Leucine Carboxyl Methyltransferase 1 (LCMT-1) Methylates Protein Phosphatase 4 (PP4) and Protein Phosphatase 6 (PP6) and Differentially Regulates the Stable Formation of Different PP4 Holoenzymes

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Leucine Carboxyl Methyltransferase 1 (LCMT-1) Methylates Protein Phosphatase 4 (PP4) and Protein Phosphatase 6 (PP6) and Differentially Regulates the Stable Formation of Different PP4 Holoenzymes*

The protein phosphatase 2A (PP2A) subfamily of phosphatases, PP2A, PP4, and PP6, are multifunctional serine/threonine protein phosphatases involved in many cellular processes. Carboxyl methylation of the PP2A catalytic subunit (PP2Ac) C-terminal leucine is regulated by the opposing activities of leucine carboxyl methyltransferase 1 (LCMT-1) and protein phosphatase methylesterase 1 (PME-1) and regulates PP2A holoenzyme formation. The site of methylation on PP2Ac is conserved in the catalytic subunits of PP4 and PP6, and PP4 is also methylated on that site, but the identities of the methyltransferase enzyme for PP4 are not known. Whether PP6 is methylated is also not known. Here we use antibodies specific for the unmethylated phosphatases to show that PP6 is carboxyl-methylated and that LCMT-1 is the major methyltransferase for PP2A, PP4, and PP6 in mouse embryonic fibroblasts (MEFs). Analysis of PP2A and PP4 complexes by blue native polyacrylamide gel electrophoresis (BN-PAGE) indicates that PP4 holoenzyme complexes, like those of PP2A, are differentially regulated by LCMT-1, with the PP4 regulatory subunit 1 (PP4R1)-containing PP4 complex being the most dramatically affected by the LCMT-1 loss. MEFs derived from LCMT-1 knock-out mouse embryos have reduced levels of PP2A B regulatory subunit and PP4R1 relative to control MEFs, indicating that LCMT-1 is important for maintaining normal levels of these subunits. Finally, LCMT-1 homozygous knock-out MEFs exhibited hyperphosphorylation of HDAC3, a reported target of the methylation-dependent PP4R1-PP4 complex. Collectively, our data suggest that LCMT-1 coordinately regulates the carboxyl methylation of PP2A-related phosphatases and, consequently, their holoenzyme assembly and function.

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 (1). PP2A primarily exists as heterotrimERIC complexes made up of a structural A subunit, 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 four groups: B (PPP2R2B), B’ (PPP2R5), B” (PPP2R3), and B’’ (striatin family). Combination of the PP2A A/C core dimer with one of the 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 (for examples, see Refs. 2–9). PP4 and PP6 have functions that are distinct from PP2A but also have some common or overlapping functions, such as dephosphorylation of γ-H2AX, a marker of DNA double-stranded breaks, formed during DNA replication or by DNA damage (10–15). Thus, cellular mechanisms that might coordinately regulate these phosphatases are of interest.

PP2Ac is methylated on the α-carboxyl group of its C-terminal leucine, leucine 309 (Leu-309). A major function of PP2Ac carboxyl methylation is to regulate the formation of certain PP2A heterotrimERIC forms, herein termed “methylation-dependent PP2A heterotrimers” (16–22). For example, PP2Ac methylation is required for efficient formation of heterotrimers containing the B family B-type subunits, Bo (16, 19, 23), Bβ (24), and Bδ, but is not necessary for the formation of heterotrimers containing the B’ or B’’ subunits (19, 24).

Organization and regulation of PP4 and PP6 holoenzymes is still being elucidated. PP4 and PP6 form hetero-oligomERIC complexes that consist of a catalytic subunit and one or more

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3 The abbreviations used are: PP2A, protein phosphatase 2A; PP2Ac, protein phosphatase 2A catalytic subunit; PP4, protein phosphatase 4; PP4c, protein phosphatase 4 catalytic subunit; PP4R, protein phosphatase 4 regulatory subunit; PP6, protein phosphatase 6; PP6c, protein phosphatase 6 catalytic subunit; BN-PAGE, blue native PAGE; HDAC3, histone deacetylase 3; LCMT-1, leucine carboxyl methyltransferase-1; MEFS, mouse embryonic fibroblasts; PME-1, protein phosphatase methylesterase-1; PP2A BAC, holoenzyme trimer of PP2A containing B subunit (PPP2R2B); A subunit, and C subunit; HDAC, histone deacetylase; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

regulatory/targeting subunits. Five different PP4 regulatory subunits have been reported thus far: PP4R1, PP4R2, PP4R3α and β, and PP4R4 (25–28). 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 SAP5 (Sit4-associated proteins) regulatory subunit and an ARS (ankyrin repeat subunit) regulatory subunit (5, 6). As for PP2A, enzyme activity and substrate specificity are specified by the regulatory subunits that associate with PP4c and PP6c.

Carboxyl methylation on PP2Ac Leu-309 is tightly regulated by leucine carboxyl methyltransferase 1 (LCMT-1) (20, 21, 22, 23). Because the three C-terminal residues (YFL) of PP2Ac are identical in PP4 and PP6 (35), we hypothesized that this subfamily of protein phosphatases might be coordinately regulated by methylation of their C termini. Indeed, the catalytic subunit of PP4 (PP4c) has been shown to be methylated on its C-terminal leucine, Leu-307 (36), and PP4 also specifically binds PME-1 (37). However, methylation of the catalytic subunit of PP6 (PP6c) has not been reported, and PP6 was reported to not associate with PME-1 (37). In addition, the identity of the PP4 methyltransferase is not known. Although the effect of PP2Ac methylation on PP2A heterotrimeric assembly is understood, how PP4 and PP6 holoenzymes are regulated has not been studied in detail. Therefore, a potential role for methylation of these PP2A-related phosphatases in regulating their holoenzyme assembly is of great interest.

