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Decreased Protein Phosphatase 2A Methylation Results in Elevated Tau Phosphorylation During Mouse Embryogenesis

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Abstract

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By Paul R. Evans

Protein phosphatase 2A (PP2A) is a heterotrimeric serine/threonine phosphatase with implications in many cellular processes including cell cycle and tau phosphorylation regulation. PP2A is composed of a structural A subunit, catalytic C subunit, and a variable regulatory B subunit. We have previously shown that in mammalian cells, leucine carboxyl methyltransferase-1 (LCMT-1) must methylate PP2A C for holoenzyme formation with Bα subunits, and a complete knockout of LCMT-1 in mice results in embryonic lethality (Lee and Pallas, 2007). Further, the BαAC heterotrimer is the main tau phosphatase, accounting for over 70% of total tau phosphatase activity (Liu et al., 2005). Elevated tau phosphorylation levels are a hallmark of tauopathies, human neurodegenerative disease associated with tau neurofibrillary degeneration and dementia. Given that BαAC heterotrimers only form after LCMT-1 methylates PP2A and this isoform is responsible for the majority of tau phosphatase activity, we predict that a knockout of LCMT-1 in an in vivo mammalian mouse model will result in elevated tau phosphorylation by synergizing with a tau P301L mutant transgene. We tested our hypothesis using quantitative immunoblotting and immunohistochemistry with day 12.5 mouse embryos. We report that a full, but not partial, knockout of LCMT-1 results in extremely downregulated LCMT-1 expression, PP2A methylation, and increased 3R and 4R tau phosphorylation.

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List of Abbreviations

3R Tau	Tau embryonic isoform containing three microtubule binding repeats
4R Tau	Tau adult isoform containing four microtubule binding repeats
V	Trigeminal nerve ganglion and rootlets
VIII	Vestibulocochlear ganglion
Aβ	Beta-amyloid
AD	Alzheimer's disease
Cdc55	*S. cerevisiae* gene involved in cellular morphogenesis homologous to PP2A's B subunit
DE	Diencephalon
FTDP-17	Frontotemporal dementia with Parkinsonism-linked to chromosome 17
HEAT	A repeated protein domain motif found on PP2A's A subunit, named for its elements (huntingtin-elongation-A subunit-TOR)
IHC	Immunohistochemistry
LCMT-1	Leucine carboxyl methyltransferase-1
Leu309	Amino acid (leucine) residue 309 on PP2A's C subunit
LV	Lateral Ventricle
MAP-T	Microtubule associated protein tau
MB	Midbrain
Neo	Roof of the neopallial cortex
NFT	Neurofibrillary tangles
P-M	Region of the pons-midbrain junction
P301L	Tau mutation resulting in a proline-leucine substitution of at amino acid residue 309
PME-1	Protein phosphatase methylesterase-1
PP2A	Protein phosphatase 2A
Ppm1p	Protein phosphatase methyltransferase-1; *S. cerevisiae* PP2A methyltransferase
PPMT	Protein phosphatase methyltransferase-1 (also referred to as LCMT-1)
PTPA	PP2A phosphatase activator
SCC	Region of the posterior semicircular canals
Str	Striatum
Ser	Serine
Thr	Threonine

Introduction

Protein Phosphatase 2A Structure

Protein phosphatase 2A (PP2A) is a highly conserved multifunctional serine/threonine phosphatase that dephosphorylates a large number of substrates conferring an important role for this phosphatase in several physiological processes that include cell cycle regulation, progression of human cancers, and development of tauopathies (Janssens and Goris, 2001; Virshup, DM, 2000; Mumby and Walter, 1993). The PP2A core enzyme is a dimer composed of a 65-kD structural (A) subunit and a 36-kD catalytic (C) subunit. The C subunit associates with the A subunit by recognition of its HEAT (huntingtin-elongation-A subunit-TOR) repeats 11-15 (Xing et al., 2006). The PP2A core enzyme interacts with a variable regulatory (B) subunit to create the PP2A holoenzyme. To date, five families of B-type PP2A regulatory subunits have been identified as B (B55/PR55), B' (B56/PR61), B'' (PR72), putative B'''(striatin), and viral antigens with each family containing multiple subunit isoforms. PP2A exists *in vivo* as either an A/C heterodimer or as a BAC heterotrimer. Holoenzyme formation with different B-type regulatory subunits is an important mechanism of determining PP2A activity, localization, and substrate specificity (Sontag et al., 1995; McCright et al., 1996, Griswold-Prenner et al., 1998).

Protein Phosphatase 2A Regulation

Aside from B-type subunit association, several other regulatory processes act on PP2A including PP2A specific physiological inhibitors, a PP2A phosphatase activator (PTPA), lipid activation, and posttranslational modifications (Bialojan and Takai, 1988;

Jordens et al., 2006; Widau et al., 2010; Pallas et al., 1986; Pallas et al., 1990; Lee and Pallas, 2007). Regulatory events have been implicated in altering the phosphatase activity, subcellular localization, protein-protein interactions, and heterotrimer assembly of PP2A (Mumby, 2001). Of these processes, much focus has been placed on the posttranslational modifications that regulate PP2A's assembly and function (Lee and Pallas, 2007; Liu et al., 2008)

This study focuses on the role of reversible carboxymethylation of PP2A C subunit by a specific methyltransferase. Protein methylation has been shown to be reversible at carboxyl groups, and it can have varying effects depending on its target (Clarke, 1985). Reversible carboxymethylation of the PP2A catalytic subunit has been shown to occur in a cell cycle dependent manner and to regulate heterotrimer formation in both *Saccharomyces cerevisiae* and in mammalian cells (Turowski et al., 1995; Tolstykh et al., 2000; Wu et al., 2000; Yu et al., 2001; Wei et al., 2001; Lee and Pallas, 2007). Methylation of the catalytic subunit occurs at the carboxy-terminal Leucine 309 (Leu309) in the conserved $TPDYFL_{309}$ motif (as reviewed by Shi, 2009). We have previously reported that deletion of the *S. cerevisiae* methyltransferase homolog (Ppm1p) results in greatly decreased PP2A trimer formation with the B family homolog (Cdc55) (Wei et al, 2001). We have also shown that carboxymethylation at Leu309 of the PP2A C subunit is required for formation of BαAC heterotrimers but not with other regulatory subunits (Yu et al., 2001).

It is known that Leu309 carboxymethylation occurs at the α-carboxyl group of this leucine residue by leucine carboxyl methyltransferase-1 (LCMT-1) in mammalian cells (Xie and Clarke, 1993; Lee and Stock, 1993; Xie and Clarke, 1994; Favre et al.,

1994; Li and Damuni, 1994; Floer and Stock, 1994; De Baere et al, 1999). As shown in Figure 1, LCMT-1 is opposed by protein phosphatase methylesterase-1 (PME-1), which functions to demethylate Leu309 of PP2A C subunit (Ogris et al., 1999; Xie and Clarke, 1994; Lee et al., 1996).

Most recently, we have demonstrated using small hairpin knockdown cell lines that LCMT-1 is the major mammalian PP2A methyltransferase, and LCMT-1 is required for formation of BαAC heterotrimers (Lee and Pallas, 2007). Further we have shown that homozygous knock-out of LCMT-1 using gene trap techniques results in embryonic lethality in mice (Lee and Pallas, 2007). This is the first in vivo model of decreased PP2A catalytic subunit methylation resulting from a decrease in LCMT-1, which normally performs this function. Thus, carboxymethylation of the PP2A C subunit is a critical regulatory feature of cellular pathways that involve BαAC heterotrimers.

Tau: Function and Regulation

Microtubule associated protein tau (MAP-T) is mainly expressed neuronally and normally functions *in vivo* and *in vitro* to stabilize and promote the assembly of microtubules by interactions with tubulin (Weingarten et al., 1975). Although encoded by a single gene, tau is found in six isoforms in the adult human brain due to alternative splicing (Goedert et al., 1989). These tau isoforms differ by the number of carboxy terminal microtubule binding repeats (three repeats (3R) or four repeats (4R)) and the number of amino terminal inserts (zero (0N), one (1N), or two (2N) inserts). 4R tau isoforms are both more efficient at binding tubulin and are preferentially sequestered by abnormally phosphorylated tau (pTau) relative to 3R isoforms (Sontag et al., 1999; Iqbal et al., 2009).

Forty-four tau serine/threonine phosphorylation sites have been identified as targets by the major tau kinases (Gong et al., 2005). Normal neuronal tau contains 2-3 moles of phosphate per mole of the protein, which is thought to relate to its ability to bind and regulate microtubules *in vivo* (Kopke et al., 1993; Matsuo et al., 1994). 0N3R tau is the only isoform expressed in the fetal brain, and 0N3R tau is reversibly hyperphosphorylated during development when tubulin levels are the highest (Goedert et al., 1989).

Disregulation of Tau and Tauopathies

Tau phosphorylation is normally a dynamic process, but if tau is abnormally hyperphosphorylated it can aggregate and form neurofibrillary tangles of paired helical fragments and/or straight filaments followed by truncation of the protein (Iqbal et al., 2009). Aberrant phosphorylation can result from either upregulated kinase activity or downregulated phosphatase activity. Glycogen synthase kinase-3 (GSK-3), cyclin dependent protein kinase-5 (cdk5), and protein kinase A (PKA) have been shown, among other kinases, to be connected with tau hyperphosphorylation (Hanger et al., 2009). PP2A is the major neuronal tau phosphatase accounting for 71% of all total tau phosphatase activity *in vitro* (Liu et al., 2005). Liu et al. (2005) also showed that pSer262 is the most preferred substrate of PP2A. It has also been shown that BαAC heterotrimers are the brain dominant PP2A isoform, and BαAC heterotrimers have the most tau phosphatase activity of PP2A holoenzymes and may directly regulate tau phosphorylation *in vivo* (Goedert et al., 1995; Sontag et al., 1996).

