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*

Received for publication, May 27, 2016, and in revised form, August 5, 2016. Published, JBC Papers in Press, August 9, 2016, DOI 10.1074/jbc.M116.739920

Juyeon Hwang, Jocelyn A. Lee1, and David C. Pallas2
From the Department of Biochemistry, Winship Cancer Center, and the Biochemistry, Cell, and Developmental Graduate Program, Emory University School of Medicine, Atlanta, Georgia 30322

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-PP4c complex. Collectively, our data suggest that LCMT-1 coordinately regulates the carboxyl methylation of PP2A-related phosphatases and, consequently, their holoenzyme assembly and function.

*This work was supported by National Institutes of Health Grant R01 CA57327 (NCI). This work was also supported by an award from the Emory University Research Committee. Dr. David Pallas is entitled to royalties from the sale of products related to the research described in this paper by Millipore Inc., Santa Cruz Biotechnologies Inc., Invitrogen, 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. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

1 Supported by the National Institutes of Health Predoctoral Fellowship Grant 5F31CA123640 (NCI).

2 To whom correspondence should be addressed: Dept. of Biochemistry, Emory University School of Medicine, 1510 Clifton Rd., Atlanta, GA 30322. Tel.: 404-727-5620; Fax: 404-727-2738; E-mail: dpallas@emory.edu.

Protein phosphatase 2A (PP2A)3 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 (PPP2R2), 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), BB (24), and BB’ (5), 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

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 trimmer of PP2A containing B subunit (PPP2R2), A subunit, and C subunit; HDAC, histone deacetylase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

4 K. A. Rahman and D. C. Pallas, unpublished information.
LCMT-1 Differentially Regulates Different PP4 Holoenzymes

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 (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.

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 SAPS (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 methyl transferase 1 (LCMT-1) (20, 29–33) and protein phosphatase methylesterase 1 (PME-1) (21, 22, 34). Because the three C-terminal residues (YFL) of PP2A 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). 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.
LCMT-1 Differentially Regulates Different PP4 Holoenzymes

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.

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 knockout 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 coimmunoprecipitation 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 B subunit was decreased by ~50% in LCMT-1 homozygous KO MEFs but not in LCMT-1 hemizygous MEFs as compared with WT MEFs (Fig. 2, B and C). It is noteworthy that B subunit protein level is also decreased in lysates from LCMT-1 KO MEFs (Fig. 2A), probably due to destabilization of B subunits in the absence of LCMT-1. Considering both the amount of reduction in the steady-state level of cellular B subunit in lysates (49 ± 18% of 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 E) (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.
LCMT-1 Differentially Regulates Different PP4 Holoenzymes

WT level; \( p = 0.008 \) and the reduced binding of the remaining B subunit (~50%; Fig. 2C), we calculate that B subunit heterotrimers are decreased ~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), PP2A C (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 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 \( \alpha_4 \) because \( \alpha_4 \) interacts with PP2Ac in the absence of the A and B subunits. Indeed, immunoblotting for \( \alpha_4 \) on similar BN-PAGE gels of MEF lysates shows that \( \alpha_4 \) 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 \( \alpha_4 \) band in Fig. 4A, marked in the same way). Moreover, the intensity of the \( \alpha_4 \) 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 \( \alpha_4 \) 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 \( \alpha_4 \)-PP2Ac complex. To test this, HA-tagged WT PP2Ac and \( \Delta \)Leu-309 mutant PP2Ac were analyzed by BN-PAGE (Fig. 4B). Although
LCMT-1 Differentially Regulates Different PP4 Holoenzymes