Despite high sequence identity (~60%) between PP2Ac, PP4c, and PP6c, most regulatory subunits for these phosphatases are specific for their respective catalytic subunit. One exception is a protein called α4 that binds to PP2Ac, PP4c, and PP6c mutually exclusively with other regulatory or structural subunits, alters their activity, and is important for their stability and function (Refs. 7 and 38–43 and references therein). Interestingly, α4 has been reported to bind both methylated and unmethylated PP2Ac, although its binding to PP2Ac is influenced by modifications that alter PP2Ac methylation (44, 45). Thus, α4 binds the catalytic subunits of the PP2A subfamily of phosphatases and regulates their function.

In this study we tested and confirmed the hypotheses that PP6 is methylated on its C terminus and that LCMT-1 is the major methyltransferase for the PP2A subfamily of protein phosphatases. By investigating the importance of LCMT-1 for PP2A subfamily phosphatase complex formation using blue native polyacrylamide gel electrophoresis (BN-PAGE) and immunoblotting, we showed that this approach can be used for analysis of PP2A subfamily phosphatase complexes. Then we used this method to analyze α4, PP4, and PP6 complexes, revealing methylation-dependent effects on an α4 complex and differential effects of LCMT-1 loss on different PP4 complexes. Moreover, we used coimmunoprecipitation and BN-PAGE electrophoretic mobility shift analysis (BN-PAGE EMSA) of PP4 complexes on BN-PAGE to identify components of PP4 complexes affected by LCMT-1. Together, our results suggest that LCMT-1 coordinately regulates the methylation of the entire PP2A subfamily of phosphatases and possibly their function.

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. For the same reason we also hypothesized that LCMT-1 is the methyltransferase for these PP2A family phosphatases. To test these hypotheses, we first developed Western blot-based assays for PP4c and PP6c methylation analogous to the assay currently used routinely for determining the methylation state of PP2Ac (19). Based on our previous experience with making C-terminal antibodies to PP2Ac (19), we reasoned that antibodies generated against 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 a commercial source 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 specificity for unmethylated PP4c and PP6c peptides with almost no reactivity to the corresponding methylated peptides (Fig. 1, A and B), 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 PP6c.

To use these antibodies for quantitation of PP4c and PP6c methylation on Western blots, it is critical that they specifically only recognize the corresponding phosphatase or that the phosphatases can be clearly separated. Although the PP6 antibody specifically recognized PP6, we found that the C-terminal PP4c antibody demonstrated some cross-reactivity to PP2Ac (Fig. 1C). Thus, we pre-adsorbed this antibody with an unmethylated PP2Ac C-terminal peptide, resulting in an antibody highly specific for unmethylated PP4c (Fig. 1C).

We next used these antibodies to analyze PP4c and PP6c methylation in cells to determine the importance of LCMT-1 for their methylation. Aliquots of cell lysates from LCMT-1 wild type (WT) and knock-out (KO) mouse embryonic fibroblasts (MEFs) were either treated with base, which removes all carboxyl methylation, and then neutralized or treated with a pre-neutralized base solution to preserve the level of methylation that exists in the cells. These treated lysates were then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting was performed using the methylation-sensitive antibodies tested in Fig. 1, A and B. The results showed that PP4c is highly methylated (75% ± 13) in WT MEFs but almost unmethylated (7% ± 4) in LCMT-1 KO MEFs (Fig. 1, D and F), showing that LCMT-1 is the major PP4c methyltransferase in these cells. Similarly, PP6c was also highly methylated (77% ± 11) in WT MEFs, whereas its methylation level decreased to 13% ± 2 in LCMT-1 KO MEFs (Fig. 1, E and F), consistent with the hypothesis that PP6c is also highly methylated in MEFs by LCMT-1. Therefore, like PP2Ac and PP4c, PP6c is indeed methylated, and LCMT-1 is the major methyltransferase in these cells for all three PP2A subfamily phosphatases, PP2Ac, PP4c, and PP6c.
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A. Peptide

Me-PP4c

Demeth-PP4c

B. Peptide

Me-PP6c

Demeth-PP6c

C. Preadsorbed on PP2Ac peptides

Endogenous PP2Ac

Endogenous PP4c

D. Base

WT

KO

Demethylated PP4c

α-Tub

E. Base

WT

KO

Demethylated PP6c

α-Tub

F. % Methylation

WT

KO

PP4c

PP6c

FIGURE 1. PP4c and PP6c are highly methylated by LCMT-1 in MEFs. A and B, testing methylation-sensitive PP4c and PP6c antibodies. PP4c and PP6c peptides corresponding to their 16 C-terminal amino acid residues including a methylated C-terminal leucine were synthesized, and equal amounts of methylated and demethylated PP4c (A) and PP6c (B) peptides on nitrocellulose membranes were probed by Western blotting with methylation-sensitive PP4c (A) and PP6c (B) antibodies. C, preadsorption of methylation-sensitive PP4c antibody with PP2Ac peptide. PP4c methylation-sensitive antibody (anti-PP4c) was pre-adsorbed with PP2Ac C-terminal peptide for 1 h rocking at 4 °C or not preadsorbed and then used for immunoblotting of stripped blots along with PP2Ac antibody (control). Lane 1, immunoblotting of lysate with PP2Ac antibody. Lane 2, immunoblotting of identical lysate with non-preadsorbed PP4c antibody. Lane 3, immunoblotting of the same lysate with preadsorbed PP4c antibody. Lane 4, immunoblotting of lysate with PP2Ac antibody preadsorbed with PP2Ac C-terminal peptide. D and E, examples of methylation assays for PP4c and PP6c on MEFs performed as described under “Experimental Procedures.” Equal amounts of cell lysates from LCMT-1 WT and KO MEFs were treated with (+) or without (−) base, and then proteins were resolved by SDS-PAGE. Western blotting was performed using methylation-sensitive PP4c (D) or methylation-sensitive PP6c (E) antibodies. α-Tubulin (α-Tub) was immunoblotted for a loading control. The low ratio of signal in the minus (−) lane compared with the plus (+) lane in WT cells in both panel D and panel E indicates that both PP4c and PP6c are highly methylated. The nearly equal levels of signal in the minus and plus lanes in KO cells indicates that LCMT-1 is the major methyltransferase for both PP4c and PP6c. In addition, comparison of PP6c levels in the plus lanes shows that the level of PP6c is increased in KO cells relative to WT cells. Results from four independent experiments using methylation-independent PP6c antibodies to measure PP6c protein levels indicate that PP6c is increased 31 ± 20% in KO MEFs compared with WT MEFs (p = 0.02). Thus, LCMT-1 may regulate the steady-state level of PP6c.

