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Regulation of protein phosphatase 2A methylation by LCMT1 and PME-1 plays a critical role in differentiation of neuroblastoma cells

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Abstract

Neuritic alterations are a major feature of many neurodegenerative disorders. Methylation of protein phosphatase 2A (PP2A) catalytic C subunit by the leucine carboxyl methyltransferase LCMT1, and demethylation by the methylesterase PME-1, is a critical PP2A regulatory mechanism. It modulates the formation of PP2A holoenzymes containing the B α subunit, which dephosphorylate key neuronal cytoskeletal proteins, including tau. Significantly, we have reported that LCMT1, methylated C and B α expression levels are down-regulated in Alzheimer disease-affected brain regions. Here, we show that enhanced expression of LCMT1 in cultured N2a neuroblastoma cells, which increases endogenous methylated C and B α levels, induces changes in F-actin organization. It promotes serum-independent neuritogenesis and development of extended tau-positive processes upon N2a cell differentiation. These stimulatory effects can be abrogated by LCMT1 knockdown and S-adenosylhomocysteine, an inhibitor of methylation reactions. Expression of PME-1 and the methylation-site L309 Δ C subunit mutant, which decrease intracellular methylated C and B α levels, block N2a cell differentiation and LCMT1-mediated neurite formation. Lastly, inducible and non-inducible knockdown of B α in N2a cells inhibit process outgrowth. Altogether, our results establish a novel mechanistic link between PP2A methylation and development of neurite-like processes.

Keywords

Protein phosphatase 2A; methyltransferase; methylesterase; methylation; differentiation

INTRODUCTION

Ser/Thr protein phosphatase 2A (PP2A or PPP2) is a family of major multimeric enzymes that are abundant in the brain and other tissues. In the typical mammalian holoenzyme, the catalytic C subunit (PPP2CA or PPP2CB) is usually in complex with a scaffolding A subunit (PPP2R1A or PPP2R1B), and a third variable regulatory “B” subunit. Several B subunit gene families (B/B55/PPP2R2, B'/B56/PPP2R5, B"/PPP2R3 and striatins) have

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been identified, each comprising several isoforms and/or splice variants that are differentially expressed in tissues and cells. PP2A subunit composition plays a key role in ensuring PP2A substrate specificity. In addition, PP2A can be regulated by many cellular factors, subunit phosphorylation, methylation or ubiquitination, and interaction with a wide variety of cellular or foreign proteins and inhibitors (Reviewed in Sontag 2001; Janssens *et al.* 2008; Virshup and Shenolikar 2009). Notably, methylation of the catalytic C subunit on the Leu-309 residue has emerged in recent years as a highly-conserved mechanism that modulates the assembly of PP2A heterotrimeric complexes (Reviewed in Janssens *et al.* 2008). It is catalyzed by the leucine carboxyl methyltransferase LCMT1 (or PPMT1) (Lee and Stock 1993; Leulliot *et al.* 2004). In mammalian cells, methylation promotes the biogenesis and stabilization of major PP2A holoenzymes containing the B α (or PPP2R2A) subunit (Ogris *et al.* 1997; Bryant *et al.* 1999; Tolstykh *et al.* 2000; Yu *et al.* 2001; Schild *et al.* 2006a; Nunbhakdi-Craig *et al.* 2007; Longin *et al.* 2007). Significantly, knockdown and/or inactivation of LCMT1 are associated with reduced formation of B α -containing PP2A heterotrimers and a net loss of intracellular B α amounts (Sontag *et al.* 2007; Lee and Pallas 2007; Sontag *et al.* 2008). On the other hand, the dedicated PP2A methylesterase PME-1 (Ogris *et al.* 1999) binds to the active site of PP2A, resulting in both PP2A demethylation and inactivation (Xing *et al.* 2008). The complex between PME-1 and inactive PP2A may prevent the inappropriate activation of PP2A C during PP2A biogenesis (Hombauer *et al.* 2007). Thus, there is strong evidence that changes in C subunit methylation state can critically influence PP2A biogenesis and intracellular subunit composition, thereby affecting its substrate specificity. In support for the pathophysiological significance of this mechanism, we have previously reported that LCMT1, methylated PP2A and B α can become down-regulated *in vivo* in response to alterations in one-carbon metabolism (Sontag *et al.* 2007; Sontag *et al.* 2008), and in Alzheimer disease (AD) neurons bearing neurofibrillary tangles (Sontag *et al.* 2004b). Yet, the precise role and regulation of neuronal PP2A methylation are not well understood.

Notably, neuritic abnormalities and disruption leading to axonal transport defects are associated with pathological lesions in AD and other neurodegenerative disorders (Hashimoto and Masliah 2003; Stokin and Goldstein 2006). A critical role for general PP2A activity in axonogenesis and axonal transport was recently brought to light (Yang *et al.* 2007; Zhu *et al.* 2010). Moreover, specific PP2A isoforms participate in the process of neuronal differentiation (Strack 2002; Schild *et al.* 2006b; Van Kanegan and Strack 2009) and dendritic branching (Brandt *et al.* 2008). To gain some fundamental new insights into the functional significance of PP2A methylation for normal neuronal homeostasis and AD pathogenesis, we thus chose here to assess how deregulating PP2A methylation affects neuritogenesis in a widely used neuroblastoma cell model.