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 comigrating 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 PP4c and PP6c 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 MEFs (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, 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.
Although the co-migration results above are suggestive of which PP4 holoenzymes might constitute the 450- and 300-kDa PP4 complexes, we devised an additional approach termed BN-PAGE EMSA to determine 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 before being analyzed by BN-PAGE. Immunoblotting was then employed to determine whether the antibodies are capable of retarding the migration of (shifting) one or both of the PP4 complexes detected by immunoblotting with the PP4c antibody. 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. 7, A and B, where the first lane in each panel is a control lane that shows the 450-kDa (filled arrowhead) and 300-kDa (asterisk) PP4 bands described in Fig. 5A that are seen if no antibody is added to the lysate before electrophoresis. When PP4R1 antibody was used (second lane; Fig. 7A), it shifted the majority of the 300-kDa PP4 complex (asterisk) without disturbing the upper band of PP4 complex (filled arrowhead). The shifted 300-kDa PP4c band can be seen in part just above the 450-kDa PP4c band in the PP4R1 antibody lane (second lane). This result indicates that PP4R1-associated PP4c constitutes at least a large portion of the 300-kDa PP4 complex whose formation is largely lost in LCMT-1 KO MEFs. The fact that some of the 300-kDa 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 at a minimum and another containing PP4c and an unidentified PP4-associated protein or proteins.

Although PP4R1 antibody shifted the 300-kDa PP4 complex but not the 450-kDa complex, antibody to PP4R2 did the opposite (third lane; Fig. 7A). Strikingly, PP4R2 antibody bound and shifted the entire 450-kDa PP4 complex (third lane, filled arrowhead), creating higher molecular weight bands (bracket). No change in the 300-kDa PP4 complex was observed using PP4R2 antibody (third lane, asterisk; Fig. 7A). This result indicates that the entire population of the 450-kDa PP4 complex contains PP4R2.

Fig. 7B shows the results of testing the effect of PP4R3α and PP4R3β antibodies on migration of PP4 complexes. PP4R3α antibody bound and shifted the majority of the 450-kDa PP4 complex (second lane, filled arrowhead), creating higher molecular weight bands (bracket), but did not affect the 300-kDa PP4 complex for the most part (second lane, asterisk). Together, these results demonstrate that the majority of the upper band of PP4 complex consists of PP4R3α in addition to PP4R2 and PP4c. On the other hand, PP4R3β antibody bound and shifted only a fraction of the upper band of PP4 complex (third lane, filled arrowhead), giving rise to a much lower amount of the shifted higher molecular weight bands (third lane, bracket) compared with those generated by addition of the PP4R3α antibody (second lane, bracket). This indicates that only a small portion of the 450-kDa PP4 complex consists of PP4R3β in addition to PP4R2. Consistent with these results, when both PP4R3α and PP4R3β antibodies were used, all of the upper bands of PP4 complex (fourth lane, arrowhead; Fig. 7B) was shifted, corroborating our hypothesis that the majority of the PP4R2-associated PP4 complex contained PP4R3α, whereas only a small population of the PP4R2-associated PP4 complex contained PP4R3β.

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 loss 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
additional differential effects of methylation on PP4 complexes and PP4 subunit protein levels.

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.

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).
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 these two 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 knock-out 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 trimmer (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 chaperone 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
LCMT-1 Differentially Regulates Different PP4 Holoenzymes

contribute to this α4 band. Indeed, we find that endogenous or epitope-tagged PP2Ac co-migrates 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 co-migrates 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, 1:5000 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); isofrom 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 Ig IP Beads (Rockland Immunocchemicals Inc.), TrueBlot® anti-rabbit IgG (HRP) (Rockland Immunocchemicals Inc.), and Clean-Blot Detection Reagent (HRP) (Thermo Scientific) were used for immunoprecipitation and detection.

Peptides corresponding to C-terminal residues of PP4c (Ac-CTRG1P55KPVADYFL-Me) and PP6c (Ac-CSRVP1PPR-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 Chemilumimag (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 preneutralized 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
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 70 V for 1 h at 4 °C. Membranes were blocked with 5% (w/v) non-fat milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% w/v Tween 20) for 1 h at room temperature and then incubated with antibodies against PP4 regulatory subunits for 1 h at room temperature. Membranes were washed 3 times with TBST buffer and incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Blots were developed using the manufacturer’s instructions (ECL, GE Healthcare). The densities of bands were quantified using the ImageJ software. 

