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Jocelyn Anne Lee Date

The Role of Leucine Carboxyl Methyltransferase 1 in Cell Growth and Mouse Development

By

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Graduate Division of Biological and Biomedical Science
Biochemistry, Cell, and Developmental Biology

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The Role of Leucine Carboxyl Methyltransferase 1 in Cell Growth and Mouse Development

By

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M.S. Biology, Virginia Commonwealth University, 2004
B.A. Biology, University of Virginia, 2000

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Abstract

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By

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Protein Phosphatase 2A (PP2A), a multifunctional Serine/Threonine phosphatase, has been implicated in the regulation of cell growth and proliferation, primarily at the G2/M transition, and in the development of human cancers. Because PP2A is involved in so many diverse processes, it is highly regulated by both non-covalent and covalent mechanisms that are still being defined. PP2A is methylated on its catalytic subunit C-terminal leucine alpha-carboxy group by Leucine Carboxyl Methyltransferase-1 (LCMT-1) and is demethylated by Protein Methylesterase-1 (PME-1). Reversible methylation is the most specific cellular mechanism for regulating PP2A, and thus may have promise as a mechanism-based therapeutic target. Little is known about the molecular mechanisms and regulation of the PP2A C subunit methylation by LCMT-1 that is specific to cell proliferation and development. In this study we have analyzed the importance of LCMT-1 for PP2A methylation and function in cell cycle regulation and mouse development. We have found that downregulation of LCMT-1 in cultured cells, results in cell cycle defects specifically during mitosis, including mitotic arrest and apoptosis. Moreover, we have found that homozygous gene-trap knockout of *lcmt-1* in mice results in embryonic lethality due to severe defects in the developing liver and brain. Loss or downregulation of LCMT-1 results in up to a 60% reduction in PP2A methylation in the developing embryo as well as cultured cells, demonstrating that LCMT-1 is the sole mammalian PP2A methyltransferase. We have observed a dramatic reduction of PP2A heterotrimers containing the Bα B family regulatory B-type subunit, the subunit that is the most affected by methylation. Lastly, we have observed a dramatic increase in the phosphorylation of microtubule-associated protein tau, a hallmark of Alzheimer's disease, in the developing embryo brain at known PP2A-regulated residues. Taken together, our results indicate that LCMT-1 is a key regulator of growth and development and is important for cell proliferation and differentiation.

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Table of Contents

List of Tables

Table Page

List of Figures

Introduction

Chapter 1: Leucine Carboxyl Methyltransferase 1 is Necessary for Normal Progression Through Mitosis in Mammalian Cells

Chapter 2: Loss of Leucine Carboxyl Methyltransferase 1 Results in Embryonic Lethality and Fetal Liver Apoptosis

List of Abbreviations

A subunit	Protein Phosphatase 2A Catalytic Scaffolding A Subunit
AD	Alzheimer's disease
Bα subunit	Protein Phosphatase 2A B alpha Regulatory B-type subunit
CFU	Colony formation unit
C subunit	Protein Phosphatase 2A Catalytic Subunit
DNA	Deoxyribonucleic acid
GEMM	Granulocyte/Erythrocyte/Monocyte/Megakaryocyte
GM	Granulocyte/Monocyte
G1/S	Growth 1 phase/Synthesis phase
G2/M	Growth 2 phase/Mitotic phase
HA	Hemagglutinin
H&E	Hematoxylin and eosin
HSC	Hematopoietic stem cell
E	Erythrocyte
ES	Embryonic stem cell
F1	First filial generation
LCMT-1	Leucine Carboxyl Methyltransferase -1
LCMT-2	Leucine Carboxyl Methyltransferase -2
Leu	Leucine
kDa	Kilodalton
MPF	Mitotic promoting factor
MPM2	Mitotic Protein Monoclonal 2
mRNA	Messenger ribonucleic acid
MT	Polyomavirus middle tumor antigen
NFT	Neurofibrillary tangle
PCR	Polymerase chain reaction
PME-1	Protein Phosphatase Methylesterase -1

PP2A	Protein Phosphatase 2A
$PP2A_{BAC}$	Protein Phosphatase 2A heterotrimers containing catalytic, scaffolding, and the B family B-type regulatory subunit
$PP2A_{B\alpha AC}$	Protein Phosphatase 2A heterotrimers containing catalytic, scaffolding, and the B alpha B-type regulatory subunit
PP4	Protein Phosphatase 4
PP6	Protein Phosphatase 6
RNA	Ribonucleic acid
Ser	Serine
shRNA	small hairpin RNA
ST	Polyomavirus and SV40 small tumor antigens
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Introduction

Sections 1-5 were adapted from Lee, J.A. and D.C. Pallas. Lcmt-1. *UCSD Molecule Pages* (2006). (doi.1038/mp.a003167.01)

LCMT-1 and PP2A Methylation

Leucine Carboxyl Methyltransferase-1 (LCMT-1) is one of several types of carboxyl methyltransferases that modify the activity of various prokaryotic and eukaryotic proteins (1-3). The only known substrate of LCMT-1 is the catalytic (C) subunit of protein phosphatase 2A (PP2A), a major Serine/Threonine protein phosphatase involved in a variety of cellular processes including growth, metabolism, apoptosis, and DNA damage repair just to name a few (3-9). PP2A modulates the activity of several protein kinases including but not limited to the members of the mitogen-activated protein kinase cascade (MAPK/ERK), Protein Kinase A, Protein Kinase B, Protein Kinase C, and Cyclin-dependent kinases (1). The ability of PP2A to form different heterotrimers and alter its substrate specificity and subcellular localization provides PP2A with the ability to influence many essential cellular pathways and processes. In 1987, K. Rundell reported the methylation of an approximately 32 kDa SV40 small tumor antigen (ST)-associated protein in cell extracts incubated with radiolabeled S-adenosylmethionine (10). In 1990, this ST-associated protein was identified as the PP2A catalytic subunit (11). A few years later, it was determined that the methyl esterification of the PP2A catalytic subunit occurs on its carboxy-terminal leucine α-carboxy group, that both heterodimeric (A/C, where A is a structural subunit) and

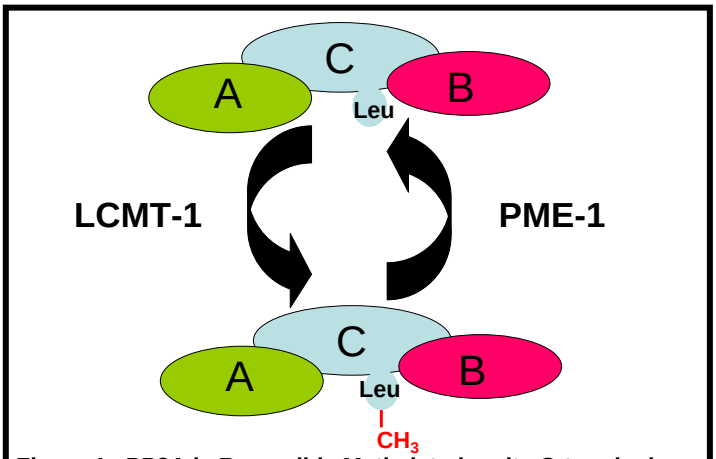

Figure 1. PP2A is Reversibly Methylated on its C-terminal Leucine. PP2A is methylated on its carboxy terminal leucine by LCMT-1 and is demethylated by PME-1.

heterotrimeric (BA/C, where B is a regulatory subunit) forms of PP2A can be methylated *in vitro*, that the methylation is reversible in extracts, and that PP2A in mammalian cells is highly methylated (3,5,6,12). The methylation of PP2A catalytic subunit by LCMT-1 is opposed by the demethylating activity of Protein Phosphatase Methylesterase-1 (PME-1) (Figure 1) (12-14). Together, these two proteins regulate the level of methylation of the PP2A catalytic subunit methylation.

Although conflicting results exist as to whether methylation directly alters the phosphatase activity of PP2A, taken together the data suggest there is little to no direct effect (6,9,15-18). Rather, methylation indirectly regulates PP2A by altering its holoenzyme formation, and thus the subcellular targeting and specificity of PP2A. This mechanism of action was first suggested by results using carboxy-terminal mutants of the PP2A C subunit, and later proven directly in yeast and mammalian systems (18-24). A variety of heterotrimeric PP2A holoenzymes exist that are composed of a catalytic subunit, a structural (A) subunit, and a regulatory/targeting "B" or "B-type" subunit. B-type subunits include cellular [B (PR55/B55), B' (PR61/B56), B" (PR72/120), or B"' (putative; striatin family)] and viral [polyomavirus and SV40 small tumor antigens (STs) and polyomavirus middle tumor antigen (MT)] subunits (Figure 2) (11,25-27).

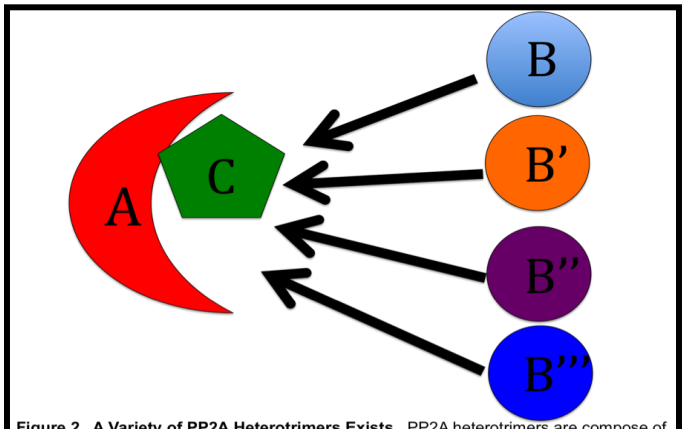

Figure 2. A Variety of PP2A Heterotrimers Exists. PP2A heterotrimers are compose of a structural (A) subunit, a catalytic (C) subunit, and one of many B-type subunits. B subunits are divided into four families: B, B', B'', and B'''.

PP2A methylation is the most selective mechanism known for regulating PP2A. Methylation of the C subunit has a differential effect on incorporation of B-type subunits into PP2A holoenzymes. In yeast, methylation increases the formation of heterotrimers containing B subunit by 20-fold while it only increases B' heterotrimer formation by approximately 2-fold (28). Moreover, in mammalian cells methylation greatly influences assembly of B subunit-

containing heterotrimers (18), but it is not necessary for formation of PP2A complexes containing striatin family members or polyomavirus MT (29). In addition to the positive effects of PP2A methylation on the formation of stable B and B' PP2A heterotrimeric complexes, there is also evidence for positive effects of demethylation on the formation of other PP2A complexes, such as striatin family/PP2A complexes and the Tor pathway dimeric PP2A complex, Tap42(α4)/C subunit (22,29,30). These effects may be due in part to an increased effective concentration of free A/C dimer that occurs when other B-type subunits dissociate upon demethylation (28). To date, no data has been published showing functional downstream consequences of PP2A methylation changes on the Tor pathway or on striatin family function.

Methylation of PP2A has been reported to vary across the cell cycle and to differ in the nucleus and cytoplasm (16,31). Therefore, PP2A methylation may function to regulate various cellular processes such as cell cycle checkpoints and DNA replication in a cell cycle-dependent manner (32). Disruption of the gene for the *Saccharomyces cerevisiae* (*S. cerevisiae*) B subunit (Cdc55p) or the gene for PPM1p, the S. *cerevisiae* LCMT-1 homolog, results in loss of spindle/mitotic exit checkpoints as evidenced by sensitivity to microtubule depolymerizing drugs (22,24,33-35). This indicates that C subunit methylation is necessary for B subunit heterotrimer formation and function in this checkpoint. A role for PP2A C subunit methylation has also been suggested in the nutrient-induced insulin secretion pathway in pancreatic β-cells (15,17). The data are consistent with the idea that glucose gets converted to phosphorylated metabolites that promote insulin exocytosis by inhibiting PP2A methylation. Recently, reduction in LCMT-1 protein levels, PP2A methylation, and BAC PP2A heterotrimers has been found in brains of Alzheimer's disease patients (36,37). We have obtained similar results with different antibodies (Davis, A., Rees, H., Levey, A. and D. C. Pallas, unpublished). Given that PP2A BAC heterotrimers are a major tau phosphatase and are regulated by methylation, these data suggest that alterations in PP2A methylation may contribute to the development of

Alzheimer's disease (38). The important roles of PP2A BAC and B'AC complexes in the regulation of cell cycle, apoptosis and tau phosphorylation suggest that drugs that modulate PME-1 or LCMT-1 may have therapeutic benefit for important diseases such as cancer and Alzheimer's disease.

Interactions of LCMT-1 with Ligands and Other Proteins

LCMT-1 was first identified and cloned due to its ability to catalyze the methyl esterfication of the catalytic subunit (C) subunit of PP2A on its carboxy-terminal leucine residue (4,5,9,21). PP2A C subunit is the only known substrate for LCMT-1. *In vitro*, purified PP2A dimers composed of A and C subunits have been shown to form tight complexes with LCMT-1 in the absence of the methyl donor, S-adenosyl-l-methionine (AdoMet) (18). Upon addition of AdoMet to these complexes, LCMT-1 methylates and dissociates from PP2A. Addition of the PP2A inhibitor, okadaic acid, can also disrupt these PP2A/LCMT-1 complexes *in vitro*, providing a molecular basis for the ability of okadaic acid to inhibit methylation of PP2A by LCMT-1 (7,18).

Mutational studies have shown that efficient methylation of the PP2A catalytic subunit *in vivo* depends on the presence of the carboxy-terminal leucine, a tyrosine residue in the carboxy-terminus, and certain highly conserved amino acids in the active site (21,29). Moreover, the methylation of PP2A by LCMT-1 *in vitro* can be inhibited by an antibody directed to the carboxyl terminus of the PP2A catalytic subunit, but not by competing peptide derived from this same region (5,8). Similarly, synthetic carboxy-terminal peptides of 8 or 17 amino acids also could not serve as a substrate for LCMT-1 (5,9). These pharmacological, mutational, peptide, and antibody data suggest that LCMT-1 and/or PME-1 may interact both with carboxy-terminal residues of the PP2A

subunit and residues in or near the active site.

The x-ray crystallographic structure of the *S. cerevisiae* LCMT-1 homolog PPM1p has been solved for the enzyme alone and for the enzyme bound to AdoMet or to S-adenosyl homocysteine (AdoHCys), the demethylated product of AdoMet (39). The x-ray structure revealed the presence of a common SAM-dependent methyltransferase fold and allowed the identification of the co-substrate binding site for SAM. Moreover, a potential binding pocket on LCMT-1 for the six PP2A C subunit carboxy-terminal residues (TPDYFL) was proposed.

Regulation of LCMT-1 Activity

LCMT-1 is a 38 kDa leucine carboxy methyltransferase that contains a central protein core domain shared by other class I *S*-adenosyl-L-methionine (AdoMet/SAM)-dependent methyltransferases (9,39-41). The catalytic activity of LCMT-1 is dependent on the binding of the methyl donor and co-factor, AdoMet, to this central domain. The methylation state of the C subunit is rapidly reversible *in vivo* (in combination with the demethylating activity of the PP2A methylesterase, PME-1), suggesting a role for LCMT-1 in the regulation of PP2A (7). Mutational studies have shown that efficient methylation of the PP2A catalytic subunit in cell extracts depends on the presence of the known site of methylation, a carboxy-terminal leucine, a tyrosine residue in the carboxy-terminus, and certain highly conserved amino acids in the active site (21,29). Moreover, the methylation of PP2A by LCMT-1 *in vitro* can be inhibited by an antibody directed to the carboxyl terminus of the PP2A catalytic subunit, but not by competing peptide derived from this same region (5,8). These mutational, peptide, and antibody data

suggest that LCMT-1 and/or PME-1 may interact both with carboxy-terminal residues of the PP2A subunit and residues in or near the active site.

Regulation of LCMT-1 activity *in vivo* is poorly understood. Carboxyl methylation of PP2A in *Xenopus* egg extracts is stimulated by cAMP but not by calcium, calcium/calmodulin, or phorbol ester (8). However, it is not known whether cAMP affects PP2A methylation by regulating LCMT-1 or PME-1, both enzymes, or by some other mechanism. In contrast to data reported for yeast and mammalian cell extracts, no PP2A demethylating activity was detected in the *Xenopus* interphase extracts and it appeared that the PP2A in these extracts was largely unmethylated. However, whether PME-1 was absent from these extracts, present in an inactive state, or perhaps even inactivated by the lysis conditions is not known. In mammalian cells, methylation has been reported to vary in the cell cycle (16,31), but whether this is due to regulation of LCMT-1 or PME-1 or both is not known. Some data suggest that inhibition of LCMT-1 by phosphorylated glucose metabolites may play a role in the nutrient-induced insulin secretion pathway in pancreatic β-cells (15,17).

Methylation of PP2A catalytic subunit by LCMT-1 (PPM1p) can be inhibited by the general methyltransferase inhibitor, *S*-adenosyl-L-homocysteine (AdoHCys) (9,39), which competes with AdoMet for its binding site on LCMT-1 (PPM1p) (39). Adenosine dialdehyde (AdOx), an inhibitor of *S*-adenosyl homocysteine hydrolase, reduces PP2A methylation by increasing the concentration AdoHCys in the cell, thus reducing LCMT-1 catalytic activity (29,39,42). Milimolar levels of sinefungin, a non-hydrolyzable AdoMet analog and general methyltransferase inhibitor, inhibits PP2A methylation in cell extracts and inhibits the ability of purified yeast LCMT-1 to methylate PP2A (8,9,15). In addition, the methylation of the catalytic subunit of PP2A by LCMT-1 is blocked by the toxin, okadaic acid (7-9,15). Okadaic acid, which binds tightly to the PP2A catalytic

subunit, has been postulated to inhibit methylation by hindering LCMT-1's access to PP2A or by altering the conformation of PP2A (7,8,43). Despite the fact that okadaic acid also inhibits the catalytic activity of the PP2A methylesterase, PME-1, addition of okadaic acid to mammalian cells results in the progressive demethylation of PP2A, perhaps due in part to the synthesis of new, unmethylated PP2A C subunit (13) (14,29,43).

Major Sites of LCMT-1 Expression

LCMT-1 is highly conserved and homologs are found from yeast to human. LCMT-1 was originally isolated from bovine brain (4) and the cDNA of the human homolog was cloned subsequently using sequence obtained from tryptic peptides of LCMT-1 purified from porcine brain (9). LCMT-1 mRNA is expressed as early in development as the pre-implantation stage. Based on NCBI Unigene EST expression profiles, LCMT-1 is likely to be expressed ubiquitously. The few tissue sources for which ESTs were not found tended to have lower numbers of total ESTs analyzed so the sample size may not have been sufficient to detect LCMT-1 mRNA. However, different cell types may differ in the relative amount of LCMT-1 protein expressed. Immunohistochemical detection of LCMT-1 in human brain slices suggests that LCMT-1 concentration is higher in some types of neurons (such as pyramidal neurons) and lower in others and in glial cells (37) (A. Davis, H. Rees, A. Levey, and D.C. Pallas, unpublished).

Phenotypes of loss of LCMT-1

Deletion of the LCMT-1 homolog in *S. cerevisiae*, *PPM1,* causes greatly increased sensitivity to microtubule depolymerizing drugs (22,24). This appears to be primarily due to the almost complete loss of formation of PP2A trimers containing the Cdc55p (B family) regulatory subunit, which is known to function in spindle/mitotic exit checkpoints. Deletion of the *PPM1* gene also causes rapamycin resistance; Cdc55p overexpression rescues the rapamycin resistance phenotype of *ΔPPM1* (22), suggesting that reduced formation of PP2A heterotrimers containing Cdc55p is the underlying molecular cause of rapamycin resistance. Before our current study, there were no reports of knockdown or loss of LCMT-1 in mammalian cells. We have found that reduction of LCMT-1 in HeLa cells results in improper mitotic progression that eventually results in the activation of apoptotic pathways. Moreover, loss of *lcmt-1* results in mid-gestation embryonic lethality in mice due to defects in liver formation, hematopoiesis, and brain/head development. Therefore, we conclude that LCMT-1 is essential for growth, development, and proliferation in mammalian cells.

PP2A and the Cell Cycle

Cancer is a devastating disorder that is estimated to affect nearly 600,000 new Americans in 2011 and is second only to cardiovascular disease as the major cause of death of Americans (American Cancer Society website). The ability of a normal cell to become cancerous is often defined by the ability of that cell to acquire six "hallmarks of cancer" (44). These hallmarks, which include the ability of a cell to evade apoptosis, maintain limitless replicative capacity, and sustained growth in the absence of growth factors, have made developing novel and useful cancer treatments a difficult task.

Cancer therapies in the past have centered on use of drugs that directly inhibit cell cycle-dependent processes such as DNA replication and spindle formation, but cause a great deal of side affects for cancer patients. Within the past few decades, cancer research has taken a novel and promising turn by instead focusing on the mechanism and regulation of proteins that monitor these very processes at cell cycle checkpoints. Consistent with its roles in cell cycle regulation and apoptosis, PP2A has been implicated in the development of human cancers (45-48). Several lines of evidence indicate that drugs capable of specifically modulating PP2A activity are able to selectively kill cancer cells or inhibit metastasis (49-63). The adenovirus protein E4orf4 kills cancer cells, but not normal cells, in a PP2A BAC trimer-dependent manner (64,65), further supporting the possibility that specific modulation of PP2A activity and/or localization may be useful for cancer chemotherapy.

In addition to its role in the development of human cancers, PP2A has been implicated in the regulation of key cellular processes including cell growth and proliferation, apoptosis, DNA repair, and cell cycle regulation (32). Mutational studies in yeast have shown specifically that formation of the BAC form of PP2A, the form of PP2A implicated in Mitosis Promoting Factor (MPF/Cdk1-cyclin B1) regulation, is most affected by methylation (24). PP2A has been implicated as a key regulatory protein in all stages of the cell cycle, playing a role in signaling cascades that control DNA replication and repair and cell cycle progression (32,66). Research over the past 20 years, including data from the Pallas lab, has implicated PP2A as a possible tumor suppressor (11,45,66,67). Mutation, deletion, and overexpression of certain PP2A subunits have been shown to cause cell cycle defects including improper chromosome segregation and production of multinucleated cells (66,68,69).

PP2A and Mitotic Entry

PP2A negatively regulates entry into mitosis by regulating several key proteins at the G2/M transition of the cell cycle (for review, see (32)). The cyclin-dependent kinase (Cdk)-cyclin complex formed between Cdk1 (Cdc2) and cyclin B1 in G2, Mitotic Promoting Factor (MPF) (Cdk1-cyclin B1), is the primary Cdk-cyclin complex that regulates the G_2/M transition in eukaryotic cells. When Cdk1 complexed with cyclin B1 is phosphoryated on residues Thr-14, Thr-15, and Thr-161, the Cdk1-cyclin B1 complex remains in its inactive form termed the pre-MPF. When Cdk1 is dephosphorylated on Thr-14 and Thr-15 by the phosphatase Cdc25, Cdk1-cyclin B1 becomes active and phosphorylates a variety of substrates including other cyclins and microtubule associated proteins (MAPs) to initiate mitotic progression. PP2A has been proposed to indirectly regulate the dephosphorylation of Cdk1 on Thr-161 and ultimately its activation by regulating Cdk-activating kinase (CAK), the kinase responsible for Thr-161 phosphorylation. Addition of the PP2A inhibitor okadaic acid to cells from multiple species including mammalian cells causes premature activation of MPF and entry into mitosis (70-72) . In addition, the BAC heterotrimeric form of PP2A is postulated to negatively regulate Cdc25 directly by catalyzing its dephosphorylation on residues phosphoryated by a Polo-like kinase (Plx1), keeping Cdc25 in an inactive state. If PP2A is inhibited, then Cdc25 is hyperphosphoryated by Plx1 on residues, and Cdc25 becomes active and initiates mitotic progression by dephosphoryating Cdk1. This activation of Cdk1 by Cdc25 initiates a positive feedback loop between the two proteins that results in the rapid activation of Cdk1 and mitotic progression. In addition, the B' subunit of PP2A has been shown by yeast-2-hybrid assays to associate with Cyclin G, a p53 DNA damage response gene which is thought to contribute to G2/M arrest (73,74).

PP2A and the Metaphase-Anaphase Transition

PP2A has also been shown to be a major player in the mitotic metaphase-anaphase transition. For example, the mitotic protein securin, important in the maintenance and proper segregation of chromosomes during mitosis, is regulated by PP2A (75). In order for cells to progress from metaphase to anaphase during mitosis, sister chromatids need to separate and migrate to their proper poles to insure each daughter cell receives a copy of the maternal DNA. Securin, a negative regulator of this transition, is responsible for the maintenance of the chromatid pair at the spindle checkpoint until conditions within the cell are favorable for mitotic exit (76). The PP2A regulatory subunit B55δ was found to protect cohesin from degradation by stabilizing securin by keeping securin hypophosphorylated (75). In addition, a recent publication from the Nasmyth lab specifically shows that fission yeast homologs of the B'AC form of PP2A (SpPar1$^{B'}$-SpPaa1^{A}-SpPpa2^{C}) are required to maintain sister chromatid cohesion integrity during meiosis I (77). Deletion of SpPar1$^{B'}$or SpPpa2^{C} resulted in delayed or missegregation of sister chromatids. In another recent publication from the Watanabe lab, PP2A was implicated in the protection of cohesin by directly regulating shugoshin, a protein that maintains cohesin stability by regulating its phosphorylation (78). When shugoshin is specifically complexed with the BAC form of PP2A, cohesin remains in a dephosphorylated and stable state, allowing cohesin to remain attached to the chromosome at the centromere and protected from separase cleavage and subsequent premature mitotic/meiotic progression in cells. This indicates that C subunit methylation is necessary for B-subunit heterotrimer formation and function in this checkpoint.

PP2A and Mitotic Exit

Several forms of PP2A have been shown to be instrumental in mitotic exit in both yeast and mammalian cells, supporting the role of PP2A as a tumor supressor. The PP2A complex containing B56δ has been shown to negatively regulate the mitotic phosphatase Cdc25c during interphase as well as mitosis. Cdc25c is responsible for the dephosphorylation and thus inactivation of Cdk1 and the MPF during anaphase, resulting is the initiation of mitotic exit. Knockdown or germline knockout of B56δ in cultured cells or mice results in hyperphosphorylation of Cdk1 and persistent MPF activity, and delayed mitotic exit (79). In addition, a recent publication employing a large scale genome-wide live-cell imaging screen using RNA interference (RNAi) to identify human protein phosphatases involved in mitotic exit identified B55α as a key player in mitotic spindle disassembly, post-mitotic nuclear membrane and Golgi apparatus assembly, and chromatin condensation (80). In this case, B55α was suggested to work downstream of Cdk1 and MPF. In yeast, the binding and downregulation of the BAC form of PP2A again by the protein separase has been shown to be important for proper mitotic exit (81). The data from these studies are additional support for the role of PP2A as a possible tumor suppressor and a negative regulator of both G2/M cell cycle progression and mitotic exit and should be used as basis to design new experiments and develop possible drug therapies that target regulators of PP2A.

LCMT-1, PP2A, and Apoptosis

PP2A has also been shown to be a positive regulator of apoptosis in eukaryotic cells (82). Apoptosis is the process of programmed cell death and is often characterized by

membrane blebbing, chromatin condensation and DNA fragmentation, and activation of key biochemical events including cytochrome c release from the mitochondria and caspase cleavage (82). Apoptosis is instrumental in many processes including embryonic development, tissue and cell regeneration, and elimination of dysregulated cells. Improper regulation of proteins involved in cellular processes such as growth and development usually through loss of protein function, often results the cell initiating apoptosis and ultimately can be responsible for the development of many chronic diseases including cancer, Alzheimer's disease, and hepatitis.

Apoptosis occurs through two distinct pathways: 1) the Fas-FasL (Fas ligand) and TNF (tumor necrosis factor) induced pathways (extrinsic, death receptor-dependent) and 2) the B-cell lymphoma 2 (Bcl-2) pro-apoptotic family pathway (intrinsic, mitochonidia localized, includes cytochrome c release) (83). The Bcl-2 family consists of both pro- and anti-apoptotic proteins, which are subdivided into three subclasses of proteins: 1) Bcl-2 subfamily (pro-survival): Bcl-2, Bcl-XL, Bcl-w, Mcl-1, and A1; 2) Bax subfamily (pro-apoptotic): Bax, Bak, and Bok; 3) BH3 subfamily (pro-apoptotic): Bad, Bid, Bik, Blk, Hrk, BNIP3, and BimL (84). Bcl-2 was first identified as an oncogene in B-cell lymphomas involved in chromosome translocations (85). Bcl-2 proteins are localized to the outer mitochondrial membrane and are regulated by their ability to homo- or hetero-dimerize with other family members (86). For example, when the pro-apoptotic protein Bad (Bcl-2-associated death promoter) is in a dephosphorylated state, it can bind to and inactivate the pro-survival proteins Bcl-2 and Bax, thus preventing cytochrome c release and caspase activation (86).

