of a temperature-sensitive Sit4 (homolog of mammalian PP6c) mutant in yeast [107]. High copy number of Tap42 also suppressed temperature-sensitive mutants of Pph21/22 (yeast PP2Ac homologs), and was thus named Tap42 ('two A and related phosphatase-associated protein' of 42kDa) [107].

α4 binds to C subunits of PP2A family phosphatases independently of the previously identified phosphatase subunits. Evidence in yeast showed that the binding of Tap42 (yeast α4) and Tpd3/Cdc55 (yeast A subunit homolog/B subunit homolog) to Pph21/22 are mutually exclusive [113]. Consistent with this, Tap42 interacts with Pph21 in the absence of Tpd3 [107]. In mammalian cells, PP2Ac was also found to form at least two mutually exclusive complexes, one with α4 and the other with A subunit [112]. Likewise, A subunit was not detected in co-immunoprecipitates of α4 containing PP2Ac

[108]. Thus, α4 and the regulatory/structural phosphatase subunits bind to C subunits of PP2A family phosphatases in a mutually exclusive manner. Although it is not clear how post-translational modifications of PP2Ac play a role in PP2Ac-α4 association, the PP2Ac double mutant (Y307F and L309Q) preferentially interacted with α4 over PP2A A/C and PP2A heterotrimer complexes [114]. In addition, enhanced interaction of Tap42 and Pph22 has been seen in PPM1 (yeast LCMT-1)-deficient yeast cells [31]. Other than α4, PP2A family phosphatase C subunits, despite their high sequence similarities, do not for the most part share regulatory/structural subunits with each other. For example, in yeast, Tpd3/Cdc55 only interacts with Pph21/22 while Sap155, one of yeast PP6 regulatory subunits, interacts only with Sit4 (PP6c homolog) [107]. Similarly, PP2A A subunit generally only interacts with PP2Ac but not with C subunits of PP2A-related phosphatases (PP4 and PP6) [112]. However, more recently, few, minor interactions between PP4c and PP2A structural/regulatory subunits have been reported. For examples, PP4c complexes containing PP2A A subunit and either Bα or B'δ were detected [72, 74] while another study showed interactions between PP4c and Aα, Bδ, or B'δ [73].

Binding of α4 to PP2A family phosphatases plays significant roles in regulation of PP2A family phosphatases as well as in cellular processes including apoptosis. While cells produce PP2Ac proteins in inactive forms as a means to protect themselves from promiscuous phosphatase activity [116], free PP2Ac monomers are protected from degradation by interacting with α4. α4 functions as an adaptor protein to protect free PP2A C subunits from degradation and helps maintain proper PP2A function and assembly in both stressed and non-stressed conditions [117]. In addition to PP2A A and C subunits, C subunits of PP4 and PP6 levels were significantly decreased upon α4

deletion [117]. Interestingly, the level of methylated PP2Ac was also decreased by α4 deletion [117]. α4 is a ubiquitin-binding protein that possesses a ubiquitin-interacting motif (UIM) [118]. The UIM of α4 protects the ubiquitin chains of PP2Ac from further elongation and therefore, in the absence of α4 UIM, PP2Ac polyubiquitination occurs.

α4 mediates the interaction between PP2Ac and Mid1. Mid1 is a microtubule-associated protein that is mutated in an X-linked, human genetic disease called Opitz syndrome (OS) [119]. In OS patients, Mid1 mutant loses its binding to the microtubule network and thus shows cytoplasmic localization. The inability of Mid1 to associate with the microtubule network is one potential mechanism underlying OS pathogenesis [120]. The C-terminal region of α4 binds Mid1 while the N-terminal region of α4 binds PP2Ac [121]. MAP kinase-mediated Mid1 phosphorylation promotes its binding to microtubules while α4-mediated Mid1 binding to PP2Ac leads to Mid1 dephosphorylation and thus release from microtubules [122]. Mid1 is also an E3 ubiquitin ligase targeting PP2Ac for proteasome degradation [123]. In OS, the Mid1 defect in ubiquitin ligase activity leads to accumulation of PP2Ac [123]. In turn, elevated PP2Ac causes dephosphorylation of microtubule-associated proteins and defective microtubule dynamics [123]. Thus, it appears that Mid1 regulates PP2Ac degradation while PP2Ac regulates Mid1 dephosphorylation and thus its association with microtubules.

α4 complexes containing C subunits of PP2A family phosphatases have been implicated in the target of rapamycin (TOR) signaling pathway. In yeast, the association of Tap42 and PP2A family phosphatase C subunits is stimulated by nutrient signals and inhibited by rapamycin, implicating Tap42 and PP2A family phosphatases in rapamycin-sensitive TOR pathway [107]. In addition, mutations in Tap42 or PP2A family

phosphatases rendered cells rapamycin-resistant [107]. Tap42 mutants also showed defects in translation initiation [107]. However, the effect of rapamycin on the interaction between PP2A family phosphatases and α4 in mammalian cells is still controversial. While there are studies showing that rapamycin reduced the interaction of α4 with PP2Ac [108, 110, 115], there is also evidence showing that the interaction of α4 with PP2Ac as well as with PP4c and PP6c is rapamycin-insensitive [109-112]. This inconsistency of results regarding rapamycin action on α4 complexes containing C subunits of PP2A family phosphatases may be cell-type specific since rapamycin-sensitivity varied among different cell lines [110]. Yet, conflicting data were obtained using the same cell line by different groups of researchers [108-110], suggesting that other issues may be involved. The effect of α4 or yeast Tap42 binding on PP2A family phosphatase activity is controversial, too. In some instances, α4 binding increased the phosphatase activity [108] while in other cases, α4 binding had a negative impact on the phosphatase activity of PP2A family phosphatases [112]. So far, it seems like the effect of α4 binding on the phosphatase activity differs depending on the type of substrate tested [111].

Just as Tap42 is essential in yeast, loss of α4 in mice is embryonic lethal [115]. α4 is an anti-apoptotic protein. In α4 knockout MEFs, loss of α4-induced apoptosis is accompanied by enhanced cleavage of caspase-3 and PARP [115]. Knockdown of α4 led to apoptosis while overexpression of α4 enhanced anchorage-independent growth which was suppressed by a dominant-negative mutant of α4, supporting the idea that α4 is a pro-survival and anti-apoptotic protein [124]. Together, these results suggest that interfering with α4 function might be a potential therapeutic strategy against cancer [124]. In agreement with this, α4 was found to be overexpressed in multiple human

cancers and its overexpression has been correlated with the stage of lung adenocarcinomas [125, 126].

The striatin family of proteins

The striatin family consists of three highly homologous proteins, striatin, S/G_2 nuclear autoantigen (SG2NA), and zinedin. Striatin family members function as scaffolding proteins with no intrinsic catalytic activity and organize a variety of multiprotein complexes including PP2A complexes. Striatin family members comprise the newest subfamily of PP2A regulatory B-type subunits, B’’’, and assemble into a PP2A heterotrimer by associating with PP2A A/C heterodimer [127]. Members of the striatin family are mainly expressed in the central and peripheral nervous systems and are likely to be important for brain function. However, their expression has been observed in other tissues as well. While these proteins share similar domain structures and localizations, their functions do not appear to be entirely redundant.

Striatin

The founding member of the striatin family, striatin was named after the striatum where it is found most abundantly in the brain [128]. Striatin is a protein of 780 amino acids with four unique protein-protein interaction domains including a caveolin-binding domain, a coiled-coil domain, a Ca^{2+}-calmodulin (CaM)-binding domain and a WD-repeat domain and these domains are conserved throughout the striatin family (Fig. 1-4)[129]. Consistent with the presence of these domains, striatin has been reported to bind

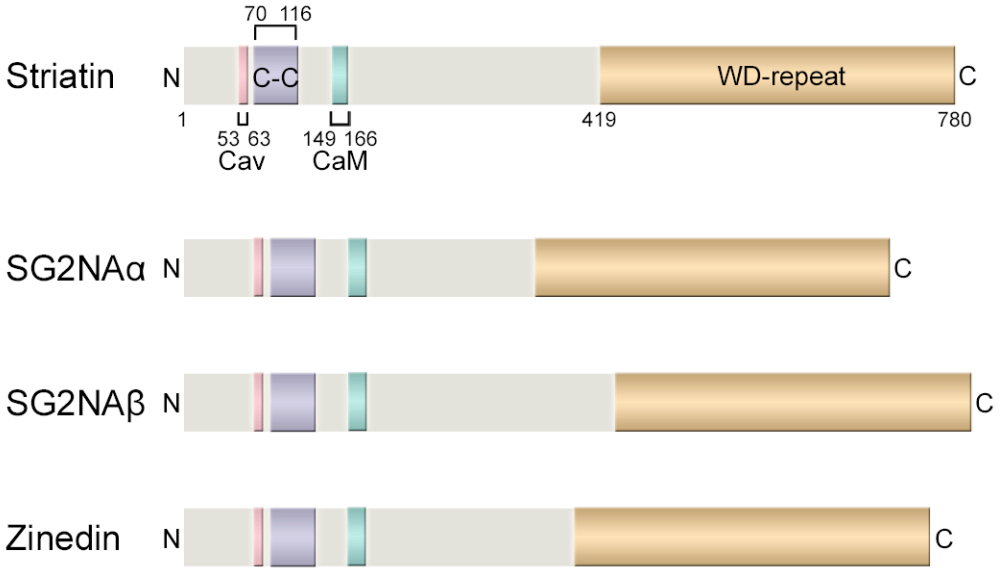

Figure 1-4. Domain structure of the striatin family members.

The domain structure of the human striatin family members including striatin (780 amino acids), two major isoforms of SG2NA, SG2NAα and SG2NAβ (713 and 797 amino acids, respectively), and zinedin (753 aa) is shown drawn to scale. The four highly conserved protein-protein interaction domains are color-coded for comparison. Some of the domains depicted in SG2NA and zinedin are only predicted regions based on sequence comparisons and have not been experimentally verified. Cav, caveolin-binding domain; C-C, coiled-coil domain; CaM, Ca^{2+}-calmodulin binding domain; WD-repeat, WD-repeat domain. (Figure and legend were taken and modified from [130]).

caveolin-1 (Cav-1) and binds CaM in a Ca^{2+}-dependent manner (Fig. 1-5)[128, 131]. Striatin is expressed throughout the central and peripheral nervous systems, especially in the striatum and motor neurons; however, it is also found in various tissues including lung, liver, kidney, skeletal muscles, and testes [128, 129, 132]. Striatin is exclusively localized to dendrites, especially in dendritic spines, and absent in axons, displaying a somato-dendritic localization in neurons [128]. The distribution of striatin in brain regions important for motor control led to a speculation that striatin may have a role in control of motor function [133]. Indeed this point was proven by a study in which downregulation of striatin in rat brains resulted in decreased nocturnal locomotor activity [134]. In addition, downregulation of striatin in motor neurons impaired the growth of dendrites without affecting axons, demonstrating the important role of striatin in dendritic growth regulation [134].

SG2NA

SG2NA was first described as a nuclear protein whose expression peaked during the S and G_2 phases of the cell cycle, hence its name [135]. Afterward, SG2NA was shown to be mainly cytosolic and membrane-bound like striatin [129, 136]. SG2NA is highly expressed in the cerebellum and cortex of the brain and exhibits somato-dendritic localization in neurons with enrichment in dendritic spines [129, 136]. Expression of SG2NA is also detected in many other tissues. Similar to striatin, SG2NA is characterized by the four protein-protein interaction domains and binds to CaM in a Ca^{2+}-dependent manner (Fig. 1-4)[127, 129]. There are two major isoforms of SG2NA, SG2NAα and SG2NAβ, which are generated by alternative splicing, in addition to some

minor variants (Fig. 1-4)[137]. SG2NAα is a 713 amino acid protein which excludes exons 8 and 9 while SG2NAβ is a full-length protein of 797 amino acids [138]. A novel, nuclear-localized splice variant of rat SG2NA, rSTRN3γ, lacking all but one WD-repeat was identified and this isoform assembles an estrogen-inducible PP2A complex with estrogen receptor α (ERα) (Fig. 1-5) [139].

Zinedin

Zinedin is a protein of 753 amino acids and was identified through a homology search for proteins highly homologous to striatin and SG2NA [129]. Like striatin and SG2NA, zinedin is characterized by the four protein-protein interaction domains and binds CaM in the presence of Ca^{2+} (Fig. 1-4)[129]. Similarly, zinedin exhibits somato-dendritic localization in neurons with enrichment in dendritic spines. Additionally, zinedin is highly expressed in the hippocampus of the brain and is also detected in various tissues [129, 140, 141].

Domain structure of the striatin family proteins

As stated above, striatin family members share a unique domain structure composed of a caveolin-binding domain, a coiled-coil domain, a Ca^{2+}-calmodulin (CaM)-binding domain and a WD-repeat domain, listed from N-terminus (Fig. 1-4). The domains and their roles in striatin family functions will be discussed briefly below.

The caveolin-binding domain

Striatin family members interact with caveolin-1 (Cav-1) via the caveolin-binding

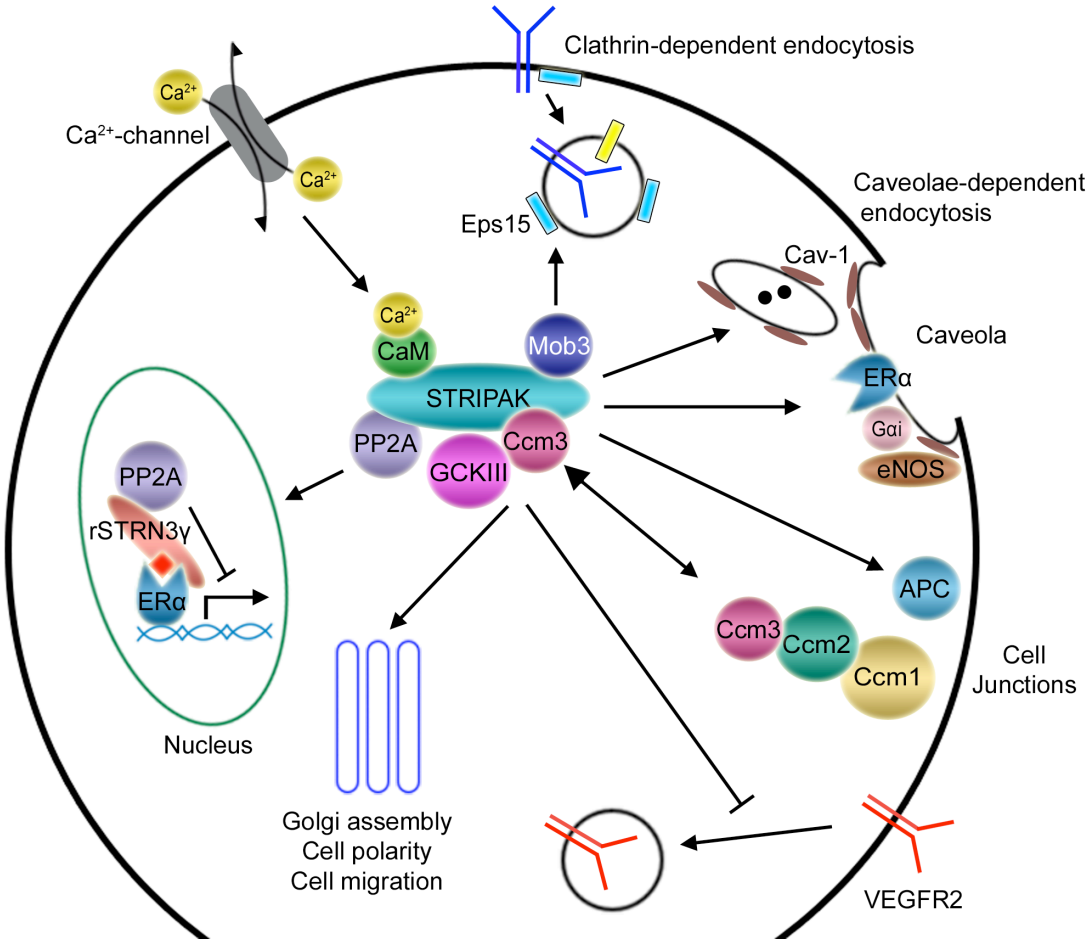

Figure 1-5. Model of the STRIPAK (Striatin-interacting phosphatase and kinase) complex in signaling pathways.

The core STRIPAK complex is depicted in the center of the diagram with some of the STRIPAK components that are thought to mediate several functions of the STRIPAK complex. Other STRIPAK components are omitted for simplicity of the figure. Some of the known or potential connections of STRIPAK components to signaling pathways and cellular events are depicted by arrows. Through Mob3 the STRIPAK complex may associate with components of clathrin-dependent endocytosis (for example, Eps15) and function in this process. The STRIPAK complex might also function in caveolae-

dependent endocytosis by interacting with Cav-1. Striatin targets ERα to membranes, probably caveolae, and forms a STRIPAK-like complex containing ERα, Gαi, and eNOS to regulate rapid nongenomic ERα signaling. Striatin binds APC and regulates organization of tight junctions (TJs). Ccm3 forms dynamic complexes with either STRIPAK or other Ccm proteins to regulate GCKIII activity and function and cell junction stability. Ccm3 stabilizes VEGFR2 on the cell surface by inhibiting its internalization. GCKIII kinases, Ccm3, and STRIPAK function in Golgi assembly, cell polarity, and cell migration. Rat STRN3γ and PP2A modulate ERα-mediated transcriptional activity in nucleus. The STRIPAK complex is predicted to be involved in Ca^{2+} signaling by binding to Ca^{2+}-CaM through the Ca^{2+}-CaM-binding domain, thus responding to changing Ca^{2+} concentrations in cells. (Figure was taken from [130] and legend was modified from [130]).

domain [131]. Caveolins are the major components of caveolae, specialized, invaginated compartments found in the plasma membrane of cells. The caveolin scaffolding domain found in the N-terminus of caveolins mediates the interaction between caveolins and many signaling proteins [142]. Striatin family members interact with the caveolin scaffolding domain of caveolins via the consensus sequence ΦXXXXΦXXΦ, where Φ is an aromatic amino acid and X is any amino acid, found in their caveolin-binding domains [129, 143]. It is now believed that caveolae may be the center of cellular signaling pathways by recruiting molecules involved in multiple signaling pathways. In fact, nongenomic effects of estrogen are mediated by ERα localized in caveolae [144]. During this process, striatin, which is likely targeted to caveolae through its interaction with Cav-1 organizes a PP2A complex containing ERα, Gαi, and eNOS for rapid, non-genomic ERα signaling (Fig. 1-5)[145].

The coiled-coil domain

The striatin family members form homo- and hetero-oligomers using their coiled-coil domains [136, 138, 140]. The coiled-coil domain is made up of heptad repeats that favor homo- and hetero-oligomerization of the striatin family of proteins [140]. In addition to being important for oligomerization, this oligomerization domain has been shown to be necessary for targeting of SG2NA to dendritic spines, suggesting that the striatin family oligomerization might be a necessary step for their targeting to the dendritic spines [140].

The Ca^{2+}-calmodulin (CaM)-binding domain

The striatin family members interact with calmodulin (CaM) in a Ca^{2+}-dependent manner [127-129]. The CaM-binding region within striatin was identified by utilizing deletion mapping and site-directed mutagenesis [146]. Although it is currently not clear how the striatin family members function in Ca^{2+} signaling, it has been proposed that they might function as Ca^{2+} sensors responding to changes in intracellular Ca^{2+} concentration and to convey the signals to other proteins (Fig. 1-5)[138]. For instance, changes in intracellular Ca^{2+} concentration appear to modulate striatin interaction with other proteins and thus affect its subcellular localization. It has been demonstrated that the interaction of striatin with Cav-1 was decreased in the presence of Ca^{2+} [131]. In a separate study, the presence of Ca^{2+} during cell lysis increased the fraction of striatin found in cytosol [146].

The WD-repeat domain

The WD-repeat domain consists of four or more copies of a conserved sequence motif of about 40 amino acids characterized by a glycine-histidine (GH) dipeptide at the N-terminus and a tryptophan-aspartate (WD) dipeptide at the C-terminus [147, 148]. The WD-repeat domains create a stable platform made up of β-propeller structures for interaction with various proteins involved in multiple cellular processes. Among all WD-repeat proteins, the striatin family members are the only proteins that associate with CaM [128]. The WD-repeat domain of the striatin family has been demonstrated to be important for association of adenomatous polyposis coli (APC) and CCT proteins. The armadillo repeat domain of APC protein has been shown to bind striatin through interacting with the WD-repeat domain [149]. The striatin proteins also interact with

CCT complex proteins that are involved in folding of WD-repeat-containing proteins [150, 151]. Thus, it is hypothesized that CCT proteins bind WD-repeat domains of the striatin family and help them fold properly.

STRIPAK (Striatin-interacting phosphatase and kinase) complexes

In addition to the previously mentioned proteins that bind to the striatin family via the four protein-protein interaction domains within striatin protein, many other proteins have been reported to interact with the striatin proteins, forming multiprotein, striatin-associated complexes. A recent proteomic analysis revealed that striatin family-associated complexes consist of not only PP2A but also germinal center kinase III (GCKIII) kinases in addition to many other proteins, and hence the name striatin-interacting phosphatase and kinase (STRIPAK) complexes [150]. While functions of the STRIPAK complexes still remain to be elucidated, numerous clues about their functions have come from the study of striatin proteins themselves as well as striatin-interacting proteins. Some of these functions are illustrated in Figure 5. Of note, there are also STRIPAK-like complexes that have not yet been determined to contain both PP2A and a kinase within the same complex.

The core mammalian STRIPAK complex consists of PP2A A and C subunits, monopolar spindle-one-binder family 3 (Mob3; Phocein; HUGO Gene Nomenclature Committee approved symbol, MOB4), members of the GCKIII subfamily of the mammalian sterile 20-like (Mst) kinases including Mst3 (Stk24), Mst4 (MASK), and Ysk1 (Sok1/Stk25), cerebral cavernous malformation 3 (Ccm3/PDCD10), and striatin-interacting proteins 1 and 2 (STRIP1/2) (Fig. 1-6) [150, 152]. Additional proteins bind to

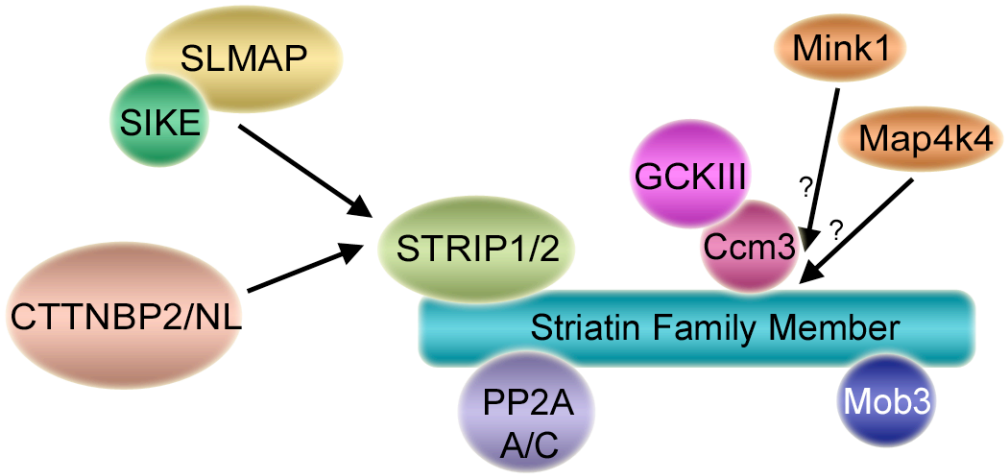

Figure 1-6. Mammalian STRIPAK complexes.

The core STRIPAK components include a striatin family member, the PP2A A/C heterodimer, Mob3, STRIP1 or STRIP2, and a GCKIII kinase bound via Ccm3. Binding of different, mutually exclusive accessory proteins to the STRIPAK core results in the formation of different STRIPAK complexes. For example, SLMAP and SIKE are not detected in STRIPAK complexes containing CTTNBP2/NL and vice versa. In addition, some STRIPAK complexes contain alternative kinases such as Mink1, Map4k4, and Tnik (latter not shown in figure), likely in a mutually exclusive manner. For this reason their binding is shown as competitive (arrows). However, whether Mink1 and Map4k4 bind via Ccm3 or directly to striatin family members is not known. (Figure was taken from [130] and legend was modified from [130]).

the core complex in a mutually exclusive manner forming distinct STRIPAK complexes containing either a cortactin-binding protein 2 family member (CTTNBP2 or CTTNBP2NL) or sarcolemmal membrane-associated protein (SLMAP) and a suppressor of IKKε (SIKE) family member [150]. Some STRIPAK complexes also appear to contain alternative kinases such as members of the GCKIV kinases, Misshapen-like kinase 1 (Mink1), TRAF2- and NCK-interacting kinase (Tnik), and Map4k4 [153] (Fig. 1-6).

Negative regulation of kinases by STRIPAK-associated PP2A

The fact that a phosphatase and one of several kinases exist in STRIPAK complexes suggests that functions of STRIPAK complexes are regulated by reversible phosphorylation processes carried out by these enzymes. In fact, many of the STRIPAK components are phosphoproteins [150], and it has been shown that PP2A inhibition by OA enhanced phosphorylation of several striatin family-associated proteins including striatin, SG2NA, and Mob3 [136]. GCKIII kinases include Mst3, Mst4, and Ysk1, and are one of major constituents of the STRIPAK complexes. While Ysk1 has been shown to be dephosphorylated and inactivated by purified PP2A *in vitro* [154], data regarding other GCKIII members remain unknown. Since GCKIII kinases are important in various cellular processes including cell cycle, cell survival, apoptosis, Golgi assembly, and cell migration [155], it would be key to determine if STRIPAK is involved in these processes through PP2A-dependent regulation of these STRIPAK-associated kinases.

It appears that STRIPAK-associated PP2A also regulates members of GCKIV kinases. Recently, one of the GCKIV kinases, Mink1 was identified as a novel STRIPAK component [153]. Mink1 plays an important role in abscission process during cytokinesis

and knockdown of either Mink1 or zinedin led to abnormal abscission [153]. While zinedin enhanced dephosphorylation of Mink1 by PP2A *in vitro*, enhanced phosphorylation of Mink1 was observed during mitosis when PP2A is known to be inhibited [153, 156]. Together, these results suggest that STRIPAK-associated PP2A negatively regulates Mink1 function in abscission.

Other GCKIV kinases were also found to interact with the striatin family members. For example, Map4k4 was identified to interact with SG2NA and zinedin [153, 157]. Map4k4 is a mediator of tumor necrosis factor α and interleukin-1β production in response to an inflammatory stimulus and is also involved in cancer cell motility and invasion [158, 159]. Although the significance of the interaction between STRIPAK and Map4k4 remains unclear, it would be interesting to investigate whether STRIPAK-associated PP2A regulates Map4k4 in these cellular events. Lastly, another GCKIV kinase, Tnik also interacts with zinedin [153]. Tnik is an effector of the small G protein Rap2A to induce brush border formation upon polarization of intestinal epithelial cells [160]. When activated by Rap2A, Tnik induces relocalization of Mst4 from Golgi to apical membrane of polarized intestinal epithelial cells, leading to phosphorylation of its target ezrin and microvilli formation [160]. It would be interesting to determine how the interaction of Tnik and STRIPAK-associated zinedin plays a role in brush border formation. So far, STRIPAK complexes seem to regulate members of GCKIII and GCKIV kinases that are involved in various cellular processes. At the same time, it is also possible that the STRIPAK complexes themselves are regulated by the kinases that they interact with.

STRIPAK and vesicular trafficking

Several lines of evidence propose possible roles for the STRIPAK components in endocytosis and vesicular trafficking. For instance, inhibition of vesicular trafficking by brefeldin A treatment rapidly altered the association of SG2NA and Mob3 with Golgi [161]. Mammalian striatin and SG2NA also associate with GAIP-interacting protein, C terminus (GIPC) that is involved in receptor endocytosis and trafficking such as TrkA [162]. Interestingly, Mob3 has sequence homology with the σ light chain subunit of clathrin adaptor complexes, suggesting that Mob3 might be involved in vesicular transport. Additionally, Mob3 interacts with proteins involved in clathrin-dependent endocytosis, including epidermal growth factor receptor substrate 15 (Eps15), dynamin I, and nucleoside-diphosphate kinase (NDPK). Eps15 is an endocytic adaptor protein involved in endocytosis of receptor tyrosine kinases such as epidermal growth factor receptor (EGFR) [163]. Eps15 has also been shown to associate directly with dynamin I [164], a GTPase that is critical in the fission of clathrin-coated vesicles from the plasma membrane during endocytosis [165]. Similarly, NDPK was also shown to interact directly with dynamin I [165]. In a study using *Drosophila*, NDPK was shown to regulate endocytosis by providing a local pool of GTP for dynamin I [166]. Additional studies with the *Drosophila* Mob3, dMob4 revealed that its function is important for microtubule organization, axonal transport, synapse assembly, and neurite growth and branching [167, 168]. Moreover, dMob4 mutants exhibited phenotypes that were common to other endocytic mutants such as *Drosophila* Eps15 [167]. Together, all these findings suggest that STRIPAK may function in endocytosis and vesicular trafficking via Mob3.

Homologs of STRIPAK components found in yeast and several filamentous fungi also support the idea of STRIPAK complexes function in vesicular trafficking. Many of these STRIPAK components homologs were localized to vacuole or vacuolar structures including Golgi and endoplasmic reticulum [169-171]. Also, mutations in these homologs resulted in abnormal vacuole phenotypes. For instance, mutations in Ham-2 (STRIP1/2 homolog), Ham-3 (striatin homolog), or Ham-4 (SLMAP homolog) in *Neurospora crassa* caused enlarged vacuole phenotypes [172]. The yeast SLMAP homolog Far9 has been shown to be important for proper sorting of proteins to the vacuole and for secretion of α-factor [173]. Thus, evidence from multiple organisms implicates a role for STRIPAK complexes in endocytosis and vesicular trafficking.

STRIPAK and cerebral cavernous malformation (CCM) disease

The identification of Ccm3 as a STRIPAK complex core component implicated the STRIPAK complexes in cerebral cavernous malformation (CCM) [150], which can result from Ccm3 mutation [174]. CCM is characterized by abnormally enlarged capillary cavities that are predominantly found in the central nervous system, especially the brain. CCM lesions consist of clusters of dilated blood vessels lined by a single layer of endothelium lacking supportive smooth muscle cells. These characteristics of CCM lesions make them prone to recurrent hemorrhages. Symptoms include headache, stroke, seizure, or even death, while many carriers remain asymptomatic (for reviews, see [175-178]). Approximately one in 200-250 people are affected by CCM, among which the majority is sporadic cases [179, 180]. Familial CCM accounts for the rest of the cases. Loss-of-function mutations in one of three *CCM* loci leads to familial CCM. The three

CCM genes are *Ccm1* (*K-Rev interaction trapped 1; Krit1*) [181, 182], *Ccm2* (*Osmosensing Scaffold for MEKK3; OSM; Malcavernin*) [183, 184], and *Ccm3* [174]. Since Ccm3 is the only Ccm proteins that associate with STRIPAK, the known functions of Ccm3 and how they relate to CCM pathogenesis will be discussed below.

Ccm3 functions and their relation to CCM pathogenesis

Ccm3 is implicated in a variety of cellular processes, including apoptosis, cell proliferation, cell migration, cell-cell adhesion, cell polarity, and vascular development. It has been shown that overexpression of Ccm3 wildtype but not CCM patient-derived Ccm3 mutants induced apoptosis, suggesting that the aberrant apoptosis due to loss of Ccm3 might lead to the disease [185]. However, the role of Ccm3 in apoptosis is controversial as some studies reveal a proapoptotic role of Ccm3 while others reported an anti-apoptotic function.

