Global loss of Leucine Carboxyl Methyltransferase-1 causes severe defects in fetal liver hematopoiesis

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Running Title: LCMT-1 Loss Causes Defects in Fetal Hematopoiesis

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Abstract

Leucine Carboxyl Methyltransferase-1 (LCMT-1)3 methylates the carboxy-terminal leucine α-carboxyl group of the catalytic subunits of the protein phosphatase 2A (PP2A) subfamily of protein phosphatasess, PP2Ac, PP4c, and PP6c. LCMT-1 differentially regulates the formation and function of a subset of the heterotrimeric complexes that PP2A and PP4 form with their regulatory subunits. Global LCMT-1 knockout causes embryonic lethality in mice, but LCMT-1 function in development is unknown. In the current study, we analyzed the effects of global LCMT-1 loss on embryonic development. LCMT-1 knockout causes loss of PP2Ac methylation, indicating that LCMT-1 is the sole PP2Ac methyltransferase. PP2A heterotrimers containing the Bα and Bδ B-type subunits are dramatically reduced in whole embryos, and the steady-state levels of PP2Ac and the PP2A structural A subunit are also down ~30%. Strikingly, global loss of LCMT-1 causes severe defects in fetal hematopoiesis and death by embryonic day 16.5 (E16.5). Fetal livers of homozygous lacmt-1 knockout embryos display hypocellularity, elevated apoptosis, and greatly reduced numbers of hematopoietic stem and progenitor cell-enriched Kit Lin Sca1+ (KLS) cells. The percent cycling cells and mitotic indexes of wild-type and lacmt-1 knockout fetal liver cells are similar, suggesting that hypocellularity may be due to a combination of apoptosis and/or defects in specification, self-renewal, or survival of stem cells. Indicative of a possible intrinsic defect in stem cells, non-competitive and competitive transplantation experiments reveal that lacmt-1 loss causes a severe multi-lineage hematopoietic repopulating defect. Therefore, this study reveals a novel role for LCMT-1 as a key player in fetal liver hematopoiesis.

Introduction

LCMT-1 is an enzyme found in all eukaryotes that was originally identified as the protein methyltransferase responsible for methylating the α-carboxyl group of the carboxy-terminal leucine (L309) of the protein phosphatase 2A catalytic subunit (C subunit or PP2Ac) (1-7). PP2A exists mainly as heterotrimeric forms of PP2A C subunit, a structural A subunit, and one of a variety of B-type regulatory/targeting subunits. In all, well over eighty different heterotrimeric forms of PP2A likely exist. Reversible methylation of PP2A C subunit L309 by LCMT-1 and a methylesterase, PME-1 (8-10), regulates the formation of certain PP2A heterotrimeric forms, herein termed ‘methylation-dependent PP2A heterotrimers’ (11-17). The dependence of different PP2A heterotrimers on PP2A C subunit methylation has not been fully elucidated yet. However, it is clear from existing data that in mammalian cells PP2A C subunit methylation is
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required for efficient formation of heterotrimers containing the B/PPP2R2 family B-type subunits, Bα/PPP2R2A (11,14,18), Bβ/PPP2R2B (17), and Bδ/PPP2R2D (K.A. Rahman and D.C. Pallas, unpublished), but is not necessary for the formation of heterotrimers containing the B''/PPP2R3 or B''''/STRN family B-type subunits (14,17). Thus, LCMT-1 likely regulates a subset of PP2A functions carried out by methylation-dependent PP2A heterotrimers.

Recently, LCMT-1 was shown to be the methyltransferase responsible for methylation of not just PP2A, but of all three members of the PP2A subfamily of serine/threonine protein phosphatases, PP2A, PP4, and PP6 (19). Based on this finding, LCMT-1 was proposed to be a master regulator of these phosphatases, which share ~60% sequence identity, including the C-terminal Leucine, the site of methylation by LCMT-1. Similar to the case for PP2A, LCMT-1 differentially regulates the formation and function of different PP4 heterotrimers, but effects on PP6 heterotrimers were not seen (19).

Based on data from mutational, x-ray crystallographic, and biochemical studies, LCMT-1 and PME-1 are specific for these three PP2A subfamily phosphatases, and likely have no additional substrates. Neither LCMT-1 nor PME-1 can recognize peptides corresponding to the PP2A C subunit C-terminus (3,9,20,21). Instead, LCMT-1 and PME-1 must interact both with highly conserved active site residues of these phosphatases and with specific, highly conserved residues at the C-terminus (10,14,20-23). Consistent with this, LCMT-1 methylation of PP2A C subunit and PME-1 demethylation of PP2A C subunit can both be inhibited by PP2A inhibitors that bind to the PP2A active site (2,5,9). Thus, only proteins that have conservation of active site residues with PP2A and a conserved C-terminus may serve as substrates for LCMT-1 and only PP2A, PP4, and PP6 share these criteria. Therefore, LCMT-1 specifically regulates a subset of PP2A subfamily phosphatase functions by regulating the formation of their methylation-dependent complexes (19). Correspondingly, LCMT-1 likely plays key roles in the functions of these phosphatases in cellular processes such as cell growth and proliferation, apoptosis, DNA repair, neuronal differentiation, and in diseases related to these processes such as cancer and Alzheimer’s Disease (24), to name a few.

To date, direct analysis of LCMT-1 function has been mostly limited to cell lines. For example, LCMT-1 is important for proper mitotic progression in yeast as well as mammalian cells, playing a key role in mitotic spindle checkpoint at least in yeast (12,13,18). In addition, LCMT-1 loss induces apoptosis in some transformed cell lines (17,18). In neuronal cell lines, LCMT-1 promotes the association of PP2A and Tau protein with cell membranes (25) and has a positive role in neurite outgrowth (26). Regarding signaling, LCMT-1 methylation of the PP2A C subunit is stimulated by multiple GiPCR agonists in adult rat ventricular myocytes (27) and references therein; LCMT-1 is a negative regulator of Akt and p70/p85 S6 kinase (28); and LCMT-1 helps prevent anchorage-independent growth of cells (28).

Although LCMT-1 is involved in these and other important cellular processes, the larger role LCMT-1 plays in growth and development has only begun to be elucidated. Previously, we reported that homozygous global knockout of LCMT-1 in mice via a gene trap approach causes embryonic lethality, indicating that LCMT-1 is essential for mouse development (18). Subsequently, it was reported that global hypomorphic knockdown of LCMT-1 resulted in some viable homozygous LCMT-1 knockout mice that showed decreased glucose tolerance and increased glucose-stimulated insulin secretion, consistent with a possible insulin resistance phenotype (29). However, no other phenotypes were reported.

In the current study, we have analyzed embryonic development in our gene trap LCMT-1 knockout mouse model to further investigate the role(s) of LCMT-1 in mouse development. We report that LCMT-1 is nearly undetectable in embryo tissue, and that its loss leads to nearly complete loss of methylation of PP2A C subunit, indicating that LCMT-1 is the sole PP2A methyltransferase. LCMT-1 loss dramatically reduces the steady-state levels of methylation-dependent PP2A heterotrimers containing the Bα and Bδ B-type subunits in whole embryos, but in addition also reduces the steady-state levels of the PP2A A and C subunits. Global loss of LCMT-1 results in severe defects in fetal hematopoiesis in the fetal liver and embryonic death by embryonic day 16.5 (E16.5). Fetal livers of homozygous lcmt-1 knockout embryos display hypocellularity, high levels of apoptosis, and greatly reduced numbers of both colony forming progenitor cells and the hematopoietic stem (HSC) and progenitor (HPC) cell-enriched Kit+LinSca1+ (KLS) cell population. As for wild-type (WT) control cells, nearly 100%
of homozygous lcmt-1 knockout fetal liver cells are cycling and WT and lcmt-1 knockout fetal liver cells have similar mitotic indexes, suggesting that hypocellularity is due to a combination of apoptosis and/or defects in HSCs and HPCs. Indicative of a possible intrinsic defect in stem cells, non-competitive and competitive transplantation experiments reveal that lcmt-1 loss also causes a severe multi-lineage hematopoietic repopulating defect. Therefore, this study reveals a novel role for LCMT-1 as a key player in fetal liver hematopoiesis.

