Targeted Disruption of the PME-1 Gene Causes Loss of Demethylated PP2A and Perinatal Lethality in Mice

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Abstract

Background: Phosphoprotein phosphatase 2A (PP2A), a major serine-threonine protein phosphatase in eukaryotes, is an oligomeric protein comprised of structural (A) and catalytic (C) subunits to which a variable regulatory subunit (B) can associate. The C subunit contains a methyl ester post-translational modification on its C-terminal leucine residue, which is removed by a specific methylesterase (PME-1). Methylesterification is thought to control the binding of different B subunits to AC dimers, but little is known about its physiological significance in vivo.

Methodology/Principal Findings: Here, we show that targeted disruption of the PME-1 gene causes perinatal lethality in mice, a phenotype that correlates with a virtually complete loss of the demethylated form of PP2A in the nervous system and peripheral tissues. Interestingly, PP2A catalytic activity over a peptide substrate was dramatically reduced in PME-1(−/−) tissues, which also displayed alterations in phosphoproteome content.

Conclusions: These findings suggest a role for the demethylated form of PP2A in maintenance of enzyme function and phosphorylation networks in vivo.

Introduction

Reversible protein phosphorylation regulated by the coordinated action of protein kinases and phosphatases is an essential signaling mechanism for most cellular processes. Post-translational phosphorylation is predicted to occur on more than 30% of all eukaryotic proteins [1]. While phosphorylation is catalysed by more than 500 characterized kinases, a relatively small number of phosphatases are responsible for removing protein phosphorylation sites [2]. In mammalian cells, phosphoprotein phosphatase 2A (PP2A), together with PP1, account for >90% of the total serine/threonine phosphatase activity [3]. PP2A is a ubiquitously expressed multimeric protein that is highly conserved across eukaryotes ranging from yeast to human. PP2A is involved in numerous cellular processes, including signal transduction pathways that regulate mitogenic and survival signals, transcriptional and translational events, and the cell cycle [4,5]. Moreover, several studies have identified alterations in specific PP2A subunits in human cancers, which has led to the hypothesis that PP2A serves as a tumor suppressor [6,7].

Despite the central role that PP2A plays in cell signaling, the biochemical mechanisms that regulate PP2A activity in vivo are poorly understood. This is in part due to the complex composition and post-translational modification of PP2A. Structurally, it is a heterotrimERIC complex typically consisting of a catalytic subunit (C), a scaffolding subunit (A or PR65) and one of an array of different regulatory subunits. In mammalian cells, the A and C subunits each have two isoforms (α and β), which share high sequence similarity. However, the most variable component of the holoenzyme is the regulatory subunit, which can belong to one of four different families termed B (or PR55), B′ (or B56 or PR61), B″ (or PR72) and B‴ (or PR93/PR110). Each one of these gene families encodes multiple isoforms and splice variants. The different families of B subunits bind to overlapping regions on the AC dimer so their association to the core enzyme is mutually exclusive. Combinatorially, this variety of B subunits could generate as many as 70 different holoenzyme assemblies [4].

The current model for regulation of PP2A suggests that heterotrimmers containing different regulatory subunits have distinct functions in vivo [8]. More specifically, B subunits, which exhibit cell- and tissue-specific expression, are considered to be a major determining factor of substrate specificity by, for example, targeting PP2A to different intracellular locations [4,9]. For example, the B subfamily has been shown to target PP2A to microtubules [10] while the B36 subfamily interacts with cyclin G and mediates PP2A functions in the Wnt/β-catenin and Erk signalling cascades [11–13]. Further support for this model stems from recent three-dimensional structural information obtained for the AC core enzyme [14] and the trimeric holoenzyme [15,16]. The intrinsic flexibility of the A scaffolding subunit [14] as well as...
the AC core dimer, which experiences profound conformational rearrangements after binding of the regulatory subunit [15,16], has been proposed to be important for binding different regulatory subunits and for performing catalysis on a wide variety of substrates.

A further level of PP2A regulation is provided by post-translational modification of the catalytic subunit, which can be phosphorylated on Tyr307, as well as on a threonine residue [17,18]. In addition, a unusual type of reversible methylation occurs at the carboxyl group of the C terminal Leu residue (Leu309) [19]. Carboxymethylation of Leu309 (referred to hereafter as “methylation”) is catalysed by a specific S-adenosymethylmethionine-dependent methyltransferase, termed leucine carboxymethyltransferase (LCMT) [20,21]. The demethylation reaction is catalysed by a specific PP2A methyltransferase (PME-1), which has been purified and molecularly characterized [22,23]. Whereas phosphorylation of the catalytic subunit of PP2A inhibits enzyme activity by modulating the binding of different regulatory B subunits, methylation is predicted to alter the targeting of PP2A to certain substrates and, as a consequence, potentially impact a wide range of signalling pathways [24]. Consistent with this model, disruption of the major methyltransferase responsible for methylating PP2A in yeast leads to severe growth defects [26]. On the other hand, deletion of the yeast PME-1 gene did not result in an observable cellular phenotype, even though PP2A methylation levels were enhanced [26]. More recent studies have proposed new models about the role of PME-1 in the regulation of PP2A. In the yeast, PME-1 seems to control the generation of active C subunit biogenesis and holoenzyme assembly [31]. In mammalian cells, and consistently with its predominant nuclear location, the major in vivo function of PME-1 has been proposed to be the stabilization of the inactive nuclear PP2A pool [32].

