CORRECTED 18 FEBRUARY 2020; SEE FULL TEXT

BIOCHEMISTRY

Antibodies recognizing the C terminus of PP2A catalytic subunit are unsuitable for evaluating PP2A activity and holoenzyme composition

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The methyl-esterification of the C-terminal leucine of the protein phosphatase 2A (PP2A) catalytic (C) subunit is essential for the assembly of specific trimeric PP2A holoenzymes, and this region of the C subunit also contains two threonine and tyrosine phosphorylation sites. Most commercial antibodies—including the monoclonal antibody 1D6 that is part of a frequently used, commercial phosphatase assay kit—are directed toward the C terminus of the C subunit, raising questions as to their ability to recognize methylated and phosphorylated forms of the enzyme. Here, we tested several PP2A C antibodies, including monoclonal antibodies 1D6, 7A6, G-4, and 52F8 and the polyclonal antibody 2038 for their ability to specifically detect PP2A in its various modified forms, as well as to coprecipitate regulatory subunits. The tested antibodies preferentially recognized the nonmethylated form of the enzyme, and they did not coimmunoprecipitate trimeric holoenzymes containing the regulatory subunits B or B′, an issue that precludes their use to monitor PP2A holoenzyme activity. Furthermore, some of the antibodies also recognized the phosphatase PP4, demonstrating a lack of specificity for PP2A. Together, these findings suggest that reinterpretation of the data generated by using these reagents is required.

INTRODUCTION

Protein phosphatase 2A (PP2A) is a major phosphoserine and phosphothreonine protein phosphatase present in all eukaryotic cells. It is involved in the regulation of diverse cellular processes including signaling cascades, cell cycle regulation, apoptosis, and development. Mutations in PP2A subunits are associated with human diseases, including cancer and neurodegenerative diseases (*1*, *2*). PP2A exists as multimeric complexes consisting of a catalytic C subunit (PPP2CA or PPP2CB, also called PP2A C α or PP2A C β), a structural A subunit (PPP2R1A or PPP2R1B, also called PP2A A α or PP2A A β), and one of many B-type regulatory subunits. B subunits are encoded by different genes, some with multiple splice variants, and are classified into four groups: B (PPP2R2A, PPP2R2B, PPP2R2C, and PPP2R2D; also known as B55α, B55β, B55γ, and B55δ), B′ (PPP2R5A, PPP2R5B, PPP2R5C, PPP2R5D, and PPP2R5E; also known as B56α, B56β, B56γ, B56δ, and B56ε), B″ (PPP2R3A, PPP2R3B, and PPP2R3C; also known as PR130, PR70, PR72, and G5PR), and B‴ (STRN, striatin family). The binding of certain B subunits to the core dimer composed of PP2A A and PP2A C (PP2A A/C) determines enzyme activity, substrate specificity, and subcellular localization (*3*).

PP2A C has a highly conserved C terminus that plays an important role in regulating the phosphatase activity. The terminal six residues, TPDYFL, are identical from yeast to human and can undergo various posttranslational modifications that activate or inhibit the function of PP2A (Fig. 1A) (*4*). Methyl-esterification of the carboxyl group of the C-terminal leucine at position 309 in mammalian PP2A (Leu^{309}) is catalyzed by the *S*-adenosylmethionine–dependent enzyme leucine carboxyl methyl transferase-1 (LCMT-1) (*5*, *6*) and reversed by protein phosphatase methylesterase-1 (PME-1) (*7*). In a mammalian cell, more than 90% of PP2A C is present in the carboxymethylated state (*8*). The major function of PP2A C carboxyl methylation is thought to be the stimulation of PP2A activity and the promotion of PP2A heterotrimeric complex assembly (*8*–*12*). For example, methylation is required for formation of B subunit–containing heterotrimers in mammalian cells (*13*). Methylation similarly facilitates mammalian B′ (B56δ) and B″ (PR72) trimer assembly in cells, although it does not seem to be strictly required for complex assembly in vitro (*14*–*17*). C-terminal methylation is, however, dispensable for trimer formation with striatin and the viral B-type subunits, SV40 small and polyomavirus small and middle tumor antigens (Fig. 1A) (*8*, *18*, *19*). The interaction with other PP2A regulatory proteins such as α4, PTPA, and Tiprl is negatively affected by posttranslational modifications of the C terminus of PP2A C, pointing to its central role in PP2A holoenzyme biogenesis (*20*–*24*).

Dysregulation of PP2A methylation has important biological consequences. Reduced PP2A methylation is associated with an increased risk of Alzheimer's disease (*25*) because it leads to a loss of PP2A B–containing trimers and, consequently, to reduced PP2A-mediated tau dephosphorylation (*26*). In addition, PME overexpression sensitizes mice to β-amyloid–induced neurodegenerative impairments, whereas LCMT-1 overexpression protects the mice from neurodegenerative impairments (*27*). In addition, PP2A C methylation appears to be important for the tumor-suppressive role of PP2A because reduction in LCMT-1 (or overexpression of PME-1) enhances the transformation of human cells in an Akt-dependent manner (*19*). Thus, studies of PP2A need to take this critical posttranslational modification into account.

The analysis of PP2A C subunit methylation is complicated by the fact that PP4 and PP6 are structurally closely related to PP2A and share four or three , respectively, identical amino acids at their C termini. Both phosphatases are also carboxymethylated by LCMT-1 (*28*). Methylation of the PP4 catalytic subunit is also crucial for

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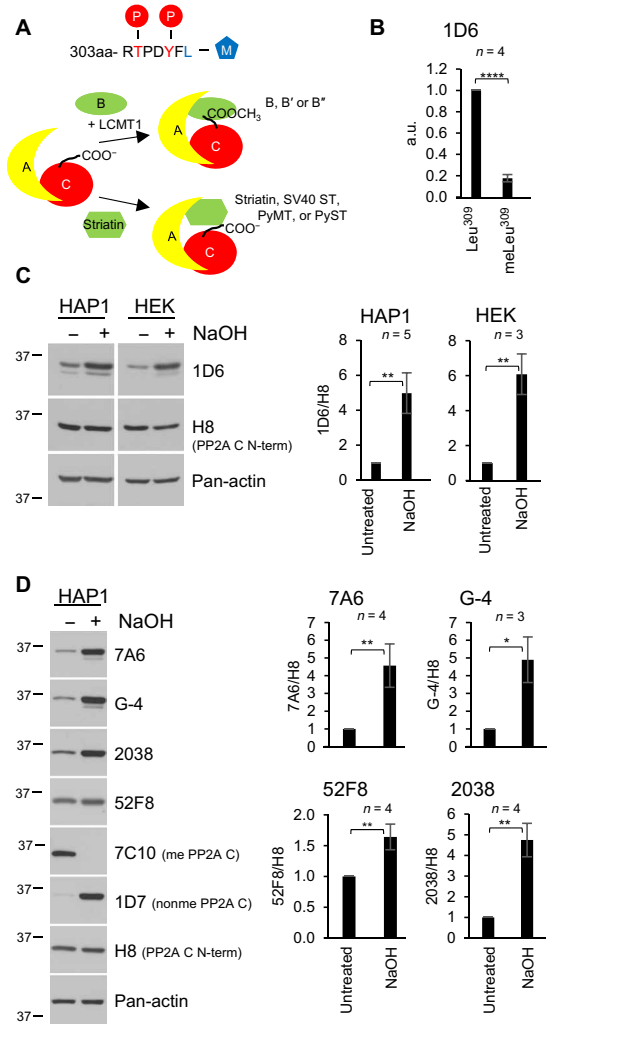