Caspases belong to a family of cysteine or aspartic acid-specific proteinases that are involved in both apoptosis and the cell's inflammatory response (87). Apoptotic caspases can be activated either by death receptors belonging to both the Fas-FasL and

TNF family or by the apoptosome (caspase 9/cytochrome c/Apaf-1), that is activated by cytochrome c release (87,88). Activation of apoptotic caspases involves a post-translational cleavage cascade of multiple family members that ultimately results in a compromised nuclear membrane and DNA fragmentation (87,88). While caspases 1, 4, and 5 are involved in cytokine activation (inflammatory caspases), the other caspase family members are activated by the two distinct apoptosis pathways (87,88). Caspases 8 and 10 are activated by signals transduced by the activation of death receptors, which ultimately result in the cleavage and activation of the pro-apoptotic protein Bid, cytochrome c release, and activation of the effector caspase, caspase 9, a member of the intrinsic apoptotic pathway. The auto-cleavage and resultant activation of caspase 9 by the apoptosome results in the cleavage of caspase 3 and caspase 7, and thus the activation of downstream effectors and caspases that are responsible for facilitating the characteristic morphology of apoptotic cells (87,88).

Multiple PP2A subunits have been implicated in the regulation of key apoptotic proteins and pathways. PP2A has been shown to positively regulate the pro-apoptotic protein Bad. When dephosphorylated by PP2A, Bad can bind and inactivate the pro-survival protein Bcl-2, leading to activation of the cell's pro-apoptotic pathway. PP2A can also directly inactivate Bcl-2 by dephosphorylating it, repressing its anti-apoptotic activity in mammalian cells (89). The latter mechanism is activated by the second messenger ceramide, which induces co-localization of the B'/PR61α form of PP2A with Bcl-2 at the mitochondrial membrane, association of these two proteins, and dephosphorylation of Bcl-2 (90). In addition, PP2A A subunit is a substrate of caspase 3 (91). When PP2A A subunit is cleaved by caspase 3, the catalytic activity of PP2A increases both *in vitro* and *in vivo* (91). Although this activation of apoptosis is suggested to be a result of increased PP2A activity towards pro-apoptotic substrates,

these substrates have not been identified. Finally, the non-catalytic regulatory PP2A subunit Tap42/alpha4 inhibits apoptosis by working upstream of Bcl-XL in mouse cells. When PP2A A subunit is either knocked down in cultured cells or knocked out in mice, C subunit forms a complex with Tap42/alpha 4, and this complex represses the activation of the transcription factors c-Jun and p53, thus repressing their transcriptional regulation of several pro-apoptotic genes (30).

PP2A is a target of several viral proteins that elicit their anti-apoptotic activity by selectively displacing or binding to specific PP2A regulatory subunits. In order to hijack and block the cell's pro-apoptotic machinery, viruses have evolved to express viral pro-survival Bcl-2 mimetics or caspase inhibitory proteins (82). When the adenoviral protein E4orf4 binds to PP2A containing the B/PR55 B-type regulatory subunit, the PP2A regulatory subunit that has been shown to be the most affected by methylation, it activates the pro-apoptotic activity of PP2A (92-95). How E4orf4 elicits apoptosis in a PP2A B/PR55 specific manner in not fully understood. In contrast, E4orf4 has also been shown to interact with PP2A containing B'/PR61 but this complex does not result in apoptosis and may participate in other viral non-apoptotic mechanisms. In the case of simian virus 40 (SV40), the interaction of the viral B-type regulatory subunit, SV40 small tumor antigen (SV40 ST), with PP2A has been shown to induce or inhibit apoptosis, depending on the cell type. Since all viral PP2A B-type subunits modulate PP2A's activity and substrate specificity by displacing cellular B-type subunits, small T is proposed to both positively and negatively regulate PP2A substrates by leaving them in a phosphorylated state, thus altering their pro- or ant-apoptotic activity.

Moreover, the PP2A related phosphatases PP4 and PP6 have also been implicated in apoptosis. Both PP4 and PP6 exhibit a high-degree of sequence homology to PP2A and PP4 has been shown to be reversibly methylated on its carboxy terminal

leucine residue (96), presumably by LCMT-1. It is not currently known if PP6 can be methylated, but we hypothesize that is it also regulated by LCMT-1. Studies involving either overexpression or downregulation of PP4 in mammalian cells support a pro-survival role for PP4 as well (97,98). The Pallas lab recently found that PP6 is important for cell viability in the presence of the viral protein E4orf4, which has been shown to selectively kill cancer cells in a PP2A-dependent manner by initiating G2/M arrest and p53-independent apoptosis (65,95,99,100). Moreover, PP6 was identified in a RNA interference (RNAi) screen of human kinases and phosphatases having a positive role in cell survival in HeLa cancer cells (101). In our current study, we have found LCMT-1 to have anti-apoptotic/pro-survival role in both cultured cells and in the developing mouse embryo, similar to what has been reported for several forms PP2A, PP4, and PP6. Therefore, LCMT-1 may be a major regulator of apoptosis, and may elicit its effects indirectly by regulating variety of proteins that may not just be targets of PP2A.

Liver Development and Hematopoiesis

The liver is an important organ in the adult mammal, performing many essential functions, including metabolism and excretion. As the largest organ in the adult mammal, the liver is responsible for the production of plasma proteins, digestive enzymes, hormones, and bile (102). In addition, the liver is responsible for the homeostasis of blood glucose levels by regulating the storage of glycogen and the detoxification of the body (102).

The architecture of the adult liver is distinctly designed to provide an environment appropriate for performance of all of its necessary functions including the absorption of metabolites and toxins. The primary functional structure found in the

adult liver is the portal triad (102). The portal triad is composed of the intrahepatic bile duct, the portal vein, and the hepatic artery. Together, these three structures work as a unit to facilitate the efficient uptake and release of nutrients and toxins. The mammalian adult liver is primarily composed of two types of cells, hepatocytes and biliary epithelial cells (103,104). Both cell types are produced from a common bi-potential progenitor called a hepatoblast. Around embryonic day 13, genes associated with the differentiation of the hepatoblast into the two functionally different cell types are turned on and this period of differentiation and division continues until just after birth. Hepatocytes comprise of over 70% of the mass of the adult liver and are derived from the early endoderm (102). Hepatocytes are responsible for performing many of the essential liver functions including the production and storage of proteins, the modification of carbohydrates, as well as the production and secretion of bile (102). Hepatocytes are primarly found surrounding both portal veins and the intrahepatic bile ducts in single-cell sheets called hepatic plates. The basal surface of hepatocytes is responsible for the absorption of metabolites and toxins from the blood plasma flowing through the portal vein. The apical surface is where bile is secreted into the intrahepactic bile duct where it ultimately is transported to the gall badder and then to the duodenum for excretion. In contrast, biliary epithelial cells, also known as cholangiocytes, are derived from the early mesoderm and eventually form the lining of the lumens of the intrahepatic bile ducts. Early in mammalian embryonic development, the liver becomes the largest organ and the location of blood cell production, called hematopoiesis, as well as hematopoietic cell maturation (102).

Hematopoiesis is the production of all the components of blood, including all the myeloid (red cells) and lymphoid (white cell) cell types. Myeloid cell lineages include erythrocytes (red blood cells), mast cells, the various types of granulocytes (basophils, neutrophils, and eosinphils), macrophages, and thromocytes (megakaryocytes) (30,105-

107). Myeloid cells are further sub-divided into myelocytes, which include granulocytes, megakayocytes, and macrophages, that are all derived from a common myeloid progenitor (30,105-107). These cells ultimately participate in innate and adaptive immunity as well as blood clotting. Lymphoid cells, which are responsible for the majority of the body's adaptive immune response, include B-cells, T-cells, and natural killer cells. All hematopoietic cell types are derived from a common multi-potential progenitor, the hematopoietic stem cell (HSC) (30,105-107).

In mammals, hematopoiesis originates in the embryonic yolk sac. As well as providing nutrition to the developing embryo, the yolk sac functions as a primitive circulatory system before the heart begins beating. The yolk sac contains areas called blood islands that support hematopoietic stem cell development and differentiation. The blood islands are derived from angioblasts, which ultimately aggregate to form primitive blood vessels and eventually produce hemoglobin. Termed "primitive hematopoiesis", the multi-potential HSC progenitors are released from the blood islands within the yolk sac into circulation and begin to differentiate in the blood stream and also begin to seed the fetal liver. In mice, around embryonic day 12, the site of hematopoiesis switches from the yolk sac to the fetal liver, thus beginning the process of "definitive hematopoiesis". In the fetal liver, HSCs begin to form and differentiate into the various myeloid and lymphoid cell types within structures similar to blood islands called macrophage islands. Near the end of mammalian gestation, HSCs migrate to their final location, the newly formed bone marrow. The marrow will support hematopoiesis for the remainder of the life of the organism. Although hemotopoiesis occurs in three structurally distinct locations in the developing embryo, there are many common genes, stem cell factors, and cytokines that are necessary for the maintenance, and ultimately, the differentiation of the multipotential HSC into the various hematopoietic cell lineages (30,105-107).

As a multipotential progenitor, non-committed HSCs have the ability to form any cell type within both the myeloid and lymphoid cell lineages. Early in hematopoiesis, many genes are silenced in HSCs, keeping the cells in a non-committed state. Stem cell factor (SCF), along with granulocyte-macrophage colony stimulating factor (GM-CSF) and the cytokines interlukin-1, interlukin-3, and interlukin-6, are some of the first genes turned on and expressed that signal the differentiation of HSCs into the common myeloid progenitor. The common myeloid progenitor then can differentiate into erythrocytes (red blood cells) by expressing the cytokine erythropoietin or into megakarocytes by expressing thrombopoietin, or default into differentiating into myeloblasts, the common progenitor to both macrophages and granulocytes, by just expressing GM-CSF. Granulocytes and monocytes are ultimately formed from myeloblasts by the expression of various other cytokines in combination with either macrophage colony stimulating factor (M-CSF) or granulocyte colony stimulating factor (G-CSF) (30,105-107). The assay to test the ability of cells isolated from fetal liver to form all the cell lineages, called Colony Formation Assays, is the gold standard of identifying genes that are necessary for proper hematopoiesis.

There are very few studies that address the role and regulation of PP2A in the liver. Yoo and colleagues found that C subunit, PP2A activity, and C subunit methylation were all higher in fetal rat liver extracts as compared to adult liver and that the majority of the methylated PP2A C subunit was found in BAC heterotrimers at both times of development (108,109). In an additional study, α4, the mammalian homolog of yeast Tap42 that regulates PP2A and decreases its catalytic activity by binding alone to C subunit, was found to complex with both methylated and unmethylated C subunit in both the fetal and adult rat liver (108). Moreover, zebrafish that were exposed to the PP2A inhibitor microcystin-LR for thirty days experienced significant hepatocyte liver

damage and alteration of expression of genes involved in oxidative stress, cytoskeleton rearrangement, and metabolism (110). Although these studies were the first to analyze the composition of PP2A in the developing liver as well as the effect of PP2A inhibition on liver homeostasis, they did not address the role of LCMT-1 and PP2A methylation in liver development.

PP2A, Tau, and Alzheimer's Disease

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by loss of memory and reduction in intellectual ability. AD currently accounts for 60-80% of all dementia cases and often is diagnosed in individuals over the age of 65 (Alzheimer's Association website). AD is a progressive disease in which the symptoms drastically worsen over time and is currently the sixth leading cause of death in the United States (Alzheimer's Association website). AD is predicted to affect 1 in 85 people worldwide by 2050 (111). AD patients often first experience mild memory loss that eventually progresses to the inability to carry on conversations, loss of independence, and a reduced quality of life for both patients and caregivers.

AD neuropathology is characterized by two major disease phenotypes: beta amyloid senile plaques derived from the amyloid precursor protein (APP) and neurofibrillary tangles (NFT). Beta amyloid senile plaques are composed of extracellular deposits of the APP in microglia and astocytes. NFTs are aggregates of paired-helical filaments formed from hyperphosphorylated forms of the microtubule-associated protein tau (112). When tau becomes hyperphosphorylated, it can no longer bind microtubules and causes their instability (113). The complete mechanism underlying of formation of these NFTs in not completely understood nor is whether tau hyperphosphorylation is the cause or an effect of AD progression.

The regulation of tau phosphorylation is a synergistic balance between the activity of tau kinases and phosphatases (114,115). When this delicate balance of activity becomes disrupted through either the changes in expression of these protein kinases and/or phosphatases, their dysregulation, or changes in the level or expression of accessory proteins (116), tau hyperphosphorylation is the result (117). Moreover, tau can itself become mutated, which also leads to the formation of NFTs (118). The most common of these tau mutations, P301L (Proline to Leucine mutation) is associated with frontotemporal dementia in humans and leads to the formation of NFTs and pretangle formations in mice (118).

Through the work of several labs, PP2A has been identified as the major tau phosphatase (119-124). The PP2A subunits C subunit, B/PR55, and B'/PR61 are highly expressed in the brain and are predicted to have an important neuronal function. The brains from AD patients exhibit reduced amount of tau phosphatase activity [Gong, 1995 #4901], suggesting that reduced PP2A tau phosphatase activity may contribute to tau hyperphosphorylation and AD progression. Transgenic mice expressing the dominant-negative PP2A C subunit mutant, L199P, exhibited hyperphosphorylated tau in neurons, which formed tau aggregates [Kins, 2001 #3814]. In addition, the PP2A heterotrimers containing both the Bα and Bβ B family B-type subunits, which are significantly affected by PP2A methylation, associate with neuronal microtubules (125). Moreover, not only have $PP2A_{B_{\alpha}AC}$ heterotrimers been shown to be the major form of PP2A that regulates tau phosphorylation, they are also reduced in neurons that display AD-like characteristics (36).

Recently, it was shown that elevated levels of homocysteine in the blood, common for many age-related disorders, was also found in AD patients (126,127). Homocysteine, a key metabolite intermediate in S-adenosylmethionine and S-

adenosylhomocyseine metabolism, also can modulate methyltransferase activity by acting through feedback inhibitory mechanism. The Sontag lab made the interesting discovery that elevated levels of blood homocysteine result in not only decreased PP2A methylation, but downregulation of the B family B-type subunit Bα and accumulation of the AD hallmarks hyperphosphorylated tau and APP plaques, establishing a direct connection between LCMT-1, PP2A methylation, and AD (126). Most importantly, the Sontag lab made the interesting finding that LCMT-1 protein levels are significantly reduced in AD-affected brains (37).

As stated above, LCMT-1 is responsible for methylating PP2A C subunit [De Baere, 1999 #3338]. Thus, loss of LCMT-1 or defects in its activity might reduce PP2A methylation and the amount of functional $PP2A_{B_{\alpha}AC}$ enzyme, contributing to the pathophysiology of AD. Based on these findings and the role of $PP2A_{BAC}$ heterotrimers in regulating tau phosphorylation, we have proposed that defects in PP2A methylation may contribute to major brain defects in the developing mouse embryo.

To gain understanding of the role of LCMT-1 in brain development in mice, we disrupted the murine *lcmt-1* gene, which results in a recessive lethal phenotype. *Lcmt-1* knockout embryos die during midgestation (E11.5-E14.5) with severe defects in head formation. The heads of *lcmt-1* knockout embryos display reduced PP2A methylation and alterations of tau phosphorylation at known PP2A-regulated residues, Serine 262 and Serine 356. Our report is the first to describe a direct role for LCMT-1 in the regulation of tau phosphorylation *in vivo*. In addition, we are the first to describe a role for LCMT-1 in brain development during embryogenesis. Therefore, LCMT-1 and PP2A methylation are key regulators of brain development and tau phosphorylation.

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Chapter 1

Leucine Carboxyl Methyltransferase 1 is Necessary for Normal Progression Through Mitosis in Mammalian Cells

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Running title: LCMT-1 is Needed for Normal Mitotic Progression

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Introduction

Protein Phosphatase 2A (PP2A), a multifunctional Serine/Threonine phosphatase, has been implicated in the regulation of cell growth and proliferation, primarily at the G_2/M transition, and in the development of human cancers (1). PP2A is composed of three functionally distinct subunits: A (structural), B-type (regulatory/targeting), and C (catalytic). Binding of different B-type subunits to the core PP2A heterodimer (A/C) alters its substrate specificity and targets PP2A to different substrates and subcellular locations. While there are two genes each for the C and A subunits in mammalian cells, there are multiple families of B-type subunits ((B (B55/PR55), B' (B56/PR61), B" (PR72)) and a putative B"' (striatin) family, each of which has multiple members (1). Thus, a variety of heterotrimeric PP2A holoenzymes exist that differ primarily in their regulatory/targeting B-type subunits. In addition, polyomavirus middle tumor antigen (MT) and polyomavirus and SV40 small tumor antigens (ST) act as viral B-type subunits, displacing certain cellular B-type subunits and binding to the PP2A core heterodimer, altering its activity ((2-9) and references therein).

Reversible methylation is one of the most specific cellular mechanisms known for regulating PP2A, and thus may have promise as a mechanism-based therapeutic target. In mammalian cells, PP2A is methylated on its catalytic subunit C-terminal leucine (L309) alpha-carboxyl group by Leucine Carboxyl Methyltransferase-1 (LCMT-1) (10-16) and is demethylated by Protein Phosphatase Methylesterase-1 (PME-1) (17-19). Methylation indirectly regulates PP2A function by altering the formation of PP2A holoenzymes, thus influencing the subcellular targeting and specificity of PP2A (8,20-25). C subunit methylation has a differential effect on incorporation of different B-type subunits into PP2A holoenzymes. In *Saccharomyces cerevisiae* where there is only one B subunit (Cdc55p) and one B' subunit (Rts1p), methylation increases the formation of heterotrimers containing B subunit 20-fold while increasing B' heterotrimer formation less than 2-fold (26). In mammalian cells, methylation is important for efficient assembly of BαAC heterotrimers (22,24) but is not necessary for formation of PP2A complexes containing striatin family members or the viral B-type subunit, polyomavirus MT (24). Based on experiments with a PP2A C subunit lacking leucine 309, methylation may differentially affect different B' family members in mammalian cells as well (27).

Based on these data, loss of PP2A methylation would be predicted to have the greatest effect on the function of PP2A heterotrimers containing the B/B55 family of regulatory subunits ($PP2A_{BAC}$). $PP2A_{BAC}$ trimers have been implicated in Mitosis Promoting Factor (MPF/Cdk1-cyclin B1) regulation in *Xenopus* (28) and in the regulation of mitotic exit in yeast (29-31). Therefore, PP2A methylation may regulate entry into mitosis as well as mitotic progression and exit. Consistent with this possibility, the level of PP2A methylation has been reported to change in a cell cycle-dependent manner in rat and human cells (32,33). $PP2A_{BAC}$ heterotrimers have also been shown to be essential in yeast for spindle checkpoint (34) and to regulate phosphorylation and degradation of human securin (35). In accord with this necessity for $PP2A_{BAC}$

heterotrimers, we and others have shown that PP2A methylation is required for spindle checkpoint in yeast as indicated by increased sensitivity of PP2A methyltransferase-disrupted cells to spindle-targeting drugs like nocodazole (23,25). However, it is not known if PP2A methylation is important for regulation of mitosis in mammalian cells. In fact, nothing is known about the role of LCMT-1 or PME-1, in mammalian cell growth and proliferation, including whether LCMT-1 is the major PP2A methyltransferase *in vivo*. In this study, we used lentiviral shRNA vectors to knock down LCMT-1 expression and investigate the importance of LCMT-1 in mammalian cells. We also tested whether LCMT-1 was necessary for embryonic development in mice. Collectively, our results indicate that LCMT-1 is the major PP2A methyltransferase in mammalian cells, that LCMT-1 is important for normal progression through mitosis and for cell survival, and it is required for mouse embryonic development.

Methods and Materials

Antibodies- LCMT-1 was detected using an affinity-purified rabbit anti-LCMT-1 polyclonal antibody, RK3110, generated against a 17-amino acid peptide corresponding to residues 173-189 of human LCMT-1. PP2A Bα and C subunit were detected with mouse monoclonal antibodies from Upstate Biotechnology (clone 2G9) and BD Transduction Laboratories, respectively. Unmethylated PP2A C subunit was detected with a previously described (24) mouse monoclonal antibody (clone 4B7; available from Upstate Biotechnology, Stratagene, Santa Cruz Biotechnology, or Invitrogen). Methylated PP2A C subunit was detected with 2A10 mouse monoclonal antibody (Upstate Biotechnology, Inc.). HA-tagged proteins were immunoprecipitated and immunoblotted with the mouse monoclonal antibodies, 12CA5 (available from Santa Cruz Biotechnology, Inc.) and 16B12 (Covance Research Products), respectively.

Cell culture and the creation of stable lines that express LCMT-1- and Bα-directed shRNAs- HeLa cervical carcinoma cells were obtained from American Type Culture Collection. HCT116 colon cancer cells were obtained from Dr. Eva Schmeltz. Both lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). To create HeLa and HCT116 lines that stably express LCMT-1- or PP2A Bα-directed shRNAs, cells were infected with lentiviruses expressing shRNAs. These lentiviruses were generated using a three plasmid–based lentivirus system (36) (available from The RNAi Consortium (TRC) at the Broad Institute). The targeted sequences in the shRNA lentiviruses we used are: L2, 5' GCCATGTTCATAAACTACGAA3' (TRC ID: TRCN0000035060); L3, 5'CGTCGACATGATGGAGTTGTA3' (TRC ID: TRCN0000035061); and Bα 5'GCAAGTGGCAAGCGAAAGAAA3' (TRC ID: TRCN0000002493). After infection with

the appropriate lentivirus, cells were selected with puromycin (2ug/ml) until a canary dish of uninfected parental cells was completely dead (usually three days). After incubating one additional day to allow the cells to recover from puromycin treatment, cells were then used for experiments and aliquots of cells were frozen down for later use. Cells were used within ten days after selection/thawing.

Determination of the level of PP2A$_c$ methylation- The steady state level of PP2A catalytic subunit methylation was measured in lysates with a monoclonal antibody specific for unmethylated PP2A C subunit (4b7) using our published method (24), which is described in the legend to Fig. 1B and 1C in the current study. Quantitation of the 4b7 signal was performed with a BioRad Fluor S-Max Chemilumimager and BioRad Quantity One Software.

Cell transfections and immunoprecipitations- The pCEP4 HA-tagged Bα construct was obtained from the lab of Dr. David M. Virshup (Department of Pediatrics and of Oncological Sciences, University of Utah). Vector control HeLa cells and HeLa cells that stably express shRNAs directed to LCMT-1 (L3) were plated at a density of 3.5 X 10^5 cells/60mm dish and then transfected with pCEP4 HA-tagged Bα using FuGENE6 transfection reagent (Roche Diagnostics). At 72 hrs post-transfection, cells were lysed in an NP40-containing lysis buffer (10% glycerol, 20mM Tris pH 8.0, 137mM NaCl, 1% NP40) and immunoprecipitation of N-terminally HA-tagged Bα was carried out with anti-HA tag monoclonal antibody, 12CA5, covalently crosslinked to protein A sepharose beads. To determine the amount of PP2A C subunit associated with each of the immunoprecipitated samples, the immunoprecipitates were analyzed by SDS-PAGE, immunoblotted with 16B12 anti-HA tag antibody and with anti-PP2A C subunit antibody, and then the relative amount of HA-tagged Bα and PP2A C subunit were

determined by quantitation using a BioRad Fluor S-Max Chemilumimager and BioRad Quantity One Software.

Analysis of cell viability by trypan blue exclusion- Vector control cells and cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 2 X10^4 cells/well in a 12-well dish, allowed to adhere overnight, washed to remove dead cells, and then incubated for 24 hours at 37°C. Cells were then harvested and checked for viability by trypan blue exclusion (0.4% trypan blue).

Analysis of DNA condensation and fragmentation using DAPI stain- Vector control HeLa cells and HeLa cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 2 X10^4 cells/well in a 12-well dish, allowed to adhere overnight, washed to remove dead cells, and then incubated for 24 hours at 37°C. Cells were then incubated with DAPI stain for 5 minutes at 37°C. Cells were subsequently observed by fluorescence microscopy using an Olympus IX81 phase/fluorescence microscope and analyzed using Slidebook Microscope Analysis software (Intelligent Imaging, Inc.).

Caspase 3 in cell detection assay- Vector control HeLa cells and HeLa cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 1.5 X10^5 cells/well in a 6-well dish, allowed to adhere overnight, washed to remove dead cells, and then incubated for 24 hours at 37°C. Cells were then harvested and gently pelleted, washed in media, and then incubated in media containing Red-DEVD-FMK caspase-3 activity indicator (DEVD-FMK conjugated to sulfo-rhodamine; Calbiochem) and 10% FCS for 1 h in a 37°C incubator per the manufacturer's instructions. Cells were subsequently placed on a coverslip and observed by fluorescence microscopy using an Olympus IX81 phase/fluorescence microscope and analyzed using Slidebook Microscope Analysis software (Intelligent Imaging, Inc.).

DEVD-AFC Caspase 3 in vitro activity assay- Vector control HeLa cells and HeLa cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 1.5 X10^5 cells/well in a 6-well dish, allowed to adhere overnight, washed to remove dead cells, and then incubated for 24 hours at 37°C. Cells were then washed 3 times with 1X PBS and incubated in an NP40-containing lysis buffer (10% glycerol, 20mM Tris pH 8.0, 137mM NaCl, 1% NP40) for 20 min. on ice. The cells were then sonicated on ice for 5 X 2 sec. bursts. The supernatant was then centrifuged for 10 minutes at 13,000 x g. The protein concentration of the supernatant was assayed by the Lowry method (BioRad). 25μg of protein was incubated with z-DEVD-AFC (25μM) (Calbiochem substrate IV) at 37°C for 1 hr. Caspase activity was measured using a Wallac Victor^2 1420 Multilabel Counter fluorimeter (Perkin Elmer) at 405nm excitation and 535nm emission wavelengths.

Effect of caspase inhibition on cell viability- Vector control HeLa cells and HeLa cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 2 X10^4 cells/well in duplicate 12-well dishes, allowed to adhere overnight, washed to remove dead cells, and then incubated with z-VAD-FMK pan-caspase inhibitor (50μM; BioMol) or with vehicle only for 24 hours at 37°C. Cells were then harvested and checked for viability by trypan blue exclusion (0.4% trypan blue) and the viability of each line ± caspase inhibitor was compared.

Phase time-lapse microscopy- Vector control HeLa cells and HeLa cells that stably express shRNAs directed towards LCMT-1 (L2 or L3) or Bα were plated at a density of 4 X10^4 cells/well in a 6-well dish, allowed to adhere overnight, washed to remove dead cells, and then incubated at 37°C in a temperature and atmosphere-controlled microscope stage incubator (M6; Zeiss Instruments) on an Olympus IX81 phase/fluorescence microscope equipped with a computer-driven motorized stage (ASI).

Phase images were captured every 6 minutes for 24 to 36 hours. Images were analyzed using Slidebook Microscope Analysis software (Intelligent Imaging, Inc.).

Nocodazole treatment and analysis of cell viability- Cells were plated at a density of 2 X10^4 cells/well into duplicate 12-well dishes, allowed to adhere overnight, washed to remove dead cells, and then one dish was incubated with nocodazole (200ng/mL) and the other with vehicle control for 24 hours at 37°C. Cells were then harvested and assayed for viability by trypan blue exclusion (0.4% trypan blue) to determine any change in cell death for each line due to the presence of nocodazole.

Thymidine block and analysis of cell viability- Cells were plated at a density of 2 X10^4 cells/well into triplicate wells in duplicate 12-well dishes, allowed to adhere overnight, washed to remove dead cells, and then incubated with thymidine (2mM) or vehicle only for 24 hours at 37°C. Cells were then harvested and checked for viability by trypan blue exclusion (0.4% trypan blue) to determine any change in cell death for each line due to G1/S arrest induced by Thymidine treatment.

Generation of LCMT-1 knockout mice and genotyping- LCMT-$1^{+/-}$embryonic stem cells containing a gene trap insertion in the first intron of the LCMT1 locus were obtained from German Gene Trap Consortium, Neuherberg, Germany. These cells were expanded in culture and were injected into blastocysts from C57BL/6 donors using standard techniques by the Emory University School of Medicine Transgenic Mouse and Gene Targeting Core Facility. Resultant chimeric mice were bred to generate heterozygous F1 animals that were then intercrossed to determine if homozygous knockout of LCMT-1 was embryonic lethal.