To more clearly understand the impact of methylation on PP2A function in mammalian biology, we have created mice that lack PME-1 by targeted gene disruption (PME-1(+/−) mice). Deletion of PME-1 resulted in post-natal lethality, a phenotype that seems to control the generation of active C subunit biogenesis and holoenzyme assembly [31]. In mammalian cells, and consistently with its predominant nuclear location, the major in vivo function of PME-1 has been proposed to be the stabilization of the inactive nuclear PP2A pool [32].

Materials and Methods

Generation of PME-1(−/−) Mice

The PME-1 gene was obtained as part of a commercial BAC clone (Invitrogen). The gene disruption construct was generated using PCR-amplified 5’ and 3’ homologous recombination fragments surrounding exon 7, which codes for amino acids 134–185 (including the catalytic serine nucleophile Ser156) of the PME-1 gene. The 5’ and 3’ homologous recombination fragments were subcloned into the NotI and BamHI sites (5’) and XhoI and internal HindIII sites (3’) of the pKO scrambler NTKV-1901 vector. Primers for 5’ homologous end: 5’-GC CTCGAG GAG ACT CAT ATT GGA AGC TGGA-3’ (forward, with XhoI site) and 5’-CAA GAG GGC TGC TAA CAC AGG-3’ (reverse). Homologous recombinant 129SvJ embryonic stem cell clones were identified by Southern blot analysis, and two such clones were used to generate chimeric mice on a C57BL/6 background. Chimeras from one of the two clones gave germline transmission of the mutated gene. All mice used in this study were first or second generation offspring from intercrosses of 129SvJ-C57BL/6 PME-1(+/−). All work performed in mice was done in accordance with the guidelines of the Institutional Animal Care and Use Committee of The Scripps Research Institute.

Genotyping of PME-1(+/+), (+/−) and (−/−) mice

For Southern blotting, genomic DNA was digested with HindIII and separated on a 1% agarose gel. Fragments were transferred to nylon membrane and probed with 5’ external probe. An external ~330 bp probe was generated by PCR using the PME-1 genomic BAC clone as template and the following primers: 5’-CAG TTA GCT AGG ATG TGC-3’ (forward primer 3’), 5’-CCA GAG GAA GTA AAC AGG-3’ (reverse primer 3’), 5’-TCT GGT GGG CTT ATA CGG-3’ (forward primer 5’), and 5’-TTT TCT GTG CTT CCT TCC-3’ (reverse primer 5’). 32P-labeled probe was hybridized overnight at 65°C.

PCR genotyping was performed using the following primers:
PME-1(+/+) primer set: 5’-G GTG TCT TCC TCC AGC ACT C-3’ and 5’-CCA TAC CAG GGG ACC TCC TAC-3’; PME-1(+/−) primer set: 5’-GTC ACA GGG GCA AAA CTG TC-3’ and 5’-GCC CCC GAT GAG ACT CAT ATT GGA AGC TGG-3’; and 5’-GCT CAC GAT GGG GCA AAT GGA AAA CTG TC-3’ and 5’-GCC CCC GAT TCG CAG GGC ATC-3’ that gave PCR products of 259 and 360 bp, respectively. After initial denaturation at 94°C for 4 min, PCR amplification was performed at a denaturing temperature of 94°C for 30 sec and followed by annealing at 60°C for 30 sec and extension at 72°C for 30 sec (35 cycles).

Western Blotting

Tissue homogenates were centrifuged at 100,000 g for 45 min at 4°C degrees to generate membrane and soluble fractions. Soluble fraction from each tissue was analysed by standard SDS-PAGE (10% NuPAGE Bis-Tris gel, Invitrogen) and western blotting fraction from each tissue was analysed by standard SDS-PAGE (10% NuPAGE Bis-Tris gel, Invitrogen) and western blotting with anti-tubulin (Sigma) were carried out as appropriate for loading control (not shown in the figures for the sake of clarity) and taking into account for band quantification as detailed in the corresponding legends.

PP2A Activity Assays

Phosphopeptide substrate assay. Phosphatase activity of E18 tissue homogenates was determined using the phosphopeptide KRPuRR as a substrate with a PP2A immunoprecipitation phosphatase assay kit (Upstate) following the manufacturers recommendations. Free PO42− generated was quantified by measurement of the absorbance at 595 nm after addition of malachite green-molibdate reagent. A standard curve with free phosphate was used to determine the amount of phosphate generated. p-Nitrophenylphosphate (pNPP) assay. Phosphatase
activity was determined following similar protocols to those previously described [25,33]. PP2A was immunoprecipitated with a PP2A antibody (Upstate) and protein A-agarose beads (Upstate) from 2 mg of E18 tissue homogenates. Washed beads were resuspended in assay buffer (50 mM Tris-HCl pH=8.5, 1.0 mM DTT, 10 mM MgCl2 and 0.10 mg/mL BSA) and incubated in presence of 10 mM pNPP at 37°C for 20 min. The reaction was terminated by addition of an equal volume of Na2CO3 1.0 M and absorbance was measured at 405 nm.