Loss of LCMT-1 Dramatically Reduces the Steady-state Level of PP2A BAC Holoenzyme Complexes in MEFs—Previously, it was shown that association of PP2Ac with the methylation-dependent B subunits is decreased by LCMT-1 knockdown in HeLa cells (23). To examine whether this association is also compromised in MEFs isolated from LCMT-1 KO embryos and to quantitate the effect, B subunit/C subunit association was assayed by immunoprecipitation using cell lysates from LCMT-1 WT, hemizygous, and KO MEFs (Fig. 2, A and B). Immunoprecipitation and Western blotting for the B subunit was performed with the anti-B subunit monoclonal antibody, 2G9, which detects Bα and to a lesser extent the other members of the B family of B-type subunits. Consistent with the previous finding in HeLa cells, association of PP2Ac with the B subunit was decreased by ~50% in LCMT-1 homozygous KO MEFs but not in LCMT-1 hemizygous MEFs as compared with WT MEFs (p = 0.02). Thus, LCMT-1 may regulate the steady-state level of PP6c.

In addition, comparison of PP6c levels in the plus lanes of Fig. 1E reveals an increase in the level of PP6c in KO cells relative to WT cells. Results from four independent experiments using methylation-independent PP6c antibodies to measure PP6c protein levels indicate that PP6c is increased 31 ± 20% in KO MEFs compared with WT MEFs (p = 0.02). Thus, LCMT-1 may regulate the steady-state level of PP6c.

PP6c is increased in KO cells relative to WT cells. Of note, the C subunits of all PP2A subfamily phosphatases migrate sometimes as singlets and sometimes as doublets (e.g. for PP6 in panel B) (Refs. 57–59 and unpublished data); whether double or single bands are seen can vary for the same sample from gel to gel. F, quantification of the percent methylation of PP4c and PP6c in WT and KO cells. Error bars represent the S.D. of at least three independent experiments. **, p ≤ 0.01 when compared with WT using Student’s t test.
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WT level; \( p = 0.008 \) and the reduced binding of the remaining B subunit (\( \sim 50\% \); Fig. 2C), we calculate that B subunit heterotrimers are decreased \( \sim 75\% \) in LCMT-1 knock-out MEFs relative to WT MEFs.

To study the effects of LCMT-1 loss on the PP2A subfamily of phosphatases, we employed BN-PAGE, which preserves the integrity of complexes while separating them by apparent size (46). We first used this method to examine changes in PP2A complexes upon LCMT-1 knock-out. Non-denatured cell lysates from LCMT-1 WT and KO MEFs were resolved by BN-PAGE and then Western-blotted with antibodies specific for PP2A B subunit (Fig. 3A), PP2Ac (Fig. 3B), or PP2A A subunit (Fig. 3C) to evaluate the PP2A complexes. PP2A B and C subunits formed a number of complexes with a wide range of molecular sizes. Strikingly, a substantial amount of PP2A B subunit in both WT and KO MEFs is present in a novel large complex (or complexes) of a little more than \( 720\)-kDa apparent molecular weight (open arrow; Fig. 3A) that apparently lacks PP2Ac (compare position in Fig. 3B). The remainder of the B subunit in WT MEFs is present in smaller bands of \( \sim 180\) kDa (asterisk; Fig. 3A), 110 kDa (upper filled arrow; Fig. 3A), and \( \sim 55\) kDa (lower filled arrow; Fig. 3A), likely representing PP2A BAC heterotrimers, BA heterodimers, and B subunit monomer, respectively. Importantly, the 180-kDa PP2A BAC heterotrimetric complex (asterisks; Fig. 3, A–C) is almost absent in LCMT-1 KO MEFs, consistent with its known dependence on PP2Ac methylation for efficient assembly (Fig. 2 and Refs. 16 and 19). Finally, the majority of PP2Ac was present in a complex of \( \sim 150\) kDa that lacks B subunit (filled arrowhead; Fig. 3B) but co-migrates with A subunit (filled arrowhead; Fig. 3C). This complex was not affected by the loss of LCMT-1, suggesting that it is composed of methylation-independent PP2A heterotrimers.

**LCMT-1 Knock-out Increases an Apparent Complex of \( \alpha4 \) with PP2Ac**—Although loss of methylation due to LCMT-1 KO clearly reduced the 180-kDa PP2A BAC complex, it generated a novel band containing PP2Ac of very high molecular weight (open arrowhead; Fig. 3B) that appears to lack B subunit (compare the same position on Fig. 3A). We hypothesized that this minor but very large PP2Ac-containing complex that increases in LCMT-1 KO MEFs might contain \( \alpha4 \) because \( \alpha4 \) interacts with PP2Ac in the absence of the A and B subunits. Indeed, immunoblotting for \( \alpha4 \) on similar BN-PAGE gels of MEF lysates shows that \( \alpha4 \) is present in a few bands, including one that comigrates with the PP2Ac band that increased upon LCMT-1 loss (compare PP2Ac band in Fig. 3B marked by the open arrowhead with the \( \alpha4 \) band in Fig. 4A, marked in the same way). Moreover, the intensity of the \( \alpha4 \) band in Fig. 4A that comigrates with PP2Ac was also enhanced in LCMT-1 KO MEFs as compared with WT MEFs. Together, these results suggest that loss of PP2A BAC heterotrimers leads to increased \( \alpha4 \) association with PP2Ac.