Acknowledgments—We thank Anita Corbett, Danny Reines, and Keith Wilkinson for helpful feedback on the manuscript. We thank Jennifer Howell, Alyssa Eidbo, and Ethan Eyman for technical assistance.

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 LCMT-1 Differentially Regulates Different PP4 Holoenzymes.

References

1. Janssens, V., and Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. Biochem. J. 353, 417–439

2. Cohen, P. T., Philip, A., and Vázquez-Martín, C. (2005) Protein phosphatase 4: from obscurity to vital functions. FEBS Lett. 579, 3278–3286

3. Bastians, H., and Ponstingl, H. (1996) The novel human protein serine/threonine phosphatase 6 is a functional homologue of budding yeast Stp4p and fission yeast ppe1, which are involved in cell cycle regulation. J. Cell Sci. 109, 2865–2874

4. Filali, M., Li, S., Kim, H. W., Wadzinski, B., and Kamoun, M. (1999) Identification of a type 6 protein ser/thr phosphatase regulated by interleukin-2 stimulation. J. Cell Biochem. 73, 153–163

5. Stefansson, B., and Brautigam, D. L. (2006) Protein phosphatase 6 subunit with conserved Sit4p-associated protein domain targets IkBe. J. Biol. Chem. 281, 22624–22634

6. Stefansson, B., Ohama, T., Daughtery, A. E., and Brautigam, D. L. (2008) Protein phosphatase 6 regulatory subunits composed of ankyrin repeat domains. Biochemistry 47, 1442–1451

7. Kong, M., Ditsworth, D., Lindsten, T., and Thompson, C. B. (2009) αε is an essential regulator of PP2A phosphatase activity. Mol. Cell 36, 51–60

8. Lillo, C., Kataya, A. R., Heidari, B., Creighton, M. T., Nemie-Feyissa, D., Ginbot, Z., and Jonassen, E. M. (2014) Protein phosphatases PP2A, PP4 and PP6: mediators and regulators in development and responses to environmental cues. Plant Cell Environ. 37, 2631–2648

9. Rusin, S. F., Schlosser, K. A., Adamo, M. E., and Kettenbach, A. N. (2015) Quantitative phosphoproteomics reveals new roles for the protein phosphatase PP6 in mitotic cells. Sci. Signal 8, rs12

10. Chowdhury, D., Keogh, M. C., Ishii, H., Peterson, C. L., Buratowski, S., and Lieberman, J. (2005) γ-H2AX dephosphorylation by protein phosphatase 2A facilitates DNA double-strand break repair. Mol. Cell 20, 801–809

11. Keogh, M. C., Kim, J. A., Downey, M., Fillingham, J., Chowdhury, D., Harrison, J. C., Onishi, M., Datta, N., Galicia, S., Emili, A., Lieberman, J., Shen, X., Buratowski, S., Haber, J. E., Durocher, D., Greenblatt, J. F., and Krogan, N. J. (2006) A phosphatase complex that dephosphorylates γ-H2AX regulates DNA damage checkpoint recovery. Nature 439, 497–501

12. Nakada, S., Chen, G. I., Gingras, A. C., and Durocher, D. (2008) PP4 is a γ H2AX phosphatase required for recovery from the DNA damage checkpoint. EMBO Rep. 9, 1019–1026

13. Chowdhury, D., Xu, X., Zhong, X., Ahmed, F., Zhong, J., Liao, J., Dykhooorn, D. M., Weinstock, D. M., Pfefer, G. P., and Lieberman, J. (2008) A PP4 phosphatase complex dephosphorylates γ-H2AX generated during DNA replication. Mol. Cell 31, 33–46