RESULTS

Downregulation of LCMT-1 Causes a Substantial Decrease in PP2Ac Methylation---To determine whether LCMT-1 was necessary for cell viability and normal cell cycle progression in mammalian cells, we decreased LCMT-1 expression in HeLa cells by stably expressing small hairpin RNAs (shRNAs) to LCMT-1 via lentiviral vectors. Figure 1A shows that two different LCMT-1 shRNAs, designated L2 and L3, were able to dramatically reduce LCMT-1 protein levels.

In yeast, loss of the LCMT-1 homolog, Ppm1p, results in loss of PP2A (Pph21p/Pph22p) methylation, indicating that Ppm1p is probably solely responsible for methylating PP2A under normal growth conditions (23,25,37). Although LCMT-1 has been shown to methylate PP2A *in vitro* (10-16), its contribution to PP2A methylation *in vivo* has not been quantitatively examined in mammalian cells. To determine the contribution of LCMT-1 to PP2A methylation in mammalian cells, we quantitated the steady state levels of PP2Ac methylation in L2 and L3 LCMT-1 knockdown cells using our monoclonal antibody, 4b7, which is specific for unmethylated PP2A catalytic subunit (see Materials and Methods and legend for Figure 1) (24). Figures 1B and 1C show that the knockdown of LCMT-1 by L2 and L3 shRNAs caused a substantial (32 or 71%, respectively) reduction in PP2A catalytic subunit methylation in HeLa cells as compared to vector control (VC) cells. Interestingly, although LCMT-1 could not be detected easily in either the L2 or L3 cells (Fig. 1A), L3 was more efficient than L2 at reducing PP2A methylation (Fig. 1C). To further corroborate our results with the 4b7 anti-unmethylated PP2A catalytic subunit antibody, we performed a separate experiment in which we analyzed vector control and L3 LCMT-1 knockdown cells with another monoclonal antibody (2A10) that is specific for methylated PP2A catalytic subunit. The results in

Figure 1D show that 2A10 also detected a large reduction in methylated PP2A catalytic subunit in L3 LCMT-1 knockdown cells. Quantitation of results from two separate experiments showed that PP2A C subunit methylation was reduced >3-fold. Collectively, these results show that mammalian LCMT-1 is responsible for the majority of PP2A methylation, similar to the case for the *S. cerevisiae* LCMT-1 homolog, Ppm1p. They also demonstrate that the small amount of residual LCMT-1 protein in the L2 and L3 knockdown cells may be sufficient for methylating 25% to 60% of the PP2A in these cells.

Knockdown of LCMT-1 Greatly Reduces PP2A Bα Binding to C subunit---In yeast, deletion of the gene encoding Ppm1p dramatically reduces the binding of PP2A B subunit to C subunit. We wanted to determine if knockdown of LCMT-1 had similar effects on Bα binding to C subunit in mammalian cells. Because no antibody capable of immunoprecipitating endogenous Bα/C subunit complexes was available, we assayed the ability of transfected HA-tagged Bα (HA-Bα) to associate with endogenous C subunit in our vector control and L3 LCMT-1 shRNA HeLa cells. Figures 2A and 2B show that methylation was greatly reduced in the L3 cells used for this experiment. Figures 2C and 2D show that downregulation of LCMT-1 by L3 shRNA greatly reduced C subunit association with HA-Bα. Thus, LCMT-1 is important for $PP2A_{B_{\alpha}AC}$ trimer formation in mammalian cells.

Knockdown of LCMT-1 Induces Cell Death---In mammalian cells PP2A plays important roles in both cell cycle control and in apoptosis. We therefore analyzed the LCMT-1 knockdown cells and control cells to determine if there was an effect of LCMT-1 knockdown on cell viability. After infection with L2 and L3 shRNA-expressing lentiviruses, we noticed that a portion of the HeLa cells knocked down for LCMT-1 was dying while little to no death was seen in the cells infected with the control lentivirus. To determine the extent of death induced by LCMT-1 knockdown, we measured the percent

of cells in each of these lines that died during a 24-hour period in culture. Figure 3A shows that both L2 and L3 shRNA knockdown HeLa cells had significantly more death (7-8%) during this period than the vector control cells (1%).

Because the stable formation of PP2A trimers containing the Bα regulatory subunit has been shown to be highly dependent on methylation of the PP2A C subunit (22,24) and we showed above that Bα/C subunit complexes were reduced in LCMT-1 knockdown cells, it was possible that the cell death seen in L2 and L3 knockdown cells was due in part to impairment of $PP2A_{B\alpha AC}$ trimer formation and function. We therefore tested whether knockdown of Bα would also induce death. Indeed, knockdown of Bα by a lentiviral shRNA vector, demonstrated in Figure 3B, also caused an increase in cell death comparable to that induced by knocking down LCMT-1 (Fig. 3A). Although the increase in cell death in Bα knockdown cells was more variable than for L2 and L3, it was statistically significant ($p<0.05$).

Knockdown of LCMT-1 Induces Caspase-dependent and Caspase-independent Cell Death---To begin to characterize the mechanism of death induced by knockdown of LCMT-1 or Bα, we analyzed our vector control, L2, L3, and Bα shRNA cells by phase time-lapse microscopy. In comparison to vector control cells, L2, L3, and Bα knockdown cells exhibited increased membrane blebbing (Fig. 4A and data not shown), a characteristic of caspase-mediated cell death (38). To further analyze this cell death, we next incubated each cell line with DAPI stain to detect condensed and fragmented DNA, which is also characteristic of apoptosis (39). Figure 4B shows that LCMT-1 or Bα knockdown induced DNA condensation and fragmentation, supporting the idea that reduction of LCMT-1 induces apoptosis, perhaps in part by inhibition of Bα function.

Since the membrane blebbing observed in our knockdown cells is a characteristic of caspase activation (38), we wanted to determine whether LCMT-1 and Bα knockdown induces caspase activation. We first assayed caspase activation in live vector control, LCMT-1 knockdown, and Bα knockdown cells. Vector control cells treated with the pan-kinase inhibitor staurosporine were used as a positive control (40). The cell-permeable substrate we used for this assay, Red-DEVD-fmk, is a preferred substrate of caspases 3 and 7 but also can serve as a substrate for a number of other caspases (41). Figure 4C shows that knockdown of LCMT-1 by either L2 or L3 shRNA caused an increase in caspase activity *in vivo*. Although knockdown of Bα consistently induced a small increase in caspase activation as detected by this assay, the increase was not statistically significant (Fig. 4C and data not shown). In a complementary approach, we assayed for caspase activity in lysates of our vector control and LCMT-1 and Bα knockdown cells using the caspase substrate z-DEVD-AFC. Again, staurosporine-treated vector control cells were used as a positive control. The results we obtained (Fig. 4D) are consistent with our results from the *in vivo* caspase assay. Knockdown of LCMT-1 by either L2 or L3 shRNA caused significant activation of caspases in three independent experiments. Knockdown of Bα on the other hand caused some caspase activation in all experiments, but only in one experiment was this increase significant (Fig. 4D and data not shown).

Although this result indicates that caspases are activated at least in the case of LCMT-1 knockdown cells, it does not show whether caspase activation is necessary for the observed apoptosis. To test whether the observed death requires caspase activation, we treated our LCMT-1 (L2 and L3) and Bα knockdown cells with the pan-caspase inhibitor z-VAD-fmk (50μM) to see if it would reduce cell death. Interestingly, we observed a reduction in cell death in all three knockdown cell lines (L2, L3 and Bα shRNA cells) as compared to vector control, but, consistent with our caspase activation

results, the reduction in cell death was always the least for the Bα knockdown cells (Fig. 4E and data not shown). These results suggest that downregulation of LCMT-1 or Bα causes death by both caspase-dependent and caspase-independent pathways but that LCMT-1 knockdown causes more caspase-dependent cell death than Bα knockdown.

LCMT-1 or Bα Knockdown Causes Nocodazole Sensitivity--- In our initial experiments using phase time-lapse microscopy, we found that a subset of LCMT-1 knockdown cells round up as if to enter mitosis, sometimes with a visible metaphase plate, and remain rounded for longer than expected for a normal mitosis, eventually undergoing apoptosis. Figure 5 shows an example of such a cell that enters mitosis (metaphase plate is clearly visible in the cell at the 1.6h time-point), and remains rounded until membrane blebbing begins approximately 14h later. In yeast, we and others have shown that deletion of the gene, *PPM1*, which encodes the yeast homolog of LCMT-1, or deletion of the gene, *CDC55,* which encodes the yeast homolog of Bα, has no effect on cell viability under normal growth conditions unless the cells are sensitized by the microtubule-depolymerizing drug nocodazole, a phenotype characteristic of a spindle checkpoint defect (23,25,29,34). To determine if LCMT-1 and/or Bα knockdown results in a similar mitotic defect in mammalian cells as in yeast, we treated our LCMT-1 (L2 and L3) and Bα knockdown cells for 24 hours with nocodazole (200nM) and then assayed these cells to determine if there were any changes in the amount of death as compared to untreated cells. The results in Figure 6A show that while nocodazole treatment only caused a 1% increase in cell death in vector control cells, it induced a much greater increase in death in cells with reduced LCMT-1 levels (10-13%). Moreover, reduction of Bα levels also resulted in increased death in the presence of nocodazole (Fig. 6A).

Thymidine treatment Rescues Cell death Caused by LCMT-1 or Bα Knockdown -- To determine if the increased death caused by nocodazole was specifically caused by arresting cells in mitosis (and thus perhaps a spindle checkpoint defect) or just due to cell cycle arrest, we also treated our vector control, LCMT-1 and Bα knockdown cells with thymidine, which has been used as a cell synchronization agent to arrest cells in G_1/S. Strikingly, thymidine treatment caused a dramatic reduction in cell death in both LCMT-1 (L2 and L3) and Bα knockdown cells (Fig. 6), indicating that the increased cell death observed when cells are treated with nocodazole is specific to mitosis.

LCMT-1 Knockdown Also Causes Increased Death of HCT116 Colon Cancer Cells in Nocodazole—Increased sensitivity of HeLa LCMT-1 knockdown cells to nocodazole (Fig. 6A) suggested that LCMT-1 plays a role in spindle checkpoint and that drugs targeting LCMT-1 might have potential in combination chemotherapy with microtubule-targeting drugs. We wanted to determine next whether LCMT-1 knockdown would cause increased killing of another cancer cell type in nocodazole. The results presented in Figures 7A-7C show that L2 and L3 shRNAs also reduce LCMT-1 protein levels and PP2A methylation in HCT116 cells. Decreased methylation in the L2 and L3 cells was detected both by immunoblotting with 2A10 antibody specific for methylated PP2A catalytic subunit (Fig. 7A) and by using our methylation assay employing our monoclonal antibody specific for unmethylated PP2A catalytic subunit (Fig. 7B and C). Importantly, Figure 7D shows that HCT116 L2 and L3 LCMT-1 knockdown cells were more sensitive to nocodazole than were vector control cells. Similar to the results obtained for HeLa cells (Fig. 6A), an average increase in percent killing of 9% in nocodazole was seen with L2 and L3 shRNAs in three separate experiments while vector control cells only showed an average increase in killing of 1.5% in the same experiments (Fig.7D and data not shown).

Homozygous knockout of LCMT-1 in mice causes embryonic lethality --- Because our data indicated that LCMT-1 was necessary for normal progression through mitosis we predicted that LCMT-1 knockout would be embryonic lethal. To test this hypothesis, we generated an LCMT-1 knockout mouse using LCMT-$1^{+/-}$ embryonic stem (ES) cells created by The German Gene Trap Consortium and tested whether live LCMT-$1^{-/-}$ pups could be obtained from crossing LCMT-$1^{+/-}$ mice with each other. Our results are shown in Figure 8. Figure 8A shows that our genotyping primer sets for wild-type LCMT-1 and for gene trap knockout LCMT-1 both produce robust products using DNA from the original LCMT-$1^{+/-}$ embryonic stem (ES) cells or from progeny LCMT-$1^{+/-}$ or LCMT-$1^{+/+}$ mice obtained from breeding. Figures 8B and 8C show that LCMT-1 protein was reduced about 2-fold in the LCMT-$1^{+/-}$ ES cell line relative to control parental ES cells. Finally, Figure 8D shows the average number of pups with each possible genotype obtained from eight LCMT-$1^{+/-}$ x LCMT-$1^{+/-}$ matings. If homozygous LCMT-1 loss had no effect on development, one would expect equal numbers of LCMT-$1^{+/+}$ and LCMT-$1^{-/-}$ mice. However, while 19 LCMT-$1^{+/+}$ mice and 26 LCMT-$1^{+/-}$ pups were born from these +/- x +/- crosses, no LCMT-$1^{-/-}$ pups were obtained from any of the crosses, indicating that loss of LCMT-1 is indeed embryonic lethal.

DISCUSSION

Carboxyl methylation of PP2A was discovered well over a decade ago but its function remained obscure until the discovery that the addition and removal of the single methyl group on PP2A's carboxy-terminal leucine could differentially regulate the assembly of certain PP2A heterotrimers (22-25). The cloning of LCMT-1 as a mammalian PP2A methyltransferase by De Baere and colleagues has facilitated the investigation of this enzyme's contribution towards PP2A methylation and function (16). However, until the recent advent of siRNA approaches for mammalian cells much advancement in our understanding has come from experiments performed in yeast because of the ease of performing genetic analyses. In fact, prior to this study nothing was known about the importance of LCMT-1 for normal cell cycle progression and cell survival. In this report, we have utilized lentiviral shRNA and genetrap knockout approaches to investigate LCMT-1 function in mammalian cells. We show that a strong reduction in LCMT-1 not only results in a substantial reduction in PP2A methylation in mammalian cells, but also causes a decrease in Bα subunit association with C subunit, induces apoptosis as evidenced by caspase activation, membrane blebbing, nuclear condensation and fragmentation and cell death, and sensitizes HeLa and HCT116 cancer cells to the microtubule targeting drug, nocodazole.

Although LCMT-1 was originally purified as a PP2A methyltransferase, only a small percentage of PP2A methyltransferase activity in the original cell extract was recovered (16), leaving open the question as to whether LCMT-1 is the major PP2A methyltransferase in mammalian cells. Our result showing that reduction of the steady-state level of PP2A C subunit methylation an average of 70% in HeLa cells and 63% in HCT116 cells by the LCMT-1 L3 shRNA clearly indicates that it is. Our observation that

cells with approximately 10% of their normal amount of LCMT-1 protein have 30-70% of their normal amount of PP2A methylation suggests that either there is a second PP2A methyltransferase that is a minor contributor to PP2A methylation under normal growth conditions or cells have an excess of LCMT-1 for maintaining steady-state methylation levels of PP2A under cell culture conditions. The most obvious candidate for an additional PP2A methyltransferase would be the only known homolog of LCMT-1, LCMT-2. LCMT-2 was originally cloned by De Baere and colleagues, who showed that it could not methylate PP2A *in vitro* (16). Consistent with this *in vitro* mammalian result, a LCMT-2 homolog exists in yeast that does not seem to contribute detectably to PP2A methylation *in vivo* (23,25,37). These results suggest that LCMT-2 is not a PP2A methyltransferase. However, it still needs to be tested *in vivo* whether LCMT-2 contributes to PP2A methylation in mammalian cells. One possible explanation for the apparent excess of LCMT-1 is that it may be necessary for dynamic responses to cellular needs or for methylation of a currently unknown additional LCMT-1 substrate. Other possible LCMT-1 substrates include the PP2A-related phosphatases, PP4 and PP6. Both of these phosphatases share approximately 60% amino acid identity with PP2A and have identical carboxy-terminal amino acids, including a carboxy-terminal leucine, the known site of carboxyl methylation for both PP2A and PP4 (42). Whether PP6 is methylated is not known. Further experiments will be necessary to determine if LCMT-1 (or LCMT-2) methylates PP4 and/or PP6.

We have previously shown that Bα is the PP2A B-type regulatory subunit whose assembly into heterotrimers is most dependent on PP2A C subunit methylation (24). Our current coimmunoprecipitation results provide further support for this conclusion. LCMT-1 has been shown previously to associate with PP2A in vitro (22,24) and to methylate PP2A on its carboxyl terminal leucine's alpha carboxyl group (10-16). In this

study we have demonstrated further that LCMT-1 downregulation in mammalian cells reduces both PP2A C subunit methylation and the formation of $PP2A_{B\alpha AC}$ heterotrimers, and that downregulation of these same heterotrimers by Bα knockdown can cause part of the phenotypes seen with LCMT-1 knockdown. Moreover, we have previously shown that loss of $PP2A_{BAC}$ heterotrimer formation results in selective loss of PP2A activity towards a $PP2A_{BAC}$-specific substrate (8,20-25). Thus, it is possible that the cell death induced by LCMT-1 downregulation may be due in part to dysregulation of Bα heterotrimer function. A similar amount of apoptosis resulted when protein levels of either Bα or LCMT-1 were greatly reduced. Moreover, the pan-caspase inhibitor, z-VAD-fmk, could prevent a portion of the cell death induced by downregulation of either LCMT-1 or Bα, indicating that in both cases, caspase-dependent and caspase-independent death pathways contribute to cell death. This similarity in the cell death observed with either LCMT-1 or Bα reduction is again consistent with the possibility that LCMT-1 downregulation induces death to some degree through Bα dysregulation. However, the cell death induced by downregulation of these two proteins does not appear to be identical. It was more difficult to detect caspase activation *in vivo* and *in vitro* with Bα knockdown cells and the rescue by z-VAD-fmk was consistently lower for the Bα knockdown cells than for LCMT-1 knockdown cells. These differences suggest that LCMT-1 knockdown has other effects independent of Bα that contribute to cell death. In yeast, deletion of the gene that encodes the LCMT-1 homolog, Ppm1p, not only reduces the level of $PP2A_{BAC}$ heterotrimers 20-fold but also causes a smaller reduction (~2-fold) in $PP2A_{B'AC}$ heterotrimers (23,25,26). Therefore, one possible effect unique to LCMT-1 knockdown may be reduction of $PP2A_{B'AC}$ heterotrimers. Other possible targets of LCMT-1 which have yet to be tested also exist, such as PP2A heterotrimers formed by other members (β, γ, δ, ε) of the B/B55 regulatory subunit family or by PP2A B"

regulatory subunits, and PP4 and/or PP6 complexes. Although apoptosis-relevant targets of LCMT-1 other than Bα remain to be determined, our results clearly indicate that they exist.

The increased sensitivity of both LCMT-1 and Bα knockdown cells to the spindle-targeting drug, nocodazole, suggests that both of these proteins are important in mammalian cells for the spindle checkpoint, which ensures proper spindle formation and chromosomal attachment prior to progression from metaphase to anaphase. The fact that cell death was largely rescued by thymidine block-induced arrest in G_1/S indicates that cell cycle arrest, which is induced in HeLa cells by either nocodazole or thymidine, is not the sensitizing factor. Instead, the effect is specific to mitosis. This death in nocodazole- and not in thymidine-treated cells is consistent with previous results from yeast showing that loss of the Bα homolog, Cdc55p, or of the LCMT-1 homolog, Ppm1p, causes increased sensitivity to nocodazole. However, it was not known with the increased complexity of mammalian cells whether this phenotype would be found in this system, where there are multiple members of each B-type regulatory subunit family. In mammalian cells, the mechanistic details of the roles of B-type subunits in spindle checkpoint and mitotic exit are just beginning to emerge. Both the B family isoform, Bδ, and the B' family isoform, B'γ, have recently been identified as negative regulators of sister chromatid separation (35,43). Bδ was found to protect cohesins from degradation by stabilizing securin, a negative regulator of the cohesin protease, separase. B'γ was reported to protect cohesin degradation by binding to Shugoshin (hSgo1) and maintaining cohesins in a more stable, hypophosphorylated state. Our data suggest that Bα is also a negative regulator of the spindle checkpoint in mammalian cells. Whether it plays yet another distinct role or shares responsibility for dephosphorylation of cohesins or securin is not known. Overexpression data such as

those presented in the Bδ/securin study do not rule out a similar role for Bα. Likewise, B'γ identification as an hSgo1 partner leaves open the possibility that other B-type subunits might also associate with Shugoshin proteins. Finally, a role for LCMT-1 in spindle checkpoint is consistent with a role for PP2A methylation in mitotic progression. Experiments are underway to explore this possibility further.

PP2A regulates apoptotic pathways both positively and negatively by targeting different substrates via its diverse regulatory subunits. Consequently, PP2A inhibitors and siRNA downregulation of PP2A C and A subunits have been reported to induce apoptosis or block apoptosis, depending on the system (44-50). In both *Drosophila* and mammalian cells, B56/B' subunits have been reported to have an anti-apoptotic role (45,46,48). Some evidence has also been reported in mammalian cells for an anti-apoptotic role for B" and B subunit families (48). In this latter study, Strack and coworkers presented results of four different assays analyzing the importance of B55/B family subunits for cell survival. In seeming contrast to our results, one of the assays that showed little effect was siRNA knockdown of Bα. However, the efficiency of their Bα knockdown was not reported, making comparison to our results difficult. Moreover, in concert with our data, results from two of their other three assays supported an anti-apoptotic role for Bα. Our finding that Bα downregulation induces a small amount of apoptosis that is dependent on progression through mitosis is also consistent with the ability of the adenovirus protein, E4orf4, to arrest cells in G2/M and induce apoptosis in cancer cells in a manner dependent on its ability to bind PP2A Bα (51-54). Our results indicate a pro-survival function for LCMT-1 as well. This function may be mediated to some extent through modulation of $PP2A_{B\alpha AC}$ heterotrimer formation but our data suggest that Bα-independent roles should also be considered.

The fact that knockdown of either Bα or LCMT-1 caused an enhancement of cell killing by nocodazole in HeLa cells raises the possibility that Bα heterotrimers and LCMT-1 may have potential as targets for combination chemotherapy with microtubule targeting drugs like taxanes. Importantly, both Bα and LCMT-1 knockdown HeLa cells were greatly protected from cell death by arrest in G_1/S, presumably by blocking the progression of the knockdown cells into mitosis, where apoptosis was triggered due to a defective spindle checkpoint. One would expect that knockdown of these proteins would not have the same sensitizing effect to nocodazole in all cell types, given that some cancer cells already have defects in checkpoints and cell cycle control. HCT116 cells also showed sensitization to nocodazole (Fig. 7). However, initial experiments with H1299 lung cancer cells gave a different result. While these cells exhibit significant death when LCMT-1 is knocked down by either L2 or L3, this cell line is already more sensitive to nocodazole than HeLa or HCT116 cells and, not unexpectedly, does not show increased nocodazole sensitivity upon LCMT-1 knockdown by the L2 and L3 shRNAs (data not shown). Further experimentation will be necessary to investigate the potential of these proteins as novel drug targets.

Consistent with the observed effects of LCMT-1 reduction on cell viability, homozygous gene trap knockout of LCMT-1 results in embryonic lethality. The expected ratio of LCMT-$1^{+/+}$: LCMT-$1^{+/-}$: LCMT-$1^{-/-}$ progeny mice from a cross of LCMT-$1^{+/-}$ mice is 1:2:1. We obtained a ratio of 1:1.4:0 (Fig. 8D) with sufficient numbers of progeny to clearly indicate that homozygous loss of LCMT-1 is lethal during development. This result could not arise simply from inviable gametes because both male and female LCMT-$1^{+/-}$ mice are fertile when bred to wild-type mice (J.A. Lee and D.C. Pallas, unpublished). Based on our results with cells, this lethality could result from defects in cell cycle or increased apoptosis, but it is also possible that additional signaling defects may also contribute. To

our knowledge, this is the first report that LCMT-1 is essential for development. In addition, the lower than expected numbers of LCMT-1$^{+/-}$ mice also raise the possibility that hemizygous loss of LCMT-1 may cause some embryonic lethality as well. Future experiments will be aimed at elucidating the nature and timing of developmental defects that lead to the death of embryos lacking this important methyltransferase enzyme.

FIGURE LEGENDS

Fig. 1. **Analysis of LCMT-1 knockdown and PP2A methylation in LCMT-1 shRNA HeLa cells.** *A.* Two different LCMT-1 shRNAs (L2 and L3) efficiently knockdown LCMT-1 protein levels in HeLa cells. Vector control cells (VC) and cells stably expressing the L2 and L3 LCMT-1 shRNAs were lysed and lysates were probed for the steady state levels of LCMT-1 and PP2A C subunit (C subunit; loading control) by immunoblotting. *B.* Knockdown of LCMT-1 by expression of L2 or L3 shRNAs reduces PP2A C subunit methylation in HeLa cells. Vector control (VC) cells and LCMT-1 L2 and L3 shRNA cells were lysed and the steady-state level of PP2A methylation in each cell line was determined using our previously published assay (24). Briefly, since base treatment demethylates PP2A catalytic subunit, one aliquot of lysate from each line was treated for 5min at 4°C with 0.2N NaOH to completely demethylate PP2A C subunit and was then neutralized (+ lanes; 100% demethylated control) while another equal aliquot of lysate from each line was combined with preneutralized buffer (- lanes; reflect endogenous methylation level). Then the untreated and base-treated aliquots were analyzed side by side on a 10% SDS-polyacrylamide gel followed by immunoblotting with a monoclonal antibody (4b7) specific for unmethylated C subunit. Demethylation of PP2A C subunit induced by L2 and L3 shRNA expression can be seen as an increase in the relative intensity of 4b7 signal in the minus versus plus lanes as compared to vector control cells. Also shown is an immunoblot of total PP2A C subunit showing that each pair of – and + lanes was loaded equally. *C.* The percent unmethylated PP2A catalytic subunit was determined by quantitatively comparing the amount of 4b7 signal in untreated samples (reflects level of endogenous unmethylated PP2A C subunit in each line) to that in the matched base-treated samples (100%

demethylated controls) using a BioRad Fluor-S Max chemilumimager. Percent methylation was calculated by subtracting the percent of unmethylated PP2A from one hundred. The graph shows the averages and standard deviations (error bars) of three independent experiments. Asterisks indicate significance versus vector control as assayed by T test (*, p=0.0499; **, p=0.0001). C subunit can migrate as either singlets or doublets; whether double or single bands are seen can vary for the same sample from gel to gel. This pattern of migration in SDS-PAGE has been noted previously for endogenous PP2A C subunits (6,8,24,32) and does not appear to be due to degradation. *D.* Immunoblotting with the anti-methylated PP2A catalytic subunit antibody, 2A10, further strengthens the conclusion that LCMT-1 is the major PP2A methyltransferase *in vivo*. Lysates prepared from vector control (VC) and L3 LCMT-1 knockdown cells were analyzed by immunoblotting with LCMT-1 antibody to demonstrate LCMT-1 knockdown and with 2A10 monoclonal antibody (Methylated C subunit) to show L3 LCMT-1 knockdown causes a substantial reduction in PP2A catalytic subunit methylation. Also shown is an immunoblot of total PP2A C subunit showing that the lanes were loaded equally. Quantitation of the 2A10 signals from two separate experiments showed that there was greater than 3-fold reduction in methylation in the L3 LCMT-1 shRNA knockdown cells.

Fig. 2. **LCMT-1 knockdown reduces C subunit-Bα association**. Vector control cells (VC) and cells stably expressing an shRNA directed against LCMT-1 (L3) were transfected with a construct expressing HA-tagged Bα (HA-Bα) or an empty vector. *A.* The steady-state level of PP2A methylation in VC and L3 lysates (5% of the input of each immunoprecipitate) was determined using our previously described assay using our 4b7 methylation-sensitive monoclonal antibody (See legend for Fig. 1B). The reduction

in methylation induced by expression of the L3 shRNA can be seen by the increased signal in the "–" lane. Also shown is an immunoblot of total PP2A C subunit showing that each pair of – and + lanes was loaded equally. *B.* Percent methylation of the PP2A catalytic subunit was determined as described in the legend to Figure 1C. The graph shows the averages and standard deviations (error bars) of three independent experiments. The asterisk indicates significance versus vector control as assayed by T test (*, p=0.02). *C.* HA-Bα immunoprecipitates of VC and L3 shRNA cells were probed for HA-tagged Bα (HA-Bα) and C subunit. The image shown is a representative immunoblot of 3 independent experiments. *D.* Each band in panel B was quantitated using a BioRad Fluor-S Max chemilumimager. The relative amount of C subunit bound to HA-Bα (Relative C subunit association) was calculated as a measure of the efficiency of C subunit association with HA-Bα. The graph shown displays the average C subunit association with HA-Bα in the three experiments. Error bars show standard deviations of the three independent experiments. Asterisks indicate significance versus vector control as assayed by T test (**, p=0.0038).