MATERIAL AND METHODS

N2a cell culture, transfection and generation of stable clones

Control (American Type Culture Collection, Manassas, VA) and transfected Neuro-2a (N2a) cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 2.5 mM Hepes, pH 7.4, 10% fetal bovine serum (HyClone, Logan, UT) and 10 μ g/ml gentamycin (Invitrogen). Unless indicated, all chemicals used in this study were from Sigma-Aldrich, St. Louis, MO. Cell transfection was performed using Metafectene ProTM reagent following the manufacturer's instructions (Biontex laboratories, Munich, Germany). N2a cells stably overexpressing either hemagglutinin (HA)-tagged wild-type C (N2a-Wt C), HA-tagged L309 Δ C (N2a-L309 Δ), HA-tagged LCMT1 (N2a-LCMT1), Myc-tagged PME-1 (N2a-PME1) or HA-tagged B α (N2a-B α) have been fully characterized in previous studies (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2007; Sontag *et al.* 2008). Double stable clones expressing both HA-tagged LCMT1 and either HA-tagged Wt C (N2a-LCMT1 + Wt C) or

HA-tagged L309Δ (N2a-LCMT1 + L309Δ) were obtained after re-transfection of N2a-LCMT1 clones with either pcDNA 3.1 expressing Wt C (Goedert *et al.* 2000) or the L309Δ C mutant (Sontag *et al.* 2007), followed by selection with 200 μg/ml hygromycin (Roche, Indianapolis, IN) and 600 μg/ml G418 (Invitrogen). Double stable clones expressing both LCMT1 and PME-1 (N2a-LCMT1 + PME-1) were obtained after re-transfection of N2a-LCMT1 clones with pBABE encoding Myc-tagged mouse PME-1 (Sontag *et al.* 2007) followed by selection with 200 μg/ml hygromycin and 1 μg/ml puromycin (Sigma). Cells stably re-transfected with the corresponding empty vector alone were used as “controls”. In all our experiments, we found that control cells behaved like untransfected N2a cells. At least 3 distinct stable clones and 2 mixed populations resulting from pooling selected clones together were used throughout our studies with similar results. The expression levels of transfected proteins were constantly monitored by both immunoblotting and/or immunofluorescence. Of note, we verified that ectopically expressed proteins could still be detected after incubation of cells in differentiation medium.

Knockdown experiments

Specific knockdown of LCMT1 or PME-1 was carried out in the indicated N2a cell clone after transfection with selective siRNA exactly as reported previously; control cells were transfected with corresponding mismatched siRNAs (Sontag *et al.* 2008). Under our experimental conditions, specific protein knockdown was obtained 72 h post-transfection and persisted for another 72 h (Sontag *et al.* 2008). Thus, differentiation studies were started 72 h post-transfection. Specific PP2A Bα subunit knockdown was performed in N2a cells using transfection with the pSIREN-DNR-DsRed-Express-shBα vector targeting sh-Bα, exactly as described previously (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2008). N2a cell-derived clones allowing inducible Bα knockdown were generated as follows. N2a were transfected with the vector pcDNATM6/TR (Invitrogen) containing the tetracycline repressor (TR) gene under the control of the human cytomegalovirus (CMV) immediate early promoter and a blasticidin resistance gene. Single clones were isolated and a cell line stably expressing TR, termed N2aTRex14, was established. N2aTRex14 were transfected with pNTO^{Puro} containing a shRNA targeting PP2A Bα subunit (shBα, AAGTGGCAAGCGAAAGAAAGA) or a corresponding shRNA mismatch sequence (shControl, GTGACAAGCTAAAGATAGA) (Van Kanegan *et al.* 2005). The pNTO^{Puro} vector was obtained by replacing the neomycin with the puromycin resistance gene in the pNTO^{Neo} vector (Strack *et al.* 2004). Stable N2aTRex14-shBα and N2aTRex14-shControl clones were obtained following selection with 5 μg/ml puromycin (Sigma) and single cell expansion. They were grown in DMEM supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin, 100 μg/ml streptomycin and 5 μg/ml blasticidin (Invitrogen).

The efficiency of protein knockdown was systematically verified by Western blotting at the beginning and end of each differentiation experiment performed in this study. Since maximal knockdown of LCMT1, PME-1 or Bα can ultimately lead to apoptosis (Lee and Pallas 2007; Sontag *et al.* 2008), the experimental conditions were optimized to allow for protein knockdown while minimizing potential cell loss.

Determination of protein expression and PP2A methylation levels

Total cell extracts were prepared and analyzed by gel electrophoresis and Western blotting for LCMT1, PME-1 and PP2A subunit expression levels exactly according to our published protocols (Sontag *et al.* 2007; Sontag *et al.* 2008). Primary antibodies utilized in this study included anti-HA clone 16B12 (Covance Research Products Inc., Berkeley, CA), anti-Myc clone 4A6 and anti-B55 clone “2G9” (Millipore, Temecula, CA). Anti β-actin antibodies (Sigma) were utilized to normalize for protein loading. Monoclonal methylation- or demethylation-specific (Millipore), and methylation-independent (BD Biosciences, San

Jose, CA) anti-C antibodies were utilized to quantify PP2A methylation levels by Western blotting and densitometry exactly as described previously (Sontag *et al.* 2007; Sontag *et al.* 2008).