Results

Disruption of the murine lcmt-1 gene--The murine lcmt-1 gene is encoded by eleven exons within 1.3 megabases of genomic DNA on mouse chromosome 7. Embryonic stem cells with a linearized pT1βgeo gene trap plasmid inserted in an unknown location within the first intron of the lcmt-1 locus were obtained from the German Gene Trap Consortium, Neuherberg, Germany (see Experimental Procedures). Insertion of pT1βgeo 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 (Supplementary Fig. 1A). Embryonic stem (ES) cells hemizygous for this insertion were previously shown to express a reduced level of LCMT-1 protein (18). We injected these ES cells into C57BL/6 blastocysts, backcrossed the resultant chimeric mice to generate hemizygous F1 animals, and tested for germline transmission of the mutant lcmt-1 allele in progeny by PCR analysis of tail DNA (Supplementary Fig. 1B). Hemizygous lcmt-1 mice were then backcrossed to C57BL/6 mice for eleven generations prior to the experiments described in this study.

Homozygous gene trap knockout of lcmt-1 results in nearly complete loss of LCMT-1 protein and >95% embryonic lethality by E16.5. We showed previously that gene trap knockout of lcmt-1 results in embryonic lethality, based on the fact that no lcmt-1<sup>-/-</sup> progeny were produced from early lcmt-1<sup>1<sup>1/2</sup> × lcmt-1<sup>1/2</sup></sup> matings (18). In the current study, after backcrossing to a C57BL/6 background eleven times to move it from a C129 strain background to C57BL/6 background, this phenotype persisted, with no live lcmt-1<sup>1/2</sup> births observed in over 200 lcmt-1<sup>1/2</sup> X lcmt-1<sup>1/2</sup> litters.

To determine the effect of homozygous lcmt-1 knockout on LCMT-1 protein expression in the whole embryo, the time of lethality during embryonic development, and the type and severity of developmental defects present, dissections of embryos produced from time-mating lcmt-1<sup>1/2</sup> X lcmt-1<sup>1/2</sup> crosses were performed. Embryo genotypes determined by PCR analysis of yolk sac DNA definitively identified live lcmt-1<sup>1/2</sup> embryos (Fig. 1A). Analysis of E12.5 whole embryo homogenates by Western blotting showed that hemizygous gene trap inactivation of the lcmt-1 gene results in an ~60% decrease in LCMT-1 protein while homozygous inactivation results in almost complete loss of LCMT-1 protein (Fig. 1B-C). Genotyping of live embryos at successive stages of embryonic development revealed that homozygous lcmt-1<sup>1/2</sup> embryos die primarily between E14.5 and E16.5 (Fig. 1D). Thus, LCMT-1 is required for survival during this period. In addition, analysis of 509 embryos of gestation stages E11.5-E14.5 indicated that approximately 20% of these embryos were lcmt-1<sup>1/2</sup>, less than the 25% value expected from Mendelian genetics, raising the possibility that lcmt-1 loss may also affect meiosis, gametes, fertilization, or cause a low level of early gestation lethality.

Homzygous lcmt-1 gene trap knockout results in a dramatic reduction in PP2A C subunit methylation--Previous work from several labs supports the idea that the yeast LCMT-1 homolog, Ppm1p, is the sole PP2A methyltransferase in yeast (12,13,30). Our previous data also indicate that LCMT-1 is likely the only PP2A methyltransferase in mouse embryonic stem cells since loss of one lcmt-1 allele in those cells resulted in >50% loss of PP2A C subunit methylation (18). However, whether LCMT-1 is the sole mammalian PP2A methyltransferase in the bulk of mammalian cell tissues is not known. To determine the effect of gene trap knockout of LCMT-1 on PP2A C subunit methylation in mouse embryos, we quantitated the steady-state levels of PP2A C subunit methylation in E12.5 WT, hemizygous, and homozygous whole embryo homogenates using anti-demethylated PP2A monoclonal antibody, 4b7, which is specific for unmethylated PP2A C subunit (see Experimental Procedures). Gene trap inactivation of the lcmt-1 gene results in >95% reduction in PP2A C subunit methylation (Fig. 2A-B), indicating that LCMT-1...
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may be solely responsible for PP2A methylation in the bulk of mouse embryo tissues.

Knockout of lcmt-1 greatly reduces PP2A Bα/δ subunit-A subunit-C subunit (PP2A<sub>BAC</sub>) heterotrimer formation and the steady-state level of the PP2A Bα/δ, A, and C subunits--Deletion of the LCMT-1 ortholog, PPM1, in yeast or knockdown of LCMT-1 in mammalian cultured cells dramatically reduces the formation of PP2ABAC heterotrimers as measured by the reduction in the amount of PP2A C subunit associated with B subunit (12-14,18). To assess the importance of LCMT-1 for PP2ABAC heterotrimer formation in an animal model, we immunoprecipitated PP2A Bα/δ subunits 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 WT levels while loss of one lcmt-1 allele has no significant effect (Fig. 2C-D). However, this only represents the effect of lcmt-1 loss on the efficiency of PP2ABAC heterotrimer formation of the B subunit still present in the lcmt-1<sup>−/−</sup> cells. We also observed a ~80% reduction in total B subunit protein in lcmt-1<sup>−/−</sup> whole embryo homogenates (Fig. 2E-F), presumably due to increased B subunit instability due to reduced PP2ABAC heterotrimer formation (31-33). Thus, the overall reduction in PP2ABAC heterotrimers in lcmt-1<sup>−/−</sup> whole embryos is ~92%, comparable to the loss we found upon PPM1 disruption in yeast (13). 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<sup>−/−</sup> homogenates (Fig. 2G-H). This reduction in the PP2A subunits that compose the core heterodimer (PP2A<sub>AC</sub>) indicates that the methylation of PP2A by LCMT-1 is not only essential for efficient PP2ABAC heterotrimer formation, but is also important for maintaining steady state levels of core heterodimers. Thus, the severe disruption of LCMT-1 expression in this gene trap mouse model has dramatic effects on a known LCMT-1 substrate and its methylation-dependent complexes.

Loss of lcmt-1 results in defective fetal liver hematopoiesis--Gross examination of homozygous lcmt-1<sup>−/−</sup> 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<sup>−/−</sup> embryos as compared to WT littermates (Fig. 3A; black arrows). Hemizygous littermates appeared normal (Fig. 3A), consistent with the fact that healthy, live births of hemizygous lcmt-1 mice are obtained. In addition to a defect in hematopoiesis, there was a 100% frequency of misshapen eyes resembling microphthalmia in lcmt-1<sup>−/−</sup> knockout embryos compared to WT and hemizygous littermates (Fig. 3A; blue arrowhead), indicating that LCMT-1 is necessary for normal eye development in this strain background. Also, an indentation of the back of the head was consistently noted in lcmt-1<sup>−/−</sup> embryos compared to WT or hemizygous littermates (Fig. 3A, red arrowhead), consistent with the possibility of effects on brain or head development. Finally, we observed a significant reduction of ~20% in the weight of E12.5 lcmt-1<sup>−/−</sup> embryos (Fig. 3B), but only a small, statistically non-significant reduction in crown-to-rump length when compared to WT littermates (data not shown).