Methylation-dependent PP2A BAC Heterotrimers Can Also Be Disrupted by Loss of the PP2Ac C-terminal Leucine (19). Thus, loss of PP2Ac Leu-309 should disrupt the 180-kDa PP2A complex on BN-PAGE and increase the amount of \( \alpha4 \)-PP2Ac complex. To test this, HA-tagged WT PP2Ac and \( \Delta \)Leu-309 mutant PP2Ac were analyzed by BN-PAGE (Fig. 4B).
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Figure 5. Loss of LCMT-1 differentially affects different PP4 protein phosphatase complexes. A and B, the importance of LCMT-1 for stable PP4 and PP6 complex formation was investigated by BN-PAGE analysis of non-denatured lysates from two independent sets (M1 and M2) of WT and LCMT-1 KO MEFs followed by immunoblotting with antibodies against PP4c (A) and PP6c (B). A: asterisk, methylation-dependent PP4 complex; filled arrowhead, PP4 complex less affected by LCMT-1 loss; bracket, PP4c-containing bands increased in KO. B: filled arrowhead, the major PP6 complex. Data in each panel are representative of at least three experiments. WB, Western blot.

HA-tagged PP2Ac WT formed both methylation-dependent and methylation-independent heterotrimers similar to endogenous PP2Ac (compare WT bands indicated by asterisk and filled arrowhead in Fig. 4B with those in Fig. 3B), the PP2Ac ΔLeu-309 mutant formed only methylation-independent heterotrimers (filled arrowhead; Fig. 4B). Interestingly, the PP2Ac ΔLeu-309 mutant had a great increase in free C subunit (arrow; Fig. 4B) compared with WT PP2Ac, consistent with a previous report that a small population of a similar methylation-deficient PP2Ac L309A mutant exists as free C subunits (47). Moreover, the high molecular weight PP2Ac complex co-migrating with α4 was enhanced in the PP2Ac ΔLeu-309 mutant as compared with WT (open arrowhead; Fig. 4B), supporting the hypothesis that loss of PP2A BAC heterotrimers due to loss of PP2Ac methylation or loss of PP2A Leu-309 leads to increased association between PP2Ac and α4.

LCMT-1-mediated PP4 Methylation Differentially 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 (Fig. 3 and Refs. 19, 24, and 48). To determine whether LCMT-1-mediated methylation regulates the steady-state levels of PP4 and PP6 complexes, PP4c and PP6c antibodies were used to detect PP4 and PP6 complexes in matched WT and LCMT-1 KO knock-out MEFs using BN-PAGE followed by immunoblotting. As shown in Fig. 5A, PP4c formed a large complex at ~450 kDa (filled arrowhead) that was found in similar amounts in WT and LCMT-1 KO MEFs, suggesting that this complex is mainly methylation-independent (hereafter referred to as “450-kDa PP4 complex”). Of note, this PP4 complex always migrates slightly slower on BN-PAGE in LCMT-1 KO MEF lysates, suggesting that some alteration may have been induced by loss of LCMT-1. PP4c formed a second large complex(es) migrating as a broad band between ~250 kDa and 350 kDa (asterisk, Fig. 5A; hereafter referred to as “300-kDa PP4 complex”), and much of this complex was lost in LCMT-1 KO MEFs, indicating that it is methylation-dependent. Corresponding with the substantial loss of the 300-kDa PP4 complex, new, smaller PP4c bands appeared in LCMT-1 KO MEF lysates (bracket; Fig. 5A), suggesting that there is a redistribution of the PP4c population caused 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, whereas the largest band in the bracket may be a PP4c complex that lost one or more interacting proteins. Therefore, similar to the case for PP2A, PP4 methylation appears to differentially affect the stable assembly of different PP4 holoenzymes. 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 migrated as one major band of ~250 kDa. This PP6 band did not change upon LCMT-1 KO (filled arrowhead; Fig. 5B), suggesting that the major complex(es) of PP6 in MEFs is methylation-independent.

The 450-kDa PP4 Holoenzyme Complex Is Composed of PP4c, PP4R2, and Either PP4R3α or PP4R3β—Whereas the 300-kDa PP4 Complex Contains PP4R1—To determine the components of the 450-kDa and 300-kDa PP4 holoenzyme complexes identified in Fig. 5, cell lysates of WT and LCMT-1 KO MEFs were resolved by BN-PAGE and analyzed using antibodies against PP4 regulatory subunits. PP4R1 signal co-migrated with the 300-kDa PP4 complex that was greatly reduced in LCMT-1 KO (asterisk; Fig. 6), whereas PP4R2 co-migrated with the 450-kDa PP4 complex that was shifted slightly in LCMT-1 KO MEF lysates (filled arrowhead; Fig. 6). These results suggest that PP4R1 is a component of the methylation-dependent complex, whereas PP4R2 is a component of the largely methylation-independent 450-kDa PP4 complex. BN-PAGE analysis also showed that the majority of PP4R3α co-migrates with the 450-kDa PP4 complex (filled arrowhead; Fig. 6), whereas a minor population of PP4R3α co-migrated with the lower part of the broad 300-kDa PP4 band (asterisk; Fig. 6). Lastly, PP4R3β was found in the upper band of the PP4 complex (filled arrowhead; Fig. 6). Unfortunately, PP4R4 could not be studied by this approach due to a lack of commercially available antibody with sufficient specificity. Thus, based on co-migration, these experiments suggest that the 450-kDa PP4 band is minimally composed of PP4c, PP4R2, and either PP4R3α or PP4R3β, whereas the 300-kDa PP4 complex is minimally composed of PP4c and PP4R1 and/or PP4R3α.