Phosphoproteomic analysis of mouse brain tissue

E18 brains from either PME-1(+/-) or (-/-) mice were homogenized in Trizol (Invitrogen) and proteins isolated following the manufacturers directions. The protein pellet was reconstituted in 7.5 μL of 6M guanidine-HCl (Gu-HCl) with phosphatase inhibitors (Sigma) and solubilized at 60°C for 30 min. Debris was pelleted by centrifugation and protein concentration was measured in the supernatant using a Bradford-based protein assay (BioRad). One milligram of total protein was brought to pH 8 by addition of 100 mM NH4HCO3, reduced with 10 mM dithiothreitol by incubation for 45 min at 60°C and alkylated with 20 mM iodoacetamide for 45 min in the dark. Proteomes were diluted with 100 mM NH4HCO3 to a final Gu-HCl concentration of 1 M and digested with trypsin (Promega, Madison, WI) overnight at 37°C. As an internal standard, the peptide FLAPGTDGAR was added to each extract. Peptides were desalted using SPE columns (Waters, Milford, MA), spiked with a second standard peptide (LIEDEAEPYTAG), and concentrated by vacuum centrifugation to a volume of 50 μL. Reductive alkylation was performed as described [34], with modifications. Briefly, desalted peptides (50 μL) from PME-1(+/-) were added to 450 μL 1 M HEPES buffer (pH 7.5), mixed with 40 μL formaldehyde (4% in water), vortexed, and then mixed immediately with 40 μL of freshly prepared cyanoborohydride (260 mM). The mixture was vortexed again and then allowed to react for 10 min. Tryptic peptides from PME-1(-/-) were labelled in a similar manner using d2-formaldehyde and sodium cyanoborohydride (Sigma-Aldrich). Afterwards, peptides were desalted, combined and then subjected to strong cation exchange (SCX) chromatography [35]. SCX fractions containing phosphopeptides were then analyzed using an automated immobilized metal affinity chromatography (IMAC)-HPLC/MS platform [36] coupled to an LTQ Orbitrap mass spectrometer (San Jose, CA). The mass spectrometer was operated in data dependent mode such that the top 5 most abundant ions in each MS scan (resolution = 100,000) were then analyzed using an automated immobilized protein database of human, mouse and rat proteins derived from the NCBI non-redundant protein database.

Results

Targeted disruption of the PME-1 gene

To generate mice lacking PME-1 [PME-1(-/-) mice], the exon that encodes amino acids 134–185, including the conserved G154HSMG158 motif that contains the catalytic serine nucleophile, was deleted by homologous recombination (Fig. 1A). Two homologously recombined 129S6/SvEv embryonic stem cell clones were identified by Southern blotting (Fig. 1B) and used to generate chimeric mice on a C57BL/6 background. One of these clones gave germline transfer of the mutated gene (Fig. 1C) and was used to create PME-1(-/-) mice on an outbred background. Western blotting with an anti-PME-1 antibody confirmed the absence of PME-1 protein in PME-1(-/-) mice (Fig. 2A).

Figure 1. Generation of PME-1(-/-) mice. (A) Genomic structure surrounding the deleted exons-7 that encodes amino acids 134–185 of the PME-1 protein. Only relevant restriction sites are designated. (B) Southern blot analysis of HindIII-digested genomic DNA using the indicated external probe, where the 4.6 and 8.8 kb bands correspond to PME(+/-) and (-/-) genotypes, respectively. (C) PCR analysis of mouse genomic DNA, where the 250 and 360 bp bands correspond to PME-1(+/-) and (-/-) genotypes, respectively. doi:10.1371/journal.pone.0002486.g001

Postnatal Lethality in PME-1(-/-) Mice

At the moment of birth, PME-1(-/-) mice showed no obvious abnormalities. However, these animals did not commence normal breathing or suckling behaviour and invariably died during first day of birth (Fig. 2B, upper panel). Further examination of animals on the day prior to birth (embryonic day 18.5) revealed the expected Mendelian frequency of genotypes [total pups: 128; PME(+/-) = 34 (26.6%), PME(-/-) = 64 (50%), PME(-/-) = 41 (24.2%)], with all genotypes appearing phenotypically normal. (Fig. 2B, lower panel). These data indicate that PME-1(-/-) mice develop normally in utero, but are incapable of surviving postnatal.