Ccm3 is important for vascular development. Depletion of Ccm3 in human umbilical vein endothelial cells (HUVECs) increased angiogenic sprouting and led to failure in lumen formation, while morpholino KD of Ccm3 in zebrafish showed defects in cranial vasculature lumenization [186-188]. In mice, loss of Ccm3 caused defective cell-cell junctions and vascular integrity, and also vascular defects including increased venous size and venous rupture [186, 189]. Similar phenotypes were also reproduced by using endothelial cell lines in which Ccm3 loss caused increased vessel size, decreased vessel density, compromised vessel integrity, and disruption of tight junctions (TJs) and adherens junctions (AJs) [189]. Thus, these observed phenotypes of Ccm3 loss in cell lines as well as model organisms may contribute to the characteristics of CCM lesions.

Ccm3 has been shown to stabilize vascular endothelial growth factor receptor 2 (VEGFR2) by blocking its endocytosis and degradation [189]. Interestingly, expression of CCM patient-derived Ccm3 mutants with C-terminal truncation enhanced VEGFR2 internalization and degradation, suggesting that Ccm3 function in VEGFR2 stabilization may be linked to CCM pathogenesis [189]. While one study failed to show the connection between Ccm3 and VEGF signaling, it would be interesting to further investigate the functional significance of Ccm3 regulation of VEGFR2 and its relation to STRIPAK and CCM pathogenesis.

Several lines of evidence suggest that Ccm3 is important for STRIPAK-associated PP2A-mediated regulation of GCKIII kinases and that misregulation of GCKIII by Ccm3 mutants lead to CCM development. Ccm3 binds GCKIII kinases via its dimerization domain at N-terminus [190]. Ccm3 also binds to the striatin family members of STRIPAK complexes [150, 152], binds Ccm2 forming a Ccm1-Ccm2-Ccm3 complex (Ccm complex) [191-194], and binds paxillin as well via its C-terminal FAT-homology domain [192]. In line with this, Ccm3 has been shown to recruit GCKIII kinases to the STRIPAK complexes [152]. Additionally, association between GCKIII kinases and Ccm2 has been detected [194, 195], suggesting a possible role of Ccm3 in recruiting GCKIII kinases to the Ccm complex. In addition to the previously discussed role of STRIPAK in negatively regulating GCKIII kinases probably through Ccm3, which tethers the kinases to STRIPAK, Ccm3 also regulates Golgi assembly, Golgi and centrosome polarization, and cell migration by stabilizing GCKIII kinases [196]. Ccm3 knockdown resulted in ubiquitination and degradation of GCKIII kinases and defects in Golgi assembly, Golgi and centrosome polarization, and cell migration [196]. More

importantly, CCM patient-derived Ccm3 mutants that do not interact with GCKIII kinases did not rescue the phenotypes due to Ccm3 depletion, indicating the functional significance of Ccm3 and GCKIII interaction in Golgi function and cell polarity and migration may be key to CCM pathology [196].

Studies in zebrafish also implicate the potential importance of the interaction between Ccm3 and GCKIII kinases in CCM pathogenesis. A CCM patient-derived Ccm3 mutant with a small N-terminal in-frame deletion and/or the equivalent deletion mutant in zebrafish does not bind GCKIII kinases while still forming the Ccm complex with Ccm1 and Ccm2 [197, 198]. Inactivation of *Ccm3* genes (*Ccm3a* and *Ccm3b*) or morpholino in-frame skipping of GCKIII binding site in *Ccm3a* with inactivation of *Ccm3b* in zebrafish led to severe cardiovascular phenotypes including cardiac dilation and pericardial edema [197, 198]. Also, depletion of all GCKIII kinases or of a combination of Ccm3 with either Mst3 or Ysk1 resulted in similar cardiovascular defects [198]. Together, these data support the idea that the interaction between Ccm3 and GCKIII kinases are critical in proper cardiovascular development in zebrafish as well as in understanding the mechanisms underlying CCM pathology [197, 198].

Goals of the Dissertation

For the last few decades, PP2A has been the focus of numerous studies which found that the heterotrimeric nature of PP2A forms potentially over 90 different complexes that regulate a plethora of cellular processes. These studies also revealed that complex regulatory mechanisms control PP2A. Among these, carboxyl methylation on the C-terminal leucine residue of PP2Ac was found to be critical for formation of

methylation-dependent holoenzyme complexes and thus regulates specificity and function of the enzyme. Evading normal cellular control of PP2A by PP2Ac methylation was also demonstrated to be a means for tumor viruses to promote tumorigenesis [199]. As a result, ways to increase PP2Ac methylation levels such as using inhibitors of PME-1 may be an alternative strategy to combat cancer where PP2Ac methylation is defective. However, similar knowledge on the methylation-dependency of PP2A-related phosphatases (PP4 and PP6) is lacking. The C subunits of PP4 and PP6 contain the completely conserved leucine residue that is the site of carboxyl methylation in PP2Ac, but only PP4c has been shown to be methylated. The organization of PP4 and PP6 holoenzymes has just begun to be explored as well. Therefore, it remains to be determined whether similar mechanisms to PP2A exist for regulation of PP4 and PP6 methylation and function. Specifically, whether PP6 is methylated needs to be determined and, if it is, the methyltransferase(s) responsible for PP4c and PP6c methylation needs to be identified. Moreover, the mechanistic role of methylation in the regulation of these phosphatases would then need to be uncovered.

Among the many heterotrimeric complexes of PP2A found in normal cells are complexes that are methylation-independent. The most well established of these are PP2A heterotrimeric complexes containing B''' (striatin family) B-type subunits. Recently, there was an explosion of research focused on these striatin-associated PP2A complexes to determine their molecular organization and function. Intriguingly, the striatin-associated PP2A complexes have been implicated in a number of human diseases, especially cerebral cavernous malformations (CCM). In addition to the presence of striatin and PP2A, the striatin-associated PP2A complexes consist of a number of core

proteins, including Mob3, GCKIII kinases, and Ccm3 and *Ccm3*, the gene encoding Ccm3 protein, is one of the three *CCM* family genes mutated in CCM. However, the mechanisms that regulate the molecular structure and function of the striatin-associated PP2A complexes and the functional significance of the co-existence of a kinase and a phosphatase in this one functional unit remain largely unexplored.

To address these gaps, studies presented in this dissertation were performed to investigate the control of PP2A family phosphatase holoenzyme assembly and function by methylation (Fig. 1-7A). In addition, the molecular architecture of the striatin family-associated PP2A complexes were studied in great detail (Fig. 1-7B). The findings reveal that PP6c, like PP2Ac and PP4c, is methylated and that LCMT-1 is the major methyltransferase for all three of these PP2A family phosphatases. Also, similar to PP2A, PP4 forms both methylation-dependent and methylation-independent complexes. The importance of PP6c methylation for PP6 holoenzyme assembly remains to be further explored. In addition, studies in this dissertation on the striatin-associated PP2A complex indicate that striatin brings PP2A and GCKIII kinases together to negatively regulate GCKIII kinase activity by PP2A-mediated dephosphorylation of GCKIII kinases. Overall, these studies provide important insights into the mechanisms that regulate the formation and function of complexes formed by both PP2A and PP2A-related phosphatases (PP4 and PP6). These insights may relate to pathogenesis of some human diseases where dysfunction of these enzymes is observed.

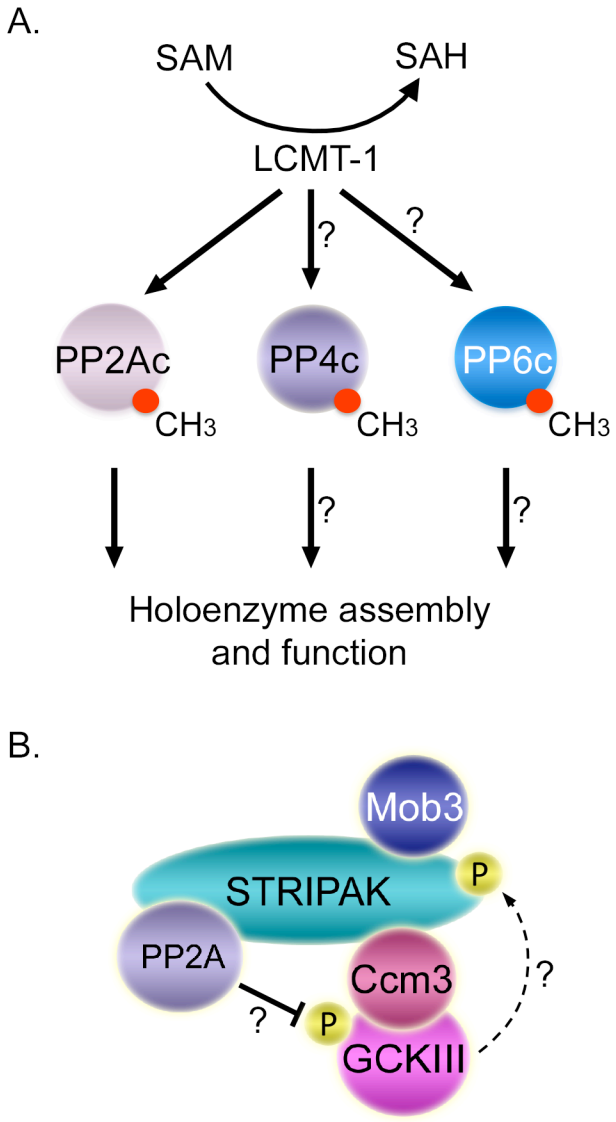

Figure 1-7. Hypothetical models illustrating the regulation mechanisms of PP2A family phosphatases and the STRIPAK complex.

(A) LCMT-1 is the methyltransferase for PP2Ac. PP2Ac methylation by LCMT-1 regulates the holoenzyme assembly and function of PP2A. While PP4c methylation has been reported, no evidence exists for PP6c methylation. Since both PP4c and PP6c possess the conserved C-terminal leucine, the site of methylation in PP2Ac, it was

hypothesized that LCMT-1 is the major methyltransferase for both PP4c and PP6c. In addition, it seems highly likely that LCMT-1 also regulates the holoenzyme assembly and function of these PP2A family phosphatases. (B) The striatin-associated PP2A complex was found to interact with a number of proteins, including Mob3, GCKIII kinases, and Ccm3, and forms a multiprotein complex called STRIPAK. Aims of this study were to identify the mechanisms that regulate the molecular structure and function of the striatin-associated PP2A complexes. Additionally, it is intriguing that GCKIII kinases and PP2A co-exist in this one functional unit and it led to a hypothesis that phosphorylation/dephosphorylation by these enzymes plays an important role in regulating the striatin-associated PP2A complex.

CHAPTER 2

Results

Carboxyl Methylation of Protein Phosphatase 4 by LCMT-1 is important for PP4 holoenzyme assembly and function

Introduction

Protein phosphatase 2A (PP2A) is a multifunctional serine/threonine protein phosphatase that is involved in a variety of cellular processes including cell cycle regulation, cell signaling, apoptosis, and development [4]. PP2A primarily exists as heterotrimeric complexes made up of a structural A subunit (PP2A A), a catalytic C subunit (PP2Ac), and one of many B-type regulatory/targeting subunits. B-type subunits are encoded by different genes, some with multiple splice variants, and are classified into 4 groups: B (PPP2R2), B' (PPP2R5), B'' (PPP2R3), and B''' (Striatin family). Combination of the PP2A A/C core dimer with one of many B-type subunits determines enzyme activity, substrate specificity, and subcellular localization.

The PP2A-related phosphatases, protein phosphatase 4 (PP4) and protein phosphatase 6 (PP6), are also involved in numerous cellular processes including cell cycle regulation and cell signaling pathways [85, 200, 201]. PP4 and PP6 have functions that are distinct from PP2A but also some common or overlapping functions. For example, one common function of PP2A, PP4, and PP6 is to dephosphorylate γ-H2AX, a

marker of DNA double-stranded breaks, formed during DNA replication or by DNA damage [89-97].

PP2Ac is methylated on its carboxyl-terminal leucine, leucine 309 (L_{309}). Carboxyl methylation on Leu_{309} is tightly regulated by leucine carboxyl methyltransferase 1 (LCMT-1) [15-20] and protein phosphatase methylesterase 1 (PME-1) [21-23]. The C-terminal residues ($_{307}YFL_{309}$) of PP2Ac are completely conserved in PP2A-related phosphatases, PP4 and PP6 [202]. While the catalytic subunit of PP4 (PP4c) is methylated on its conserved C-terminal leucine, Leu_{307} [62], methylation of the catalytic subunit of PP6 (PP6c) has not yet been described. In addition, the identities of the PP4 methyltransferase and methylesterase remain unknown.

One major function of PP2Ac carboxyl methylation is to regulate PP2A holoenzyme assembly. Depending on the methylation state of PP2Ac, PP2Ac binds different sets of B-type subunits creating either methylation-dependent or methylation-independent complexes. Organization of PP4 and PP6 holoenzymes is not fully understood and has only recently begun to be elucidated. PP4 and PP6 form hetero-oligomeric complexes that consist of a catalytic subunit and one or more regulatory/targeting subunits. Five different PP4 regulatory subunits have been reported thus far: PP4R1, PP4R2, PP4R3α and β, and PP4R4 [69-72]. PP4c associates with one or two of these regulatory subunits forming either a heterodimeric or heterotrimeric complex. PP6 is known to assemble into a heterotrimeric complex formed by PP6c binding to a SAPS (Sit4-Associated Proteins) regulatory subunit and an ARS (ankyrin repeat subunit) regulatory subunit [27, 83]. As for PP2A, enzyme activity and substrate specificity are specified by the regulatory subunits that associate with PP4c and PP6c.

Despite high sequence similarities, each PP2A family phosphatase has a set of regulatory subunits that are generally not shared with each other. One exception is a protein called α4, a common binding partner of PP2A family phosphatases that binds to C subunits independently from other regulatory or structural subunits [107-112]. While the role of PP2Ac methylation on PP2A heterotrimeric assembly is understood, how PP4 and PP6 holoenzymes are assembled has not been studied in detail. Therefore, how methylation of these PP2A-related phosphatases relates to their holoenzyme assembly is of fundamental interest.

Here we show that as for PP2Ac and PP4c, PP6c is methylated *in vivo*. Like for PP2Ac, LCMT-1 is the major methyltransferase for PP4c and PP6c. As previously reported for PP2A and Tap42 in yeast, loss of LCMT-1 leads to increased PP2Ac interaction with α4. Intriguingly, loss of LCMT-1 altered distribution of PP4 complexes as observed by blue native polyacrylamide gel electrophoresis (BN-PAGE), indicating that PP4 forms both methylation-dependent and methylation-independent complexes. Further analyses of PP4 holoenzyme assembly reveal that the methylation-dependent PP4 complex that is lost in LCMT-1 KO MEFs contains PP4R1 while the largely methylation-independent PP4 complex is composed of PP4R2 with either PP4R3α or PP4R3β. Lastly, hyperphosphorylation of HDAC3, a substrate of PP4R1-PP4c complex, was observed by LCMT-1 loss. Thus, our data indicate that LCMT-1 not only regulates carboxyl methylation of PP2A family phosphatases but also their holoenzyme assembly and function.

Methods and materials

Antibodies and other reagents

Methylation-sensitive antibodies were used to detect the level of demethylated PP2Ac (clone 4b7; available from EMD Millipore), PP4c (Bethyl Laboratories, Inc.), and PP6c (Bethyl Laboratories, Inc.). Methylation-insensitive antibodies were used to detect the total level of PP2Ac (BD Transduction Laboratories), PP4c (Bethyl Laboratories, Inc. and R&D systems), and PP6c (Proteintech). Other antibodies used include anti-α-tubulin mouse monoclonal antibody (Calbiochem), anti-GAPDH mouse monoclonal antibody (Novus Biologicals), anti-actin goat polyclonal antibody (Santa Cruz Biotechnology), anti-HA tag mouse monoclonal antibody 16B12 (Covance), anti-α4 mouse monoclonal antibody (EMD Millipore), anti-PP2A B subunit mouse monoclonal antibody clone 2G9 (EMD Millipore), anti-HDAC3 rabbit monoclonal antibody (EMD Millipore), and anti-phospho-HDAC3 (Ser_{424}) rabbit polyclonal antibody (Cell Signaling). All rabbit antibodies against PP4 regulatory subunits were from Bethyl Laboratories, Inc. Protein G-Sepharose 4B beads (Invitrogen), TrueBlot® anti-Rabbit Ig IP Beads (Rockland Immunochemicals Inc.), TrueBlot® anti-Rabbit IgG (HRP) (Rockland Immunochemicals Inc.), and Clean-Blot Detection Reagent (HRP) (Thermo Scientific) were used for immunoprecipitation and detection.

Peptides corresponding to C-terminal residues of PP4c (Ac-CTRGIPSKKPVADYFL-Me) and PP6c (Ac-CSERVIPPRTTTPYFL-Me) were synthesized by NeoBioScience (Cambridge, MA) and used to characterize the

methylation-sensitive PP4c and PP6c antibodies. PP2A C-terminal peptide was used to pre-adsorb PP4c antibody.

Cell culture and transfection

LCMT-1 wildtype, hemizygous, and homozygous knockout mouse embryonic fibroblasts (MEFs) were isolated from E14.5 or older mouse embryo torsos and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, and nonessential amino acids at 37°C in 5% CO_2. QBI293 (QBI-HEK-293 from QBiogene) cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in 10% CO2. QBI293 cells were transfected using FuGENE™ 6 transfection reagent (Promega) and Opti-MEM® I Reduced Serum Medium (Gibco®) according to the manufacturer's protocol.

Cell lysis, immunoprecipitation, SDS-PAGE, and immunoblotting

Cells were washed with ice-cold phosphate-buffered saline and IP wash buffer (0.135 M NaCl, 10% glycerol, 20 mM Tris, pH 8.0) before being lysed with IP lysis buffer (IP wash buffer containing 1% Nonidet P-40, 0.04 trypsin inhibitor units/ml aprotinin, 1 mM phenylmethylsufonyl fluoride, 50 mM sodium fluoride, and 1mM sodium orthovanadate) by rocking for 20 minutes at 4°C. Lysates were cleared by centrifugation at 13,000 × g for 10 minutes at 4°C. Protein concentration was determined by using Bio-Rad Protein Assay Dye Reagent Concentrate according to the manufacturer's instruction. In order to co-immunoprecipitate protein complexes, cell lysates were incubated either with crosslinked antibody-sepharose beads or with

immunoprecipitating antibody and sepharose beads for 1.5 hours at 4°C. Protein lysate samples and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes for western blotting. Bands from immunoblotting were visualized by enhanced chemiluminescence and a Fluor S-Max Chemilumimager (Bio-Rad), which directly measures band intensities via a supercooled CCD camera that provides linear data over 4.8 orders of magnitude. Quantity One software from Bio-Rad was used to quantify band intensities.

Methylation assay

Methylation assays for PP4c and PP6c were performed as previously reported for PP2A [34]. Briefly, two equal aliquots of cell lysates were prepared. One aliquot of each cell lysate was treated with pre-neutralization solution to preserve methylation state of PP4c and PP6c. The other aliquot was treated with 200mM NaOH base solution for 5 minutes on ice, which removes all C-terminal carboxyl methylation. Samples were analyzed by SDS-PAGE and immunoblotting using methylation-sensitive antibodies to determine the level of carboxyl methylation on PP4c and PP6c.

Blue native polyacrylamide gel electrophoresis (BN-PAGE)

To preserve native protein complexes, cells were lysed in non-denaturing lysis buffer containing 1% Triton X-100. 20ug of cell lysates per lane were resolved by NativePAGE™ Novex 4–16% Bis-Tris Gels (Life Technologies) according to the manufacturer's instruction. Proteins were transferred to PVDF (polyvinylidene difluoride) membranes for immunoblotting. For BN-PAGE experiments, freshly prepared cell

extracts were always used instead of frozen cell lysates to preserve the native state of protein complexes.

Results

LCMT-1 is the major methyltransferase for PP4c and PP6c

We hypothesized that PP6c is methylated because of its similarity to PP2Ac and PP4c, including the identity of their C-terminal YFL residues, which include the C-terminal leucine methylated in PP2Ac and PP4c (Fig. 2-1A). For the same reason we also hypothesized that LCMT-1 was the methyltransferase for these PP2A family phosphatases. To test this hypothesis, we first developed western blot-based assays for PP4c and PP6c methylation analogous to the assay we use routinely for determining the methylation state of PP2Ac [34]. Based on our previous experience with antibodies to PP2Ac, we reasoned that antibodies raised to unmethylated C-terminal peptides of PP4c and PP6c may be largely specific for unmethylated PP4c and PP6c. To test this, we obtained C-terminal antibodies to these phosphatase catalytic subunits from Bethyl laboratories and tested their specificity for peptides corresponding to the C-termini of methylated and unmethylated PP4c and PP6c. Both the PP4c and PP6c C-terminal antibodies showed high specificity for unmethylated PP4c and PP6c peptides and almost no reactivity to the corresponding methylated peptides (Fig. 2-1B and C), demonstrating that the binding of these antibodies is indeed inhibited by methylation. Thus, these antibodies provide valuable reagents for analyzing the methylation state of PP4c and

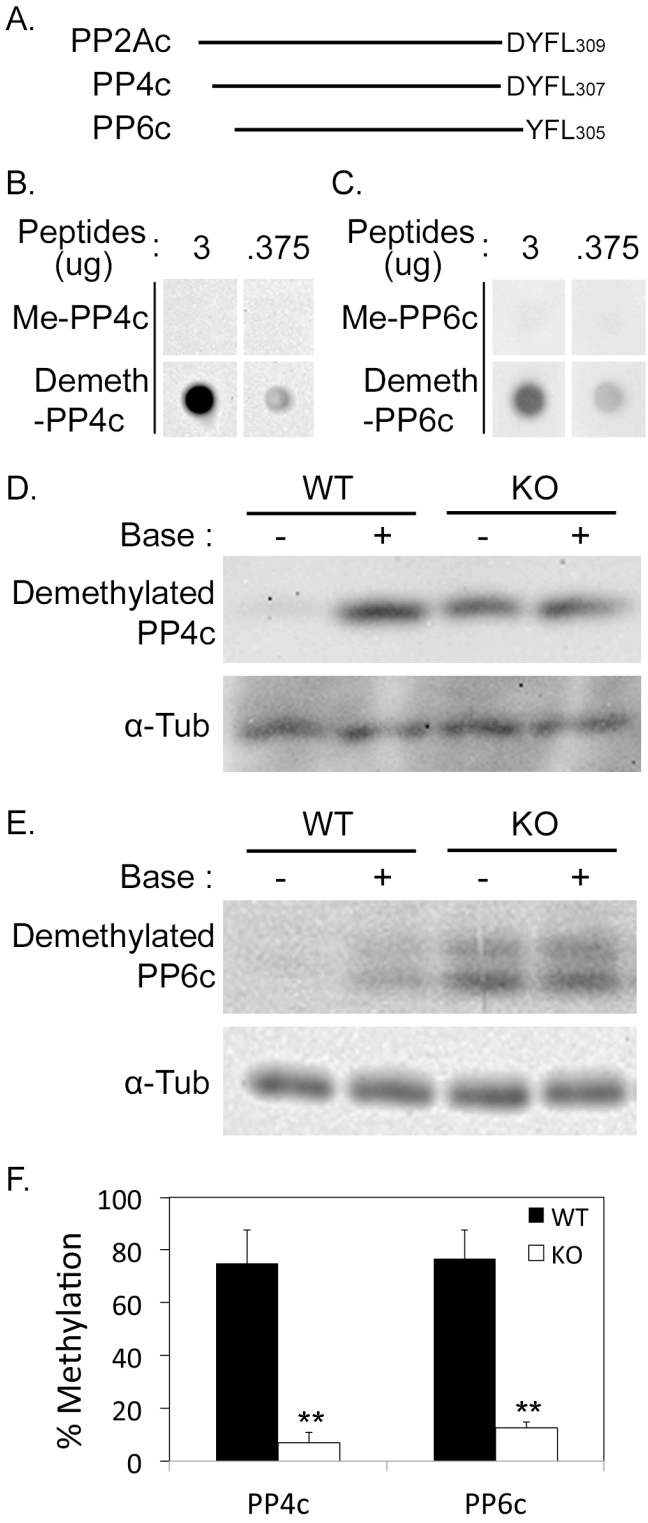

Figure 2-1. PP4c and PP6c are highly methylated *in vivo* by LCMT-1.

(A) A stick diagram illustrating PP2Ac, PP4c, and PP6c proteins. The conserved C-terminal YFL sequence is shown where the leucine residue is the known methylation

site in PP2Ac and PP4c. (B-C) Testing methylation-sensitive PP4c and PP6c antibodies. PP4c and PP6c peptides of 16 C-terminal amino acid residues were synthesized to characterize the methylation-sensitive PP4c and PP6c antibodies. Two different concentrations of the PP4c (B) and PP6c (C) peptides were demethylated by base treatment and spotted onto nitrocellulose membranes along with methylated peptides. Western blotting was performed using the methylation-sensitive PP4c (B) and PP6c (C) antibodies. (D-F) Equal amounts of cell lysates from LCMT-1 WT and KO MEFs were treated with or without base. Cell lysates were resolved by SDS-PAGE and western blotting was performed using the methylation-sensitive PP4c (D) and PP6c (E) antibodies. α-Tubulin (α-Tub) serves as a loading control. Base treatment removes all carboxyl methylation in proteins and thus the plus base lane (+) represents total amount of demethylated proteins of interest in reactions. Normalizing the amount of demethylated protein in the minus base lane (-) to that of the plus base lane (+) gives the percent demethylation of the protein, which can then be converted to the percent methylation (100 - % demethylation). The percent methylation of PP4c and PP6c are quantified in (F). The error bars represent the standard deviation of at least three independent experiments. **, $p \leq 0.01$.

PP6c and the same lot of them that was characterized in Figure 2-1B and C was used throughout this study.

To use these antibodies for quantitation of PP4c and PP6c methylation on western blots, it is critical that they specifically only recognize the corresponding protein or that the phosphatases can be clearly separated. While the PP6 antibody specifically recognized PP6 (data not shown), we found that the C-terminal PP4c antibody demonstrated some cross-reactivity to PP2Ac. While we could usually resolve PP4c and PP2A on gels, to produce a more specific antibody specific for unmethylated PP4c, we pre-adsorbed this antibody with a PP2Ac C-terminal peptide, resulting in an antibody highly specific for unmethylated PP4c (Fig. 2-2).

We next used these antibodies to analyze PP4c and PP6c methylation *in vivo* and to determine the importance of LCMT-1 for their methylation. Cell lysates from LCMT-1 wildtype (WT) and knockout (KO) mouse embryonic fibroblasts (MEFs) were treated with or without base, which removes all carboxyl methylation. Lysates were then resolved by SDS-PAGE and western blotting was performed using the methylation-sensitive antibodies tested in Fig. 2-1B and C. The results showed that PP4c is highly methylated (75.0%±12.8) in WT MEFs but almost unmethylated (6.9% ±4.2) in LCMT-1 KO MEFs (Fig. 2-1D and F). These results indicate that PP4c is highly methylated *in vivo* and that LCMT-1 is the major PP4c methyltransferase. Similarly, PP6c was also highly methylated (76.6%±11.0) in WT MEFs while its methylation level decreased to 12.6%±2.4 in LCMT-1 KO MEFs (Fig. 2-1E and F), indicating that PP6c is also highly methylated *in vivo* by LCMT-1. Therefore, PP6c is indeed methylated *in vivo* and

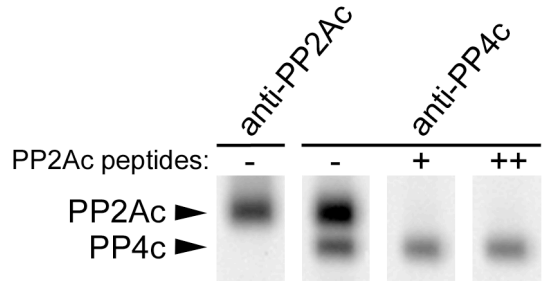

Figure 2-2. Pre-adsorption of the PP4c antibody with PP2Ac peptides.

PP4c methylation-sensitive antibody was pre-adsorbed with PP2Ac C-terminal peptides for an hour rocking at 4°C. While the untreated PP4c antibody detected both endogenous PP2Ac and PP4c, pre-adsorption of PP4c antibody helped clear out PP2Ac signal without affecting PP4c detection. +, 20ug of PP2Ac peptides; ++, 50ug of PP2Ac peptides.

LCMT-1 is the major methyltransferase for all three PP2A-related phosphatases PP2Ac, PP4c, and PP6c.

Loss of LCMT-1 affects formation of PP2A holoenzyme complexes

Previously, our lab has shown that association of PP2Ac with the methylation-dependent Bα subunit is decreased by LCMT-1 knockdown in HeLa cells [203]. To examine whether this association is also compromised by LCMT-1 knockout in MEFs isolated from embryos, co-immunoprecipitation was performed using cell lysates from LCMT-1 WT, hemizygous, and KO MEFs. Consistent with our previous finding, the association of PP2Ac with B subunit was decreased by more than 50% in LCMT-1 KO but not in hemizygous MEFs as compared to WT MEFs (Fig. 2-3B and C). It is noteworthy that B subunit protein level is also decreased in LCMT-1 KO MEFs, probably due to destabilization of B subunit in the absence of LCMT-1 (Fig. 2-3A). Considering both the reduction of B subunit amount and the reduced binding of the remaining B subunit, we calculate that B subunit trimers are decreased over 90% in LCMT-1 knockout MEFs (not shown).

To study the effects of LCMT-1 loss on PP2A family phosphatases, we employed BN-PAGE, which preserves the integrity of complexes while separating them by apparent size. To characterize BN-PAGE for this purpose, we first used it to examine changes in PP2A complexes upon LCMT-1 knockout. Non-denatured cell lysates from two independently derived sets of LCMT-1 WT and KO MEFs (M1 and M2) were resolved by BN-PAGE, and then western blotted with antibodies specific for PP2A B subunit and PP2Ac (Fig. 2-4A and B, respectively) to compare the PP2A complexes.

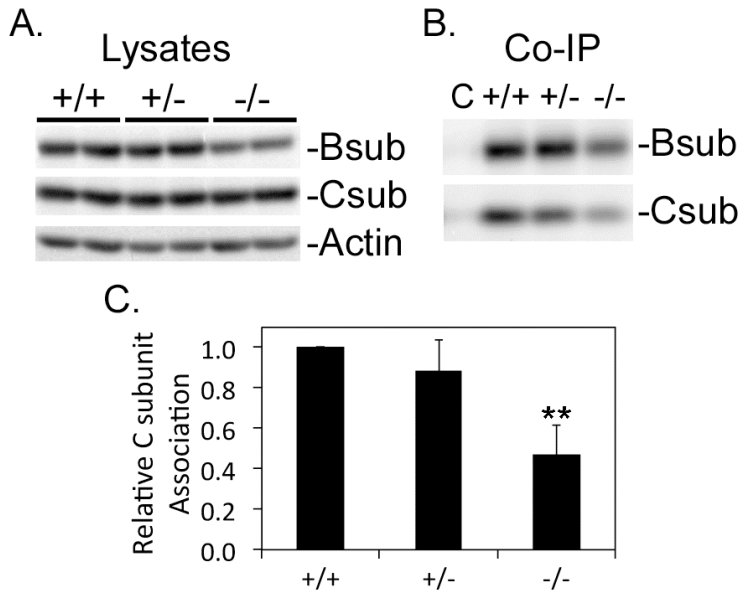

Figure 2-3. Association of PP2A C subunit and B subunit is decreased in LCMT-1 KO MEFs.