Cell differentiation

To study the ability of cells to grow neurites, all cells were plated in regular cell culture medium at equivalent low density ($\sim 5 \times 10^4$ cells/cm²) onto poly-L-Lysine (Sigma)-coated multi-well dishes (for morphological analysis), glass coverslips (for immunocytochemistry) or filter inserts (for neurite outgrowth quantification assays). For N2a knockdown experiments, cells were seeded on poly-L-lysine-coated dishes 72 h post-transfection. Once attached (after 4–6 h), the cells were quickly washed with phosphate-buffered saline (PBS) and incubated for 24–96 h in differentiation medium (DMEM containing 0.1% FBS). Control cells were left in regular cell culture medium. When indicated in some experiments, 10 μ M retinoic acid (RA; Sigma) was added to the differentiating medium. For differentiation studies of stable N2aTRex14 clones, cells were first incubated for 72 h in regular cell culture medium with or without 1 μ g/ml Doxycyclin (Dox, Sigma) then seeded onto poly-L-Lysine-coated multi-well dishes. On the next day, cells were incubated for 24–48 h in differentiation medium containing 1% serum in the presence or absence of 10 μ M RA and 1 μ g/ml Dox. Note that B α knockdown was induced in N2a-TRex-shB α clones by supplementing the cell culture medium for 72 h with 1 μ g/ml Dox, and could be maintained by continuing to supplement the medium with Dox for the whole duration of the differentiation experiment.

Analysis of cell morphology and neurite-like cell processes

Cells were analyzed by phase-contrast microscopy for their morphology and the presence and length of neurite-like processes, which were defined here as thin protrusions that were at least one cell diameter in length (Dehmelt *et al.* 2003). At least 100 cells/condition and 3 distinct stable cell clones or transfection experiments were analyzed in each condition. Just prior to examination, the cell medium was carefully replaced with phenol red-free DMEM medium (Invitrogen). Cells were examined by phase contrast (15 $\times$ magnification) on a Nikon Eclipse TE2000-U microscope equipped with a digital camera. Images were directly acquired using the MetaMorph software and transferred to Adobe Photoshop/Illustrator CS4 (Adobe Systems Incorporated, San Jose, CA). The length of neurite-like processes was determined after manual tracing. Statistical analysis was performed using Student's *t*-test. Differences with *p* values < 0.05 were considered statistically significant. In some experiments, quantification was performed using the Neurite Outgrowth Quantification Assay kitTM (Millipore, Temecula, CA). Briefly, cells were plated in duplicate filter inserts of Transwell plates and processed 72 h after incubation in differentiation medium. Quantification of process outgrowth was performed after staining and extraction of the cell processes following the manufacturer's instructions. In other experiments, duplicate batches of cells were plated on glass coverslips and analyzed by immunofluorescence microscopy for the presence of neurite-like processes. Cells were washed quickly at 37°C with PEM buffer (0.1 M PIPES, pH 6.9, 2 mM EGTA, 5 mM MgCl₂) and fixed for 5 min at –20°C with absolute methanol (Nunbhakdi-Craig *et al.* 2007). Cells were stained for 1 h with mouse anti- β III-tubulin antibody (Covance Research Products, Berkeley, CA) followed by incubation for 1 h with Alexa Fluor 488-conjugated goat antibodies (Invitrogen). The samples were mounted with Fluoromount (Fisher Scientific, Pittsburgh, PA) and examined on a Zeiss microscope (Carl Zeiss Inc., Thornwood, NY) using a 63 $\times$ objective. Captured images were transferred to Adobe Photoshop/Illustrator CS4.

Analysis of tau and F-actin by confocal microscopy

Cells were fixed for 20 min with 4% paraformaldehyde, permeabilized for 5 min in phosphate buffered saline containing 0.1% Triton X-100, then washed and labeled exactly as described previously (Sontag *et al.* 1995; Nunbhakdi-Craig *et al.* 2007). Cells were stained for 2 h at room temperature with rabbit anti-tau (1:1,000; rPeptide, Bogart, GA) or monoclonal Tau-1 (1:100; Millipore; North Ryde, Australia) antibodies followed by incubation for 1 h with Alexa Fluor 488-conjugated goat antibodies (Invitrogen). To visualize actin, cells were directly stained for 1 h with Alexa Fluor 488-phalloidin (Invitrogen). The samples were mounted with Fluoromount and examined on an Olympus FluoView FV1000 confocal microscope (Olympus, North Ryde, Australia) using a 60x objective. Captured images were transferred to Adobe Photoshop and Illustrator CS4.