PME-1 is the primary enzyme responsible for the in vivo demethylation of the catalytic subunit of PP2A

PME-1 has been described as the enzyme that specifically demethylates the catalytic subunit of mammalian PP2A based on in vitro data using either reconstituted systems [22] or in lysates from PME-1-expressing bacteria [23]. However, in vitro verification of this hypothesis has remained lacking. We compared the levels of methylated and demethylated PP2A in tissues from PME-1(+/-) and (-/-) mice using methylation-specific antibodies. Brain extracts from PME-1(-/-) mice contained essentially no demeth-
Role of PME-1 in PP2A Function

Figure 2. Deletion of PME-1 leads to postnatal lethality in mice. (A) Western blot analysis showing the absence of PME-1 protein in tissues from PME-1(−/−) mice. (B) Upper panel, P0 littermates with genotypes shown [note dead pup for PME-1(−/−)]; lower panel, E18 embryos with genotypes shown. Scale bar, 1 cm.

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Decreased PP2A activity in PME-1(−/−) tissues

We next investigated the influence of the methylation state of PP2A on catalytic activity using a phosphopeptide substrate (KRpTIR). Immunoprecipitated PP2A from PME-1(−/−) tissues showed significant reductions in phosphatase activity, which were most dramatic in brain tissue (~4-fold reduction) (Fig. 4A). More than 80% of the phosphatase activity measured in this assay for both PME-1(+/+)(−/−) and (−/−) tissues was inhibited by the PP2A inhibitor okadaic acid, confirming that it derived from the PP2A enzyme (Fig. 4B). Previous research has not revealed a direct link between the methylation state of PP2A and catalytic activity, although none of these studies were conducted with a fully methylated enzyme expressed in native mammalian tissues. Instead, it has been proposed that PP2A methylation might affect phosphorylation at Tyr307, which has been found to inactivate PP2A [17]. We therefore attempted to measure the contribution of phosphorylated PP2A to the total phosphatase activity in PME-1(+/+) and (−/−) brains by immunoprecipitation with an anti-phosphotyrosine antibody. Western blotting of the beads and the supernatant with an antibody that specifically recognizes phospho-tyrosine 307 of PP2A, as well as with antibodies for the different forms of the catalytic PP2A subunit (total, methylated and demethylated) did not yield any appreciable signal in immunoprecipitated samples from either PME-1(+/+) or (−/−) tissue. Instead, signals for total, methylated and demethylated PP2A were exclusively observed in the supernatant fraction (no signal was observed for phospho-tyrosine 307 PP2A in any sample). These data indicate that phosphorylation of Tyr307 is not a major steady-state modification on PP2A in brain tissue of PME-1(+/+) and (−/−) mice.

Another mode of regulation of PP2A occurs through binding to the PP2A phosphatase activator (PTPA) protein. PTPA is highly conserved from yeast to human and was initially named based on its ability to stimulate the basal level of phosphotyrosyl phosphatase activity of PP2A [39–41]. However, recent findings suggest that the more likely physiological function of PTPA is to reactivate the Ser/Thr phosphatase activity of an inactive form of PP2A [33,42,43]. Therefore, we analyzed whether the observed differences in PP2A activity between PME-1(+/+) and (−/−) tissues could be attributed to variations in the levels of PTPA. However,
no significant changes in the levels of PTPA were observed in brain tissue from PME(+/+) and (-/-) mice (Fig. 4C).

Taken together, these data suggest that the decreased phosphopeptide phosphatase activity observed in PME-1(-/-) mice may be directly related to the absence of demethylated forms of the PP2A catalytic subunit. In order to establish whether the defective activity of PP2A was observed with other classes of substrates, we tested the activity of immunoprecipitated PP2A

Results represent mean values±SE for at least three independent experiments (**p<0.001, *p<0.01 for PME-1(+/+) versus (−/−) samples). (B) Inhibition of phosphatase activity of brain immunoprecipitated PP2A by okadaic acid. Results represent mean values±SE of two independent experiments performed in duplicate (**p<0.001 for phosphatase activity in absence versus presence of okadaic acid). (C) PTPA expression in brains from PME-1(+/+) and (−/−) E18 embryos. Blot is representative of two independent samples. (D) PP2A was immunoprecipitated from brain of PME-1(+/+) and (−/−) E18 embryos and phosphatase activity towards p-nitrophenylphosphate (pNPP) in presence or absence of okadaic acid was determined. Results are expressed in nmol of p-nitrophenol (pNP) released per 20 min and 2 mg of protein and represent mean values±SE for three independent experiments. Inset shows that the okadaic acid-sensitive fraction (obtained by subtraction of okadaic acid non-sensitive pNPPase activity from total pNPPase activity) of pNPPase activity (like the total pNPPase activity) was equivalent between PME-1(+/+) and (−/−) samples (**p<0.01 for phosphatase activity in absence versus presence of okadaic acid).