Co-immunoprecipitation was performed using crosslinked 2G9-Protein G sepharose beads and cell lysates from LCMT-1 WT (+/+), hemizygote (+/-), and KO (-/-) MEFs. Inputs (A) and immunoprecipitates (B) were resolved by SDS-PAGE and analyzed by immunoblotting. Actin serves as a loading control. C, control, beads only. The relative association of PP2Ac and B subunit is quantified in (C). Error bars represent S.D. of three independent experiments. **, $p \leq 0.01$.

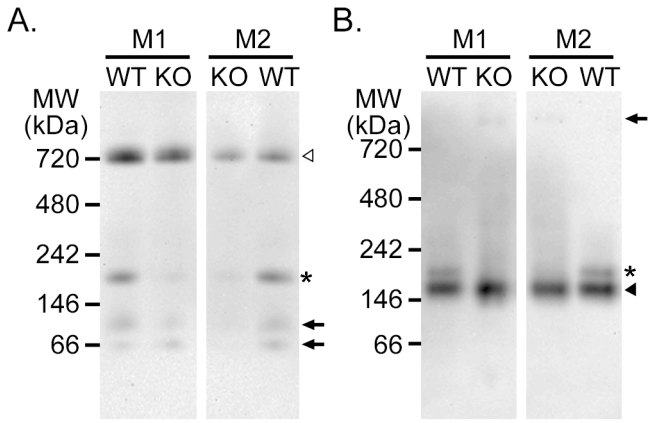

Figure 2-4. Loss of methylation-dependent PP2A heterotrimer in LCMT-1 KO MEFs.

Two sets (M1 and M2) of LCMT-1 WT and KO MEFs were lysed in non-denaturing lysis buffer and equal amounts (20ug/well) of lysates were resolved by BN-PAGE. Formation of the PP2A complex in the presence and absence of LCMT-1 was analyzed by immunoblotting with various antibodies including antibodies against the B subunit (2G9) (A) and total PP2Ac (B). (A) Asterisk, the decreased methylation-dependent PP2A heterotrimer; arrows, the decreased B subunit-containing complex in KO; open arrowhead, the high molecular weight PP2A complexes containing A and B subunits plus unknown binding partner(s). (B) Asterisk, the methylation-dependent PP2A heterotrimer; solid arrowhead, the methylation-independent PP2A heterotrimer; arrow, the high molecular weight PP2A complex containing PP2Ac plus unknown binding partner(s).

The results were striking. PP2A B and C subunits formed a number of major complexes with a wide range of molecular weights. First, a substantial amount of PP2A B subunit in both WT and KO MEFs is present in a novel large complex of approximately 720kDA apparent molecular weight (open arrowhead; Fig. 2-4A) that apparently lacks PP2Ac. PP2A A subunit was detected co-migrating with this complex by western blotting (data not shown), suggesting that this novel complex contains B subunit and A subunit complexed with other yet unidentified proteins in the absence of PP2Ac. Reciprocally, the majority of PP2Ac was present in a complex of approximately 150kDa that lacks B subunit (solid arrowhead; Fig. 2-4B) but co-migrates with A subunit (not shown). This complex is not affected by the loss of LCMT-1, suggesting that this is a methylation-independent PP2A heterotrimer(s) (solid arrowhead in Fig. 2-4B). The remainder of the B subunit in WT MEFs is present mainly in smaller bands of approximately 180kDa (asterisk; Fig. 2-4A) and 110kDa, sizes consistent for BAC heterotrimers and BA heterodimers, respectively, and a small band of approximately 55kDa, likely B subunit monomer (arrows; Fig. 2-4A). Consistent with these possible assignments, the 180 kDa complex immunoblots with PP2Ac antibody in WT cells (Fig. 2-4B; asterisk) and A subunit antibody (not shown), while the 110kDa complex (solid arrowhead; Fig. 2-4) immunoblots only with A subunit (Fig. 2-4B and data not shown). Moreover, the BAC PP2A heterotrimer complex (asterisk; Fig. 2-4A and B) is nearly lacking in LCMT-1 KO MEFs, consistent with its known dependency on PP2Ac methylation for efficient assembly [34, 35].

While loss of methylation due to LCMT-1 KO clearly reduced the PP2A BAC complex, it generated a novel band containing PP2Ac of very high molecular weight

(arrow; Fig. 2-4B). Although only a weak band is shown in Fig. 2-4B due to low abundance of this complex, we have consistently observed existence of a small amount of this high molecular weight PP2A complex in LCMT-1 WT MEFs as well. This PP2A complex will be discussed in more detail below.

Loss of PP2Ac methylation by LCMT-1 knockout redistributes PP2A complexes: Loss of PP2Ac methylation by LCMT-1 knockout increases co-migration of α4 with PP2Ac

To determine the identity of the minor but very large PP2Ac-containing complex that increased in LCMT-1 KO MEFs (arrow in Fig. 2-4B), we searched for candidate proteins that interact with PP2Ac in the absence of the A and B subunits. Alpha4 (α4) is known to associate with PP2Ac independently of A and B subunits [107, 108, 112, 113]. In yeast, Tap42 (yeast α4)-associated PP2Ac accounts for about 2% of the total cellular PP2A [4]. As shown in Fig. 2-5A, α4 forms a number of complexes as analyzed by BN-PAGE. Intensity of the α4 band with the highest molecular weight was enhanced in LCMT-1 KO MEFs as compared to WT MEFs (open arrowhead in Fig. 2-5A). Interestingly, this α4 complex co-migrated with the PP2Ac band in Fig. 2-4B (arrow) that also increases in LCMT-1 KO MEFs, suggesting that α4 and PP2Ac assemble into a very large molecular weight complex with other unknown interactors and that the interaction between α4 and PP2Ac is enhanced by LCMT-1 KO.

To confirm that the loss of the methylation-dependent PP2A heterotrimer and redistribution of PP2A complexes in LCMT-1 KO MEFs are indeed due to loss of LCMT-1 and therefore loss of PP2Ac methylation, HA-tagged PP2Ac WT and ΔLeu_{309}

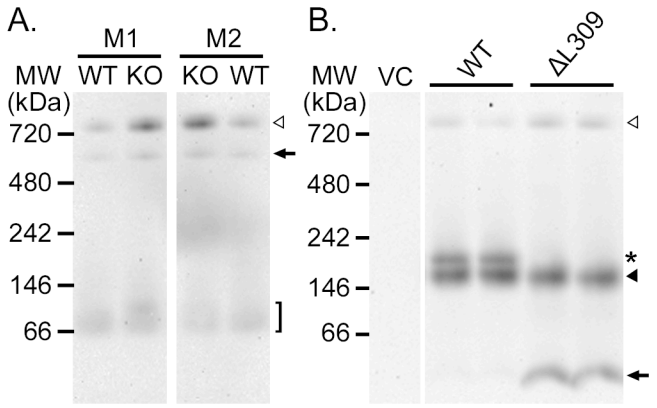

Figure 2-5. Loss of PP2Ac methylation by LCMT-1 knockout increases co-migration of α4 with PP2Ac.

(A) Cell lysates from two sets (M1 and M2) of LCMT-1 WT and KO MEFs were prepared under non-denaturing conditions and resolved by BN-PAGE for further analysis. To determine overall distribution of α4-contatining complexes, antibody against α4 was used for immunoblotting. Open arrowhead, α4 co-migrating with PP2Ac; arrow, the α4 band that does not co-migrate with PP2A components; bracket, additional α4-specific bands. (B) QBI293 cells expressing HA-tagged PP2Ac WT and ΔLeu_{309} mutant were lysed in non-denaturing conditions and cell lysates were resolved by BN-PAGE. Antibody against HA-tag (16B12) was used to determine PP2A complexes containing PP2Ac WT and ΔLeu_{309} mutant. Asterisk, the methylation-dependent PP2A heterotrimer; solid arrowhead, the methylation-independent heterotrimer; arrow, the free PP2Ac; open arrowhead, co-migration with α4.

mutant were analyzed by BN-PAGE. While HA-tagged PP2Ac WT formed both methylation-dependent and methylation-independent heterotrimers similar to endogenous PP2Ac (asterisk and solid arrowhead, respectively; Fig. 2-5B), the PP2Ac ΔLeu_{309} mutant formed only methylation-independent heterotrimers (solid arrowhead in Fig. 2-5B). Interestingly, the PP2Ac ΔLeu_{309} mutant had a great increase in free C subunit (arrow in Fig. 2-5B) compared to WT PP2Ac, consistent with a previous report that a small population of a similar methylation-deficient PP2Ac L309A mutant exists as free C subunits [36]. Moreover, the high molecular weight PP2Ac complex associated with α4 was enhanced in PP2Ac ΔLeu_{309} mutant as compared to WT (open arrowhead; Fig. 2-5B), confirming that loss of PP2Ac methylation leads to increased association between PP2Ac and α4. Our results from Fig. 2-5A and 2-5B are consistent with the previous finding in yeast that the interaction of Tap42 (yeast α4) with Pph22 (yeast PP2Ac) increased in PPM1 (yeast LCMT-1)-deficient yeast cells [31], demonstrating that the conservation of this phenomenon between different species.

LCMT-1-mediated PP4 methylation regulates the stable formation of certain PP4 complexes

PP2Ac methylation greatly enhances the formation of certain PP2A heterotrimers, enhances the formation of others to a lesser degree, and has no effect on still others [32-34]. To determine whether LCMT-1-mediated methylation regulates the steady-state levels of PP4 and PP6 complexes, PP4 and PP6 complexes were examined in matched WT and LCMT-1 knockout MEFs using BN-PAGE. As shown in Fig. 2-6A, PP4c formed a large complex at about 450kDa (filled arrowhead) that was found in similar

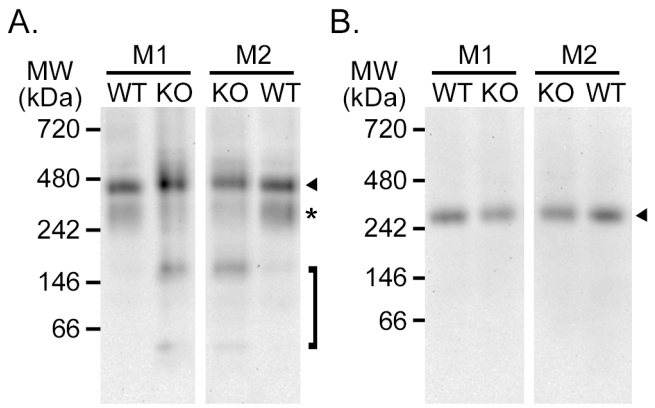

Figure 2-6. Loss of LCMT-1 affects formation of the PP4 protein phosphatase complexes.

Formation of the PP4 and PP6 complexes in the presence and absence of LCMT-1 was analyzed by BN-PAGE and immunoblotting with antibodies including antibodies against total PP4c (A) and total PP6c (B). Cell lysates of two sets (M1 and M2) of LCMT-1 WT and KO MEFs that were prepared under non-denaturing conditions were used. (A) Asterisk, the methylation-dependent complex; filled arrowhead, the methylation-independent complex; bracket, increased in KO. (B) Filled arrowhead, the major PP6 complex.

amounts in WT and LCMT-1 KO MEFs, indicating that this complex is mainly methylation-independent (hereafter referred to as “450kDa PP4 complex”). Of note, this PP4 complex always migrates slightly slower in LCMT-1 KO MEF lysates, suggesting that post-translational modification of one or more components or gain or exchange of one or more binding partners may have been induced by loss of LCMT-1. Interestingly, PP4c formed a second large complex(es) migrating as a broad band between approximately 250kDa and 350kDa (asterisk) and much of this complex was lost in LCMT-1 KO MEFs (hereafter referred to as “300kDa PP4 complex”), indicating that this complex is largely methylation-dependent. Corresponding with the great loss of the 300kDa PP4 complex, new, smaller PP4c complexes appeared only in LCMT-1 KO MEFs (bracket; Fig. 2-6A), suggesting that there is a redistribution of PP4c population by LCMT-1 loss. Based on its size, the smallest band indicated by the bracket probably represents free PP4c monomers, which are absent in WT MEFs, while the larger band in the bracket may be a PP4c complex that lost or exchanged one or more interacting protein(s). Therefore, similar to the case for PP2A, PP4 methylation appears to differentially affect the stable assembly of PP4 holoenzymes. Interestingly, however, in apparent contrast to PP2A and PP4 protein complexes, similar analysis of PP6 complexes in WT and LCMT-1 KO MEFs showed that PP6 formed one major complex of about 250kDa that did not change upon LCMT-1 KO (filled arrowhead; Fig. 2-6B), suggesting that PP6 forms one major methylation-independent protein complex in MEFs. Whether this complex is in fact a mixture of co-migrating PP6 complexes or is a single complex predominating in MEFs remains to be determined. Thus it is possible that methylation-sensitive PP6 complexes exist but were not amenable to detection in this system.

PP4R1-associated PP4 holoenzyme complex is methylation-dependent

To determine the components of the 450kDa and 300kDa PP4 holoenzyme complexes identified in Fig. 2-6, cell lysates of WT and LCMT-1 KO MEFs were resolved by BN-PAGE and analyzed using antibodies against PP4 regulatory subunits (PP4Rs). PP4R1 co-migrated with the 300kDa PP4 complex that was greatly reduced in LCMT-1 KO (asterisk; Fig. 2-7A). Consistent with the hypothesis that PP4R1 is part of the methylation-dependent 300kDa PP4 complex, the 300kDa PP4R1 band was substantially decreased in LCMT-1 KO MEFs as compared to WT (asterisk; Fig. 2-7B). On the other hand, PP4R2 was found to co-migrate with the 450kDa PP4 complex that was mostly intact in LCMT-1 KO MEF lysates (filled arrowhead; Fig. 2-7A). As was the case for the 450kDa complex containing PP4c (filled arrowhead; Fig. 2-6A), PP4R2 migration was slightly retarded in LCMT-1 KO MEF lysates (filled arrowhead; Fig. 2-7C). This result supports the idea that PP4R2 is indeed a component of the 450kDa PP4 complex and that PP4R2 and PP4c remain in the same complex even in the absence of LCMT-1 and PP4c methylation (i.e., that PP4R2-PP4c association is not regulated by methylation). BN-PAGE analysis also showed that the majority of PP4R3α co-migrates with the 450kDa PP4 complex while a minor population of PP4R3α co-migrates with the 300kDa PP4 complex (Fig. 2-7A). Lastly, PP4R3β was found in the upper band of PP4 complex (filled arrowhead; Fig. 2-7A). Finally, PP4R4 could not be studied by this approach due to lack of commercially available antibody with sufficient specificity. Thus, based on co-migration and in the case of PP4R2 its altered electrophoretic mobility in LCMT-1 KO MEF lysates, these experiments suggest that the 450kDa PP4 band is

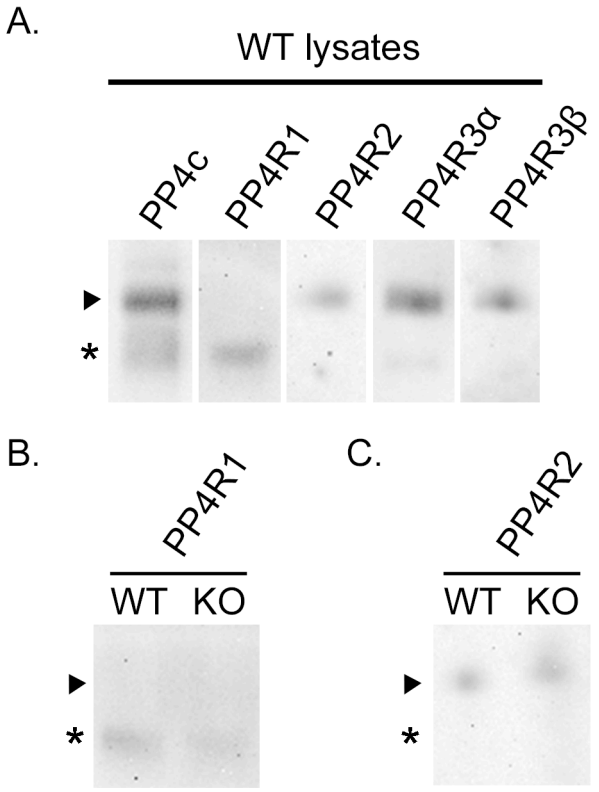

Figure 2-7. PP4R1-associated PP4 holoenzyme complex is methylation-dependent.

Cell lysates of LCMT-1 WT MEFs were analyzed by BN-PAGE and immunoblotting using antibodies against the total PP4c and PP4 regulatory subunits (PP4R1, PP4R2, PP4R3α, and PP4R3β) (A). Migration of PP4R1 (B) and PP4R2 (C) subunits was compared between LCMT-1 WT and KO MEFs. Filled arrow indicates the migration of the 450kDa PP4 complex, while asterisk indicates the migration of the 300kDa PP4 complex.

minimally comprised of PP4c, PP4R2, and either PP4R3α or PP4R3β, while the 300kDa PP4 band is minimally composed of PP4c and PP4R1 and/or PP43α.

While the co-migration results above are suggestive of which PP4 holoenzymes might constitute the 450kDa and 300kDa PP4 complexes, we devised an additional approach termed BN-PAGE EMSA (blue native polyacrylamide gel electrophoretic migration shift assay) to test directly which PP4 regulatory subunits are found in these two PP4 complexes. For this assay, cell lysates from WT MEFs were incubated with different PP4 regulatory subunit immunoprecipitating antibodies prior to being analyzed by BN-PAGE to determine if the antibodies are capable of retarding the migration of ("shifting") one or both of the PP4 complexes. If an antibody shifts a PP4c band, it would imply that the target of that antibody is a component of that PP4c band. The results of our analysis are shown in Fig. 2-8A and 2-8B and the first lane in each panel is a control lane that shows the 450kDa (filled arrowhead) and 300kDa (asterisk) PP4 bands described in Fig. 2-6A) that are seen if no antibody is added to the lysate prior to electrophoresis.

When PP4R1 antibody was used (lane 2; Fig. 2-8A), it shifted the majority of the 300kDa PP4 complex (asterisk) without disturbing the upper band of PP4 complex (filled arrowhead). The shifted 300kDa PP4 band can be seen in part just above the 450kDa PP4 band in the PP4R1 antibody lane. This indicates that PP4R1-associated PP4c constitutes at least a large portion of the 300kDa PP4 complex whose formation is largely lost in LCMT-1 KO MEFs. The fact that some of the 300kDa PP4 complex was not shifted by PP4R1 antibody suggests that this complex may be a mixture of two co-migrating complexes, one containing PP4c and PP4R1 and another PP4c and an unidentified PP4 regulatory subunit. PP4R4 is a potential candidate for the unidentified PP4 regulatory

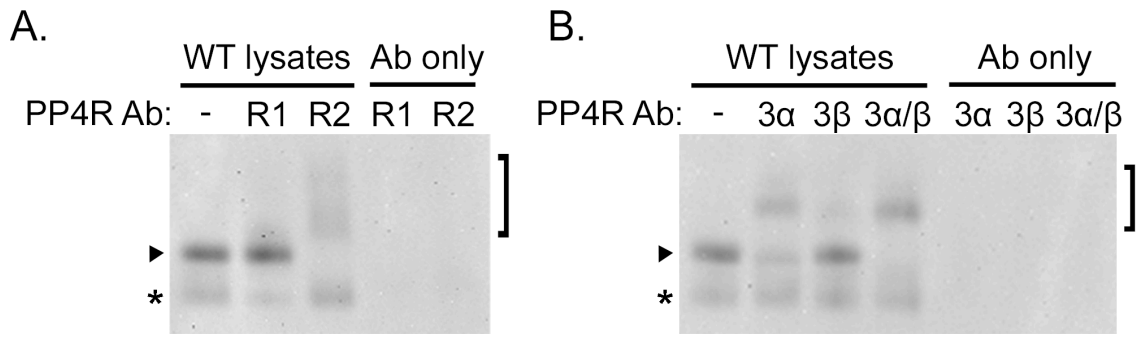

Figure 2-8. The 300kDa PP4 band is minimally composed of PP4c and PP4R1, while the 450kDa PP4 complex is minimally comprised of PP4c, PP4R2, and either PP4R3α or PP4R3β.

Cell lysates of LCMT-1 WT MEFs were incubated with PP4R-immunoprecipitating antibodies which would then bind and retard the migration of corresponding PP4 complexes. The resulting supershifts of the PP4 complexes were analyzed by BN-PAGE. (A) Supershift of PP4 complexes by PP4R1 or PP4R2 antibodies. (B) Supershift of PP4 complexes by PP4R3α, PP4R3β, or both PP4R3α/β antibodies. Asterisk, the methylation-dependent complex (the 300kDa PP4 band); filled arrowhead, the methylation-independent complex (the 450kDa PP4 band); bracket, migration of PP4 complexes whose migration slowed down.

subunit since epitope-tagged PP4R4 exhibited similar mobility as the lower band of PP4 complex when analyzed by BN-PAGE (data not shown).

While PP4R1 antibody shifted the 300kDa PP4 complex but not the 450kDa complex, antibody to PP4R2 did the opposite (lane 3; Fig. 2-8A). Strikingly, PP4R2 antibody bound and shifted the entire 450kDa PP4 complex (lane 3, filled arrowhead), creating higher molecular weight bands (bracket). The variability of the size of the shifted bands likely results from the use of a polyclonal R2 antibody, which might allow the binding of a variable number of antibodies to R2. No change in the 300kDa PP4 complex was observed using PP4R2 antibody (lane3, asterisk; Fig. 2-8A). This reveals that the entire population of the 450kDa PP4 complex contains PP4R2.

Figure 2-8B shows the results of testing the effect of PP4R3α and PP4R3β antibodies. PP4R3α antibody bound and shifted the majority of the upper band of PP4 complex (lane 2, filled arrowhead), creating higher molecular weight bands (bracket). Binding of the PP4R3α antibody did not affect the lower band of PP4 complex for the most part (lane 2, asterisk; Fig. 2-8B). These results demonstrate that the majority of the upper band of PP4 complex consists of PP4R3α in addition to PP4R2. On the other hand, PP4R3β antibody bound and shifted only a fraction of the upper band of PP4 complex (lane 3, filled arrowhead; Fig. 2-8B), giving rise to much less higher molecular weight bands (bracket) as compared to those generated by addition of the PP4R3α antibody (lane 2, bracket). This indicates that only a small portion of the upper band of PP4 complex consists of PP4R3β in addition to PP4R2. It is therefore clear that the PP4R2-associated PP4 complex is assembled into two mutually exclusive complexes either with PP4R3α or PP4R3β, where a larger portion of the PP4R2-associated PP4 complex is bound to

PP4R3α. Indeed, when both PP4R3α and PP4R3β antibodies were used, all of the upper band of PP4 complex (lane 4, arrowhead; Fig. 2-8B) was shifted, corroborating our hypothesis that the majority of the PP4R2-associated PP4 complex has PP4R3α while only a small population of the PP4R2-associated PP4 complex is with PP4R3β. This is in agreement with a previous report that identified PP4 heterotrimeric complexes composed of PP4c, PP4R2, and either one of PP4R3 subunits, PP4R3α or PP4R3β [71].

The methylation-dependent PP4R1-associated PP4 holoenzyme complex is largely disrupted by LCMT-1 knockout

In order to confirm that loss of PP4c methylation by LCMT-1 KO leads to disruption of the methylation-dependent PP4 holoenzyme assembly, co-immunoprecipitation experiment was performed using PP4R antibodies and the association of PP4Rs with PP4c was examined in the presence and absence of LCMT-1. As shown in Figure 2-9A and D, the association of PP4R1 with PP4c was dramatically decreased to 51% as compared to that of wildtype upon LCMT-1 KO. Considering the fact that the PP4R1 protein level is downregulated (by 19%; Fig. 2-9A and E) upon LCMT-1 KO, the association between PP4c and PP4R1 may be further diminished. However, the interaction of PP4R2 with PP4c remained mostly unaffected (by 13%) in the absence of LCMT-1 (Fig. 2-9B and D). Interestingly, the protein level of PP4R2 was upregulated (1.62 fold increase) by LCMT-1 loss (Fig. 2-9B and E). Binding of PP4R3β to PP4c showed about 23% of decrease by LCMT-1 loss (Fig. 2-9C and D). Similar to PP4R1, the PP4R3β protein level is downregulated (by 19%; Fig. 2-9C and E) upon LCMT-1 KO. Interestingly, PP4c protein expression was also upregulated in LCMT-1

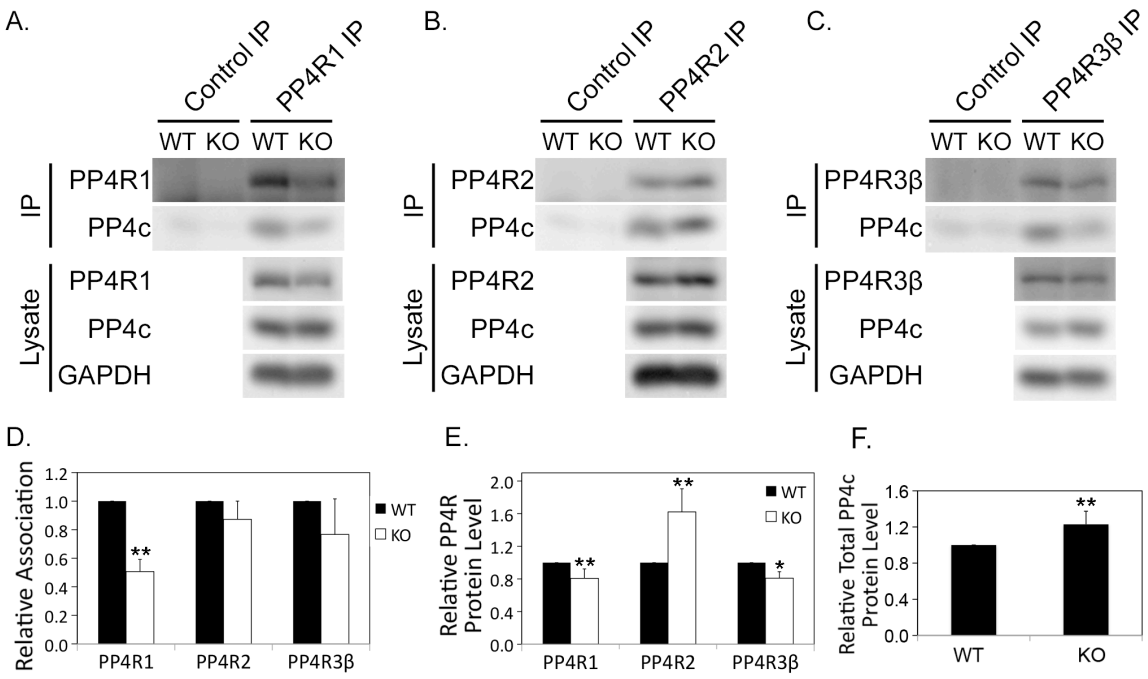

Figure 2-9. Formation of PP4R1-associated PP4 holoenzyme complexes is decreased by LCMT-1 knockout.

(A-C) Co-immunoprecipitation was performed using PP4R antibodies and cell lysates from LCMT-1 WT and KO MEFs. Inputs and immunoprecipitates were resolved by SDS-PAGE. Relative association of PP4c with PP4R1 (A), PP4R2 (B), and PP4R3β (C) in the presence and absence of LCMT-1 were analyzed by immunoblotting. The relative association of PP4c and PP4R1, PP4R2, or PP4R3β is quantified in (D). Protein levels of PP4Rs and PP4c in the presence and absence of LCMT-1 are quantified in (E) and (F), respectively. Error bars represent S.D. of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$.

KO (Fig. 2-9F). These results corroborate the findings that loss of PP4c methylation by LCMT-1 KO leads to disruption of the methylation-dependent PP4R1-associated PP4 holoenzyme complexes primarily without disturbing the methylation-independent PP4 complexes.

Loss of LCMT-1 results in hyperphosphorylation and activation of HDAC3

Since our experiment showed that loss of LCMT-1 have a profound effect on the PP4R1-associated PP4 complex formation, we examined the downstream substrates of PP4R1-associated PP4 complex. HDAC3 is one of known substrates of the PP4R1-associated PP4 complex. It has been reported that PP4R1-PP4c complex downregulates HDAC3 activity by dephosphorylation at Ser^{424} [75]. To determine whether loss of the methylation-dependent PP4R1-associated PP4 complex due to LCMT-1 knockout leads to hyperphosphorylation of HDAC3, cell lysates of LCMT-1 WT and KO MEFs were analyzed for phosphorylation of HDAC3 at Ser^{424}. Indeed, LCMT-1 KO MEFs exhibited hyperphosphorylation at Ser^{424} as compared to WT (Fig. 2-10). Therefore, it appears that since phosphorylation of HDAC3 on Ser^{424} stimulates its HDAC activity, disruption of the PP4R1-associated PP4 complex due to loss of LCMT-1 results in hyperphosphorylation and activation of HDAC3.

Discussion

Here we provide the first evidence that indicates PP6c is carboxyl methylated *in vivo* (Fig. 2-1E). The use of methylation-sensitive antibody against PP6c in methylation

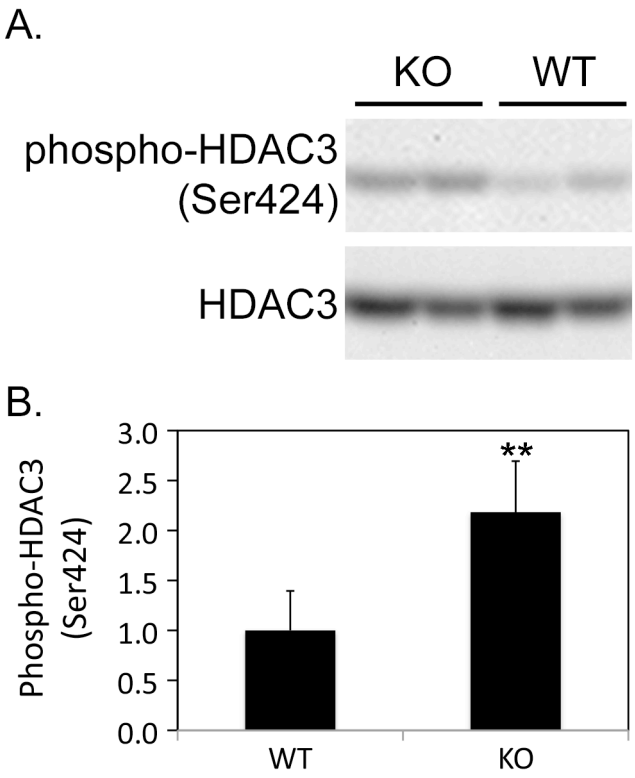

Figure 2-10. Loss of LCMT-1 results in hyperphosphorylation and activation of HDAC3. (A) Cell lysates of LCMT-1 WT and KO MEFs were resolved by SDS-PAGE and analyzed by immunoblotting to determine the level of phpspho-HDAC3 (Ser^{424}). Total HDAC3 antibody was used to compare the amount of HDAC3 protein in each sample. The relative amount of phpspho-HDAC3 is quantified in (B).

assay proved that PP6c is highly methylated. The increase of antibody sensitivity to base treatment suggests that it is indeed the carboxyl methylation since no other modifications have been shown to be base-sensitive other than carboxyl methylation. Methylation assay using the methylation-sensitive PP4c antibody also confirmed the carboxyl methylation of PP4c reported first elsewhere (Fig. 2-1D)[62]. In addition, we first reveal the identity of the methyltransferase for PP4 and PP6. It is thus clear that LCMT-1 is the major methyltransferase for all PP2A family phosphatases.