All tauopathies, human neurodegenerative diseases that involve neurofibrillary degeneration, are marked by neurofibrillary tangle formations (NFTs) that are composed

of abnormally hyperphosphorylated tau and their occurrence in the neocortex is associated with dementia. Examples of tauopathies include Alzheimer's disease (AD), Down syndrome, Pick disease, and Frontotemporal dementia with Parkinsonism-linked to chromosome 17 (FTDP-17). The cause of tauopathies such as AD have been debated in great detail, and the Amyloid Cascade Hypothesis is currently the predominant hypothesis for the cause of AD positing that Aβ is the primary cause of AD, with NFT formation and dementia as secondary events (Savioz et al., 2009). However, several lines of evidence contradict this hypothesis. The main fault in this hypothesis is that cognitive impairment best correlates with the degree of neurofibrillary damage, not the number of senile plaques, and β-amyloidosis alone does not cause clinical dementia (Arriagada et al., 1992).

Alzheimer's Disease and PP2A

Studies have reported post mortem PP2A-related variations in human AD, the most predominant tauopathy, and control brains (Sontag et al., 2004a; Sontag et al., 2004b). Sontag et al. (2004a) demonstrated with immunoblot and immunohistochemical analysis that BαAC heterotrimers had overall reduced expression in AD post mortem brains, excluding cerebellum, relative to post mortem brains of control and non-AD dementia cases, and the immunohistochemistry indicated that BαAC heterotrimers expression was specifically decreased in AD-affected regions and NFT positive neurons. Decreased PP2A C subunit methylation was then shown to correlate regionally with a decrease in LCMT-1 (referenced PPMT) expression and an increase in the extent of tau neuropathology in post mortem human AD but not control and non-AD dementia case brains (Sontag et al., 2004b). This study suggests that a decrease in PP2A C subunit

methylation resulting from decreased LCMT-1 expression could be responsible for tau pathology in AD.

Subsequent *in vitro* studies reported possible contributing factors to deregulation of PP2A C subunit methylation (Sontag et al., 2007; Sontag et al., 2008). In one study, deficient homocysteine metabolism was analyzed by incubating neuroblastoma cells with S-adenosylhomocysteine (SAH), a byproduct of elevated blood homocysteine levels (Sontag et al., 2007). This treatment as well as increased PME-1 expression resulted in a decrease in PP2A methylation, an increase in tau and amyloid precursor protein phosphorylation, and altered secretase pathway function. Increased SAH levels resulting from a folate deficient diet created the same results as well as downregulated LCMT-1 (Sontag et al., 2007). Sontag et al. (2008) further showed that folate starved neuroblastoma cells exhibit downregulated LCMT-1, decreased PP2A C methylation and cell death. These results were exaggerated with PME-1 overexpression and rescued with LCMT-1 or Bα overexpression. Also, mice fed folate deficient diets showed similar results: downregulation of LCMT-1, PP2A C methylation, and Bα expression and increased tau phosphorylation.

Hypotheses and Approach

Based on previous knowledge of the role of LCMT-1 in regulating BαAC heterotrimer formation and the role of this PP2A holoenzyme in regulating tau phosphorylation, we hypothesized that downregulation of LCMT-1 expression in a mammalian *in vivo* model will result in increased tau phosphorylation. We specifically predicted that embryonic day 12.5 tau transgenic (P301L) LCMT-1 full knock-out (-/-) mouse embryos will display: (1) increased 3R and 4R tau phosphorylation levels, (2) no

LCMT-1 expression, and (3) severely decreased PP2A catalytic subunit methylation, relative to tau transgenic (P301L) LCMT-1 full wild-type (+/+) mouse embryos. We also predicted that embryonic day 12.5 tau transgenic (P301L) LCMT-1 partial knock-out (+/-) mouse embryos will exhibit similar, but much less pronounced, results relative to tau transgenic (P301L) LCMT-1 full wild-type (+/+) mouse embryos.

Several methods were used in our approach to test this hypothesis. First, tau transgenic mice expressing human tau with a P301L mutation (Lewis et al., 2000; Taconic Farms, Inc.) were crossed with LCMT-1 mice to assess the effect of LCMT-1 partial-knockout and full-knockout with the presence of the transgenic tau. The P301L mutation is a familial, missense point mutation found in FTDP-17 resulting in a proline-leucine substitution at the 301st amino acid (numbered according to the 441-residue tau isoform). This P301L tau mutation has been shown to cause NFT formation with selective deposition of this mutant tau (Spillantini et al., 2000; Lewis et al., 2000; Miyasaka et al., 2001). Of particular interest, this mutation has been shown to reduce the binding of tau *in vitro* to BαAC heterotrimers by 25% and to microtubules by 30% (Goedert et al., 2000).

In this study we tested whether loss, of one or both alleles, of LCMT-1 was sufficient to increase tau phosphorylation levels. We expected that tau phosphorylation levels would increase due to decreased BαAC heterotrimer formation, and thus tau phosphatase activity. Quantitative immunoblotting and immunohistochemistry were used to assess the effects of LCMT-1 deficiency in day 12.5 mouse embryos.

Methods

Breeding

As previously described, LCMT-1 (+/-) embryonic stem cells containing a gene trap insertion were obtained from German Gene Trap Consortium, Neuherberg, Germany (Lee and Pallas, 2007). The splice acceptor construct of the gene trap vector was found to have inserted approximately 10107 base pairs within intron one of the LCMT-1 gene. Following expansion in culture, these cells were injected into donor C57BL/6 blastocysts using standard techniques by the Emory University School of Medicine Transgenic Mouse and Gene Targeting Core Facility. This colony maintains both wild-type (+/+) and partial knockout (+/-) LCMT-1 genotypes. A knockout of both LCMT-1 alleles (-/-) is embryonically lethal. The LCMT-1 knockout line has been established and bred down 11 generations maintained exclusively in the C57BL/6 mouse background.

A male mouse homozygous for P301L transgenic tau (P301L/P301L) of a C57BL/6, DBA/2, SW mixed background was acquired from Taconic Farms, Inc. This model expresses human microtubule-associated protein tau (MAP-T) with a P301L P301Lation under the mouse prion promoter (MoPrP). Mice heterozygous (+/P301L) and homozygous (P301L/P301L) for the P301L transgene develop NFTs at 6.5 and 4.5 months, respectively (Lewis et al., 2000). This male [LCMT-1 (+/+) Tau (P301L/P301L)]was crossed with LCMT-1 partial knockout females [LCMT-1 (+/-) Tau (+/+)] creating offspring with Tau (+/P301L) and either LCMT-1 (+/+) or (+/-) genotypes. These F1 offspring were then intercrossed to establish a separate LCMT-1/Tau mouse colony to use in timed mating dissections. Mice were maintained under guidelines approved by the Institutional Animal Care and Use Committee and in

accordance with regulations of Emory University School of Medicine Department of Animal Resources.

Genotyping

Genomic DNA was isolated from tails of postnatal mouse pups by incubation in tail lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.2% sodium dodecyl sulfate (SDS), 200 mM NaCl, 100 μg Proteinase K/mL) overnight in a shaking incubator at 56°C. Tails were then centrifuged in a Microfuge 18 Centrifuge (Beckman Coulter) for 5 min. at 18,000 x g to pellet debris. Supernatants were transferred to separate microcentrifuge tubes, mixed with an equal volume of 100% isopropanol, and centrifuged in a Microfuge 18 Centrifuge (Beckman Coulter) for 5 min. at 18,000 x g to pellet genomic DNA. Supernatants were removed, and DNA pellets were resuspended in nuclease free water and stored at -20°C pending genotype analysis.

Genomic DNA was isolated from yolk sacs of mouse embryos by incubation in PCR lysis buffer (50 mM Tris pH 8.8, 1 mM EDTA, 0.5% Tween 20, 100 μg Proteinase K/mL) overnight in a shaking incubator at 56°C. Yolk sacs were agitated for 5 min. in a Super Mixer II (Baxter Scientific) and heated for 10 min. at 99°C. Samples were centrifuged in a Microfuge 18 Centrifuge (Beckman Coulter) for 5 min. at 18,000 x g to pellet debris. Supernatants containing genomic DNA were transferred to separate microcentrifuge tubes and stored at -20°C pending genotype analysis.

Genotypes were determined by PCR analysis using GoTaq Green Master Mix (Promega) carried out on a C1000 Thermocycler (Bio-Rad). Each LCMT-1 PCR reaction contains a primer pair specific for a wild-type LCMT-1 (production of the corresponding 200bp band indicates the presence of at least one wild-type LCMT-1 allele without a

gene trap insertion) and one primer pair specific for an LCMT-1 allele containing the gene trap insertion (Genetrap; forward primer matching the LCMT-1 intron and reverse primer matching the gene trap insert producing a 254bp band) (Lee and Pallas, 2007). The transgenic P301L tau allele was detected using a primer pair provided by Taconic Farms, Inc. The presence of a 254 base pair band indicates the presence of the P301L tau allele (either one or two copies assigned the genotype Tau (P301L)), and the absence of the allele indicates that the mouse is wild-type. A primer pair was also included specific to a housekeeping gene (sequence provided by Taconic Farms, Inc.) producing a 200 bp band, indicating that the PCR reaction was successful.

Timed Mating Dissection

Due to the embryonic lethality that results from a full knock-out of LCMT-1, timed-mating dissections were performed at embryonic day 12.5 to analyze this genotype (Lee and Pallas, 2007). This stage of development was selected because non-tau transgenic LCMT-1 (-/-) embryos are still seen at an average frequency of approximately 20% relative to the total litter (D. Pallas Laboratory, Unpublished data). All embryos used in this study carried at least one transgenic tau allele (P301L); distinctions between heterozygous and homozygous tau transgenic mice could not be made.

Timed mating cages were established intercrossing LCMT-1 (+/-) Tau (+/P301L) genotypes. The females were inspected daily, morning and afternoon, for presence of a vaginal copulation plug to indicate time of conception. On day 12.5, females were sacrificed, and concepti were immediately collected. The placenta, yolk sac, and embryos were mechanically separated under a dissection microscope. Embryos were washed in 0.4% bovine serum albumin (BSA) in phosphate buffered saline (PBS), photographed

under 16X magnification, and weighed. The yolk sac was retained and washed in PBS, and DNA was isolated for PCR analysis to determine embryo genotype. If used for western blot analysis, embryo heads were mechanically separated from the torso, flash-frozen in liquid nitrogen, and stored at -80°C pending genotype analysis. If used for western blot analysis, embryos were fixed in 4% paraformaldehyde (PFA) in PBS at 4°C for 24 hours.