Fig. 1. Antibodies raised against the C terminus of PP2A C preferentially recognize the nonmethylated C subunit of PP2A. (**A**) Posttranslational modifications of the PP2A C-terminal tail and schematic of PP2A holoenzyme maturation. The PP2A core enzyme consists of a structural A subunit and a catalytic C subunit. LCMT-1 methylates PP2A C. The methylated PP2A core enzyme associates with B, B′, or B″ regulatory subunits. The unmethylated core enzyme associates with striatin, SV40 ST, PyST, or MT regulatory subunits. (**B**) Quantification of monoclonal antibody 1D6 binding to the peptides Leu^{309} (Ac-HVTRRTPDYFL) and meLeu^{309} (Ac-HVTRRTPDYFL-Me) by ELISA. Antibody binding data are shown as the average and SD of $n = 4$ independent ELISA experiments. The signals were normalized to Leu^{309} peptide, which was arbitrarily set to 1. (**C**) Immunoblotting of lysates from untreated or NaOH-treated HAP1 (HAP) and HEK293Trex (HEK) cells using the indicated antibodies. The panel originates from two different blotting membranes used for the C subunit antibodies (1D6 and H8). The 1D6 blot was incubated with a pan-actin antibody as a loading control. The blots are representative of $n = 5$ (HAP) or $n = 3$ (HEK) independent immunoblotting experiments. The blots were quantified using a dilution series of the NaOH-treated sample (fig. S1B). (**D**) Immunoblotting of lysates from untreated or NaOH-treated HAP1 cells using the indicated antibodies. The panel originates from seven different blotting membranes used for the C subunit–specific antibodies. The H8 blot was reincubated with pan-actin antibody as a loading control. The blots are representative of $n = 3$ or $n = 4$ independent immunoblotting experiments. The blots were quantified using a dilution series of the NaOH-treated sample and normalized to the H8 signal. Statistical significance of ELISA and immunoblotting quantifications were assessed using Student's *t* test. *$P < 0.05$, **$P < 0.01$, ****$P < 0.001$; a.u., arbitrary units.

complex formation with the PP4 regulatory subunit PP4R1 but not for complex formation with PP4R2, whereas PP6 complex formation has not been shown to be affected by methylation (*28*). The PP2A C subunit also has additional important posttranslational modification sites. In addition to the methylation of Leu^{309}, the C terminus of PP2A C has two reported phosphorylation sites at Thr^{304} and Tyr^{307}, which have mainly been associated with the inactivation of PP2A and the regulation of holoenzyme assembly (Fig. 1A) (*18*, *29*, *30*), indicating that the highly conserved C terminus of PP2A C is a major regulatory hub for PP2A holoenzyme maturation.

Antibodies are among the most important tools in the biological sciences. They are used to identify and isolate proteins and are supposed to be highly specific for the protein of interest. Unfortunately, it is becoming clear that many commercial antibodies are not sufficiently validated and may cross-react with other proteins or even detect entirely different proteins than what the label suggests (*31*). Therefore, it is not surprising that antibodies have been identified as one of the most common causes for the misinterpretation of results and the lack of reproducibility. Antibodies are frequently raised against the N and C termini of proteins because these termini are thought to be often surface exposed and therefore accessible for antibody binding (*32*). Many of the currently used and commercially available antibodies specific for the PP2A C subunit were originally raised against synthetic nonmethylated peptides from the C terminus of the protein. In the meantime, however, it has become clear that up to 90% of total PP2A C is methylated on its C terminus in vivo (*8*, *19*). Thus, many of the currently used antibodies might therefore not efficiently detect the carboxymethylated C subunit. Two antibodies, 1D6 and 7A6, have already been shown to be methyl sensitive and have been used to detect nonmethylated C subunit (*11*, *19*, *33*). In addition, potential cross-reactivity of these antibodies with PP4 or PP6, or both, cannot be ruled out because the last four or three amino acids, respectively, are identical to those in PP2A (*28*).

Here, we set out to systematically examine the methylation sensitivity of antibodies raised against the C terminus of PP2A C. In particular, we focused on clone 1D6, which is used in a commercial phosphatase assay kit, to immunoprecipitate PP2A and to determine PP2A activity from cell lysates. Our analysis revealed that 1D6 and several other antibodies raised against the C terminus of PP2A C were not suited to analyze PP2A holoenzyme activity because of their preference for the nonmethylated C subunit, their sensitivities to phosphorylated Thr^{304} or Tyr^{307}, their cross-reactivity with PP4 or PP6, and their inability to immunoprecipitate B and B′ holoenzymes. For the analysis of these PP2A holoenzymes, antibodies that target and immunoprecipitate specific regulatory PP2A subunits in complex with the A and C subunit, such as the B-specific monoclonal 2G9, should be used.

RESULTS

Antibodies raised against the C terminus of PP2A C preferentially recognize nonmethylated PP2A C

To determine the catalytic activity of PP2A but no other phosphoserine and threonine phosphatases, PP2A needs to be immunoprecipitated from cell lysates using subunit-specific antibodies. The enzymatic activity of immunoprecipitated PP2A is then measured either by colorimetric phosphatase assays using, for example, para-nitrophenyl phosphate (pNPP) or phosphorylated substrate peptides and malachite green, or by assays using ^{32}P-labeled protein substrates, such

as phosphorylase a and histone H1 (*12*, *34*). The antibody most commonly used to immunoprecipitate PP2A for activity assays is the mouse monoclonal antibody 1D6, which was raised against a 15-residue nonmethylated PP2A C subunit C-terminal peptide (RGEPHVTRRTPDYFL) and has already been shown to have a preference for nonmethylated PP2A C (*11*). To confirm the methyl sensitivity of 1D6, we performed an enzyme-linked immunosorbent assay (ELISA) on a methylated versus a nonmethylated PP2A C-terminal undecapeptide. 1D6 detected the nonmethylated peptide with a 5.6-fold higher signal than it did the methylated peptide (Fig. 1B). Two control antibodies—an antibody specific for the methylated C terminus, clone 7C10, and an antibody that preferentially detects nonmethylated PP2A C, clone 1D7—detected the corresponding peptides with 42- and 15-fold higher signals than they did their nonmethylated or methylated counterparts, respectively (fig. S1A). To confirm this result with methylated and nonmethylated full-length protein, we chemically removed the methyl group from cellular PP2A by treating lysates of two different human cell lines, HAP1 and human embryonic kidney (HEK) 293Trex (HEK) with NaOH (*35*). The 1D6 signals for PP2A C increased fivefold (HAP1) or sixfold (HEK) in NaOH-treated compared with untreated cell lysates, confirming the methyl sensitivity of 1D6 observed in the ELISA (Fig. 1C). The nonmethylated C–specific antibody, 1D7, gave an 8.9 times higher PP2A signal on the NaOH-treated lysates of HAP1 cells, suggesting that almost 90% of the PP2A C subunit was methylated in these cells (fig. S1, B and C). In HEK cells, the PP2A signal in the untreated lysate was even lower than that in the HAP1 cells, suggesting that more than 90% of the PP2A C subunit was methylated in these cells (fig. S1, B and C). The methyl-PP2A–specific antibody 7C10 only detected methylated PP2A in the untreated cell lysates but not in the NaOH-treated lysates, whereas actin and total C subunit abundances were unchanged by NaOH treatment (Fig. 1C and fig. S1B). These results raised concerns that other antibodies generated against the C terminus of the PP2A C subunit might be similarly hampered by methylation at Leu^{309}. Using the same experimental setup with NaOH-treated versus untreated HAP1 cell lysates, we found that the mouse monoclonal antibody 7A6, which was generated by our laboratory and has been licensed to several companies, rabbit polyclonal antibody 2038 (Cell Signaling Technology), and the mouse monoclonal antibody G-4 (Santa Cruz Biotechnology) were four to fivefold worse in detecting methylated PP2A C, whereas the rabbit monoclonal antibody 52F8 (Cell Signaling Technology) was only hampered by methylation 1.6-fold (Fig. 1D).

Additional posttranslational modifications to the C terminus of PP2A include phosphorylation at two reported sites, Thr^{304} and Tyr^{307}. To test whether methylation-sensitive antibodies directed against the C terminus were affected by modifications at these residues, we performed ELISAs on nonmethylated PP2A C-terminal undecapeptides either nonphosphorylated, phosphorylated on Thr^{304} ($pThr^{304}$), or phosphorylated on Tyr^{307} ($pTyr^{307}$). Signals from the 1D6, G-4, and 7A6 antibodies decreased to about 70, 45, or 65%, respectively, on the nonmethylated $pThr^{304}$ peptide compared with the nonmethylated, nonphosphorylated peptide and gave almost no signal on the nonmethylated $pTyr^{307}$ peptide (fig. S2A), indicating that phosphorylation at Tyr^{307} blocked PP2A detection by these antibodies. 52F8 and 2038 did not recognize any of the undecapeptides in ELISAs and were therefore not included in this analysis. The nonmethyl-specific antibody 1D7 was hardly affected by phosphorylation of Tyr^{307} but severely impaired (4.7-fold) when Thr^{304} was phosphorylated (fig. S2B). In contrast, detection by the methyl-specific antibody 7C10 was not impaired but increased 1.6-fold by the phosphorylation of Thr^{304}. A slight increase observed with the $pTyr^{307}$ peptide was not statistically significant (fig. S2C).