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Figure 4. PP2A phosphatase activity in tissues from PME-1(+/+) and (−/−) mice. (A) PP2A was immunoprecipitated from different tissues of PME-1(+/+) and (−/−) E18 embryos and phosphatase activity towards the phosphopeptide substrate KRpTIRR was determined. Results are expressed in pmol of phosphate released per 10 min and 250 μg of protein and represent mean values±SE for at least three independent experiments (**p<0.001, *p<0.01 for PME-1(+/+) versus (−/−) samples). (B) Inhibition of phosphatase activity of brain immunoprecipitated PP2A by okadaic acid. Phosphatase activity (like the total pNPPase activity) was equivalent between PME-1(+/+) and (−/−) samples. (C) PTPA expression in brains from PME-1(+/+) and (−/−) E18 embryos. Blot is representative of two independent samples. (D) PP2A was immunoprecipitated from brain of PME-1(+/+) and (−/−) E18 embryos and phosphatase activity towards p-nitrophenylphosphate (pNPP) in presence or absence of okadaic acid was determined. Results are expressed in nmol of p-nitrophenol (pNP) released per 20 min and 2 mg of protein and represent mean values±SE for three independent experiments. Inset shows that the okadaic acid-sensitive fraction (obtained by subtraction of okadaic acid non-sensitive pNPPase activity from total pNPPase activity) of pNPPase activity (like the total pNPPase activity) was equivalent between PME-1(+/+) and (−/−) samples (**p<0.01 for phosphatase activity in absence versus presence of okadaic acid).

towards the small-molecule substrate p-nitrophenylphosphate (pNPP). Interestingly, no significant differences were observed in pNPPase activity in brain tissue from PME-1(+/+) and (−/−) mice (Fig. 4D). Similar results were obtained when the okadaic acid-sensitive portion of pNPPase activity was taken into account (Fig. 4D, inset), which accounted for approximately 60% of the total pNPPase activity in brain tissue from PME-1(+/+) and (−/−) mice. These data suggest that the methylation state of PP2A selectively impacts the productive binding/recognition of phosphopeptide substrates rather than causing inherent defects in catalytic activity. A possibility that cannot be excluded from the present results is that the decrease in activity can be due to differential composition of the immunoprecipitates between the PME-1(+/+) and (−/−) samples. Although statistically significant variations in the levels of the immunoprecipitated C subunit (total and methylated) were not detected by western blot (data not shown), their different composition cannot be ruled out, especially since this phenomenon has been widely described to influence PP2A activity [4,8–13]. Therefore, instead of pursuing further characterization of in vitro activity towards a wide range of substrates (which may or may not reflect the physiological situation), we focused our efforts on profiling differentially phosphorylated proteins in the PME-1(+/+) and (−/−) mice.

Analysis of candidate PP2A substrates by phosphoproteomic mapping in brain tissue from PME-1(+/+) and (−/−) mice

Methylation state of PP2A has been previously involved in the specific recognition of substrates [4,26]. This fact, together with our data indicating impaired hydrolysis of a phosphopeptide substrate in tissues from PME-1(−/−) mice, suggested that this animal model might represent a suitable model to identify PP2A substrates for which PP2A demethylation (or methylation/ demethylation cycling) is required. To begin to test this idea, we conducted a comparative phosphoproteomic mapping of brain tissue from PME-1(+/+) and (−/−) mice. Proteins were extracted from whole brain with Trizol, reduced, alkylated and digested with trypsin. To allow quantification, the mixture of peptides was isotopically labeled as detailed in the materials and methods section. After labeling, the PME-1(+/+) and (−/−) proteomes were combined, subjected to SCX chromatography [35] and analyzed using an automated IMAC-HPLC/MS platform [36] coupled to an LTQ Orbitrap mass spectrometer. Following this methodology, phosphopeptides present in both proteomes appear in the mass spectrum as doublets separated by 6 m/z units per modified primary amine group in the peptide. Phosphopeptides that are unique to one or the other proteome appear as singlets. After phosphopeptide identification using SEQUEST [37], the ratio between the area of the peaks of the heavy and light version of the same phosphopeptide was used for quantification. Reproducibility of the peptide extraction, derivatization and analytical methodology was ensured by analyzing three d0- and d1- labelled independent proteomes. The proteins identified as up- or down-regulated in at least two of three independent experiments with a difference between PME-1(+/+) and (−/−) samples of at least two fold were considered significant hits and were manually validated (Tables 1 and 2).

Using the aforementioned criteria, we successfully sequenced 40 phosphopeptides corresponding to 38 proteins that differed between PME-1(+/+) and (−/−) samples. The majority of the identified proteins are known phosphoproteins (with the exception of phosphocalan). Interestingly, in addition to detecting some known PP2A interacting proteins and/or substrates [e.g., MAP1B [44,45], neurofilament protein [46] and Cdc2 [4,47], we have also characterized previously unidentified phosphorylation sites as candidates for PP2A action. Identified phosphoproteins mainly fell into three categories based on predicted function: proteins involved in cellular communication, transcriptional regulation and cellular structure. A limited number of proteins with roles in the DNA replication and protein synthesis (BM28 homologue and cellular structure). A limited number of proteins with roles in the DNA replication and protein synthesis (BM28 homologue and cellular structure). A limited number of proteins with roles in the DNA replication and protein synthesis (BM28 homologue and cellular structure).