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. While lcmt-1<sup>−/−</sup> embryos showed no significant increase in cleaved caspase 3 compared to WT embryos, we observed an approximately 2.5-fold increase in cleaved caspase-3 levels in lcmt-1<sup>−/−</sup> embryos (Fig. 3C-D), suggesting that differences in apoptosis may be partially responsible for the differences in embryo size.

Defective fetal liver hematopoiesis in LCMT-1 knockout embryos is caused in part by increased apoptosis, but not by reduced cell division--Dissection of mutant embryos revealed that lcmt-1 knockout results in smaller, anemic livers (Fig. 4A), indicating at a minimum a defect in erythrocyte production (34). E12.5 lcmt-1<sup>−/−</sup> fetal livers weighed ~2-fold less than WT fetal livers, even when normalized to total embryo weight (Fig. 4B), indicating that loss of LCMT-1 protein has a more dramatic effect on fetal liver development than the development of whole embryo on average.

To ascertain whether an increase in apoptosis might contribute to the reduced fetal liver size of lcmt-1<sup>−/−</sup> embryos, we performed histological and biochemical analyses of fetal livers from E12.5 WT and lcmt-1<sup>−/−</sup> embryos. A
number of fragmented pyknotic nuclei, characteristic of cells undergoing apoptosis, were abundant in \textit{lcmt-1}\textsuperscript{−/−} fetal livers as compared to WT and hemizygote littermates (Fig. 4C). Moreover, \textit{lcmt-1}\textsuperscript{−/−} fetal livers exhibited an increase in cells staining positive for cleaved-caspase 3 (Fig. 4D) as well as TUNEL immunoreactivity (Fig. 4E), hallmarks of both intrinsic and extrinsic apoptosis pathways. Parallel Western blotting experiments demonstrated a nearly 10-fold increase in cleaved caspase 3 (Fig. 5). These data support the hypothesis that increased apoptosis contributes to the impaired development of \textit{lcmt-1}\textsuperscript{−/−} fetal livers.

To determine if a decrease in cell proliferation also contributed to the reduced size of \textit{lcmt-1}\textsuperscript{−/−} fetal livers, we examined the level of cellular proliferation within the fetal livers of embryos using the cell proliferation marker Ki67 and the mitotic marker MPM-2. Ki67 immunoreactivity of WT fetal livers showed that almost all cells were proliferating, consistent with the rapid growth of the fetal liver at this stage of development (Fig. 6A and 6C). Parallel analysis of \textit{lcmt-1}\textsuperscript{−/−} fetal livers revealed that nearly all live, non-apoptotic cells were Ki67-positive as well (Fig. 6A and 6C). In addition, no significant difference in the percentage of MPM-2 positive fetal liver cells was found between \textit{lcmt-1}\textsuperscript{−/−} and WT embryos (Fig. 6B and 6D), indicating that the mitotic index of fetal liver cells in WT and \textit{lcmt-1}\textsuperscript{−/−} embryos was identical. Together, these results indicate that decreased fetal liver size in E12.5 \textit{lcmt-1}\textsuperscript{−/−} embryos may be due in part to an increase in cell death but not to a decrease in proliferation, at least at this stage of gestation. Moreover, they indicate that LCMT-1 is essential for proper development of mouse fetal liver.

\textit{Lcmt-1 loss causes multi-lineage defects in fetal liver hematopoiesis, and a corresponding reduction in colony forming units (CFUs) and the HSC/HPC-enriched KLS population—At E12.5, the vast majority of fetal liver cells are of hematopoietic origin and reduced fetal liver size is indicative of reduced numbers of hematopoietic cells. To determine the actual reduction in cellularity caused by loss of LCMT-1 and to ascertain whether fetal liver cell death was also occurring at a stage later than E12.5, E12.5 and E14.5 WT and \textit{lcmt-1}\textsuperscript{−/−} fetal liver cells were counted from at least ten matched (same mother) WT and \textit{lcmt-1}\textsuperscript{−/−} embryo pairs. Dramatic reductions in fetal liver cells of 5.6-fold and 7.8-fold were seen at E12.5 and E14.5 stages, respectively (Fig. 7A-B). Between these embryonic stages the WT liver cellularity increased 8-fold due to rapid expansion of hematopoietic cells (Fig. 7A). Substantially increased cell death in \textit{lcmt-1}\textsuperscript{−/−} fetal liver cells was seen at both E12.5 and E14.5 (Fig. 7C), indicating that fetal liver cells continue to die through this period of gestation. Especially at E12.5, Ter119\textsuperscript{+} fetal liver erythroid cells were disproportionately reduced in \textit{lcmt-1}\textsuperscript{−/−} fetal livers relative to the total cell number (Fig.7D), probably due in part to a higher fold increase in death over WT fetal liver cells (compare Fig. 7E to Fig. 7C). Importantly, analysis of additional cell types in WT and \textit{lcmt-1}\textsuperscript{−/−} fetal livers revealed that LCMT-1 loss causes a multi-lineage reduction in fetal liver hematopoietic cells (Fig.8A).

Another potential cause of the hypocellularity in \textit{lcmt-1}\textsuperscript{−/−} embryos is reduced amounts or reduced function of stem and/or progenitor cells. During early mouse embryogenesis, the yolk sac is the initial location of hematopoiesis, termed primitive hematopoiesis (35). Then in mid-gestation, hematopoiesis shifts to the fetal liver as part of the process of definitive hematopoiesis. To assess the importance of LCMT-1 for fetal liver hematopoietic progenitor cell proliferation and differentiation, we performed \textit{in vitro} colony formation unit (CFU) assays in semisolid media with cells from E12.5 and E14.5 fetal livers. Single cell suspensions of \textit{lcmt-1}\textsuperscript{−/−} and WT fetal liver cells 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 erythroid and myeloid progenitor cells, including erythrocyte precursors (Burst-Forming Unit Erythrocytes; BFU-E), progenitors capable of producing monocytes and/or granulocytes (CFU-GM), and multi-potential progenitors that can produce granulocytes, erythrocytes, monocytes, and megakaryocytes (CFU-GEMM). The results of the assays showed that there were approximately 4-fold and 3-fold decreases in the total number of competent progenitor colony forming cells at E12.5 and E14.5, respectively (Fig. 8B and data not shown). All types of hematopoietic progenitor colony
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forming units were strikingly reduced in E14.5 lcmt-1/- fetal livers as compared to WT littermates (Fig. 8B). Given that 1) these assays are performed for lcmt-1/- and WT fetal liver cells with the same number of viable nucleated cells, and 2) there are 7.8-fold less viable cells in an E14.5 lcmt-1/- fetal liver compared to an E14.5 WT fetal liver, the absolute numbers of colony forming progenitors per fetal liver are even more strikingly reduced. Adjusting the E14.5 data from Figure 8B for average relative WT fetal liver cellularity (Fig. 7A-B), lcmt-1/- E14.5 BFU-E, CFU-GM, CFU-GEMM, and total colonies would be ≤6% of WT levels, respectively, on a per fetal liver basis. Consistent with these results, the percentage of CD45+ cells that are in the HSC- and HPC-enriched KLS population in LCMT-1/- fetal livers is decreased 2.6-fold from WT levels (Fig. 8C). Combined with the overall decrease in CD45+ cells in LCMT-1/- fetal livers (Fig. 8A), this result indicates a >9-fold decrease in this HSC/HPC-rich population relative to WT livers on a per liver basis. Thus, reduced HSCs and HPCs could be the major explanation for the small LCMT-1/- fetal livers, and these results are consistent with the possibility of a major role for LCMT-1 in HSC generation, self-renewal, and/or survival.