Antibodies raised against the C terminus of PP2A C only weakly immunoprecipitate B or B′ holoenzymes

1D6 is used in a commercial kit to assess PP2A activity from cellular lysates. Given the methylation sensitivity of this antibody and the requirement for methylation for the assembly and/or activity of certain PP2A holoenzymes, we sought to determine which PP2A holoenzymes could be immunoprecipitated by 1D6. Methylation of the C terminus of PP2A C is an important prerequisite for the assembly of PP2A holoenzymes containing B and B′ regulatory subunits but not for holoenzymes containing the B″′ regulatory subunit (striatin) (*8*). All of these PP2A holoenzymes, however, can efficiently be isolated by N-terminal epitope tagging of the PP2A C subunit subsequent immunoprecipitation of the tag (*18*, *36*). We therefore expressed N-terminally hemagglutinin (HA)–tagged PP2A C subunit α isoform in HEK cells and analyzed the immunoprecipitates by Western blotting. The preference of 1D6 and 7A6 for the nonmethylated PP2A C subunit was less pronounced in the immunoprecipitation than in the ELISA and immunoblotting experiments. 1D6 immunoprecipitated 146%, and 7A6 immunoprecipitated 228% of the amount of nonmethylated PP2A C immunoprecipitated by the HA tag antibody and 68 or 34% of the amount of methylated PP2A C, respectively (Fig. 2A). The amounts of the structural PP2A A subunit decreased to 56% in the 1D6 and to 18% in the 7A6 immunoprecipitates. Critically, the B-type subunits B and B′ were almost undetectable in the 1D6 and 7A6 immunoprecipitates (Fig. 2A). Conversely, 1D6, but not 7A6, copurified ninefold increased amounts of striatin complexes compared with HA tag immunoprecipitates (Fig. 2A and fig. S3B). The general PP2A C antibody H8, which binds to a fragment encompassing amino acids 46 to 90, did not immunoprecipitate PP2A C, probably because large parts of its epitope on PP2A C are not surface exposed or are masked by A subunit binding and therefore not accessible to the antibody (fig. S3A) (*15*–*17*). The rabbit monoclonal 52F8 immunoprecipitated exclusively nonmethylated PP2A C but coimmunoprecipitated hardly any A subunit and no B and B′ subunits (fig. S3A). The methyl-PP2A–specific antibody 7C10 weakly immunoprecipitated methylated PP2A C and A subunits but no trimers containing B and B′ (fig S3A). 2G9, the antibody specific for α and δ B subunits, efficiently coimmunoprecipitated trimeric PP2A holoenzymes consisting of PP2A B, PP2A A, and exclusively the methylated form of PP2A C subunit, reflecting the requirement of methylation for B subunit–containing holoenzymes (fig. S3, A and B).

B-type regulatory subunits determine the activity and specificity of PP2A toward its substrates (*37*). The inability of 1D6 to efficiently immunoprecipitate two major PP2A holoenzyme classes, the B and B′ trimers, and the fact that 1D6 is used to determine PP2A activity from cell extracts made us wonder about the PP2A activity present in 1D6 immunoprecipitates. Therefore, we determined the catalytic activity of HA-PP2A C, 1D6 PP2A C, and 2G9 PP2A B immunoprecipitates toward two different in vitro substrates, phosphorylase a and histone H1 (Fig. 2B). Phosphorylase a is a general substrate for mono-, di-, and trimeric PP2A complexes, with the monomeric C subunit showing the highest activity and B and B′ trimers showing