RESULTS

Expression of the methylation-site mutant of PP2A catalytic subunit prevents N2a cell differentiation

We have previously shown that endogenous PP2A methylation levels can be selectively manipulated in N2a cells using ectopic expression or knockdown of PP2A methyltransferase and methylesterase, and expression of the PP2A methylation-site L309 Δ C subunit mutant (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2007; Sontag *et al.* 2008). Similar strategies were used here to investigate the role of PP2A methylation in N2a cell differentiation. Like other neuroblastoma cells, N2a cells can be induced to differentiate following serum starvation and addition of retinoic acid (RA) (Wu *et al.* 2009). Enhanced expression of the neuronal-specific class III β -tubulin accompanies neurite outgrowth during differentiation, so that neurite-like processes can be labeled using antibodies to β III-tubulin (Aletta 1996), or directly visualized by phase contrast microscopy. As expected, control N2a cells plated on Poly-L-lysine and grown in regular cell culture medium exhibited the typical undifferentiated morphology characterized by round-shaped cell bodies with absent or tiny cell processes (Fig. 1a). When they were switched from high to low serum-containing cell culture medium, they started to become pyramidal and grew several neurite-like processes. About 20–25% of control N2a cells ($n = 3$) harbored processes after 24 h incubation in differentiation medium. However, ~80–90% ($n = 3$) of N2a-Wt C cells stably expressing PP2A wild-type C subunit and 0–2% ($n = 3$) of N2a-L309 Δ clones stably expressing the methylation-defective L309 Δ C subunit mutant were differentiated under the same experimental conditions. In this context, it is worth mentioning that we have previously reported that total C subunit levels are increased by ~30% in N2a-Wt C and N2a-L309 Δ cells relative to controls, and that HA-tagged wild-type or mutated C subunit levels represent ~40% of total C subunit levels in these clones (Sontag *et al.* 2007). Overexpression of PP2A Wt C subunit, which increases total endogenous PP2A activity by ~30% relative to controls (Sontag *et al.* 2007), also appeared to stimulate formation of neurite-like processes. After 48 h incubation in low serum medium, N2a-Wt C cells were well-differentiated, but N2a-L309 Δ cells failed to develop processes (Fig. 1b). A quantitative assay further confirmed that expression of Wt C subunit stimulated outgrowth of neurite-like processes by ~3-fold in N2a cells incubated for 72 h in low serum-containing medium, while expression of the L309 Δ C mutant dramatically prevented it (Fig. 1c). Similar stimulatory effects of expressed Wt C on differentiation were observed in N2a cells incubated with RA, which is known to accelerate neuritogenesis in serum-starved cells. At the same time, incubating N2a-L309 Δ cells in RA-containing differentiation medium not only prevented differentiation but ultimately led to compromised cell survival (data not shown).

Expression of PME-1 in N2a cells inhibits cell differentiation while expression of LCMT1 promotes the formation of elongated neurite-like processes

We next assessed the effects of modulating PP2A methylesterase and methyltransferase expression levels in N2a cells. As illustrated in Fig. 2a, stable overexpression of PME-1 in N2a cells, which was accompanied by a ~3-fold increase in total intracellular PME-1 amounts relative to control cells (Sontag *et al.* 2007), prevented cell differentiation. While extended neurite-like processes were prominent in control cells, only marginal sprouting was observed in N2a-PME1 clones even after 72 h incubation in low serum medium. Conversely, selective PME-1 knockdown in N2a cells, which decreases endogenous PME-1 expression levels by ~68–88% (Sontag *et al.* 2008), promoted the formation of very elongated and thin neurite-like projections (Fig. 2b). In contrast to PME-1, stable overexpression of LCMT1, which is associated with a ~2-fold increase in total intracellular LCMT1 levels relative to controls (Sontag *et al.* 2007), was sufficient to induce neuritogenesis in the presence of serum. This was evidenced by the presence of long and thick projections extending from the cell bodies (Fig. 3a), and clearly distinguished N2a-LCMT1 cells from the typical undifferentiated phenotype of control N2a cells grown in regular serum-rich culture medium (Fig. 2a). The outgrowth of neurite-like processes was further accelerated after incubating N2a-LCMT1 cells in low serum-containing differentiating medium. Similar effects were observed in N2a-LCMT1 clones incubated in RA-containing differentiation medium (data not shown). Interestingly, while multiple neurite-like processes per cell body and secondary branching were commonly observed in differentiated control cells, N2a clones expressing high levels of LCMT1 primarily harbored either a single neurite-like protrusion or straight bipolar projections without interstitial branching. Compared to controls, the mean process length of N2a-LCMT1 cells was significantly increased in both high and low serum-containing culture media (Fig. 3b). Notably, these effects were abrogated following LCMT1 knockdown (Fig. 3c), which reduces endogenous LCMT1 levels by ~77–95% (Sontag *et al.* 2008), or supplementation of the cell medium with S-adenosylhomocysteine (SAH) (Fig. 3d), an inhibitor of LCMT1 methyltransferase activity (Leulliot *et al.* 2004; Sontag *et al.* 2007).