Lcmt-1 loss causes multi-lineage defects in hematopoietic repopulating function that vary with lineage—Because LCMT-1 has been knocked out in all tissues of this mouse model, defects resulting from LCMT-1 loss could result from loss of LCMT-1 function(s) intrinsic to hematopoietic cells, LCMT-1 functions extrinsic to those cells such as in niche cells that support hematopoiesis, or both. One way to test for an intrinsic function of LCMT-1 in hematopoietic cells is to determine if fetal liver HSCs lacking LCMT-1 are defective in their ability to repopulate lethally irradiated WT congenic BoyJ (CD45.1+) mice. Therefore, we performed non-competitive transplantation experiments in which WT and LCMT-1-/- E14.5 mouse embryo fetal liver cells (CD45.2+) were transplanted into CD45.1+ BoyJ recipients. We found that LCMT-1-/- fetal liver cells could repopulate the recipient mice, but severe defects in numbers of white blood cells and in most lineages were seen, supporting a critical and possibly intrinsic role for LCMT-1 in hematopoietic repopulating function (Fig. 9). B220+ B cells and CD4+ T cells were especially defective, as can be seen from their greatly reduced absolute cell numbers (Fig. 9). Competitive transplantation experiments also support a critical role for LCMT-1 in hematopoietic repopulating activity. LCMT-1-/- fetal liver cells injected at a 1:5 donor to competitor ratio into lethally irradiated BoyJ mice made no contribution to repopulating the irradiated recipients while WT fetal liver cells injected in parallel at the same ratio constituted 83±5% (average) of the recipient hematopoietic cells at 3 months (Fig. 10A-B).

Discussion

In this study, we have utilized a gene trap knockout of the lcmt-1 gene to investigate LCMT-1 function in mouse embryo development. Examination of developing lcmt-1/- embryos revealed that certain developmental processes and tissues were more dramatically affected than others, suggesting specific roles for LCMT-1 rather than a global negative effect on growth. Global LCMT-1 loss on a C57BL/6 background leads to a significantly smaller embryo, a slight but consistent alteration in head shape, misdeveloped eyes, severe defects in fetal hematopoiesis, anemia, and death of >95% of embryos by E16.5. Fetal livers of lcmt-1/- knockout embryos displayed multi-lineage hypopcellularity, elevated apoptosis, greatly reduced numbers of CFUs, and dramatically reduced numbers of the HSC- and HPC-enriched KLS cell population. Indicative of a possible intrinsic defect in hematopoietic stem cells, LCMT-1 loss caused a severe multi-lineage defect in hematopoietic repopulating function. Thus, our data reveal novel critical roles for LCMT-1 in mouse fetal development, fetal liver hematopoiesis, and hematopoietic repopulating function.

The knockout efficiency of our gene trap construct is very high based on the low amount of residual LCMT-1 protein (<1%; Fig. 1B). Any residual LCMT-1 probably results from a very small percentage of untrapped functional LCMT-1 mRNAs from the lcmt-1-pTβgeo gene trap cassette. The fact that global gene trap knockout of LCMT-1 results in nearly complete loss of PP2A C subunit methylation in whole embryo lysates is consistent with the hypothesis that LCMT-1 is the sole methyltransferase for the PP2A subfamily of protein phosphatases in all tissues. However, based on whole embryo analysis, we cannot rule out the possibility of higher LCMT-1 methylation...
in a minor tissue. Interestingly, embryos that are hemizygous for \textit{lcmt-1} knockout displayed >50% reduction of LCMT-1 protein levels (Fig. 1B-C), but no evidence of haploinsufficiency (Figs. 2 and 3A and data not shown). Consistent with this, \textit{lcmt-1}\textsuperscript{-/-} mice develop normally through adulthood. Thus, loss of one \textit{lcmt-1} allele has no detectable consequences, suggesting that LCMT-1 is normally expressed in excess amount over what is needed for most cellular and developmental functions, while nearly complete loss of LCMT-1 expression has dramatic consequences.

Global \textit{lcmt-1} homozygous knockout in mice reduces total PP2A C, A and B\textsubscript{a} family protein levels (Fig. 2E-H). In agreement with previously reported observations that used cells from multiple species (31-33), this result suggests that both the B subunit and the core heterodimer are destabilized when not in heterotrimeric complexes. Because the loss of LCMT-1 in our mouse model is nearly complete, we are able for the first time to conclude that the percentage of all PP2A complexes that are methylation-dependent in whole embryos is >30%, the amount of reduction in the A and C subunits in \textit{lcmt-1}\textsuperscript{-/-} embryos.

Previously, we reported results from initial litters from global LCMT-1 knockout on a mixed C129:C57BL/6 background showing that global LCMT-1 loss was embryonic lethal (18). After backcrossing 11 generations to C57BL/6 strain background, we now find that LCMT-1 loss still results in embryonic lethality. In addition, by analyzing a large number of embryos, we find a significant difference between the expected (25%) and observed (~20%; Fig. 1D) frequency of \textit{lcmt-1}\textsuperscript{-/-} midgestation embryos resulting from crossing hemizygous parents. This result suggests that global LCMT-1 loss may also affect meiosis, gametes, fertilization, or may cause a low level of early gestation lethality. Recent reports of changes in PP2A C subunit carboxymethylation during spermatid development (36) and male sterility caused by conditional knockout of the major isoform (\textit{\alpha}) of the PP2A C subunit (37) are consistent with the possibility that loss of LCMT-1 may have a effect on fertility, but this will need to be investigated further.

During normal development, waves of hematopoiesis occur in the developing mouse embryo (for review, see (38)). The first (primitive) wave of hematopoiesis begins with the transient production of primitive erythrocytes, macrophages, and megakaryocytes at E7 in the yolk sac, while the second (definitive) wave involves the emergence of bipotential erythroid-myeloid progenitors (EMPs) at E8.25 in the yolk sac and cells with lymphoid potential at E8.5/E9.5. The third (also definitive) wave of hematopoiesis begins with the generation of the first HSCs at E10.5 from the aorta-gonad mesonephros (AGM) region. HSCs self-renew, are transplantable, and produce all the cells for the erythroid, myeloid, and lymphoid lineages. The fetal liver provides the niche for growth, expansion, and differentiation of definitive hematopoiesis HSCs and EMPs. Then, shortly before birth, HSCs migrate to the bone marrow, which provides supportive niches for their growth and differentiation for the life of the mouse. Global LCMT-1 loss severely impairs definitive hematopoiesis in the fetal liver, as evidenced by severe fetal liver hypocellularity (Fig. 7A-B and 8A), reduction in KLS cells (Fig. 8C), and multi-lineage defects seen in the fetal liver lineage analysis (Fig. 8A), as well as the CFU assays (Fig. 8B and data not shown), and the transplantation assays (Figs. 9-10) (39,40). While fetal liver cell lineage analysis (Fig. 8A) and colony forming assays (Fig. 8B) showed substantial effects on erythroid and myeloid lineage cells, results of transplantation experiments indicate strong defects in lymphoid cells as well (Fig. 9). Reduction in KLS cells, CFUs, and hematopoietic repopulating function strongly point to a role(s) for LCMT-1 in generation of HSCs, HSC self-renewal, and/or survival of HSCs and HPCs. Importantly, the defects seen when equal numbers of fetal liver white blood cells were transplanted into WT hosts suggest that LCMT-1 has an intrinsic role in HSC function. However, we cannot rule out the possibility that global loss of LCMT-1 has irreversible extrinsic effects on HSCs as has been reported for the Wnt pathway (41). Future study of conditional knockout mice for LCMT-1 will be necessary to fully sort out intrinsic and extrinsic effects.