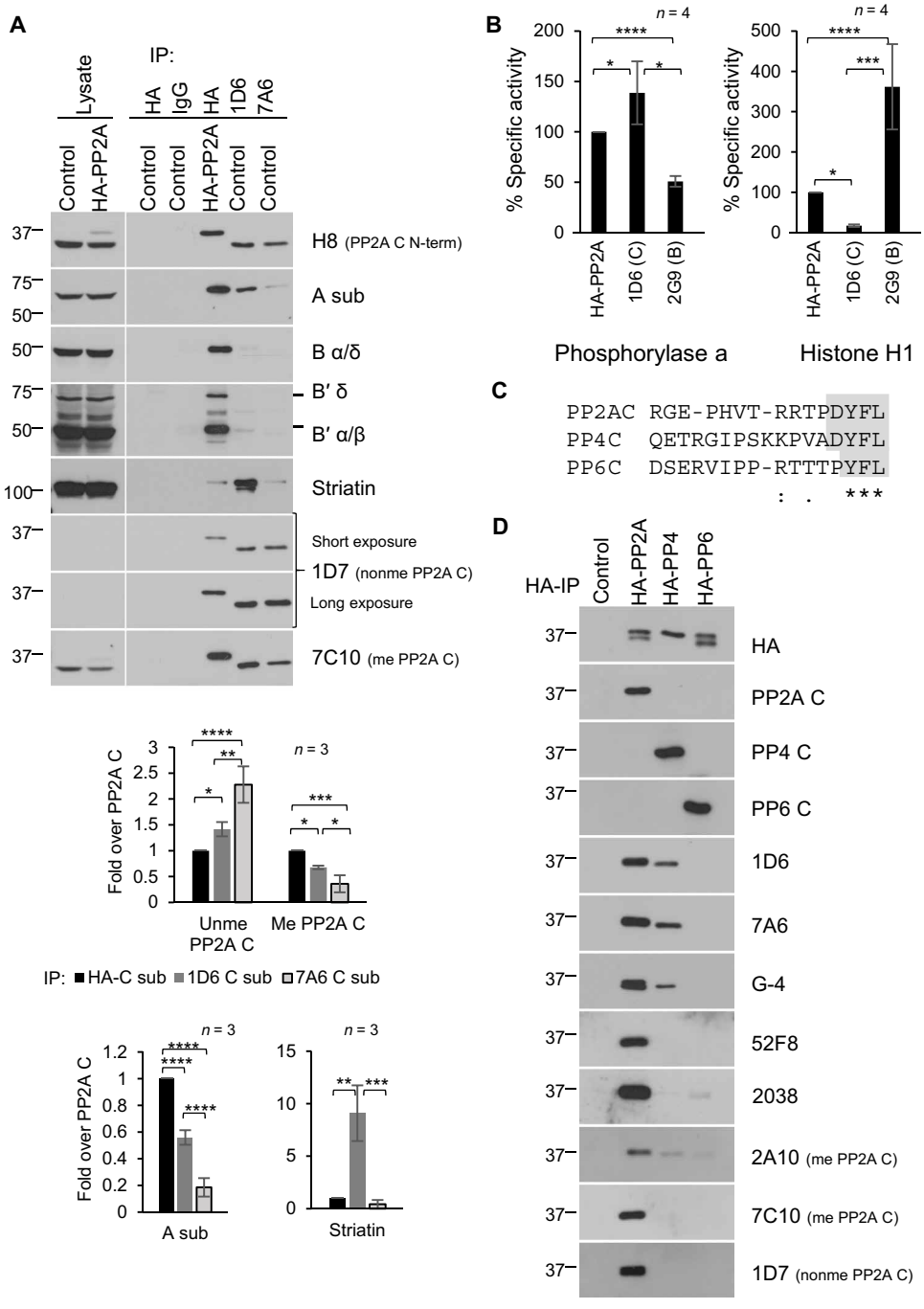

Fig. 2. Antibodies raised against the C terminus of PP2A C weakly immunoprecipitate B or B′ holoenzymes and cross-react with PP4 C. (**A**) Immunoblotting of HA, 1D6, 7A9, and IgG (immunoglobulin G; control) immunoprecipitates (IP) and lysates ($^{1}/_{10}$ of input) from HEK293Trex cells transfected with either empty vector (control) or vector encoding HA-PP2A C subunit using antibodies H8, 1D7, or 7C10 or antibodies directed against the indicated A and B subunits. The panels originate from three different blotting membranes. The blots are representative of $n = 3$ independent immunoprecipitation experiments. The A subunit and striatin coimmunoprecipitations were quantified from three independent experiments relative to the amount of immunoprecipitated PP2A C, and the HA-PP2A C IP was arbitrarily set to 1. (**B**) Quantification of the catalytic activity of HA, 1D6, or 2G9 immunoprecipitates from lysates of HEK293Trex cells transfected with either vector encoding HA-PP2A C subunit (for HA-PP2A immunoprecipitation) or control vector (for 1D6 and 2G9 immunoprecipitations) analyzed by phosphatase assays toward phosphorylase a or CDK1-phosphorylated histone H1. The measured activity was normalized to the amount of immunoprecipitated PP2A C subunit, and the specific activity of HA-PP2A C IP was set to 100%. $n = 4$ independent immunoprecipitation and phosphatase assay experiments. (**C**) Alignment of the C termini of mammalian C subunits of PP2A, PP4, and PP6. (**D**) Immunoblotting of HA immunoprecipitates from lysates of NIH3T3 cells infected with either retroviral supernatants (control) or retrovirus carrying HA-PP2A C, HA-PP4 C, or HA-PP6 C using the indicated antibodies. To equilibrate HA amounts, two times more of the HA-PP4 and three times more of the HA-PP6 C immunoprecipitates were loaded compared with HA-PP2A. The panel originates from 11 different blotting membranes that are representative of $n = 3$ independent immunoprecipitation experiments. Statistical significance of immunoblotting and phosphatase assay quantifications was assessed using ANOVA followed by Tukey's HSD as a post hoc test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, $^{****}P < 0.001$; C, PP2A C subunit; B, PP2A B subunit.

the lowest activity but highest affinity toward this substrate (*38*). In contrast, histone H1 that has been phosphorylated by the cyclin-dependent kinase CDK1 has been shown to be specifically dephosphorylated by heterotrimeric complexes containing a B subunit (*18*, *39*–*42*). Compared with the HA-PP2A C immunoprecipitate, 1D6-precipitated PP2A showed a 5.5-fold reduction in activity toward CDK1-phosphorylated histone H1, reflecting the nearly complete absence of B-containing trimers (Fig. 2B and fig. S3), whereas the activity toward the general substrate phosphorylase a increased 1.4-fold. As expected, 2G9-immunoprecipitated B holoenzymes had 360% of activity toward its specific substrate histone H1 compared with HA-PP2A C immunoprecipitates (of which only a fraction represents B-containing holoenzymes) and 50% of activity toward phosphorylase a (Fig. 2B).

Antibodies raised against the C terminus of PP2A C cross-react with PP4 and PP6

PP4 and PP6 are also type 2A phosphatases and share about 50% sequence identity with PP2A. The C termini of the PP2A, PP4, and PP6 catalytic subunits share the DYFL (PP2A and PP4) or YFL (PP6) motif (Fig. 2C). We therefore asked whether antibodies raised against the PP2A C terminus would cross-react with PP4 C and/or

PP6 C. We immunoprecipitated HA-tagged human PP2A C α, HA-tagged mouse PP4 C, or HA-tagged mouse PP6 C from NIH3T3 mouse fibroblasts stably expressing each of these HA-tagged proteins individually and analyzed the precipitated proteins by immunoblotting using several antibodies purportedly specific for PP2A. We used three antibodies raised in our laboratory against sequences unique to PP2A, PP4, and PP6 as specific control antibodies. 1D6, 7A6, and G-4 all cross-reacted with PP4 C, but not with PP6 C (Fig. 2D). The rabbit polyclonal antibody, 2038 (Cell Signaling Technology), detected primarily PP2A C and only weakly detected PP6 C (Fig. 2D). The rabbit monoclonal antibody, 52F8 (Cell Signaling Technology), only recognized PP2A C (Fig. 2D). We also tested two antibodies generated in our laboratory against the methylated PP2A C subunit: Whereas clone 7C10 was specific for methylated PP2A, clone 2A10 (available from Millipore since 1999) also recognized PP4 C (*43*) and even weakly recognized PP6 C (Fig. 2D). Consistent with what we observed for the PP2A C subunit, both antibodies recognized their targets in a methylation-dependent manner (fig. S4).

1D6 methylation sensitivity is the source of a detection bias in the 1D6 immunoprecipitation phosphatase assay kit

To determine whether the methylation sensitivity of 1D6 might influence the results and interpretation of experiments in studies using 1D6-immunoprecipitated PP2A, we analyzed PP2A complex assembly and activity in wild-type HAP1 cells and HAP1 cells, in which *LCMT-1* was knocked out. It was already reported that deletion of *LCMT-1* in mouse embryonic fibroblasts decreases methylation of PP2A C by >95%, methylation of PP4 C by ~93%, and methylation of PP6 C by ~87% (*28*). Furthermore, deletion of *LCMT-1* in mammalian cells, or of its ortholog (PPM1) in yeast, reduces the formation of PP2A C/A/B heterotrimers, impairs catalytic activity, and reduces the steady-state abundance of the PP2A Bα, A, and C subunits (*10*–*13*).

We used HAP1 wild-type cells, which are near-haploid derivates of the chronic myelogenous leukemia cell line KBM-7, and HAP1 *Lcmt-1*$^{-}$ cells and measured the PP2A activity using the PP2A immunoprecipitation assay kit as directed by the manufacturer. The activity of PP2A in the *Lcmt-1*$^{-}$ cells appeared to be unchanged compared with the wild-type cells (Fig. 3A, left panel), although these cells contained about 40% less PP2A C (Figs. 3B and 4A). However, because of its preference for nonmethylated PP2A C, 1D6 immunoprecipitated 2.5 times higher amounts of PP2A C from *Lcmt-1*$^{-}$ cells than it did from wild-type cells (Fig. 3B). When we normalized the measured activity to the amount of immunoprecipitated PP2A C subunit, the specific activity of PP2A C from *Lcmt-1*$^{-}$ cells was reduced to 35% (Fig. 3A, right panel), indicating that the activity of PP2A C was hampered in the absence of the PP2A methyltransferase, which is consistent with data obtained in yeast lacking the LCMT-1 homolog PPM1 (*12*). Without methylation of the C subunit, certain trimeric PP2A complexes—in particular, those exerting tumor-suppressive functions such as B- and B′-containing trimers—do not assemble efficiently and are very much reduced in *Lcmt-1*$^{-/-}$ cells (*8*, *19*, *28*). This difference between wild-type and *Lcmt-1*$^{-}$ cells was not detectable by 1D6 because it immunoprecipitated almost no B subunit–containing trimers from wild-type or *Lcmt-1*$^{-}$ cells (Fig. 4A). In contrast to the high preference for nonmethylated C observed by ELISA and Western blot analyses, we detected substantial amounts of methylated PP2A C in the 1D6 immunoprecipitates from wild-type cells, suggesting that methylated PP2A C can be precipitated by ID6 but is not accessible for antibody binding when it is associated with B subunits. The B subunit–specific antibody 2G9 immunoprecipitated high amounts of B-containing trimers from wild-type cells. These contained exclusively methylated C subunits, but these amounts were greatly reduced in *Lcmt-1*$^{-}$ cells, confirming defective holoenzyme biogenesis in these cells. The almost complete absence of B-containing trimers in *Lcmt-1*$^{-}$ cells was also evident when we measured the specific activity of immunoprecipitated PP2A C toward the B-specific substrate CDK1-phosphorylated histone H1. The specific activity of 2G9-immunoprecipitated PP2A C from wild-type cells was 21-fold higher toward this B-specific substrate compared with 1D6-precipitated PP2A C from wild-type as well as *Lcmt-1*$^{-}$ cells, both of which displayed low activities. Unfortunately, in *Lcmt-1*$^{-}$ cells, we could not normalize the 2G9-precipitated activity to the precipitated C subunit amounts (and therefore could not compare to the activity in a wild-type cell) because the amounts of PP2A C subunit were below the detection range of the general PP2A C antibody H8 (Fig. 4A). However, even without normalization, we could only detect very little