Further immunofluorescent analysis of N2a-LCMT1 extended cell processes revealed that they were immuno-positive for β III-tubulin (Fig. 4a) and the axon-specific marker tau (Fig. 4b), indicating that these elongated protrusions resemble neurites and share axonal-like properties of primary neurons (Dehmelt and Halpain 2004). Significantly, the actin filament (F-actin) network also appeared to be reorganized in differentiated N2a-LCMT1 cells, relative to controls (Fig. 4c). F-actin-rich filopodia were abundant in differentiated N2a cells with multiple neurite-like projections or secondary branching. In contrast, the overall actin staining pattern was punctuated and disorganized in differentiated N2a-LCMT1 cells, with only very few short and thin actin filaments visible along the elongated neurite-like processes. These results suggest that enhanced expression of LCMT1 in N2a cells affects F-actin dynamics, which is known to play a critical role in neurite initiation, elongation and branching (Reviewed in Da Silva and Dotti 2002; Luo 2002).

Enhanced intracellular amounts of methylated PP2A correlate with the initiation and elongation of neurite-like processes

Interestingly, concomitant ectopic expression of either PME-1 or the Leu309 Δ C subunit mutant in N2a-LCMT1 clones blocked LCMT1-induced neuritogenesis (Fig. 5). On the other hand, expression of Wt C in N2a-LCMT1 clones had a synergistic effect on process outgrowth. As observed in N2a-LCMT1 clones, N2a-LCMT1 + Wt C cells were able to develop long processes in both high and low serum media. Compared to the parental N2a-LCMT1 cells (Fig. 3a), there was a ~1.3- to 1.6-fold ($n = 3$) increase in mean process length in these double clones upon differentiation. However, unlike N2a-LCMT1 cells,

differentiated N2a-LCMT1 + Wt C cells frequently exhibited secondary branching, so that their morphological appearance was more closely related to that of well-differentiated N2a-Wt C clones (Fig. 1c).

To further support the hypothesis that PP2A methylation plays a major role in neurite-like process initiation and outgrowth, we compared PP2A methylation state in the single and double clones that were analyzed here for their ability to differentiate (Fig. 6). Stable expression of the L309 Δ C mutant or PME-1 in N2a cells, which inhibit differentiation (Figs. 1 and 2), reduced endogenous levels of methylated PP2A by ~45% and ~60%, respectively, according to our previous studies (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2007). Conversely, total methylated PP2A levels were increased by ~35% (Sontag *et al.* 2007) and neuritogenesis was readily stimulated (Fig. 3) following stable overexpression of LCMT1 in N2a cells. Expression of Wt C, which enhances total C subunit amounts and PP2A activity by ~30% in N2a cells (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2007), also increased the total pool of intracellular PP2A C available for LCMT1-dependent methylation. Not surprisingly, the highest levels of methylated PP2A were present in clones overexpressing both LCMT1 and PP2A Wt C subunit. Relative to controls, the ~2-fold increase in endogenous methylated PP2A content in these double clones was associated with marked process outgrowth either in the absence or presence of serum (Fig. 5b). On the other hand, LCMT1-mediated neuritogenesis was abolished following expression of either the L309 Δ mutant or PME-1 (Fig. 5), which both induced a large drop in intracellular methylated PP2A amounts relative to the parental N2a-LCMT1 clones. Of note, N2a-LCMT1 + PME-1 double clones expressed higher levels of the methyltransferase than N2a-PME1 single clones, which likely explains why total levels of methylated C were lower in these cells. Altogether, our results suggest that enhancing PP2A methylation stimulates the formation and elongation of neurite-like processes. They also support the hypothesis that functional levels of LCMT1 and PME-1, and a fine balance between intracellular methylated and demethylated PP2A pools, are critically required for proper N2a cell differentiation.

B α is critically required for formation of neurite-like processes

We have previously shown in N2a cells that LCMT1-dependent PP2A methylation promotes the formation and stabilization of B α -containing PP2A holoenzymes; conversely, down-regulation of LCMT1 and methylated C is associated with a loss of endogenous B α subunits (Sontag *et al.* 2008). The results of our PP2A methylation studies thus raised the possibility that B α -containing PP2A holoenzymes, which expression is highly sensitive to changes in C subunit methylation state, participate in N2a cell differentiation. To address this hypothesis, we examined the behavior of N2a cells in response to changes in B α expression levels. As illustrated in Fig. 7a-b, overexpression of B α in N2a cells, which results in a mean ~180% increase in endogenous B α levels relative to controls (Sontag *et al.* 2008), stimulated neurite-like process extension. Conversely, selective knockdown of B α in N2a cells, which reduces endogenous protein levels by ~half (Nunbhakdi-Craig *et al.* 2007; Sontag *et al.* 2008), inhibited cell differentiation. To further support the role of B α in process formation, we also generated stable N2a-derived, N2a-Trex14 cell lines in which B α knockdown could be induced by incubation of cells with doxycycline (Dox) (Fig. 7c). As observed in N2a cells transiently transfected with vectors encoding B α silencing (shB α) target sequences (Fig. 7a), the induction of B α knockdown in N2a-Trex14 clones resulted in marked down-regulation of B α expression levels. The decrease in B α levels was sufficient to block the neuritogenesis normally triggered by incubating cells in medium containing low serum with or without RA (Fig. 7d). At the same time, N2a-Trex14-shB α clones maintained in the absence of Dox, or N2a-Trex-shControl clones stably transfected with vectors encoding shControl mismatch B α target sequences, were able to extend processes when switched to

differentiation media. Together, these results support the hypothesis that Ba-containing PP2A isoforms play an essential role in N2a cell differentiation.