A closer examination of the list of putative direct substrates of PP2A (i.e., phosphoproteins elevated PME(−/−) brain tissue)
### Table 1. Phosphoproteins increased in PME-1(+/−) brains

| General class                        | Name, accession number (gi) and phosphorylated residue | Fold change<sup>†</sup> | Known to interact with PP2A |
|--------------------------------------|--------------------------------------------------------|--------------------------|-----------------------------|
| **Cellular communication and signal transduction** | | | |
| Adaptor Molecules | Gab1 (10433473) (Ser 98) | 2.5 | Yes<sup>a</sup> |
| GEF | Dock7 (Rac GEF) (12698087) (Ser 600) | 2.5 | No |
| Protein kinases | Cdc2 (4502709) (Tyr 15) | 2.7 | Yes<sup>b</sup> |
| | SN12 (12643489) (Ser 437) | 3.33 | No |
| DNA replication | BM28 homolog (2183319) (Ser 27) | 2.6 | No |
| **Transcription factors and transcriptional regulators** | | | |
| | TF1B (3183181) (Ser 501) | 3.3 | No |

<sup>†</sup>Fold change is defined as the ratio between the area of the peaks in PME-1(+/−) to (+/+) samples.

See references [54]; [4], [47]; [55]; [44], [45]; [46]; [56].

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### Table 2. Phosphoproteins decreased in PME-1(+/−) brains

| General class                        | Name, accession number (gi) and phosphorylated residue | Fold change<sup>†</sup> | Known to interact with PP2A |
|--------------------------------------|--------------------------------------------------------|--------------------------|-----------------------------|
| **Cellular communication and signal transduction** | | | |
| Adaptor/Scaffold Molecules | X11 (423056) (Tyr 135) | 0.3 | No |
| Neuronal protein 4.1 (2224617) (Ser 784) | 0.4 | No |
| Palmitylated membrane protein 2 (4885493) (Ser 42) | 0.4 | No |
| Receptors | Mglu7 (547904) (Ser 900) | 0.4 | No |
| | Phosphacan (6755250) (Ser 276) | 0.1 | No |
| GAPs | GAP120 (6753930) (Ser 225) | 0.3 | No |
| Vesicle Trafficking | Syntaxin (9297065) (Ser 287 and 288) | 0.3 | Yes<sup>c</sup> |
| | Reticulon (12643485) (Ser 352) | 0.4 | No |
| **Transcription factors and transcriptional regulators** | | | |
| | RYBP (9790205) (Ser 201) | 0.4 | No |
| | PC4 AND SFRS1 (11024645) (Ser 106) | 0.5 | No |
| | Lbh (12052926) (Ser 163) | 0.2 | No |
| | MeCP2 (12083609) (Ser 80) | 0.3 | |
| | ZBP89 (12585539) (Ser 306) | 0.3 | No |
| Structural/Cytoskeletal | MAP1B (5174525) (Ser 1260) | 0.4 | Yes<sup>d</sup> |
| | Neurofilament protein (8393823) (Ser 766) | 0.1 | Yes<sup>e</sup> |
| | Drehlin (2498314) (Ser 659) | 0.3 | No |
| | Drehlin-like (7304993) (Thr 296 and Ser 274) | 0.3 | No |
| Actin binding proteins | Synaptopodin (2654323) (Ser 19) | 0.1 | No |
| Metabolism | CTPU (12643330) (Ser 315 and 319) | 0.5 | No |
| Unkown | KIAA1582 (10047239) (Ser 515) | 0.3 | |
| | AK003611 (12834382) (Ser 57) | 0.4 | |
| | AK009886 (12844957) (Ser 43) | 0.4 | |
| | AK011522 (12847703) (Ser56) | 0.3 | |
| Unclassified | GAP43 (8393415) (Ser 96) | 0.3 | Yes<sup>f</sup> |
| | HASPP28 (12018258) (Tyr 17) | 0.3 | |

<sup>†</sup>Fold change is defined as the ratio between the area of the peaks in PME-1(+/−) to (+/+) samples.

See references [54]; [4], [47]; [55]; [44], [45]; [46]; [56].

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suggests some molecular and cellular hypotheses potentially related to the pre-mature death observed in PME-1(−/−) mice. Gab1 plays an essential role in several steps of mammalian development. For example, in Gab1(−/−) mice, migration of myogenic precursor cells is impaired and muscles in the diaphragm are missing [40]. Dock 7 plays a critical role in axon development [49]. B-type lamins are found in all cell types and are expressed throughout development. In the nucleus, lamin B1 binds directly to chromatin and histones and has a direct role in DNA synthesis. An essential role for lamin B was confirmed by the analysis of mice deficient in this protein, which die in the perinatal period with defects in lung and bone [50,51]. Two additional proteins with altered phosphorylation in PME-1(−/−) mice, Cdc2 and the translation initiation factor 5 (eIF5), exert a broader influence over cellular processes by governing entrance into mitosis [47] and initiation of protein synthesis [52,53], respectively.