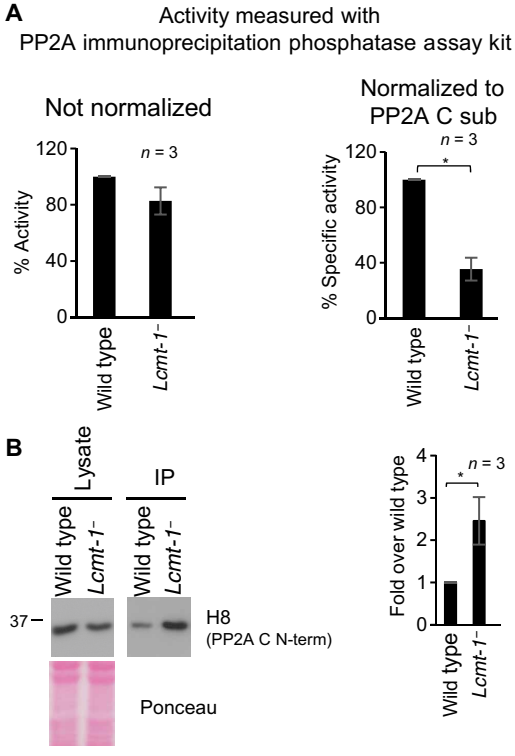

Fig. 3. Methylation sensitivity is the source of a detection bias in the 1D6-based commercial immunoprecipitation phosphatase assay kit. (**A**) The catalytic activity of 1D6 immunoprecipitates from wild-type and *Lcmt-1*$^{-}$ HAP1 cells was analyzed with the PP2A immunoprecipitation phosphatase assay kit (Millipore). The activity of wild type was set to 100% and was calculated both without normalization to the immunoprecipitated PP2A C subunit and with normalization to the immunoprecipitated PP2A C subunit. $n = 3$ independent experiments. (**B**) 1D6 immunoprecipitates (IP) from the PP2A phosphatase activity kit on wild-type and *Lcmt-1*$^{-}$ HAP1 cells were immunoblotted using the indicated antibodies. The panels that are shown originate from the same blotting membrane and are representative of three independent experiments. Statistical significance of immunoblotting and phosphatase assay quantifications was assessed using Student's *t* test. *$P < 0.05$.

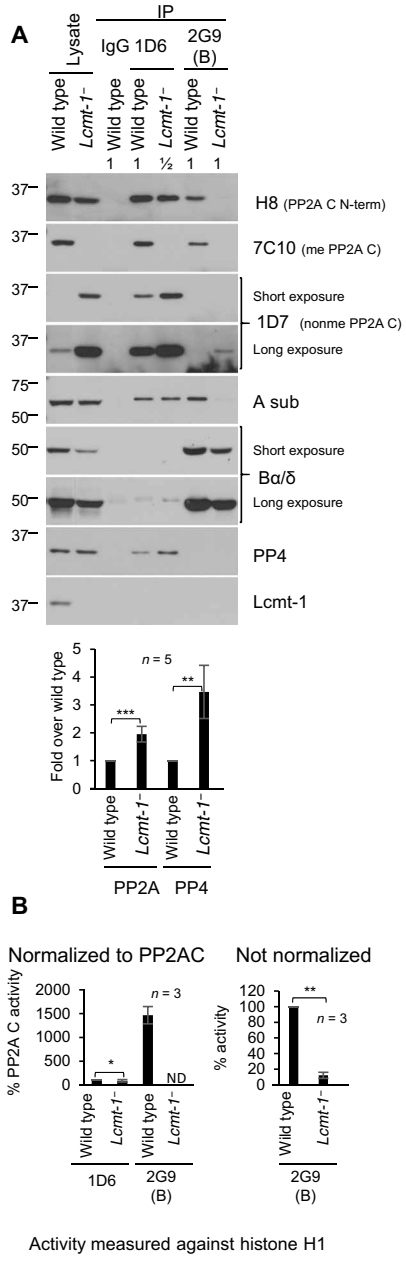

Fig. 4. Analysis of 1D6 immunoprecipitates fails to detect the nearly complete absence of B-containing trimers in *Lcmt-1*⁻ cells. (**A**) Lysates and 1D6 and 2G9 immunoprecipitates (IP) of wild-type or *Lcmt-1*⁻ HAP1 cells were analyzed by immunoblotting with the indicated antibodies. IgG immunoprecipitates are the control. Blots that are shown originate from three different blotting membranes and are representative of $n = 5$ (1D6) or $n = 3$ (2G9) independent immunoprecipitation experiments. The PP2A C and PP4 C immunoprecipitation was quantified from $n = 5$ independent experiments, and the immunoprecipitations from wild-type cells were set to 1. (**B**) The catalytic activities of 1D6 and 2G9 immunoprecipitates from wild-type and *Lcmt-1*⁻ HAP1 cells were analyzed by phosphatase activity toward histone H1. The measured activity was normalized to activity immunoprecipitated with 1D6 from wild-type cells, which was arbitrarily set to 100% and was calculated by either normalization to the immunoprecipitated PP2A C subunit or normalization to activity immunoprecipitated with 2G9 from wild-type cells without normalization to PP2A C abundance. Statistical significance of immunoblotting and phosphatase assay quantifications was assessed using Student's t test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$; ND, not determined.

activity in the 2G9 immunoprecipitates from *Lcmt-1*⁻ cells compared with wild-type cells (Fig. 4B, right panel).

Another factor complicating the interpretation of the catalytic activity measurements with 1D6 is its cross-reactivity with PP4. We detected substantial amounts of the PP4 catalytic subunit in the 1D6 immunoprecipitates (Fig. 4A). Eight percent of total PP4 (8 ± 2%) was coimmunoprecipitated from wild-type cells, and 3.5-fold higher amounts from *Lcmt-1*⁻ cells, suggesting a nonmethyl preference of 1D6 also for the PP4 catalytic subunit.

In summary, the 1D6-based PP2A immunoprecipitation phosphatase assay kit did not accurately measure total PP2A activity because trimers containing B and B′ were minimally coimmunoprecipitated with PP2A C by 1D6. In addition, 1D6 showed a preference for the nonmethylated forms of the PP2A and PP4 catalytic subunits. Hence, measuring PP2A activity with the PP2A immunoprecipitation phosphatase assay measured a combined activity of PP2A and PP4, which, in addition to failing to precipitate major holoenzyme classes, makes the meaningful interpretation of the obtained results nearly impossible.

DISCUSSION

In this study, we tested several PP2A C subunit–specific antibodies for their usefulness as PP2A analysis tools. Our study revealed that antibodies directed against the C terminus of PP2A C were hampered to various extents by methylation and phosphorylation of this region (Table 1) and were incapable of immunoprecipitating the majority of PP2A holoenzymes. A further complication with some of the C-terminal PP2A C antibodies is their cross-reactivity with the catalytic subunits of PP4 and, to a lesser extent, PP6. Considering that 1D6 was and still is used in a commercial PP2A immunoprecipitation assay kit, this cross-reactivity is particularly concerning. The phosphorylated peptide substrate KRpTIRR used in the assay kit is not PP2A specific and is dephosphorylated by PP4 as well (*44*, *45*). Thus, with this peptide, the activity measured in the 1D6 immunoprecipitates cannot be assigned to PP2A only. One would have to assess and compare the catalytic efficiencies of PP4 and PP2A toward the KRpTIRR peptide, and these differences would have to be considered in all the activity measurements using this substrate. With phosphorylase a as the substrate, PP4 activity can be neglected in activity measurements because PP4 C has an about 85-fold lower phosphatase activity toward this substrate than does PP2A C (*46*). All these considerations are very complex and make the interpretation of results achieved through the use of the 1D6-based immunoprecipitation assay kit nearly impossible. Our analyses of other commercial PP2A antibodies directed against the C terminus of PP2A C revealed that almost all of these antibodies also have limitations and are not well suited for analyses of PP2A holoenzymes or their activity. Even 7C10, the antibody that is highly specific for methylated PP2A C, did not immunoprecipitate holoenzymes, probably because the methylated C terminus is masked by the interaction of the PP2A C subunit with the A and B subunits (*17*).