DISCUSSION

In agreement with a recent report showing that up-regulation of PP2A catalytic subunit promotes axonal formation and elongation while PP2A inhibition prevents differentiation (Zhu *et al.* 2010), we found that neurite-like process outgrowth is facilitated in N2a-WT C cells. However, instead of looking at the effect of modulating PP2A activity, we chose here to focus our attention on assessing the potential role of PP2A methylation in N2a cell differentiation, which have been widely used to study formation of neurite-like processes. In the context of these studies, it is important to note that C subunit methylation likely modulates PP2A substrate specificity (Reviewed in Janssens *et al.* 2008) through its role in PP2A biogenesis (Hombauer *et al.* 2007). We and others have shown that the methylation-site L309 Δ mutant cannot bind to regulatory B subunits, but can still associate with other regulatory subunits from the B' and B'' families (Nunbhakdi-Craig *et al.* 2007; Longin *et al.* 2007). In addition, down-regulation of LCMT1 and methylated PP2A expression levels lead to a loss of Ba and compensatory changes in intracellular PP2A subunit composition that can affect its substrate specificity and targeting (Schild *et al.* 2006a; Gentry *et al.* 2005; Lee and Pallas 2007; Longin *et al.* 2007; Sontag *et al.* 2008). Our data provide the first evidence that modulating PP2A methylation can dramatically affect the differentiation process in N2a cells. We have also obtained similar results in preliminary studies performed in human SH-SY5Y neuroblastoma and rat PC12 cells (E. Sontag, unpublished results). Notably, enhancing LCMT1 expression was able to trigger serum-independent differentiation. Data from the double N2a-LCMT1 + L309 Δ clones indicated that the ability of LCMT1 to drive the initiation and extension of neurite-like processes was primarily mediated by PP2A. Most importantly, the extent of both process formation and elongation appeared to be highly correlated with the total levels of methylated PP2A enzymes present in our single and double clones. Interestingly, protein methylation is involved in neurite outgrowth in PC12 cells (Cimato *et al.* 1997) and general inhibition of methyltransferase activity interferes with neuronal differentiation of P19 embryonal cells (Hong *et al.* 2008). Accordingly, the ability of LCMT1 to promote neuritogenesis was impeded by incubating N2a cells with 100 μ M SAH, a universal inhibitor of cellular methylation reactions. In agreement with the role of this metabolite in inhibiting LCMT1 methyltransferase activity (Leulliot *et al.* 2004), we have previously reported that supplementing N2a cell culture medium with 100 μ M SAH induces a 40–50% decrease in endogenous methylated C levels (Sontag *et al.* 2007). Thus, together with our findings that the L309 Δ mutant blocks neuritogenesis in N2a and N2a-LCMT1 cells, these observations strongly support the hypothesis that PP2A is one of the methylated proteins critically required for commencement of the neuronal differentiation process.

Results from PME-1 overexpression studies in N2a and N2a-LCMT1 cells also indicate that increased PP2A demethylation has an adverse effect on the initiation of neurite-like process formation. This may be in part because PME-1 overexpression induces a loss of methylated PP2A and subsequent loss of Ba in N2a cells (Sontag *et al.* 2008), and the resulting down-regulation of Ba prevents differentiation (Fig. 7). Ba-containing PP2A holoenzymes are very abundant in neurons where they regulate microtubule stability (Nunbhakdi-Craig *et al.* 2007) and the phosphorylation state of tau (Sontag *et al.* 2008; Xu *et al.* 2008), amyloid precursor protein (APP) (Sontag *et al.* 2007) and neurofilament proteins (Strack *et al.* 1997). It is well recognized that the microtubule cytoskeleton (Fukushima *et al.* 2009), tau (Stoothoff and Johnson 2005), APP (Stokin and Goldstein 2006) and neurofilament proteins (Szabo and Strong 2010) all play a critical role in axonal growth. In addition, many signaling pathways, including the MAP kinase (ERK) pathway (Van Kanegan *et al.* 2005), are

regulated by B α -containing PP2A holoenzymes. Based on these observations, it is difficult to pinpoint the precise mechanisms that directly or indirectly contribute to B α -dependent effects on N2a cell differentiation in our study.