As for PP2A, in-depth knowledge of PP2A holoenzyme assembly is available, including the role of carboxyl methylation in formation of PP2A methylation-dependent and methylation-independent complexes. However, comparable studies on PP4 and PP6 holoenzyme assembly are lacking. In an attempt to learn about the function of carboxyl methylation on PP4 and PP6 holoenzyme assembly, epitope-tagged PP4c and PP6c mutants lacking the C-terminal leucine residue were created. For PP2A, the methylation-deficient ΔLeu_{309} C subunit mutant failed to form the methylation-dependent complex (Fig. 2-5B). By contrast, analysis of the epitope-tagged PP4c and PP6c constructs was hindered as the epitope-tagging alone interfered with methylation of PP4c and PP6c wildtype proteins (data not shown). Thus, in this paper, BN-PAGE served as a valuable tool for analyzing PP2A family phosphatase complexes and helped us advance our understanding of the PP2A family phosphatase holoenzyme assemblies and the role that methylation has in the process.

Our BN-PAGE analysis detected the methylation-dependent PP2A heterotrimer containing Bα as its B subunit at about 180kDa (Fig. 2-4 and 2-5), which is consistent with a previous detection of PP2A native complex containing methylated PP2Ac of about

178kDa by gel filtration [15]. This demonstrates that our method of examination of PP2A family phosphatase native complexes was reliable. Moreover, this BN-PAGE method offered interesting perspectives on analyzing native complexes of PP2A family phosphatases that have not been tried before. As shown in Figure 2-4 and 2-5, PP2A forms not only complexes of estimated sizes (PP2A heterotrimeric complex made up of PP2Ac, A, and B subunits, for example) but also incredibly large molecular weight complexes. A number of PP2A complexes whose molecular weights exceed 700kDa were detected. The composition of these large multiprotein complexes has not been determined in our study but remains as an interesting area of further investigation. Study of these large complexes, their composition and the identity of the binding proteins, would help us further understand PP2A family phosphatase holoenzyme assembly and unexplored functions.

We proposed α4 as one of the potential binding partners of the PP2A high molecular weight complexes and that the association between PP2Ac and α4 strengthens upon LCMT-1 knockout (Fig. 2-5). While additional components found in this high molecular weight complex were not determined, it is highly possible that CCT (Chaperonin containing TCP-1) complex may be present in this multiprotein complex. It has been reported that PP4c forms a mutually exclusive complex with α4 and CCT complex without regulatory subunits [71]. CCT is a large, heterooligomeric complex of over 800kDa, explaining the huge molecular weight.

In addition to the involvement in TOR (target of rapamycin) signaling pathway, the interaction between PP2Ac and α4 plays a significant role in PP2A degradation and regulation. It has been reported that α4 is involved in PP2Ac ubiquitination and

degradation via E3 ubiquitin ligase Mid1 [123]. α4 is also a ubiquitin-binding protein and possesses a ubiquitin-interacting motif (UIM). UIM of α4 opposes polyubiquitination of PP2Ac and thus prevents PP2Ac from degradation [118]. Moreover, it is believed that α4 functions as a reservoir protecting the free C subunits from degradation and preparing them to be assembled promptly into complexes when necessary [117]. Therefore, it is possible that the free PP2Ac subunits not assembled into complexes due to loss of their methylation in LCMT-1 KO may be bound by α4, which then prepares the C subunits to be assembled quickly into complexes. In addition to the PP2A A and C subunits, C subunits of PP4 and PP6 levels were significantly decreased upon α4 deletion [117]. Since α4 is a common interacting partner of the PP2A family phosphatases, it is highly likely that α4 also regulates degradation of PP4c and PP6c. However, more investigation is necessary to confirm this hypothesis.

We present the first indication that, similar to PP2A, PP4 forms both methylation-dependent and methylation-independent complexes (Fig. 2-6). Based on our results, PP4 formed two major complexes, one at about 450kDa and the other, broader band at about 300kDa. The larger 450kDa complex was proposed to be largely methylation-independent since its presence was not lost by LCMT-1 knockout (Fig. 2-6A). However, this upper band of PP4 complex appeared to be little shifted in LCMT-1 KO, which may be due to PP4R2 shift in LCMT-1 KO (Fig. 2-7C). In addition, this 450kDa PP4 complex also co-migrated with PP4R3α and PP4R3β (Fig. 2-7A). The broad 300kDa PP4 complex was primarily lost in LCMT-1 KO MEFs, suggesting that it is the major methylation-dependent PP4 complex (Fig. 2-6A). Further analysis showed that PP4R1 was present in this 300kDa PP4 complex (Fig. 2-7A). Similar to our findings, PP4

complexes from bovine testis protein extracts by gel filtration detected two major PP4c-containing complexes of approximately 270-300 and 400-450 kDa [69], again ensuring the reliability of our assay.

In contrast, PP6 complex formation did not seem to be affected by loss of LCMT-1. As revealed by our BN-PAGE analysis, PP6 formed essentially one major complex that was largely methylation-independent (Fig. 2-6B). To ensure that it is not the cell line- or tissue-specific issue, we have also assessed PP6 holoenzyme assembly in other cell lines as well as in mouse brain lysates and found no clear evidence of methylation dependency, at least in our system. It would be important to discover the functional significance of carboxyl methylation of PP6c since it does not seem to be playing a major role in PP6 holoenzyme assembly. However, we cannot completely rule out the possibility of methylation-dependency of PP6 holoenzyme assembly just based on our results. Using reliable PP6 regulatory subunit antibodies in BN-PAGE and co-immunoprecipitation experiments in the presence and absence of LCMT-1 would help establish whether PP6c methylation is important for PP6 holoenzyme assembly.

As shown in Figure 2-3, the protein level of the B subunit is decreased by LCMT-1 KO. Similarly, expression level of the methylation-dependent PP4R1 was decreased upon LCMT-1 loss (Fig. 2-9E), although the reduction was minimal as compared to that of PP2A B subunits. Surprisingly, PP4R2 protein expression was upregulated by more than two fold (Fig. 2-9E). Additionally, protein expression of PP4c was increased upon LCMT-1 KO (Fig. 2-9F). These data suggest that LCMT-1 regulates protein expression of PP4c and its regulatory subunits.

Finally, LCMT-1 loss resulted in enhanced phosphorylation of PP4R1 substrate HDAC3 at Ser^{424} which is critical for HDAC activity (Fig. 2-10), suggesting that downregulation of PP4c methylation by LCMT-1 loss and disruption of the PP4R1-associated complex increase HDAC3 activity (Fig. 2-11). HDAC3 has been implicated in cancer due to several reasons. Increased HDAC3 mRNA and proteins levels were detected in various tumors such as squamous cell lung carcinomas and astrocytic glial tumors. Interestingly, depletion of HDAC3 in colon cancer cell lines rescued the cancerous phenotypes, leading to inhibition of cell growth and increased programmed cell death. Recently, it has been shown that LCMT-1 KD results in cancer phenotypes including anchorage-independent growth [199]. It would be interesting to see if HDAC3 inhibition would rescue the anchorage-independent growth phenotype in LCMT-1 KD cells. Together, HDAC3 is a potential target for cancer therapy and selective targeting of HDAC activity might provide therapeutic approaches to some cancers.

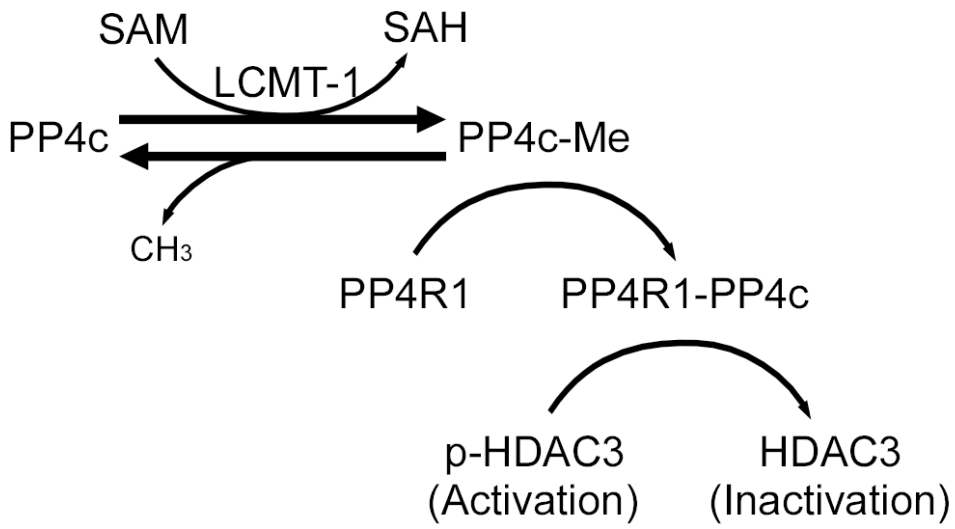

Figure 2-11. Proposed model for the regulation mechanism of PP4c methylation in PP4R1-associated PP4 complex formation and function.

LCMT-1 is the major methyltransferase for PP4c. Downregulation of PP4c methylation by LCMT-1 loss leads to disruption of the PP4R1-associated PP4 complex. HDAC3 is a known substrate of PP4R1-associated PP4 complex. Thus, disruption of the PP4R1-associated PP4 complex results in hyperphosphorylation of HDAC3 on Ser^{424}, which is critical for the HDAC3 activity.

CHAPTER 3

Results

Protein Phosphatase 2A (PP2A) Binds Within the Oligomerization Domain of Striatin and Regulates the Phosphorylation and Activation of the Mammalian Ste20-Like Kinase Mst3

(The material contained in Chapter 2.2 was originally published [204] and was included in this dissertation. See reference [204])

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Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3

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Abstract

Background: Striatin, a putative protein phosphatase 2A (PP2A) B-type regulatory subunit, is a multi-domain scaffolding protein that has recently been linked to several diseases including cerebral cavernous malformation (CCM), which causes symptoms ranging from headaches to stroke. Striatin association with the PP2A A/C (structural subunit/catalytic subunit) heterodimer alters PP2A substrate specificity, but targets and roles of striatin-associated PP2A are not known. In addition to binding the PP2A A/C heterodimer to form a PP2A holoenzyme, striatin associates with cerebral cavernous malformation 3 (CCM3) protein, the mammalian Mps one binder (MOB) homolog, Mob3/phocein, the mammalian sterile 20-like (Mst) kinases, Mst3, Mst4 and STK25, and several other proteins to form a large signaling complex. Little is known about the molecular architecture of the striatin complex and the regulation of these sterile 20-like kinases.

Results: To help define the molecular organization of striatin complexes and to determine whether Mst3 might be negatively regulated by striatin-associated PP2A, a structure-function analysis of striatin was performed. Two distinct regions of striatin are capable of stably binding directly or indirectly to Mob3–one N-terminal, including the coiled-coil domain, and another more C-terminal, including the WD-repeat domain. In addition, striatin residues 191-344 contain determinants necessary for efficient association of Mst3, Mst4, and CCM3. PP2A associates with the coiled-coil domain of striatin, but unlike Mob3 and Mst3, its binding appears to require striatin oligomerization. Deletion of the caveolin-binding domain on striatin abolishes striatin family oligomerization and PP2A binding. Point mutations in striatin that disrupt PP2A association cause hyperphosphorylation and activation of striatin-associated Mst3.

Conclusions: Striatin orchestrates the regulation of Mst3 by PP2A. It binds Mst3 likely as a dimer with CCM3 via residues lying between striatin's calmodulin-binding and WD-domains and recruits the PP2A A/C heterodimer to its coiled-coil/oligomerization domain. Residues outside the previously reported coiled-coil domain of striatin are necessary for its oligomerization. Striatin-associated PP2A is critical for Mst3 dephosphorylation and inactivation. Upon inhibition of PP2A, Mst3 activation appears to involve autophosphorylation of multiple activation loop phosphorylation sites. Mob3 can associate with striatin sequences C-terminal to the Mst3 binding site but also with sequences proximal to striatin-associated PP2A, consistent with a possible role for Mob 3 in the regulation of Mst3 by PP2A.

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Background

Protein phosphatase 2A (PP2A) is a multifunctional serine/threonine phosphatase found in all eukaryotes that regulates a host of cellular processes [1,2]. It is composed of a core heterodimer consisting of a structural A subunit and a catalytic C subunit [1], which further complexes with regulatory B-type subunits to form heterotrimeric PP2A holoenzymes. The B-type subunits direct PP2A to specific signaling complexes and subcellular locations [1] and regulate the activity of the C subunit towards different substrates (for examples, see [3-8]). While there are only two isoforms of the A and C subunits (α, β), there are several families of B-type subunits (B55/B, B56/B', and B'') [8,9]. In addition to these three families of B-type subunits, the striatin family of scaffolding proteins (striatin, S/G_2 nuclear autoantigen (SG2NA), and zinedin) represents a possible fourth family of PP2A B-type subunits in that they bind PP2A A/C heterodimers in the absence of other B-type subunits [8,9] and alter the activity of the bound PP2A A/C heterodimer [8].

Striatin family members serve as molecular scaffolds that organize large signaling complexes. Striatin, SG2NA, and zinedin contain multiple protein-binding domains: a caveolin-binding domain [10], a coiled-coil domain [11], a Ca^{2+}-calmodulin-binding domain [12], and a WD-repeat domain [13] (Figure 1A). Consistent with the presence of these domains, striatin family members have been reported to associate with calmodulin in a Ca^{2+}-dependent manner [8,11,13] and with caveolin-1 [10]. In addition, striatin family members oligomerize with each other [14] and the coiled-coil domain has been shown to mediate this interaction [15]. Other proteins found in complexes with striatin family members include, but are not limited to, Mob3/phocein (called Mob3 herein) [14,16], which is involved in vesicular trafficking [16-18]; Mst3, Mst4, and STK25 [9], members of the Germinal Center Kinase-III (GCK-III) subfamily of sterile 20-like kinases recently implicated in control of cell migration, cell cycle, Golgi assembly, and cell polarity [19-22]; cerebral cavernous malformation 3 (CCM3) protein [9], which is required for stabilization of the GCK-III kinases and thus for their function [21]; and striatin-interacting proteins (STRIP) 1 and 2 [9]. In addition, striatin family complexes containing PP2A, Mob3, GCK-III kinases, CCM3, and STRIP 1 and 2 can also associate mutually exclusively with additional components [9]. Finally, striatin has been found to bind Gαi and estrogen receptor α, facilitating rapid non-genomic signaling of estrogen receptor α [23].

Several lines of evidence suggest that a common function of striatin family complexes is their involvement in endocytosis and vesicular trafficking. Striatin, SG2NA and the common associated protein Mob3 are localized to the Golgi, cytoplasm, and plasma membrane [14,16] and the Golgi localizations of SG2NA and Mob3 are rapidly altered by exposure of cells to brefeldin A, an inhibitor of Arfs, small G proteins known to regulate vesicular trafficking [16]. In addition, striatin, SG2NA, and Mob3 all associate with proteins implicated in endocytosis and vesicular trafficking [17,24]. Finally, both CCM3 and the Drosophila Mob3/phocein homolog, DMob4, have been directly implicated in endocytosis and/or vesicular trafficking [18,25].

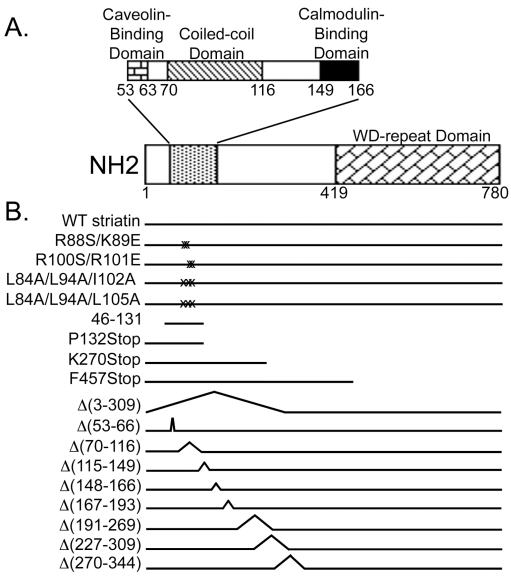

Figure 1 Schematic of the structures of human wild-type and mutant striatin proteins. **(A)** Schematic of wild-type striatin, drawn to scale, showing the locations of its previously published protein-interaction domains. White boxes represent regions of unknown function. **(B)** Stick diagrams of wild-type and mutant striatins used in this study. Point mutations are denoted by X's, and deletions are shown as peaks in the stick diagrams or absence of a line. Positions of mutants are noted relative to wild-type striatin domains in panel A.

Striatin complexes have been linked to several clinical conditions. The CCM3 protein found in striatin complexes is one of three gene products mutated in cerebral cavernous malformation, a common type of angioma [9,26-28]. Moreover, a small deletion in the 3' untranslated region of striatin that leads to lower levels of striatin mRNA was recently implicated in a canine model of arrhythmogenic right ventricular cardiomyopathy [29]. Finally, the striatin gene was also found in one of twenty-two loci containing common variants associated with QRS interval length and cardiac ventricular

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conduction [30]. Thus, the study of striatin family complexes is of great interest because of their potential roles in vesicular trafficking, Golgi assembly, cell polarity, cell migration, cell cycle, signaling, and disease.

Despite the identification of a large number of components of striatin family complexes, little is known about the architecture of these complexes and their regulation. Defining the molecular organization of striatin family complexes is critical to understanding how they function. Equally important is the elucidation of the targets, roles, and regulation of the phosphatase and kinase enzymes in these complexes. Striatin association with the PP2A A/C heterodimer alters PP2A's substrate specificity [8], but the targets and role(s) of striatin-associated PP2A are not known. Treatment of cells with okadaic acid at concentrations known to inhibit PP2A induces hyperphosphorylation of striatin, SG2NA, Mob3, and other unidentified striatin family binding partners, including proteins that migrate at the size and isoelectric point of Mst3 and Mst4 [14]. Thus, striatin-associated PP2A may regulate the function of striatin-family complexes by modulating the phosphorylation state of striatin and its associated proteins, including the Mst3 and Mst4 kinases. However, this hypothesis has not been directly tested.

To help define the molecular organization of striatin complexes and to determine whether Mst3 might be regulated by striatin-associated PP2A, we performed a structure-function analysis of striatin, defining domains of striatin important for the binding of a number of striatin binding partners and determining the effect of PP2A-deficient striatin mutants on the activation state of Mst3. To gain insight into the mechanism of Mst3 activation by autophosphorylation, we also performed a mutational analysis of Mst3 activation loop phosphorylation sites. Our results greatly enhance our understanding of the architecture of striatin family complexes, uncover unique binding requirements for PP2A, and provide new insight into the oligomerization of striatin family members and into the mechanism of activation of Mst3 by autophosphorylation. Overall, they support a model in which striatin orchestrates the regulation of Mst3 by PP2A to regulate Mst3 function in the cell.

Results

Generation of striatin mutants for structure-function analysis of striatin complexes

To facilitate dissection of the molecular organization of striatin complexes, we generated a set of deletion mutants and a complementary set of point mutants of striatin for use in our experiments. Because all cells tested to date contain endogenous striatin, all striatin mutants were constructed with an N-terminal Hemagglutinin (HA)-epitope tag to allow specific immunoprecipitation and detection of the exogenously expressed wild-type and mutant striatins. Figure 1A shows the known domain organization of striatin while Figure 1B shows a schematic of the different striatin mutants used in this study on the same scale as Figure 1A.

The WD-repeats of striatin are not required for PP2A binding but contribute to Mob3 association

The A and C subunits of PP2A are known to bind striatin [8] but the region of striatin that binds to PP2A is unknown. As a first approach to identifying striatin sequences important for PP2A association, we compared the relative ability of HA-epitope tagged wild-type striatin and two HA-tagged striatin C-terminal deletion mutants, K270Stop and F457Stop (Figure 1), to bind PP2A in vivo. Both bind PP2A (Figure 2A). Quantitation of the ratio of PP2A bound to striatin shows that loss of seven of eight of striatin's WD-repeats (F457Stop) has no effect on PP2A association while loss of all eight WD-repeats plus the central region of striatin (K270Stop) has only a small effect on PP2A binding that was not statistically significant (Figure 2B). These data indicate that residues 270-780 of human striatin are largely dispensable for PP2A association and that the first 269 amino acids of striatin contain the primary PP2A binding domain.

To determine the importance of C-terminal striatin sequences for Mob3/striatin complex formation, the same immunoprecipitates were probed for Mob3. Mob3, like PP2A, also binds to both of the C-terminal deletion mutants (Figure 2A). Thus, amino acids 1-269 in striatin also contain a domain that binds Mob3, either directly or indirectly. However, quantitation of results from multiple experiments shows that Mob3 binds at reduced levels to both of the C-terminal mutants (Figure 2B). Loss of seven WD-repeats (F457Stop) or all eight WD-repeats plus the central region of striatin (K270Stop) results in a 29% or 45% reduction in Mob3 binding, respectively. These results demonstrate that the WD-repeats are important for efficient association of Mob3 and that residues between 270 and 457 may also contribute. Thus, striatin residues 1-269 contain binding sites for both Mob3 and PP2A.

Both N- and C-terminal sequences of striatin associate with Mob3 but only N-terminal striatin sequences associate stably with PP2A

The reduced binding of Mob3 to both of the striatin C-terminal deletion mutants suggested that striatin sequences beyond residue 269 might bind Mob3. To test for this possibility, immunoprecipitates of Δ(3-309) striatin, an N-terminal deletion mutant of striatin (Figure 1), were probed for the presence of Mob3. The Δ(3-309) striatin mutant bound Mob3 at approximately 40% the level of wild-type striatin (Figure 2C-D). Thus, there are at least two distinct domains within striatin (aa1-269 and aa310-780) capable of interacting directly or

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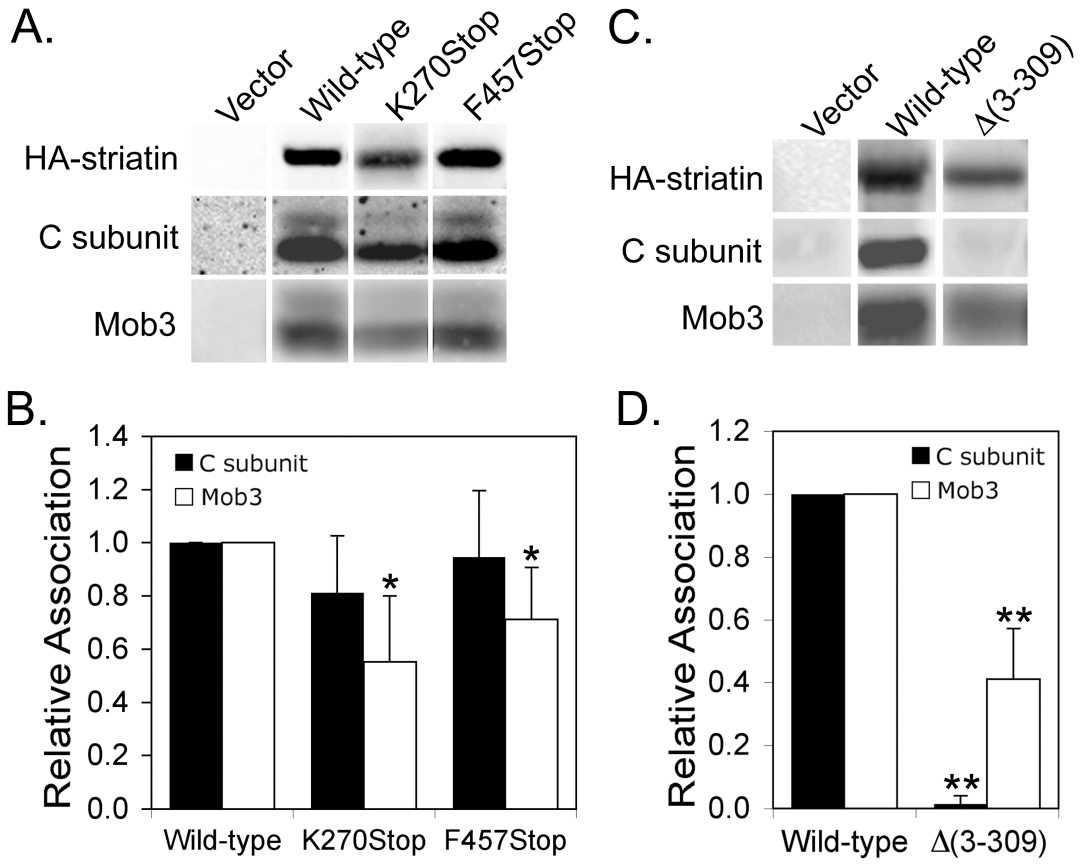

Figure 2 The N-terminal region of striatin binds PP2A while Mob3 associates with both N-terminal and more C-terminal striatin sequences. **(A)** HEK293 cells transfected with HA-tagged wild-type and mutant striatins were lysed and HA-striatin immune complexes were isolated, separated by SDS-PAGE, and proteins contained in the immunoprecipitates were detected by immunoblotting using antibodies that recognize the HA-epitope tag, PP2A C subunit, and Mob3. **(B)** Relative binding of PP2A C subunit and Mob3 to wild-type striatin and C-terminal truncation mutants of striatin was determined by quantitatively comparing the C subunit/HA-striatin and Mob3/HA-striatin ratios of wild-type striatin to those of the mutants using a Bio-Rad Fluor S-Max chemilumimager (see Methods). **(C)** Immunoblot showing that Mob3 also associates with residues 310-780 of striatin while PP2A does not. **(D)** Relative binding of PP2A C subunit and Mob3 to wild-type and Δ(3-309) striatins was measured in the same manner as described in *B*. The HA-striatins migrated at different positions due to their size differences but are shown side by side in panels A and C for comparison of their levels. C subunit can migrate as either singlets or doublets; whether double or single bands are seen can vary for the same sample from gel to gel. This pattern of migration in SDS-PAGE has been noted previously for endogenous PP2A C subunits [40-43] and is not due to degradation. The error bars in panels B and D represent the standard deviation of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type.

indirectly with Mob3. PP2A C subunit, on the other hand, did not bind to Δ(3-309) striatin (Figure 2C-D), demonstrating that PP2A associates stably only with residues located in the N-terminal region of striatin.

The coiled-coil and caveolin-binding domains of striatin, but not the calmodulin-binding domain of striatin, are necessary for oligomerization and for PP2A binding

To further localize the PP2A-binding domain within the N-terminal 269 amino acids of striatin, HA-tagged striatin mutants lacking previously identified N-terminal protein-interaction domains (caveolin-binding, coiled-coil, or calmodulin-binding) were created (Figure 1). Co-immunoprecipitation was utilized to test the abilities of these mutants to bind PP2A C subunit (Figure 3). The coiled-coil domain of striatin is required for binding PP2A, since a coiled-coil deletion mutant, Δ(70-116), is unable to specifically co-precipitate PP2A C subunit (Figure 3A-B). It has been reported that the coiled-coil domain is the oligomerization domain of the striatin

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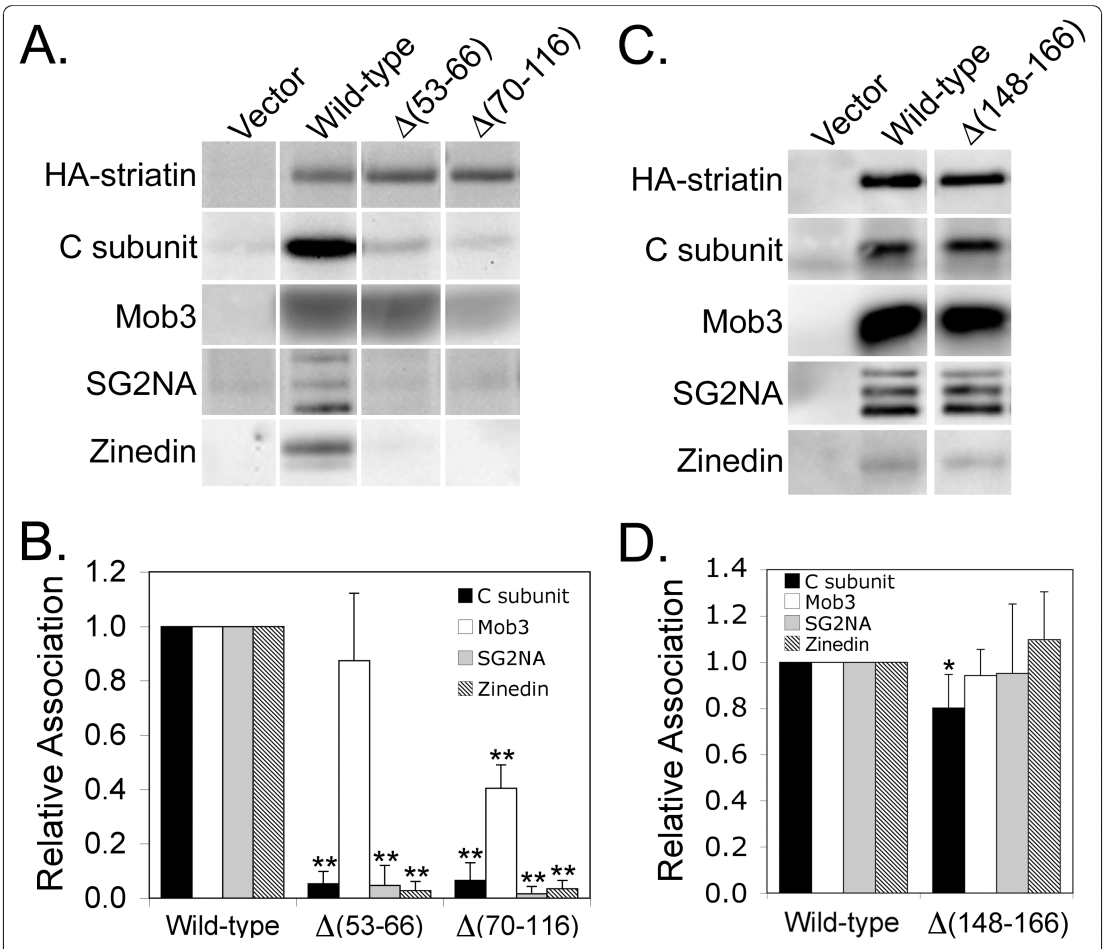

Figure 3 The coiled-coil and caveolin-binding domains are required for oligomerization and PP2A binding but the calmodulin-binding domain is not. **(A)** HEK293 cells transfected with HA-tagged wild-type and mutant striatins were lysed and HA-striatin immune complexes were isolated, separated by SDS-PAGE, and proteins contained in the immunoprecipitates were detected by immunoblotting using antibodies that recognize the HA-epitope tag, PP2A C subunit, Mob3, SG2NA, and zinedin. Deletion of either the coiled-coil domain [Δ(70-116) striatin] or the caveolin-binding domain [Δ(53-66) striatin] of striatin resulted in loss of oligomerization and PP2A binding. **(B)** Relative binding of wild-type and deletion mutant striatins to PP2A C subunit, Mob3, SG2NA, and zinedin was measured as described in the legend to Figure 2B. **(C)** A similar experiment to (A) was conducted with wild-type striatin and Δ(148-166) striatin, which deletes the striatin calmodulin-binding motif. **(D)** Relative binding of wild-type and mutant striatins to PP2A C subunit, Mob3, SG2NA, and zinedin was measured as described in the legend to Figure 2B. The HA-striatins migrated at different positions due to their size differences but are shown side by side in panels A and C for comparison of their levels. The error bars in panels B and D represent the standard deviation of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type.

family of proteins [15]. In agreement with this, Δ(70-116) striatin fails to oligomerize with SG2NA and zinedin, as demonstrated by the absence of SG2NA and zinedin in immunoprecipitates of this mutant (Figure 3A-B). Δ(70-116) also shows reduced ability to bind Mob3, binding only 40% of wild-type levels. Considering the fact that there is a Mob3 binding domain in aa1-269 of striatin that can bind approximately 55% of the wild-type level of Mob3 (Figure 2B), these results suggest that the coiled-coil domain contains binding sites for both PP2A and Mob3.