Given these findings, we suggest that alternative approaches be used to measure PP2A activity. The simplest approach relies on the ectopic expression of N-terminally epitope-tagged C, A, and B subunits, followed by tag antibody immunoprecipitation, and the analysis of the immunoprecipitate by Western blot (to determine the amount of precipitated PP2A subunits) and activity measurements toward general phosphatase substrates such as phosphorylase a. One caveat

Table 1. Summary of results with C-terminal antibodies. This table shows an overview of the properties of the tested C-terminal PP2A C antibodies. For details, please see results and figures. ND, not determined

Clone	Sensitive to meLeu^{309}	Sensitive to pThr^{304}	Sensitive to pTyr^{307}	Cross-reacts with PP4	Cross-reacts with PP6	Immunoprecipitates PP2A holoenymes
1D6	Yes	Yes	Yes	Yes	No	No
7A6	Yes	Yes	Yes	Yes	No	No
G-4	Yes	Yes	Yes	Yes	No	ND
52F8	Yes	ND	ND	No	No	No
2038	Yes	ND	ND	Yes	Yes	ND
	Detects meLeu^{309}	**Sensitive to pThr^{304}**	**Sensitive to pTyr^{307}**	**Cross-reacts with PP4**	**Cross-reacts with PP6**	**Immunoprecipitates PP2A holoenymes**
7C10	Yes	No	No	No	No	No
2A10	Yes	ND	ND	Yes	Yes	ND
1D7	No	Yes	No	No	No	ND

of this approach is that ectopic overexpression of PP2A subunits disturbs the endogenous subunit homeostasis [although high overexpression of PP2A C cannot be achieved due to an autoregulatory control mechanism (*47*)]; another is the undefined holoenzyme composition of the C or A subunit immunoprecipitates. For example, the types and amounts of holoenzymes present in tagged C subunit immunoprecipitates may differ markedly between cell types and cell states, but the measured activity might seemingly be equal because the substrate used in the assay is not holoenzyme specific and, thus, cannot detect increased or decreased abundance or activity of certain PP2A holoenzymes. Major alterations in PP2A holoenzyme assembly, such as that in *LCMT-1* knockout cells, can be detected with tagged C subunit immunoprecipitation assays, but more subtle changes in holoenzyme homeostasis will be missed. Here, more laborious assays are necessary, such as consecutive immunoprecipitations, which, however, require coexpression of tagged A and B subunits in the same cells (*48*). Alternatively, B subunit–specific immunoprecipitating antibodies, such as the 2G9 monoclonal antibody used in our study, and B-specific substrates such as CDK1-phosphorylated histone H1 or a CDK1-phosphorylated peptide from retinoblastoma protein (pRb) can be used (*41*). The limitations of such assays are the availability of B-type isoform–specific antibodies that are capable of immunoprecipitating the specific B holoenyzme, as well as the limited knowledge on the B subunit–specific substrates and the kinases involved in the phosphorylation of these substrates. One possible—although elaborate—solution to antibody limitations is epitope tagging of the N- or C-terminal domains of endogenous individual B subunits using CRISPR-Cas9 technology. This could be done in standard cell lines that could then be shared between laboratories or in embryonic stem cells to generate knock-in mice that express tagged PP2A subunits in all tissues. The discovery of short linear binding motifs in the substrates of B′ holoenzymes (*49*–*51*) will facilitate the identification of B-type specific substrates that can be used in B-type subunit–specific catalytic assays.

Dysregulation of PP2A is detrimental for cells and organisms. A potential tumor suppressor function was first described for PP2A in a study linking missense mutations in the PP2A A subunit to colorectal cancer (*52*). Since then, extensive evidence has been provided for the tumor-suppressive function of PP2A, in particular those holoenzymes that depend on methylation for assembly and negatively regulate the RAS-Akt, Myc, and Wnt signaling (*19*, *53*, *54*). However, many studies that had reported PP2A activity changes in cancer cells relied on the 1D6 PP2A immunoprecipitation kit to determine total PP2A activity (>150 papers by March 2019) (*55*–*58*). In the light of our results, these published PP2A activity data need to be revisited because any changes in PP2A methylation may have markedly affected the assay results and their interpretation. For example, a decrease in methylated C subunit—and therefore a decrease in tumor-suppressive PP2A holoenzymes—concomitant with an increase in nonmethylated C subunit would be read out by the 1D6 assay as increased PP2A "total" activity because of the antibody's strong preference for nonmethylated C subunit. Conversely, an increase in methylated C—and therefore an increase in tumor-suppressive PP2A holoenzymes—would be measured by the 1D6 assay as decreased PP2A activity. In conclusion, our data with 1D6 and other PP2A C-terminal antibodies strongly suggest that researchers must be very careful in using these antibodies for PP2A detection in whole-cell extracts, immunoprecipitation, and measurement of phosphatase activity.

MATERIALS AND METHODS

Mammalian tissue culture and transfection

HAP1 wild-type and leucine carboxyl methyltransferase 1 (*Lcmt-1*) CRISPR-Cas9 deletion cells (Horizon Discovery, #C631 and #HZGHC004373c001) were grown in Iscove's modified Dulbecco's medium (Thermo Fischer Scientific, Life Technologies, #12440053) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma, #F7524, lot 104M3333), GlutaMAX (Thermo Fischer Scientific, Life Technologies, #35050-38, lot 1895829), and penicillin-streptomycin solution (Sigma, #P4333, lot 125M4781V). HEK293Trex, HEK293, NIH3T3, and BOSC-23 cells were grown in Dulbecco's modified Eagle's medium (Sigma, #D5671, lot RNBG4527) supplemented with 10% (v/v) FCS, GlutaMAX, and penicillin-streptomycin solution. NIH3T3 cells were infected using a viral supernatant produced in BOSC-23 cells. Cells were transfected using Lipofectamine 2000 (Invitrogen). Puromycin (Sigma) was used at a concentration of 5 μg/ml, blasticidin (GE Healthcare) at 5 μg/μl, and G418-sulfate (MedChemExpress) at 300 μg/ml. Cells were tested for mycoplasma

infections using the MycoAlert Mycoplasma Detection kit (Lonza, #LT07-418); cell lines were free of mycoplasma or other contaminations.

Plasmids

pcDNA3 puro (this study) was derived by substituting in the pcDNA3 neo plasmid (Thermo Fisher, Invitrogen) the neo cassette, which was removed by cutting with Bst BI and Sma I, with the puro cassette from pBabe puro (*59*) also cut with Bst BI/Sma I. This resulted in a doubled SV40 promoter, of which one copy was removed by cutting with Avr II. pcDNA3 puro HA-PP2A C (this study) was generated by inserting the HA-PP2A C subunit cassette, derived from pGRE5 HA p36 (*7*) by cutting with Hind III/Xho I, into pcDNA3 puro, which was cut with Hind III/Xho I (*59*). pBabe hygro new polylinker (NP) (this study) was generated by destroying the Hind III site in pBabe hygro (*59*) by cutting with Hind III and filling up the 5′ overhangs with Klenow. An NP consisting of Bam HI, Hind III, Nde I, Xho I, Apa I, Eco RI, Sna BI, and Sal I was inserted into the pBabe hygro by cutting with Bam HI/Sal I. pBabe hygro HA-PP2A (this study) was generated by inserting the HA-PP2A C subunit cassette from pGRE5 HA p36 (*7*) into the Hind III/Xho I cut pBabe hygro NP plasmid. pBabe hygro HA-PP4 (this study) was generated by amplifying PP4 from NIH3T3 cDNA with primers: PP4sense (5′-CGGGATCCACCATGGCGGAGATCAGCG-3′) adding a Bam HI site 5′ to the start codon, and PP4antisense (5′-GCCGCTCGAGATCACAGGAAATAGTCGGCCACTG-3′) adding an Xho I site 3′ to the stop codon. The digested polymerase chain reaction (PCR) fragment was cloned into pBabe hygro HA, by cutting with Bam HI and Xho I. pBabe hygro HA-PP6 (this study) was derived by amplifying PP6 from NIH3T3 cDNA with primers: mPP6 sense (5′-CGGGATCCACCATGGCGCCGCTGGATCTG-3′) adding a Bam HI site 5′ to the start codon, and mPP6 antisense (5′-GCCGCTCGAGTCAAAGGAAATACGGCGTCGTG-3′) adding an Xho I site 3′ to the stop codon. The digested PCR fragment was cloned into pBabe hygro HA by cutting with Bam HI and Xho I. The exact DNA sequences can be obtained upon request.