It has been reported that differentiation of SH-SY5Y cells or PC12 cells does not affect B α mRNA or protein expression levels. In contrast, the mRNA and/or protein levels of B β , B γ , and B δ , which also belong to the PP2A regulatory B subunit family, but are much less abundant than B α , become either up- or down-regulated during the differentiation process in those cells. As a result, it had been proposed that those are more likely to be specifically required for early neurite formation (Strack 2002; Schild *et al.* 2006b). Indeed, B γ plays a key role in MAP kinase-dependent PC6-3 cell differentiation (Strack 2002). Recently, PP2A holoenzymes containing the B β and B δ subunits have also been implicated in nerve growth factor-mediated PC12 cell differentiation (Van Kanegan and Strack 2009), and dendritic branching (Brandt *et al.* 2008). While B α protein expression levels do not fluctuate during the differentiation process, our results indicate that, nevertheless, these basal amounts are critically required for proper neuritogenesis. Indeed, B α knockdown, which also leads to a subsequent loss of LCMT1 expression levels in N2a cells (Sontag *et al.* 2008), inhibited N2a cell differentiation (Fig. 7), thereby mimicking the effects of LCMT1 knockdown (Fig. 3). On the other hand, overexpression of B α , which is associated with down-regulation of demethylated C levels (Sontag *et al.* 2008), promoted process elongation in serum-starved N2a cells (Fig. 7). Thus, there is a very close interrelationship between LCMT1-dependent PP2A methylation, B α expression levels and process formation. Yet, we cannot exclude the possibility that variations in intracellular B α amounts also indirectly influence neuritogenesis by inducing a compensatory increase or decrease in the levels of other regulatory “B” subunit-containing PP2A holoenzymes that play a role in the differentiation process. While many PP2A isoforms have the potential to regulate neuronal differentiation, it is likely that they are recruited in response to distinct extracellular or intracellular stimuli, are functioning in well-defined signaling pathways, and have a differential cellular and subcellular distribution. Further studies will be needed to elucidate their respective contribution to each step of the differentiation process by identifying their specific substrates. However, the lack of suitable B-specific antibodies, and the complex regulation and widespread cellular effects exerted by various PP2A isoforms make this a quite difficult prospect.

Interestingly, tau- and microtubule-containing straight projections reminiscent of neuronal axons were the hallmark of differentiated N2a-LCMT1 cells (Fig. 4). Secondary neuritic branching was observed in differentiated N2a, N2a-Wt C and N2a-LCMT1 + Wt C, but not in N2a-LCMT1 cells. These discrepancies reinforce the central dogma that PP2A activity and methylation differentially modulate PP2A function, and have divergent effects on the growth of primary and secondary cellular processes. Significantly, we observed that the organization of F-actin was altered in N2a-LCMT1 cells relative to controls. Together with microtubules, the actin cytoskeleton plays a critical role in neuritic development (Dehmelt and Halpain 2004). Axon formation, elongation and branching are highly sensitive to local perturbation of microtubule and actin dynamics (Luo 2002). It is thus tempting to speculate that local changes in actin stability could underlie the observed differentiated phenotype of N2a-LCMT1 cells. However, in-depth studies are required to fully understand the mechanisms by which PP2A methylation affects neuronal morphogenesis.

As observed with LCMT1 overexpression, PME-1 knockdown led to the formation of elongated processes. However, they appear somehow anomalous, being quite thin compared to the standard neurites of differentiated N2a cells. We have previously reported that PME-1 silencing in N2a cells does not significantly affect B α and LCMT1 expression levels (Sontag *et al.* 2008). While enhanced PME-1 expression inhibits N2a cell differentiation, the

aberrant process formation induced by artificially decreasing intracellular PME-1 levels suggests that threshold levels of this PP2A methylesterase need to be maintained for ensuring normal neurite-like process development. Indeed, while LCMT1 and PME-1 selectively regulate PP2A methylation state, combined data from our differentiation experiments and earlier studies (Sontag *et al.* 2008) indicate that they do not simply exert opposite actions on PP2A regulation. When examined altogether, our results suggest that a subtle equilibrium between LCMT1 and PME-1 expression levels and activity, and their subsequent modulation of the methylation/demethylation state of specific PP2A isoforms, is required for proper coordinated development of the neuritic network.

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Abbreviations used

AD	Alzheimer disease
APP	amyloid precursor protein
Dox	Doxycyclin
F-actin	actin filaments
LCMT1	leucine carboxyl methyltransferase 1
PME-1	protein phosphatase methylesterase 1
PP2A	protein phosphatase 2A
RA	retinoic acid
SAH	S-adenosylhomocysteine
Wt C	wild-type C subunit

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Fig. 1.

Expression of the PP2A methylation-defective L309 Δ C subunit mutant inhibits N2a cell differentiation. Control N2a and N2a cells stably expressing the wild-type (N2a-Wt C) or mutated L309 Δ (N2a-L309 Δ) PP2A C subunit were incubated in regular cell culture medium containing 10% serum (High serum), or incubated in differentiation medium containing 0.1% serum (Low serum). (a) Cells were analyzed after 24 h by immunofluorescence for the presence of neurite-like processes after staining with anti- β III-tubulin antibody. (b) Cells were analyzed by phase contrast for the presence of processes 48 h after differentiation. For (a) and (b), representative photographs are shown. Similar results were obtained in three separate experiments and with three distinct clones. Bars = 40 μ m. (c) Neurite-like process outgrowth was quantified using a colorimetric assay kit 72 h after cell differentiation (mean $\pm$ SEM, $n = 3$).

Fig. 2. PME-1 expression affects N2a cell differentiation. (a) Control N2a and N2a cells stably expressing PME-1 (N2a-PME1) were incubated for the indicated time in medium containing either 10% (High serum) or 0.1% (Low serum) serum and analyzed by phase contrast microscopy. Note that same results were obtained in N2a-PME1 cells cultured at higher density. (b) N2a cells were either mock-transfected (N2a-Mock) or transfected with siRNA targeted for PME-1 (N2a-siPME1) or a control mismatch siRNA (N2a-siControl). After 72 h, cells were switched to differentiation medium containing 0.1% FBS (Low serum) and incubated for another 48 h. For (a) and (b), representative pictures are shown. Similar results were obtained in three separate experiments and N2a-PME1 clones. Bars = 40 μ M.