In addition to the coiled-coil domain, striatin has been reported to bind caveolin-1 through a caveolin-binding domain found within amino acid residues 53-63 [10]. To determine the role, if any, of the caveolin-binding domain in striatin association with PP2A and Mob3, a

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mutant of striatin lacking the caveolin-binding domain, Δ(53-66) striatin, was analyzed. Co-immunoprecipitation studies revealed that Δ(53-66) striatin binds little to no PP2A (Figure 3A-B). In contrast, the Δ(53-66) striatin mutant could co-precipitate Mob3 at near wild-type levels, confirming that loss of PP2A binding is specific and not due to protein misfolding. Interestingly, this mutant is completely defective in oligomerization as indicated by its inability to bind SG2NA and zinedin (Figure 3A-B), indicating that one or more residues within 53-66 are essential for striatin oligomerization.

Finally, we examined the importance of the calmodulin-binding domain in the N-terminus of striatin by analyzing the striatin deletion mutant, Δ(148-166), which deletes this entire domain (Figure 1). Loss of striatin's calmodulin-binding domain has only a small effect on PP2A C subunit binding, and no significant effect on Mob3 binding or oligomerization with SG2NA and zinedin (Figure 3C-D).

Residues in the coiled-coil domain of striatin are critical for PP2A C subunit association independent from their role in oligomerization

The fact that the coiled-coil domain of striatin is required for its association with PP2A suggested that PP2A might bind directly to this region. If so, the introduction of point mutants in the coiled-coil domain may disrupt PP2A binding without interfering with striatin oligomerization. To test this hypothesis, we compared the peptide sequence of the coiled-coil domain of striatin from several species and identified residues that were completely conserved. Four such residues were Arg^{88}, Lys^{89}, Arg^{100} and Arg^{101}. Pairs of these residues were substituted with uncharged serine residues or residues of opposite charge in an attempt to disrupt striatin/PP2A association. The resulting double point mutants, R88S/K89E and R100S/R101E striatin, were then analyzed for PP2A, Mob3, and SG2NA binding. The mutants R88S/K89E and R100S/R101E are able to bind only 40% and 10% of wild-type levels of PP2A C subunit, respectively (Figure 4A-B). Binding of Mob3 was not reduced. Both mutants retained the ability to efficiently bind SG2NA at wild-type (R88S/K89E) or near wild-type (81%; R100S/R101E) levels (Figure 4A-B), indicating that effects on PP2A C subunit can be separated from effects on oligomerization. These results support the hypothesis that PP2A binds directly to the coiled-coil domain of striatin.

To further investigate PP2A binding in the coiled-coil domain, four conserved hydrophobic residues (leucines 84, 94, and 105, and isoleucine 102) were replaced with alanine. Two triple mutants (L84A/L94A/I102A and L84A/L94A/L105A) reduced PP2A binding to 32% to 17% of wild-type levels, while striatin oligomerization remained intact (Figure 4C-D). No statistically significant reduction in the binding of Mob3 was seen. Together, these results show that critical residues in the PP2A binding domain of striatin are within the central portion of the coiled-coil domain between residues 84 and 105.

To define whether the coiled-coil domain was sufficient to bind PP2A and to test the importance of the amino acids preceding the caveolin-binding domain for PP2A binding, two additional mutants (P132Stop and 46-131) were constructed (Figure 1). Both of these mutants contain the coiled-coil domain. Because the caveolin-binding domain is also required for PP2A binding (Figure 3) both mutants were designed to retain this domain as well, but mutant 46-131 lacks most of the amino acids that precede it. PP2A binds efficiently to both mutants, demonstrating that the binding site for PP2A is within striatin amino acids 46-131 and that the first forty-five amino acids of striatin are dispensable for PP2A binding (Figure 4E).

Determinants within striatin residues 191-344 are critical for binding to Mst3 and Mst4 kinases and to CCM3

We next investigated the association of striatin with Mst3 kinase, a recently discovered component of striatin family complexes [9]. Initial experiments showed that Mst3 does not bind to Δ(3-309) striatin (Figure 5A), indicating that striatin residues before 310 are critical for its association. However, Mst3 bound well to Δ(70-116) striatin (Figure 5A) and Δ(53-66) (data not shown), indicating that Mst3 association with striatin does not require the coiled-coil or caveolin-binding domains or oligomerization of striatin. This result also indicates that the loss of PP2A binding to Δ(70-116) seen in Figure 3A-B is not simply due to misfolding of this mutant.

To further delineate the striatin residues needed for Mst3 binding, the ability of Mst3 to associate with a set of deletion mutants spanning regions of unknown function between the coiled-coil domain and residue 344 of striatin (Figure 1) was measured. The results of this analysis (Figure 5B-C) indicate that deletion of the residues between the coiled-coil domain and the calmodulin domain and deletions after the calmodulin-binding domain up to residue 193 cause no reduction in Mst3 binding. However, deletion of residues 191-269 causes a ~33% reduction in Mst3 binding while loss of residues 270-344 causes an ~85% loss of Mst3 binding. Deletion of striatin amino acids 191-269 and 270-344 also reduced Mst4 binding by 33 ± 5% and 75 ± 3%, respectively (average ± range of two experiments). These results indicate that the Mst3 and Mst4 binding sites probably span across amino acid 269, with the strongest interactions being C-terminal to this residue.

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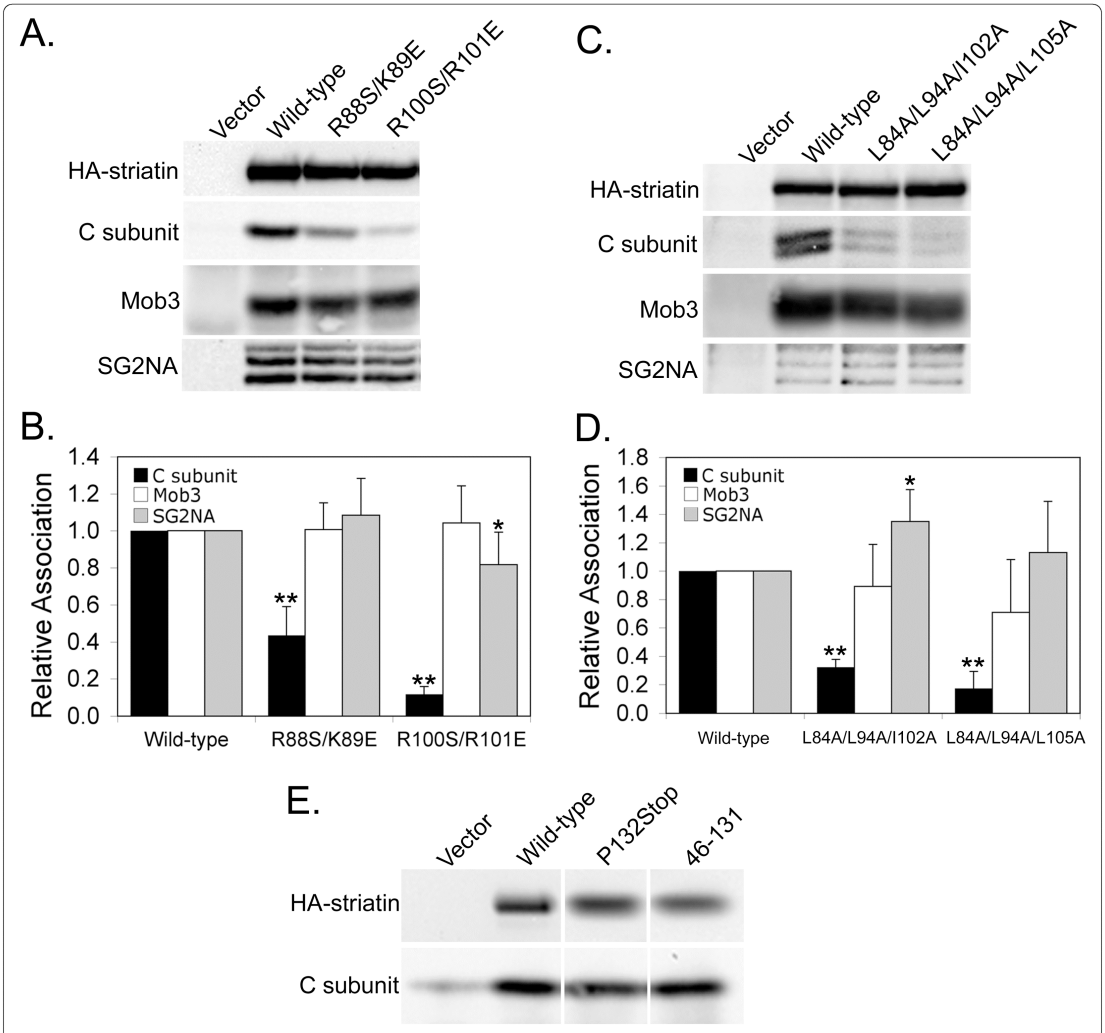

Figure 4 Conserved residues within the coiled-coil domain of striatin are critical for association with PP2A but not with Mob3. **(A)** HEK293 cells transfected with HA-tagged wild-type and coiled-coil mutant striatins were lysed and HA-striatin immune complexes were isolated and proteins contained in the immunoprecipitates were detected by immunoblotting using antibodies that recognize the HA-epitope tag, PP2A C subunit, Mob3, and SG2NA. **(B)** Relative binding of wild-type and point mutant striatins to PP2A C subunit, Mob3, and SG2NA was measured as described in the legend to Figure 2B. The error bars represent the standard deviation of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type. **(C)** A similar experiment to (A) was conducted with wild-type striatin and striatin mutants with hydrophobic coiled-coil residue substitutions. **(D)** Relative binding of wild-type and point mutant striatins to PP2A C subunit, Mob3, and SG2NA was measured as described in the legend to Figure 2B. The error bars represent the standard deviation of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type. The difference in C subunit association between L84A/L94A/I102A and L84A/L94A/L105A was also significant ($p = 0.029$). **(E)** A similar experiment to (A) was conducted with wild-type striatin and small, coiled-coil-containing mutants, P132Stop striatin and 46-131 striatin. The HA-striatins migrated at different positions but are shown side by side for comparison of their levels. Although some non-specific sticking of C subunit was seen in the vector control lane, both mutants bound wild-type levels of PP2A C subunit.

Because CCM3 has been reported to bind and stabilize Mst3 and Mst4 [21], the importance of striatin residues 191-269 and 270-344 for CCM3 binding was tested. Since commercially available CCM3 antibodies did not consistently detect CCM3, we established a stable FLAG-tagged CCM3-expressing cell line as described in Methods and analyzed the ability of this CCM3 to associate with HA-tagged wild-type and

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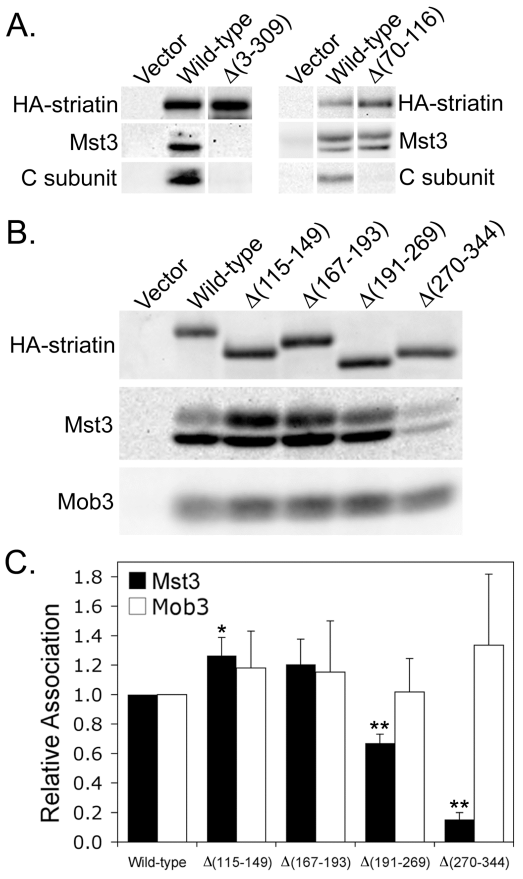

Figure 5 Characterization of Mst3 association with striatin. **(A)** Three days after transfection with HA-tagged wild-type and mutant striatins, HEK293 cells were lysed and HA-striatin immune complexes were isolated, separated by SDS-PAGE, and immunoblotted. Representative immunoblots are shown. *Left*, Mst3 binding, like PP2A C subunit binding, requires N-terminal sequences of striatin. *Right*, unlike PP2A C subunit, Mst3 binding does not require the striatin coiled-coil domain or striatin oligomerization. **(B)** A similar experiment to (A) was conducted to compare the abilities of Mst3 and Mob3 to bind wild-type striatin and a set of internal striatin deletion mutants localized to regions of unknown function. **(C)** Relative binding of wild-type and mutant striatins to Mst3 and Mob3 was measured as described in the legend to Figure 2B. The error bars represent the standard deviation of at least three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type.

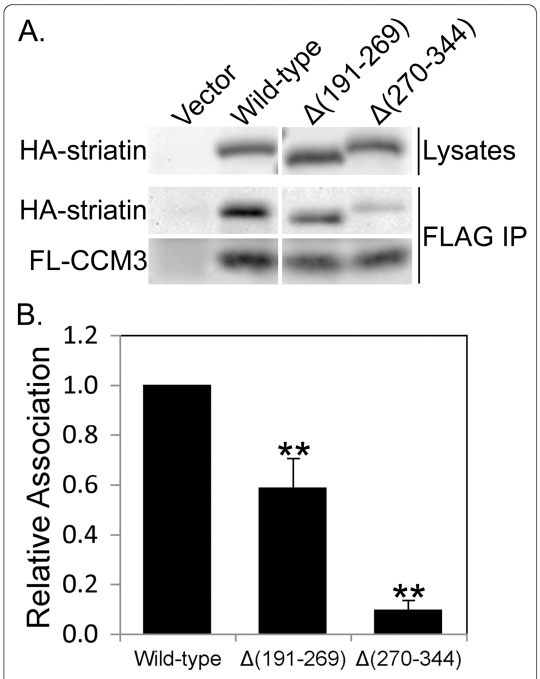

Figure 6 CCM3 binding to striatin is affected by the same deletions that reduce Mst3 binding. **(A)** Three days after transfection with HA-tagged wild-type and mutant striatins, HEK293 cells stably expressing FLAG-CCM3 were lysed and FLAG-CCM3 immune complexes were isolated, separated by SDS-PAGE, and immunoblotted. Lysate levels of the transfected HA-tagged striatins are shown for comparison to the amount of HA-striatins that coimmunoprecipitated with FLAG-CCM3. **(B)** Relative binding of wild-type and mutant striatins to FLAG-CCM3 was measured as described in the legend to Figure 2B, except that in this experiment the values were normalized to expression of the HA-constructs in the cell lysates. The error bars represent the standard deviation of three independent experiments. **, $p \leq 0.01$ relative to wild-type.

mutant striatins by co-immunoprecipitation. Deletion of striatin residues 191-269 and 270-344 reduced CCM3 binding ~40% and ~90%, respectively (Figure 6A-B), suggesting that CCM3 and the Mst3 and Mst4 kinases may bind as a complex to this region of striatin.

The calmodulin-binding domain of striatin negatively regulates association with the Mst3 and Mst4 kinases

The deletion mutant, Δ(115-149) striatin, had a statistically significant ~25% increase in the amount of Mst3 bound (Figure 5C). Since this region is just before the calmodulin-binding domain of striatin, we tested the ability of Mst3 to bind to Δ(148-166) striatin, in which the calmodulin-binding domain has been deleted. At the same time, we tested an additional mutant, Δ(227-309) striatin, spanning the region that seemed to affect Mst3 binding the most. The results indicate that Δ(227-309) striatin is almost completely defective in Mst3 binding, while still binding Mob3 at 75% of wild-type levels (Figure 7A-B). In striking contrast, Mst3 bound ~3.5-fold better when the calmodulin-binding domain of striatin was deleted (Figure 7A-B).

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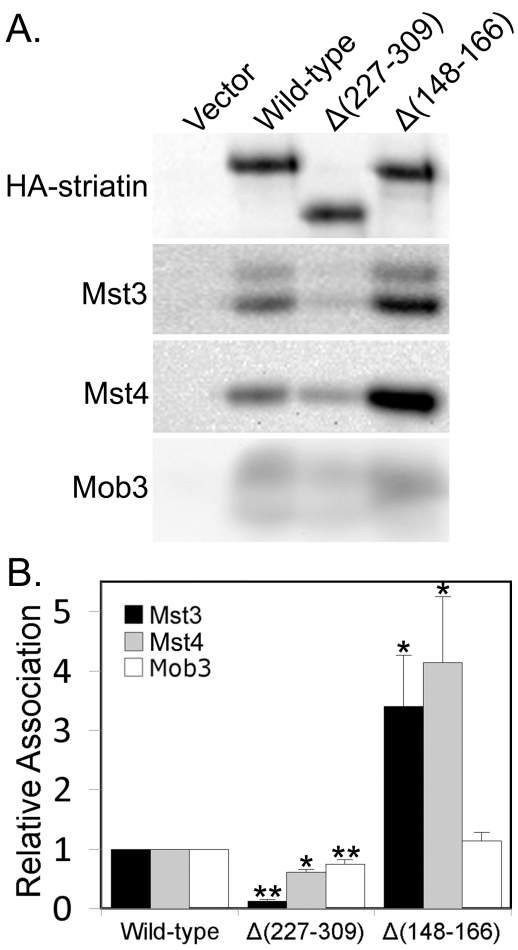

Figure 7 Loss of striatin residues 227-309 nearly abolishes Mst3 binding while loss of the calmodulin domain greatly enhances the binding of both Mst3 and Mst4. **(A)** Three days after transfection with HA-tagged wild-type and mutant striatins, HEK293 cells were lysed and HA-striatin immune complexes were isolated, separated by SDS-PAGE, and immunoblotted. The Mob3 panel shown has been compressed 25% in the vertical dimension to save space. **(B)** Relative binding of wild-type and mutant striatins to Mst3, Mst4, and Mob3 was measured as described in the legend to Figure 2B. The error bars represent the standard error of four independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type.

Interestingly, probing of the same immunoprecipitates for the related striatin-associated kinase, Mst4, revealed a less dramatic reduction (38%) in Mst4 binding to Δ(227-309) striatin but a similar ~4-fold increase in binding to the calmodulin-binding domain deletion mutant.

Striatin-associated PP2A negatively regulates the phosphorylation of Mst3 kinase

Previously, two-dimensional analysis of SG2NA complexes from ^{32}P-inorganic phosphate-labeled cells revealed an ~52 kDa unknown protein whose phosphorylation increased dramatically upon treatment of cells with okadaic acid at concentrations known to inhibit PP2A [14]. The size and estimated isoelectric point of that phosphoprotein are similar to Mst3, raising the possibility that striatin-associated PP2A regulates the phosphorylation state of striatin-associated Mst3. To test this possibility, we took advantage of the fact that phosphorylation slows the mobility of many proteins. Figure 8A shows that upon incubation with ATP and manganese in vitro, a portion of Mst3 undergoes a gel shift on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel shift does not occur in the absence of ATP or if the kinase inhibitor staurosporine is included (Figure 8A), indicating that the shift occurs due to autophosphorylation of Mst3 or phosphorylation of Mst3 by a tightly associated kinase.

Mst3 in lysates from untreated human 293 cells exists predominantly as one major band and a minor, more slowly migrating, upper band (Figure 8B, first lane). Upon treatment with 100 nM okadaic acid, the Mst3 in the lower band shifts to the upper band over time until at 4 h the upper band becomes the predominant band (Figure 8B). It was previously shown that treatment of mammalian cells with 100 nM okadaic acid for 6 h-24 h inhibits most cellular PP2A without significantly inhibiting PP1 because of the slow rate at which okadaic acid enters cells [31]. Considered together, these results indicate that Mst3 phosphorylation may be regulated by PP2A.

Okadaic acid-induced phosphorylation of Mst3 could be due to inhibition of PP2A in the striatin complex or to inhibition of another PP2A holoenzyme responsible for regulating the phosphorylation state of Mst3. To distinguish between these two possibilities, we tested whether selectively reducing the amount of PP2A associated with striatin would increase the steady-state phosphorylation level of Mst3 in those striatin complexes. We reasoned that if PP2A in the striatin complex were responsible for dephosphorylation of striatin-associated Mst3, then mutant striatins with reduced PP2A binding would bind phosphorylated Mst3 but dephosphorylate it at a reduced rate, causing the accumulation of hyperphosphorylated Mst3 in those striatin complexes.

To test this prediction, the four PP2A-deficient striatin coiled-coil domain point mutants were used. In cells expressing these mutants, PP2A that is not complexed with striatin (~98% of PP2A in the cell [8]) will be unaffected; only the amount of PP2A associated

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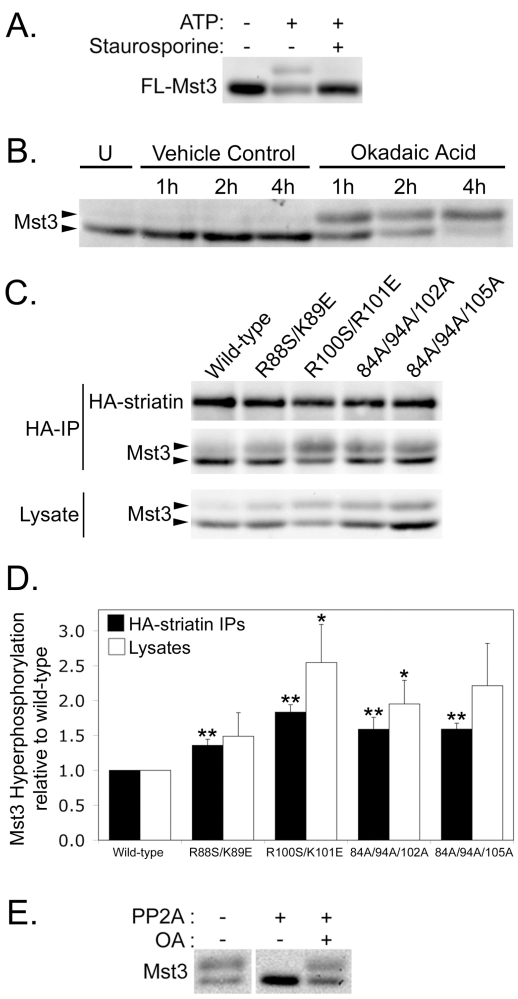

Figure 8 Loss of PP2A binding to striatin causes Mst3 hyperphosphorylation. **(A)** FLAG-Mst3 (FL-Mst3) immunoprecipitates were prepared, and then aliquots were incubated without ATP, with ATP, or with ATP plus the kinase inhibitor, staurosporine. FLAG-Mst3 was visualized by immunoblotting. **(B)** Mst3 hyperphosphorylation caused by incubation of HEK293 cells with 100 nM okadaic acid for the times indicated retards the migration of Mst3 in SDS-PAGE. U, untreated. Vehicle Control, DMSO. **(C)** Three days after transfection of HEK293 cells with HA-tagged wild-type striatin and striatin mutants deficient in PP2A binding, HA-striatin immune complexes (HA-IP) and lysates were prepared and immunoblotted. **(D)** Relative hyperphosphorylation of Mst3 in striatin complexes and lysates of transfected cells was quantitated by measuring the ratios of the upper and lower bands of Mst3 using a chemilumimager and normalizing to wild-type HA-striatin. The error bars represent the standard error of at least four independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to wild-type. The p values for R88S/K89E and 84A/94A/105A mutant effects in lysates were 0.19 and 0.06, respectively. **(E)** Three days after transfection of HEK293 cells with HA-tagged R100S/R101E striatin, HA-striatin immune complexes were prepared in the absence of phosphatase inhibitors, denatured, and divided into three equal portions. Samples were incubated without PP2A or with purified PP2A plus either DMSO (vehicle control) or 100 nM okadaic acid. After incubation, the Mst3 protein bands were detected by immunoblotting. All lanes are from the same gel and exposure but the first two lanes were originally separated by a blank lane.

with HA-striatin will be affected. We compared the ratio of the upper (hyperphosphorylated) and lower bands of Mst3 associated with the PP2A-deficient striatin mutants with the ratio of these Mst3 bands associated with wild-type striatin. Figure 8C (HA-IP) shows the results of a representative experiment. Mst3 associated with striatin mutants deficient in binding PP2A has a greater proportion of the upper band than Mst3 associated with wild-type striatin, indicating that there is an increased amount of hyperphosphorylated Mst3 associated with the PP2A-deficient striatin mutants. Quantitative analysis of several experiments showed that Mst3 hyperphosphorylation was significantly increased in all four of our PP2A-deficient striatin complexes (Figure 8D; black columns), with the greatest increase in the R100S/R101E mutant shown in Figure 4 to be the most defective in binding PP2A. The reduction of PP2A binding to striatin causes the hyperphosphorylation (gel shift) of a large fraction of the associated Mst3, as can be seen most clearly by visual comparison of the Mst3 bands in the wild-type and R100S/R101E lanes in the HA-IP panel of Figure 8C. More than half of Mst3 associated with the R100S/R101E mutant is in the upper band.

To determine the effect on the total Mst3 population in the cell, we also analyzed the amount of hyperphosphorylation (upper band) of Mst3 in lysates from cells expressing exogenous wild-type or PP2A-deficient mutant striatins. Figure 8C-D (lysate panel and white columns) show that expression of PP2A-deficient striatin does cause an increase in total hyperphosphorylated Mst3 in cells. The most robust increase, ~2.5-fold, was again induced by expression of the striatin mutant most deficient in binding PP2A, R100S/R101E.

To definitively prove that the gel shift in Mst3 observed with PP2A-deficient striatin mutants is due to increased phosphorylation and not some other modification, we tested whether gel-shifted Mst3 associated with R100S/R101E striatin could be eliminated by treatment with purified PP2A. The slower migrating form of Mst3 disappeared when denatured R100S/R101E striatin immunoprecipitates were incubated with purified PP2A but not when they were incubated with PP2A and okadaic acid (Figure 8E). Together, these results indicate

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that PP2A in the striatin complex regulates the phosphorylation state of striatin-bound Mst3.

PP2A negatively regulates the activation of striatin-associated Mst3 kinase

To gain insight into the functional significance of the PP2A-regulated phosphorylation of Mst3 in the striatin complex, we determined the sites of phosphorylation on Mst3 necessary for the observed gel shift. To do this, the three reported activation loop phosphorylation sites of Mst3, threonine residues 172, 178, and 182 in Mst3 isoform b (PhosphoSitePlus [32]), were individually mutated to alanine to prevent their phosphorylation. After transfection of constructs expressing FLAG-tagged wild-type and mutant Mst3 isoform b proteins into HEK293 cells and treatment of the cells with okadaic acid or vehicle control (DMSO), lysates were analyzed by immunoblotting with anti-FLAG antibody (Figure 9; compare top two panels). The results show that mutation of threonine 172 causes a small reduction in the ratio of shifted to unshifted Mst3 bands while mutation of either threonine 178 or 182 to alanine abolishes the okadaic-acid induced gel shift of Mst3.

Next an autophosphorylation site-specific (pT178) antibody was used to further analyze the T172A, T178A, and T182A Mst3 mutants. Because Mst3, Mst4, and STK25 have similar sequences in their activation loops, this antibody reacts with all of these kinases when activated by autophosphorylation. To specifically examine Mst3, lysates of okadaic acid-treated cells expressing the FLAG-tagged Mst3 isoform b proteins were first denatured to disrupt complexes by heating with SDS and reducing agent as described in Methods. Then anti-FLAG immunoprecipitates of wild-type and mutant Mst3 proteins were prepared and immunoblotted with anti-phospho-Mst3 (pT178) antibody and with anti-FLAG antibody (Figure 9; two middle panels). The results confirm the specificity of the anti-phospho-Mst3 (pT178) antibody since only IgG background is seen in the T178A pMst3 immunoblot lane while a strong band of the T178A mutant protein is seen in the FLAG-Mst3 (FL-Mst3) immunoblot panel. The results also reveal that the T182A Mst3 mutant is phosphorylated robustly on threonine 178 and that Mst3 phosphorylated on threonine 178 is present in both the lower and upper bands of the Mst3 doublet in the wild-type and T172A lanes. Thus, phosphorylation of threonine 178 is not sufficient to generate the upper Mst3 band, but appears to be a prerequisite for its formation. No threonine 178 phosphorylation was detected on any of the FLAG-tagged Mst3 proteins when FLAG-Mst3 immunoprecipitates were prepared from vehicle control (DMSO)-treated cells (Figure 9; bottom two panels), indicating that the Mst3 autophosphorylation on threonine 178 seen with okadaic acid-treated cells was induced by okadaic acid and not pre-existing. Together, the results in this section show that both the upper and lower Mst3 bands contain phosphorylated Mst3 species but generation of the upper, hyperphosphorylated band appears to require at a minimum phosphorylation of both threonine 178 and threonine 182 in the activation loop of Mst3. Thus, the gel shift seen with PP2A-deficient striatin mutants is indicative of, but probably an underestimate of, activation and autophosphorylation of Mst3.

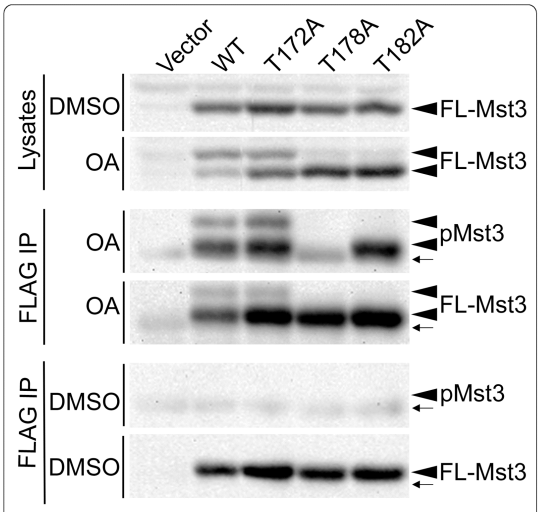

Figure 9 Mutation of either threonine 178 or threonine 182 to alanine in the activation loop of Mst3 prevents the hyperphosphorylation-induced gel shift induced by okadaic acid (OA) treatment. Three days after transfection with empty vector or FLAG epitope-tagged wild-type (WT) or mutant Mst3 isoform b-expressing plasmids, HEK293 cells were treated with either DMSO or OA (100 nM) for four hours and then lysed. Lysates were denatured as described in Methods and FLAG-Mst3 was immunoprecipitated with anti-FLAG antibody. Cell lysates (Lysates) and FLAG-Mst3 immunoprecipitates (FLAG IP) were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody (FL-Mst3) and phospho-specific antibody recognizing the threonine 178 autophosphorylation site of Mst3 (pMst3). The small arrows denote the position of IgG heavy chain in the immunoprecipitates. In anti-FLAG immunoblots of lysates from DMSO-treated cells (top panel), a background band is seen at the expected position of hyperphosphorylated Mst3 in all lanes. The fact that this band is present in the vector control lysate lane and absent in anti-FLAG Mst3 immunoprecipitates prepared from these lysates (bottom panel) shows that this band is not Mst3. Okadaic acid induces the presence of the upper band of FLAG-tagged Mst3 in wild-type (WT) and T172A Mst3-expressing cells (clearly darker than the vector control background band), but not in T178A and T182A Mst3-expressing cells (second panel from the top).

Discussion

The results of this study help define the architecture and regulation of the striatin family complexes. A model

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for the organization of the striatin complex is presented in Figure 10. The creation of striatin point mutants deficient in PP2A association facilitated a set of experiments clearly identifying striatin-associated PP2A as the phosphatase negatively regulating Mst3 activation in striatin family complexes. Moreover, mutational analysis of Mst3 autophosphorylation sites and studies with the PP2A inhibitor, okadaic acid, provided new insight on Mst3 activation.