14. Douglas, P., Zhong, J., Ye, R., Moorhead, G. B., Xu, X., and Lees-Miller, S. P. (2010) Protein phosphatase 6 interacts with the DNA-dependent protein kinase catalytic subunit and dephosphorylates γ-H2AX. Mol. Cell. Biol. 30, 1368–1381

15. Zhong, J., Liao, J., Liu, X., Wang, P., Liu, J., Hou, W., Zhu, B., Yao, L., Wang, J., Li, J., Stark, J. M., Xie, Y., and Xu, X. (2011) Protein phosphatase Pp6 is required for homology-directed repair of DNA double-strand breaks. Cell Cycle 10, 1411–1419

16. Tolstykh, T., Lee, J., Vafai, S., and Stock, J. B. (2000) Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. EMBO J. 19, 5682–5691

17. Wu, J., Tolstykh, T., Lee, J., Boyd, K., Stock, J. B., and Broach, J. R. (2000) Carboxyl methylation of the phosphoprotein phosphatase 2A catalytic subunit promotes its functional association with regulatory subunits in vivo. EMBO J. 19, 5672–5681

18. Wei, H., Ashby, D. G., Moreno, C. S., Ogris, E., Yeong, F. M., Corbett, A. H., and Pallas, D. C. (2001) 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. 276, 1570–1577

19. Yu, X. D., Xu, X., Moreno, C. S., Green, R. E., Ogris, E., Feng, Q., Chou, L., McQuoid, M. J., and Pallas, D. C. (2001) Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Be regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. Mol. Biol. Cell 12, 185–199

20. De Baere, I., Derua, R., Janssens, V., Van Hoof, C., Waelkens, E., Merlevede, W., and Goris, J. (1999) Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. Biochemistry 38, 16539–16547

21. Lee, J., Chen, Y., Tolstykh, T., and Stock, J. (1996) A specific protein carboxyl methyltransferase that demethylates phosphoprotein phosphatase 2A in bovine brain. Proc. Natl. Acad. Sci. U.S.A. 93, 6043–6047

22. Ogris, E., Xu, X., Nelson, K. C., Mak, E. K., Yu, X. X., Lane, W. S., and Pallas, D. C. (1999) A protein phosphatase methyltransferase (PME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. J. Biol. Chem. 274, 14382–14391

23. Lee, J. A., and Pallas, D. C. (2007) Leucine carboxyl methyltransferase-1 is necessary for normal progression through mitosis in mammalian cells. J. Biol. Chem. 282, 30974–30984

24. Longin, S., Zwaenepoel, K., Louis, J. V., Dilworth, S., Goris, J., and Janssens, V. (2006) Selection of protein phosphatase 2A regulatory subunits is mediated by the C terminus of the catalytic subunit. J. Biol. Chem. 282, 26971–26980

25. Kloecker, S., and Wadzinski, B. E. (1999) Purification and identification of a novel subunit of protein serine/threonine phosphatase 4. J. Biol. Chem. 274, 5339–5347

26. Hastie, C. J., Carnegie, G. K., Morrice, N., and Cohen, P. T. (2000) A novel 50-kDa protein forms complexes with protein phosphatase 4 and is located at centrosomal microtubule organizing centres. Biochem. J. 347, 845–855

27. Gingras, A. C., Caballero, M., Zarske, M., Sanchez, A., Hazbun, T. R., Fields, S., Sonenberg, N., Hafen, E., Raught, B., and Aebersold, R. (2005) A
novel, evolutionarily conserved protein phosphatase complex involved in cispaltin sensitivity. Mol. Cell Proteomics 8, 1725–1740.

28. Chen, G. I., Tsayakorn, S., Jorgensen, C., D’Ambrosio, L. M., Goudreault, M., and Gingras, A. C. (2008) PIP4K1AIA/12 forms a novel stable cytosolic complex with phosphoprotein phosphatase 4. J. Biol. Chem. 283, 29273–29284.