Tissue Homogenization

Flash-frozen embryo heads were retrieved from storage at -80°C, and immediately homogenized on ice in an equal volume of lysis buffer (700mM glycerol, 10mM Tris pH 8.0, 70mM NaCl, 0.025 mM Aprotonin (Sigma-Aldrich), 2mM PMSF (Sigma-Aldrich)). Homogenates were then centrifuged in a Microfuge 18 Centrifuge (Beckman Coulter) for 10 min. at 18,000 x g at 4°C to pellet tissue and debris, and the supernatant was kept on ice pending methylation assay and tau enrichment.

PP2A Catalytic Subunit Methylation Assay

As previously described, the methylation assay is utilized to determine the *in vivo* state of PP2A C subunit methylation (Yu et al., 2001). 5 µL aliquots of lysate were separated into two tubes containing lysis buffer on ice. The (-) aliquot was treated with a pre-neutralized solution (2:2:1/200mM NaOH: 200mM HCl: 1M Tris 6.8) and immediately heated 3 min. at 99°C with 1:4 GSD sample buffer (4.5 M glycerol, 230 mM SDS, 330 mM dithiothreitol (DTT), 2mM Tris-HCl pH 8.8) to preserve the *in vivo* state of PP2A C subunit methylation. The (+) aliquot was treated with 200mM NaOH on ice for 5 minutes to cause complete PP2A C subunit demethylation. This sample was then

treated with a neutralization solution (2:1/200mM HCl: 1M Tris pH 6.6), 1:4 GSD was added, and the sample was heated 3 min. at 99°C.

Methylation analysis lysates were separated by SDS-PAGE, proteins were transferred to nitrocellulose (Millipore), and membranes were blocked in 5% non-fat dry milk in 1X TBST (20 mM Tris, 0.14M NaCl, 0.1% Tween 20) to promote specific epitope binding. The samples were immunoblotted with 4B7 (Yu et al., 2000, 1:5000), anti-PP2A C subunit (Transduction Laboratories, 1:20,000), and anti-LCMT-1 antibody RK3110 (Lee and Pallas, 2007; 1:5000). 4B7 is a monoclonal antibody specific for unmethylated PP2A C subunit. Percent *in vivo* PP2A C methylation was found by calculating the ratio of demethylation in lysates from quantitative immunobloting with 4B7 and subtracting this value from 1 [1-(-):(+)]. The Pallas Laboratory raised antibody RK3110 in rabbit against a 17 amino acid peptide sequence at the N-terminus of human LCMT-1; the antibody was affinity purified and specifity confirmed by immunoblot analysis. Immunoblots were quantitated using a Bio-Rad Fluor S-Max Chemilumimager and Bio-Rad Quantity One Software.

Tau Assay

For the tau enrichment, approximately half the lysate was heated for 5 minutes at 99°C without buffer. The samples were then centrifuged in a Microfuge 18 Centrifuge (Beckman Coulter) for 10 min. at 18,000 x g at 4°C to pellet non-heat stable proteins. The samples were finally heated for 3 min. at 99°C after adding 1:4 GSD. Tau lysates were separated by SDS-PAGE, proteins were transferred to nitrocellulose (Millipore), and membranes were blocked in 5% non-fat dry milk in 1X TBST. The samples were immunobloted with 12E8 (Seubert et al., 1995, 1:5000), DOVE anti-4 repeat tau (B.

Hyman Laboratory, Unpublished data, 1:10,000), and STORM anti-3 repeat tau (B. Hyman Laboratory, Unpublished data, 1:10,000). 12E8 detects tau phosphorylated at serine 262 and to a lesser extent serine 356. To ensure specificity of immunoblot signal, we stripped tau immunoblots immediately following visualization with Horse Radish Peroxidase conjugated secondary antibodies using Restore Western Blot Stripping Buffer (Thermo Scientific) according to standard protocol. Immunoblots were quantitated using a Fluor S-Max Chemilumimager (Bio-Rad) and Quantity One Software (Bio-Rad).

Immunohistochemistry

Embryos were removed from 4% PFA in PBS at 4°C, and were gradually dehydrated through serial incubations of increasing ethanol concentration. Following the last ethanol wash, embryos were stored at -20°C overnight in 100% ethanol. Embryos were washed in Xylene and paraffin embedded. Embryos were sliced in 9 micron sections and mounted on slides. Immunohistochemistry was then performed according to standard methods (de Silva et al., 2003; Togo et al., 2002). Slides were probed with primary antbodies: 4B7 (1:500), RK3110 (1:250), 12E8(1:250), anti-3 repeat tau RD3 (Upstate, 1:1000), and anti-4 repeat RD4 (Upstate, 1:100). All slides were probed with secondary biotinylated antibodies (1:500), and deposition was visualized using 3,3'-Diaminobenzidine (DAB) staining.

Data Analysis

All records of colony maintenance, genotyping, and timed mating dissections were maintained and analyzed using Microsoft Excel. Immunoblots were quantitated using Quantity One Software (Bio-Rad). All experiments reported, except full LCMT-1

knockout data, were repeated at least 3 times to ensure reliability of results. A two-tailed student's T-test was used to determine significance of results, defined as a $p \leq .05$. A chi-square test was used to determine significance, defined as a $p \leq .05$, of results in the genotype frequency analysis. Immunohistochemical and phenotypic observations are considered qualitative data for the purpose of this thesis.

Results

Embryo Phenotypes and Genotyping

Timed-mating dissections revealed that LCMT-1 (+/-) embryos do not differ phenotypically from LCMT-1 (+/+) mice (Fig. 2A). LCMT-1 (-/-) embryos exhibit several phenotypic differences from wild-type: figure eight eye formation, decreased vasculature, decreased size, humped head morphology, and a darkened, smaller liver. Fig. 2B shows LCMT-1 genotyping results corresponding to the phenotypes. Production of the 200bp band indicates the presence of at least one wild-type LCMT-1 allele without a gene trap insertion while the 254bp band indicates the presence of at least one LCMT-1 allele containing a gene trap insert.

LCMT-1 Expression is Down-regulated in Partial- and Full-knockout Mouse Embryos

Quantitative immunoblotting of mouse embryo head lysates was used to determine the level of LCMT-1 expressed in day 12.5 mouse embryos. Primary antibodies 3110 and anti-PP2A C were used to detect LCMT-1 and PP2A C respectively. Fig. 3A shows that LCMT-1 expression is down-regulated or absent in LCMT-1 (+/-) and (-/-) respectively, relative to wild-type embryos. Fig. 3B shows that LCMT-1 expression is significantly reduced to 50% in LCMT-1 (+/-) embryos relative to wild-type ($P = 0.003$). This figure also shows that no LCMT-1 expression is detected in full-knockout day 12.5 mouse embryos. However, significance cannot be determined for full-knockout embryos because this value was obtained from only two blots containing full knockout embryos. LCMT-1 expression was normalized to PP2A C as a loading control.

Knockout of one LCMT-1 allele does not result in a significant decrease in PP2A C Methylation

Quantitation of immunoblots with demethylation sensitive, monoclonal antibody 4B7 was used to determine the percent PP2A C methylation. Fig. 3A shows a representative immunoblot of mouse embryo head lysates treated with either 200 mM NaOH (Fig. 4A +) to induce complete PP2A demethylation or a pre-neutralized solution (Fig. 4A -) to preserve the *in vivo* steady-state PP2A C methylation (Yu et al., 2001). After normalizing to total PP2A C, the ratio of steady-state demethylated PP2A C was calculated by dividing the (-) sample by the (+) sample; this value was then subtracted from one to yield the fraction, and percent, of PP2A C methylation. Fig. 3B shows that both LCMT-1 (+/+) and (+/-) embryos are approximately 100% steady-state methylated at PP2A C. Fig. 4B shows that full-knockout of LCMT-1 results in decreased levels of PP2A C methylation. The percentage shown for LCMT-1 (-/-) methylation (approximately 38%) is believed to be higher than the true steady-state value because of a loading error on one of the two immunoblots containing full-knockout embryos.

Three and Four Repeat Tau (3R and 4R Tau) Isoform Phosphorylation is Increased in LCMT-1 full-knockout mouse embryos

Fig. 5A shows immunoblot results for total 3R Tau, the endogenous embryonic mouse isoform, and 3R Tau phosphorylated at 12E8 specific residues, phosphorylated serine 262 and to a lesser extent phsosphorylated serine 356. 3R Tau phosphorylation is increased in LCMT-1 (-/-) but not LCMT-1 (+/-) embryos. Quantitation of these

immunoblots reveals that 3R Tau exhibits a three-fold increase in phosphorylation with a full knockout of LCMT-1 relative to wild-type (Fig. 5B). Immunoblot results for total and phosphorylated 4R Tau, the transgenic, human P301L isoform, are shown in Fig. 6A. 4R Tau was found to be almost four-fold more phosphorylated at 12E8 sites in the LCMT-1 (-/-) relative to the wild-type (Fig. 6B). Again, the LCMT-1 (+/-) did not show any significant change in 4R Tau phosphorylation relative to wild-type.

Immunohistochemical Results

Immunohistochemistry (IHC) was performed on day 12.5 mouse embryos to determine the regional relationship in the brain between LCMT-1, PP2A C methylation, and tau. Only LCMT-1 (+/+) and (-/-) embryos were shown because no difference was observed between LCMT-1 wild-type and (+/-) embryos. Day 12.5 mouse embryos were paraffin embedded, sectioned sagitally in 9 micron slices, and stained using the DAB method (see Methods). Incubation with only biotinylated mouse secondary antibody (no primary antibody) yielded no deposition, confirming that the 4B7 and RD3 deposition observed was specific (data not shown).

We probed embryos with primary antibody 4B7 to determine localization of demethylated PP2A C. As a positive control, one LCMT-1 (+/-) embryo section was treated with 200mM NaOH for 5 min. at 4°C to cause PP2A C demethylation. However, this base treatment is a commonly used to expose epitopes for IHC that other samples were not subjected to. Therefore, the positive control may also have stronger antibody binding due to additional epitope exposure yielding stronger deposition than complete PP2A C demethylation alone.