Fig. 3. **LCMT-1 or Bα knockdown causes cell death**. *A.* Asynchronous vector control cells (VC), LCMT-1 shRNA knockdown cells expressing one of two different shRNAs to LCMT-1 (L2 or L3), and PP2A Bα regulatory subunit knockdown cells (Bα) were plated and allowed to adhere, washed to remove dead cells, and then harvested after 24 hours and assayed for viability by trypan blue exclusion. Error bars show standard deviations of five independent experiments. Asterisks indicate significance versus vector control as assayed by T test (L2**, p=0.000005; L3**, p=0.00004; Bα *, p=0.014). *B.* PP2A Bα shRNA dramatically reduces Bα protein levels in HeLa cells. Vector control cells (VC) and cells stably expressing shRNAs directed

against Bα were lysed and lysates were probed for the steady state levels of Bα and PP2A C subunit (loading control) by immunoblotting.

Fig. 4. **Characterization of cell death induced by knockdown of LCMT-1 or Bα.** *A.* LCMT-1 knockdown causes membrane blebbing. Asynchronous LCMT-1 shRNA knockdown cells (L2) were observed for 24 to 36 hours by phase time-lapse microscopy. Six representative images of membrane blebbing in L2 knockdown cells are shown. *B.* Knockdown of LCMT-1 or Bα causes DNA condensation and fragmentation in HeLa cells. Asynchronous unselected vector control cells (VC) (data not shown), LCMT-1 shRNA knockdown cells (L2 or L3), and Bα knockdown cells infected with an MOI of 3 were incubated with DAPI DNA stain to detect DNA condensation and fragmentation by immunofluorescence microscopy. Three representative images (top row, DAPI image; bottom row, DIC image) are shown for each cell line. *C.* LCMT-1 knockdown HeLa cells contain active caspases. Twenty-four hours after removal of dead cells by washing, asynchronous untreated vector control cells (VC), staurosporine-treated vector control cells (ST; positive control), LCMT-1 shRNA knockdown (L2 or L3), and Bα knockdown cells were harvested, incubated with Red-DEVD-fmk (Calbiochem) for 1h at 37°C, and assayed by immunofluorescence microscopy. Error bars show standard deviations of results obtained from a minimum of three fields. Asterisks indicate significance versus vector control as assayed by T test (*, p=0.021; **, p=0.002). Similar results were obtained in two independent experiments. *D.* LCMT-1 knockdown HeLa cell lysates exhibit caspase activity. The same cells as used in panel C were harvested, lysed by sonication, and incubated with z-DEVD-AFC (Calbiochem) for 1h at 37°C, and assayed for caspase activation using a fluorimeter. Caspase activity shown is in relative fluorescence units. Error bars show standard deviations of results obtained

triplicate wells. Asterisks indicate significance versus vector control as assayed by T test (ST**, p=0.0003; L2**, p=0.003; L3**, p=0.004). Similar results were obtained in three independent experiments except that Bα had a statistically significant increase in caspase activity in one experiment. *E.* Caspase inhibitor partially rescues cell death in LCMT-1 and Bα knockdown cells**.** The same cells as used in panel C were plated into duplicate plates and dead cells were removed by washing. One plate of cells was treated with 50μM z-VAD-fmk for 24h while the other was treated with vehicle only. Then the cells were harvested and assayed for trypan blue exclusion. A decrease in cell death in z-VAD-fmk-treated cells (black bars) as compared to untreated cells (vehicle control; white bars) can be seen. Error bars show standard deviations of triplicate wells. Asterisks indicate significance versus vector control as assayed by T test (L2*, p=0.027; L3*, p=0.022; **, p=0.006). Similar results were obtained in two independent experiments.

Fig. 5. **Some LCMT-1 knockdown cells die after entering mitosis.** Asynchronous vector control cells (VC), LCMT-1 shRNA knockdown cells (L2 or L3), and Bα subunit knockdown cells were observed for 24 to 36 hours by phase time-lapse microscopy. A representative image of L3 knockdown cells at 0, 1.6, and 16 hours is shown. The black arrow indicates the cell of interest through 3 panels. The metaphase plate (vertical white line) of this cell is visible in panel 2 (1.6h time-point).

Fig. 6. **LCMT-1 or Bα reduction causes nocodazole sensitivity in HeLa cells**. *A.* Vector control HeLa cells (VC), LCMT-1 shRNA knockdown cells (L2 or L3), and Bα shRNA cells were plated into duplicate plates and dead cells were removed by washing. One plate of cells was treated with nocodazole for 24h while the other was

treated with vehicle only. Then the cells were harvested and assayed for trypan blue exclusion. Shown is the net increase in cell death for each line resulting from nocodazole treatment (% death of each line in nocodazole minus % death of the same line in vehicle control). Error bars show standard deviations of three different experiments. Asterisks indicate significance versus vector control as assayed by T test (*, p=0.022; L3**, p=0.004; Bα**, p=0.002). *B.* Thymidine rescues cell death in LCMT-1 and Bα knockdown HeLa cells**.** The same cells as used in panel A were plated into duplicate plates and dead cells were removed by washing. One plate of cells was treated with thymidine for 24h while the other was treated with vehicle only. Then the cells were harvested and assayed for trypan blue exclusion. Shown is the cell death in untreated (control; white bars) and thymidine-treated cells (black bars). Error bars show standard deviations for triplicate wells. Asterisks indicate significance versus vector control as assayed by T test (L2**, p=0.002; L3**, p=0.003; Bα**, p=0.002). Similar results were obtained two independent experiments.

Fig. 7. **LCMT-1 knockdown in HCT116 colon cancer cells also causes decreased PP2A C subunit methylation and increased nocodazole sensitivity.** *A.* Two different LCMT-1 shRNAs (L2 and L3) efficiently knockdown LCMT-1 protein levels and reduce PP2A C subunit methylation in HCT116 cells. Lysates from vector control cells (VC) and cells stably expressing the L2 and L3 LCMT-1 shRNAs were probed by immunoblotting for the steady state levels of LCMT-1, PP2A C subunit methylation (methylated PP2A C subunit; using an anti-methyl PP2A C subunit monoclonal antibody, 2A10). Total PP2A C subunit was also immunoblotted as a loading control. *B***.** The steady-state level of PP2A methylation in VC, L2 and L3 lysates was also analyzed with our previously described assay using our 4b7 methylation-sensitive

monoclonal antibody (See legend for Fig. 1B). The untreated (-) and base-treated (+; 100% unmethylated control) aliquots were analyzed side by side on a 10% SDS-polyacrylamide gel followed by immunoblotting with 4b7, which is specific for unmethylated C subunit. Demethylation of PP2A C subunit induced by L2 and L3 shRNA expression can be seen as an increase in the relative intensity of 4b7 signal in the minus versus plus lanes of L2 and L3 as compared to the relative intensity of the minus and plus lanes in vector control. Also shown is an immunoblot of total PP2A C subunit showing that each pair of – and + lanes was loaded equally. *C.* Quantitation of PP2A C subunit methylation reduction in HCT116 L2 and L3 LCMT-1 shRNA cells. The percent of unmethylated PP2A catalytic subunit in HCT116 cell lysates was determined by quantitatively comparing the amount of 4b7 signal in untreated samples (endogenous unmethylated PP2A C subunit) to that in the matched base-treated samples (100% demethylated controls) using a BioRad Fluor-S Max chemilumimager. Percent methylation was calculated by subtracting the percent of unmethylated PP2A from one hundred. The graph shows the averages of the results from two assays ± range. *D.* LCMT-1 knockdown in HCT116 cells causes increased sensitivity to nocodazole. HCT116 vector control (VC) and L2 and L3 LCMT-1 shRNA knockdown cells were plated into duplicate plates and dead cells were removed by washing. One plate of cells was treated with nocodazole for 24h while the other was treated with vehicle only. Then the cells were harvested and assayed for trypan blue exclusion. Shown is the net increase in cell death for each line resulting from nocodazole treatment (% death of each line in nocodazole minus % death of the same line in vehicle control). Percent death in the absence of nocodazole in this experiment was: VC, 1.74±0.43; L2, 9.2±1.5, p=0.012; L3, 3.9±0.7, p=0.025). Error bars show standard deviations for triplicate wells. Asterisks indicate significance versus vector control as assayed by T test (*, p=0.028; **,

p=0.002). Similar results were obtained in three independent experiments, except in one case the p-value for L3 was 0.06.

Fig. 8. **Homozygous knockout of LCMT-1 in mice is embryonic lethal**. *A*. DNA was prepared from *lcmt-1*$^{+/-}$ mouse embryonic stem cells (ES) obtained from The German Gene Trap Consortium and from progeny mice (M13, M26) obtained from crossing of chimeric knockout mice with C57BL/6 breeders. Each DNA was used in two PCR reactions, one using a primer pair specific for the wild-type *lcmt-1* gene (WT; if positive, it indicates at least one *lcmt-1* allele without gene trap insertion is present) and another using a primer pair specific for *lcmt-1* having the gene trap insertion (Genetrap; forward primer matching the *lcmt-1* intron and reverse primer matching the gene trap insert). Strong bands specific for the *lcmt-1* genetrap knockout are seen in the *lcmt-1*$^{+/-}$ ES cells (control) and in the *lcmt-1*$^{+/-}$ mouse (M13) while the *lcmt-1*$^{+/+}$ mouse (M26) clearly lacks the knockout allele. *B*. LCMT-1 protein level is reduced in the *lcmt*$^{+/-}$ ES cells. Equal numbers of *lcmt-1*$^{+/-}$ ES cells (+/-) and control parental ES cells (+/+) were lysed and analyzed by SDS-PAGE and immunoblotting with LCMT-1 antibody. *C*. The results from one experiment from Fig. 7B performed in triplicate were quantitated and the average and standard deviations of the LCMT-1 levels in the *lcmt-1*$^{+/-}$ ES cells normalized to the control *lcmt-1*$^{+/+}$ ES cells are shown. Two separate experiments showed a reduction of over 50%. *D*. No pups with homozygous gene trap knockout of *lmct-1* were obtained from *lcmt-1*$^{+/-}$ x *lcmt-1*$^{+/-}$ mouse crosses. Eight crosses of *lcmt-1*$^{+/-}$ x *lcmt-1*$^{+/-}$ mice were performed and pups were genotyped. Forty-five pups were obtained, 19 of which were *lcmt-1*$^{+/+}$ and 26 of which were *lcmt-1*$^{+/-}$. No LCMT-1$^{-/-}$ mice were obtained. Shown are the average number of pups in each litter that were *lcmt-1*$^{+/+}$, *lcmt-*

1$^{+/-}$, or *lcmt-1*$^{-/-}$. Error bars indicate standard deviation. Asterisks indicate 99.8% confidence determined by T test that homozygous knockout of *lcmt-1* causes embryonic lethality (p=0.002).

Figure 1

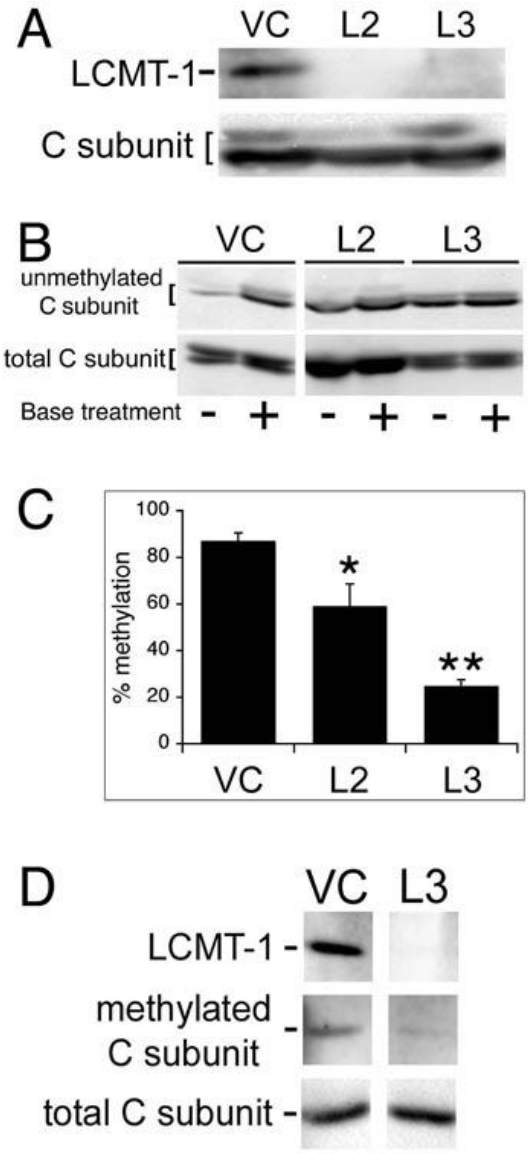

A
VC
L2
L3
LCMT-1
C subunit
B
VC
L2
L3
unmethylated C subunit
total C subunit
Base treatment
- + - + - +
C
% methylation
100
80
60
40
20
0
*
**
VC
L2
L3
D
VC
L3
LCMT-1
methylated C subunit
total C subunit

Figure 2

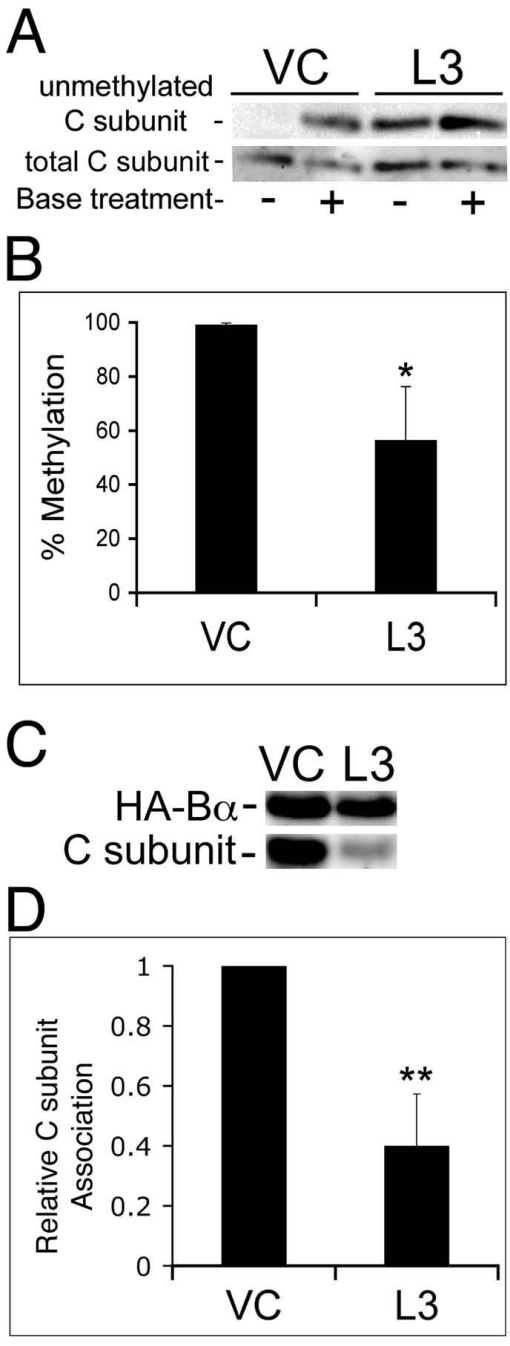

A
VC
L3
unmethylated
C subunit -
total C subunit -
Base treatment- - + - +
B
% Methylation
100
80
60
40
20
0
*
VC
L3
C
VC L3
HA-Bα -
C subunit -
D
Relative C subunit Association
1
0.8
0.6
0.4
0.2
0
**
VC
L3

Figure 3

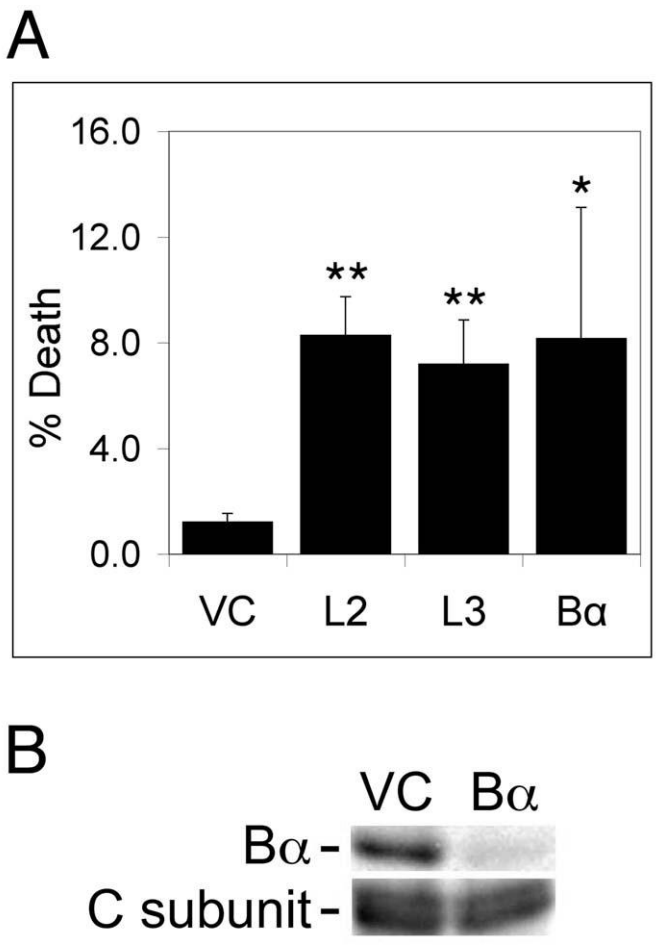


Figure 4

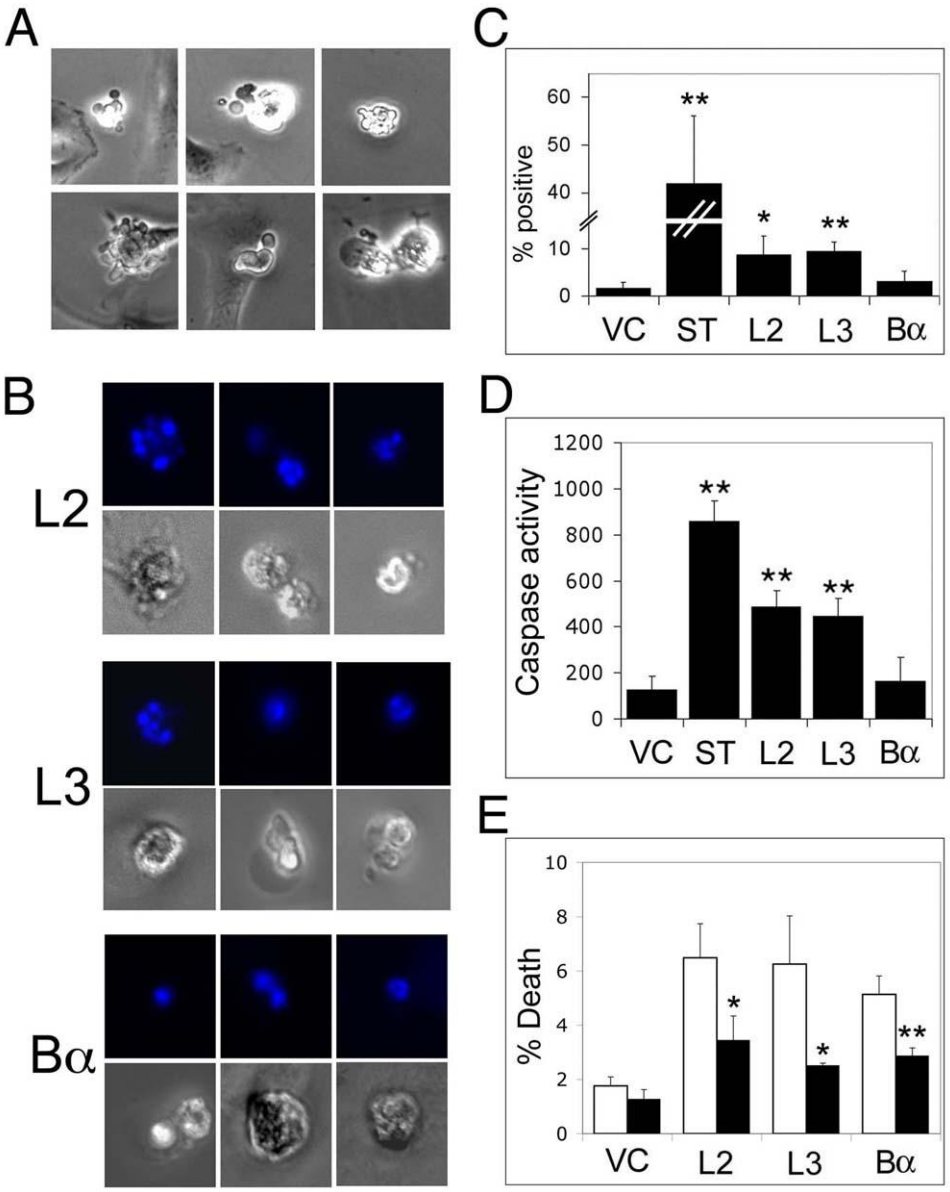

A
B
L2
L3
Bα
C
% positive
60
50
40
10
0
**
*
**
VC
ST
L2
L3
Bα
D
Caspase activity
1200
1000
800
600
400
200
0
**
**
**
VC
ST
L2
L3
Bα
E
% Death
10
8
6
4
2
0
*
*
**
VC
L2
L3
Bα

Figure 5

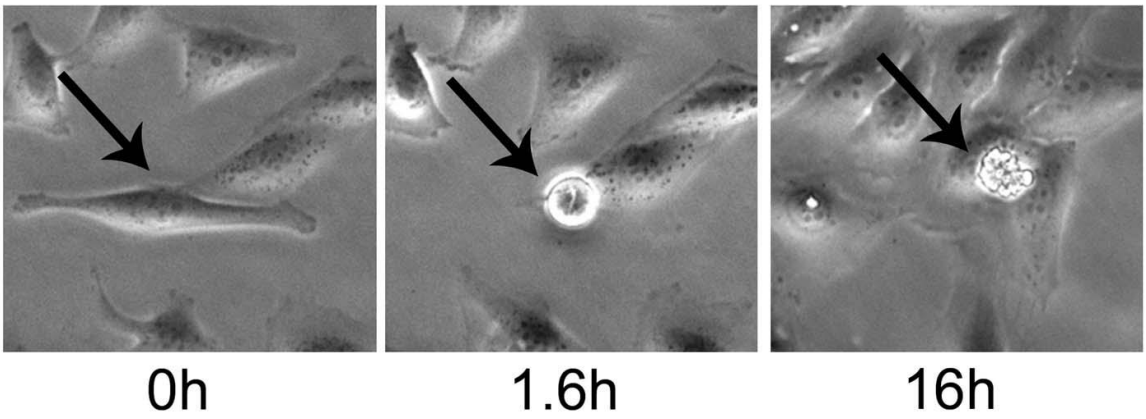


Figure 6

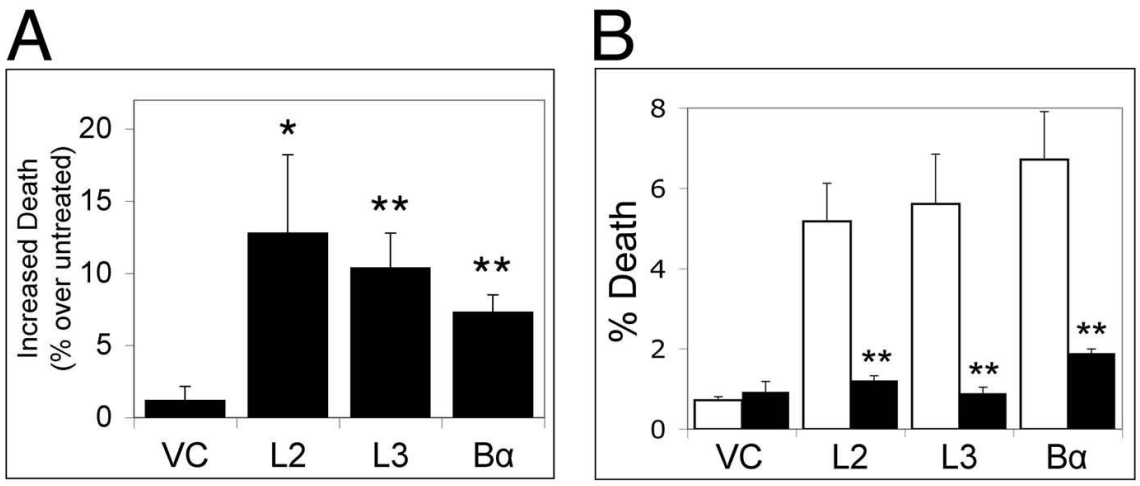

A
Increased Death
(% over untreated)
20
15
10
5
0
*
**
**
VC
L2
L3
Bα
B
% Death
8
6
4
2
0
**
**
**
VC
L2
L3
Bα

Figure 7

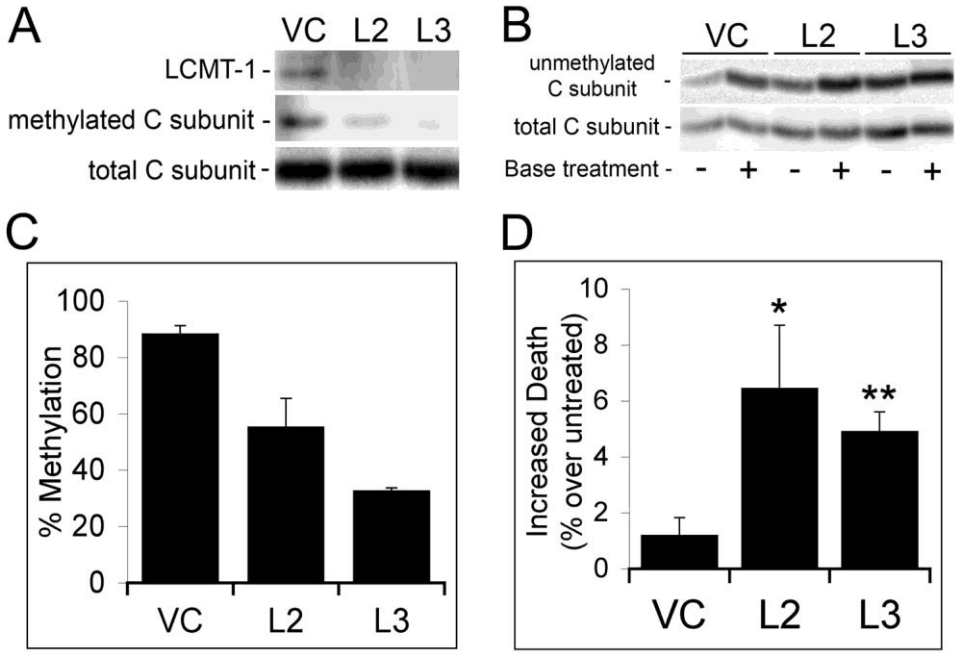
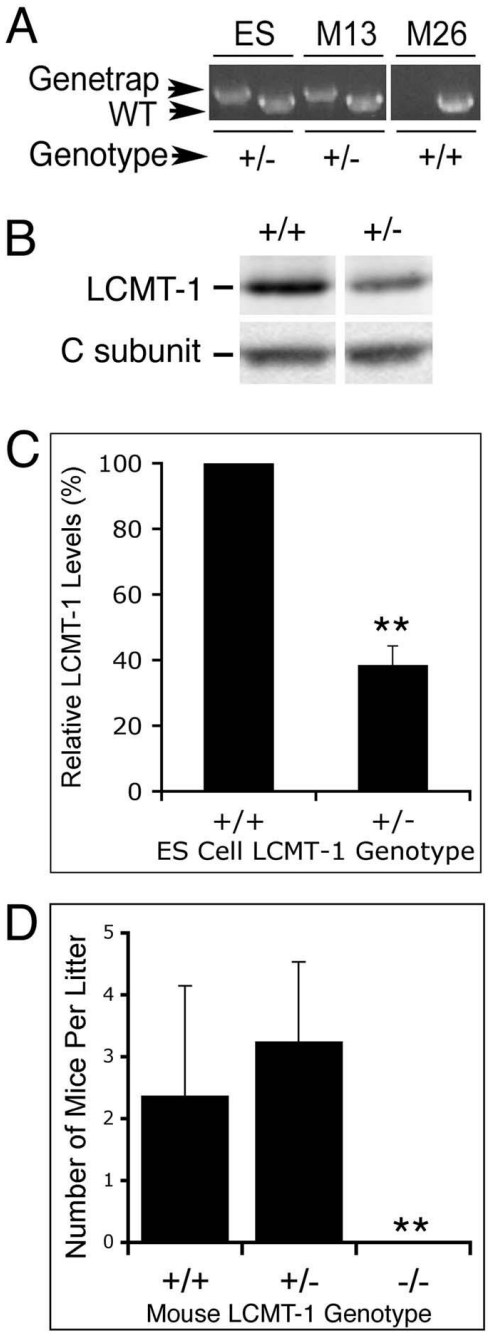

A
VC L2 L3
LCMT-1 -
methylated C subunit -
total C subunit -
B
VC L2 L3
unmethylated C subunit -
total C subunit -
Base treatment - - + - + - +
C
% Methylation
100
80
60
40
20
0
VC L2 L3
D
Increased Death (% over untreated)
10
8
6
4
2
0
*
**
VC L2 L3

Figure 8


A
ES
M13
M26
Genetrap
WT
Genotype
+/-
+/-
+/+
B
+/+
+/-
LCMT-1
C subunit
C
Relative LCMT-1 Levels (%)
100
80
60
40
20
0
**
+/+
+/-
ES Cell LCMT-1 Genotype
D
Number of Mice Per Litter
5
4
3
2
1
0
**
+/+
+/-
-/-
Mouse LCMT-1 Genotype

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Chapter 2

Loss of Leucine Carboxyl Methyltransferase 1 Results in Embryonic Lethality and Fetal Liver Apoptosis

Introduction

The role of protein phosphatases in many signaling cascades that regulate cell growth and development are just being uncovered. Once thought to be primarily regulated by the activity of protein kinases, recent data has placed protein phosphatases as the molecular switches that are key to the regulation of many important signaling cascades.