We observed a significant level of apoptosis in fetal livers from \textit{lcmt-1} homozygous knockout mice as evidenced by increased cleaved-caspase 3 and TUNEL immunoreactivity, but also by the presence of fragmented, pyknotic nuclei,
characteristic of cells undergoing apoptosis (Figs. 4C-E and 6). Percent cycling cells and mitotic indexes of WT and lcmt-1 knockout fetal liver cells are similar, suggesting that hypocellularity may be due to a combination of apoptosis and defects in the generation, self-renewal, or survival of HSCs. However, a reduction in cell proliferation of stem and early progenitor cells cannot be ruled out without further experiments examining those specific populations. 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 (42), 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 erythroblasts 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 (43,44). 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 fetal liver.

We propose that LCMT-1 normally functions in hematopoietic cells by methylating the catalytic subunits of PP2A subfamily phosphatases, promoting the formation of methylation-dependent holoenzymes, which in turn play important roles in fetal hematopoiesis and hematopoietic repopulating function (Fig. 11). It is well established that members of the B/PPP2R2 family of regulatory subunits are the main methylation-dependent PP2A regulatory subunits in yeast as well as in mammalian cells (11-15,17,18). Considering reduction in Bα/δ subunit levels (Fig. 2E-F) as well as loss of PP2A_{B\in\delta}AC heterotrimer formation (Fig 2D-E), we show global loss of LCMT-1 causes a 92% global loss of PP2A_{B\in\delta}AC heterotrimers in whole embryos. This result further strengthens previous conclusions made on a cellular level as to the dependence of these heterotrimers on methylation and extends them to a whole animal. These results are in contrast to in vitro studies that have found that PP2A_{B\in\delta}AC heterotrimers can form in vitro in the absence of methylation (45,46). The methylation-independence of these heterotrimers in vitro probably represents an in vitro artifact or the consequence of the absence of a cellular factor required for methylation-dependent regulation, such as a competing, methylation-independent binding protein of lower affinity. Recent data obtained using mouse embryo fibroblasts (MEFs) from our global LCMT-1 knockout mouse model show that LCMT-1 loss also dramatically reduces carboxyl methylation of the PP4 and PP6 catalytic subunits, and differentially reduces formation of different PP4, but not PP6, complexes. Collectively, these results suggest that the defects we observe due to LCMT-1 loss in the current study are due to a combination of defects in methylation-regulated PP2A, PP4, and PP6 function (Fig. 11).

Consistent with the above hypothesis, LCMT-1 and PME-1, like PP2A, PP4, and PP6, are ubiquitously expressed, and, according to the expressed sequence tag (EST) database, Unigene (47), are both expressed in cells from blood, bone marrow (BM), spleen, and thymus. Moreover, methylation-dependent PP2A B-type subunits Bα and Bδ (but little to no Bβ and Bγ) and the methylation-dependent PP4 subunit, PP4R1, are also expressed in these same tissues (47,48). Thus, regulation of methylation-dependent PP2A and PP4 complexes by LCMT-1 and PME-1 likely occurs in a variety of hematopoietic cells.

The specific signaling pathway or pathways through which LCMT-1 signals to regulate hematopoiesis remain to be determined. LCMT-1 is a negative regulator of Akt and p70/p85 S6 kinase (28), likely through its regulation of methylation-dependent PP2A_{BAC} complexes. PP2A_{BAC} heterotrimers also positively regulate Raf (49), and thus LCMT-1 loss is predicted to inhibit the Raf/MEK/Erk pathway. Thus, LCMT-1 loss may have a direct impact upon HSC and their response to proliferative stimuli. Other possible connections also exist that may affect cell survival, cell growth, differentiation, as well as other aspects of HSC function. Methylation-dependent PP4 complexes may also provide important links. PP4R1 has been implicated in the negative regulation of NF-κB signaling in T cells and may be a suppressor of aberrant NF-κB signaling in some Cutaneous T cell Lymphomas (CTCLs) (50,51). Given that in mice both canonical and non-canonical NF-κB signaling regulate HSC self-
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renewal intrinsically (52,53) and enhanced NF-κB signaling can impair HSC self-renewal (54), we hypothesize that the reduction in PP4R1 PP4 complexes caused by LCMT-1 loss (19) enhances NF-κB signaling, impairing HSC self-renewal and function. NF-κB activation has been shown to be required intrinsically for HSC emergence from the hemogenic endothelium as well (55,56). Thus, it is possible that LCMT-1 is important for both specification of HSC and for HSC self-renewal and function. Together, these mechanisms might explain the severe multi-lineage effects we see upon loss of LCMT-1, and are of high priority for future investigations.

While the current study was being conducted, Chen and colleagues reported that an endothelial/hematopoietic knockout of PP2Aα, the major catalytic isofrom of PP2A C subunit in cells, selectively depletes committed erythroid cells via increased apoptosis, causing severe anemia at E12.5 and embryonic lethality (57). Their study used the Tie2 endothelial cell driver for Cre expression, which knocks out PP2Aα expression in endothelial cells and in the majority of hematopoietic cells. In striking contrast to our findings, they found no decrease in absolute numbers of any other lineage tested, of CD45+ cells, or of KLS cells. The Tie2-Cre PP2Aα conditional knockout would be expected to reduce all PP2A heterotrimers in the fraction of hematopoietic cells with PP2Aα knocked out, so it is difficult to compare directly to our system, where only methylation-regulated PP2A heterotrimers are reduced. However, their results support the idea that the increased erythroid cell death we observe (Fig. 7D-E) in LCMT-1−/− fetal liver cells may be due to loss of methylation-dependent PP2A heterotrimers containing PP2Aα and that this effect may be distinct from the dramatic, multi-lineage defect and loss of CD45+ cells and KLS cells that we observe. Thus, we propose that there are at least two distinct defects in hematopoietic fetal liver cells lacking LCMT-1: 1) increased death of Ter119+ lineage cells, at a minimum involving defective methylation-dependent PP2Aα complexes, and 2) defective generation, self-renewal, and/or survival of HSCs, which may involve loss of PP4 and/or PP6 methylation-dependent function, potentially in combination with loss of PP2A methylation-dependent heterotrimer function. Future experiments will be necessary to dissect the relative contributions of each of these phosphatases and their different holoenzyme forms.

Given our results showing a critical role for LCMT-1 in mouse fetal hematopoiesis and the similarities between human and mouse hematopoiesis, it is possible that a defect in LCMT-1 amount or activity could contribute to hematopoietic disorders in humans. Conversely, enhancing LCMT-1 levels or activity or inhibiting the opposing methylsterase, PME-1, may have promise for therapeutic-based targets in hematopoietic disease. If LCMT-1 indeed functions at the level of HSC generation, self-renewal, or survival, then enhancing LCMT-1 function or inhibiting PME-1 may prove beneficial for expanding HSCs in vivo or ex vivo, which would have a number of clinical applications. We are currently pursuing these possibilities.

Finally, 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 of the defects we report here. The gene trap knockout mouse model we have generated should be very useful for these investigations.