4B7 showed a strong signal in all brain regions in the positive control except for the medial portion of the pons-midbrain junction. The LCMT-1 (+/+) exhibited minimal reactivity to 4B7, showing only slight deposition in the roof of the neopallial cortex (Fig. 7A, Fig. 7B) and rootlets of the trigeminal nerve ganglion (Fig. 7A, Fig. 7C). The LCMT-1 full-knockout reacted strongly for 4B7 in many areas: the region of the pons-midbrain junction, striatum, roof of the neopallial cortex (Fig.7A, Fig. 7B), rootlets of the trigeminal nerve ganglion, left trimenal ganglion, choroid plexus extending into the lateral ventricle, and region of the posterior semicircular canals (Fig. 7A, Fig. 7C).

We probed embryos with primary antibody RD3 to determine localization of 3R Tau. Strong deposition was observed in the wild-type and (-/-) embryo in the region of the pons-midbrain junction (Fig. 8A, 8D). The wild-type embryo also showed strong deposition on the wall of the diencephalon (Fig. 8C) and slight deposition in the roof of the neopallial cortex (Fig. 8B). In the (-/-) embryo, strong immunoreactivity was detected in the roof of the midbrain (Fig. 8C), vestibulocochlear ganglion (Fig. 8D), and the regions ventral and caudal to the striatum extending to the ventral region of the third ventricle (Fig. 8B). The differential deposition between wild-type and knockout embryos could be a result of varying slice angle that revealed different structures in the embryos.

Due to variability of results, data from mouse embryos stained with primary antibodies 3110 and 12E8 are not shown. Based on previous studies, it is expected that LCMT-1 will show immunoreactivity in all brain regions of wild-type and LCMT-1 (+/-) embryos and be undetected in (-/-) embryos (Sontag et al., 2004b). Further, it is expected that regions exhibiting demethylated PP2A C deposition with 4B7 will overlap with regions that show deposition for phosphorylated tau using 12E8 (Sontag et al., 2004a).

Also, primary antibody RD4 yielded no result for IHC or immunoblotting at recommended concentrations.

Genotype Frequency Analysis

Due to the low number of LCMT-1 (-/-) embryos found in these experiments, a genotype frequency analysis was performed comparing day 12.5 embryo genotype frequencies from timed matings between LCMT-1 mice only (LCMT-1 TM) and between LCMT-1/Tau P301L mice (Tau TM). The raw data and analysis of these frequencies are displayed in Table 1. The average frequency of knockouts found LCMT-1 TM litters was 0.206, while the frequency of knockouts found in Tau TM litters was 0.933. A chi-square test was performed between the embryo genotypes found in Tau TM litters using the genotype frequencies found in LCMT-1 TM litters. The chi-square test revealed a P-value of 0.1 for (-/-) embryos indicating an insignificant difference. However, given that the frequency of (-/-) embryos remains at this level, it is expected that once more Tau TM dissections are performed that this result will become significant because the difference between expected and observed frequencies will increase.

Discussion

Methylation is an important physiological method of regulating several cellular processes. For PP2A, this carboxymethylation has been shown differentially to regulate the formation of certain PP2A holoenzymes, and it is critical for promoting the assembly of BαAC heterotrimers (Tolstykh et al., 2000; Wu et al., 2000, Yu et al., 2001; Wei et al., 2001; Lee and Pallas, 2007). More recently, it was found that these BαAC heterotrimers are the main neuronal PP2A holoenzyme and represents the majority of tau phosphatase activity, critical for regulation of tau proteins and prevention of tauopathies (Goedert et al., 1995; Sontag et al., 1996). Taken together with reports of decreased LCMT-1 expression and regional correlation with decreased PP2A C methylation and NFT formation, PP2A C subunit methylation may have a critical role in the maintenance of homeostatic tau phosphorylation levels and dementia prevention (Sontag et al., 2004a; Sontag et al., 2004b, Arriagada et al., 1992).

In this study, we report that loss of both, but not one, of the alleles for LCMT-1 is required to produce decreased PP2A C methylation and increased 3R and 4R tau phosphorylation. Because LCMT-1 is the major mammalian PP2A methyltransferase and and no change in PP2A C methylation is detected by immunoblot or IHC in partial knockout mouse embryos, we can infer that LCMT-1 must be either largely abundant relative to PP2A C or must be very efficient at methylating PP2A C.

The P301L tau model we used to examine the effects of decreased PP2A C methylation also provides insight into the mechanism by which PP2A regulates the phosphorylation state of tau. Studies on the P301L mutation have suggested that this tau mutant becomes hyperphosphorylated because PP2A binds it less efficiently (Goedert et

al., 2000). Previously, it has also been suggested that BαAC heterotrimers may directly regulate tau phosphorylation because they have the most tau phosphatase activity of all PP2A holoenzymes and are the major neuronal isoform (Goedert et al., 1995; Sontag et al., 1996). If this were true, we would not expect the full-knockout of LCMT-1 to produce a much larger increase in 4R tau phosphorylation because PP2A already binds the P301L mutant tau less efficiently. Therefore, a reduction in the number of BαAC heterotrimers would not be expected to have a large additional effect. Our results suggest that the indirect regulation of tau by PP2A may also play a significant role in maintaining the phosphorylation state of tau.

As previously stated, PP2A has implications in many signaling pathways PP2A both directly and indirectly regulates the same substrate (Hanger et al., 2009). PP2A has been shown to bind directly to and dephosphorylate tau, but PP2A also functions upstream of several kinases that also target tau (Sontag et al., 1999; Hanger et al., 2009; Lin et al., 2007). For example, PP2A was shown to indirectly regulate the activity of glycogen synthase kinase (GSK3β) by activating protein kinase B (AKT) through the PP2A-regulated PI-3-kinase pathway resulting in inactivation of GSK3β by phosphorylation at serine 9 (Lin et al., 2007). The upstream regulation of GSK3β by PP2A is important because GSK3β is the major tau kinase (Martin et al., 2009). PP2A also directly regulates the activity of peptidyl-prolyl isomerase Pin1, which causes the cis/trans conformational change of many proteins including tau, by removing the activity-dependent phosphorylation from serine 16 of Pin1 (Lim et al., 2005; Galas et al., 2006).

Maintentance of tau phosphorylation by PP2A C methylation, specifically by LCMT-1, has direct application to human disease, especially tauopathies. A stuy of post-

mortem AD brains have already revealed a regional correlation between a decrease in LCMT-1, decreased PP2A C methylation, decreased BαAC heterotrimers, and an increase in tau related neuropathology (NFT positive neurons) (Sontag et al., 2004a). In this study, we show that a knockout LCMT-1 directly results in decreased PP2A C methylation and hyperphosphorylated 3R and 4R tau isoforms in a mammalian *in vivo* model. These results that a deficiency in LCMT-1 could directly cause tau disregulation and the subsequent tauopathies.

The role of PP2A C methylation also has future applications in translational research for human diseases. For example, a PME-1 inhibitor may be a possible neuroprotective drug. PME-1 opposes the action of LCMT-1 by removing the carboxymethylation from Leu309 on PP2A C, exaggerating the effect of decreased LCMT-1 expression (Fig. 1). If a small drug screen revealed a specific inhibitor of this methylesterase, it could be used as a therapeutic drug to prevent tau pathology in tauopathies such as AD.

In summary, PP2A C methylation is a critical regulatory process of many cellular signaling pathways, and it is achieved primarily by LCMT-1 in mammalian cells. Our results indicate that PP2A is a vital regulator of the tau phosphorylation pathway, and methylation of PP2A C is necessary to maintain normal tau phosphorylation levels.

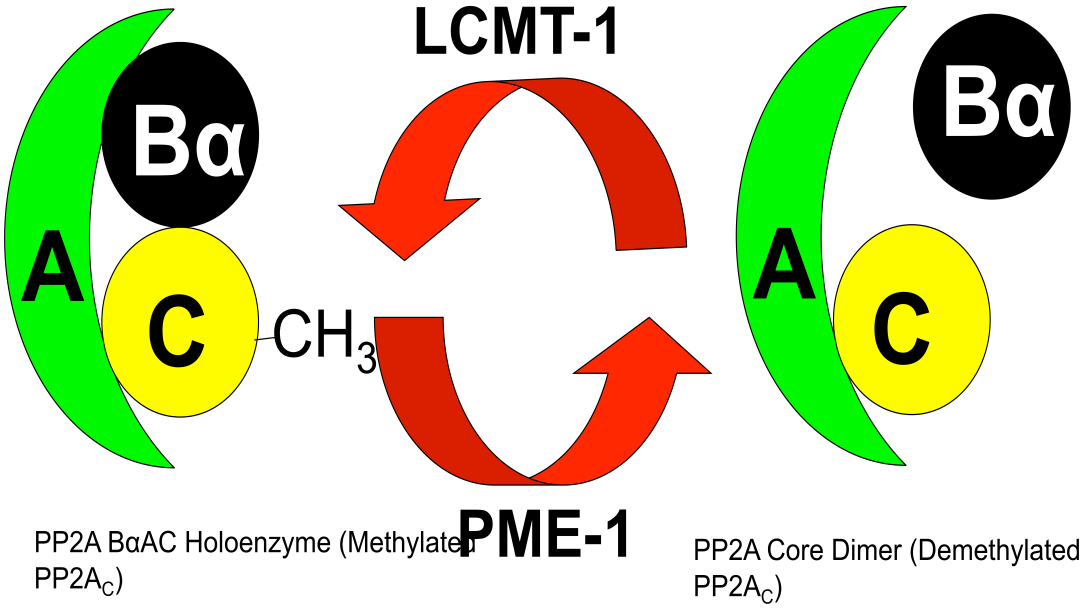

Figure 1: PP2A Structure and Regulation

Carboxymethylation at Leu309 of the PP2A C subunit is required for formation of BαAC heterotrimers but is not necessary for holoenzyme formation with other regulatory subunits (Yu et al., 2001). It is known that Leu309 carboxymethylation occurs at the α-carboxyl group of this leucine residue by leucine carboxyl methyltransferase-1 (LCMT-1) in mammalian cells (Xie and Clarke, 1993; Lee and Stock, 1993; Xie and Clarke, 1994; Favre et al., 1994; Li and Damuni, 1994; Floer and Stock, 1994; De Baere et al, 1999). As shown in Figure 1, LCMT-1 is opposed by protein phosphatase methylesterase-1 (PME-1), which functions to demethylate Leu309 of PP2A C subunit (Ogris et al., 1999; Xie and Clarke, 1994; Lee et al., 1996).