Protein Phosphatase 2A (PP2A) is a multifunctional serine/threonine protein phosphatase that is a key regulator of many cellular processes including growth and proliferation and is a positive regulator of apoptosis (1). Importantly, negative regulation of PP2A through either downregulation of PP2A, mutation of PP2A subunits, or overexpression of PP2A inhibitory proteins has shown to contribute to many forms of cancer. PP2A, which is composed of three functionally distinct subunits [A (structural), B (regulatory), and C (catalytic)], interacts with a variety of substrates and proteins that directly regulate its holoenzyme formation. A number of heterotrimeric PP2A holoenzymes exist that differ in their regulatory/targeting "B-type" subunits. Although there are two genes each for the C and A subunits in mammalian cells, there are multiple families of B-type subunits ((B (B55/PR55), B′ (B56/PR61), B″ (PR72)) and a putative B‴ (striatin) family, each of which has multiple members (1).

Reversible methylation is the most specific cellular mechanism for regulating PP2A. In yeast as well as mammalian cells, PP2A is methylated on its catalytic subunit C-terminal leucine alpha-carboxy group by Leucine Carboxy Methyltransferase (LCMT-1) and is demethylated by Protein Phosphatase Methylesterase-1 (PME-1), originally

cloned and characterized in the Pallas lab (2-8). Together, these two proteins regulate the methylation of the C subunit, indirectly regulating PP2A by altering its holoenzyme formation, and thus its subcellular targeting and substrate specificity. In mammalian cells, methylation is necessary for the proper assembly of heterotrimers containing the Bα (PR55α) and Bβ (PR55β) B-family members, but does not significantly affect the formation of PP2A complexes containing members of the B' (PR61), B" (PR72), B"' (striatin) family members, or the viral B-type subunit, Polyomavirus middle tumor antigen (9,10). Therefore drugs that modulate PP2A methylation may be the most efficient manner of targeting substrates of PP2A heterotrimers containing B family regulatory subunits, which include βcatenin, the mitotic spindle regulatory proteins securin and shugoshin, the microtubule associated protein tau, and TGFβ receptors (11-14).

The *lcmt-1* gene is found in all eukaryotes from yeast to humans. In mammals, there is one functional gene for LCMT-1 that encodes a 38-kDa protein. The Pallas lab and others have shown that LCMT-1 is a pro-survival protein that is important for proper mitotic progression in yeast as well as mammalian cells, playing a key role in mitotic spindle checkpoint (15,16). In addition, LCMT-1 has been implicated in apoptosis in several transformed cells lines (17,18). Although LCMT-1 is involved all these important cellular processes, the larger role LCMT-1 plays in growth and development due to its ability to modulate these processes has not been elucidated. To gain understanding of the role of LCMT-1 and PP2A signaling in mouse development, we disrupted the murine *lcmt-1* gene, which results in a recessive lethal phenotype. *Lcmt-1* knockout embryos die during midgestation (E11.5-E14.5) with several defects, including a severe disruption of proper liver development and hematopoiesis. The fetal livers of *lcmt-1* knockout embryos display hypocellularity, high levels of apoptosis, and an inability to produce hematopoietic myeloid progenitor cells that will eventually populate the bone marrow.

Through this study, we have identified LCMT-1 as the sole PP2A methyltransferase, and thus loss of LCMT-1 dramatically affects the formation of PP2A heterotrimers containing the B family of B-type subunits. Moreover, we have found that loss of LCMT-1 results in the instability of both the catalytic (C) and structural (A) PP2A subunits. Therefore, LCMT-1 and PP2A methylation are key regulators of liver development and necessary for proper mouse development.

Materials and Methods

Generation of lcmt-1 gene-trap *knockout mice and genotyping*

Embryonic stem cells with a gene trap insertion in an unknown location within the first intron of the *lcmt-1* locus were obtained from the German Gene Trap Consortium, Neuherberg, Germany. These *lcmt-1+/- stem* cells were expanded and injected into blastocysts from C57BL/6 donors and then the blastocysts were implanted into foster mothers. Resultant chimeric mice were bred to generate heterozygous F1 animals, which were then backcrossed for twelve generations to C57BL/6 mice to create a homogenous line for study. The exact integration site of the gene-trap vector within the ~22kb intron 1 of the *lcmt-1* gene was determined by using 22 forward primers (one primer approximately every 1kb) and 3 reverse primers that spanned the length of the HindIII-digested pT1betageo plasmid used to make the gene trap knockout collection. PCR products were sequenced to determine the location of the pT1betageo integration site, facilitating the design of primers for genotyping.

To genotype mice for *lcmt-1*, DNA was first extracted from tail biopsies digested in tail lysis buffer (100mM Tris-HCl, pH 8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl, 100ug/mL Proteinase K) overnight at 56°C. Then DNA samples were subjected to 35 cycles of PCR (94°C for 30 s; 56°C for 30 s; 72°C for 1 min) using one forward primer and two reverse primers: 1 (forward), 5'-AACATGAAGTGGTGTTCTCCGGGCGCC-3'; 2 (reverse), 5'-TGGCTGTGTGCCCTTGGAACCTCAGCACC-3'; and 3 (reverse), 5'-ATGCACTGAAATGAAAACGTGAAGACGACG-3'. Primers 1 and 2 amplify an ~200 bp fragment of the endogenous *lcmt-1* allele, whereas primers 1 and 3 amplify an approximately 300 bp region between the *lcmt-1* intron 1 and LacZ in the pT1betageo gene trap cassette.

Biochemical Analysis of lcmt-1 Embryos

E12.5 embryos were dissected free from the uterus and extraembryonic membranes, flash frozen in liquid nitrogen, and then stored at -80°C until use. Yolk sacs were saved for genotyping. Whole embryos or livers were dounce homogenized in a Nonidet P-40-containing lysis buffer (10% glycerol, 20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40) containing 1mM phenylmethylsufonyl fluoride and 0.04 trypsin inhibitor units/ml aprotinin. Homogenates were cleared by centrifugation at 13,000 x g and in some cases immunoprecipitated with anti-PP2A B subunit antibody, 2G9 (Millipore), covalently cross-linked to protein A or G sepharose beads. Homogenates and immunoprecipitates were analyzed by SDS-PAGE. Relative proteins levels were determined by quantitation using a Fluor S-Max Chemilumimager and Quantity One Software (Bio-Rad) and lysate protein levels were normalization to actin. Antibodies used for western blotting include mouse monoclonal antibodies against PP2A A subunit (clone 4g7; Santa Cruz Biotechnologies or Millipore), PP2A B subunit (2G9; Millipore), PP2A C subunit (BD Transduction Laboratories), and cleaved caspase 3 (Cell Signaling), a goat polyclonal antibody against actin (Santa Cruz Biotechnologies) and an affinity-purified rabbit anti-LCMT-1 polyclonal antibody, RK3110, generated against a 17-amino acid peptide corresponding to residues 173-189 of human LCMT-1 (17).

Determination of the steady-state level of PP2A C subunit methylation

The steady state level of PP2A catalytic subunit methylation was measured in whole embryo homogenates with a monoclonal antibody specific for unmethylated PP2A C subunit (4b7) using our published method (17). Quantitation of the 4b7 signal was performed with a BioRad Fluor S-Max Chemilumimager and BioRad Quantity One Software. Lysates were also probed with Actin as a loading control.

Histological Analysis of lcmt-1 Embryos

E12.5 embryos were dissected free from the uterus and extraembryonic membranes and fixed in 4% paraformaldehyde in phosphate buffered saline at 4°C overnight. Yolk sacs were saved for genotyping. The following day, the embryos were dehydrated through an ethanol series, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin (H&E). Immunohistochemistry was performed using a polyclonal anti-cleaved caspase 3 antibody (1:100; Biocare Medical), a polyclonal rabbit anti-Ki67 antibody (1:200; Abcam), and a monoclonal mouse anti-MPM-2 antibody (1:200; Millipore). The Vectastain elite avidin biotinylated enzyme complex (ABC) staining kit was used on paraffin sections according to the protocol specified by the supplier (Vector labs). Pre-treatment in 100°C 10mM citric acid buffer pH 6.0 containing 0.25% Triton-X 100 was performed for all immunostainings. Control sections were treated with non-related isotype-matched secondary immunoglobulin instead of primary antibody. TUNEL assays were performed using the ApopTag Peroxidase in Situ Apoptosis Detection Kit (Millipore). All sections were co-stained with hematoxylin.

Clonogenic Progenitor Cell Assays

E12.5 and E14.5 fetal livers were dissected free in 2% heat-inactivated fetal calf serum in phosphate-buffered serum followed by dissaggregation into single cell suspension using a 21-gauge syringe. The cells were then washed twice in 2% fetal bovine serum in phosphate buffered saline and counted. An aliquot of cells were diluted in 2% acetic acid to lyse non-nucleated mature erythrocytes and viable cells were counted using trypan blue. Then 2 x 10^4 viable cells were plated in methylcellulose media containing 3 units/mL Epo, 10 ng/mL mouse recombinant IL-3, 10 ng/mL human recombinant IL-6, and 50 ng/mL mouse recombinant stem-cell factor (MethoCult M3434, Stem Cell Technologies, Vancouver, BC). At 7 days, assays were scored for the number of burst-

forming unit–erythroid (BFU-E), colony-forming unit–granulocyte/macrophage (CFU-GM), and CFU–granulocyte/erythrocyte/monocyte/macrophage (CFU-GEMM) colonies. Benzidine-staining for hemoglobin was used to confirm or clarify BFU-E and CFU-GEMM colonies.

Microarray analysis of lcmt-1 knockout embryos – Total RNA was extracted from E12.5 wild-type, hemizygous, and *lcmt-1* knockout embryos using the Absolutely RNA Miniprep Kit (Strategene). For each sample replicate, 100 ng of total RNA were amplified using the Ambion WT Expression Kit (Applied Biosystems) and labeled using the Affymetrix GeneChip Terminal Labeling Kit. Affymetrix GeneChip Mouse Gene 1.0 ST arrays were hybridized with labeled sense DNA, washed, stained, and scanned using the Affymetrix GeneChip Expression Wash, Stain and Scan kit. Data was extracted, normalized, and summarized with the robust multi-average array (RMA) method (19,20). This work was supported in part by the Emory Winship Cancer Genomics Shared Resource.

Statistical Analysis

All statistical analyses were done using student t test, and $p \leq 0.05$ was considered significant. All error bars shown are the standard deviation. The number of replicates and statistical significance are indicated in results and in the figure legends.

Results

Disruption of the murine lcmt-1 gene

As described in Materials and Methods, we constructed a mouse model for studying the function of LCMT-1 in mouse development by utilizing the gene trap approach (21). The murine *lcmt-1* gene is encoded by eleven exons within 1.3mb of genomic DNA on mouse chromosome 7. Insertion of a linearized pT1βgeo gene trap plasmid within the first intron of the *lcmt-1* gene creates a "trapped" or truncated LCMT-1 transcript because of the splice acceptor present in pT1βgeo (Figure 1A). Embryonic stem (ES) cells hemizygous for this insertion were previously shown to express a reduced level of LCMT-1 protein (17). We injected these ES cells into C57BL/6 blastocysts, backcrossed the resultant chimeric mice to generate heterozygous F1 animals, and tested for germline transmission of the mutant *lcmt-1* allele in progeny by PCR analysis of tail DNA (Figure 1B). Finally, we backcrossed hemizygous *lcmt-1* mice to C57BL/6 mice for twelve generations prior to the experiments described in this study.

Homozygous genetrap knockout of lcmt-1 results in nearly complete loss of LCMT-1 protein and embryonic lethality between E14.5 and E16.5 days of gestation

We showed previously that gene trap knockout of *lcmt-1* results in embryonic lethality, based on the fact that no *lcmt-1*$^{-/-}$ progeny were produced from early *lcmt-1*$^{+/-}$ X *lcmt-1*$^{+/-}$ matings (17). In this study, after backcrossing to C57BL/6 background twelve times, we found that this phenotype persisted. To determine the effect of homozygous knockout of *lcmt-1* on LCMT-1 protein expression, the time of lethality during embryonic development, and the type and severity of developmental defects caused by homozygous gene trap knockout of *lcmt-1*, we performed time-mating dissections of embryos

produced from *lcmt-1*$^{+/-}$ X *lcmt-1*$^{+/-}$ matings. Embryo genotypes were determined by PCR analysis of yolk sac DNA (Figure 2A). Homozygous inactivation of the *lcmt-1* gene results in an almost complete loss of LCMT-1 protein as assayed from homogenates made from E12.5 whole embryos (Figure 2C). Examination of embryos at successive stages of embryonic development revealed that homozygous *lcmt-1*$^{-/-}$ embryos die primarily between embryonic days 14.5 and 16.5 of gestation (E14.5-E16.5) (Figure 2B). Thus, LCMT-1 is required for development during this period.

Loss of lcmt-1 results in defects in fetal liver hematopoiesis

Gross examination of homozygous *lcmt-1*$^{-/-}$ mutant embryos revealed that the most obvious consequence of homozygous knockout of the *lcmt-1* gene was a severe impairment of hematopoiesis, observed as a smaller more pale liver in *lcmt-1*$^{-/-}$ embryos as compared to wild-type littermates (Figure 3A; black arrowheads). This defect was also seen at embryonic day 11.5 (Supplemental Figure S1). No defects were found in hemizygous littermates (Figure 2A) consistent with the fact that we obtain healthy, live births of hemizygous *lcmt-1* mice. In addition to this defect in hematopoiesis, there was a high frequency of misshapen eyes in *lcmt-1*$^{-/-}$ knockout embryos compared to wild-type and hemizygous littermates, indicating that LCMT-1 is necessary for normal eye development. Moreover, we observed a significant reduction in the weight of freshly isolated *lcmt-1*$^{-/-}$ embryos (Figure 3B), although we did not observed a significant difference in length (crown-to-rump length) when compared to wild-type littermates (Figure 3C). To determine if increased apoptosis might contribute to the reduction in embryo weight, we assayed for cleaved caspase-3 levels in whole embryo homogenates from viable embryos (Figure 3D). We observed a greater than 2.5-fold increase in cleaved caspase-3 levels in *lcmt-1*$^{-/-}$ embryos, suggesting that differences in apoptosis may be partially responsible for the significant differences in embryo size.

Homozygous lcmt-1 knockout results in a dramatic reduction in PP2A methylation

Previous work from several labs supports the idea that the yeast LCMT-1 homolog, Ppm1p, is the sole PP2A methyltransferase in yeast (15,16,22). Our previous data also indicate that LCMT-1 is the major PP2A methyltransferase in mammalian cells since shRNA knockdown of LCMT-1 in cultured cells resulted in up to a 71% decrease in PP2A methylation (17), but whether LCMT is the sole mammalian PP2A methyltransferase is not known. To determine the effect of gene trap knockout of LCMT-1 on PP2A methylation in mouse embryos, we quantitated the steady-state levels of PP2A methylation in E12.5 wild-type, hemizygous, and homozygous whole embryo homogenates using anti-demethylated PP2A monoclonal antibody, 4b7, which is specific for unmethylated PP2A catalytic subunit. Gene trap inactivation of the *lcmt-1* gene results in >95% reduction in PP2A C subunit methylation (Figure 4A), indicating that mammalian LCMT-1 is probably solely responsible for PP2A methylation in mouse embryos.

Knockout of lcmt-1 greatly reduces $PP2A_{BAC}$ heterotrimer formation and the steady-state level of PP2A subunits

Deletion of *PPM1* in yeast or knockdown of LCMT-1 in mammalian cultured cells dramatically reduces the formation of $PP2A_{BAC}$ heterotrimers as measured by the reduction in the amount of PP2A C subunit associated with B subunit (10). To assess the importance of LCMT-1 for $PP2A_{BAC}$ heterotrimer formation in an animal model, we immunoprecipitated PP2A B subunit from whole embryo homogenates and probed for C subunit. Homozygous gene-trap knockout of LCMT-1 reduces the relative amount of C subunit associated with B subunit to ~40% of wild-type levels while loss of one *lcmt-1*

allele has no significant effect (Figure 4B). However, this only represents the effect of *lcmt-1* loss on the efficiency of $PP2A_{BAC}$ heterotrimer formation of the B subunit still present in the *lcmt-1*$^{-/-}$ cells. We also observed a ~80% reduction in total B subunit protein in *lcmt-1*$^{-/-}$ whole embryo homogenates (Figure 3C), presumably due to increased B subunit instability due to reduced $PP2A_{BAC}$ heterotrimer formation. Thus, the overall reduction in $PP2A_{BAC}$ heterotrimers in *lcmt-1*$^{-/-}$ embryos is ~92%, comparable to the loss we found upon *PPM1* disruption in yeast (16). Importantly, in addition to the reduction in B subunit, we also observed an ~30% reduction of both the PP2A C and A subunits in *lcmt-1*$^{-/-}$ homogenates (Figure 4D). This reduction in the PP2A subunits that compose the core heterodimer ($PP2A_{AC}$) suggests that the methylation of PP2A by LCMT-1 is not only essential for efficient $PP2A_{BAC}$ heterotrimer formation, but is also important for core heterodimer subunit stability.

The development of the liver, the site of hematopoiesis in midgestation, is impaired in lcmt-1$^{-/-}$ *embryos*

Comparison of mutant embryos revealed that *lcmt-1* knockout results in smaller, anemic livers (Figure 5A). No differences were observed between wild-type and hemizygous littermates. *Lcmt-1* mutant embryo livers appeared more pale than wild-type embryo livers as early as day 11.5 (data not shown), an indication of a defect in erythrocyte production (23). Embryonic day 12.5 *lcmt-1*$^{-/-}$ fetal livers were ~2-fold smaller in weight than wild-type livers, even when normalized to total embryo weight (Figure 5B), indicating that loss of LCMT-1 protein has a more dramatic effect on liver development than the development of whole embryo on average.

To ascertain whether an increase in apoptosis might contribute to the reduced liver size of *lcmt-1*$^{-/-}$ embryos, we performed histological examination of livers from E12.5 wild-type and *lcmt-1*$^{-/-}$ embryos. A number of fragmented pyknotic nuclei,

characteristic of cells undergoing apoptosis, were abundant in *lcmt-1*$^{-/-}$ fetal livers as compared to wild-type and hemizygote littermates (Figure 5C). Moreover, *lcmt-1*$^{-/-}$ fetal livers exhibited an increase in cleaved-caspase 3 (Figure 5D) as well as TUNEL immunoreactivity (Figure 5E), hallmarks of both intrinsic and extrinsic apoptosis pathways. These data support the hypothesis that increased apoptosis contributes to the impaired liver development of *lcmt-1*$^{-/-}$ fetal livers.

To determine if the reduced size of *lcmt-1*$^{-/-}$ fetal livers was due to a decrease in cell proliferation in addition to apoptosis, we examined the level of cellular proliferation within the livers of embryos using the cell proliferation marker Ki67 and the mitotic marker MPM-2. Ki67 immunoreactivity of wild-type livers showed that essentially all cells were proliferating, consistent with the rapid growth of the liver at this stage of development (Figure 6A). Parallel analysis of *lcmt-1*$^{-/-}$ fetal livers revealed that the only cells that were not Ki67-positive and thus not proliferating exhibited fragmented, pyknotic nuclei characterisitic of apoptosis (Figure 6A). No significant difference in the percentage of MPM-2 positive liver cells was found between *lcmt-1*$^{-/-}$ and wild-type embryos (Figure 6B and C), indicating that the mitotic index of liver cells in wild-type and *lcmt-1*$^{-/-}$ embryos was identical. Together, these results indicate that decreased liver size in *lcmt-1*$^{-/-}$ fetal livers is due to an increase in cell death and not an overall decrease in proliferation. Moreover, they indicate that LCMT-1 is essential for proper development of mouse fetal liver.

Lcmt-1 is necessary for hematopoietic progenitor cell proliferation and differentiation

During early mouse embryogenesis, the yolk sac is the initial location of hematopoiesis, termed primitive hematopoiesis (24-27). Around E12, hematopoiesis shifts to the fetal liver, which then begins the process of definitive hematopoiesis (24-27). Since E12.5

lcmt-1$^{-/-}$ fetal livers appear anemic, we wanted to determine if this defect in hematopoiesis might be due to reduced numbers of competent myeloid progenitor cells. Therefore, to assess the importance of LCMT-1 for hematopoietic progenitor cell proliferation and differentiation, we performed *in vitro* colony formation unit (CFU) assays on cells from E12.5 and E14.5 fetal livers. Single cell suspensions of *lcmt-1*$^{-/-}$ and wild-type fetal livers from the two developmental stages were counted and equal numbers of nucleated cells were plated in methylcellulose media supplemented with cytokines and stem cell factors that support the proliferation and differentiation of all the myeloid cell progenitor cells, including erythrocyte precursors (Burst-Forming Unit Erythrocytes; BFU-E), progenitors capable of producing monocytes and/or granulocytes (CFU-GM), and multipotential progenitors that can produce granulocytes, erythrocytes, macrophages, and megakaryocytes (CFU-GEMM). Consistent with the reduced liver size in *lcmt-1*$^{-/-}$ embryos, we observed a 3-fold reduction in the number of nucleated cells in both E12.5 and E14.5 *lcmt-1*$^{-/-}$ fetal livers as compared to wild-type (Figure 7A). The results of the assays showed that there was a 4- and 5-fold decrease, respectively, in the total number of competent progenitor cells at E12.5 and E14.5 (Figure 7B and 7C). All hematopoietic progenitors were strikingly reduced in *lcmt-1*$^{-/-}$ fetal livers as compared to wild-type littermates at both developmental stages (Figure 5B and 5C). Interestingly, the percentage of BFU-E colonies was 4-fold lower at E14.5 than at E12.5, consistent with the fact that embryos appear more anemic later in gestation. Not only was the number of colonies obtained from *lcmt-1*$^{-/-}$ fetal livers affected but the size of the colonies that were formed was smaller on average than colonies from wild-type fetal livers. Therefore, LCMT-1 deficiency significantly decreases the number and proliferation of erythroid, granulocyte/macrophage, as well as multipotential immature progenitors. Together, these data indicate that LCMT-1 is important for definitive hematopoiesis because it is

required for the generation and maintenance of sufficient competent hematopoietic progenitor cells capable of producing all myeloid lineages.

Genechip analysis of lcmt-1 knockout embryos reveals a crucial role for LCMT-1 in liver development and hematopoiesis- To determine the effect of loss of *lcmt-1* of the global expression of genes in the mouse embryo, we subjected E12.5 embryos to gene array analysis. We chose to use the Affymetrix Genechip Mouse Gene 1.0 ST Array due to its ability to accurately cover the entire mouse transcriptome. The Affymetrix Genechip Mouse Gene 1.0 ST Array contains probes to accurately survey the expression of over 28,000 of the most commonly expressed mouse genes. In addition, the Affymetrix Genechip Mouse Gene 1.0 ST Array contains approximately 27 probes that span each individual gene, reducing the possibility of false positives. To reduce the effects of individual embryo variation, three wild-type, *lcmt-1*$^{+/-}$, or *lcmt-1*$^{-/-}$ embryos were pooled. Four replicate arrays were performed for each genotype to increase the confidence of the results. We found that loss of *lcmt-1* results in the downregulation of 134 genes, but the upregulation of only 4 genes. Changes in gene expression in *lcmt-1-/-* embryos were defined as significant if they experienced a greater than 1.3-fold change (10% false-positive rate) when compared to wild-type embryos. The major functional network that was affected in *lcmt-1*$^{-/-}$ embryos consists of genes implicated in hematological diseases, organismal injury and abnormalities, and small molecule biochemistry (Table 1), confirming our previous result that loss of *lcmt-1* reduces the number and proliferation of hematopoietic progenitors. Amongst other genes involved in liver development/homeostasis and hematopoiesis (Table 1), these genes include glycophorin A (1.75-fold reduction), hemogen (1.70-fold reduction), and kruppel-like factor 1 (Klf-1) (1.62-fold reduction). Glycophorin A, a sialoglycoprotein that spans the membrane of erythrocytes, is postulated to aid in the prevention of blood cell

aggregation during circulation (28). Hemogen is a nuclear protein that is the mouse homolog of the human protein, EDAG. Hemogen is found in the blood islands, primitive hematopoietic cells, and fetal liver during mouse embryogenesis (29) and is thought to have a role in the proliferation and differentiation of hematopoietic cells. Moreover, Hemogen is proposed to prevent apoptosis through the activation of nuclear factor-kappa B (NF-kB) (30,31). Klf-1 encodes a hematopoietic cell-specific transcription factor that regulates the expression of several key hematopoietic genes including globin genes and it thought to be instrumental in hemoglobin switching during development (32). Several of the targets of these proteins and transcription factors, including NF-kB and GATA1, a transcription factor that binds Klf-1, are regulated by PP2A (33,34). In many cases, the reduction in messenger RNA for these proteins may reflect a reduction in the number of hematopoietic cells in *lcmt-1*$^{-/-}$ embryos. Identification of the critical events leading to hematopoietic defects observed in this study will be critical to understanding the role of LCMT-1 in this process.

Discussion

Carboxyl methylation of PP2A is the most specific manner of regulating PP2A activity by altering the formation of PP2A heterotrimers, and thus their subcellular localization and substrate specificity. A number of studies have addressed the role of PP2A methylation in yeast and mammalian cells in culture. For example, recently our lab and others have addressed the role of LCMT-1 in the cell cycle, cell proliferation, and several other cell processes in culture (1,10,16-18,35-37). However, the roles of LCMT-1 and PP2A methylation *in vivo* in the development of tissues and organs, and ultimately the development of an organism have not been reported. In this study, we have utilized a gene-trap homozygous knockout of the *lcmt-1* gene to investigate LCMT-1 function in mammalian cells and mouse embryo development. Consistent with our previous finding that LCMT-1 reduction in cultured cancer cells induces a portion of the cells to undergo apoptosis (17), homozygous gene trap knock-out of *lcmt-1* results in embryonic lethality. Examination of developing lcmt-1$^{-/-}$ embryos, revealed certain developmental processes were more dramatically affected than others. Most strikingly, hematopoiesis and development of the fetal liver were greatly impaired, implicating LCMT-1 for the first time in these developmental processes.