Figure 6. Immunoblotting analysis to investigate the PP4 regulatory subunit composition of the 300- and 450-kDa PP4 complexes. Nondenatured WT MEF cell lysates were analyzed by BN-PAGE and immunoblotting using antibodies against PP4c and PP4 regulatory subunits. WB Ab, immunoblotting antibody for that lane. Filled arrowhead, 450-kDa PP4 complex; asterisk, 300-kDa PP4 complex. PP4R2, PP4R3α, and PP4R3β co-migrated with the 450-kDa PP4 band, whereas PP4R1 and a small amount of PP4R3α co-migrated with the 300-kDa PP4 band. Data show representative results from at least two experiments.
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Coimmunoprecipitation Studies Confirm That PP4R1 Is a Component of a Methylation-dependent Complex and Reveal Differential Effects of LCMT-1 Loss on Different PP4 Complexes—To confirm that loss of PP4c methylation caused by LCMT-1 KO leads to disruption of a methylation-dependent PP4 holoenzyme containing PP4R1, coimmunoprecipitation experiments were performed. PP4 regulatory subunit immunoprecipitates prepared from WT and LCMT-1 KO MEFs were probed with PP4c and PP4 regulatory subunit antibodies to measure the relative association of PP4 regulatory subunits with PP4c. As shown in Fig. 8, A and D, association of PP4R1 with PP4c was decreased to ~50% that of the WT level upon LCMT-1 KO. Correcting for the reduction in the steady-state PP4R1 protein level of ~20% upon LCMT-1 KO (Fig. 8, A and E), the amount of PP4c-PP4R1 complex was reduced overall to ~40% of that of the WT level upon LCMT-1 loss. The relative association of PP4R2 with PP4c, on the other hand, was not significantly altered by LCMT-1 loss (Fig. 8, B and D). However, the steady-state level of PP4R2 was increased 1.6-fold by LCMT-1 KO as compared with the WT level (Fig. 8, B and E), suggesting that there are more PP4c-PP4R2 complexes in LCMT-1 KO cells. Binding of PP4R3β to PP4c appears to be mildly reduced, but the reduction was not statistically significant (Fig. 8D). The steady-state protein level of PP4R3β was reduced ~20% (Fig. 8, C and E) upon LCMT-1 KO, suggesting that there is a small reduction in PP4R3β complexes. Interestingly, the steady-state level of PP4c protein is increased ~20% in LCMT-1 KO MEFs (Fig. 8F). These results further support our conclusion that loss of PP4c methylation by LCMT-1 KO leads to a great reduction of the methylation-dependent PP4R1-associated PP4 holoenzyme complex. They also reveal...
Loss of LCMT-1 increased the phosphorylation of HDAC3 on an activating site—Because our experiments showed that loss of LCMT-1 has the greatest effect on PP4R1-associated PP4 complex formation, we examined the effect of LCMT-1 KO on the phosphorylation of a known PP4R1-PP4c substrate, histone deacetylase 3 (HDAC3). The PP4R1-PP4c complex down-regulates HDAC3 activity by dephosphorylation at Ser-424 (49). To determine whether reduction in the methylation-dependent PP4R1-PP4c complex due to LCMT-1 knock-out has physiological consequences on HDAC3, cell lysates of WT and LCMT-1 KO MEFs were analyzed for phosphorylation of HDAC3 at Ser-424. LCMT-1 KO caused a 2-fold increase in phosphorylation of HDAC3 Ser-424 (Fig. 9), indicating that LCMT-1 loss has physiological consequences on a known downstream PP4 regulatory subunit (E) and PP4c (F) in WT and LCMT-1 KO MEFs. Error bars represent S.D. of at least three independent experiments. *, p ≤ 0.05; **, p ≤ 0.01.

Discussion

The data in this study provide the first evidence that PP6c, like PP2Ac and PP4c, is carboxyl-methylated in cells (Fig. 1). They also establish that LCMT-1 is the major methyltransferase for the entire PP2A subfamily of protein phosphatases. BN-PAGE analyses revealed LCMT-1-dependent dynamics of PP2A subfamily complexes and dissection of the effects of LCMT-1 loss on PP4c complexes revealed differential regulation of different PP4 complexes by LCMT-1 as well as effects on a function-relevant downstream phosphorylation. Together, these results suggest that LCMT-1 coordinately regulates the methylation of the entire PP2A subfamily of phosphatases and possibly their function (Fig. 10).
PP6c more efficiently than did wild type PME-1, suggesting that catalytically inactive PME-1 coimmunoprecipitated PP2Ac and not be detected in analogous immunoprecipitates. Importantly, lytically inactive PME-1, but a PP6c/PME-1 association could PP4c could be coimmunoprecipitated with wild type and catalytically inactive PME-1, indicating that LCMT-1 does not require distinct colocalization or scaffolding of LCMT-1 with a particular PP2A subfamily phosphatase for the entire PP2A subfamily of phosphatases, we hypothesize that these phosphatases may share this methylesterase. However, it remains to be seen whether PP4c and PP6c are indeed actively demethylated in cells and whether PME-1 is a shared methylesterase for the entire PP2A subfamily of phosphatases.

Our results show that BN-PAGE combined with Western blotting is a powerful tool for analyzing the regulation of phosphatase holoenzyme complexes. BN-PAGE gels have the advantage over traditional native gels in that complexes are largely separated by size. The ability to analyze complexes via this approach in wild type and LCMT-1 KO cells confirmed previous findings of methylation-dependent and methylation-independent PP2A complexes and revealed new information about PP2A subfamily phosphatase complexes and the differential regulation of those complexes by LCMT-1. For example, BN-PAGE analysis detected methylation-dependent PP2A heterotrimer containing B subunit at ~180 kDa (Figs. 3 and 4), consistent with a previous detection of a native PP2A complex containing methylated PP2Ac of ~178 kDa by gel filtration (29). Quantitative analysis of the consequences of LCMT-1 loss revealed an ~75% net reduction in methylation-dependent PP2A BAC complexes in LCMT-1 KO MEFs (Fig. 2), providing one downstream mechanism to explain the effects of loss of LCMT-1 on cellular and organismal function. On the other hand, these data show that a small amount of PP2A BAC complexes remain in the absence of LCMT-1, indicating that LCMT-1 loss is not as severe as a knockout of all methylation-dependent PP2A subunits. BN-PAGE analysis also revealed a separate, methylation-independent complex of ~150 kDa (Fig. 4), consistent with previous reports of differential effects of methylation on different PP2A complexes (19, 24, 48).