**Discussion**

The post-translational carboxylmethylation of the catalytic subunit of PP2A appears to exist in all eukaryotic organisms from yeast to human and, therefore, likely represents a key mechanism for regulating PP2A activity. Methylation has been hypothesized to influence the association of the PP2A heterodimer with different B regulatory subunits, which in turn control PP2A intracellular location and recognition of substrates. This model has been supported by various *in vitro* biochemical studies [14,27,28,30] and genetic experiments in yeast [25,26,29]. The latter results illuminated an important role for the primary PP2A methyltransferase in survival, but yeast lacking the major PP2A methyltransferase (Pme1) were without apparent phenotypic defect [26]. The endogenous functions of methylated/demethylated forms of PP2A in mammalian systems have not yet been explicitly tested.

Here we have investigated the function of mammalian PME-1 gene by deleting it from mice. PME-1 gene deletion resulted in perinatal lethality, a phenotype that correlated with essentially complete loss of the demethylated form of PP2A in brain tissue. Further studies revealed that PME-1(−/−) brain tissue also possessed significantly reduced PP2A activity with phosphopeptide substrates and diminished quantities of PP2A holocomplexes. To begin to assess the net biochemical and cellular effects of these changes in PP2A activity and complex assembly, we performed a comparative phosphoproteomic analysis of brain tissue from PME-1(+/+), PME-1(−/−), and (−/−) mice. Several phosphoproteins were identified that exhibited either elevated or reduced signals in PME-1(+/+) and (−/−) brains, suggesting that the absence of demethylated PP2A invokes widespread alterations in phosphorylation networks. Collectively, we interpret these results to indicate that demethylated PP2A plays essential non-redundant functions that cannot be undertaken by the methylated pool of this protein. This statement implicitly assumes that methylated PP2A is the main, if not the unique, PME-1 substrate. However, the data available about the absolute substrate specificity of PME-1 are far from being conclusive. Therefore, the possibility of the existence of alternative substrates that could potentially play a role in the lethality and/or differential PP2A activity can not be unequivocally ruled out. Having said this, and even in the presence of other potential PME-1 substrates, it is clear that no other enzyme can undertake the role of PME-1 in PP2A demethylation, since non-methylated PP2A is virtually absent from PME-1(−/−) tissues. Considering the importance that has been attributed to the methylation state of PP2A, it seems logical to hypothesize that at least part of the effects are due to the disruption of the PP2A methylation state. Precisely how the absence of demethylated PP2A leads to reductions in enzyme activity or differential recognition of substrates remains unclear. Possible explanations could be that proper functioning and stability of PP2A complexes requires the dynamic ability to switch between methylated and demethylated forms during the substrate binding/catalytic cycle. In the absence of PME-1, this cycling would be blocked, resulting in an imbalanced accumulation of methylated forms of PP2A. Another (and not necessarily mutually exclusive) explanation is that PME-1 is, itself, a key component of PP2A complexes *in vivo*. Potentially consistent with this latter idea, PME-1 has been shown to regulate active PP2A C subunit generation and holoezyme assembly [31] and to stably associate with “inactive” forms of PP2A complexes in tissues [32,42], suggesting that this protein may confer a regulatory effect on PP2A activity through both binding interactions as well as catalysis. This final hypothesis could be directly tested by attempting to rescue the biochemical and cellular phenotypes observed in PME-1(−/−) mice with catalytically active and inactive (e.g., Ser156-to-Ala) forms of the PME-1 enzyme. Such next-generation studies should further refine our understanding of the evidently critical role that post-translational methylation plays in regulating PP2A activity *in vivo*.

**Supporting Information**

**Figure S1** Expression of PP2A structural and regulatory subunits (A, B, and B′) in PME-1(+/+) and (−/−) tissues. Tissues were harvested from E10 embryos.

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**Author Contributions**

Conceived and designed the experiments: BC SO. Performed the experiments: SO SF. Analyzed the data: SO SF EP. Contributed reagents/materials/analysis tools: SO DL. Wrote the paper: BC SO.

**References**

1. Hubbard MJ, Cohen P (1993) On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem Sci 18: 172–177.
2. Hunter T (2000) Signaling—2000 and beyond. Cell 100: 113–127.
3. Oliver CJ, Shenolikar S (1998) Physiologic importance of protein phosphatase inhibitors. Front Biosci 3: D961–972.
4. Janssens V, Goris J (2005) Protein phosphatase 2A: a panoply of enzymes. Curr Opin Genet Dev 15: 34–41.
5. Millward TA, Zolnierowicz S, Hemmings BA (1999) Regulation of protein kinase cascades by protein phosphatase 2A. Trends Bioi Sci 24: 106–111.
6. Janssens V, Goris J, Van Hoof C (2005) PP2A: the expected tumor suppressor. Janssens V, Goris J, Van Hoof C (2005) PP2A: the expected tumor suppressor. PLos ONE 2008;3(7):e2486.
7. Li HH, Cai X, Shouke GP, Piluso LG, Liu X (2007) A specific PP2A regulatory subunit, B56c, mediates DNA damage-induced dephosphorylation of p53 at Thr55. EMBO J 26: 402–411.
8. Silverstein AM, Barrow CA, Davis AJ, Mumby MC (2002) Actions of PP2A on the MAP kinase pathway and apoptosis are mediated by distinct regulatory subunits. Proc Natl Acad Sci USA 99: 4221–4226.
9. Virshup DM (2000) Protein phosphatase 2A: a panoply of enzymes. Curr Opin Cell Biol 12: 180–185.
10. Sontag E, Nunbhakdi-Craig V, Bloom GS, Mumby MC (1995) A novel pool of protein phosphatase 2A to dephosphorylate Mdm2. Mol Cell 9: 761–771.
12. Seeling JM, Miller JR, Gil R, Moon RT, White R, et al. (1999) Regulation of beta-catenin signaling by the B56 subunit of protein phosphatase 2A. Science 283: 2089–2091.