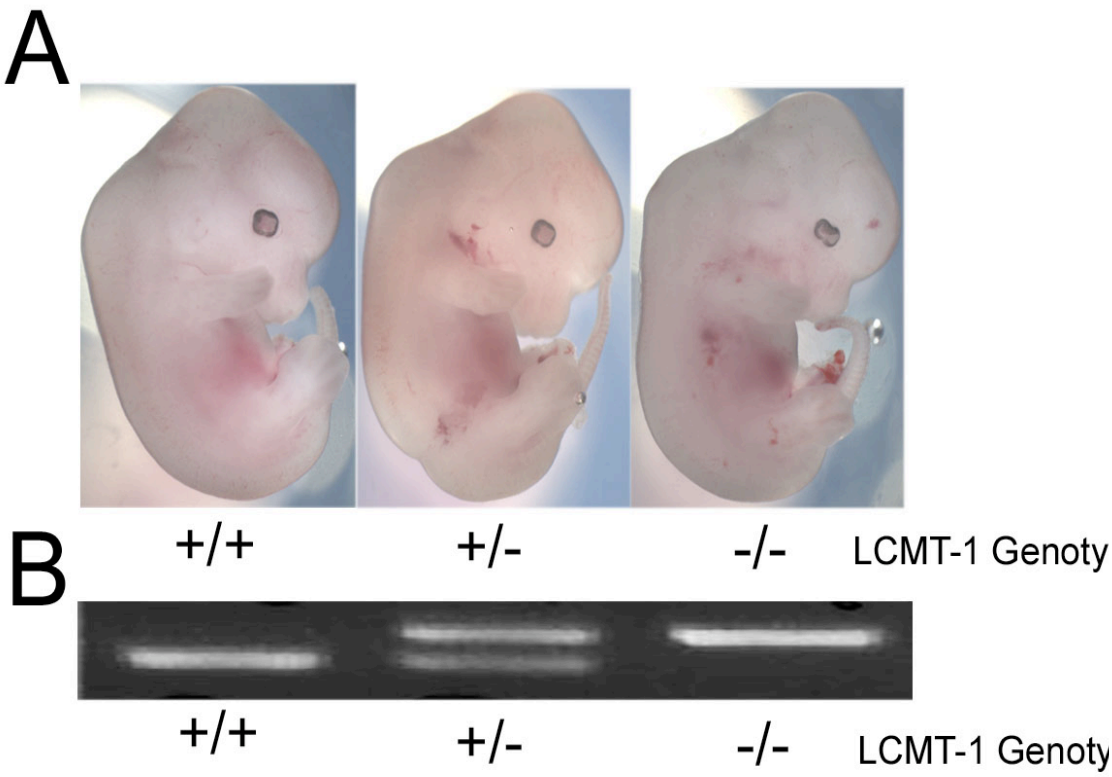

Figure 2 : Embryo Phenotypes and Genotyping
Figure 2A shows images captured of day 12.5 mouse embryos immediately following mechanical extraction from the uterus. Both the wild-type and LCMT-1 (+/-) embryos show no phenotypic abnormalities. LCMT-1 (-/-) embryos exhibit several phenotypic differences from wild-type: figure eight eye formation, decreased vasculature, decreased size, humped head morphology, and a darkened liver. Fig. 2B shows corresponding LCMT-1 genotyping results to the above phenotypes. Production of the 200bp band indicates presence of at least one wild-type LCMT-1 allele without a gene trap insertion while the 254bp band indicates the presence of at least one LCMT-1 allele containing a gene trap insert.

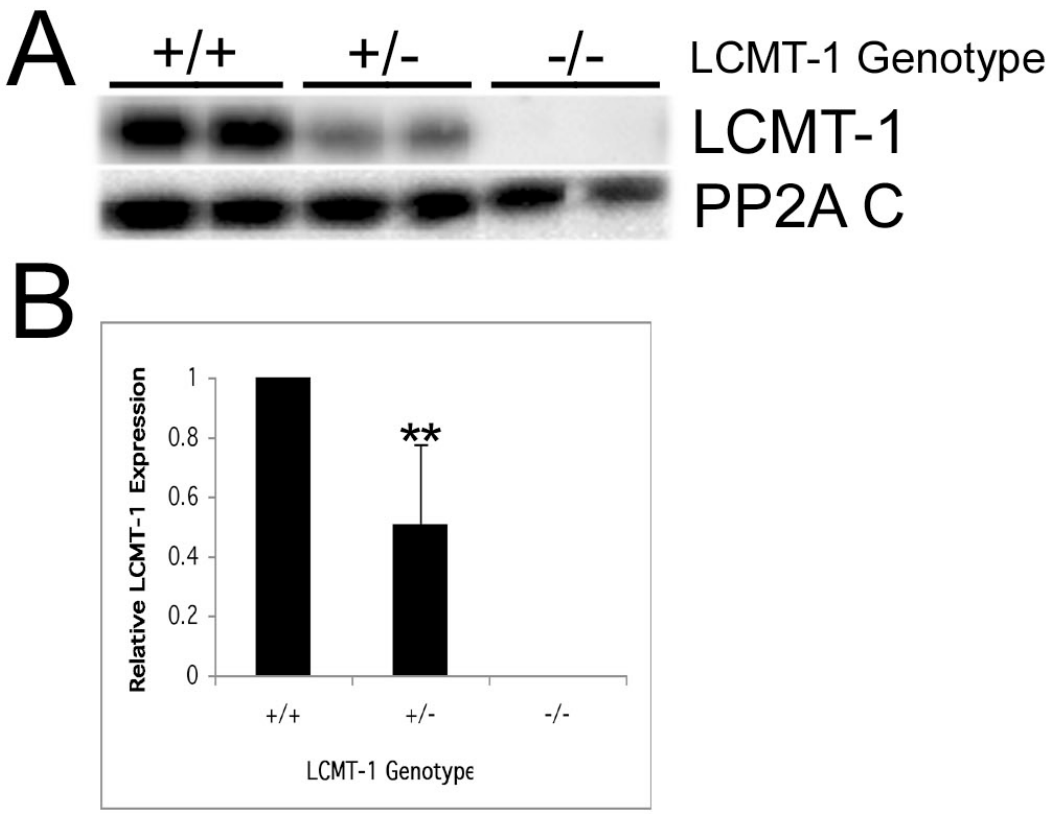

Figure 3: LCMT-1 Expression is Down-regulated in Partial- and Full-knockout Mouse Embryos

Fig. 3A shows immunoblot results with primary antibodies against LCMT-1 (3110) normalized to levels of PP2A C subunit (PP2AC). Quantitative immunobloting (B) revealed that LCMT-1 is expression is significantly down-regulated 50% in the LCMT-1 (+/-) relative to wild-type (P value = 0.003). Almost 0% LCMT-1 is expressed in the full-knockout mouse embryo.

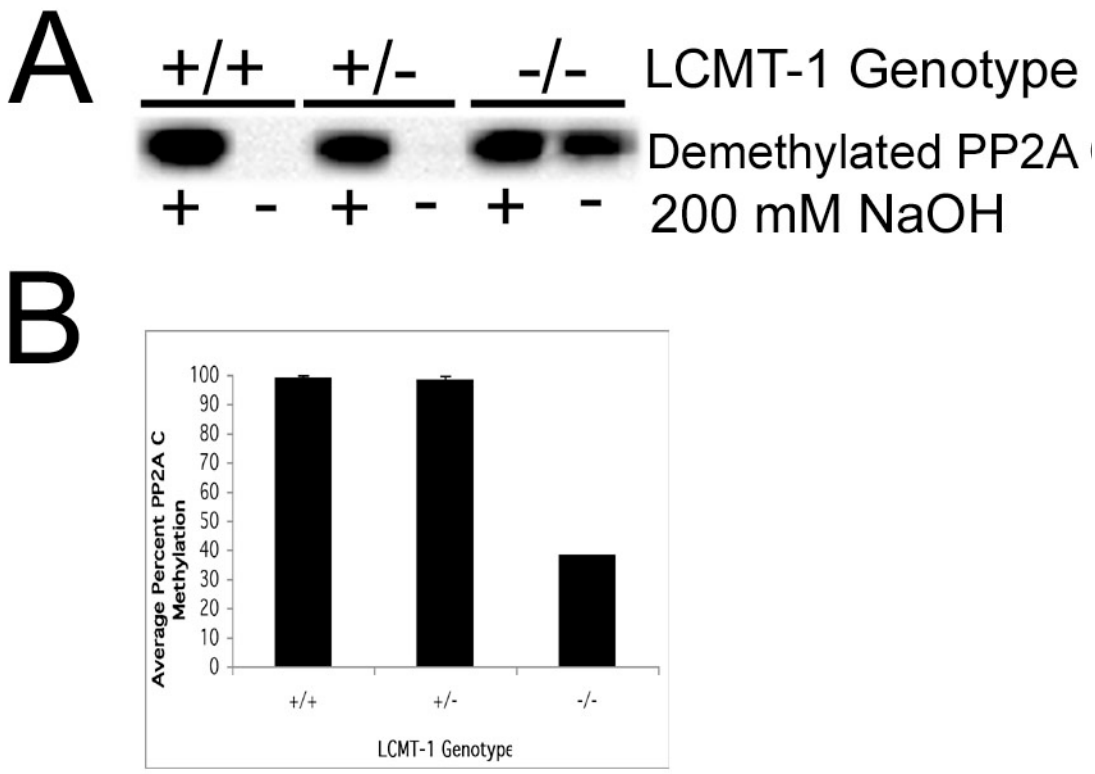

Figure 4: Knockout of one LCMT-1 allele does not result in a significant decrease in PP2A C methylation

Quantitation of immunoblots with demethylation sensitive, monoclonal antibody 4B7 was used to determine percent PP2A C methylation. Fig. 3A shows a representative immunoblot of mouse embryo head lysates treated with either 200 mM NaOH (Fig. 4A +) to induce complete PP2A demethylation or a pre-neutralized solution (Fig. 4A -) to preserve the *in vivo* steady-state PP2A C methylation (Yu et al., 2001). After normalizing to total PP2A C, the ratio PP2A C demethylation was calculated by dividing the (-) sample by the (+) sample; this value was then subtracted from one to yield the ratio, and percent, of PP2A C methylation. Fig. 3B shows that both LCMT-1 (+/+) and (+/-) embryos are approximately 100% steady-state methylated at PP2A C. Fig. 4B shows that full-knockout of LCMT-1 results in decreased levels of PP2A C methylation. The percentage shown for LCMT-1 (-/-) methylation (approximately 38%) is believed to be higher than the true steady-state value because of a slight loading error on one of the two immunoblots containing full-knockout embryos.