The large amount of cellular PP2A B subunit contained in a high molecular weight complex(es) lacking detectable C subunit is very striking (Fig. 3A). The complex appears to be a little more than 720 kDa in size. We propose that this complex likely represents PP2A B subunit bound to the CCT/TriC chaperonin complex (TCP-1 ring complex, or TRiC). CCT/TriC is known to migrate ~700 kDa on native gels (50) and helps fold a number of proteins, including many WD-repeat proteins (51), a class to which PP2A B subunit belongs. The abundance of the B subunit in this complex lacking the C subunit (open arrow, Fig. 3A) relative to that in a PP2A BAC trimer (asterisk, Fig. 3A) or lower molecular weight forms suggests that up to half or more of total cellular B subunit may not be in complex with C subunit in MEFs. No change in the amount of this band is seen upon LCMT-1 KO, suggesting that it may be a biogenesis intermediate to heterotrimeric PP2A BAC formation and not a reservoir for disassembled B subunit.

Like PP2A B subunit, α4 also exists in a large complex of >720 kDa (Fig. 4A), but this complex is larger than the one containing the B subunit. In addition, α4 is present in another complex of ~700 kDa and in a smaller form that may represent monomer. Based on the size of the >720-kDa α4 band, we hypothesize that this band corresponds to a complex detected previously by mass spectrometric analysis that contains the TRiC chaperonin complex, PP4c, and α4 but no PP4 regulatory subunits (27). However, because α4 binds all PP2A subfamily phosphatases, analogous complexes that contain PP2Ac or PP6c in place of PP4c may comigrate at this same position and

**FIGURE 10.** LCMT-1 differentially regulates the methylation and likely the function of the PP2A subfamily of protein phosphatases. Black arrows show previously established connections. Blue arrows indicate relationships established in this study. The pink arrow with a question mark indicates the open question of whether there are PP6 methylation-dependent complexes regulated by LCMT-1. If they do not exist, methylation of PP6c still might regulate PP6 function via another mechanism. In this model loss of LCMT-1 could lead to dysfunction of all three PP2A subfamily phosphatases, whereas in normal cells regulation of LCMT-1 might coordinately regulate these phosphatases in common (e.g. DNA repair) functions. Also based on this model, independent regulation of these phosphatases by LCMT-1 would likely require distinct colocalization or scaffolding of LCMT-1 with a particular PP2A subfamily phosphatase. SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine.

Several lines of evidence support the idea that PP6c is carboxyl-methylated on its C terminus. First, a C-terminal modification could be detected on PP6c by its ability to prevent the binding of an antibody raised against an unmethylated C-terminal peptide. Second, the removal of the modification by 5-min incubation in ice-cold base solution is consistent with carboxyl methylation but not other modifications such as phosphorylation. Third, loss of this modification upon loss of the methyltransferase, LCMT-1, also supports this conclusion, especially as the three C-terminal amino acids of PP6, YFL, are identical to those of the previously established LCMT-1 substrate, PP2A, and include the site of methylation on PP2A, the C-terminal leucine. The use of methylation-sensitive antibody directed against PP6c in a quantitative methylation assay showed that PP6c is highly methylated. Similarly, the results of a methylation assay using a methylation-sensitive PP4c antibody (Fig. 1) confirmed the carboxyl methylation of PP4c reported first elsewhere (36) but additionally indicated that PP4c is highly methylated in MEFs.

Reversible methylation is an established paradigm for regulation of PP2A. Given that LCMT-1 is the major methyltransferase for the entire PP2A subfamily of phosphatases, we hypothesize that these phosphatases are coordinately regulated by reversible methylation. However, whether the entire PP2A subfamily of phosphatases shares the same methyltransferase enzyme remains to be determined. PP2A is demethylated by the methyltransferase, PME-1 (22). Wandzioch et al. (37) showed that PP4c could be communoprecipitated with wild type and catalytically inactive PME-1, but a PP6c/PME-1 association could not be detected in analogous immunoprecipitates. Importantly, catalytically inactive PME-1 communoprecipitated PP2Ac and PP4c more efficiently than did wild type PME-1, suggesting that...
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Contribute to this α4 band. Indeed, we find that endogenous or epitope-tagged PP2Ac comigrates with the >720-kDa band of α4 (open arrowhead in Figs. 3B and 4).

BN-PAGE analysis shows that PP4 forms two major complexes in wild type MEFs, one at ~450 kDa and another broader band at ~300 kDa (Fig. 5). These likely correspond to PP4c-containing complexes of ~270–300 kDa and 400–450 kDa previously identified from bovine testis extracts by gel filtration (25). BN-PAGE EMSA experiments (Fig. 7) were very useful for identifying components of PP4 complexes in this study. They demonstrated that the 450-kDa PP4 band is composed of PP4c, PP4R2, and either PP4R3α or PP4R3β, in agreement with a previous report that identified these PP4 heterotrimeric complexes by mass spectrometric analysis in HEK293 cells (27).

BN-PAGE EMSA experiments also showed that the PP4c-PP4R2-PP4R3α complex is much more abundant in MEFs than is the PP4c-PP4R2-PP4R3β complex. Furthermore, these experiments demonstrated that PP4R1 is a major component of the 300-kDa PP4 band. Importantly, a portion of the 300-kDa band was not shifted by the PP4R1 antibody, suggesting that it may be a mixture of PP4 complexes. A small amount of PP4R3α comigrates with the lower portion of that band (Fig. 6), so it may be in complex with PP4R1 and PP4c or in a separate PP4 complex migrating at that position. HA-tagged PP4R4 is a candidate for an additional component of a methylation-dependent PP4 complex. However, no commercially available PP4R4 antibodies were of sufficient quality to either immunoblot native PP4R4 or to function in BN-PAGE EMSA experiments so the methylation dependence of PP4R4-PP4c complex formation remains to be determined.