The A and C subunits of PP2A were previously shown to bind to striatin family complexes but the region of striatin to which the A/C heterodimer bound was unknown [8]. Our current data show that the coiled-coil domain of striatin mediates the formation of this PP2A heterotrimer (Figure 10). Although we cannot rule out a direct contribution of the caveolin-binding motif, the fact that double or triple point mutations in the middle of the coiled-coil/oligomerization domain of striatin almost completely disrupt PP2A association suggests that the primary determinants for PP2A binding are near the middle of the coiled-coil domain (residues 84-105).

Our data also suggest that PP2A association with striatin is dependent on oligomerization of striatin complexes. Of the charged coiled-coil residues that were mutated to disrupt PP2A binding to striatin, only arginine 88 is predicted to be at the dimerization interface when either NCOILS [33] or Paircoil2 [34] prediction programs are used, while the other residues are predicted to be more available to potentially interact with PP2A. However, arginine 101, mutated in the R100S/R101E mutant, is part of a signature in striatin called a trimerization motif [35], and could have an effect on topology as well. Of the hydrophobic coiled-coil mutations, leucine 84, isoleucine 102, and leucine 105 are predicted to be at the dimerization interface, while leucine 94 may be more accessible. Thus, it is interesting that the striatin mutant L84A/L94A/L105A is significantly more defective in PP2A binding than the mutant L84A/L94A/I102A ($p = 0.029$), because the only difference between these two mutants is in amino acids predicted to be at the interface of the helices. These results are consistent with the idea that association of the PP2A A/C heterodimer is sensitive to local alterations in the dimerization interface of striatin. We hypothesize that this sensitivity results from PP2A binding asymmetrically across the dimerization interface to more than one striatin family coiled-coil domain. Further support for this model comes from the fact that deletion of the caveolin-binding motif, which leaves the residues mutated above intact, abolishes oligomerization of striatin and its association with PP2A. Thus, although a precise understanding of the effects of the different striatin mutants on PP2A association may await crystallization studies, our current data are consistent with a model in which the PP2A A/C heterodimer binds to the coiled-coil domain of striatin family members in an oligomerization-dependent manner. Because we have shown that Mst3 binding to striatin is not dependent on striatin family oligomerization, this model provides an attractive feature of restricting the regulation of striatin-associated Mst3 by PP2A until oligomerization occurs.

A surprising result from this study was the complete loss of oligomerization caused by deletion of the caveolin-binding motif within striatin (mutant Δ(53-66)). A previous study [15] reported that a fusion protein consisting of residues 81-131 of mouse SG2NA (corresponding to striatin amino acids 65-115) fused to the C-

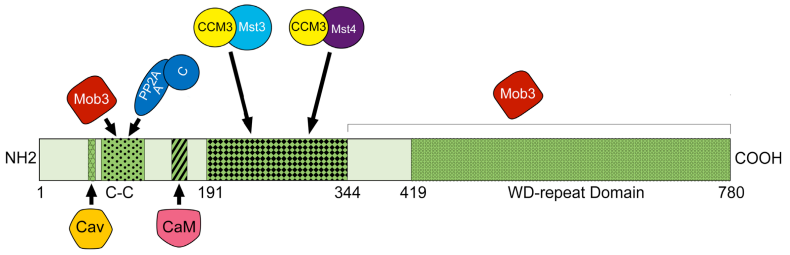

Figure 10 Model of human wild-type striatin complex, based on results from previous studies and the current study. Schematic of wild-type human striatin, drawn to scale, showing the locations of its previously published protein-interaction motifs/domains and residues or domains determined in this study to be important for binding of the striatin-associated proteins. Previously published domains: Caveolin (Cav)-binding motif (aa53-63); C-C, coiled-coil region (aa70-116); Calmodulin (CaM)-binding domain (aa149-166); WD-repeat domain (aa419-780). The Cav-binding motif and CaM-binding domain are indicated by arrows with corresponding proteins below. Estrogen receptor alpha (not shown) has been found to bind to striatin amino acids 1-203 [23]. Upper labels (from this study): Names are as indicated. Residues important for the binding of these proteins are indicated by arrows or by brackets. Mst4 binding was not affected as dramatically as Mst3 by Δ(227-309) so it may have an overlapping, but not identical, binding site. Note: The amino acid stretch in striatin important for Mst3/CCM3 association contains a small region of amino acid homology with PP2A B' B-type subunits that we previously identified [8]. Whether these residues play a role in binding Mst3/CCM3 remains to be tested.

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terminus of GFP did not oligomerize for unknown reasons. One possible explanation for both of these results is that striatin family oligomerization requires binding of caveolin. This would have to be a transient requirement, however, because we could find no associated caveolin even in wild-type striatin immunoprecipitates (data not shown) and no caveolin was found in striatin family complexes in a recent proteomics study [9]. Alternatively, loss of oligomerization could result from one or more of the deleted residues being directly required for oligomerization. Analyses using NCOILS [33] and Paircoil2 [34] prediction programs suggest that the N- and C-terminal limits previously assigned to the coiled-coil domain of striatin (70-116; [11]) may need to be extended. Using a 21 residue window with NCOILS, which is recommended for locating the ends of coil-coils, and an unweighted analysis (heptad residues a-g given equal weight), probabilities of almost 1 are obtained for residues 61-69 being part of the coiled-coil of human striatin. Weighted analysis (residues a and d given 2.5-fold more weight than the other heptad residues) using the same window still assigns these residues high probability (0.621 for residues 61-63 and 0.956 for residues 64-69) of being part of the coiled-coil. Results from similar analyses of human SG2NA suggest that SG2NA residues corresponding to striatin aa61-69 (unweighted) or aa64-69 (weighted) have a high probability of being part of the coiled-coil. Finally, for human zinedin, NCOILS predicts unweighted and weighted N-terminal ends corresponding to striatin aa56 and aa61, respectively. For striatin, SG2NA, and zinedin, these analyses also suggest that the C-terminal end of the coiled-coil may be around aa120-121. Paircoil2 analysis of these proteins predicts a similar end to the coiled-coil but an even earlier start to it, ranging from striatin aa56-61, potentially including much of the caveolin-binding motif, residues 53-63. The results of these analyses are consistent with the idea that our caveolin deletion mutant and the GFP-SG2NA fusion protein published by others may have removed amino acids critical to the formation of striatin coiled-coils. Since the missing residues common to both of these constructs are residues 53-64, it is possible that these are the critical amino acids. In fact, the complete loss of coiled-coil formation caused by our small caveolin-binding motif deletion is reminiscent of "trigger" sequences, sequences absolutely required for coiled-coil formation [36]. Additional analyses will be needed to firmly define the striatin coiled-coil and resolve these possibilities.

There are at least two distinct domains within striatin (aa1-269 and aa310-780) that interact directly or indirectly with Mob (Figure 10). The C-terminal striatin residues important for Mob3 binding were further narrowed down by the fact that deletion of striatin residues 270-344 had no significant effect on Mob3 binding (Figure 5C). This result suggested that the most important C-terminal determinants of striatin for Mob binding likely occur after residue 344 and include the WD domain. However, because of the larger standard deviation of Mob3 binding to the Δ(270-344) mutant, a contribution of striatin residues 310-344 cannot be completely ruled out. In the N-terminus of striatin, Mob3 binding seems to be largely restricted to the coiled-coil domain because deletion mutants collectively spanning residues 115-269 and a mutant deleting the caveolin-binding motif had no significant effect on Mob3 binding, while deletion of the coiled-coil domain had the same reduction of Mob3 binding as the Δ(3-309) striatin mutant. The fact that Mob3 binds much better to the caveolin-binding motif deletion mutant than to the coiled-coil deletion mutant and close to wild-type levels also supports the idea that Mob3 binding to the striatin N-terminus is not highly dependent on oligomerization. This result suggests that Mob3 binding to the striatin N-terminus is not solely due to indirect binding of Mob3 through oligomerization of endogenous striatin family members with mutants. However, additional studies are necessary to strengthen this conclusion. It has not been firmly established whether Mob3 binds directly or indirectly to striatin. A previous study identified Mob3 (phocein) by its binding to striatin in a two-hybrid assay but no additional data was presented to rule out the possibility that the interaction was mediated by a conserved protein in yeast (e.g., PP2A A and C subunits) bridging the association. Mob3 binds striatin independently of PP2A C subunit, Mst3, and CCM3 because when close to 85% of the latter associations were disrupted by various mutations little to no reduction in Mob3 binding was seen. Since PP2A C subunit, Mst3, and CCM3 are other core striatin binding partners, the results support the model that Mob3 binds directly to striatin. However, the question of whether striatin binds two molecules of Mob3 or uses two binding domains to interact with a single molecule of Mob3 still needs to be resolved. Given our other data on the organization of the striatin complex, it is tempting to speculate that binding of two striatin domains to one Mob3 molecule might function in part to bring Mst3 and PP2A into proximity in the 3D structure of striatin.

The results of this study show that striatin-associated PP2A is the phosphatase responsible for negatively regulating the phosphorylation and activation of Mst3 in striatin family complexes. Four separate striatin point mutants deficient in PP2A binding showed hyperphosphorylation of associated Mst3. Moreover, hyperphosphorylated, gel-shifted Mst3 could also be detected in lysates from cells expressing these mutants, indicating that these mutants cause the hyperphosphorylation of a

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significant portion of the Mst3 in the cell. Using Mst3 activation loop point mutants, autophosphorylation site T178-specific antibody, and okadaic acid to inhibit PP2A and activate Mst3 in vivo [37], we demonstrated that gel-shifted Mst3 was indicative of Mst3 activation and autophosphorylation in vivo. This conclusion is further supported by previous studies that reported a gel shift of Mst3 upon autophosphorylation in vitro [19,38], and a consequential increase in kinase activity after autophosphorylation [38]. Our data further show that the Mst3 gel shift represents an underestimation of the amount of Mst3 autophosphorylated on threonine 178, the reported site of Mst3 autophosphorylation for Mst3b [19], because phosphorylation at both threonines 178 and 182 on Mst3b appears to be required to obtain the observed gel shift. Thus, striatin-associated PP2A negatively regulates the phosphorylation and activation of Mst3, and likely of the other related kinases in striatin family complexes, Mst4 (which is also gel-shifted upon okadaic acid treatment) and STK25, as well. The fact that reduction in striatin-associated PP2A is sufficient to cause activation of Mst3 suggests that one way the Mst3 and the other GCKIII kinases could be regulated is by modulation of striatin-associated PP2A activity or modulation of PP2A's access to Mst3 in striatin family complexes. An important goal of future research on these complexes will be to determine if these mechanisms exist, and if so, to elucidate them.

Previous data suggested that threonine 182 was not a major autophosphorylation site on Mst3 [19]. However, this data was largely based on peptide phosphorylation studies. We (Figure 8A) and others [19,38] have found that incubation of immunoprecipitated Mst3 in the presence of ATP and manganese generates the hypershifted form of Mst3 in vitro. Considered together with the current result that mutation of threonine 182 to alanine completely prevents the gel-shift of Mst3 without blocking the phosphorylation of threonine 178, this finding supports the notion that threonine 182 is a major autophosphorylation site of Mst3. The fact that there is no detectable gel shift when either threonine 178 or threonine 182 is mutated to alanine is intriguing. One possible explanation is that simultaneous phosphorylation at both of these sites is required to produce the gel shift. Another possibility is that there may be a preference for sequential phosphorylation of Mst3 activation loop phosphorylation sites, with phosphorylation at threonine 178 being a prerequisite for phosphorylation at threonine 182, which in turn is required for the observed gel shift to occur. Evidence for an interdependence in activation loop phosphorylations has been noted previously for other kinases such as Chk2 [39]. Availability of a phospho-specific antibody for threonine 182 of Mst3 would be helpful for distinguishing between these and other possibilities.

Deletion of the calmodulin-binding domain (Δ(148-166) striatin), which has no effect on striatin oligomerization or binding of Mob3 to striatin and little effect on PP2A association with striatin (Figure 3C-D), caused a 3- to 4-fold increase in both Mst3 and Mst4 association with striatin. This result is consistent with the possibility that the calmodulin-binding domain negatively regulates the binding of Mst3 and Mst4 in striatin family complexes. The effect of this deletion mutant is not simply due to the removal of amino acids in this general area of the striatin sequence because adjacent deletions on either side of the calmodulin-binding domain had either no effect or only a modest 25% increase in Mst3 binding. Given that we and others have shown that striatin family members associate with calmodulin in a calcium-dependent manner [8,11,12], calcium may regulate Mst3 and Mst4 recruitment to striatin complexes by regulating calmodulin binding to striatin. However, it is also possible that removal of these specific sequences modifies Mst3 binding in a manner that will not be recapitulated by changes in calcium. Further experimentation will be required to distinguish between these possibilities.

Conclusions

The results of this study help define the architecture of striatin complexes by identifying residues of striatin important for complex formation with Mob3, PP2A, Mst3, Mst4, and CCM3. Our data support the novel hypothesis that PP2A binding to the coiled-coil domain requires striatin family oligomerization, which has implications for the assembly of striatin family complexes and the ability of PP2A to regulate other components of these complexes. The finding of residues critical for striatin oligomerization N-terminal to its previously assigned coiled-coil domain combined with results from analyses using coiled-coil prediction programs suggest that striatin's coiled-coil domain may begin earlier in the striatin sequence than previously thought. In addition, our results support a model in which striatin binds Mst3 and CCM3 likely as a dimer via residues lying between striatin's calmodulin-binding and WD-domains and recruits the PP2A A/C heterodimer to its coiled-coil/oligomerization domain to regulate Mst3. Striatin-associated PP2A is critical for efficient dephosphorylation and inactivation of striatin-associated Mst3. Upon inhibition of PP2A by okadaic acid, Mst3 activation appears to involve autophosphorylation of its activation loop phosphorylation sites, resulting in a gel shift on SDS-PAGE. Mob3 can associate with striatin sequences C-terminal to the Mst3 binding site but also with sequences proximal to striatin-associated PP2A, consistent with a possible role for Mob 3 in the regulation of Mst3 by PP2A.

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Methods

Antibodies

Anti- HA-tag antibodies, 12CA5 and F7 (available from Santa Cruz Biotechnology) were used for immunoprecipitation of HA-tagged proteins, while anti-HA antibody 16b12 (Covance) was used for immunoblotting. Other antibodies used include mouse monoclonal anti-FLAG epitope tag antibody M2 (Sigma), PP2A C subunit antibody (BD Transduction Labs), anti-SG2NA mouse monoclonal antibody S68 [14] (available from Millipore and Santa Cruz Biotechnology or by request to the corresponding author), anti-Mob3 rabbit polyclonal antibody RK130 [14], and rabbit monoclonal antibodies anti-Mst3, anti-Mst4, and anti-Mst4(pT178)/Mst3 (pT190)/STK25 (pT174) (Epitomics, Inc.). Mst3 has two isoforms, a longer isoform, referred to as isoform a, and a shorter isoform, isoform b. The anti-phospho Mst4/Mst3/STK25 antibody mentioned above detects Mst3 isoform a that is phosphorylated on threonine 190 in its activation loop or Mst3 isoform b phosphorylated on its corresponding activation loop threonine, threonine 178. Because we used FLAG epitope-tagged Mst3 isoform b for experiments utilizing this antibody, we have referred to this antibody as anti-Mst3 (pT178) in this report.

Plasmids, mutagenesis, and creation of stable cell lines

A human striatin cDNA clone was assembled from ESTs by standard approaches, sequenced in its entirety to confirm that it contained no mutations, and inserted into pEGFP-N3 with a modified multiple cloning site to make pEGFP-N3-wild-type striatin. Standard restriction cloning and PCR mutagenesis techniques were then used to make point mutations or small deletions in pEGFP-N3-wild-type striatin, except that in most cases PCR was carried out using Herculase® II polymerase (Stratagene) because this enzyme was necessary for efficient PCR of GC-rich striatin sequences. The striatin deletion mutants containing only the coiled-coil domain or coiled-coil and caveolin-binding domains were made by standard cloning procedures in a modified lentivirus plasmid (pLenti6/V5-D-Topo; Invitrogen) with an N-terminal double HA epitope tag, but were used as plasmids (i.e., transfected). The empty vector and wild-type striatin expressed in the same construct as the mutants were used as controls. All striatin mutants were sequenced.

The cDNA of human Mst3 isoform b (Mst3b; Clone ID HsCD00042929) was obtained from the Dana-Farber/Harvard Cancer Center DNA Resource Core. The hMst3b ORF was PCR-amplified and ligated into HindIII-EcoRI sites of pcDNA3.1 with a HindIII-HindIII N-terminal FLAG epitope tag, excised with the tag by cutting with NheI and ApaI, and ligated into an XbaI-ApaI digested backbone from the plasmid pLenti6/V5-D-Topo. Standard PCR mutagenesis procedures were used to introduce the threonine to alanine point mutations in the activation loop. The cDNA of human CCM3 (Catalog No. SC320246) was obtained from OriGene Technologies, Inc. and the hCCM3 ORF was PCR-amplified and inserted into a modified pLenti6/V5-D-Topo plasmid with an N-terminal FLAG epitope tag. To create HEK293 cells stably expressing FLAG-tagged hCCM3, cells were infected with lentiviruses that express either empty lentivirus plasmid with a FLAG epitope or lentivirus plasmid expressing N-terminally FLAG-tagged hCCM3. Infected cells were incubated in media supplemented with 7 ug/ml of blasticidin to select cells that stably express FLAG-tagged CCM3. The selected stable cell lines were used to perform co-immunoprecipitation experiments after transfecting with a set of striatin deletion mutants and controls.

Cell culture, transfections, and cell lysis

HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5 or 10% fetal calf serum at 37°C in 10% CO_2. For experiments, HEK293 cells were plated into 60 mm dishes at 15% confluence and grown at 37°C for 16-24 hours. The cells were then transfected using FuGENE™ 6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol. Control inclusion of a small amount (one-tenth of other DNAs) of GFP expressing plasmid in some experiments showed > 50% of cells were routinely being transfected. After a minimum of 48 h to allow the wild-type and mutant striatins to express and bind associated proteins in the cells, the cells were washed with ice cold phosphate-buffered saline and then with IP wash buffer (0.135 M NaCl, 10% glycerol, 20 mM Tris, pH 8.0) prior to being lysed with IP lysis buffer (IP wash buffer containing 1% Nonidet P-40, 1 mM phenylmethylsufonyl fluoride, 0.04 trypsin inhibitor units/ml aprotinin, and in the case of phosphorylation-related experiments, 50 mM sodium fluoride and 100 nM okadaic acid (LC Laboratories) to prevent dephosphorylation in lysates) by rocking for 20 minutes at 4°C. Lysates were cleared by centrifugation at 13,000 × g.

Immunoprecipitation, gel electrophoresis, and immunoblotting

To immunoprecipitate HA-tagged proteins and complexes, cleared lysates were incubated with 12CA5 anti-HA antibody and protein A-Sepharose 4B beads (Invitrogen) or F7 anti-HA-agarose conjugate (when probing for Mst3, Mst4, or pMst3; Santa Cruz Biotechnology # sc-7392 AC) for 1.5 hours at 4°C with rocking. FLAG epitope-tag immunoprecipitates were prepared in the same manner with M2 anti-FLAG antibody (Sigma-Aldrich) and recombinant protein G-Sepharose 4B beads (Invitrogen). Immune complexes were harvested

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by centrifugation, washed twice in phosphate buffered saline, twice in IP lysis buffer, and heated at near boiling in sample buffer for 5 minutes. Immune complexes were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose for immunoblotting. Clean Blot (Pierce; 1:1000) was used for detection of Mst3, Mst4, or pMst3 in most cases to reduce background from the immunoprecipitating IgG heavy chain. Bands from immunoblotting were visualized by enhanced chemiluminescence and a Fluor S-Max Chemilumimager (Bio-Rad). The chemilumimager directly measures band intensities without the use of film via a supercooled CCD camera that provides linear data over 4.8 orders of magnitude. This method yields highly reproducible results that do not vary with image capture times. For mapping experiments in which the amount of associated proteins were being compared, only experiments in which similar expression of wild-type and mutants were obtained were used for quantitation to avoid an artificial bias from different levels of expression. Quantitation of striatin levels in vector control cells and HA-wild-type striatin transfected cells indicates that 1 ug striatin transfected into a 60 mm dish of cells results in close to a 1:1 level of expression of HA-wild-type striatin to endogenous striatin (data not shown). The amount of DNA used for experiments in this study ranged from 0.5-2 ug.

Phosphorylation of Mst3 in vitro

Three days after transfection of HEK293 cells with a plasmid encoding FLAG-tagged wild-type Mst3, FLAG immunoprecipitates were prepared and washed twice with phosphate buffered saline (pH 7.3), twice with lysis buffer, and once with tris buffered saline. The immune complexes were combined, divided equally into three tubes, and then suspended in a buffer containing 40 mM 1,4 Piperazine-diethanesulfonic acid (pH7.0) and 2 mM $MnCl_2$. Two samples were incubated with DMSO (vehicle control) while the other sample was incubated with 1 μM staurosporine, all for 30 min at room temperature. Then ATP was added to a final concentration of 20 μM to one vehicle control aliquot and to the staurosporine-containing aliquot. After incubation for 30 min at room temperature all three reactions were analyzed by SDS-PAGE and Mst3 protein bands detected by immunoblotting.

Dephosphorylation of Mst3 in vitro

Three days after transfection with HA-tagged R100S/R101E striatin, HEK293 cells were lysed in the absence of phosphatase inhibitors and HA-striatin immune complexes were prepared. The immune complexes were denatured by heating at near boiling for 5 minutes in lysis buffer containing 0.5% SDS and 5 mM β-mercaptoethanol, centrifuged to remove the protein A sepharose beads, and then the supernatant was divided into three equal portions. Each sample was then diluted with a four-fold excess of lysis buffer to capture excess SDS into mixed micelles with Nonidet P-40. For two of the samples, the lysis buffer contained purified PP2A (Millipore) plus DMSO (vehicle control) or 100 nM okadaic acid. After incubation for 60 minutes at 30°C, the samples were analyzed by SDS-PAGE and Mst3 protein bands were detected by immunoblotting.

Immunoprecipitation of denatured lysates

To denature lysates prior to immunoprecipitation, cells were first lysed in lysis buffer as described above and then, after clearing the lysates, SDS and β-mercaptoethanol were added to final concentrations of 0.5% and 5 mM, respectively. The lysates were then heated at near boiling for 5 minutes, diluted 1:4 with IP lysis buffer to capture excess SDS into mixed micelles, and then immunoprecipitated as described above.

Acknowledgements

We thank Danita Ashby, Sameer Patel, and Courtney Plattner for excellent technical assistance, and Jocelyn Lee, Jennifer Jackson, Anita Corbett, and Richard Cummings for critical reading of the manuscript. We are also grateful to Dr. Oskar Laur and the Emory University Custom Cloning Core Facility for the construction of some of the mutants used in this study and to Michael Konomos for graphic design. This work was supported by a grant from the NIH/NCI to DCP (CA57327) and by NIH/NIGMS IRACDA grant number K12 GM000680.

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Authors' contributions

JG and JH carried out most of the study, contributed ideas towards its design and execution, and contributed to the preparation of the manuscript. KC, CAJ, QLK, and CSM participated in execution, analysis, and troubleshooting of the striatin structure-function experiments. CSM and RHK contributed ideas to the study and participated in the drafting of the manuscript. DCP conceived of the study, and participated in its design, coordination and execution, and helped draft the manuscript. All authors read and approved the final manuscript.

Competing interests

Dr. David Pallas is entitled to royalty from the sale of products related to the research described in this paper by Millipore Inc., Santa Cruz Biotechnologies Inc., Invitrogen Corp., and Novus Biologicals Inc. In addition, this same author serves as a consultant to Millipore. The terms of these arrangements have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

Received: 25 March 2011 Accepted: 10 October 2011
Published: 10 October 2011

References

1. Janssens V, Goris J: **Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.** *Biochem J* 2001, **353(Pt 3)**:417-439.
2. Virshup DM, Shenolikar S: **From promiscuity to precision: protein phosphatases get a makeover.** *Mol Cell* 2009, **33(5)**:537-545.
3. Sola MM, Langan T, Cohen P: **p34cdc2 phosphorylation sites in histone H1 are dephosphorylated by protein phosphatase 2A1.** *Biochim Biophys Acta* 1991, **1094(2)**:211-216.
4. Agostinis P, Derua R, Sarno S, Goris J, Merlevede W: **Specificity of the polycation-stimulated (type-2A) and ATP,Mg-dependent (type-1) protein phosphatases toward substrates phosphorylated by P34cdc2 kinase.** *Eur J Biochem* 1992, **205(1)**:241-248.
5. Ferrigno P, Langan TA, Cohen P: **Protein phosphatase 2A1 is the major enzyme in vertebrate cell extracts that dephosphorylates several physiological substrates for cyclin-dependent protein kinases.** *Mol Biol Cell* 1993, **4(7)**:669-677.
6. Kamibayashi C, Estes R, Lickteig RL, Yang SI, Craft C, Mumby MC: **Comparison of heterotrimeric protein phosphatase 2A containing different B subunits.** *J Biol Chem* 1994, **269(31)**:20139-20148.
7. Mayer-Jaekel RE, Ohkura H, Ferrigno P, Andjelkovic N, Shiomi K, Uemura T, Glover DM, Hemmings BA: **Drosophila mutants in the 55 kDa regulatory subunit of protein phosphatase 2A show strongly reduced ability to dephosphorylate substrates of p34cdc2.** *Journal of Cell Science* 1994, **107**:2609-2618.
8. Moreno CS, Park S, Nelson K, Ashby DG, Hubalek F, Lane WS, Pallas DC: **The WD40 Repeat Proteins Striatin and SG2NA are Members of a Novel Family of Calmodulin-Binding Proteins that Associate with PP2A.** *Journal of Biological Chemistry* 2000, **275(8)**:5257-5263.
9. Goudreault M, D'Ambrosio LM, Kean MJ, Mullin MJ, Larsen BG, Sanchez A, Chaudhry S, Chen GI, Sicheri F, Nesvizhskii AI, Aebersold R, Raught B, Gingras AC: **A PP2A phosphatase high density interaction network identifies a novel striatin-interacting phosphatase and kinase complex linked to the cerebral cavernous malformation 3 (CCM3) protein.** *Mol Cell Proteomics* 2009, **8(1)**:157-171.
10. Gaillard S, Bartoli M, Castets F, Monneron A: **Striatin, a calmodulin-dependent scaffolding protein, directly binds caveolin-1.** *FEBS Lett* 2001, **508(1)**:49-52.
11. Castets F, Rakitina T, Gaillard S, Moqrich A, Mattei MG, Monneron A: **Zinedin, SG2NA, and striatin are calmodulin-binding, WD repeat proteins principally expressed in the brain.** *J Biol Chem* 2000, **275(26)**:19970-19977.
12. Bartoli M, Monneron A, Ladant D: **Interaction of calmodulin with striatin, a WD-repeat protein present in neuronal dendritic spines.** *J Biol Chem* 1998, **273(35)**:22248-22253.
13. Castets F, Bartoli M, Barnier JV, Baillat G, Salin P, Moqrich A, Bourgeois JP, Denizot F, Rougon G, Calothy G, Monneron A: **A novel calmodulin-binding protein, belonging to the WD-repeat family, is localized in dendrites of a subset of CNS neurons.** *J Cell Biol* 1996, **134(4)**:1051-1062.
14. Moreno CS, Lane WS, Pallas DC: **A mammalian homolog of yeast MOB1 is both a member and a putative substrate of striatin family-protein phosphatase 2A complexes.** *J Biol Chem* 2001, **276**:24253-24260.
15. Gaillard S, Bailly Y, Benoist M, Rakitina T, Kessler JP, Fronzaroli-Molinieres L, Dargent B, Castets F: **Targeting of proteins of the striatin family to dendritic spines: role of the coiled-coil domain.** *Traffic* 2006, **7(1)**:74-84.
16. Baillat G, Moqrich A, Castets F, Baude A, Bailly Y, Benmerah A, Monneron A: **Molecular cloning and characterization of phocein, a protein found from the golgi complex to dendritic spines.** *Mol Biol Cell* 2001, **12(3)**:663-673.
17. Baillat G, Gaillard S, Castets F, Monneron A: **Interactions of Phocein with Nucleoside-Diphosphate Kinase, Eps15, and Dynamin I.** *J Biol Chem* 2002, **277(21)**:18961-18966.
18. Schulte J, Sepp KJ, Jorquera RA, Wu C, Song Y, Hong P, Littleton JT: **DMob4/Phocein regulates synapse formation, axonal transport, and microtubule organization.** *J Neurosci* 2010, **30(15)**:5189-5203.
19. Lu TJ, Lai WY, Huang CY, Hsieh WJ, Yu JS, Hsieh YJ, Chang WT, Leu TH, Chang WC, Chuang WJ, Tang MJ, Chen TY, Lu TL, Lai MD: **Inhibition of cell migration by autophosphorylated mammalian sterile 20-like kinase 3 (MST3) involves paxillin and protein-tyrosine phosphatase-PEST.** *J Biol Chem* 2006, **281(50)**:38405-38417.
20. Preisinger C, Short B, De Corte V, Bruyneel E, Haas A, Kopajtich R, Gettemans J, Barr FA: **YSK1 is activated by the Golgi matrix protein GM130 and plays a role in cell migration through its substrate 14-3-3zeta.** *J Cell Biol* 2004, **164(7)**:1009-1020.
21. Fidalgo M, Fraile M, Pires A, Force T, Pombo C, Zalvide J: **CCM3/PDCD10 stabilizes GCKIII proteins to promote Golgi assembly and cell orientation.** *J Cell Sci* 2010, **123(Pt 8)**:1274-1284.
22. Comils H, Kohler RS, Hergovich A, Hemmings BA: **Human NDR kinases control G1/S Cell Cycle transition by directly regulating p21 stability.** *Mol Cell Biol* 2011.
23. Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH: **Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha.** *Proc Natl Acad Sci USA* 2004, **101(49)**:17126-17131.
24. Varsano T, Dong MQ, Niesman I, Gacula H, Lou X, Ma T, Testa JR, Yates JR, Farquhar MG: **GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling.** *Mol Cell Biol* 2006, **26(23)**:8942-8952.
25. He Y, Zhang H, Yu L, Gunel M, Boggon TJ, Chen H, Min W: **Stabilization of VEGFR2 signaling by cerebral cavernous malformation 3 is critical for vascular development.** *Sci Signal* 2010, **3(116)**:ra26.
26. Bergametti F, Denier C, Labauge P, Arnoult M, Boetto S, Clanet M, Coubes P, Echenne B, Ibrahim R, Irthum B, Jacquet G, Lonjon M, Moreau JJ, Neau JP, Tremoulet M, Tournier-Lasserve E, Société Française de Neurochirurgie : **Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations.** *Am J Hum Genet* 2005, **76(1)**:42-51.
27. Guclu B, Ozturk AK, Pricola KL, Bilguvar K, Shin D, O'Roak BJ, Gunel M: **Mutations in apoptosis-related gene, PDCD10, cause cerebral cavernous malformation 3.** *Neurosurgery* 2005, **57(5)**:1008-1013.
28. Robinson JR, Awad IA, Little JR: **Natural history of the cavernous angioma.** *J Neurosurg* 1991, **75(5)**:709-714.
29. Meurs KM, Mauceli E, Lahmers S, Acland GM, White SN, Lindblad-Toh K: **Genome-wide association identifies a deletion in the 3' untranslated region of striatin in a canine model of arrhythmogenic right ventricular cardiomyopathy.** *Hum Genet* 2010, **128(3)**:315-324.
30. Sotoodehnia N, Isaacs A, de Bakker PI, Dorr M, Newton-Cheh C, Nolte IM, van der Harst P, Muller M, Eijgelsheim M, Alonso A, Hicks AA, Padmanabhan S, Hayward C, Smith VA, Polasek O, Giovannone S, Fu J, Magnani WJ, Marciante DK, Pfeufer A, Gharib AS, Teumer A, Li M, Bis CJ, Rivadeneira F, *et al*: **Common variants in 22 loci are associated with QRS duration and cardiac ventricular conduction.** *Nat Genet* 2010, **42(12)**:1068-1076.
31. Favre B, Turowski P, Hemmings BA: **Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin.** *J Biol Chem* 1997, **272(21)**:13856-13863.
32. Hornbeck PV, Chabra I, Kornhauser JM, Skrzypek E, Zhang B: **PhosphoSite: A bioinformatics resource dedicated to physiological protein phosphorylation.** *Proteomics* 2004, **4(6)**:1551-1561.
33. Lupas A: **Prediction and analysis of coiled-coil structures.** *Methods Enzymol* 1996, **266**:513-525.
34. McDonnell AV, Jiang T, Keating AE, Berger B: **Paircoil2: improved prediction of coiled coils from sequence.** *Bioinformatics* 2006, **22(3)**:356-358.
35. Kammerer RA, Kostrewa D, Progias P, Honnappa S, Avila D, Lustig A, Winkler FK, Pieters J, Steinmetz MO: **A conserved trimerization motif controls the topology of short coiled coils.** *Proc Natl Acad Sci USA* 2005, **102(39)**:13891-13896.
36. Ciani B, Bjelic S, Honnappa S, Jawhari H, Jaussi R, Payapilly A, Jowitt T, Steinmetz MO, Kammerer RA: **Molecular basis of coiled-coil oligomerization-state specificity.** *Proc Natl Acad Sci USA* 2010, **107(46)**:19850-19855.
37. Stegert MR, Hergovich A, Tamaskovic R, Bichsel SJ, Hemmings BA: **Regulation of NDR protein kinase by hydrophobic motif phosphorylation mediated by the mammalian Ste20-like kinase MST3.** *Mol Cell Biol* 2005, **25(24)**:11019-11029.
38. Schinkmann K, Blenis J: **Cloning and characterization of a human STE20-like protein kinase with unusual cofactor requirements.** *J Biol Chem* 1997, **272(45)**:28695-28703.