Fig. 3. Enhanced expression of LCMT1 promotes the formation of extended neurite-like processes. (a) Control N2a and N2a cells stably expressing LCMT1 (N2a-LCMT1) were incubated for the indicated time in medium containing either 10% (High serum) or 0.1% (Low serum) serum and analyzed by phase contrast microscopy. (b) Mean process length was determined in control N2a (white bars) and N2a-LCMT1 clones (black bars) by measuring the length of protrusions in ~100 cells per experimental condition, and in three separate clones and experiments (mean $\pm$ SD; $n = 3$; * $p < 0.001$ relative to controls). (c) N2a-LCMT1 cells were transfected with either siRNA targeted for LCMT1 (siLCMT1) or a control mismatch siRNA (siControl) then incubated 72 h post-transfection in differentiation medium containing 0.1% FBS for 48 h. (d) N2a-LCMT1 clones were incubated for 24 h in differentiation medium containing 100 μM SAH. For (a), (c) and (d) representative photographs are shown. Similar results were obtained in three separate experiments and clones. Bars = 40 μM .

Fig. 4.

Characterization of N2a-LCMT1 cells reveals changes in F-actin organization. N2a and N2a-LCMT1 cells were differentiated for 48 h in DMEM containing 0.1 % serum then processed for analysis by confocal microscopy. Representative pictures from three separate labeling experiments are shown. Bars = 20 μ m. (a) N2a-LCMT1 cells were labeled with anti- β III-tubulin antibody, a marker of neurite-like processes. (b) N2a-LCMT1 cells were stained with a rabbit anti-tau antibody to detect axonal-like processes. Similar results were obtained with anti-tau “Tau-1” antibody (data not shown) (c) N2a and N2a-LCMT1 cells were labeled with fluorescent phalloidin to detect F-actin. Two separate panels are shown to illustrate distinct details of F-actin organization in those cells. Note the presence of many filopodia in N2a cells (arrowheads) and the contrasting punctuated pattern of the F-actin network in N2a-LCMT1 clones.

Fig. 5.

Expression of either L309Δ C subunit mutant or PME-1 blocks LCMT1-dependent process formation. (a) N2a-LCMT1 cells were stably transfected with plasmids expressing either HA-tagged Wt C (N2a-LCMT1 + Wt C), HA-tagged Leu309Δ C (N2a-LCMT1 + L309Δ), myc-tagged PME-1 (N2a-LCMT1 + PME-1) or the corresponding empty vector (N2a-LCMT1 + vector). After selection, the clones were analyzed by Western blotting with either anti-HA or anti-Myc antibodies to detect ectopically expressed protein levels. Single clones are shown for reference. (b–c) The clones were incubated for the indicated time in medium containing either 10% (High serum) or 0.1% (Low serum) serum, and analyzed by phase contrast microscopy. Representative photographs are shown. Similar results were obtained in three separate experiments performed with three separate double clones. Note that the morphology of N2a-LCMT1 clones transfected with corresponding empty vectors was undistinguishable from that of untransfected or mock-transfected N2a-LCMT1 clones (data not shown). Bars = 40 μm.

Fig. 6.

PP2A methylation status in single and double N2a clones. Total homogenates were prepared from four separate N2a single or double clones and analyzed by Western blotting for PP2A methylation state using a combination of methylation-specific, demethylation-specific and methylation-independent monoclonal anti-C antibodies. Total cellular methylated C subunit levels (mean $\pm$ SD, $n=4$, percent of methylation measured in control cells) were determined after quantitative densitometry analysis of the immunoblots. Similar methylated C levels were observed in untransfected or mock-transfected N2a cells (data not shown).

Fig. 7.

Changes in B α expression levels affect neurite-like process formation. (a) N2a cells were mock-transfected (N2a-Mock) or transfected with either sh-B α (N2a-shB α) or HA-tagged B α (N2a-B α). Representative phase contrast images show the effects of B α overexpression or knockdown on process formation in N2a cells ~40 h after incubation in differentiation medium containing 0.1% FBS (Low serum). Bar = 40 μ m. (b) Mean length of cell processes was determined in ~100 cells per condition and in 3 separate experiments (mean $\pm$ SD; $n = 3$; * $p < 0.001$). (c) Inducible N2aTRex14 cell lines stably expressing shB α (shB α) or a mismatch control (shControl) were analyzed by Western blotting for B α expression levels 3 days after incubation in regular cell culture medium in the absence (- Dox, white bars) or presence (+ Dox, black bars) of 1 μ g/ml doxycycline. The corresponding bar graph shows B α expression levels in the clones after normalization for β -actin levels. (d) N2aTRex14-shControl and N2aTRex14-shB α clones were analyzed by phase contrast 48 h after incubation in differentiation medium containing 1% serum without (Low serum) or with 10 μ M RA (Low serum + RA). Bar = 40 μ m.