Our data show that, as for PP2A complexes, methylation-dependent PP4 complexes exist, and different PP4 complexes are differentially regulated by LCMT-1. The most striking requirement for LCMT-1 was seen with the 300-kDa complex containing PP4R1, where loss of LCMT-1 caused an ~60% net loss in PP4R1-PP4c complexes (Figs. 5 and 8). There appears to be a more subtle change in the 450-kDa PP4R2-PP4R3-PP4c complex upon LCMT-1 loss with only a small but consistent increase in size noted on BN-PAGE. However, this small change could represent something as dramatic as an exchange of two similarly sized but different subunits. Thus, the underlying cause of this change is important to determine. In addition, the ~60% increase in the steady-state level of PP4R2, the ~20% decrease in the steady-state level of PP4R3β, and the ~20% increase in PP4c in LCMT-1 KO MEFs (Fig. 8) suggest that PP4 complexes are affected in other ways by loss of LCMT-1 either directly or indirectly and that there is a net increase in association of PP4R2 with PP4c. Together, these results show that LCMT-1 differentially affects distinct PP4 complexes.

In contrast to our findings reported here that methylation of Leu-307 in PP4c has differential effects on PP4 complexes, a recent study using a mutational approach to explore the function of PP4c methylation concluded that loss of PP4c methylation has a severe global effect on PP4 complexes and function (52). In that study the authors changed the C-terminal leucine (Leu-307) of PP4c to alanine (L307A mutant), which alters the side chain of the C-terminal amino acid and may affect methylation. They used coimmunoprecipitation to analyze the effects of this amino acid substitution on the association of epitope-tagged PP4c L307A mutant with PP4R1, PP4R2, PP4R3α, and PP4R3β. They found that the L307A mutation nearly abolished PP4c interaction with all of the regulatory subunits and inhibited multiple PP4c functions. A major caveat of their study is that there was no way to know whether the changes observed were due to altered methylation or the change in the R group of PP4c amino acid 307. In the current study the identity of PP4c Leu-307 was unchanged, and PP4c methylation status was manipulated by removing LCMT-1. Thus, the changes we observed are due specifically to altered methylation. We find that loss of PP4c methylation has differential effects on different PP4c complexes, with an ~50% decrease in PP4c-PP4R1 association (versus nearly abolished with the L307A mutation in the other study) being the most severe effect detectable by coimmunoprecipitation assays (Fig. 8). Moreover, we find no statistically significant loss of PP4c association with PP4R2 or PP4R3β upon complete loss of PP4c methylation and in fact find evidence for a net increase in PP4c-PP4R2 association when expression levels and coimmunoprecipitation results are considered together. Thus, it is likely that the severe effects the previous study reported were due to loss of the leucine side chain and not loss of methylation. Consistent with this possibility, we showed previously that C-terminal PP2Ac amino acid substitutions that block methylation can have more severe effects than does simple loss of methylation on regulatory subunit associations (19, 48). In the case of the current study we cannot rule out the possibility that LCMT-1 has indirect effects on PP4 complex formation through another substrate such as PP2A. However, our results strongly suggest that methylation of Leu-307 of PP4c has differential effects on stable formation of distinct holoenzymes as has been previously documented for PP2Ac (see for example, Ref. 19).

In contrast to PP4, BN-PAGE analysis of PP6 complex formation suggests that PP6 complexes in MEFs are methylation-independent (Fig. 5B). Whether the single PP6 complex detected in Fig. 5B is in fact a mixture of co-migrating PP6 complexes or is a single complex predominating in MEFs remains to be determined. Methylation-sensitive PP6 complexes may exist that were not detected in this system. Analyses with PP6 regulatory subunit antibodies did not detect specific bands (not shown) because of the low quality and/or low sensitivity of commercially available antibodies and the restricted loading capacity of BN-PAGE. Using reliable and sensitive PP6 regulatory subunit antibodies in BN-PAGE, other cell types and coimmunoprecipitation experiments in the presence and absence of LCMT-1 would help establish whether PP6c methylation is important for assembly of certain PP6 holoenzymes. Although methylation-dependent PP6 complexes were not detected, loss of LCMT-1 increased PP6c steady-state protein levels. Thus, LCMT-1 regulates the protein levels of both PP4c and PP6c.

LCMT-1 loss resulted in enhanced phosphorylation of the PP4R1-PP4c substrate, HDAC3, at Ser-424 (Fig. 9), a site known to be important for activating HDAC3 activity. This result suggests that LCMT-1 is important for down-regulation of HDAC3 activity via stabilization of PP4R1-PP4c complexes.
in wild type cells. HDAC3 has been implicated in cancer (53–55). Recently, it has been shown that LCMT-1 KD promotes cancer phenotypes such as anchorage-independent growth (56). It would be interesting to determine if dysregulation of HDAC3 contributes to the anchorage-independent growth phenotype in LCMT-1 KD cells.

In summary, our results show that LCMT-1 is a master regulator of PP2A subfamily phosphatase methylation and thus may coordinately regulate their functions (Fig. 10). An important implication of these findings is that many functions of LCMT-1 that have been attributed to LCMT-1 methylation of PP2A may be due instead, or at least in part, to methylation of PP4 and possibly of PP6. It will be interesting to study the role and regulation of LCMT-1 in cellular processes such as DNA repair where the PP2A subfamily of phosphatases function together. Future studies will also help unravel more details of the specific complexes regulated by LCMT-1, the downstream signaling pathways that are regulated, and critically, upstream regulation of LCMT-1.