Experimental Procedures

Generation of lcmt-1 gene trap knockout mice—This research was reviewed and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). A mouse model for studying the function of LCMT-1 in mouse development was constructed by utilizing the gene trap approach (58). 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 hemizygous F1 animals, which were then backcrossed for eleven generations to C57BL/6 mice to create a homogenous line for study. The integration site of the gene trap vector within the ~22k intron 1 of
the lcmt-1 gene was determined by using 22 forward primers (one primer approximately every 1kb of intron sequence) 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 approximate location of the pT1betageo integration site, facilitating the design of primers for genotyping capable of distinguishing WT, hemizygous KO, and homozygous KO mice.

Genotyping--For standard genotyping of mice or embryos for lcmt-1 status, DNA was extracted from tail biopsies or yolk sacs 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 (95°C for 60 s; 52°C for 60 s; 72°C for 90 s) using 0.25 units/sample of taq polymerase (Promega) and one forward primer and two reverse primers: primer 1 (forward), 5'-CCAGATATGATTCAAGGTTGAA-3'; primer 2 (reverse), 5'-CCAATCACTCTGTATTGCTGCAT-3'; and primer 3 (reverse), 5'-AAGAAAAGTAGGCTAGCTCAA-3'. Primers 1 and 2 amplify an approximately 200 bp fragment of the endogenous lcmt-1 allele, whereas primers 1 and 3 amplify an approximately 250 bp amplicon between the lcmt-1 intron 1 and LacZ in the pT1betageo gene trap cassette. For rapid genotyping of embryos, DNA was extracted from yolk sacs digested 1h in tail lysis buffer containing 500ug/ml Proteinase K or from torsos digested 1h in torso lysis buffer (500 mM KCl, 100 mM Tris pH 8.3, 0.01% gelatin, 1% Nonidet P-40, 1% Tween 20). DNA samples were subjected to 29 cycles of PCR (95°C for 1 minute; 52°C for 1 minute; 72°C for 30 seconds using GoTaq Green Master Mix (Promega)) using the primers described above.

Timed-matings, embryo dissections, and imaging--Timed matings of lcmt-1+/- mice were conducted and the day of vaginal plug detection was designated embryonic day 0.5 (E0.5). E10.5, E11.5, E12.5, E14.5 and E16.5 embryos were dissected free from the uterus and extraembryonic membranes, embryos were weighed, and genotyping was performed as described above. Whole embryos or isolated fetal livers were processed as described in the relevant sections below. When appropriate, embryos or fetal livers were photographed with a Nikon Digital Sight DS-Fi1 camera mounted on an Olympus SZX7 stereo dissection microscope using Nikon Elements D software.

Biochemical analysis of lcmt-1 embryos--E12.5 embryos were used fresh or were flash frozen in liquid nitrogen and then stored at -80°C until use. Yolk sacs were saved for genotyping. Whole embryos or fetal 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 phenylmethylsulfonyl 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-polyacrylamide gel electrophoresis and immunoblotting. Relative levels of individual proteins were determined by quantitation of immunoblots using a Fluor S-Max Chemilumimager and Quantity One Software (Bio-Rad). To achieve similar protein concentration in lysates, lysis buffer volume used was proportional to the wet weight of the tissue being lysed (10ul of lysis buffer per mg). Lysate protein levels were normalized to actin on Western blots. Antibodies used for western blotting include mouse monoclonal antibodies against PP2A A subunit (clone 4g7; Santa Cruz Biotechnologies or EMD Millipore), PP2A B subunit (2G9; EMD 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 (18). Biorad Precision Plus protein standards were used for all gels to provide molecular size markers for all blots.

Determination of the steady-state level of PP2A C subunit methylation--The steady state level of PP2A C subunit methylation was measured in whole embryo homogenates with a monoclonal antibody specific for unmethylated PP2A C subunit (4b7; EMD Millipore or Santa Cruz Biotechnologies) using our published method
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 in figures; 100% unmethylated control). Another equal aliquot of lysate from each embryo was combined with preneutralized buffer (- base treatment lanes in figures; reflect endogenous % unmethylated level). Then the untreated and base-treated aliquots were analyzed side by side on a 10% SDS-polyacrylamide gel followed by immunoblotting with 4b7 anti-unmethylated C subunit monoclonal antibody. To obtain the percent methylation of PP2A C subunit, the percent unmethylated PP2A C subunit in each embryo was first determined by quantitatively comparing the amount of 4b7 signal in the untreated lane for each embryo (level of endogenous unmethylated PP2A C subunit) to that in the matched base-treated samples (100% unmethylated controls) using a Bio-Rad Fluor-S Max Chemilumimager and BioRad Quantity One Software. Percent methylation was then calculated by subtracting the percent of unmethylated PP2A from 100%. Lysates were also probed with Actin as a loading control.

Histological analysis of lcmt-1 Embryos—E12.5 embryos were fixed in 4% paraformaldehyde in DPBS at 4°C overnight. The following day, the embryos were dehydrated through an ethanol series, embedded in paraffin, and sectioned at 4 µm thickness. Then the sections were de-waxed and rehydrated by incubation twice in xylene and then in a series of decreasing ethanol (100%; 75%; 50%; 25%; 0%). The rehydrated sections were stained with hematoxylin and eosin (H&E) or just hematoxylin when indicated. Pre-treatment in 100°C 10mM citric acid buffer pH 6.0 containing 0.25% Triton-X 100 was performed for all immunostainings for antigen retrieval. 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). Antibodies used for immunohistochemistry were rabbit polyclonal anti-cleaved caspase 3 antibody (1:100; Biocare Medical), rabbit polyclonal anti-Ki67 antibody (1:200; Abcam), and a monoclonal mouse anti-MPM-2 antibody (1:200; EMD Millipore). Some 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 (EMD Millipore). For TUNEL assays and anti-cleaved caspase 3 staining, sections were co-stained only with hematoxylin.

Flow cytometry analysis of fetal liver cell viability—Cells from E12.5 or E14.5 lcmt-1+/+ or lcmt-1-/- fetal livers were counted and 1 x 10^5 cells were used for each analysis. Fetal liver cells were blocked for 15 minutes in 5% normal mouse serum in DPBS. All cells were stained with 7-AAD (eBiosciences 00-6993) to identify dead cells and either TER-119 PE (eBioscience 12-5921), CD45.2 PE (BD Pharmingen 560695), Mac-1 PE (eBioscience 12-0112), or Gr-1 PE (eBioscience 12-5931) or matched isotype controls in 5% normal mouse serum in DPBS for 15 minutes. Cells were washed and analyzed in 2% heat-inactivated FBS in DPBS on an Accuri C6 cytometer. In several experiments, combinations of markers were analyzed simultaneously using FITC, PE, and APC-conjugated antibodies and 7-AAD.

Clonogenic Progenitor Cell Assays—Wild-type and lcmt-1 knockout fetal livers from E12.5 and E14.5 embryos were dissected free in 2% heat-inactivated fetal bovine serum (FBS) in Dulbecco’s phosphate-buffered (DPBS) followed by disaggregation into single cell suspension using a 21-gauge syringe. The cells were then washed in 2% FBS in DPBS and counted. An aliquot of cells was diluted in 2% acetic acid to lyse non-nucleated mature erythrocytes and then viable cells were counted using trypan blue to identify dead cells. 2 x 10^4 viable nucleated lcmt-1+/+ or lcmt-1-/- fetal liver 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). After 7 days, assays were scored for the number of burst-forming unit–erythroid (BFU-E), colony-forming unit–granulocyte/monocyte (CFU-GM), and CFU–granulocyte/erythrocyte/monocyte/megakaryocyte (CFU-GEMM) colonies. Benzidine-staining for hemoglobin was used to confirm or clarify identification of BFU-E and CFU-GEMM colonies.
colonies by adding a 1:10 v/v of benzidine solution (0.43% benzidine in 13% acetic acid containing freshly added H2O2 (4.3% final)) dropwise to the methylcellulose colony assay dish media and examining colonies under a microscope for dark blue staining of cells.