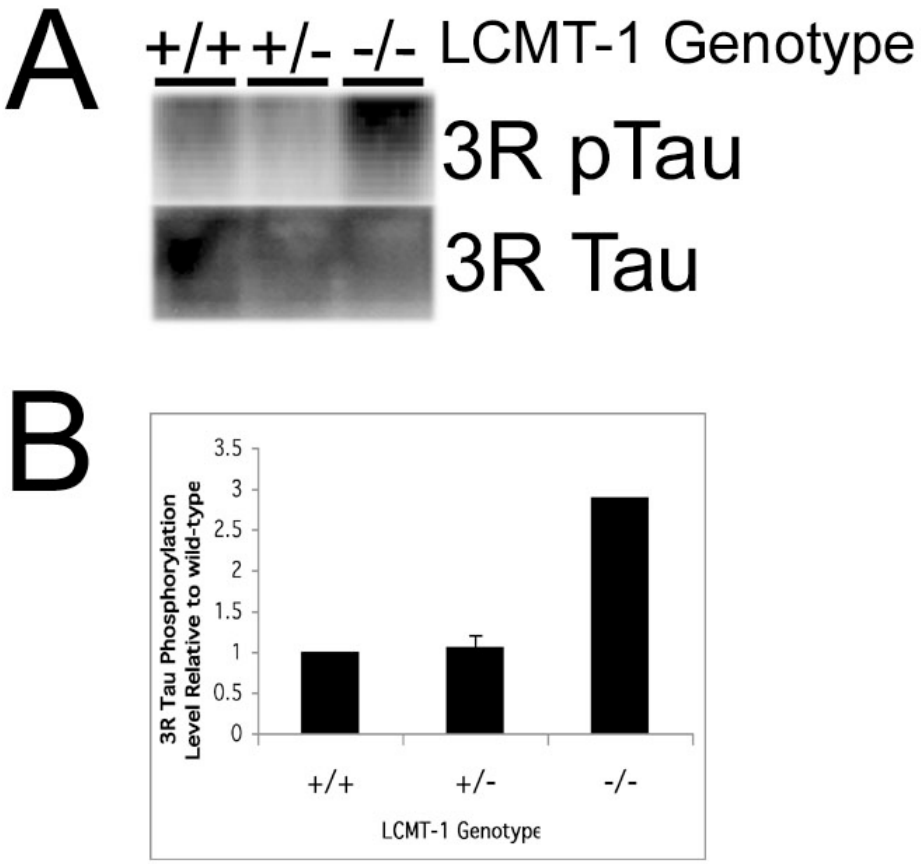

Figure 5: Three Repeat Tau (3R) Isoform Phosphorylation is Increased in LCMT-1 full-knockout mouse embryos

Fig. 5A shows immunoblot results for total 3R Tau, the endogenous embryonic mouse isoform, and 3R tau phosphorylated at 12E8 specific residues. 3R Tau is increased in LCMT-1 (-/-) but not LCMT-1 (+/-) embryos. Quantitation of these immunoblots reveals that 3R Tau exhibits a three-fold increase in phosphorylation with a full knockout of LCMT-1 relative to wild-type (Fig. 5B).

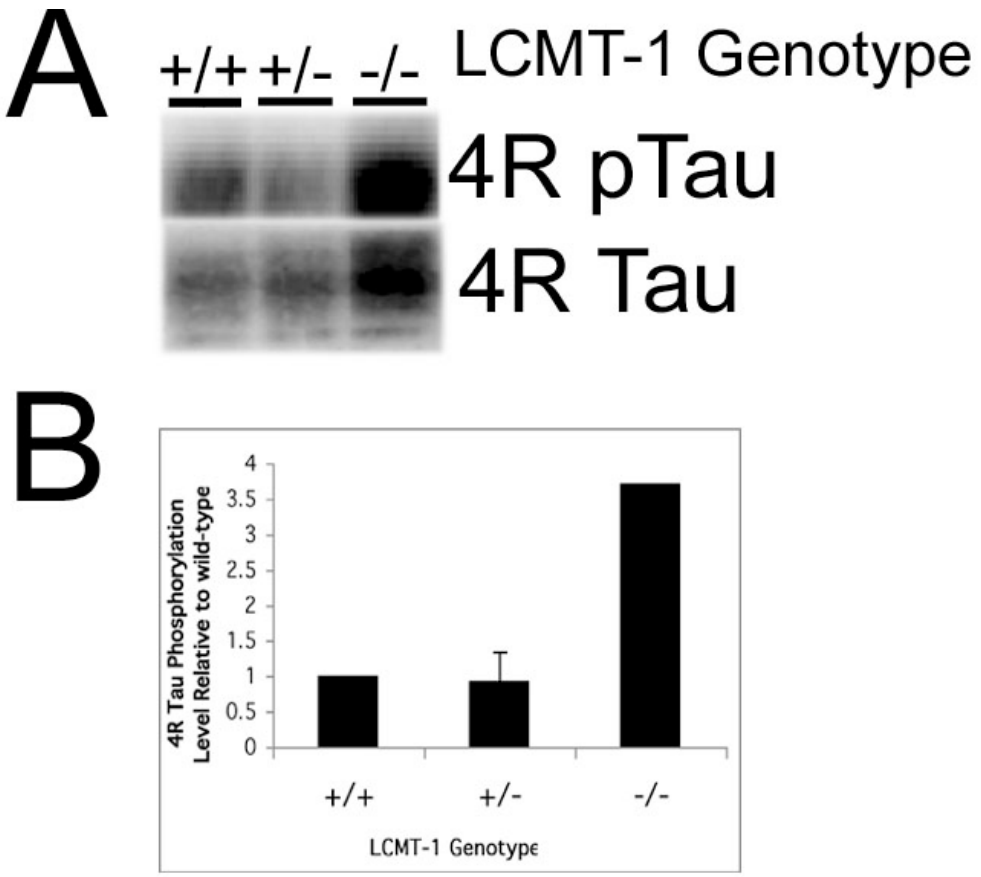

Figure 6: Four Repeat Tau (4R) Isoform Phosphorylation is Increased in LCMT-1 full-knockout mouse embryos

Immunoblot results for total and phosphorylated 4R Tau, the transgenic, human P301L isoform, are shown in Fig. 6A. 4R Tau was found to be almost four-fold more phosphorylated at 12E8 sites in the LCMT-1 (-/-) relative to the wild-type (Fig. 6B). Again, the LCMT-1 did not show any significant change in 4R Tau phosphorylation relative to wild-type.

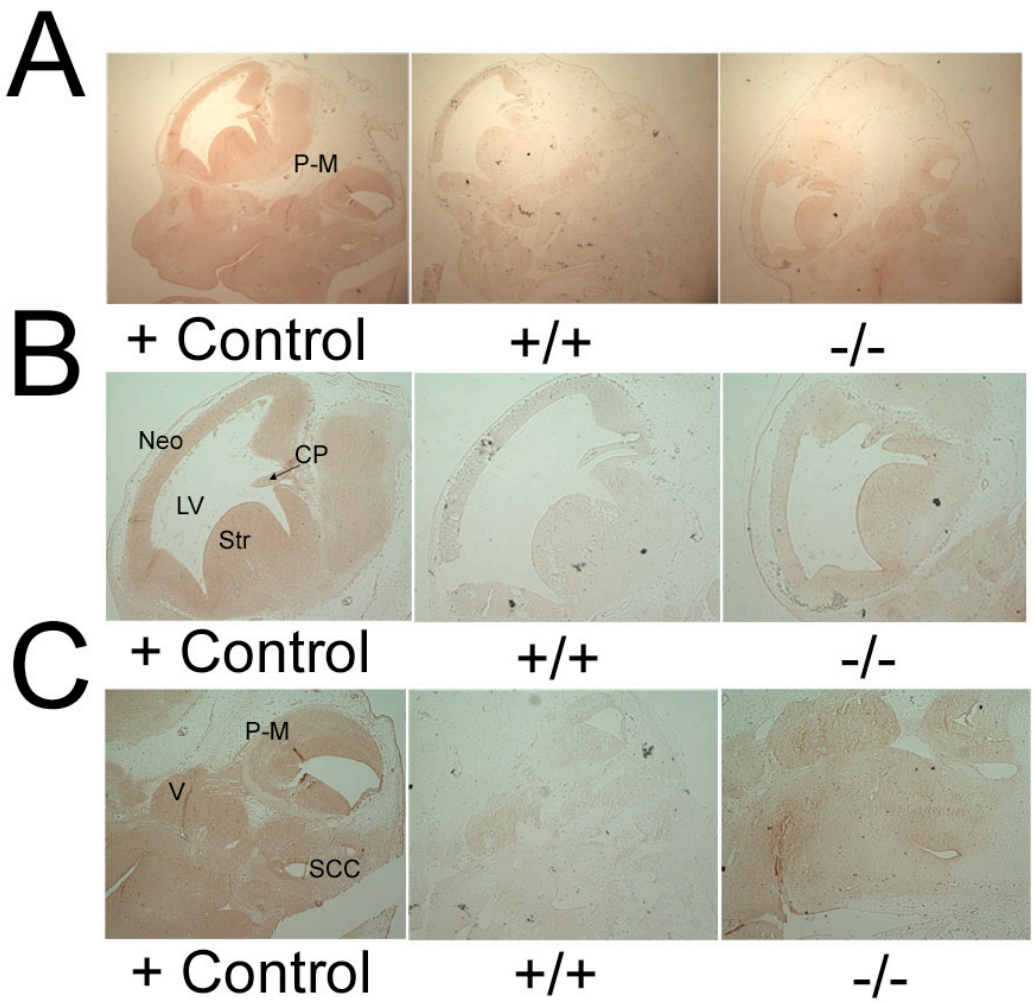

Figure 7: 4B7 Immunohistochemical Staining is Increased in Full-Knockout LCMT-1 Embryos

We stained embryos with primary antibody 4B7 to determine localization of demethylated PP2A C. 4B7 showed a strong signal in all brain regions in the positive control except for the medial portion of the pons-midbrain junction. The LCMT-1 (+/+) exhibited minimal reactivity to 4B7, only slightly staining in the roof of the neopallial cortex (Neo) (Fig. 7A, Fig. 7B) and rootlets of the trigeminal nerve ganglion (Fig. 7A, Fig. 7C). The LCMT-1 full-knockout stained strongly for 4B7 in many areas: the region of the pons-midbrain junction (P-M), striatum (Str), roof of the neopallial cortex (Fig.7A, Fig. 7B), rootlets of the trigeminal nerve ganglion (V), left trimenal ganglion, choroid plexus (CP) extending into the lateral ventricle (LV), and region of the posterior semicircular canals (SCC) (Fig. 7A, Fig. 7C). As a positive control, one LCMT-1 (+/-) embryo section was treated with 200mM NaOH for 5 min. at 4°C to cause PP2A C demethylation. However, this base treatment is a commonly used method to expose epitopes for IHC staining that other samples were not given. Therefore, the positive control may have stronger antibody binding due to additional epitope exposure causing stronger deposition than complete PP2A C demethylation alone. Figure 7A was imaged at 20X magnification, and figures 7B and 7C were imaged on 40X magnification.