29. Xie, H., and Clarke, S. (1993) Methyl esterification of C-terminal leucine residues in cytosolic 36-kDa polyepitides of bovine brain: a novel eukaryotic protein carboxyl methylation reaction. J. Biol. Chem. 268, 13364–13371.

30. Lee, J., and Stock, J. (1993) Protein phosphatase 2A catalytic subunit is methyl-esterified at its carboxyl terminus by a novel methyltransferase. J. Biol. Chem. 268, 19192–19195.

31. Xie, H., and Clarke, S. (1994) Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. J. Biol. Chem. 269, 1981–1984.

32. Favre, B., Zolnierowicz, S., Turowski, P., and Hemmings, B. A. (1994) The catalytic subunit of protein phosphatase 2A is carboxyl-methylated in vivo. J. Biol. Chem. 269, 16311–16317.

33. George, R. R., Harris, R., Nunn, C. M., Cramer, R., and Djordjevic, S. (2002) Chaperonin assisted overexpression, purification, and characterisation of human PP2A methyltransferase. Protein Expr. Purif. 26, 266–274.

34. Xie, H., and Clarke, S. (1994) An enzymatic activity in bovine brain that catalyzes the reversal of the C-terminal methyl esterification of protein phosphatase 2A. Biochem. Biophys. Res. Commun. 203, 1710–1715.

35. Chen, J. S., and Xu, W. (2007) Crystal structure of a protein phosphatase 2A heterotrimeric holoenzyme. Nature 455, 53–57.

36. Kloecker, S., Bryant, J. C., Strack, S., Colbran, R. J., and Wadzinski, B. E. (1997) Carboxymethylation of nuclear protein serine/threonine phosphatase X. Biochem. J. 327, 481–486.

37. Wanzioch, E., Pusey, M., Werda, A., Bail, S., Bhaskar, A., Nestor, M., Yang, J. I., and Rice, L. M. (2014) PME-1 modulates protein phosphatase 2A activity to promote the malignant phenotype of endometrial cancer cells. Cancer Res. 74, 4295–4305.

38. Murata, K., Wu, J., and Brautigan, D. L. (1997) B cell receptor-associated protein α6 displays rapamycin-sensitive binding directly to the catalytic subunit of protein phosphatase 2A. Proc. Natl. Acad. Sci. U.S.A. 94, 10624–10629.

39. Chen, J., Peterson, R. T., and Schreiber, S. L. (1998) α4 associates with protein phosphatases 2A, 4, and 6. Biochem. Biophys. Res. Commun. 247, 827–832.

40. Inui, S., Sanjo, H., Maeda, K., Yamamoto, H., Miyamoto, E., and Sakaguchi, N. (1998) Ig receptor binding protein 1 (α4) is associated with a rapamycin-sensitive signal transduction in lymphocytes through direct binding to the catalytic subunit of protein phosphatase 2A. Blood 92, 539–546.

41. Nanahoshi, M., Nishiuma, T., Tsujishita, Y., Hara, K., Inui, S., Sakaguchi, N., and Yonezawa, K. (1998) Regulation of protein phosphatase 2A catalytic activity by α4 protein and its yeast homolog Tap42. Biochem. Biophys. Res. Commun. 251, 520–526.

42. Nanahoshi, M., Tsujishita, Y., Tokunaga, C., Inui, S., Sakaguchi, N., Hara, K., and Yonezawa, K. (1999) α4 protein as a common regulator of type 2A-related serine/threonine protein phosphatases. FEBS Lett. 446, 108–112.

43. Prickett, T. D., and Brautigan, D. L. (2006) The α4 regulatory subunit exerts opposing allosteric effects on protein phosphatases PP6 and PP2A. J. Biol. Chem. 281, 30503–30511.

44. Chung, H., Nairn, A. C., Murata, K., and Brautigan, D. L. (1999) Mutation of Tyr-307 and Leu-309 in the protein phosphatase 2A catalytic subunit favors association with the α 4 subunit which promotes dephosphorylation of elongation factor-2. Biochemistry 38, 10371–10376.