**Experimental Procedures**

**Antibodies and Other Reagents**—Methylation-sensitive antibodies directed against the C terminus of the respective phosphatases were used to detect the level of demethylated PP2Ac (clone 4b7; Ref. 19; available from EMD Millipore, catalogue #05-577, 1:2000 dilution), PP4c (Bethyl Laboratories, Inc., catalogue #A300-893A, 1:5000 dilution), and PP6c (Bethyl Laboratories, catalogue #A300-844A, 1:5000 dilution). Methylation-insensitive antibodies were used to detect the total level of PP2Ac (BD Transduction Laboratories, catalogue #610556, 1:10,000 dilution), PP4c (Bethyl Laboratories, catalogue #A300-835A, 1:5000 dilution; R&D Systems, catalogue #MAB5074, 1:500 dilution), and PP6c (ProteinTech catalogue #15852-1-AP). Other antibodies used included 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-α mouse monoclonal antibody (EMD Millipore), anti-PP2A B subunit mouse monoclonal antibody clone 2G9 (EMD Millipore); isoform reactivity α > δ,β > γ), anti-PP2A A subunit mouse monoclonal antibodies (Santa Cruz Biotechnology), anti-HDAC3 rabbit monoclonal antibody (EMD Millipore), and anti-phospho-HDAC3 (Ser-424) rabbit polyclonal antibody (Cell Signaling). Protein G-Sepharose 4B beads (Invitrogen), TrueBlot anti-rabbit IgG IP Beads (Rockland Immunoc hemicals Inc.), TrueBlot anti-rabbit IgG (HRP) (Rockland Immunoch emicals Inc.), and Clean-Blot Detection Reagent (HRP) (Thermo Scientific) were used for immunoprecipitation and detection.

Peptides corresponding to C-terminal residues of PP4c (Ac-CTRGLPSKKPVADYFL-Me) and PP6c (Ac-CSERVIPPR-TTTPYFL-Me) were synthesized by NeoBioScience (Cambridge, MA) and used to characterize the methylation-sensitive PP4c and PP6c antibodies. Unmethylated PP2A C-terminal peptide was used to preadsorb PP4c antibody to remove any antibody that cross-reacts with PP2Ac.

**Cell Culture and Transfection**—Wild type, hemizygous, and homozygous LCMT-1 knock-out MEFs were prepared from E14.5 or older mouse embryo torsos from a previously described gene-trap LCMT-1 knock-out mouse (23) and then cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and nonessential amino acids at 37 °C in 5% CO₂. 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 then immunoprecipitation (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 phenylmethylsulfonyl fluoride, 50 mM sodium fluoride, and 1 mM sodium orthovanadate) by rocking for 20 min at 4 °C. Lysates were cleared by centrifugation at 13,000 × g for 10 min at 4 °C. Protein concentration was determined by using Bio-Rad Protein Assay Dye Reagent Concentrate according to the manufacturer’s instruction. To coimmunoprecipitate protein complexes, cell lysates were incubated either with cross-linked antibody-Sepharose beads or with immunoprecipitating antibody and Sepharose beads for 1.5 h 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 >4.8 orders of magnitude. Quantity One software from Bio-Rad was used to quantify band intensities. In the case of blots reprobed for PP2A A subunit after blotting for PP2Ac, membranes were either stripped with RESTORE Plus Western blot stripping buffer (Thermo Scientific) and reprobed or dried for an extended period of time and tested for the absence of leftover PP2Ac signal after incubation with mouse secondary antibody before reprobing with anti-PP2A antibody.

**Methylation Assay**—Methylation assays for PP4c and PP6c were performed as previously reported for PP2A (19, 56). Briefly, two 20-μl aliquots of each cell lysate were prepared. One aliquot of each cell lysate was treated with 50 μl of preeutemalized solution (80 mM NaOH, 80 mM HCl, 200 mM Tris, pH 6.8) and heated in gel sample buffer to preserve the methylation state of PP4c and PP6c. The other aliquot was treated with 20 μl of 200 mM NaOH base solution for 5 min on ice, which removes all C-terminal carboxyl methylation, and then neutralized with 30 μl of neutralization buffer (133.3 mM HCl, 333.3 mM Tris, pH 6.8) and heated in gel sample buffer. Samples were analyzed by SDS-PAGE and immunoblotting using methylation-sensitive antibodies to determine the level of endogenous demethylated PP4c and PP6c, and a loading control was immunoblotted to assure that each pair of aliquots contained equivalent lysate. Percent methylated PP4c or PP6c was calculated by calculating the ratio (%) of endogenous demethylated phosphatase to the
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100% demethylated control level (base-treated lanes) and then subtracting % demethylated phosphatase from 100%

**BN-PAGE and BN-PAGE EMSA**—To preserve native protein complexes, cells were lysed in non-denaturing lysis buffer (50 mM Bis-Tris, 0.016 M HCl, 50 mM NaCl, 10% w/v glycerol, 0.001% Ponceau S, pH 7.2) containing 1% Triton X-100. 20 μg of protein in cell lysates per lane were resolved by Native-PAGE™ Novex 4–16% Bis-Tris gels (Life Technologies) according to the manufacturer’s instructions. 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. To conduct BN-PAGE EMSA experiments, 20 μg of cell lysates were incubated with 2 μl of antibodies against PP4 regulatory subunits for 1 h on ice with intermittent gentle agitation. The mixture was then resolved by 4–16% BN-PAGE and transferred to PVDF membranes for Western blotting.

**Author Contributions**—D. C. P. conceived the initial project design and experimental approaches, supervised project execution, analyzed and interpreted the data, and helped write/edit manuscript and create figures. J. A. L. performed the experiments analyzing PP2A subunit levels in WT and KO MEFs and complex formation by immunoprecipitation, generated the relevant figures, and participated in revision of the manuscript. J. H. carried out all other experiments, made significant contributions to experimental design and interpretations, and contributed substantially to writing and editing the paper and made initial set of figures.

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