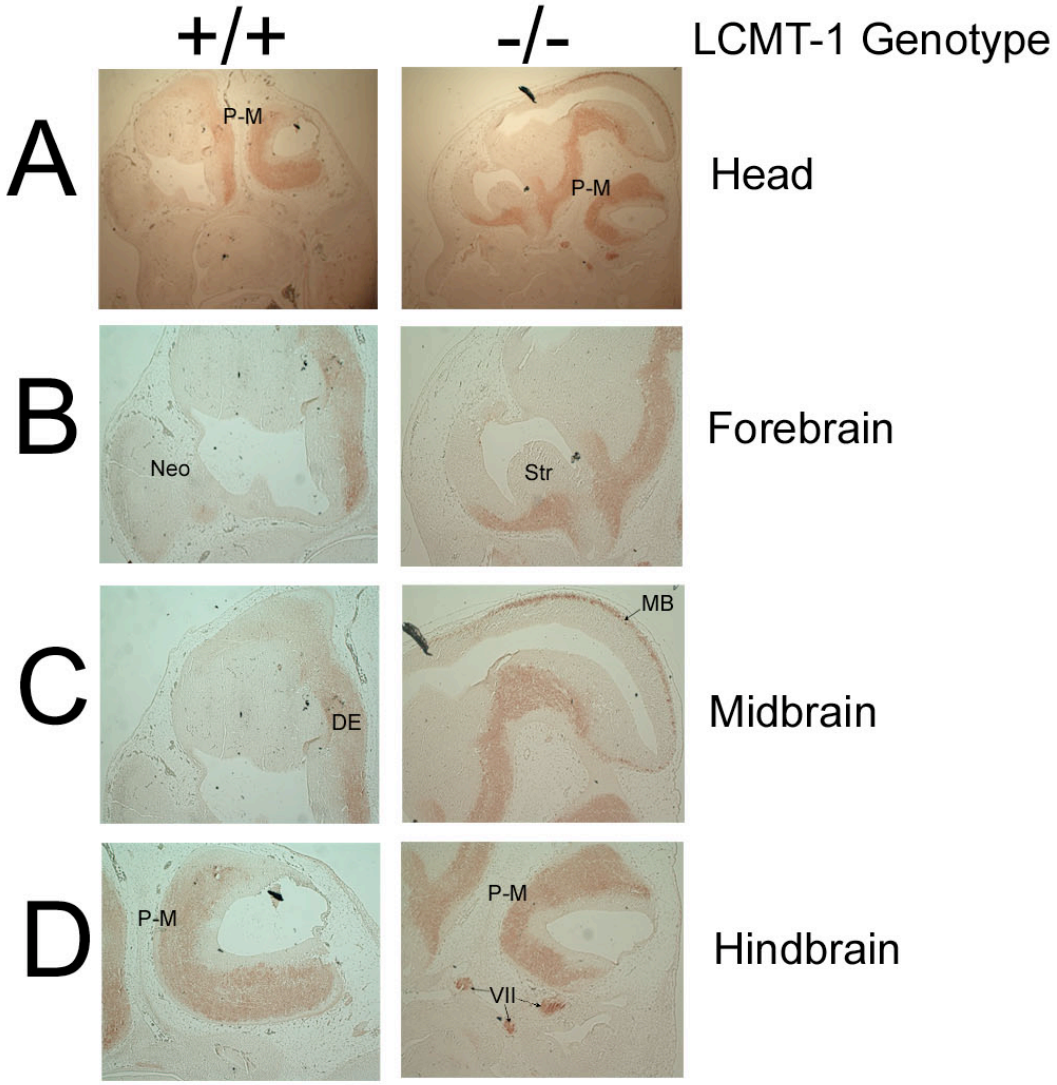

Figure 8: RD3 Immunohistochemical Staining
We stained embryos with primary antibody RD3 to determine localization of 3R Tau. Strong staining was observed in the wild-type and (-/-) embryo in the region of the pons-midbrain junction (P-M) (Fig. 8A, 8D). The wild-type embryo also showed strong staining on the wall of the diencephalon (DE) (Fig. 8C) and slight deposition in the roof of the neopallial cortex (Neo) (Fig. 8B). In the (-/-) embryo, strong immunoreactivity was detected in the roof of the midbrain (MB) (Fig. 8C), vestibulocochlear ganglion (VIII) (Fig. 8D), and the regions ventral and caudal to the striatum extending to the ventral region of the third ventricle (Fig. 8B). Figure 8A was imaged at 20X magnification, and figures 8B-D were imaged on 40X magnification.

Table 1: Timed Mating Genotype Frequency Analysis

Tau TM v. LCMT-1 TM Statistics								
	LCMT-1 Day 12.5 Embryo Genotype Stats				**Tau Total Embryos**			
	+/+	+/-	-/-	Total Embryos	+/+	+/-	-/-	Total Embryos
Total # Embryos	68	114	45	227	10	13	11	118
Genotype Ratio	0.299559471	0.502202643	0.198237885		0.084745763	0.110169492	0.093220339	
Standard Deviation	0.179425066	0.205075554	0.143015026		0.026784348	0.026784348	0.16472102	
Average Litter #	7.111111111				8.428571429			
Standard Deviation	1.897632762				3.457751286			
Total # Dissections	45				14			
Tau TM Raw Data								
	Total Embryos Observed			**Total Embryos Expected (Based on LCMT-1 TM)**				
Total Embryo #	+/+	+/-	-/-	+/+	+/-	-/-		
10	2	8	0	2.9375	5	2.0625		
4	2	1	1	1.175	2	0.825		
7	2	5	0	2.05625	3.5	1.44375		
8	0	4	4	2.35	4	1.65		
4	2	2	0	1.175	2	0.825		
5	1	4	0	1.46875	2.5	1.03125		
3	2	0	1	0.88125	1.5	0.61875		
12	3	7	2	3.525	6	2.475		
12	3	8	1	3.525	6	2.475		
13	7	6	0	3.81875	6.5	2.68125		
7	1	4	2	2.05625	3.5	1.44375		
10	3	7	0	2.9375	5	2.0625		
12	5	7	0	3.525	6	2.475		
11	5	6	0	3.23125	5.5	2.26875		
	+/+	+/-	-/-					
Chi-square test	0.667998329	0.886137017	0.104192873					

Literature Cited

1. Arriagada, P.V., Growdon, J.H., Hedley-Whyte, E.T., and Hyman, B.T. (1992). Neurofibrillary tangles but not senile plaques parallel duration and severity of Alzheimer's disease. Neurology 42:631–639

2. Bialojan C, and Takai A. (1988). Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J, 256: 283–290

3. Clarke, S. (1985) Regulation and subcellular distribution of a protein methyltransferase and its damaged aspartyl substrate sites in developing Xenopus oocytes. Annu. Rev. Biochem. 54, 479-506.

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

5. de Silva, R., Lashley, T., Gibb, G., Hanger, D., Hope, A., Reid, A., Bandopadhyay, R., Utton, M., Strand, C., Jowett, T., Khan, N., Anderton, B., Wood, N., Holton, J., Revesz, T., and Lees, A. (2003). Pathological inclusion bodies in tauopathies contain distinct complements of tau with three or four microtubule-binding repeat domains as demonstrated by new specific monoclonal antibodies. Neuropathol. Appl. Neurobiol. 29: 288-302.

6. Favre, B., Zolnierowicz, S., Turowski, P., and Hemmings, B. A. (1994). Differential inhibition and posttranslational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin-A, okadaic acid, and tautomycin. J. Biol. Chem. 269, 16311–16317

7. Floer, M., and Stock, J. (1994) Carboxyl methylation of protein phosphatase 2A from Xenopus eggs is stimulated by cAMP and inhibited by okadaic acid. Biochem. Biophys. Res. Commun. 198,372–379

8. Goedert, M., Spillantini, M.G., Jakes, R., Rutherford, D., and Crowther, R.A. (1989) Multiple isoforms of human microtubule-associated protein tau: sequences and localization in neurofibrillary tangles of Alzheimer's disease. Neuron 3:519–526

9. Goedert, M., Jakes, R., Wang, J.H., and Cohen, P. (1995c). Protein phosphatase 2A is the major enzyme in brain that dephosphorylates tau protein phosphorylated by proline-directed kinases or cyclic AMP–dependent protein kinase. J. Neurochem. 65, 2804–2807.

10. Goedert, M., Satumtira, S., Jakes, R., Smith, M.J., Kamibayashi, C., White, C.L. 3rd, Sontag, E. (2000) Reduced binding of protein phosphatase 2A to tau protein with

frontotemporal dementia and parkinsonism linked to chromosome 17 mutations. J Neurochem. 75(5):2155-62.