39. Guo X, Ward MD, Tiedebohl JB, Oden YM, Nyalwidhe JO, Semmes OJ: **Interdependent phosphorylation within the kinase domain T-loop Regulates CHK2 activity.** *J Biol Chem* 2010, **285(43)**:33348-33357.
40. Yu XX, Du X, Moreno CS, Green RE, Ogris E, Feng Q, Chou L, McQuoid MJ, Pallas DC: **Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen.** *Mol Biol Cell* 2001, **12(1)**:185-199.
41. Campbell KS, Auger KR, Hemmings BA, Roberts TM, Pallas DC: **Identification of regions in polyomavirus middle T and small t antigens important for association with protein phosphatase 2A.** *J Virol* 1995, **69(6)**:3721-3728.
42. Turowski P, Fernandez A, Favre B, Lamb NJ, Hemmings BA: **Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression.** *J Cell Biol* 1995, **129(2)**:397-410.
43. Ogris E, Gibson DM, Pallas DC: **Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen.** *Oncogene* 1997, **15(8)**:911-917.

doi:10.1186/1471-2091-12-54
Cite this article as: Gordon *et al.*: Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3. *BMC Biochemistry* 2011 12:54.

CHAPTER 4

Additional Results

PP2A negatively regulates the recruitment of Mst3 and Mst4 to striatin complexes

Increased binding of Mst3 and Mst4 to striatin upon inhibition of PP2A by okadaic acid

To determine whether PP2A has a role in regulating the recruitment of Mst3 to striatin complexes, I measured the relative amount of Mst3 association with striatin in the presence or absence of okadaic acid. Okadaic acid treatment of HEK293 cells causes a robust (~4-fold) increase in Mst3 association with striatin (Fig. 4-1). Examination of the Mst3 immunoblot of lysates from these cells (Fig. 4-1A) reveals that this increase was not simply due to an increase in the steady-state level of Mst3 protein in the okadaic acid-treated cells. Immunoblotting for the related striatin associated kinase, Mst4, shows that PP2A inhibition also increased its association with striatin over 4-fold with no increase in Mst4 seen in lysates, indicating that PP2A regulates Mst4 recruitment to striatin complexes as well. Therefore, inhibition of PP2A by okadaic acid treatment increased binding of Mst3 and Mst4 kinases to striatin complex.

Discussion

Results from Chapter 3 and Chapter 4 indicate that the striatin-associated PP2A not only regulates dephosphorylation/activation of Mst3 kinase but also its recruitment to

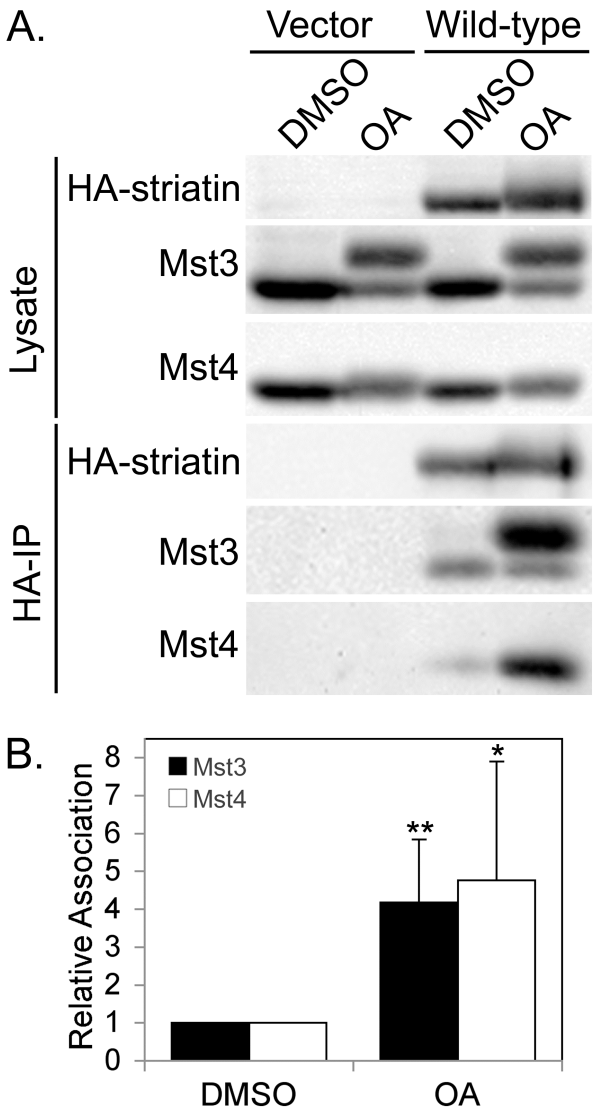

Figure 4-1. Okadaic acid (OA) treatment causes increased binding of Mst3 and Mst4 to striatin.

(A) HEK293 cells were transfected with empty vector (Vector) or HA-striatin expressing plasmid (Wild-type). 72 hours post-transfection, cells were treated with either DMSO or 100nM OA for 4 hours. Lysates and HA-striatin (HA-IP) were analyzed for the presence of HA-striatin, Mst3, and Mst4 by immunoblotting. (B) Band intensities of

HA-striatin, Mst3, and Mst4 were quantitated using a chemilumimager. Relative levels of Mst3 and Mst4 in the HA-striatin complex were determined by normalizing the amount of Mst3 and Mst4 to the amount of wild-type HA-striatin. Relative association of Mst3/Mst4 and striatin was increased by about 4 fold upon OA treatment, suggesting that PP2A may also regulate the recruitment and/or release of Mst3 and Mst4. The error bars represent the S.D. of three independent experiments. *, $p \leq 0.05$; **, $p \leq 0.01$ relative to DMSO control.

the striatin complexes. It remains to be determined whether there is increased recruitment of Ccm3 to the striatin-associated complexes in the presence of okadaic acid. Given the fact that Ccm3 brings Mst3 and Mst4 to the striatin complexes, it would not be surprising to find that the binding of Ccm3 to the striatin complexes is indeed enhanced by okadaic acid treatment. Although the striatin-associated PP2A-mediated dephosphorylation and activation of Mst4 kinase was not determined in this study (Chapter 3), it was clear that Mst4 binding to the striatin complex was increased upon PP2A inhibition. Thus, it would be interesting to investigate whether a similar negative regulation mechanism controls other GCKIII kinases including Mst4 and Ysk1.

It is also interesting to note that there was an increase of Mst3 and Mst4 recruitment to the striatin complexes without an increase in the steady state levels of these kinases. This result suggests that the hyperphosphorylated and activated forms of Mst3 and Mst4 kinases have higher affinity for the striatin complexes than the dephosphorylated forms. It is also possible that the enhanced association of Mst3 and Mst4 with the striatin complexes is due to the changes of the striatin complexes and/or Mst kinases in subcellular localization by okadaic acid. Overall, our results support a model in which striatin orchestrates both the recruitment and regulation of Mst3 and Mst4 by PP2A.

CHAPTER 5

Discussion and future directions

PME-1, PME-1 inhibitors, and cancer

While LCMT-1 was demonstrated to be the common methyltransferase for all PP2A family phosphatases, PP2A is the only known substrate for PME-1 so far (Fig. 5-1). Considering the highly conserved C-terminal residues among the C subunits of PP2A family phosphatases, it is highly likely that PME-1 is also involved in demethylation of PP4 and PP6. To prove this point, it would be necessary to show how methylation of PP4c and/or PP6c changes upon manipulation of PME-1 levels by depletion and overexpression. Using purified PME-1 enzyme could also be an alternative way to show that it could demethylate PP4c and/or PP6c *in vitro*. To corroborate this hypothesis, it would be important to further show that PME-1 wildtype overexpressing cells reduce the methylation level of PP4c and/or PP6c while catalytically inactive PME-1 mutant (S156A) overexpressing cells do not. Recently, aza-β-lactam (ABL) compounds have been discovered as selective inhibitors of PME-1 [54]. These potent PME-1 inhibitors would make useful reagents to further test if PME-1 is the major methylesterase of PP2A-related phosphatases. Our preliminary data showed that the ABL compounds were capable of reversing the action of PME-1 on PP2Ac methylation. Yet the effect on PP4c and/or PP6c is still in question.

Investigation of PME-1 and its inhibitors could be critical in cancer research since PME-1 overexpression has been reported in malignant glioblastomas as well as endometrial adenocarcinomas [52, 53]. Since PME-1 overexpression is detected in these

cancers, it is predicted that they would have greatly increased levels of demethylation of PP2A family phosphatases. Experimentally, it was also shown that loss of LCMT-1 and PP2Ac methylation led to cancerous phenotypes in cells [199]. Additionally, tumor viruses appear to replace the methylation-dependent B-type subunits and evade cellular control of PP2A by methylation to promote tumorigenesis [199]. Therefore, PME-1 inhibitors may potentially serve as valuable drugs for treatment of cancer where PME-1 overexpression and/or defective PP2A methylation are observed. Furthermore, since PP4 also appears to form both methylation-dependent and methylation-independent complexes and overexpression of PP4 has been observed in certain cancers, it would be interesting to study if similar mechanisms exist for PP4 and cancer, and whether PME-1 inhibitors would have the potential to reverse the cancerous phenotypes in these cases. Lastly, although PP6c is under tight control of methylation by LCMT-1, it was not fully determined whether PP6 shows methylation-dependency in holoenzyme assembly and it would be an interesting area of further research.

LCMT-1 negatively regulates HDAC3 phosphorylation and activation

It is of high importance to recognize the fact that some of the functions ascribed to PP2A may actually come from PP4 and/or PP6. For example, data obtained using drugs at concentrations specific for PP2A inhibition may be misleading since the concentration of the drug used may inhibit PP2A-related phosphatases as well. In the present study, LCMT-1 KO MEFs were utilized as an approach to study methylation of PP2A-related phosphatases. It was also demonstrated that LCMT-1 KO shows enhanced phosphorylation and activation of HDAC3. However, since LCMT-1 KO leads to

disruption of the methylation-dependent PP2A complexes and perhaps PP6 complexes, caution is required when interpreting the data obtained here with HDAC3 as well as other published data regarding LCMT-1 functions. Although there is no evidence yet for PP2A and PP6 in regulating HDAC3 phosphorylation and activation, it would be especially important to gain deeper insights into mechanisms involved in regulation of HDAC3 by protein phosphatases in order to assign the function as a HDAC3 phosphatase solely to PP4. To make sure that the effect of LCMT-1 knockout on HDAC3 is in fact due to PP4 dysfunction by LCMT-1 loss, it would be necessary to determine whether specific inhibition of PP2A by knocking out or depletion would alter the phosphorylation state and activity of HDAC3. Similar studies to specifically inhibit PP6 would be a good starting point to determine the connection between PP6 and HDAC3 as well, if any.

Functional significance of the PP4c methylation

This study showed that loss of LCMT-1 has a major effect on PP4R1-associated PP4 complex (Fig. 5-1). Interestingly, PP4R1 is involved in regulation of HDAC3, while other PP4 regulatory subunits have important functions in many aspects of DNA repair processes. Since PP4R1 is the major methylation-dependent PP4 complex component and other regulatory subunits seem mostly methylation-independent, I hypothesized that PP4c methylation regulates the function of PP4 enzymes in processes other than DNA repair. According to this hypothesis, dysfunction of LCMT-1 and abnormal PP4c methylation would not lead to major defects in DNA repair processes. In agreement with this, my preliminary data suggests that the methylation levels of PP4c and PP6c do not change upon DNA damage (not shown). Thus, the fact that PP4 regulatory subunits involved in

DNA damage response are mostly methylation-independent suggests that this may be a means for cells to actively repair DNA damage for cell survival without being affected by the changes in PP4c methylation state. However, considering the fact that PP4 regulatory subunits other than PP4R1 showed minor phenotypes in the absence of LCMT-1, LCMT-1 loss might show limited defects in DNA repair process as well. For instance, the function of KAP-1, a substrate of PP4R3β, in NHEJ-mediated repair of DSBs [94] and in regulation of G_2/M checkpoint [95] may be affected in LCMT-1 KO. Moreover, there was a clear reduction of migration of the native PP4R2/PP4 complexes in LCMT-1 knockout cells, and this could indicate some type of alteration in this complex that might affect its function. Therefore, more investigation will be needed to further reveal the functional significance of methylation control of PP4 holoenzymes.

As mentioned above, loss of LCMT-1 also affected the PP4R2- and PP4R3β-associated PP4 complexes. Although the interaction between PP4R2 and PP4c remained mostly intact, the migration of the native PP4R2-associated PP4 complex was slightly but clearly reduced in LCMT-1 KO. This suggests that the complex is modified either by post-translational modifications or by changes in binding partners. Considering the fact that there was a minor reduction in the PP4R3β-associated PP4 complex formation in LCMT-1 KO, the PP4R2-associated PP4 complex may have established new interactions as a substitute for PP4R3β. In this case, even though the PP4R2-associated PP4 complex still exists in LCMT-1 KO, its substrates and thus the function of the PP4R2-associated PP4 complex may be altered. To further explore the changes in the assembly of the PP4 holoenzyme complexes, AP-MS analysis could be utilized to compare and identify PP4-interacting proteins in the presence and absence of LCMT-1. The results from this

suggested study will provide more insights into the regulation mechanisms of the PP4 holoenzyme complexes and function by methylation.

Regulation of subcellular localization of the striatin complexes

In addition to the fact that deletion of the Ca^{2+}-CaM-binding domain of striatin greatly increases the association of the GCKIII subfamily kinases, Mst3 and Mst4, with striatin, PP2A inhibition also enhances the binding of both Mst3 and Mst4 to striatin. There was an increase in phosphorylation of Mst3 and Mst4 by PP2A inhibition and it was this population of Mst3 and Mst4 that showed enhanced interaction with striatin under this condition. These data indicate that in addition to the Ca^{2+}-CaM-binding domain, PP2A may negatively regulate the binding of Mst3 and Mst4 to striatin as well, and releasing Mst3 and Mst4 from striatin by dephosphorylation. Considering these and other evidence that showed changes in striatin localization due to Ca^{2+} concentration, it is tempting to speculate that Ca^{2+}-CaM may regulate binding of GCKIII kinases to striatin by altering striatin subcellular localization. A direct analysis of the effect of Ca^{2+}-CaM binding on GCKIII kinase association with striatin would be interesting. Similarly, investigation of the effect of PP2A inhibition on subcellular localization of the striatin family and the associated proteins would provide more insights into the regulation mechanisms of the striatin-associated PP2A complexes and function.

Implication of Ccm3-GCKIII and STRIPAK functions in CCM pathogenesis

It appears that the interaction between Ccm3 and GCKIII kinases are critical for normal functioning of cells and dysfunction of any of these processes due to their

disrupted interaction leads to CCM development. As mentioned in Chapter 1, several lines of evidence support this proposal. An additional study in *Drosophila* again clearly demonstrates functional connections between Ccm3 and GCKIII kinases in cellular processes that may be relevant to CCM development. In this study, loss of Ccm3 or the single dGckIII kinase resulted in a tracheal tube lumenization failure which could not be rescued by a Ccm3 mutant that cannot bind to dGckIII, indicating that Ccm3 functions through dGckIII in this process [186].

Considering the fact that loss of function of Ccm3 and GCKIII kinases seems to activate CCM-relevant pathways, mutations that increase the expression of the striatin family members or enhance the ability of STRIPAK to downregulate GCKIII kinases might contribute to CCM development. For that reason, it would be important to examine the status of STRIPAK components in CCM patients. By contrast, loss of STRIPAK function might activate Ccm3–GCKIII kinase function and not lead to CCM. In agreement with this, depletion of striatin family proteins promotes cell polarization [152], while depletion of Ccm3 or GCKIII kinases impairs it [152, 196]. Since STRIPAK is the upstream regulator of Ccm3 and GCKIII kinases, it seems unlikely that inhibition of STRIPAK function would be therapeutically beneficial against CCM disease caused by Ccm3 mutations that fails to tether GCKIII kinases to STRIPAK. However, in CCM patients where Ccm3–GCKIII signaling is at least partially intact, inhibiting STRIPAK could serve as a novel therapeutic strategy to fight CCM.

Another important area of further research would be to elucidate the role of STRIPAK complexes in RhoA signaling and its relation to CCM development. Recently, RhoA signaling was found to be upregulated in human CCM cases. In addition, depletion

of Ccm3, Mst3, or Ysk1 enhanced RhoA activation, suggesting that Ccm3 and GCKIII kinases negatively regulate RhoA signaling. Since STRIPAK is a negative regulator of GCKIII kinases, and GCKIII kinases in turn negatively regulate RhoA activation, STRIPAK may act as a positive regulator of RhoA signaling pathway. Therefore, it would be critical to determine if modulation of STRIPAK function regulates RhoA signaling as well as to investigate the possibility of targeting STRIPAK therapeutically to fight CCM disease.

Overview

This study focused on addressing some of the unanswered questions on the regulation and function of PP2A family phosphatases. Chapter 2 showed that LCMT-1 is the major methyltransferase for both PP4 and PP6, and that methylation of PP4c plays an important role in PP4 holoenzyme assembly. Loss of PP4c methylation by LCMT-1 knockout resulted in loss of methylation-dependent PP4 holoenzyme complexes. Results indicated that the PP4R1-associated PP4 complex is largely methylation-dependent while other PP4 complexes containing PP4R2, PP4R3α, and PP4R3β are may be primarily methylation-independent. Consistent with this, LCMT-1 knockout led to hyperphosphorylation of PP4R1 substrate, HDAC3 on the Ser^{424} residue which is critical for histone deacetylase activity. Therefore, these data support the hypothesis that carboxyl methylation of PP2A family phosphatase C subunits is a general strategy for regulating holoenzyme assembly and function of PP2A family phosphatases. In Chapter 3 and 4, a structure-function analysis was performed to determine the molecular organization of the striatin-associated PP2A complexes, which have recently been

implicated in human diseases, especially cerebral cavernous malformations (CCM). The results indicate that striatin-associated PP2A regulates the phosphorylation and activation of Mst3 kinase. Additionally, inhibition of PP2A by okadaic acid enhanced recruitment of Mst3 and Mst4 kinases to the striatin complexes. Altogether, these studies provide significant insights into the regulation of PP2A family phosphatase holoenzyme assembly and function by carboxyl methylation, and the molecular organization and function of the striatin-associated PP2A complexes. Overall, these research have laid the foundation for further investigation of the role of methylation in PP2A family phosphatase holoenzyme formation and function, and the complex regulation mechanisms controlling the function of the striatin-associated PP2A complexes.

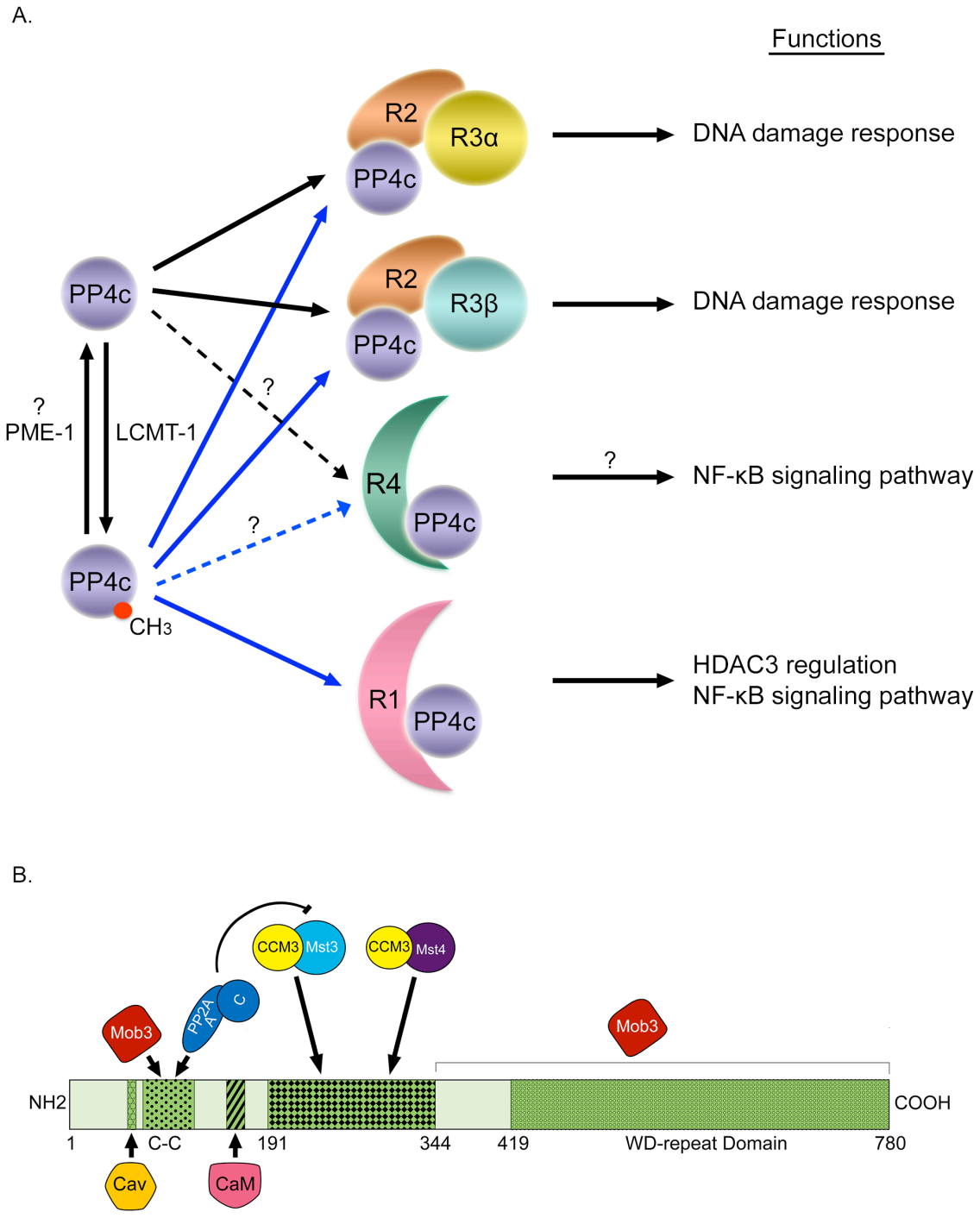

Figure 5-1. Proposed model for the regulation mechanisms of PP2A family phosphatases and the STRIPAK complex.

(A) PP4c methylation is regulated by LCMT-1. Whether PME-1 is involved in the PP4c demethylation is still in question. PP4 forms both methylation-dependent (blue arrows) and methylation-independent (black arrows) complexes. Loss of LCMT-1 results in major loss of the PP4 complex containing PP4R1, suggesting that PP4R1 is largely methylation-dependent. Thus, loss of LCMT-1 would lead to dysfunction of PP4R1-mediated pathways. The PP4R2- and PP4R3β-associated PP4 complexes remain mostly intact in the absence of LCMT-1, suggesting that they are largely methylation-independent. Methylation-dependency of the PP4R4-associated PP4 complex is not known. (B) Structure-function analysis performed in this study as well as previous studies reveal the molecular organization of the striatin-associated PP2A complex and that striatin-associated PP2A regulates the phosphorylation and activation of Mst3 kinase. (Figure was taken and modified from [204]).

CHAPTER 6

References

1. Manning, G., et al., *The protein kinase complement of the human genome.* Science, 2002. **298**(5600): p. 1912-34.
2. Moorhead, G.B., L. Trinkle-Mulcahy, and A. Ulke-Lemee, *Emerging roles of nuclear protein phosphatases.* Nat Rev Mol Cell Biol, 2007. **8**(3): p. 234-44.
3. Eichhorn, P.J., M.P. Creyghton, and R. Bernards, *Protein phosphatase 2A regulatory subunits and cancer.* Biochim Biophys Acta, 2009. **1795**(1): p. 1-15.
4. Janssens, V. and J. Goris, *Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling.* Biochem J, 2001. **353**(Pt 3): p. 417-39.
5. Walter, G., et al., *Molecular cloning and sequence of cDNA encoding polyoma medium tumor antigen-associated 61-kDa protein.* Proc Natl Acad Sci U S A, 1989. **86**(22): p. 8669-72.
6. Hemmings, B.A., et al., *alpha- and beta-forms of the 65-kDa subunit of protein phosphatase 2A have a similar 39 amino acid repeating structure.* Biochemistry, 1990. **29**(13): p. 3166-73.
7. Ruediger, R., et al., *Identification of binding sites on the regulatory A subunit of protein phosphatase 2A for the catalytic C subunit and for tumor antigens of simian virus 40 and polyomavirus.* Mol Cell Biol, 1992. **12**(11): p. 4872-82.
8. Ruediger, R., et al., *Molecular model of the A subunit of protein phosphatase 2A: interaction with other subunits and tumor antigens.* J Virol, 1994. **68**(1): p. 123-9.

9. Pallas, D.C., et al., *Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A.* Cell, 1990. **60**(1): p. 167-76.

10. Sontag, J.M. and E. Sontag, *Protein phosphatase 2A dysfunction in Alzheimer's disease.* Front Mol Neurosci, 2014. **7**: p. 16.

11. Chen, J., B.L. Martin, and D.L. Brautigan, *Regulation of protein serine-threonine phosphatase type-2A by tyrosine phosphorylation.* Science, 1992. **257**(5074): p. 1261-4.

12. Guo, H. and Z. Damuni, *Autophosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2500-4.

13. Ogris, E., D.M. Gibson, and D.C. Pallas, *Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not polyomavirus middle tumor antigen.* Oncogene, 1997. **15**(8): p. 911-7.

14. Wei, H., et al., *Carboxymethylation of the PP2A catalytic subunit in Saccharomyces cerevisiae is required for efficient interaction with the B-type subunits Cdc55p and Rts1p.* J Biol Chem, 2001. **276**(2): p. 1570-7.

15. Xie, H. and S. Clarke, *Methyl esterification of C-terminal leucine residues in cytosolic 36-kDa polypeptides of bovine brain. A novel eucaryotic protein carboxyl methylation reaction.* J Biol Chem, 1993. **268**(18): p. 13364-71.

16. Lee, J. and J. Stock, *Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase.* J Biol Chem, 1993. **268**(26): p. 19192-5.

17. Xie, H. and S. Clarke, *Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain.* J Biol Chem, 1994. **269**(3): p. 1981-4.

18. Favre, B., et al., *The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo.* J Biol Chem, 1994. **269**(23): p. 16311-7.

19. De Baere, I., et al., *Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue.* Biochemistry, 1999. **38**(50): p. 16539-47.

20. George, R.R., et al., *Chaperonin assisted overexpression, purification, and characterisation of human PP2A methyltransferase.* Protein Expr Purif, 2002. **26**(2): p. 266-74.

21. Xie, H. and S. Clarke, *An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A.* Biochem Biophys Res Commun, 1994. **203**(3): p. 1710-5.

22. Lee, J., et al., *A specific protein carboxyl methylesterase that demethylates phosphoprotein phosphatase 2A in bovine brain.* Proc Natl Acad Sci U S A, 1996. **93**(12): p. 6043-7.

23. Ogris, E., et al., *A protein phosphatase methylesterase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A.* J Biol Chem, 1999. **274**(20): p. 14382-91.

24. Leulliot, N., et al., *Structure of protein phosphatase methyltransferase 1 (PPM1), a leucine carboxyl methyltransferase involved in the regulation of protein phosphatase 2A activity.* J Biol Chem, 2004. **279**(9): p. 8351-8.

25. Sontag, E., et al., *Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation.* J Neurosci, 2007. **27**(11): p. 2751-9.

26. Sents, W., et al., *The biogenesis of active protein phosphatase 2A holoenzymes: a tightly regulated process creating phosphatase specificity.* FEBS J, 2013. **280**(2): p. 644-61.

27. Stefansson, B., et al., *Protein phosphatase 6 regulatory subunits composed of ankyrin repeat domains.* Biochemistry, 2008. **47**(5): p. 1442-51.

28. Stanevich, V., et al., *The structural basis for tight control of PP2A methylation and function by LCMT-1.* Mol Cell, 2011. **41**(3): p. 331-42.

29. Xing, Y., et al., *Structural mechanism of demethylation and inactivation of protein phosphatase 2A.* Cell, 2008. **133**(1): p. 154-63.

30. Turowski, P., et al., *Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression.* J Cell Biol, 1995. **129**(2): p. 397-410.

31. Wu, J., et al., *Carboxyl methylation of the phosphoprotein phosphatase 2A catalytic subunit promotes its functional association with regulatory subunits in vivo.* EMBO J, 2000. **19**(21): p. 5672-81.

32. Gentry, M.S., et al., *A novel assay for protein phosphatase 2A (PP2A) complexes in vivo reveals differential effects of covalent modifications on different Saccharomyces cerevisiae PP2A heterotrimers.* Eukaryot Cell, 2005. **4**(6): p. 1029-40.

33. Longin, S., et al., *Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic Subunit.* J Biol Chem, 2007. **282**(37): p. 26971-80.

34. Yu, X.X., et al., *Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen.* Mol Biol Cell, 2001. **12**(1): p. 185-99.

35. Tolstykh, T., et al., *Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits.* EMBO J, 2000. **19**(21): p. 5682-91.

36. Bryant, J.C., R.S. Westphal, and B.E. Wadzinski, *Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory Balpha subunit.* Biochem J, 1999. **339 (Pt 2)**: p. 241-6.

37. Sontag, E., et al., *Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis.* J Neuropathol Exp Neurol, 2004. **63**(10): p. 1080-91.

38. Janssens, V., J. Goris, and C. Van Hoof, *PP2A: the expected tumor suppressor.* Curr Opin Genet Dev, 2005. **15**(1): p. 34-41.

39. Wang, S.S., et al., *Alterations of the PPP2R1B gene in human lung and colon cancer.* Science, 1998. **282**(5387): p. 284-7.