13. Leitounieux C, Rocher G, Porteu F (2006) B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. EMBO J 25: 727–738.

14. Xing Y, Xu Y, Chen Y, Jeffrey PD, Chao Y, et al. (2006) Structure of protein phosphatase 2A core enzyme bound to tumor-inhibiting toxins. Cell 127: 341–353.

15. Xu Y, Xing Y, Chen Y, Chao Y, Lin Z, et al. (2006) Structure of the protein phosphatase 2A holoyzyme. Cell 127: 1239–1251.

16. Chen J, Martin BL, Braunitz DL (1992) Regulation of protein serine-threonine phosphate type-2A by tyrosine phosphorylation. Science 257: 1261–1264.

17. Guo H, Damani Z (1995) Auto phosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. Proc Natl Acad Sci U S A 90: 2300–2304.

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

19. De Baere I, Deura R, Janssens V, Van Hood C, Waelkens E, et al. (1999) Purification of porcine brain protein phosphatase 2A leucine carboxyl methyltransferase and cloning of the human homologue. Biochemistry 38: 16539–16547.

20. Leuillon N, Quevillon-Cheruel S, Soel I, de la Sierra-Gallay IL, Collinet B, et al. (2004) Structure of protein phosphatase methyltransferase 1 (PPMA1), a leucine carboxyl methyltransferase involved in the regulation of protein phosphatase 2A activity. J Biol Chem 279: 8351–8358.

21. Lee J, Chen Y, Tolstykh T, 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, Du X, Nelson KG, Mak EK, Yu XS, et al. (1999) A protein phosphatase methyltransferase (PPME-1) is one of several novel proteins stably associating with two inactive mutants of protein phosphatase 2A. J Biol Chem 274: 14382–14391.

23. Zhu T, Matsuzawa S, Mizuno Y, Kamibayashi C, Munby MC, et al. (1997) The interconversion of protein phosphatase 2A between PP2A1 and PP2A0 during retinoic acid-induced granulocytic differentiation and a modification on the catalytic subunit in S phase of HL-60 cells. Arch Biochem Biophys 339: 210–217.

24. Wu J, Tolstykh T, Lee J, Vafai S, Stock J (2000) Carboxyl methyltransferase regulates protein phosphatase 2A by controlling the association of regulatory B subunits. EMBO J 19: 5682–5691.

25. Xing Y, Xu Y, Chen Y, Jeffrey PD, Chao Y, et al. (2006) Structure and mechanism of protein phosphatase 2A regulatory subunit is mediated by the C terminus of the catalytic subunit. J Biol Chem 282: 26971–26980.

26. Cayla X, van Hoof C, Bosch M, Waelkens E, Van de Weyer J, et al. (1994) Molecular cloning, expression, and characterization of PTPA, a protein that activates the tyrosyl phosphatase activity of protein phosphatase 2A. J Biol Chem 260: 15668–15675.

27. Leulliot N, Quevillon-Cheruel S, Sorel I, de la Sierra-Gallay IL, Collinet B, et al. (2004) Structure of protein phosphatase methyltransferase 1 (PPMA1), a leucine carboxyl methyltransferase involved in the regulation of protein phosphatase 2A activity. J Biol Chem 279: 8351–8358.

28. Guo H, Damani Z (1995) Auto phosphorylation-activated protein kinase phosphorylates and inactivates protein phosphatase 2A. Proc Natl Acad Sci U S A 90: 2300–2304.

29. Wei H, Ashby DG, Moreno CS, Ogris E, Yeong FM, et al. (2001) Protein phosphatase 2A subunit. Biochem J 339: 241–246.

30. Yu XX, Du X, Moreno CS, Ogris E (1997) Generation of active protein phosphatase 2A core enzyme bound to tumor-inhibiting toxins. Cell 127: 183–196.

31. Beaudouin SA, Jedrychowski M, Schwartz D, Elias JE, Villein J, et al. (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci U S A 101: 12130–12135.

32. Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, et al. (2002) Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae. Nat Biotechnol 20: 301–416.

33. Eng JK, McCormick AL, Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 5: 976–989.

34. Longin S, Zwaanepoel K, Louis JV, Dinh Dinh S, Goris J, et al. (2007) Selection of protein phosphatase 2A regulatory subunit is mediated by the C terminus of the catalytic subunit. J Biol Chem 282: 26971–26980.

35. Letourneux C, Rocher G, Porteu F (2006) B56-containing PP2A dephosphorylate ERK and their activity is controlled by the early gene IEX-1 and ERK. EMBO J 25: 727–738.