The expected ratio of *lcmt-1*$^{+/+}$: *lcmt-1*$^{+/-}$: *lcmt-1*$^{-/-}$ progeny mice from a cross of *lcmt-1*$^{+/-}$ mice is 1:2:1. Previously, we reported a ratio of 1:1.4:0 Mendelian ratio (17) with sufficient numbers of progeny to indicate that homozygous loss of LCMT-1 is lethal during development in the mixed strain that was analyzed. We concluded that this result could not have arisen simply from inviable gametes because both male and female *lcmt-1*$^{+/-}$ mice were fertile when bred to wild-type mice. In the present study, we found that after backcrossing 12 generations to C57BL/6 mice, the Mendelian ratio is 1:2:0, indicating that LCMT-1 loss in this homogenous background also results in embryonic

lethality. However, the difference in the Mendelian ratio relative to the frequency of hemizygous knockout mice raises the possibility that the earlier Mendelian ratio may have underestimated the frequency of *lcmt-1*$^{+/-}$ due to the relatively low numbers of litters analyzed in the mixed background.

Definitive hematopoiesis, which begins around embryonic day 12, is severely impaired as evidenced by the pale liver in the *lcmt-1* homozygous gene-trap knockout mice as early as embryonic day 11.5 (38,39). We have found *lcmt-1* homozygous knockout embryos die primarily after embryonic day 14.5, and do not survive past embryonic day 16.5. We show that a gene-trap homozygous knockout of *lcmt-1* not only results in a substantial reduction in PP2A methylation in whole embryo homogenates but also causes a decrease in B subunit association with C subunit, and also induces a significant amount of apoptosis in the entire embryo as evidence by increased cleaved caspase 3 by Western blot analysis. In addition, embryos that are heterozygous for *lcmt-1* knockout displayed a significant reduction of LCMT-1 protein levels as well, but not any of the biochemical or phenotypic characteristics found in their homozygous gene-trap knockout littermates. This suggests that the amount of LCMT-1 protein produced in the heterozygous embryo does not result in haploinsufficiency. This conclusion is also supported not only by the number of *lcmt-1* heterozygous mice that survive gestation and develop normally through adulthood, but also by the LCMT-1 protein levels and the level of PP2A methylation in organs from these adult mice, including the heart, lungs, brain, and most importantly, the liver (data not shown).

The process of hematopoiesis has been extensively studied in both mice and humans. Recently, the accessibility and increased ease of creating knockout mice as well as the short gestational period of mice has facilitated a greater understanding of the signaling and timeline of hematopoiesis in mice. In mammals, the embryonic yolk sac is the first site of hematopoiesis, termed primitive hematopoiesis (38,40). By embryonic

day 12, hematopoiesis moves to the fetal liver, initiating definitive hematopoiesis (38,40). The liver remains the main site for definitive hematopoiesis until late gestation, when hematopoiesis moves to the bone marrow and remains there throughout the rest of the life of the mouse (38,39,41-43). All hematopoietic cells are derived from a common progenitor, the hematopoietic stem cell (HSC), which will go on to produce all the cells for both the myeloid and lymphoid lineages (40,41,44,45). We found that definitive hematopoiesis was drastically impaired in fetal livers from *lcmt-1* homozygous knockout mice at both embryonic day 12.5 and 14.5 when assayed by clonal cell proliferation assays in methylcellulose supplemented with all the cytokines necessary to produce all the cells of the myeloid lineage (erythrocytes, monocytes, granulocytes, and megakaryocytes) (44,45). Myeloid progenitors producing erythrocytes, granulocytes, monocytes, and megakaryocytes were significantly reduced, resulting in not only an 80% reduction in total colony number, but a noticeable reduction in colony size as well. Future experiments will need to be conducted to determine if LCMT-1 also plays a role in the proliferation and differentiation of cells in the lymphoid lineage as well. Since *lcmt-1* homozygous knockout seems to affect all myeloid lineages, we hypothesize that LCMT-1 may act upstream or on the level of the production of the common HSC, and therefore will affect both the myeloid and lymphoid hematopoietic cell lineages.

We observed a significant level of apoptosis in fetal livers from *lcmt-1* homozygous knockout mice as evidenced by increased cleaved-caspase 3 and TUNEL immunoreactivity by immunohistochemistry, but also by the presence of fragmented, pyknotic nuclei, characteristic of cells undergoing apoptosis. We did not observe any changes in cell proliferation in the fetal liver between *lcmt-1* homozygous knockout embryos and their wild-type littermates using the proliferation marker Ki67 and the mitotic marker MPM-2 as indicators. Therefore, we conclude that the large decrease in liver weight and total cell number we observe in *lcmt-1* homozygous knockout fetal livers

is largely due to an increase in cell death, but a reduction in the rate of cell proliferation and cell division cannot be ruled out as contributors without further experiments. Since differentiation of erythroblasts has been shown to involve activation of caspase 3 and nuclease cleavage of nuclear DNA that can sometimes be detected by the TUNEL assay (46), it is possible that some of the increased cleaved caspase 3 and TUNEL assay signals might be due to LCMT-1 loss inducing increased differentiation of erythoblasts in fetal liver. Since these differentiating cells cease dividing, this might account for loss of Ki67 staining as well. However, during erythroid differentiation, nuclei are not usually fragmented. Our clear observation of fragmented pyknotic nuclei in H&E stained sections as well as in cells staining in the TUNEL and caspase 3 assays suggests that we are indeed observing genuine apoptosis in the liver.

We and others have previously shown that Bα is the PP2A B-type regulatory subunit whose assembly into PP2A heterotrimers is most dependent on C subunit methylation in yeast as well as mammalian cells (10,15-18,47). We and others have previously identified LCMT-1 as the major PP2A methyltransferase in mammalian cells, methylating PP2A C subunit on its C-terminal Leucine residue. Data from this current study has caused the revision of this statement, indicating that LCMT-1 is the sole PP2A methyltransferase as homozygous knockout of the *lcmt-1* gene results in a greater than 95% reduction of PP2A methylation as compared to wild-type. We hypothesize that this result may be an underestimation due to the production of a small percentage of untrapped functional LCMT-1 mRNAs from the *lcmt-1*-pTβgeo gene-trap cassette that produce a small amount (less than 1% as indicated by the reduction of LCMT-1 protein levels in *lcmt-1* knockout embryos) of active LCMT-1 protein that is able to methylate around 5% of the available PP2A C subunits.

Lcmt-1 homozygous knockout in mice reduces total PP2A C, A and B family protein levels. It also results in a dramatic reduction in PP2A C subunit methylation and

the formation of $PP2A_{BAC}$ heterotrimers. These results suggest that both B subunit and the core heterodimer may be destabilized when not complexed. While Bα reduction upon LCMT-1 knockdown in cultured cells had been previously reported (18), reductions in the PP2A A and C subunits had not. This may be in part because the reduction in LCMT-1 achieved in this gene trap knockout mouse model is greater than that achieved in cell culture previously. The fact that an 80% reduction of B family subunits and an ~92% overall loss of $PP2A_{BAC}$ heterotrimers are seen in *lcmt-1*$^{-/-}$ knockout embryos indicates that the requirement for PP2A C subunit methylation for efficient formation of $PP2A_{BAC}$ heterotrimers is present in the vast majority of cells in the embryo. Moreover, the dramatic effects on this family of heterotrimers suggests that down-regulation of these heterotrimers may cause part of the phenotypes seen with *lcmt-1* knockout, including a reduction in embryo weight, increased caspase 3 cleavage, a smaller and paler liver, and hematopoietic defects.

Although the effect of LCMT-1 loss on hematopoiesis and liver development is striking, LCMT-1's effects are not restricted to these processes. *Lcmt-1*$^{-/-}$ embryos also frequently have misdeveloped eyes, almost resembling two eyes fused together. Moreover, although the vast majority of *lcmt-1*$^{-/-}$ embryos die after embryonic day 14.5, analysis of large numbers of embryos at E12.5 indicates that there is a small reduction (~4%) from the expected frequency of *lcmt-1*$^{-/-}$ embryos, suggesting that LCMT-1 loss either causes a small amount of death in early gestation or has an effect on gametes. Further study will likely reveal other important roles for LCMT-1 in development and additional studies into LCMT-1 function during development will be necessary to delineate in more detail the underlying mechanisms. The gene trap knockout mouse model we have generated should be very useful for these investigations.

In summary, we have shown that the absence of LCMT-1 results in embryonic lethality, probably due to at least in part a defect in definitive hematopoiesis. The data

from the clonal proliferation assays suggests that LCMT-1 is necessary for the production, proliferation, and survival of all progenitors from the myeloid lineage but may have an effect on cellular proliferation on all hematopoietic cells. This is the first study that addresses the role of LCMT-1 in the development of a multicellular organism. We hypothesize that although PP2A is the most abundant protein phosphatase in mammalian cells, the dramatic reduction of PP2A methylation may not be solely responsible for the dramatic phenotypes of *lcmt-1* homozygous knockout embryos. Mammalian cells also express two additional phosphatases, Protein Phosphatase 4 (PP4) and Protein Phosphatase 6 (PP6), which exhibit a high degree of sequence homology to PP2A. PP4 is also reversibly methylated on its carboxy terminal leucine residue (48). Although PP6 has the same three carboxy-terminal amino acids as PP2A and PP4, it has not been determined whether PP6 can also be reversibly methylated. Based on the high degree of sequence homology between PP2A, PP4, and PP6, we hypothesize that PP6 is also carboxymethylated on its carboxy-terminal leucine and that both PP4 and PP6 are most likely methylated by LCMT-1. Therefore, the severity of the defects we observe in *lcmt-1* homozygous knockout embryos may be due to the dysregulation of a variety of substrates that may not just be targets of PP2A. Given our results showing a critical role for LCMT-1 in mammalian hematopoiesis, further experiments should be conducted to determine if LCMT-1 activity or PP2A methylation may be affected in hematopoietic disorders such as aplastic anemias, leukemias, lymphomas, and hemoglobinopathies. Moreover, LCMT-1 or the opposing methylesterase, PME-1, may have promise for therapeutic-based targets in these debilitating disorders.

Figure Legends

Figure 1 Gene trap inactivation of the mouse *lcmt-1* gene. **(A)** Shown is a schematic of the gene trap targeting vector, pT1βgeo, inserted into intron 1 of the mouse *lcmt-1* gene. Sequencing indicates that pT1βgeo inserted into the ~22kb intron 1. Splicing of the first *lcmt-1* exon (E1) to the gene trap construct splice acceptor results in a truncated mRNA encoding only a short N-terminal fragment of LCMT-1 because of the absence of a splice donor site in the vector. **(B)** Shown are the approximately 250-bp and 200-bp PCR amplification products obtained from genotyping of wild-type (+/+) and hemizygous (+/-) mice.

Figure 2 Analysis of wild-type, hemizygous, and homozygous knockout *lcmt-1* mouse embryos. **(A)** Genotyping PCR analysis of DNA isolated from yolk sacs of wild-type (+/+), hemizygous knockout (+/-), and homozygous knockout (-/-) E12.5 embryos using the primers described in Methods and Materials. **(B)** The gene trap allele of lcmt-1 effectively inactivates LCMT-1 expression. Wild-type, hemizygous, and homozygous *lcmt-1* knockout E12.5 mouse embryos were dounce homogenized, and homogenates were probed for the steady-state levels of LCMT-1 and Actin (loading control) by immunoblotting. The graph shows the averages and S.D. (error bars) of LCMT-1 expression from three independent experiments. Asterisks indicate significance versus wild-type embryos as assayed by t test (+/-, $p = 3.6 \times 10^{-5}$; -/-, $p = 6.5 \times 10^{-14}$). **(B)** *Lcmt-1*$^{-/-}$ embryos die between E14.5 and E16.5. The number of embryos analyzed is indicated in parenthesis below each gestational stage. Note that less than the expected value of 25% *lcmt-1*$^{-/-}$ embryos were obtained between E11.5-E14.5, suggesting that *lcmt-1* loss may affect gametes or cause a low level of early gestation lethality.

Figure 3 Loss of *lcmt-1* results in impairment of fetal hematopoiesis, smaller embryo size, and caspase 3 cleavage. **(A)** Phenotype of wild-type (+/+), *lcmt-1* hemizygous (+/-), and *lcmt-1* homozygous knockout (-/-) embryos at E12.5. *Lcmt-1*$^{-/-}$ embryos are smaller and have smaller, more pale livers (white arrows) than their normal wild-type and hemizygous littermates. **(B)** E12.5 *lcmt-1*$^{-/-}$ embryos (-/-) weigh significantly less than their wild-type (+/+) and hemizygous (+/-) littermates. The graph shows the averages and S.D. (error bars) of 38 independent E12.5 litters. Asterisks indicate significance versus wild-type embryos as assayed by t test (-/-, $p = 1.1 \times 10^{-8}$). **(C)** E12.5 *lcmt-1*$^{-/-}$ embryos are not significantly shorter on average than their wild-type or hemizygous littermates. The graph shows the averages and S.D. (error bars) of 10 independent E12.5 litters. **(D)** *Lcmt-1*$^{-/-}$ embryos have increased levels of cleaved caspase 3. *W*ild-type (+/+), hemizygous (+/-), and homozygous (-/-) *lcmt-1* knockout E12.5 mouse embryos were dounce homogenized, and homogenates were probed for the steady-state levels of cleaved caspase 3 (Cleaved Casp3) and Actin (loading control) by immunoblotting. The graph shows the averages and S.D. (error bars) of cleaved caspase 3 levels from three independent experiments. Asterisks indicate significance versus wild-type embryos as assayed by t test (-/-, $p = 0.003$).

Figure 4 Gene trap knockout of *lcmt-1* greatly reduces PP2A methylation and $PP2A_{B\alpha AC}$ heterotrimer formation in E12.5 embryos and causes a reduction in the steady-state levels of PP2A subunits. **(A)** PP2A methylation is greatly reduced in *lcmt-1*$^{-/-}$ knockout (-/-) mouse embryos. E12.5 wild-type (+/+), hemizygous (+/-), and homozygous (-/-) *lcmt-1*$^{-/-}$ knockout mouse embryos were dounce homogenized, and the steady-state level of PP2A methylation in each embryo was determined as described in Materials and Methods using our previously published assay (17). Briefly, because base treatment demethylates the PP2A catalytic subunit, one aliquot of lysate from each embryo

genotype was treated for 5 min at 4°C with 0.2 N NaOH to completely demethylate PP2A C subunit and then was neutralized (+ base treatment lanes; 100% demethylated control), whereas another equal aliquot of lysate from each embryo was combined with preneutralized buffer (- base treatment lanes; reflect endogenous methylation level). Then the untreated and base-treated aliquots were analyzed side by side on a 10% SDS-polyacrylamide gel followed by immunoblotting with a monoclonal antibody (4b7) specific for unmethylated C subunit. Whereas PP2A C subunit was highly methylated in wild-type and hemizygous *lcmt-1* knockout mouse embryo homogenates (low 4b7 signal in '-' vs '+' lanes), the homozygous *lcmt-1* knockout mouse embryo homogenate was almost completely demethylated (similar signal in the '-' and '+' base treatment lanes. The graph shows the averages and S.D. (error bars) of methylation levels obtained by quantitating the percent of PP2A catalytic subunit that is methylated in four independent experiments (four different litters). To obtain these values, the percent unmethylated PP2A catalytic subunit in each embryo was first determined by quantitatively comparing the amount of 4b7 signal in the untreated lane for each embryo (reflects level of endogenous unmethylated PP2A C subunit in each line) to that in the matched base-treated samples (100% demethylated controls) using a Bio-Rad Fluor-S Max Chemilumimager. Percent methylation was then calculated by subtracting the percent of unmethylated PP2A from 100. Asterisks indicate significance versus wild-type as assayed by t test (**, p = 0.01). **(B)** Loss of LCMT-1 and PP2A methylation reduces $PP2A_{BAC}$ heterotrimer formation. PP2A B subunit immunoprecipitates of lysates (Co-IP) from wild-type (+/+), hemizygous (+/-), and homozygous (-/-) *lcmt-1*$^{-/-}$ knockout mouse embryos were probed for PP2A B subunit (Bsub) and C subunit (Csub). The image shown is from an immunoblot representative of three independent experiments. Bands were quantitated using a Bio-Rad Fluor-S Max Chemilumimager and the relative amount of C subunit bound to B subunit (Relative C:B Association) was calculated as a measure

of the efficiency of C subunit association with B subunit. The graph shows the average C subunit association with B subunit relative to wild-type ±S.D. in the four independent experiments. The asterisk indicates significance versus wild-type control embryos as assayed by t test (**, p = 0.006). **(C)** Loss of LCMT-1 and PP2A methylation greatly reduces the amount of B subunit in the entire embryo. The relative amount of steady-state B subunit (Bsub) in the different embryos was compared by normalizing B subunit levels in the embryo homogenates to actin levels. The graph shows the averages and S.D. (error bars) of B subunit expression, relative to wild-type embryos from three independent experiments. Asterisks indicate significance versus wild-type embryos as assayed by t test (-/-, $p = 2.4 \times 10^{-5}$). **(D)** Loss of LCMT-1 and PP2A methylation cause significant reductions in the amounts of A and C subunits expressed in embryos. *W*ild-type and hemizygous and homozygous *lcmt-1* knockout E12.5 embryos were dounce homogenized, and homogenates were probed for the steady-state levels of A subunit (Asub), C subunit (Csub), and actin (loading control) by immunoblotting. The graphs show the relative levels of A subunit and C subunit ±S.D. (error bars) from three independent experiments. Asterisks indicate significance versus wild-type control embryos as assayed by t test (Asub *lcmt-1*$^{-/-}$, p = 0.05; Csub *lcmt-1*$^{-/-}$, p = 0.02).

Figure 5 E12.5 *lcmt-1*$^{-/-}$ fetal livers are anemic and show increased apoptosis. **(A)** Dissected fetal livers from wild-type and *lcmt-1*$^{-/-}$ embryos showing reduced size and more pale appearance of the *lcmt-1*$^{-/-}$ liver. **(B)** LCMT-1 knockout embryos (-/-) have significantly smaller livers than wild-type embryos (+/+) at E12.5, even when normalized to total embryo weight. Graph represents an average ±S.D. (error bars) of three independent litters from E12.5 mothers. Liver weights were normalized to the total weight of each embryo. Asterisks indicate significance versus wild-type control livers as assayed by t test (*, p = 0.029). **(C)** Hematoxylin and eosin (H&E) stained sagittal

sections of fetal livers from wild-type and *lcmt-1*$^{-/-}$ embryos. The boxed area in center of *lcmt-1*$^{-/-}$ panel was magnified 50% and then shown as an inset in the top right of the same panel. Note the presence of apoptotic cells and fragmented, pyknotic nuclei in *lcmt-1*$^{-/-}$ embryo livers (arrowheads). **(D)** *Lcmt-1*$^{-/-}$ livers have increased cleaved caspase-3 levels. Note the significant number of cleaved caspase-3 positive cells in the *lcmt-1*$^{-/-}$ embryo livers compared with wild-type livers. The sections shown were counterstained with hematoxylin. **(E)** *Lcmt-1*$^{-/-}$ fetal livers have increased numbers of TUNEL-positive, fragmented, pyknotic nuclei. Note the significant number of TUNEL-positive cells in the *lcmt-1*$^{-/-}$ embryo livers compared with wild-type livers, especially corresponding to cells with fragmented pyknotic nuclei. The sections shown were counterstained with hematoxylin.

Figure 6 E12.5 *lcmt-1*$^{-/-}$ fetal livers exhibit no reduction in proliferation. **(A)** *Lcmt-1* knockout does not reduce the proliferation of the non-apoptotic fetal liver cells. Immunohistochemical analysis of liver proliferation using an anti-Ki67 antibody counterstained with hematoxylin is shown. Nearly all non-apoptotic cells in both *lcmt-1*$^{+/+}$ and *lcmt-1*$^{-/-}$ fetal livers show Ki-67 immunoreactivity. The result shown is representative of three independent comparisons of matched (same litter) wild-type and *lcmt-1*$^{-/-}$ embryos. **(B)** Immunohistochemical analysis of liver proliferation using an anti-MPM2 antibody shows that there is no decrease in the number of cells in mitosis in *lcmt-1*$^{-/-}$ livers compared to wild-type livers. The sections shown were counterstained with hematoxylin. The graph shows results of quantitation of MPM-2 immunoreactivity in the fetal liver. Results show the average ±S.D. (error bars) of MPM-2 immunoreactivity in the fetal livers of three independent matched (same litter) wild-type and *lcmt-1*$^{-/-}$ embryos, where three separate fields per embryo were quantitated. There was no significant difference between wild-type control livers and *lcmt-1*$^{-/-}$ livers as

assayed by t test.

Figure 7 *Lcmt-1* loss greatly reduces the number of competent hematopoietic progenitors in E12.5 and E14.5 fetal livers. **(A)** Livers from *lcmt-1*$^{-/-}$ embryos exhibit reduced number of nucleated cells. Graph represents an average ±S.D. (error bars) of total nucleated cells for three independent wild-type (+/+) and homozygous *lcmt-1*$^{-/-}$ knockout (-/-) livers. **(B and C)** LCMT-1 loss greatly reduces the number of competent hematopoietic progenitors in E12.5 and E14.5 fetal livers. 2 x 10^4 nucleated cells from single cell suspensions of E12.5 and E14.5 wild-type (+/+) and *lcmt-1*$^{-/-}$ (-/-) embryo livers were plated in duplicate cultures of methylcellulose-based medium supplemented with 3 units/mL Epo, 10 ng/mL mouse recombinant IL-3, 10 ng/mL human recombinant IL-6, and 50 ng/mL mouse recombinant stem-cell factor. The number of BFU-E, CFU-GM, and CFU-GEMM colonies were determined after 7 days culture and are shown relative to wild-type. **B)** Results from embryonic day 12.5. **C)** Results from embryonic day 14.5. The number of BFU-E, CFU-GM, and CFU-GEMM colonies were all reduced in *lcmt-1*$^{-/-}$ fetal livers compared with wild-type littermates at both times of gestation. Graphs represent an average ±S.D. (error bars) of results from three independent matched (same litter) wild-type and *lcmt-1*$^{-/-}$ analyses. Asterisks indicate significance versus wild-type control livers as assayed by t test (E12.5: BFU-E, $p = 0.003$; CFU-GM, $p = 0.0002$; CFU-GEMM, $p = 0.005$; total, $p = 0.0004$. E14.5: BFU-E, $p = 0.003$; CFU-GM, $p = 0.00003$; CFU-GEMM, $p = 0.02$; total, $p = 0.00008$.)

Table 1 Functional categorization of significantly downregulated genes in *lcmt-1* knockout embryos. E12.5 wild-type and lcmt-1 embryos were subjected to whole transcriptome gene expression analysis using the Affymetrix Genechip Mouse Gene 1.0 ST Array. Downregulated genes are divided into 6 functional networks as listed.

Changes in gene expression in *lcmt-1*$^{-/-}$ embryos were defined as significant if they experienced a greater than 1.3-fold change (10% false-positive rate) when compared to wild-type embryos.

Figure 1

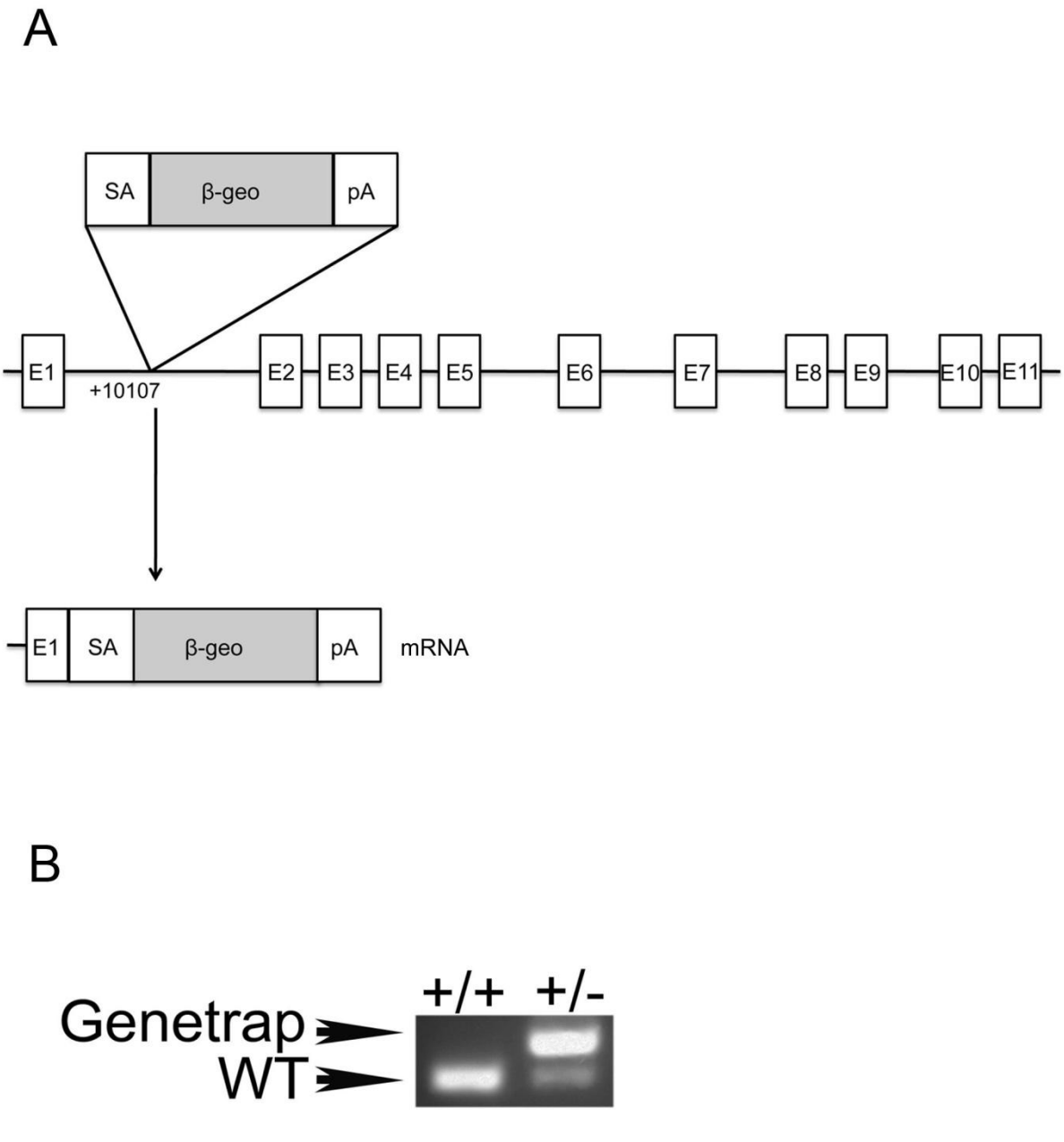
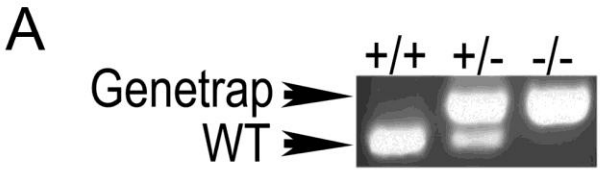
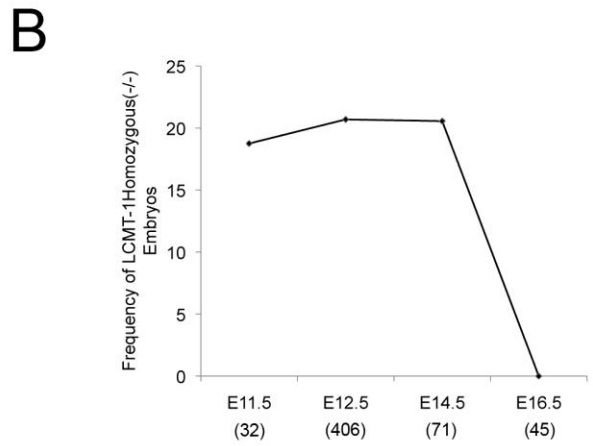
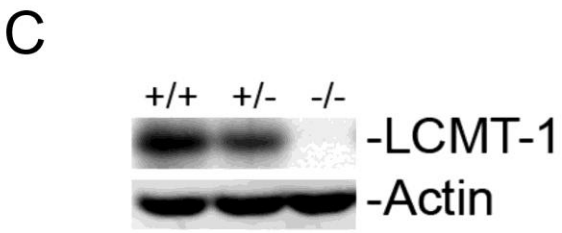

A
SA
β-geo
pA
E1
+10107
E2
E3
E4
E5
E6
E7
E8
E9
E10
E11
E1
SA
β-geo
pA
mRNA
B
+/+
+/-
Genetrap
WT

Figure 2


A
+/+ +/- -/-
Genetrap
WT


B
Frequency of LCMT-1Homozygous(-/-) Embryos
25
20
15
10
5
0
E11.5 (32)
E12.5 (406)
E14.5 (71)
E16.5 (45)