**KLS cell analysis**—For KLS determination in the fetal liver cells, mature red blood cells were lysed RBC lysis buffer (0.155 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA) and washed in 2% FBS in DPBS. The fetal liver cells were then stained with FITC-conjugated lineage markers (B220, Gr-1, Ter119, CD8, CD4 and NK1.1), anti-c-Kit-APC, anti-CD45.2-PE and anti-Sca1-Pacific blue or anti-Sca1-PE-Cy7. Stained cells were washed and analyzed on a Becton Dickinson LSR II flow cytometer.

**Fetal liver cell non-competitive and competitive transplantations**—Freshly isolated fetal liver cells from embryonic day 14.5 embryos genotyped the same day were manually dissociated into a single cell suspension in phosphate-buffered saline (PBS) with 2% fetal bovine serum (FBS) using a 1-mL syringe by sequential passage through 16-gauge (blunt) and 25-gauge needles. Cells were counted by hemocytometer and/or calibrated flow cytometry and used for transplantation experiments or analyzed as described. BM cells were obtained from adult mice by flushing both hind legs (tibias and femurs) into 3 mL PBS/2% FBS then nucleated cells were recovered by centrifugation, washed with PBS/2% FBS, and viable cells counted after staining with trypan blue. Overall engraftment of the donor cells was determined by staining peripheral blood leukocytes with APC-labeled antibody to CD45.2 and FITC-labeled antibody to CD45.1. The absolute number of engrafted donor cells was calculated as the %CD45.2 positive cells multiplied by the total white blood cell count per microliter of blood. Cells were then analyzed on a fluorescence activated cell sorter (FACS) LSR II flow cytometer (BD Biosciences). For multilineage analyses, cells were stained with APC-conjugated CD45.2 antibody, FITC-conjugated CD45.1 antibody, and with PE-conjugated Gr-1 or Ter119 and eFluor 450-conjugated Cd45R/B220 or CD4 antibodies before analysis. The absolute number of engrafted donor cells in each lineage was calculated as the %CD45.2 lineage positive cells multiplied by the total white blood cell count per microliter of blood. Peripheral blood cells were also stained with CD45.1, CD45.2 and lineage antibodies to analyze chimerism. Donor chimerism for each lineage was determined as: [%CD45.2 lineage positive cells/(%CD45.1 lineage positive cells + %CD45.2 lineage positive cells)] x 100.

**Statistical Analysis**—All initial statistical analyses were done using Student’s t test, and p≤0.05 was considered significant. Figure legends indicate whether error bars shown represent standard deviations or ±range. The number of
replicates and statistical significance are indicated in the results or the figure legends. For the competitive transplantation experiment graph shown, one outlier value of 39% for a recipient of WT cells was eliminated using the Grubbs’ Test for outliers with a significance of p<0.05; however, even with that value included, the average percent chimerism for the WT cells is still 76% and the p value for KO cells vs WT cells is highly significant at 4.4 x 10⁻⁵.

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Conflict of Interest—Dr. David Pallas is entitled to royalty from the sale of products related to the research described in this paper by Millipore Inc., Santa Cruz Biotechnologies Inc., and Novus Biologicals Inc. In addition, this same author serves as a consultant to Millipore. The terms of these arrangements have been reviewed and approved by Emory University in accordance with its conflict of interest policies.

Author Contributions—D.C.P. conceived project design and experimental approaches, supervised project execution, analyzed and interpreted data, helped execute fetal liver analysis, various flow cytometry, and other experiments, and helped write/edit manuscript and created and revised figures. J.A.L. helped D.C.P. establish and analyze mouse model, performed experiments establishing the gene trap insertion location, helped establish a genotyping method, performed phenotypic analyses, analyzed PP2A methylation, PP2A subunit levels, and complex formation, helped determine embryonic stage of death, performed IHC and caspase experiments and many flow cytometry analyses, helped carry out KLS and transplantation experiments, made significant contributions to experimental design and interpretations, generated initial relevant figures, and participated in revision of the manuscript. D.S. performed a number of flow cytometry experiments to analyze effects of LCMT-1 loss on cell populations, cell death, performed a large part of the data analysis, created figures, and participated in revision of the manuscript. Z.W. carried out KLS and transplantation experiments with the help of J.A.L. and D.C.P., designed initial figures for this data, and participated in revision of the manuscript. K.D.B. oversaw the transplantation experiments, provided advice and input on additional experiments, and participated in revision of the manuscript.

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Footnotes

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3The abbreviations used are: ABC, avidin biotinylated enzyme complex; BFU-E, burst forming unit-erythroid; BM, Bone Marrow; CFU, colony formation unit; CFU-GEMM, colony formation unit-granulocyte/erythrocyte-monocyte/macrophage; CFU-GM, colony formation unit-granulocyte/macrophage; DPBS, Dulbecco’s phosphate-buffered saline; ES cell, embryonic stem cell; EMP, erythroid-myeloid progenitor; FBS, fetal bovine serum; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; KO, knockout; LCMT-1, Leucine Carboxyl Methyltransferase 1; KLS, Kit Lineage-Sca-1+ hematopoietic cell population; L309, leucine 309; Leucine Carboxyl Methyl Transferase-1; PBS, phosphate-buffered saline; PME-1, Protein Phosphatase Methylesterase-1; PP2A, protein phosphatase 2A; PP2A\textsubscript{AC}, PP2A core heterodimer; PP2A\textsubscript{BAC}, methylation-sensitive PP2A heterotrimer with B family regulatory subunit; PP2Ac or PP2A C subunit, protein phosphatase 2A catalytic subunit; PP4, protein phosphatase 4; PP4c, protein phosphatase 4 catalytic subunit; PP4R, Protein phosphatase 4 regulatory subunit; PP6, protein phosphatase 6; PP6c, protein phosphatase 6 catalytic subunit; RU, repopulating unit; WT, wild-type.
Figure 1. Analysis of WT, hemizygous, and homozygous lcmt-1 knockout mouse embryos. (A) Genotyping PCR analysis of DNA isolated from yolk sacs of live WT (+/+), hemizygous knockout (+/-), and homozygous knockout (-/-) E12.5 mouse embryos. (B-C) The gene trap allele of lcmt-1 effectively blocks expression of LCMT-1 protein. (B) Lysates from whole E12.5 mouse embryos were probed for the steady-state levels of LCMT-1 and Actin (loading control) by immunoblotting. The LCMT-1 panel is compressed vertically to 75% of its original height and the actin panel to 50% to show more context and marker positions. As expected, LCMT-1 migrates at ~38kDa, just above the 37kDa molecular size marker, and actin migrates at ~43kDa (here and future Figure panels). (C) The graph shows the averages (% of WT level) and S.D. (error bars) of LCMT-1 protein expression from three independent experiments. Asterisks indicate significance versus WT embryos as assayed by Student’s t test (+/-, p = 3.6 x 10^{-5}; -/-, p = 6.5 x 10^{-14}). (D) Lcmt-1^-/- embryos die primarily between E14.5 and E16.5. The number of live embryos analyzed is indicated in parenthesis below each gestational stage.
Figure 2. Knockout of *lcmt-1* greatly reduces PP2A C subunit methylation and PP2A<sub>BAC</sub> heterotrimer formation in E12.5 embryos and causes a reduction in the steady-state levels of PP2A B, A, and C subunits. (A) PP2A methylation is greatly reduced in *lcmt-1<sup>−/−</sup>* mouse embryos. E12.5 WT (+/+) and hemizygous *lcmt-1* knockout mouse embryo homogenates were analyzed by IP using an antibody (4b7) that is specific for unmethylated-PP2A C subunit. Briefly, because base treatment demethylates the PP2A catalytic subunit, an increase in 4b7 reactivity can be seen and quantitated upon base treatment if PP2A is methylated (compare adjacent lanes). Whereas PP2A C subunit was highly methylated in WT and hemizygous *lcmt-1* knockout mouse embryo homogenates (low 4b7 signal in ‘−’ vs