40. Calin, G.A., et al., *Low frequency of alterations of the alpha (PPP2R1A) and beta (PPP2R1B) isoforms of the subunit A of the serine-threonine phosphatase 2A in human neoplasms.* Oncogene, 2000. **19**(9): p. 1191-5.

41. Ruediger, R., H.T. Pham, and G. Walter, *Alterations in protein phosphatase 2A subunit interaction in human carcinomas of the lung and colon with mutations in the A beta subunit gene.* Oncogene, 2001. **20**(15): p. 1892-9.

42. Takagi, Y., et al., *Alterations of the PPP2R1B gene located at 11q23 in human colorectal cancers.* Gut, 2000. **47**(2): p. 268-71.

43. Francia, G., et al., *Identification by differential display of a protein phosphatase-2A regulatory subunit preferentially expressed in malignant melanoma cells.* Int J Cancer, 1999. **82**(5): p. 709-13.

44. Ito, A., et al., *A truncated isoform of the PP2A B56 subunit promotes cell motility through paxillin phosphorylation.* EMBO J, 2000. **19**(4): p. 562-71.

45. Bialojan, C. and A. Takai, *Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics.* Biochem J, 1988. **256**(1): p. 283-90.

46. Suganuma, M., et al., *Okadaic acid: an additional non-phorbol-12-tetradecanoate-13-acetate-type tumor promoter.* Proc Natl Acad Sci U S A, 1988. **85**(6): p. 1768-71.

47. Suganuma, M., et al., *Calyculin A, an inhibitor of protein phosphatases, a potent tumor promoter on CD-1 mouse skin.* Cancer Res, 1990. **50**(12): p. 3521-5.

48. Li, M., A. Makkinje, and Z. Damuni, *The myeloid leukemia-associated protein SET is a potent inhibitor of protein phosphatase 2A.* J Biol Chem, 1996. **271**(19): p. 11059-62.

49. von Lindern, M., et al., *Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene.* Mol Cell Biol, 1992. **12**(8): p. 3346-55.

50. Yang, S.I., et al., *Control of protein phosphatase 2A by simian virus 40 small-t antigen.* Mol Cell Biol, 1991. **11**(4): p. 1988-95.

51. Chen, W., et al., *Identification of specific PP2A complexes involved in human cell transformation.* Cancer Cell, 2004. **5**(2): p. 127-36.

52. Puustinen, P., et al., *PME-1 protects extracellular signal-regulated kinase pathway activity from protein phosphatase 2A-mediated inactivation in human malignant glioma.* Cancer Res, 2009. **69**(7): p. 2870-7.

53. Wandzioch, E., et al., *PME-1 modulates protein phosphatase 2A activity to promote the malignant phenotype of endometrial cancer cells.* Cancer Res, 2014. **74**(16): p. 4295-305.

54. Bachovchin, D.A., et al., *Academic cross-fertilization by public screening yields a remarkable class of protein phosphatase methylesterase-1 inhibitors.* Proc Natl Acad Sci U S A, 2011. **108**(17): p. 6811-6.

55. Bachovchin, D.A., et al., *Discovery and optimization of sulfonyl acrylonitriles as selective, covalent inhibitors of protein phosphatase methylesterase-1.* J Med Chem, 2011. **54**(14): p. 5229-36.

56. Ruvolo, P.P., et al., *Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A.* J Biol Chem, 1999. **274**(29): p. 20296-300.

57. Ruvolo, P.P., et al., *A functional role for the B56 alpha-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function.* J Biol Chem, 2002. **277**(25): p. 22847-52.

58. Chiang, C.W., et al., *Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin- 3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation.* Blood, 2001. **97**(5): p. 1289-97.

59. Shtrichman, R., et al., *Induction of apoptosis by adenovirus E4orf4 protein is specific to transformed cells and requires an interaction with protein phosphatase 2A.* Proc Natl Acad Sci U S A, 1999. **96**(18): p. 10080-5.

60. Marcellus, R.C., et al., *Induction of p53-independent apoptosis by the adenovirus E4orf4 protein requires binding to the Balpha subunit of protein phosphatase 2A.* J Virol, 2000. **74**(17): p. 7869-77.

61. Sontag, E., et al., *Altered expression levels of the protein phosphatase 2A ABalphaC enzyme are associated with Alzheimer disease pathology.* J Neuropathol Exp Neurol, 2004. **63**(4): p. 287-301.

62. Kloeker, S., et al., *Carboxymethylation of nuclear protein serine/threonine phosphatase X.* Biochem J, 1997. **327 (Pt 2)**: p. 481-6.

63. Brewis, N.D., et al., *PPX, a novel protein serine/threonine phosphatase localized to centrosomes.* EMBO J, 1993. **12**(3): p. 987-96.

64. Hastie, C.J. and P.T. Cohen, *Purification of protein phosphatase 4 catalytic subunit: inhibition by the antitumour drug fostriecin and other tumour suppressors and promoters.* FEBS Lett, 1998. **431**(3): p. 357-61.

65. Helps, N.R., et al., *Protein phosphatase 4 is an essential enzyme required for organisation of microtubules at centrosomes in Drosophila embryos.* J Cell Sci, 1998. **111 (Pt 10)**: p. 1331-40.

66. Sumiyoshi, E., A. Sugimoto, and M. Yamamoto, *Protein phosphatase 4 is required for centrosome maturation in mitosis and sperm meiosis in C. elegans.* J Cell Sci, 2002. **115**(Pt 7): p. 1403-10.

67. Ronne, H., et al., *Protein phosphatase 2A in Saccharomyces cerevisiae: effects on cell growth and bud morphogenesis.* Mol Cell Biol, 1991. **11**(10): p. 4876-84.

68. Hastie, C.J., et al., *The Saccharomyces cerevisiae orthologue of the human protein phosphatase 4 core regulatory subunit R2 confers resistance to the anticancer drug cisplatin.* FEBS J, 2006. **273**(14): p. 3322-34.

69. Kloeker, S. and B.E. Wadzinski, *Purification and identification of a novel subunit of protein serine/threonine phosphatase 4.* J Biol Chem, 1999. **274**(9): p. 5339-47.

70. Hastie, C.J., et al., *A novel 50 kDa protein forms complexes with protein phosphatase 4 and is located at centrosomal microtubule organizing centres.* Biochem J, 2000. **347 Pt 3**: p. 845-55.

71. Gingras, A.C., et al., *A novel, evolutionarily conserved protein phosphatase complex involved in cisplatin sensitivity.* Mol Cell Proteomics, 2005. **4**(11): p. 1725-40.

72. Chen, G.I., et al., *PP4R4/KIAA1622 forms a novel stable cytosolic complex with phosphoprotein phosphatase 4.* J Biol Chem, 2008. **283**(43): p. 29273-84.

73. Glatter, T., et al., *An integrated workflow for charting the human interaction proteome: insights into the PP2A system.* Mol Syst Biol, 2009. **5**: p. 237.

74. Wepf, A., et al., *Quantitative interaction proteomics using mass spectrometry.* Nat Methods, 2009. **6**(3): p. 203-5.

75. Zhang, X., et al., *Histone deacetylase 3 (HDAC3) activity is regulated by interaction with protein serine/threonine phosphatase 4.* Genes Dev, 2005. **19**(7): p. 827-39.

76. Karagianni, P. and J. Wong, *HDAC3: taking the SMRT-N-CoRrect road to repression.* Oncogene, 2007. **26**(37): p. 5439-49.

77. Bartling, B., et al., *Comparative application of antibody and gene array for expression profiling in human squamous cell lung carcinoma.* Lung Cancer, 2005. **49**(2): p. 145-54.

78. Liby, P., et al., *Elevated and deregulated expression of HDAC3 in human astrocytic glial tumours.* Folia Biol (Praha), 2006. **52**(1-2): p. 21-33.

79. Wilson, A.J., et al., *Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon cell maturation and p21 expression and are deregulated in human colon cancer.* J Biol Chem, 2006. **281**(19): p. 13548-58.

80. Carnegie, G.K., et al., *Protein phosphatase 4 interacts with the Survival of Motor Neurons complex and enhances the temporal localisation of snRNPs.* J Cell Sci, 2003. **116**(Pt 10): p. 1905-13.

81. Bosio, Y., et al., *PPP4R2 regulates neuronal cell differentiation and survival, functionally cooperating with SMN.* Eur J Cell Biol, 2012. **91**(8): p. 662-74.

82. Gewurz, B.E., et al., *Genome-wide siRNA screen for mediators of NF-kappaB activation.* Proc Natl Acad Sci U S A, 2012. **109**(7): p. 2467-72.

83. Stefansson, B. and D.L. Brautigan, *Protein phosphatase 6 subunit with conserved Sit4-associated protein domain targets IkappaBepsilon.* J Biol Chem, 2006. **281**(32): p. 22624-34.

84. Zeng, K., et al., *Protein phosphatase 6 regulates mitotic spindle formation by controlling the T-loop phosphorylation state of Aurora A bound to its activator TPX2.* J Cell Biol, 2010. **191**(7): p. 1315-32.

85. Sutton, A., D. Immanuel, and K.T. Arndt, *The SIT4 protein phosphatase functions in late G1 for progression into S phase.* Mol Cell Biol, 1991. **11**(4): p. 2133-48.

86. Luke, M.M., et al., *The SAP, a new family of proteins, associate and function positively with the SIT4 phosphatase.* Mol Cell Biol, 1996. **16**(6): p. 2744-55.

87. Fernandez-Capetillo, O., et al., *H2AX: the histone guardian of the genome.* DNA Repair (Amst), 2004. **3**(8-9): p. 959-67.

88. Shiloh, Y., *ATM and related protein kinases: safeguarding genome integrity.* Nat Rev Cancer, 2003. **3**(3): p. 155-68.

89. Chowdhury, D., et al., *gamma-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair.* Mol Cell, 2005. **20**(5): p. 801-9.

90. Keogh, M.C., et al., *A phosphatase complex that dephosphorylates gammaH2AX regulates DNA damage checkpoint recovery.* Nature, 2006. **439**(7075): p. 497-501.

91. Nakada, S., et al., *PP4 is a gamma H2AX phosphatase required for recovery from the DNA damage checkpoint.* EMBO Rep, 2008. **9**(10): p. 1019-26.

92. Chowdhury, D., et al., *A PP4-phosphatase complex dephosphorylates gamma-H2AX generated during DNA replication.* Mol Cell, 2008. **31**(1): p. 33-46.

93. Lee, D.H., et al., *A PP4 phosphatase complex dephosphorylates RPA2 to facilitate DNA repair via homologous recombination.* Nat Struct Mol Biol, 2010. **17**(3): p. 365-72.

94. Liu, J., et al., *Protein phosphatase PP4 is involved in NHEJ-mediated repair of DNA double-strand breaks.* Cell Cycle, 2012. **11**(14): p. 2643-9.

95. Lee, D.H., et al., *Phosphoproteomic analysis reveals that PP4 dephosphorylates KAP-1 impacting the DNA damage response.* EMBO J, 2012. **31**(10): p. 2403-15.

96. Douglas, P., et al., *Protein phosphatase 6 interacts with the DNA-dependent protein kinase catalytic subunit and dephosphorylates gamma-H2AX.* Mol Cell Biol, 2010. **30**(6): p. 1368-81.

97. Zhong, J., et al., *Protein phosphatase PP6 is required for homology-directed repair of DNA double-strand breaks.* Cell Cycle, 2011. **10**(9): p. 1411-9.

98. Wang, B., et al., *Protein phosphatase PP4 is overexpressed in human breast and lung tumors.* Cell Res, 2008. **18**(9): p. 974-7.

99. Weng, S., et al., *Overexpression of protein phosphatase 4 correlates with poor prognosis in patients with stage II pancreatic ductal adenocarcinoma.* Cancer Epidemiol Biomarkers Prev, 2012. **21**(8): p. 1336-43.

100. Ivanov, S.V., et al., *Pro-tumorigenic effects of miR-31 loss in mesothelioma.* J Biol Chem, 2010. **285**(30): p. 22809-17.

101. Wu, N., et al., *MicroRNA-373, a new regulator of protein phosphatase 6, functions as an oncogene in hepatocellular carcinoma.* FEBS J, 2011. **278**(12): p. 2044-54.

102. Shen, Y., et al., *Serine/threonine protein phosphatase 6 modulates the radiation sensitivity of glioblastoma.* Cell Death Dis, 2011. **2**: p. e241.

103. Hammond, D., et al., *Melanoma-associated mutations in protein phosphatase 6 cause chromosome instability and DNA damage owing to dysregulated Aurora-A.* J Cell Sci, 2013. **126**(Pt 15): p. 3429-40.

104. Hayashi, K., et al., *Abrogation of protein phosphatase 6 promotes skin carcinogenesis induced by DMBA.* Oncogene, 2014. **0**.

105. Gold, H.L., et al., *PP6C hotspot mutations in melanoma display sensitivity to Aurora kinase inhibition.* Mol Cancer Res, 2014. **12**(3): p. 433-9.

106. Hagiwara, K., et al., *Identification of genes upregulated in the inflamed colonic lesions of Crohn's disease.* Biochem Biophys Res Commun, 2001. **283**(1): p. 130-5.

107. Di Como, C.J. and K.T. Arndt, *Nutrients, via the Tor proteins, stimulate the association of Tap42 with type 2A phosphatases.* Genes Dev, 1996. **10**(15): p. 1904-16.

108. Murata, K., J. Wu, and D.L. Brautigan, *B cell receptor-associated protein alpha4 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A.* Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10624-9.

109. Chen, J., R.T. Peterson, and S.L. Schreiber, *Alpha 4 associates with protein phosphatases 2A, 4, and 6.* Biochem Biophys Res Commun, 1998. **247**(3): p. 827-32.

110. Inui, S., et al., *Ig receptor binding protein 1 (alpha4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A.* Blood, 1998. **92**(2): p. 539-46.

111. Nanahoshi, M., et al., *Regulation of protein phosphatase 2A catalytic activity by alpha4 protein and its yeast homolog Tap42.* Biochem Biophys Res Commun, 1998. **251**(2): p. 520-6.

112. Nanahoshi, M., et al., *Alpha4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases.* FEBS Lett, 1999. **446**(1): p. 108-12.

113. Jiang, Y. and J.R. Broach, *Tor proteins and protein phosphatase 2A reciprocally regulate Tap42 in controlling cell growth in yeast.* EMBO J, 1999. **18**(10): p. 2782-92.

114. Chung, H., et al., *Mutation of Tyr307 and Leu309 in the protein phosphatase 2A catalytic subunit favors association with the alpha 4 subunit which promotes dephosphorylation of elongation factor-2.* Biochemistry, 1999. **38**(32): p. 10371-6.

115. Kong, M., et al., *The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis.* Science, 2004. **306**(5696): p. 695-8.

116. Hombauer, H., et al., *Generation of active protein phosphatase 2A is coupled to holoenzyme assembly.* PLoS Biol, 2007. **5**(6): p. e155.

117. Kong, M., et al., *Alpha4 is an essential regulator of PP2A phosphatase activity.* Mol Cell, 2009. **36**(1): p. 51-60.

118. McConnell, J.L., et al., *Alpha4 is a ubiquitin-binding protein that regulates protein serine/threonine phosphatase 2A ubiquitination.* Biochemistry, 2010. **49**(8): p. 1713-8.

119. Quaderi, N.A., et al., *Opitz G/BBB syndrome, a defect of midline development, is due to mutations in a new RING finger gene on Xp22.* Nat Genet, 1997. **17**(3): p. 285-91.

120. Schweiger, S., et al., *The Opitz syndrome gene product, MID1, associates with microtubules.* Proc Natl Acad Sci U S A, 1999. **96**(6): p. 2794-9.

121. LeNoue-Newton, M., et al., *The E3 ubiquitin ligase- and protein phosphatase 2A (PP2A)-binding domains of the Alpha4 protein are both required for Alpha4 to inhibit PP2A degradation.* J Biol Chem, 2011. **286**(20): p. 17665-71.

122. Liu, J., et al., *Phosphorylation and microtubule association of the Opitz syndrome protein mid-1 is regulated by protein phosphatase 2A via binding to the regulatory subunit alpha 4.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6650-5.

123. Trockenbacher, A., et al., *MID1, mutated in Opitz syndrome, encodes an ubiquitin ligase that targets phosphatase 2A for degradation.* Nat Genet, 2001. **29**(3): p. 287-94.

124. Prickett, T.D. and D.L. Brautigan, *Cytokine activation of p38 mitogen-activated protein kinase and apoptosis is opposed by alpha-4 targeting of protein phosphatase 2A for site-specific dephosphorylation of MEK3.* Mol Cell Biol, 2007. **27**(12): p. 4217-27.

125. Chen, L.P., et al., *alpha4 is highly expressed in carcinogen-transformed human cells and primary human cancers.* Oncogene, 2011. **30**(26): p. 2943-53.

126. Sakashita, S., et al., *Overexpression of immunoglobulin (CD79a) binding protein1 (IGBP-1) in small lung adenocarcinomas and its clinicopathological significance.* Pathol Int, 2011. **61**(3): p. 130-7.

127. Moreno, C.S., et al., *WD40 repeat proteins striatin and S/G(2) nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A.* J Biol Chem, 2000. **275**(8): p. 5257-63.

128. Castets, F., et al., *A novel calmodulin-binding protein, belonging to the WD-repeat family, is localized in dendrites of a subset of CNS neurons.* J Cell Biol, 1996. **134**(4): p. 1051-62.

129. Castets, F., et al., *Zinedin, SG2NA, and striatin are calmodulin-binding, WD repeat proteins principally expressed in the brain.* J Biol Chem, 2000. **275**(26): p. 19970-7.

130. Hwang, J. and D.C. Pallas, *STRIPAK complexes: structure, biological function, and involvement in human diseases.* Int J Biochem Cell Biol, 2014. **47**: p. 118-48.

131. Gaillard, S., et al., *Striatin, a calmodulin-dependent scaffolding protein, directly binds caveolin-1.* FEBS Lett, 2001. **508**(1): p. 49-52.

132. Moqrich, A., et al., *Cloning of human striatin cDNA (STRN), gene mapping to 2p22-p21, and preferential expression in brain.* Genomics, 1998. **51**(1): p. 136-9.

133. Salin, P., et al., *Distribution of striatin, a newly identified calmodulin-binding protein in the rat brain: an in situ hybridization and immunocytochemical study.* J Comp Neurol, 1998. **397**(1): p. 41-59.

134. Bartoli, M., et al., *Down-regulation of striatin, a neuronal calmodulin-binding protein, impairs rat locomotor activity.* J Neurobiol, 1999. **40**(2): p. 234-43.

135. Muro, Y., et al., *A cell-cycle nuclear autoantigen containing WD-40 motifs expressed mainly in S and G2 phase cells.* Biochem Biophys Res Commun, 1995. **207**(3): p. 1029-37.

136. Moreno, C.S., W.S. Lane, and D.C. Pallas, *A mammalian homolog of yeast MOB1 is both a member and a putative substrate of striatin family-protein phosphatase 2A complexes.* J Biol Chem, 2001. **276**(26): p. 24253-60.

137. Sanghamitra, M., et al., *WD-40 repeat protein SG2NA has multiple splice variants with tissue restricted and growth responsive properties.* Gene, 2008. **420**(1): p. 48-56.

138. Benoist, M., S. Gaillard, and F. Castets, *The striatin family: a new signaling platform in dendritic spines.* J Physiol Paris, 2006. **99**(2-3): p. 146-53.

139. Tan, B., et al., *Striatin-3 gamma inhibits estrogen receptor activity by recruiting a protein phosphatase.* J Mol Endocrinol, 2008. **40**(5): p. 199-210.

140. Gaillard, S., et al., *Targeting of proteins of the striatin family to dendritic spines: role of the coiled-coil domain.* Traffic, 2006. **7**(1): p. 74-84.

141. Benoist, M., et al., *Distribution of zinedin in the rat brain.* J Neurochem, 2008. **106**(2): p. 969-77.

142. Li, S., J. Couet, and M.P. Lisanti, *Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding*

negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem, 1996. **271**(46): p. 29182-90.

143. Couet, J., et al., *Identification of peptide and protein ligands for the caveolin-scaffolding domain. Implications for the interaction of caveolin with caveolae-associated proteins.* J Biol Chem, 1997. **272**(10): p. 6525-33.

144. Chambliss, K.L., et al., *Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae.* Circ Res, 2000. **87**(11): p. E44-52.

145. Lu, Q., et al., *Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha.* Proc Natl Acad Sci U S A, 2004. **101**(49): p. 17126-31.

146. Bartoli, M., A. Monneron, and D. Ladant, *Interaction of calmodulin with striatin, a WD-repeat protein present in neuronal dendritic spines.* J Biol Chem, 1998. **273**(35): p. 22248-53.

147. Li, D. and R. Roberts, *WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases.* Cell Mol Life Sci, 2001. **58**(14): p. 2085-97.

148. Smith, T.F., *Diversity of WD-repeat proteins.* Subcell Biochem, 2008. **48**: p. 20-30.

149. Breitman, M., et al., *The armadillo repeat domain of the APC tumor suppressor protein interacts with Striatin family members.* Biochim Biophys Acta, 2008. **1783**(10): p. 1792-802.

150. Goudreault, M., et al., *A PP2A phosphatase high density interaction network identifies a novel striatin-interacting phosphatase and kinase complex linked to the cerebral cavernous malformation 3 (CCM3) protein.* Mol Cell Proteomics, 2009. **8**(1): p. 157-71.

151. Valpuesta, J.M., et al., *Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT.* FEBS Lett, 2002. **529**(1): p. 11-6.

152. Kean, M.J., et al., *Structure-function analysis of core STRIPAK Proteins: a signaling complex implicated in Golgi polarization.* J Biol Chem, 2011. **286**(28): p. 25065-75.

153. Hyodo, T., et al., *Misshapen-like kinase 1 (MINK1) is a novel component of striatin-interacting phosphatase and kinase (STRIPAK) and is required for the completion of cytokinesis.* J Biol Chem, 2012. **287**(30): p. 25019-29.

154. Pombo, C.M., et al., *Activation of a human Ste20-like kinase by oxidant stress defines a novel stress response pathway.* EMBO J, 1996. **15**(17): p. 4537-46.

155. Ling, P., et al., *Biosignaling of mammalian Ste20-related kinases.* Cell Signal, 2008. **20**(7): p. 1237-47.

156. Wurzenberger, C. and D.W. Gerlich, *Phosphatases: providing safe passage through mitotic exit.* Nat Rev Mol Cell Biol, 2011. **12**(8): p. 469-82.

157. Frost, A., et al., *Functional repurposing revealed by comparing S. pombe and S. cerevisiae genetic interactions.* Cell, 2012. **149**(6): p. 1339-52.

158. Aouadi, M., et al., *Orally delivered siRNA targeting macrophage Map4k4 suppresses systemic inflammation.* Nature, 2009. **458**(7242): p. 1180-4.

159. Collins, C.S., et al., *A small interfering RNA screen for modulators of tumor cell motility identifies MAP4K4 as a promigratory kinase.* Proc Natl Acad Sci U S A, 2006. **103**(10): p. 3775-80.

160. Gloerich, M., et al., *Rap2A links intestinal cell polarity to brush border formation.* Nat Cell Biol, 2012. **14**(8): p. 793-801.

161. Baillat, G., et al., *Molecular cloning and characterization of phocein, a protein found from the Golgi complex to dendritic spines.* Mol Biol Cell, 2001. **12**(3): p. 663-73.

162. Varsano, T., et al., *GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling.* Mol Cell Biol, 2006. **26**(23): p. 8942-52.

163. van Bergen En Henegouwen, P.M., *Eps15: a multifunctional adaptor protein regulating intracellular trafficking.* Cell Commun Signal, 2009. **7**: p. 24.

164. Salcini, A.E., et al., *The Eps15 C. elegans homologue EHS-1 is implicated in synaptic vesicle recycling.* Nat Cell Biol, 2001. **3**(8): p. 755-60.

165. Baillat, G., et al., *Interactions of phocein with nucleoside-diphosphate kinase, Eps15, and Dynamin I.* J Biol Chem, 2002. **277**(21): p. 18961-6.

166. Krishnan, K.S., et al., *Nucleoside diphosphate kinase, a source of GTP, is required for dynamin-dependent synaptic vesicle recycling.* Neuron, 2001. **30**(1): p. 197-210.

167. Schulte, J., et al., *DMob4/Phocein regulates synapse formation, axonal transport, and microtubule organization.* J Neurosci, 2010. **30**(15): p. 5189-203.

168. Sepp, K.J., et al., *Identification of neural outgrowth genes using genome-wide RNAi.* PLoS Genet, 2008. **4**(7): p. e1000111.

169. Bloemendal, S., et al., *A mutant defective in sexual development produces aseptate ascogonia.* Eukaryot Cell, 2010. **9**(12): p. 1856-66.

170. Wang, C.L., W.B. Shim, and B.D. Shaw, *Aspergillus nidulans striatin (StrA) mediates sexual development and localizes to the endoplasmic reticulum.* Fungal Genet Biol, 2010. **47**(10): p. 789-99.

171. Lisa-Santamaria, P., A. Jimenez, and J.L. Revuelta, *The protein factor-arrest 11 (Far11) is essential for the toxicity of human caspase-10 in yeast and participates in the regulation of autophagy and the DNA damage signaling.* J Biol Chem, 2012. **287**(35): p. 29636-47.

172. Simonin, A.R., et al., *Genes encoding a striatin-like protein (ham-3) and a forkhead associated protein (ham-4) are required for hyphal fusion in Neurospora crassa.* Fungal Genet Biol, 2010. **47**(10): p. 855-68.

173. Bonangelino, C.J., E.M. Chavez, and J.S. Bonifacino, *Genomic screen for vacuolar protein sorting genes in Saccharomyces cerevisiae.* Mol Biol Cell, 2002. **13**(7): p. 2486-501.

174. Bergametti, F., et al., *Mutations within the programmed cell death 10 gene cause cerebral cavernous malformations.* Am J Hum Genet, 2005. **76**(1): p. 42-51.

175. Chan, A.C., et al., *Recent insights into cerebral cavernous malformations: animal models of CCM and the human phenotype.* FEBS J, 2010. **277**(5): p. 1076-83.

176. Faurobert, E. and C. Albiges-Rizo, *Recent insights into cerebral cavernous malformations: a complex jigsaw puzzle under construction.* FEBS J, 2010. **277**(5): p. 1084-96.

177. Labauge, P., et al., *Genetics of cavernous angiomas.* Lancet Neurol, 2007. **6**(3): p. 237-44.

178. Riant, F., et al., *Recent insights into cerebral cavernous malformations: the molecular genetics of CCM.* FEBS J, 2010. **277**(5): p. 1070-5.

179. Del Curling, O., Jr., et al., *An analysis of the natural history of cavernous angiomas.* J Neurosurg, 1991. **75**(5): p. 702-8.

180. Robinson, J.R., I.A. Awad, and J.R. Little, *Natural history of the cavernous angioma.* J Neurosurg, 1991. **75**(5): p. 709-14.

181. Laberge-le Couteulx, S., et al., *Truncating mutations in CCM1, encoding KRIT1, cause hereditary cavernous angiomas.* Nat Genet, 1999. **23**(2): p. 189-93.

182. Sahoo, T., et al., *Mutations in the gene encoding KRIT1, a Krev-1/rap1a binding protein, cause cerebral cavernous malformations (CCM1).* Hum Mol Genet, 1999. **8**(12): p. 2325-33.

183. Denier, C., et al., *Mutations within the MGC4607 gene cause cerebral cavernous malformations.* Am J Hum Genet, 2004. **74**(2): p. 326-37.

184. Liquori, C.L., et al., *Mutations in a gene encoding a novel protein containing a phosphotyrosine-binding domain cause type 2 cerebral cavernous malformations.* Am J Hum Genet, 2003. **73**(6): p. 1459-64.

185. Chen, L., et al., *Apoptotic functions of PDCD10/CCM3, the gene mutated in cerebral cavernous malformation 3.* Stroke, 2009. **40**(4): p. 1474-81.

186. Chan, A.C., et al., *Mutations in 2 distinct genetic pathways result in cerebral cavernous malformations in mice.* J Clin Invest, 2011. **121**(5): p. 1871-81.

187. Zhu, Y., et al., *Differential angiogenesis function of CCM2 and CCM3 in cerebral cavernous malformations.* Neurosurg Focus, 2010. **29**(3): p. E1.

188. Yoruk, B., et al., *Ccm3 functions in a manner distinct from Ccm1 and Ccm2 in a zebrafish model of CCM vascular disease.* Dev Biol, 2012. **362**(2): p. 121-31.

189. He, Y., et al., *Stabilization of VEGFR2 signaling by cerebral cavernous malformation 3 is critical for vascular development.* Sci Signal, 2010. **3**(116): p. ra26.

190. Ceccarelli, D.F., et al., *CCM3/PDCD10 heterodimerizes with germinal center kinase III (GCKIII) proteins using a mechanism analogous to CCM3 homodimerization.* J Biol Chem, 2011. **286**(28): p. 25056-64.

191. Hilder, T.L., et al., *Proteomic identification of the cerebral cavernous malformation signaling complex.* J Proteome Res, 2007. **6**(11): p. 4343-55.

192. Li, X., et al., *Crystal structure of CCM3, a cerebral cavernous malformation protein critical for vascular integrity.* J Biol Chem, 2010. **285**(31): p. 24099-107.

193. Stahl, S., et al., *Novel CCM1, CCM2, and CCM3 mutations in patients with cerebral cavernous malformations: in-frame deletion in CCM2 prevents formation of a CCM1/CCM2/CCM3 protein complex.* Hum Mutat, 2008. **29**(5): p. 709-17.

194. Voss, K., et al., *CCM3 interacts with CCM2 indicating common pathogenesis for cerebral cavernous malformations.* Neurogenetics, 2007. **8**(4): p. 249-56.

195. Costa, B., et al., *STK25 protein mediates TrkA and CCM2 protein-dependent death in pediatric tumor cells of neural origin.* J Biol Chem, 2012. **287**(35): p. 29285-9.

196. Fidalgo, M., et al., *CCM3/PDCD10 stabilizes GCKIII proteins to promote Golgi assembly and cell orientation.* J Cell Sci, 2010. **123**(Pt 8): p. 1274-84.

197. Voss, K., et al., *Functional analyses of human and zebrafish 18-amino acid in-frame deletion pave the way for domain mapping of the cerebral cavernous malformation 3 protein.* Hum Mutat, 2009. **30**(6): p. 1003-11.

198. Zheng, X., et al., *CCM3 signaling through sterile 20-like kinases plays an essential role during zebrafish cardiovascular development and cerebral cavernous malformations.* J Clin Invest, 2010. **120**(8): p. 2795-804.

199. Jackson, J.B. and D.C. Pallas, *Circumventing cellular control of PP2A by methylation promotes transformation in an Akt-dependent manner.* Neoplasia, 2012. **14**(7): p. 585-99.

200. Cohen, P.T., A. Philp, and C. Vazquez-Martin, *Protein phosphatase 4--from obscurity to vital functions.* FEBS Lett, 2005. **579**(15): p. 3278-86.

201. Stefansson, B. and D.L. Brautigan, *Protein phosphatase PP6 N terminal domain restricts G1 to S phase progression in human cancer cells.* Cell Cycle, 2007. **6**(11): p. 1386-92.

202. Cho, U.S. and W. Xu, *Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme.* Nature, 2007. **445**(7123): p. 53-7.

203. Lee, J.A. and D.C. Pallas, *Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells.* J Biol Chem, 2007. **282**(42): p. 30974-84.

204. Gordon, J., et al., *Protein phosphatase 2a (PP2A) binds within the oligomerization domain of striatin and regulates the phosphorylation and activation of the mammalian Ste20-Like kinase Mst3.* BMC Biochem, 2011. **12**: p. 54.