C
+/+ +/- -/-
-LCMT-1
-Actin

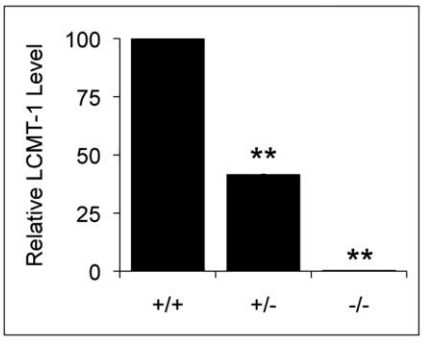

Relative LCMT-1 Level
100
75
50
25
0
**
**
+/+
+/-
-/-

Figure 3

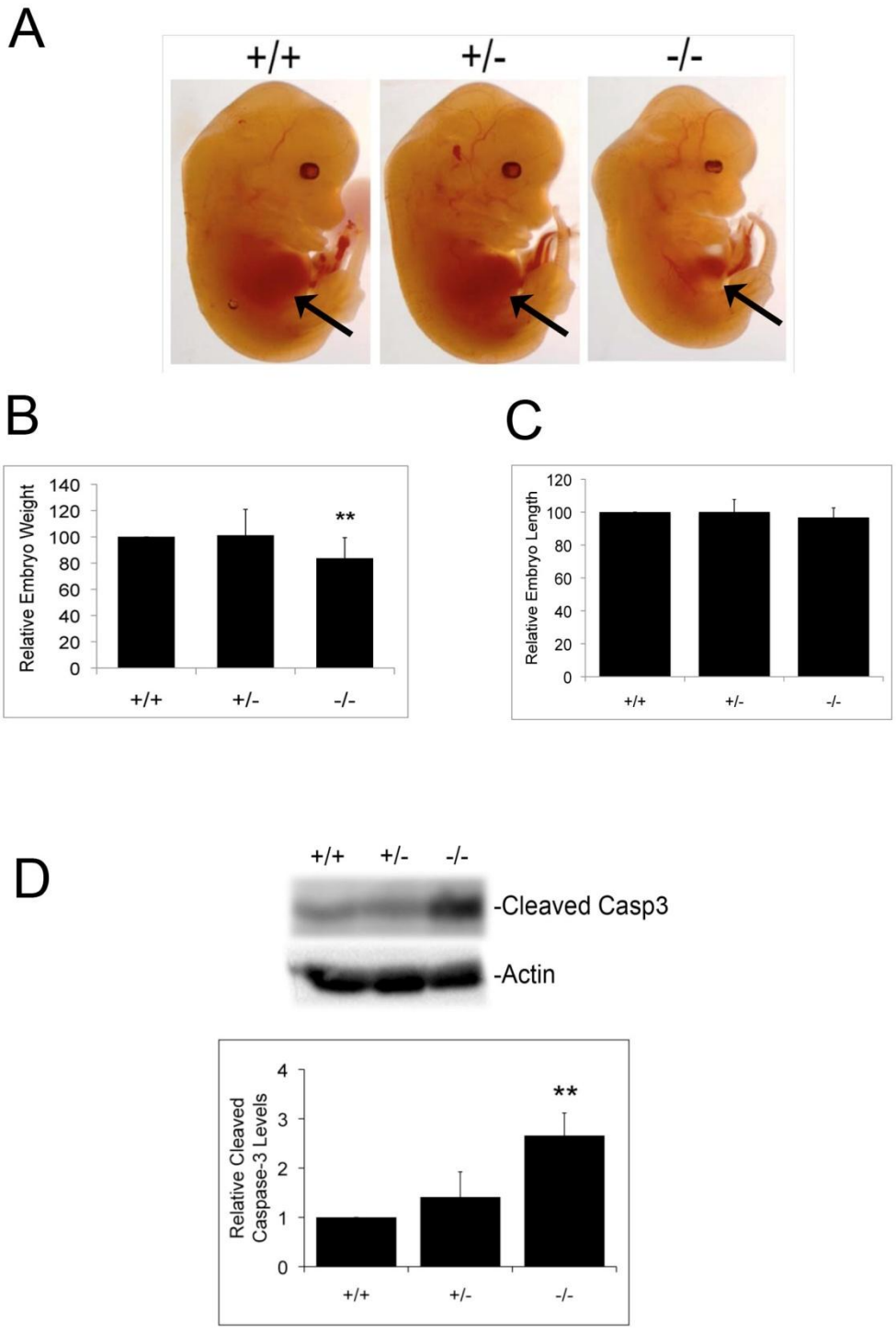

A
+/+
+/-
-/-
B
Relative Embryo Weight
140
120
100
80
60
40
20
0
**
+/+
+/-
-/-
C
Relative Embryo Length
120
100
80
60
40
20
0
+/+
+/-
-/-
D
+/+
+/-
-/-
-Cleaved Casp3
-Actin
Relative Cleaved Caspase-3 Levels
4
3
2
1
0
**
+/+
+/-
-/-

Figure 4

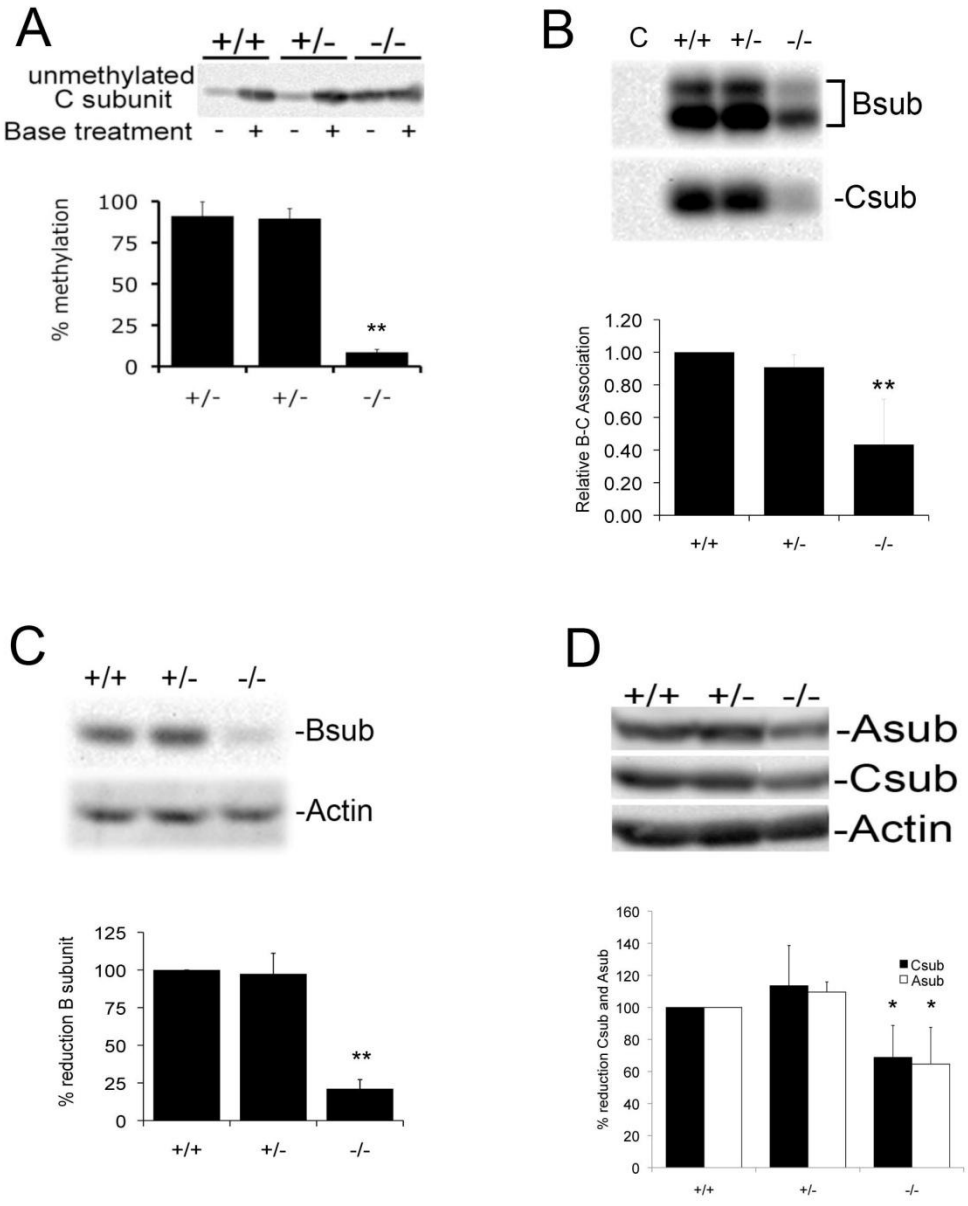

A
+/+ +/- -/-
unmethylated
C subunit
Base treatment - + - + - +
% methylation
100
75
50
25
0
**
+/- +/- -/-
B
C +/+ +/- -/-
Bsub
-Csub
Relative B-C Association
1.20
1.00
0.80
0.60
0.40
0.20
0.00
**
+/+ +/- -/-
C
+/+ +/- -/-
-Bsub
-Actin
% reduction B subunit
125
100
75
50
25
0
**
+/+ +/- -/-
D
+/+ +/- -/-
-Asub
-Csub
-Actin
% reduction Csub and Asub
160
140
120
100
80
60
40
20
0
Csub
Asub
* *
+/+ +/- -/-

Figure 5

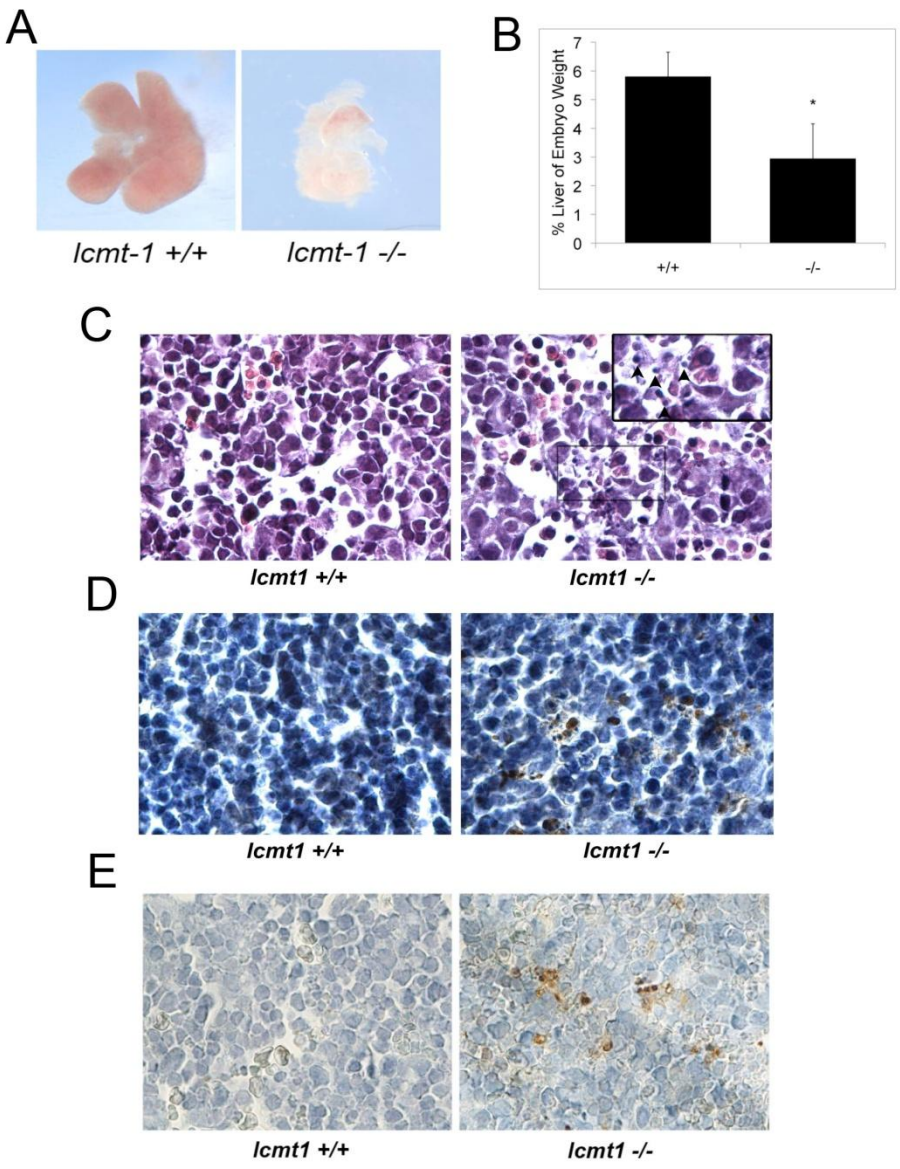

A
lcmt-1 +/+
lcmt-1 -/-
B
% Liver of Embryo Weight
0
1
2
3
4
5
6
7
*
+/+
-/-
C
lcmt1 +/+
lcmt1 -/-
D
lcmt1 +/+
lcmt1 -/-
E
lcmt1 +/+
lcmt1 -/-

Figure 6

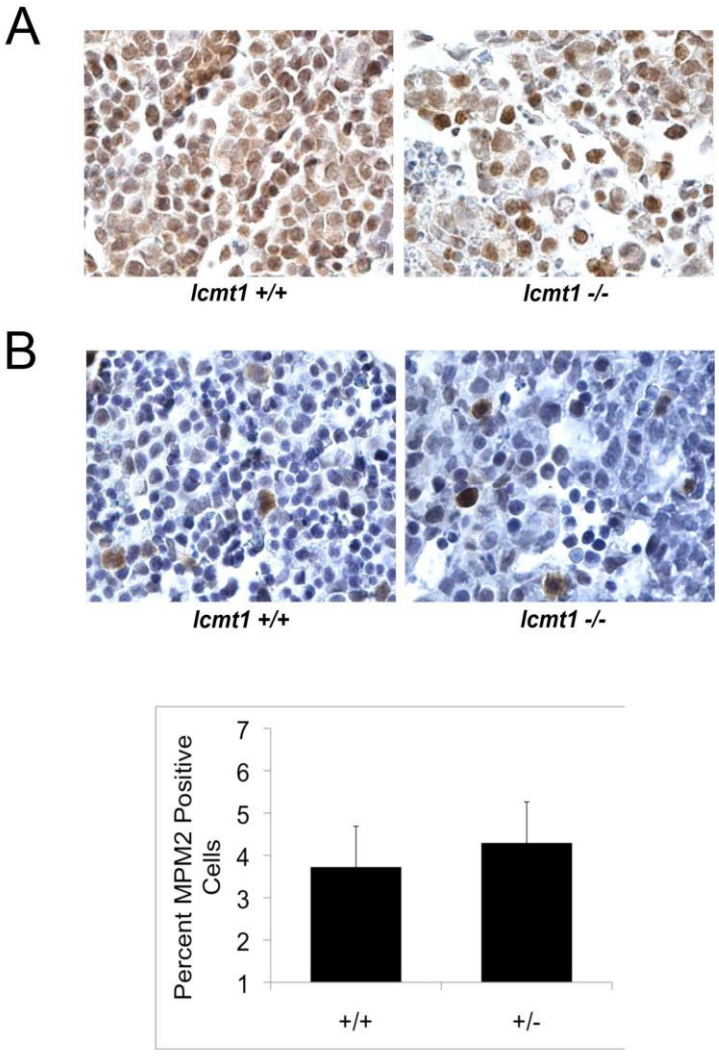

A
lcmt1 +/+
lcmt1 -/-
B
lcmt1 +/+
lcmt1 -/-
Percent MPM2 Positive Cells
7
6
5
4
3
2
1
+/+
+/-

Figure 7

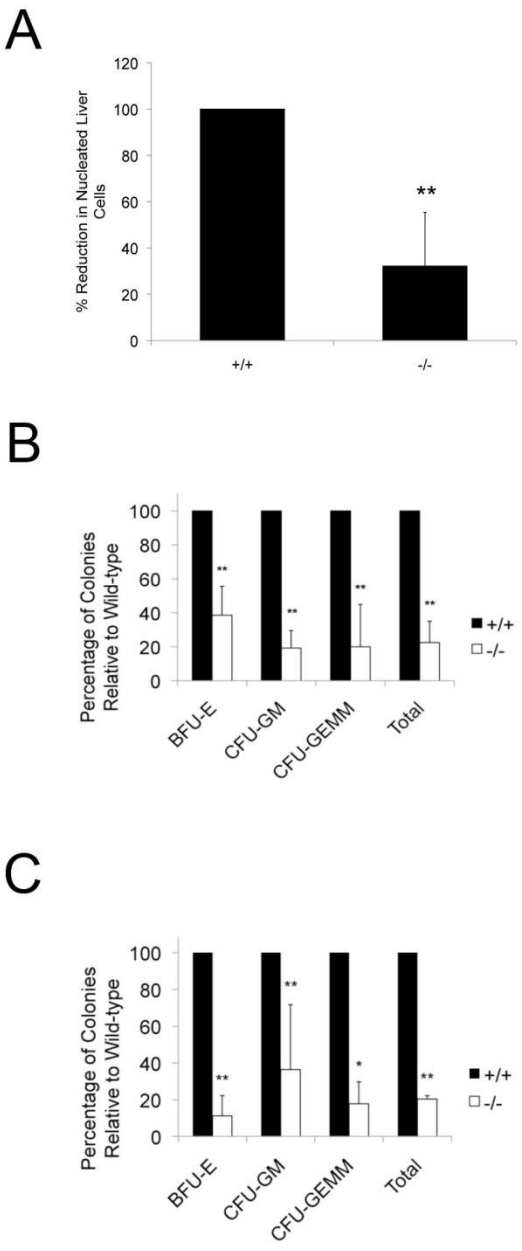

A
% Reduction in Nucleated Liver Cells
120
100
80
60
40
20
0
**
+/+
-/-
B
Percentage of Colonies Relative to Wild-type
100
80
60
40
20
0
**
**
**
**
+/+
-/-
BFU-E
CFU-GM
CFU-GEMM
Total
C
Percentage of Colonies Relative to Wild-type
100
80
60
40
20
0
**
**
*
**
+/+
-/-
BFU-E
CFU-GM
CFU-GEMM
Total

Table 1 - Functional categorization of significantly downregulated genes in *lcmt-1* knockout embryos

Functional Network	***Genes***
ematological Disease, Organismal Injury and Abnormalities, Small Molecule Biochemistry	ALAS2, ANK1, APOA1, APOB, C3, C5, CA2, CALB1, CFI, CPB2, Elastase, EPB42, FGB, FGG, Fibrin, Fibrinogen, GATA1, GYPA, Igm, IL1, KLF1, LDL, Mediator, NFkB (complex), PLG, RHAG, RHCE, SAA4, SERPINB10, SERPINF2, SLC4A1, SPTA1, SPTB, TNFAIP2, Trypsin
Cell Morphology, Lipid Metabolism, Molecular Transport	AMBP, Ap1, APOM, BLVRB, BTK, Ck2, ERK1/2, F Actin, FABP1, FGF13, GYS2, HMBS, HNF4α dimer, HPX, Hsp70, Immunoglobulin, Insulin, ITIH2, Jnk, KCNN4, KEL, KLB, P38 MAPK, Pkc(s), PKLR, Pld, Ras homolog, RBP4, RIT2, SLC25A37, SNCA, SNCG, TMEFF2, TNFRSF14, XK
Cardiovascular System Development and Function, Cell Signaling, Vitamin and Mineral Metabolism	AFP, Akt, APOM, BMP2K, C20ORF12, CDCA5, CDK12, CLDND1, Collagen type I, DENND4A, ELP3, ERK, ERMAP, FAIM2, GFI1B, HIRIP3, Histone h3, HNF4A, HSD17B2, IKZF1, KDM5C, PI3K, PLC, PLN, PNPO, PZP, RNA polymerase II, SEC31A, SLC16A6, SLC27A2, SPATS2L, SPTA1, STAT5a/b, Vegf, ZNRD1
Genetic Disorder, Cancer, Neurological Disease	ACAA2, ALDOC, ATP6V0D1, beta-estradiol, CA2, CDC123, CDH12, CRYAA, CRYBA1, DRD5, ELL2, EPO, FECH, GPR64, GRIN2B, GYPA, IFI30, IGHG1, IL5, KRT16, LGI1, LRP1, MAPK3, NEFM, PDCD2, PRPH, PTPN5, RNASE3, SCN9A, SERPIND1, SLC25A12, SNCG, SPCS2, TMF1, TPI1
Cell Cycle, Lipid Metabolism, Molecular Transport	AADAC, ADH6, ADH1B, AFP, APOM, BLZF1, CENPO, CPOX, E2F4, ENDOD1, ethanol, FXR2, GABRG2, GCKR, GPX2, HNF1A, KRT20, LCMT1, MGC12538, MGST3, MIR124-1 (includes EG:406907), NEUROD6, PDIA2, PECI, retinoic acid, RIG1, RNASE4, RNF144A, RNF144B, SSX2IP, TNFAIP2, TP53, TRAF2, UBE2L6, ZBED3
Cell Cycle, Cancer, Genetic Disorder	AFP, ASF1B, BCKDHA, CCNG1, CCNG2, CDK5RAP3 (includes EG:80279), CDKN2A, CDKN2D, CSMD3 (includes EG:114788), CTNNB1, FEN1, GABARAP, GNL3, HEMGN, ING4, KIF23, KLK2, MIR301A (includes EG:407027), NCAM2, NUAK1, PDE4B, PHC2, PODXL, PTP4A1, RAD17, RBBP5, REM2, RFC3, RRM1 (includes EG:6240), SIAH1, STK11, TBR1, TP53, TUBA4A, UCHL1

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Chapter 3

Leucine Carboxyl Methyltransferase 1 Regulates Tau Hyperphosphorylation in the Developing Mouse Embryo

Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by loss of memory and reduction in intellectual ability. AD currently accounts for 60-80% of all dementia cases and is often diagnosed in individuals over the age of 65 (Alzheimer's Association website). AD is a progressive disease in which the symptoms drastically worsen over time and is currently the sixth leading cause of death in the United States (Alzheimer's Association website). AD is predicted to affect 1 in 85 worldwide by 2050 (1). AD patients often first experience mild memory loss that eventually progresses to the inability to carry on conversations, loss of independence, and a reduced quality of life for both patients and caregivers.

AD neuoropathology is characterized by two major disease phenotypes: beta amyloid senile plaques derived from the amyloid precursor protein (APP) and neurofibrillary tangles (NFT). Beta amyloid senile plaques are composed of extracellular deposits of the APP in microglia and astocytes. NFTs are aggregates of paired-helical filaments formed from hyperphosphorylated forms of the microtubule-associated protein tau (2). When tau becomes hyperphosphorylated, it can no longer bind microtubules and causes their instability (3). The complete mechanism underlying of formation of these NFTs in not completely understood as well as whether tau hyperphosphorylation is the cause or effect of AD progression.

The regulation of tau phosphorylation is a synergistic balance between the activity of tau kinases and phosphatases (4,5). When this delicate balance of activity becomes disrupted through either the changes in expression of these protein kinases

and/or phosphatases, their dysregulation, or changes in the level or expression of accessory proteins (6), tau hyperphosphorylation is the result (7). Moreover, tau can itself become mutated, which also leads to the formation of NFTs (8). The most common of these tau mutations, P301L (Proline to Leucine mutation) is associated with frontotemporal dementia in humans and leads to the formation of NFTs and pretangle formations in mice (8).

Through the work of several labs, PP2A has been identified as the major tau phosphatase (9-14). The PP2A subunits C subunit, B/PR55, and B'/PR61 are highly expressed in the brain and are predicted to have an important neuronal function. The brains from AD patients exhibit reduced amount of tau phosphatase activity (15), suggesting that reduced PP2A tau phosphatase activity may contribute to tau hyperphosphorylation and AD progression. Transgenic mice expressing the dominant-negative PP2A C subunit mutant, L199P, exhibited hyperphosphorylated tau in neurons, which formed tau aggregates (16). In addition, the PP2A heterotrimers containing both the Bα and Bβ B family B-type subunits, which are significantly affected by PP2A methylation, associate with neuronal microtubules (17). Moreover, not only have $PP2A_{B\alpha AC}$ heterotrimers been shown to be the major form of PP2A that regulates tau phosphorylation, they are also reduced in neurons that display AD-like characteristics (18).

Recently, it was shown that elevated levels of homocysteine in the blood, common for many age-related disorders, was also found in AD patients (19,20). Homocysteine, a key metabolite intermediate in S-adenosylmethionine and S-adenoslhomocyseine metabolism, also can modulate methyltransferase activity by acting through feedback inhibitory mechanism. The Sontag lab made the interesting discovery that elevated levels of blood homocysteine result in not only decreased PP2A

methylation, but downregulation of the B family B-type subunit Bα and accumulation of the AD hallmarks hyperphosphorylated tau and APP plaques, establishing a direct connection between LCMT-1, PP2A methylation, and AD (19). Most importantly, the Sontag lab made the interesting finding that LCMT-1 protein levels are significantly reduced in AD-affected brains (21).

As stated previously, LCMT-1 is responsible for methylating PP2A C subunit (22). Thus, loss of LCMT-1 or defects in its activity might reduce PP2A methylation and the amount of functional $PP2A_{B_{\alpha}AC}$ enzyme, contributing to the pathophysiology of AD. Based on these findings and the role of $PP2A_{BAC}$ heterotrimers in regulating tau phosphorylation, we have proposed that defects in PP2A methylation may contribute to major brain defects in the developing mouse embryo.

To gain understanding of the role of LCMT-1 in brain development in mice, we disrupted the murine *lcmt-1* gene, which results in a recessive lethal phenotype. *Lcmt-1* knockout embryos die during midgestation (E11.5-E14.5) with severe defects in head formation. The heads of *lcmt-1* knockout embryos display reduced PP2A methylation and alterations of tau phosphorylation at known PP2A-regulated residues, Serine 262 and Serine 356. Our report is the first to describe a direct role for LCMT-1 in the regulation of tau phosphorylation *in vivo*. In addition, we are the first to describe a role for LCMT-1 in brain development during embryogenesis. Therefore, LCMT-1 and PP2A methylation are key regulators of brain development and tau phosphorylation.

Methods and Materials

Biochemical Analysis of lcmt-1 Embryos

E12.5 embryos were dissected free from the uterus and extraembryonic membranes, flash frozen in liquid nitrogen, and then stored at -80°C until use. Yolk sacs were saved for genotyping. Embryo heads were dounce homogenized in a Nonidet P-40-containing lysis buffer (10% glycerol, 20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P-40) containing 1mM phenylmethylsufonyl fluoride and 0.04 trypsin inhibitor units/ml aprotinin. Homogenates were cleared by centrifugation at 13,000 x g and were then analyzed by SDS-PAGE. Relative proteins levels were determined by quantitation using a Fluor S-Max Chemilumimager and Quantity One Software (Bio-Rad) and lysate protein levels were normalization to actin. Antibodies used for western blotting include mouse monoclonal antibodies against PP2A B subunit (2G9; Millipore), a goat polyclonal antibody against actin (Santa Cruz Biotechnologies) and an affinity-purified rabbit anti-LCMT-1 polyclonal antibody, RK3110, generated against a 17-amino acid peptide corresponding to residues 173-189 of human LCMT-1 (23).

Determination of Tau phosphorylation

E12.5 embryo head homogenates were prepared as stated above. Homogenates were cleared by centrifugation at 13,000 x g, and then were boiled and centrifuged to enrich for tau (24). Equivalent amounts of head homogenates determined by mass of embryos heads were analyzed by SDS-PAGE. Relative proteins levels were determined by quantitation using a Fluor S-Max Chemilumimager and Quantity One Software (Bio-Rad) and probed with the phospho-tau antibodies 12E8 (anti-phosphorylated Serine-262, Elan Pharmaceutical) and Tau1 (reacts to epitope at or near a phosphorylated site

on tau, Millipore). Phosphorylated tau levels were normalized to total 3R tau (Storm rabbit polyclonal antibody, Davies lab).

Determination of the steady-state level of PP2A$_c$ methylation

The steady state level of PP2A catalytic subunit methylation was measured in homogenates from embryo heads with a monoclonal antibody specific for unmethylated PP2A C subunit (4b7) using our published method (23). Quantitation of the 4b7 signal was performed with a BioRad Fluor S-Max Chemilumimager and BioRad Quantity One Software. Homogenates were also probed with Actin as a loading control.