11. Gong, C.-X., Liu, F., Grundke-Iqbal, I. and Iqbal, K. (2005) Post-translational modifications of tau protein in Alzheimer's disease. J. Neural Transm., 112, 813–838.

12. Griswold-Prenner, I., Kamibayashi, C., Maruoka, E.M., Mumby, M.C., and Derynck, R. (1998). Physical and functional interactions between type I transforming growth factor beta receptors and Balpha, a WD-40 repeat subunit of phosphatase 2A. Mol. Cell. Biol. 18, 6595-6604.

13. Iqbal, K., Liu, F., Gong, C.X., Alonso Adel, C., and Grundke-Iqbal, I. (2009) Brain glucose transporters, O-GlcNAcylation and phosphorylation of tau in diabetes and Alzheimer's disease. Acta Neuropathol. 118(1):53-69.

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

15. Jordens, J., Janssens, V., Longin, S., Stevens, I., Martens, E., Bultynck, G., Engelborghs, Y., Lescrinier, E., Waelkens, E., Goris, J., and Van Hoof, C. (2006). The protein phosphatase 2A phosphatase activator is a novel peptidyl-prolyl cis/trans-isomerase. J Biol Chem. 281 (10): 6349-57. Epub 2005 Dec 27.

16. Kopke, E., Tung, Y.C., Shaikh, S., Alonso, A.C., Iqbal, K., Grundke-Iqbal, I. (1993) Microtubule-associated protein tau Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. J Biol Chem 268:24374–24384

17. Lee, J., and Stock, J.(1993)J.Biol.Chem.268,19192–19195

18. Lee, J., Chen, Y., Tolstykh, T., and Stock, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6043– 6047

19. Lee J.A., and Pallas, D.C. Leucine carboxyl methyl transferase-1 is necessary for normal progression through mitosis in mammalian cells. J Biol Chem, 2007, 282: 30974-30984.

20. Lewis, J., McGowan, E., Rockwood, J., Melrose, H., Nacaraju, P., Van Slegtenhorst, M., Gwinn-Hardy, K., Murphy, M.P., Baker, M., Yu, X., Duff, K., Hardy, J., Corral, A., Lin, W-L., Yen, S-H., Dickson, D.W., Davies, P., and Hutton, M. (2000) Neurofibrillary Tangles, Amyotrophy and Progressive Motor Disturbance in Mice Expressing Mutant (P301L) Tau Protein. Nat Genet, 25(4):402-406.

21. Li, M., and Damuni, Z. (1994). I1PP2A and I2PP2A. Two potent protein phosphatase 2A-specific inhibitor proteins. Biochem. Biophys. Res. Commun. 202, 1023–1030

22. Lin, C.F, Chen, C.L, Chiang, C.W, Jan, M.S, Huang, W.C, and Lin, Y.S. (2007). GSK-3beta acts downstream of PP2A and the PI 3-kinase-Akt pathway, and upstream of caspase-2 in ceramide-induced mitochondrial apoptosis. J Cell Sci. (16):2935-43.

23. Liu, R., Zhou, X.W., Tanila, H., Bjorkdahl, C., Wang, J.Z., Guan, Z.Z., Cao,Y., Gustafsson, J.A., Winblad, B., and Pei, J.J. (2008). Phosphorylated PP2A (tyrosine 307) is associated with Alzheimer neurofibrillary pathology. J. Cell. Mol. Med. 12, 241-257.

24. Martin, L., Magnaudeix, A., Esclaire, F., Yardin, C., and Terro, F. (2009). Inhibition of glycogen synthase kinase-3beta downregulates total tau proteins in cultured neurons and its reversal by the blockade of protein phosphatase-2A. Brain Res. 2009 Feb 3;1252:66-75. Epub 2008 Nov 30.

25. Matsuo, E. S., Shin, R.-W., Billingsley, M., Van de Voorde, A., O'Connor, M., Trojanowski, J. Q., and Lee, V. M.-Y. (1994) Biopsy-derived adult human brain tau is phosphorylated at many of the same sites as Alzheimer's disease paired helical filament tau. Neuron 13, 989–1002

26. McCright, B., Rivers, A.M., Audlin, S., and Virshup, D.M. (1996) The B56 family of protein phosphatase 2A (PP2A) regulatory subunits encodes differentiation-induced phosphoproteins that target PP2A to both nucleus and cytoplasm. J. Biol. Chem. 271, 22081-22089.

27. Miyasaka, T., Morishima-Kawashima, M., Ravid, R., Kamphorst, W., Nagashima, K., and Ihara, Y. J. (2001) Selective deposition of mutant tau in the FTDP-17 brain affected by the P301L mutation. Neuropathol Exp Neurol. 2001 Sep;60(9):872-84.

28. Mumby M.C. (2001) A new role for protein methylation: switching partners at the phosphatase ball. Sci STKE 2001: PE1

29. Mumby M.C., and Walter, G. (1993). Protein serine/threonine phosphatases: structure, regulation, and functions in cell growth. Physiol Rev, 1993, 73: 673-699.

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

31. Pallas, D.C., Schley, C., Mahoney, M., Harlow, E., Schaffhausen, B.S., and Roberts, T.M. (1986). Polyomavirus small t antigen: overproduction in bacteria, purification, and utilization for monoclonal and polyclonal antibody production. J. Virol. 60, 1075-108

32. Pallas, D.C., Shahrik, L.K., Martin, B.L., Jaspers, S., Miller, T.B., Brautigan, D.L., and Roberts, T.M. (1990). Polyoma small and middle T antigens and SV40 small t antigen form stable complexes with protein phosphatase 2A. Cell 60, 167-176.

33. Savioz, A., Leuba, G., Vallet, P.G., and Walzer, C. (2009). Contribution of neural networks to Alzheimer disease's progression. Brain Res Bull. 2009 Oct 28;80(4-5):309-14.

34. Shi, Y.G. (2009). Assembly and structure of protein phosphatase 2A. Sci China Ser C-Life Sci. 52(6): 135-146.

35. Sontag, E, Nunbhakdi-Craig, V., Bloom, G.S., and Mumby, M.C. (1995) A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. 128, 1131-1144.

36. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Bloom, G.S., and Mumby, M.C. (1996). Regulation of the phosphorylation state and microtubule-binding activity of Tau by protein phosphatase 2A. Neuron. 1996 Dec;17(6):1201-7.

37. Sontag, E., Nunbhakdi-Craig, V., Lee, G., Brandt, R., Kamibayashi, C., Kuret, J., White, C.L. 3rd, Mumby, M.C., and Bloom, G.S. (1999). Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J Biol Chem. 1999 Sep 3;274(36):25490-8.

38. Sontag, E., Luangpirom, A., Hladik, C., Mudrak, I., Ogris, E., Speciale, S., White, 3rd C.L. (2004a). Altered expression levels of the protein phosphatase 2A ABaC enzyme are associated with Alzheimer disease pathology. J Neuropathol Exp Neurol 63: 287–301

39. Sontag, E., Hladik, C., Montgomery, L., Luangpirom, A., Mudrak, I., Ogris, E., and White, C.L. 3rd. (2004b). Downregulation of protein phosphatase 2A carboxyl methylation and methyltransferase may contribute to Alzheimer disease pathogenesis. J Neuropathol Exp Neurol. Apr;63(4):287-301.

40. Sontag, E., Nunbhakdi-Craig, V., Sontag, J.M., Diaz-Arrastia, R., Ogris, E., Dayal, S., Lentz, S.R., Arning, E., and Bottiglieri, T. J. (2007). Expression of protein phosphatase 2A mutants and silencing of the regulatory B alpha subunit induce a selective loss of acetylated and detyrosinated microtubules. Neurosci. 2007 Mar 14;27(11):2751-9.

41. Sontag, J.M., Nunbhakdi-Craig, V., Montgomery, L., Arning, E., Bottiglieri, T., and Sontag, E. (2008). Protein phosphatase 2A methyltransferase links homocysteine metabolism with tau and amyloid precursor protein regulation. J Neurosci. 2008 Nov 5;28(45):11477-87.

42. Spillantini, M.G., van Swieten, J. C., and Goedert, M. (2000) Tau gene mutations in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17). Neurogenetics 2, 193–205.

43. Togo, T., Sahara, N., Yen, S.H., Cookson, N., Ishizawa, T., Hutton, M., de Silva, R., Lees, A., and Dickson, D.W. (2002) Argyrophilic grain disease is a sporadic 4-repeat tauopathy. J. Neuropathol. Exp. Neurol. 61: 547-556.

44. Tolstykh, T., Lee, J., Vafai, S., and Stock, J.B. (2000). Carboxyl methylation regulates phosphoprotein phosphatase 2A by controlling the association of regulatory B subunits. EMBO J. 2000 Nov 1;19(21):5682-91.

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

46. Virshup D.M. (2000) Protein phosphatase 2A; a panoply of enzymes: Curr Opin Cell Biol, 2000, 12: 180-185.

47. Wei, H., Ashby, D.G., Moreno, C.S., Ogris, E., Yeong, F.M., Corbett, A.H., and Pallas, D.C. (2001) Carboxymethylation of the PP2A catalytic subunit in Saccharomyces cerevisiae is required for efficient interaction with the B-type subunits Cdc55p and Rts1p. J Biol. Chem. 276, 1570-1577.

48. Weingarten, M.D., Lockwood, A.H., Hwo, S.Y., and Kirschner, M.W. (1975) A protein factor essential for microtubule assembly. Proc Natl Acad Sci USA 72:1858–1862

49. Widau, R.C., Jin, Y., Dixon, S.A., Wadzinski, B.E., and Gallagher, P.J. (2010) Protein phosphatase 2A (PP2A) holoenzymes regulate death associated protein kinase (DAPK) in ceramide-induced anoikis. J Biol Chem. 2010 Mar 10. [Epub ahead of print]

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

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

52. Xie, H., and Clarke, S.(1994) Protein phosphatase 2A is reversibly modified by methyl esterification at its C-terminal leucine residue in bovine brain. J.Biol.Chem.269,1981–19

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

54. Xu, Y., Xing, Y., Chen, Y., Chao, Y., Lin, Z., Fan, E., Yu, J.W., Strack, S., Jeffrey, P.D., and Shi, Y. (2006) Structure of the protein phosphatase 2A core enzyme bound to tumor-inducing toxins. Cell, 2006, 127: 341-353.

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