45. Yoo, S. J., Jimenez, R. H., Sanders, J. A., Boylan, J. M., Brautigan, D. L., and Gruppuso, P. A. (2008) The α4-containing form of protein phosphatase 2A in liver and hepatic cells. J. Cell Biochem. 105, 290–300.

46. Schägger, H., and von Jagow, G. (1991) Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. 199, 223–231.

47. Bryant, J. C., Westphal, R. S., and Wadzinski, B. E. (1999) Methylated C-terminal leucine residue of PP2A catalytic subunit is important for binding of regulatory β subunit. Biochem. J. 339, 241–246.

48. Gentry, M. S., Li, Y., Wei, H., Syed, F. F., Patel, S. H., Hallberg, R. L., and Pallas, D. C. (2005) 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 4, 1029–1040.

49. Zhang, X., Ozawa, Y., Lee, H., Wen, Y. D., Tan, T. H., Wadzinski, B. E., and Seto, E. (2005) Histone deacetylase 3 (HDAC3) activity is regulated by interaction with protein serine/threonine phosphatase 4. Genes Dev. 19, 827–839.

50. Wells, C. A., Dingus, J., and Hildebrandt, J. D. (2006) Role of the chaperonin CCT/TRIc complex in G protein βγ-dimer assembly. J. Biol. Chem. 281, 20221–20232.

51. Valpuesta, J. M., Martin-Benito, J., Gómez-Puertas, P., Carrascosa, J. L., and Willson, K. R. (2002) Structure and function of a protein folding machine: the eukaryotic cytosolic chaperonin CCT. FEBS Lett. 529, 11–16.

52. Lee, J., and Lee, D. H. (2014) Leucine methylation of protein phosphatase PP4C at terminal is critical for its cellular functions. Biochem. Biophys. Res. Commun. 452, 42–47.

53. Bartling, B., Hofmann, H. S., Boettger, T., Hansen, G., Burdach, S., Silber, R. E., and Simm, A. (2005) Comparative application of antibody and gene array for expression profiling in human squamous cell lung carcinoma. Lung Cancer 49, 145–154.

54. Libý, P., Kostrouchová, M., Pohludka, M., Yilma, P., Hrabal, P., Sikora, J., Brozová, E., Kostouchová, M., Rall, J. E., and Kostrouch, Z. (2006) Elevated and deregulated expression of HDAC3 in human astrocytic gli tumours. Folia Biologica 52, 21–33.

55. Wilson, A. J., Byun, D. S., Popova, N., Murray, L. B., L’Italien, K., Sowa, Y., Arango, D., Velch, A., Augenlicht, L. H., and Mariadason, J. M. (2006) Histone deacetylase 3 (HDAC3) and other class I HDACs regulate colon maturation and p21 expression and are deregulated in human colon cancer. J. Biol. Chem. 281, 13548–13558.

56. Jackson, B. J., and Pallas, D. C. (2012) Circumventing cellular control of PP2A by methyltransfer promotes transformation in an Akt-dependent manner. Neoplasia 14, 585–599.

57. Campbell, K. S., Auger, K. R., Hemmings, B. A., Roberts, T. M., and Pallas, D. C. (1995) Identification of regions in polyomavirus middle T and small t antigens important for association with protein phosphatase 2A. J. Virol. 69, 3721–3728.

58. Ogris, E., Gibson, D. M., and Pallas, D. C. (1997) Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular β subunit but not polyomavirus middle tumor antigen. Oncogene 15, 911–917.

59. Turowski, P., Fernandez, A., Favre, B., Lamb, N. J., and Hemmings, B. A. (1995) Differential methylation and altered conformation of cytoplasmic and nuclear forms of protein phosphatase 2A during cell cycle progression. J. Cell Biol. 129, 397–410.