Antibodies

Antibodies used in this study are listed in table S1.

Whole-cell extracts and alkaline treatment

Cells were lysed in IP Lyse [1% NP-40, 10% (v/v) glycerol, 135 mM NaCl, 20 mM tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (0.03 U/ml; Sigma) and 1× Complete (Roche)] for 15 min with rocking at 4°C. Lysates were scraped and cleared at 13,000*g* and normalized for total protein concentration. For alkaline treatment, 100 µl of lysate was mixed with NaOH to a final concentration of 0.2 M and incubated for 10 min at room temperature (RT). The reaction was neutralized by adding HCl to a final concentration of 0.2 M and diluted to 200 µl with IP Lyse. The control reaction was treated with preneutralization solution (0.2 M NaOH and 0.2 M HCl) and diluted to 200 µl with IP Lyse. The samples were boiled with protein sample buffer (Laemmli) for immunoblot analysis.

Immunoprecipitation

Whole-cell protein lysates of cell lines were incubated either with 12CA5 antibody cross-linked to bovine serum albumin (BSA)–coated protein A–Sepharose beads (GE Healthcare) to immunoprecipitate HA epitope–tagged PP2A C, with 4 µg of clone 1D6 to immunoprecipitate endogenous C subunits, or with 50 µl of clone 2G9 hybridoma supernatant to immunoprecipitate B for 1 hour and subsequently incubated with 20 µl of protein G–Sepharose beads (GE Healthcare) for 1 hour. The immune complexes were washed once with IP Lyse, three times with tris-buffered saline [25 mM tris, 135 mM NaCl, 2.6 mM KCl (pH 7.4) with HCl]. A tenth of the immunoprecipitate was used for the phosphatase assays (for more details, see phosphatase assays section below), and the rest of the immunoprecipitate was boiled for 5 min at 95°C in protein sample buffer for immunoblot analysis.

Western blotting and data analysis

Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose. Membranes were blocked with 3% nonfat dry milk (NFDM) in PBST (phosphate-buffered saline supplemented with Tween 20) for 1 hour at RT and probed with primary antibody diluted in 0.5% NFDM in PBST overnight at 4°C. After incubation with horseradish peroxidase–coupled secondary antibody, signals were visualized by enhanced chemiluminesence (GE Healthcare) and exposure of x-ray films. X-ray films of two to three exposures were scanned using a CanoScan LiDE220 scanner (Canon), and ImageJ was used for quantification of Western blot signals. For lysate quantifications, a 3× twofold dilution series of the strongest signal was fit to a linear or logarithmic trend line by Excel. The equation of the scan with the best R^2 value was used to calculate the values of the Western blot signals (fig. S1B).

All experiments were performed at least three times (*n*), and the values were presented as means ± SD; *n* in the figure panels and/or figure legend indicates the number of independent experiments. Statistical significance of immunoblotting and ELISA data were assessed using Student's *t* test for pairwise comparisons and one way analysis of variance (ANOVA) and a Tukey's honestly significantly different (HSD) post hoc test for threefold comparisons. In all cases, *P* values of <0.05 were considered to be statistically significant and are indicated with $*P < 0.05$, $**P < 0.01$, $***P < 0.005$, $****P < 0.001$. Statistical analysis was performed in Excel using the data analysis and real statistics add-ins.

Phosphorylase a and histone H1 phosphatase assays

Phosphatase activity of PP2A immunoprecipitates was assayed toward ^{32}P-labeled phosphorylase a and ^{32}P-labeled histone H1. ^{32}P-labeled phosphorylase a was prepared according to a protocol provided by D. Brautigan. First phosphorylase kinase was activated using PKA buffer [New England Biolabs (NEB)] [0.2 mM adenosine triphosphate (ATP), 24 U of phosphorylase kinase (Sigma, #P2014), 2 µl of PKA (NEB, #P6000)] in a total volume of 25 µl at 30°C for 20 min. For the phosphorylase b kinase reaction, the reagents were added in the following order: 50 mM tris-HCl (pH 8.5), 100 µM $CaCl_2$, 0.1 M β-glycerophosphate, 2% β-mercaptoethanol, 1 mM ATP, 10 mM Mg acetate, H_2O, cOmplete tablet EDTA free (Roche, #11873580001), 1 µM microcystin-LR (Alexis, #350-012-C100), 25 µl of γ^{32}P ATP (250 µCi) (PerkinElmer, #NEG 002A), 5.5 mg of phosphorylase b (provided by D. Brautigan), and activated phosphorylase kinase. The total reaction volume of 250 µl was incubated at 30°C for 2 hours, and afterward, 50 mM natriumfluorid was added for 10 min. The reaction was stopped by adding 262.5 µl of cold 90% ammonium sulfate (saturated), incubated on ice for 30 min, and centrifuged at 14,000*g* at 4°C for 10 min. After centrifugation, the pellet was washed six times with 45% ammonium sulfate. The final pellet was dissolved in 500 µl of solubilization buffer and dialyzed

with Slide-A-Lyzer (Dialysis Kit, 10,000 MWKO, 0.5- to 3-ml capacity; Thermo Scientific, #66382) against 1 liter of solubilization buffer [50 mM Mops (pH 7.0), 0.1 mM EDTA, 15 mM caffeine, 0.1% (v/v) β-mercaptoethanol]. The next day, the substrate was removed from the Slide-A-Lyzer, and the membrane was washed four times with 250 μl of prewarmed solubilization buffer, pooled in a 2-ml tube, and stored at 4°C.

To test catalytic activity of PP2A complexes toward phosphorylase a, $^{1}/_{10}$ of the immunoprecipitate was used. The beads were resuspended in 40 μl of phosphorylase assay buffer [0.1 mM EDTA and BSA (1 mg/ml)], and 20 μl of ^{32}P-labeled phosphorylase a solution (final concentration in the reaction was 10 μM) was added. Each reaction was incubated for exactly 15 min at 30°C on a thermo shaker with 1100rpm agitation. The phosphatase reactions were stopped by adding 180 μl of ice-cold 20% (w/v) trichloroacetic acid. The tubes were vortexed and placed on ice for at least 10 min and then centrifuged at 18,000*g* for 10 min at 4°C. Last, 200 μl of the clear supernatants was directly transferred to Scintillation Counter Vials (Greiner Bio-One) containing 5 ml of Ecoscint H Scintillation Solution (National Diagnostics). Last, the cpm (counts per minute) values of the released 32Pi from the histone HI and the phosphorylase a assays were normalized to the amount of immunoprecipitated HA-tagged or endogenous C subunits. The amounts of immunoprecipitated C subunits were determined by analyzing an aliquot of the immunoprecipitates by SDS-PAGE and immunoblotting as described above.

^{32}P-labeled histone HI was phosphorylated by cdc2 protein kinase (NEB). A detailed description can be found in (*60*). To test catalytic activity of PP2A complexes toward ^{32}P-histone HI, $^{1}/_{40}$ of the immunoprecipitate was transferred to fresh 1.5-ml tubes containing 10 μl of protein A–Sepharose in tris-buffered saline (TBS) 1:1 slurry because $^{1}/_{40}$ of the C subunit beads is hardly visible and, thus, would easily be lost during further steps of the experiment. The tubes were centrifuged at 120*g* for 1 min in a tabletop centrifuge (Eppendorf, 5417C) using a swing-bucket rotor (Eppendorf, A-12-11), and the supernatants were removed. The beads were resuspended in 10 μl of histone assay buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, BSA (1 mg/ml), 0.1% β-mercaptoethanol, 0.1 mM EDTA] and 10 μl of phosphatase reaction buffer [50 mM tris-HCl (pH 7.5), BSA (1 mg/ml), 0.1% β-mercaptoethanol, and 0.1 mM EDTA]. To start the phosphatase reaction, 10 μl of ^{32}P-labeled histone HI (final concentration in the reaction was 10 μM) was added to the reaction tubes. Each reaction was shortly vortexed and incubated for exactly 15 min at 30°C on a thermo shaker with 1100rpm agitation. The reaction was stopped by adding 100 μl of ice-cold 20% (w/v) trichloroacetic acid. The precipitates were incubated on ice for at least 10 min and then centrifuged at 18,000*g* for 10 min at 4°C. One hundred microliters of the clear supernatants was transferred to fresh 1.5-ml tubes containing 700 μl of a 1:1 (v/v) toluene/isobutanol solution, 467 μl of 5 mM tungstosilicic acid hydrate (Fluka) in 1 mM H_2SO_4, and 93 μl of 5% (w/v) ammonium heptamolybdate (Merck) in 2 M H_2SO_4. The extractions were thoroughly vortexed and centrifuged at 18,000*g* for 4 min at RT in a tabletop centrifuge. Five hundred microliters of the upper phase was lastly transferred to scintillation counter vials (Greiner Bio-One) containing 5 ml of Ecoscint H Scintillation Solution (National Diagnostics) and counted in a Tri-Carb Liquid Scintillation Analyzer 1900CA (Packard) to determine the amount of radioactivity released in the assay as ^{32}Pi. Last, the assay values were analyzed in the same manner as for the phosphorylase a assay described above.