Microarray analysis of lcmt-1 knockout embryos – Total RNA was extracted from E12.5 wild-type, hemizygous, and *lcmt-1* knockout embryos using the Absolutely RNA Miniprep Kit (Strategene). For each sample replicate, 100 ng of total RNA were amplified using the Ambion WT Expression Kit (Applied Biosystems) and labeled using the Affymetrix GeneChip Terminal Labeling Kit. Affymetrix GeneChip Mouse Gene 1.0 ST arrays were hybridized with labeled sense DNA, washed, stained, and scanned using the Affymetrix GeneChip Expression Wash, Stain and Scan kit. Data was extracted, normalized, and summarized with the robust multi-average array (RMA) method (25,26). This work was supported in part by the Emory Winship Cancer Genomics Shared Resource.

Results

LCMT-1 Knockout Results in Reduced PP2A C Subunit Methylation and Enhanced Phosphorylation of Tau in the Embryonic Brain— PP2A heterotrimers containing the B family B-type subunit Bα is a major brain tau phosphatase in adult mice (18). In addition, both LCMT-1 and Bα are reduced in human Alzheimer's disease brains (18) and in hyperhomocysteinemic mice (27) and correlates with increased tau phosphorylation. However, whether loss of *lcmt-1* in an organism causes in tau phosphorylation is not know. Gross examination of homozygous *lcmt-1*$^{-/-}$ mutant embryos revealed one severe consequence of homozygous knockout of the *lcmt-1* gene was an impairment of both midbrain and hindbrain formation in *lcmt-1*$^{-/-}$ embryos as compared to wild-type littermates (Figure 1A; black arrowheads). This defect was also seen as early as embryonic day 11.5 (Supplemental Figure S2). No defects were found in hemizygous littermates (Figure 1A). To directly determine the effect of *lcmt-1* knockout on tau phosphorylation so early in development, we analyzed the level tau phosphorylation in head/brain homogenates of E12.5 embryos (Figure 1B). We found that *lcmt-1*$^{-/-}$ embryo heads/brains exhibit a significant and substantial increase in phosphorylated tau at both the 12E8 and Tau-1 epitopes (Figure 1B and C). 12E8 recognizes tau that is phosphorylated on Serine 262 and Serine 356, known PP2A-regulated resides (28,29). Tau-1 reacts to epitope at or near an unknown phosphorylated residue on tau (30). This increase in tau phosphorylation correlates with a dramatic reduction in total LCMT-1 protein and PP2Ac methylation in the same brain/head homogenates (Fig. 1D and E). Therefore, not only does LCMT-1 affect tau phosphorylation in the adult brain, but results in a significant increase in tau phosphorylation as early as mouse embryonic day 12.5.

Genechip analysis of lcmt-1 knockout embryos reveal a crucial role for LCMT-1 in brain development- To determine the effect of loss of *lcmt-1* on the expression of genes involved in neuronal and brain development, we subjected E12.5 embryos to gene array analysis. We found that loss of *lcmt-1* results in the significant downregulation several brain-specific genes, including NeuroD6 (1.72-fold change), alpha synuclein (1.63-fold change) and gamma synuclein (1.50-fold change). Changes in gene expression in *lcmt-1*$^{-/-}$ embryos were defined as significant if they experienced a greater than 1.3-fold change when compared to wild-type embryos because of the high quality of the results. At this range, there is only a 0.1% false-positive rate. NeuroD6 is a member of the basic helix-loop-helix family of transcription factors and is implicated in neuronal differentiation and maturation (31). Although the function of alpha synuclein is currently not known, it localizes to the presynaptic terminals of the brain during a synaptic rearrangement (32) and can aggregate to form Lewy bodies in synucleinopathies such as Parkinson's disease (33). In addition, alpha-synuclein significantly interacts with tubulin (34), and therefore may act on microtubule-associated protein like tau (35). Gamma synuclein is also thought to be involved in the progression of neuorodegenerative diseases. Gamma synuclein is highly expressed in the peripherial nervous system and in downregulated in the retinas of AD patients (35-36). What influence LCMT-1 activity is having on these proteins, and thus brain development and homeostasis, has not been elucidated. We hope future experiments will determine the role of LCMT-1 on these brain-specific proteins.

Discussion

Neuropathological conditions associated with the dysregulation of tau are the cause of many forms of dementia. These disorders, or tauopathies, often result in the inability of tau to properly associate with microtubules, and eventually leads to the formation of paired neurofibrillary tangles comprised of hyperphosphorylated tau (37). The most prevalent of these tauopathies is AD. It was previously found that BαAC heterotrimeric form of PP2A is the major tau phosphatase and is significantly reduced in AD brains (18). Moreover, LCMT-1, which affects the formation of $PP2A_{B\alpha AC}$ heterotrimers more than any other form of PP2A, is also dramatically reduced in AD brains (21). Because methylation of PP2A is key for $PP2A_{B\alpha AC}$ heterotrimer formation (23,38,39), we hypothesized that loss of LCMT-1 would contribute to the formation of an AD-like pathology in the developing mouse embryo. We found that *lcmt-1*$^{-/-}$ embryos exhibited improper head formation, visible at embryonic day 12.5. This defect in head formation correlated not only with a significant decrease in PP2A methylation, but an increase of tau phosphorylation at two known PP2A-regulated residues. This data is the first evidence for a direct role for LCMT-1 and PP2A methylation in regulating tau phosphorylation *in vivo*. In addition, we found that loss of *lcmt-1* results in the downregulation of several brain-specific genes which may provide some insight in the overall function of LCMT-1 in neuronal development.

Future experiments should focus on further analyzing the neuropathology of *lcmt-1*$^{-/-}$ embryos. Preliminary immunohistochemical analysis of saggital head sections from paraffin-embedded embryos does not suggests there are any differences in the number of differentiating neurons (Tuj1 immunoreactivity) or proliferating cells (Ki67 immunoreactivity) between *lcmt-1* knockout embryos and their wild-type littermates (data not shown). Therefore, LCMT-1 may regulate unknown proteins that may not affect these

pathways directly. In addition, it is not known if loss of *lcmt-1* also has the ability to form NFTs at this early of a stage in development, and thus also should be explored.

Although the current data in this study is not extensive, it provides the first direct proof that loss of LCMT-1 in an animal model causes tau hyperphosphorylation. We believe the development and implementation of drugs that modulate PP2A methylation will be an important new approach to preventing or treating AD. Recently, a potent and specific PME-1 inhibitor, ABL127, was discovered as part of the National Institutes of Health (NIH) Common Fund Molecular Libraries Program (40). This screening program currently funds medicinal chemistry-related centers at academic institutions around the United States to enable scientists to find biologically-relevant molecules (NIH website). ABL127 was found to completely block PME-1 activity in both cultured cells and in mice (40). We hypothesize that pharmacological inhibition of PME-1 may be the best route to help balance a reduction in LCMT-1 found in AD patients and be very useful for preventing hyperphosphorylation of tau and AD progression.

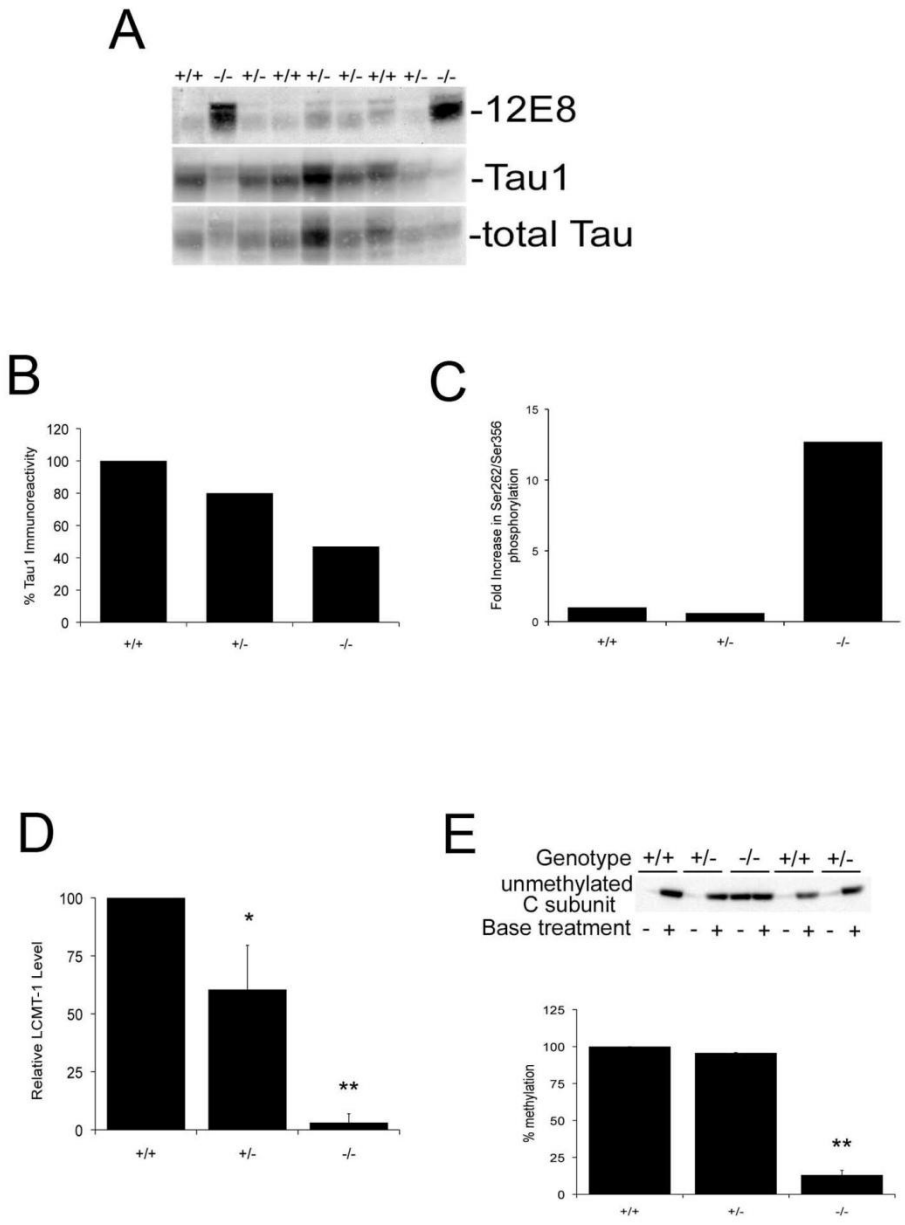
In conclusion, our studies suggest that LCMT-1 is a critical player in tau hyperphosphorylation and development of AD. Thus, counterbalancing the loss of LCMT-1 or PP2A Bα could be a valuable therapeutic approach for the treatment of AD and other tauopathies.

Figure Legends

Figure 1 Loss of *lcmt-1* in embryo heads results in tau hyperphosphorylation. **A)** *Lcmt-1*$^{-/-}$ embryos heads exhibit tau hyperphosphorylation. *W*ild-type (+/+), hemizygous (+/-), and homozygous (-/-) *lcmt-1* knockout E12.5 mouse heads embryos were dounce homogenized, cleared, and boiled to enrich for tau. Enriched homogenates were probed for the steady-state levels of phospho-Serine262/Serine356 tau (12E8), phospho-tau (Tau1), and Total Tau (loading control) by immunoblotting. **B)** The graph shows a representative experiment for Tau1 immunoreactivity. Tau1 was assayed in three separate litters, where the percent reduction in Tau1 immunoreactivity in lcmt-1 knockout embryos heads ranged between 32-60% decrease compared wild-type littermates. **C)** The graph shows a representative experiment for 12E8 immunoreactivity. 12E8 was assayed in three separate litters, where the fold-change in Serine262/Serine365 phosporylation in lcmt-1 knockout embryos heads ranged between 3 and 30 fold-increase over wild-type littermates. **D)** Wild-type, hemizygous, and homozygous *lcmt-1* knockout E12.5 mouse embryo heads were dounce homogenized, and homogenates were probed for the steady-state levels of LCMT-1 and Actin (loading control) by immunoblotting (data not shown). The graph shows the averages and S.D. (error bars) of LCMT-1 expression from three independent experiments. Asterisks indicate significance versus wild-type embryos as assayed by t test (+/-, $p = 3.6 \times 10^{-5}$; -/-, $p = 6.5 \times 10^{-14}$). **E)** PP2A methylation is greatly reduced in *lcmt-1*$^{-/-}$ knockout (-/-) mouse embryo heads. E12.5 wild-type (+/+) ,hemizygous (+/-), and homozygous (-/-) *lcmt-1*$^{-/-}$ knockout mouse embryo heads were dounce homogenized, and the steady-state level of PP2A methylation in each embryo was determined as described in Materials and Methods using our previously published assay (23). Untreated (-) and base-treated (+) aliquots were analyzed side by side on a 10% SDS-polyacrylamide gel followed by

immunoblotting with a monoclonal antibody (4b7) specific for unmethylated C subunit. Whereas PP2A C subunit was highly methylated in wild-type and hemizygous *lcmt-1* knockout mouse embryo head homogenates (low 4b7 signal in '-' vs '+' lanes), the homozygous *lcmt-1* knockout mouse embryo head homogenate was almost completely demethylated (similar signal in the '-' and '+' base treatment lanes. The graph shows the averages and S.D. (error bars) of methylation levels obtained by quantitating the percent of PP2A catalytic subunit that is methylated in three independent experiments (three different litters). To obtain these values, the percent unmethylated PP2A catalytic subunit in each embryo was first determined by quantitatively comparing the amount of 4b7 signal in the untreated lane for each embryo head (reflects level of endogenous unmethylated PP2A C subunit in each line) to that in the matched base-treated samples (100% demethylated controls) using a Bio-Rad Fluor-S Max Chemilumimager. Percent methylation was then calculated by subtracting the percent of unmethylated PP2A from 100. Asterisks indicate significance versus wild-type as assayed by t test (**, $p = 0.01$).

Figure 1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125
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Discussion of Study

The multifunctional enzyme PP2A has been previously shown to be involved in a diversity of cellular processes including metabolism, cellular transformation, cell growth, DNA repair, and gene transcription and translation (1). PP2A modulates the activity of several protein kinases including but not limited to the members of the mitogen-activated protein kinase cascade (MAPK/ERK), Protein Kinase A, Protein Kinase B, Protein Kinase C, and Cyclin-dependent kinases (1). The ability of PP2A to form different heterotrimers and alter its substrate specificity and subcellular localization provides PP2A with the ability to influence many essential cellular pathways and processes. In this study, we have used cultured cells and the developing mouse embryo to access the role of LCMT-1 and PP2A methylation growth and development. Through our current studies, we have found exciting new roles for LCMT-1 and PP2A methylation in mitotic progression, apoptosis, liver development, hematopoiesis, and embryogenesis.

Our studies of LCMT-1 knockdown by lentiviral infection of small hairpin RNAs (shRNA) in HeLa cancer cells have proven that LCMT-1, PP2A methylation, and the B family B-type subunit Bα are important for proper progression through mitosis. In addition, we found that the inability of cells in which these proteins are reduced to properly progress through mitosis results in the activation of apoptotic pathways through a mechanism that is not completely understood. We have found a similar role for LCMT-1 and PP2A methylation in growth and apoptosis in mouse development but on a much larger scale. Homozygous knockout of the *lcmt-1* gene in mice results in mid-gestation embryonic lethality, due in part to improper liver development and hematopoiesis, but also results in a smaller embryo, defects in midbrain and hinbrain formation, hyperphosphorylation of the microtubule associated protein tau, and a significant increase apoptotic markers including cleaved caspase-3 and DNA

fragmentation. We believe that reduction or loss of LCMT-1 is in part causing defects in these key signaling cascades through its regulation of $PP2A_{BAC}$ heterotrimer formation.

In both cultured cells and embryo homogenates, we observed a significant reduction in $PP2A_{BAC}$ heterotrimer formation. Our studies of exogenously expressed HA-tagged Bα in HeLa cancer cells revealed a greater than 50% reduction of $PP2A_{B_{\alpha}AC}$ heterotrimers when LCMT-1 protein was reduced. We advanced our understanding of the influence of LCMT-1 and PP2A methylation on $PP2A_{BAC}$ heterotrimer formation by assaying the ability of the endogenous B-family B subunits to be properly incorporated into PP2A heterotrimers in the developing mouse emrbyo. The B-family antibody, clone 2G9, we used to assess the efficiency of BAC heterotrimers has the ability to recognize all four members of the B-family of B-type regulatory subunits, although the antibody preferentially recognizes the Bα and Bδ isoforms (in order of increasing immunoreactivity: Bα>Bδ>Bβ>Bγ). Therefore, the greater than 92% reduction in $PP2A_{BAC}$ heterotrimers when *lcmt-1* is lost (calculation of the 80% reduction in PP2A B subunit protein levels and the 60% reduction of $PP2A_{BAC}$ heterotrimer formation for the remaining B subunit) may be the first reflection of the effect of methylation on the entire B-family, and may explain the severity of defects in *lcmt-1* knockout mice.

As stated previously, Bα is involved in G2/M transition, mitotic progression, mitotic exit, apoptosis, and the Wnt, Akt, and TGFβ signaling pathways amongst others (1-4). Bα, Bβ, and Bγ have all been shown to regulate the bone morphogenic protein (BMP) pathway signaling, which regulates dorsoventral patterning, cardiac development, ectopic cartilage and bone formation and are important for adult and embryonic stem cell fate and proliferation, by interacting with BMP receptor complexes and dephosphorylating both the C-terminus and the linker region of BMP signal transducer Smad1 (5,6). B55δ has been shown to be a negative regulator of the cyclin-dependent

kinase Cdk1 as well as to stabilize the metaphase-anaphase protein securin and therefore may have a role as a negative regulator of mitotic progression (7,8). We believe that loss of LCMT-1 is directly influencing the targets of several B-type subunits that are affected by PP2A methylation, including B55α and B55δ. As stated previously, The PP2A regulatory subunit B55δ protects cohesin from degradation by stabilizing securin by keeping securin hypophosphorylated. If securin is hyperphosphorylated due to loss of the formation of PP2A heterotrimers containing B55δ, we hypothesize that cells would lose the normal metaphase-anaphase spindle checkpoint, prematurely attempt to exit mitosis, and thus activate apoptotic pathways. In addition, the B55α form of PP2A was implicated in the protection of cohesin by directly regulating shugoshin, a protein that maintains cohesin stability by regulating its phosphorylation (9). When shugoshin is specifically complexed with the B55α form of PP2A, cohesin remains in a dephosphorylated and stable state, allowing cohesin to remain attached to the chromosome at the centromere and protected from separase cleavage and subsequent premature mitotic/meiotic progression in cells. We believe the dysregulation of this pathway is partially responsible for significant activation of apoptotic pathways as indicated by increased capsase 3 cleavage, TUNEL immunoreactivity, and fragmented nuclei when LCMT-1 is reduced in cultured cells or loss *in vivo*. In addition, preliminary experiments (data not shown) indicate that the MAP Kinase pathway, which regulates various cellular activities, such as gene expression, mitosis, differentiation, proliferation, and cell survival/apoptosis(10), is hyperactivated when LCMT-1 is lost. Constitutive or hyperactivation of the MAP Kinase pathway is responsible for the development of malignant diseases including cancer (10). It has been previously found that loss of B subunit in *Drosophila* leads to enhanced activation of the MAP Kinase component ERK as well as a loss of cell viability (11). The reduced viability of these cells correlated with the induction of markers of apoptosis including membrane blebbing and stimulation of

caspase-3-like activity. We hypothesize that loss of LCMT-1 and reduction of PP2A methylation is influencing all of these B-family B-type subunits, and therefore their substrate specificity, subcellular regulation, and negatively regulating their targeted pathways, and in the case of mice, growth and development (Figure 1 and 2).

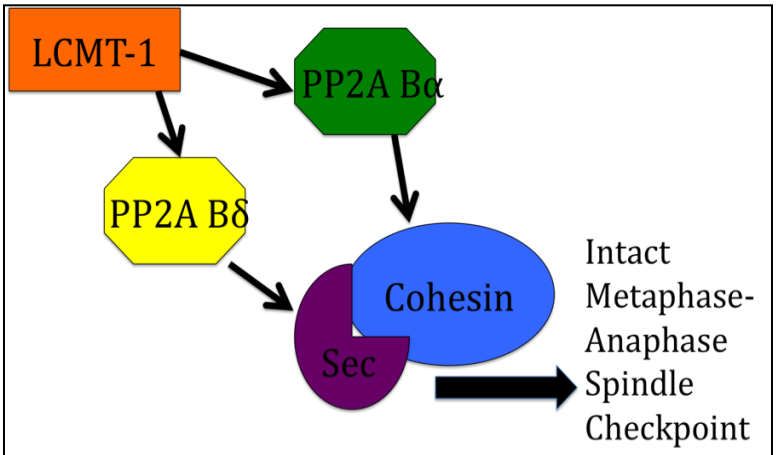

Figure 1: LCMT-1 Negatively Regulates Metaphase-Anaphase Transition by Keeping Securin and Cohesin Hypophosphorylated. The Bα and Bδ forms of PP2A are regulated by LCMT-1 and keep Securin (Sec) and Cohesin hypophosphorylated, maintaining an intact spindle checkpoint.

We should also consider the possibility that LCMT-1 may have additional targets besides PP2A and therefore may play a role in additional signaling pathways. As stated previously, mammalian cells also express two additional phosphatases, Protein Phosphatase 4 (PP4) and Protein Phosphatase 6 (PP6), which exhibit a high degree of sequence homology to PP2A. PP4 is also reversibly methylated on its carboxy terminal leucine residue (12). Although PP6 has the same three carboxy-terminal amino acids as PP2A and PP4, it has not been determined whether PP6 can also be reversibly methylated. Based on the high degree of sequence homology between PP2A, PP4, and PP6, we hypothesize that PP6 is also carboxymethylated on its carboxy-terminal leucine and that both PP4 and PP6 are most likely methylated by LCMT-1. Recently, an study attempting to diagram the PP2A protein interactome found that the PP2A subunit Aα (PPP2R1A) and the B-type subunit B'δ (PPP2R5D) associate with the catalytic subunit of PP4 (PPP4C), suggesting a certain degree of subunit interchangeablility amongst the PP2A-phosphatase family (13). PP6 has been shown to regulate cell cycle progression in

human cells at least in part through control of cyclin D1 (14). The Pallas lab found that PP6 is important for cell viability in the presence of the viral protein E4orf4, which has been shown to selectively kill cancer cells in a PP2A-dependent manner by initiating G2/M arrest and p53-independent apoptosis (15-18). Moreover, PP6 was identified in a RNA interference (RNAi) screen of human kinases and phosphatases having a positive role in cell survival in HeLa cancer cells (19). Since PP4 has been implicated in DNA repair (20,21) as well as microtubule growth and organization, apoptosis, tumor necrosis factor signaling, and pre-T-cell receptor signaling (22-25), and PP6 plays a role in cell survival (19), LCMT-1 activity may also influence these pathways and processes as well.

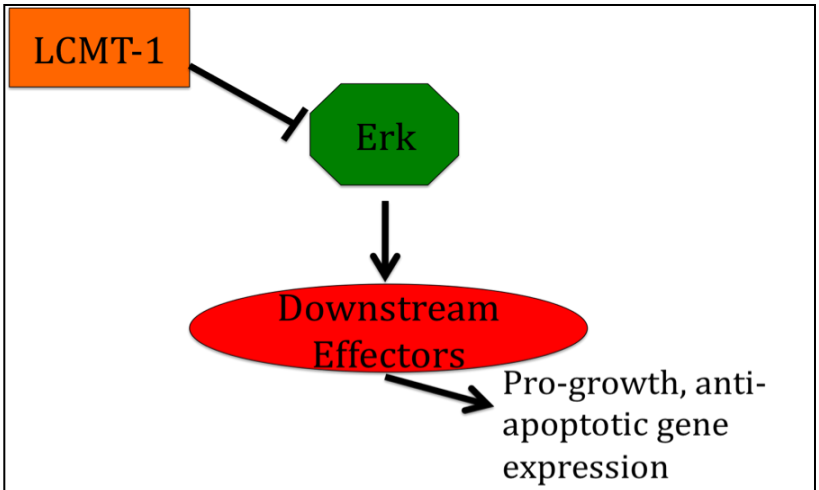

Figure 2: LCMT-1 Negatively Regulates MAP Kinase Pathway and Erk Activation. Loss of LCMT-1 results in Erk hyperphosphorylation. Erk regulates the expression of pro-growth/anti-apoptotic genes.

Understanding the activity and selectivity of LCMT-1 and thus its ability to regulate signaling pathways is of the greatest importance. High levels of the methyltransferase inhibitor, homocysteine, have been shown to impair liver hepatocyte proliferation during liver regeneration in mice (26). In addition, many PP2A targets including bone morphogenic protein 4 (BMP4) (27) and Akt are important regulators of liver development and hematopoietic cell growth and survival (4,28). There are several hematopoietic malignancies and disorders, including aplastic amemia, in which the bone marrow from patients is not able to produce new hematopoietic stem cells (HSCs) in order to repopulate the dying, worn-out, or missregulated cells (Mayo Clinic website). Patients

often feel fatigued and experience uncontrolled bleeding and an overall reduction in quality of life. Recently, clinicians and researchers have begun to design protocols to deliver clinically meaningful levels of the most common genes that are dysregulated in hematopoetic disorders into donor cells in the hope of repopulating hematopoietic stem cells in patients (Boston University Center for Regenerative Medicine). Based on our results involving the reduction of all myeloid progenitor lineages in *lcmt-1* knockout embryos, we hypothesize that LCMT-1 may influence hematopoietic cell proliferation and differentiation as early as HSC production. Therefore, by further exploring the role of LCMT-1 in hematopoiesis and liver development, we may find that targeting the PP2A methylesterase, PME-1, or overexpressing LCMT-1 in HCSs and the bone marrow may open the door to novel therapeutic approaches.

Understanding the overall role of LCMT-1 in mitosis is equally beneficial. Several PP2A subunits are mutated, overexpressed, or dysregulated in several human cancers. Both isoforms of PP2A A subunit (Aα and Aβ) are mutated in melanomas as well as breast and lung carcinomas (29). In addition, the B-type subunit B'γ (PR61g) is both overexpressed and truncated in malignant melanomas (30,31). Moreover, the nuclear protein SET's potent inhibition of PP2A leads to the formation of lymphocytic myeloid leukaemias (32,33). PP2A subunits have been found in play a role at every stage of the mitosis, including G2/M transition, metaphase-anaphase transition/spindle checkpoint, and mitotic exit (1-3,8,34-41). In addition, the viral protein E4orf4 selectively kills cancer cells in a PP2A-dependent manner (15-18). Again, if we can we can selectively target PME-1 or its substrates in cancer cells, we may be able to develop drugs that may have promise as a cancer therapeutic. There are several anti-metastatic drugs that indirectly modify PP2A's activity including cytostatin, but have many side effects (42).

We feel by only targeting a subset of PP2A subunits by targeting PME-1 may provide the specificity to produce efficient cancer drugs with little or no off-target effects.

This study also indicates that loss of *lcmt-1* results in the hyperphosphoryation of the microtubule-associated protein tau early in mouse development. Neurofibrillary tangles, which are composed of hyperphosphorylated tau, are one of two neuropathological hallarks of AD. $PP2A_{B_{\alpha}AC}$ heterotrimers are the major form of PP2A that dephosphorylates tau and the amount of this heterotrimer has recently been shown be reduced in AD-affected neurons [Sontag, 1996 #1949; Sontag, 1995 #1472. Moreover, LCMT-1 proteins levels are significantly reduced in AD brains (43). We found that tau was hyperphosphoryated on two known PP2A-reguated residues (44) in *lcmt-1* knockout embryos. As stated previously, this data provides the first direct cause and effect relationship between LCMT-1 and tau hyperphosphorylation. We believe the implementation of drugs that modulate PP2A methylation will be an important new approach to preventing or treating AD. A potent and specific PME-1 inhibitor, ABL127, was recently discovered and found to have dramatic effects on PME-1 acitvity in cultured cells and in mice (45). We believe that pharmacological inhibition of PME-1 may be the best route to help balance a reduction in LCMT-1 found in AD patients, but also should be considered as a possible cancer therapeutic. Although the discovery ABL127 is one of the most promising advances in the phosphatase field in recent years, we should continue to screen and develop new drugs that may be more specific or have less side effects.

In conclusion, this study is only the beginning of our understanding of LCMT-1 and its role in growth and development, yet it provides a foundation to branch into the areas of hematopoiesis, brain development and AD, and cancer regulation. Further studies should explore not only the role of LCMT-1 and PME-1 in these important

processes, but also the B-type subunits that are affected by methylation, as well as PP4 and PP6. Only by exploring LCMT-1's far-reaching influence in these processes can we understand the importance of the data and results in this seminal study.

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