PP2A immunoprecipitation phosphatase assays

We measured phosphate release as an index of phosphatase activity according to the manufacturer's instructions (Millipore, #17-313). Briefly, cells were lysed with imidazole buffer [20 mM imidazole-HCL (pH 7.0), 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, aprotinin (0.03 U/ml; Sigma), and 1× Complete (Roche)] with 2 × 5 s of sonication. Protein lysate (200 μg) was combined with 4 μg of 1D6 and 50 μl of protein A–agarose beads in 500 μl of TBS. The mixture was shaken for 2 hours at 4°C, and then beads were collected by centrifugation. After three washes with TBS, 60 μl of phosphopeptide KRpTIRR (750 μM) and 20 μl of Ser/Thr assay buffer were added to the beads and incubated on a shaking incubator at 30°C for 10 min. After a brief centrifugation, 25 μl of the reaction was added to a half-area microtiter plate, and 100 μl of Malachite Green Phosphate Detection Solution was added. After 15 min of incubation, the absorbance at OD_{600} was read with a 96-well plate reader (Victor^3).

Enzyme-linked immunosorbent assay

ELISA 96-well plates (Thermo Scientific, Medisorb) were coated with 50 μl of peptides (2 μg/ml in TBS) at 4°C overnight. The plate was blocked with 2% BSA in TBS for 1 hour at RT and incubated with primary antibodies (1D6 200 ng/ml, G-4 200 ng/ml, me C sub, 7C10 1:200 and nonme C sub, 1D7 1:500, and 7A6 1:100) in TBS for 2 hours at RT. Incubation with secondary peroxidase–conjugated anti-mouse was performed for 1 hour at RT followed by detection with TMB (3′,5′,5′,5′-tetramethylbenzidine; Sigma, catalog no. T2885) and H_2O_2 in a sodium acetate buffer (pH 6). The reaction was stopped by the addition of 1 N H_2SO_4, and the absorbance was measured at 450 nm; for background correction, the absorption of 560 nm was subtracted. For ELISA quantification, the signals of the antibodies were normalized to the signal of the cognate target peptide of the antibody, which was artificially set to 1.

Peptides

Peptides were purchased from piCHEM. The sequences of the peptides used in this study were as follows: Leu^{309}, Ac-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-Tyr-Phe-Leu-OH; meLeu^{309}, Ac-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-Tyr-Phe-Leu-OMe; pThr^{304}–Leu^{309}, Ac-His-Val-Thr-Arg-Arg-pThr-Pro-Asp-Tyr-Phe-Leu-OH; pThr^{304}–meLeu^{309}, Ac-His-Val-Thr-Arg-Arg-pThr-Pro-Asp-Tyr-Phe-Leu-OMe; pTyr^{307}–Leu^{309}, Ac-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-pTyr-Phe-Leu-OH; pTyr^{307}–meLeu^{309}, Ac-His-Val-Thr-Arg-Arg-Thr-Pro-Asp-pTyr-Phe-Leu-OMe.

Ethics statement

The maintenance of mice and rabbits and the experimental procedures have been conducted according to the Austrian Animal Experiments Act and have been approved by the Austrian Federal Ministry of Science and Research GZ 66.009/34-BrGT/2004, GZ 66.009/0040–II/3b/2011, GZ 66.009/0091–II/3b/2011, and BMWFW-66.009/0211-WF/V3b/2015, and the animal experiments ethics committee of the Medical University of Vienna.

SUPPLEMENTARY MATERIALS

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Fig. S1. 1D6 preferentially recognizes nonmethylated PP2A C subunit.

Fig. S2. Phosphorylation of Thr^{304} or Tyr^{307} influences epitope recognition by PP2A C-terminal antibodies.

Fig. S3. 1D6 and other C-terminal antibodies do not immunoprecipitate two major PP2A holoenzyme families.

Fig. S4. 7C10 specifically recognizes methylated PP2A C, and 2A10 also recognizes methylated PP4 C.
Table S1. Antibodies used in this study.

View/request a protocol for this paper from *Bio-protocol*.

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Acknowledgments: We thank G. Narla, J. Veis, and D. Virshup for critically reading the manuscript; the biostatistician of the Vienna BioCenter Core Facilities GmbH; A. Aszodi for statistical advice; and D. Brautigan (University of Virginia, Charlottesville, USA) for materials. **Funding:** This study was funded by royalty and service fees from antibody licensing agreements and our monoclonal antibody service facility. **Author contributions:** I.E.F. conceived, performed, and analyzed the experiments; prepared the figures; and wrote the manuscript. S.S., S.K., and I.M. performed and analyzed the experiments. E.O. conceived and analyzed the experiments and wrote the manuscript. **Competing interests:** E.O. serves as a consultant to Millipore Corporation. The Medical University of Vienna on behalf of the author E.O. is filing a patent on the PP2A methyl–C subunit–specific monoclonal antibody 7C10. The other authors declare that they have no competing interests. Monoclonal antibodies 7A6, 2A10, and 10C11 directed against the PP2A C subunit, as well as monoclonal antibodies 7B5-G4 against PP4, 7G7-A7 against PP6 C subunit, 2G9 against PP2A B, and 2A3 against pan-actin are licensed to biotech companies including Abcam, BioLegend, BioTechne/Novus, Cell Signaling Technology, EMD Millipore, ImmuQuest, Santa Cruz Biotechnology, and Sigma. **Data and materials availability:** All the data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials. Plasmids, cell lines, and antibodies used in this publication will be made available upon completion of a material transfer agreement to the Medical University of Vienna.

Submitted 10 April 2019
Accepted 17 December 2019
Published 28 January 2020
10.1126/scisignal.aax6490

Citation: I. E. Frohner, I. Mudrak, S. Kronlachner, S. Schüchner, E. Ogris, Antibodies recognizing the C terminus of PP2A catalytic subunit are unsuitable for evaluating PP2A activity and holoenzyme composition. *Sci. Signal.* **13**, eaax6490 (2020).

Antibodies recognizing the C terminus of PP2A catalytic subunit are unsuitable for evaluating PP2A activity and holoenzyme composition

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Sci. Signal. **13** (616), eaax6490.
DOI: 10.1126/scisignal.aax6490

Know thy antibodies

Knowing whether a given antibody is specific for its intended target or is sensitive to posttranslational modifications of that target is critical for interpreting experimental data generated with antibody reagents. Two Research Resources from the Ogris group highlight the importance of knowing the capabilities and limitations of antibody reagents. Frohner *et al.* found that various antibodies raised against the catalytic (C) subunit of protein phosphatase 2A (PP2A) were sensitive to methylation and phosphorylation of PP2A C, cross-reacted with related phosphatases, and failed to immunoprecipitate major subsets of trimeric holoenzymes. This implies that findings using a common commercial phosphatase assay kit that relies on one of these antibodies should be reevaluated. Schüchner *et al.* found that the recognition of Myc-tagged proteins by a widely used Myc-specific antibody varied depending on sequences adjacent to the tag. A Focus by Janes highlights the importance of systemic validation of research antibodies.