**Figure 2.**
(+• lanes), the lcmt-1−/− mouse embryo homogenate was essentially completely demethylated (similar signal in the −• and +• base treatment lanes). As expected, PP2A C subunit migrates at ~36kDa, just below the 37kDa molecular size marker, in this and other Figure panels. (B) The graph shows the averages and S.D. (error bars) of methylation levels of PP2A C subunit from four independent experiments (four different litters). Asterisks indicate significance versus WT as assayed by t test (**, p = 6 x 10^-6). (C) Loss of LCMT-1 and PP2A methylation reduces PP2A_BAC heterotrimer formation. Native PP2A B subunit immunoprecipitates of lysates (B sub IP) from lcmt-1+/+, lcmt-1+/−, and lcmt-1−/− mouse embryos were probed for PP2A B subunit and for coimmunoprecipitated C subunit. The image shown is from an immunoblot representative of three independent experiments. As expected, PP2A B subunit migrates just above the 50kDa marker. B subunit ran as a doublet in this particular gel. (D) Bands from experiments like the one in panel C 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. The graph shows the average C subunit association with B subunit relative to WT ±S.D. in the four independent experiments. Asterisk indicates significance versus WT control embryos as assayed by Student’s t test (**, p = 0.006). (E) Loss of LCMT-1 and PP2A methylation greatly reduces the amount of B subunit in the entire embryo. The relative steady-state level of PP2A B subunit in whole embryo lysate was compared by normalizing B subunit levels in the embryo homogenates to actin levels. (F) The graph shows the averages and S.D. (error bars) of B subunit expression, relative to WT embryos from three independent experiments. Asterisks indicate significance versus WT embryos as assayed by t test (−/−, p = 2.4 x 10^-5). (G) Loss of LCMT-1 and PP2A methylation causes significant reductions in the amounts of A and C subunits expressed in embryos. Homogenates from WT, lcmt-1+/+, and lcmt-1−/− E12.5 embryos were prepared and then probed for levels of A and C subunits and actin (loading control) by immunoblotting. The actin bands shown here are the same as in Figure 3C because the same blot was used to probe for cleaved caspase 3 in addition to PP2A A and C subunits and actin. The migration of actin on this particular gel in the context of molecular size markers is shown in Figure 3C. (H) The graph shows the relative levels of the A and C subunits ±S.D. (error bars) from three independent experiments, determined as described in G. Asterisk indicates significance versus WT control embryos as assayed by t test (*, Csub lcmt-1−/−, p = 0.02). The reduction of A subunit in lcmt-1−/− embryos was close to statistical significance with p = 0.06.
Figure 3. Loss of LCMT-1 results in impairment of fetal hematopoiesis, smaller embryo weight, and increased cleaved caspase 3. (A) Phenotype of WT (+/+), hemizygous (+/-), and homozygous lcmt-1 knockout (-/-) embryos at E12.5. While no effect was seen upon loss of one LCMT-1 allele, lcmt-1-/- embryos are smaller and have smaller, more pale livers (arrows) than WT and hemizygous littermates. (B) E12.5 lcmt-1-/- embryos (-/-) weigh significantly less (on average, 81% of wildtype) than their WT (+/+) and hemizygous (+/-) littermates. The graph shows the averages (% of WT level) and S.D. (error bars) of 18 independent E12.5 litters that each contained embryos with all three genotypes. Asterisks indicate significance versus WT embryos as assayed by t-test (-/-, p = 9.9 x 10^-6). (C) Lcmt-1-/- embryos have increased levels of cleaved caspase 3. Wild-type (+/+), hemizygous (+/-), and homozygous (-/-) lcmt-1 knockout E12.5 mouse embryo homogenates were probed for the steady-state levels of cleaved caspase 3 (Cleaved Casp3) and Actin (loading control) by immunoblotting. As expected, cleaved caspase 3 migrates at a molecular size of ~17kDa, and actin migrates at ~43kDa. The actin bands shown here are identical to those in Figure 2G because the same embryo lysate blot was probed both for the proteins in Figure 2G (including actin) and for cleaved caspase 3. (D) The graph shows the averages (relative to WT) ± range (error bars) of cleaved caspase 3 levels in embryo torsos from two independent experiments.
Figure 4. E12.5 lcmt-1-/- fetal livers are smaller, often anemic, and show increased apoptosis. (A) Representative dissected fetal livers from WT and lcmt-1-/- E12.5 embryos show reduced size and sometimes more pale appearance of lcmt-1-/- fetal livers. (B) LCMT-1 knockout embryos (-/-) have significantly smaller livers than WT embryos (+/+) at E12.5, even when normalized to total embryo weight. Graph represents an average ±S.D. (error bars) of three independent litters. Liver weights were normalized to the total weight of each embryo. Asterisks indicate significance versus WT control livers as assayed by t test (*, p = 0.029). (C) Hematoxylin and eosin (H&E) stained sagittal sections of fetal livers from WT and lcmt-1-/- embryos reveal fragmented, pyknotic nuclei in lcmt-1-/- embryo livers (red arrowheads in inset). The boxed area in center of the lcmt-1-/- panel was magnified 50% and shown as an inset in the top right of the same panel. (D) Lcmt-1-/- fetal livers have increased cleaved caspase-3 positive cells and fragmented nuclei (brown) compared with WT fetal livers, as seen in these representative E12.5 fetal liver sections stained with antibody to cleaved caspase 3 and counterstained with hematoxylin. (E) Lcmt-1-/- fetal livers have clearly increased numbers of TUNEL-positive (brown), fragmented, pyknotic nuclei compared to WT fetal livers. The sections shown were counterstained with hematoxylin.
Figure 5. E12.5 *lcmt-1*<sup>−/−</sup> fetal livers show greatly increased caspase 3 cleavage. (A) E12.5 *lcmt-1*<sup>−/−</sup> fetal livers have greatly increased levels of cleaved caspase 3 as assayed by immunoblotting of lysates. All embryos from a single mother were dissected, homogenates were prepared from the embryo fetal livers, and the homogenates were analyzed by SDS-PAGE and Western blotting for cleaved caspase 3, which as expected migrates at a molecular size of ~17kDa, and for actin as a loading control, which migrates at ~43kDa. (B) The graph shows the averages (relative to WT) ± S.D. (error bars) of three independent experiments, each analyzing at least one litter with all three genotypes present. Asterisks indicate significance versus WT control livers as assayed by t test (*, p = 0.01).
Figure 6. E12.5 lcmt-1<sup>−/−</sup> 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 fetal liver proliferation using an anti-Ki67 antibody counterstained with hematoxylin is shown. Nearly all non-apoptotic cells in both lcmt-1<sup>+/+</sup> and lcmt-1<sup>−/−</sup> fetal livers show Ki-67 immunoreactivity. The result shown is representative of three independent comparisons of matched (same litter) WT and lcmt-1<sup>−/−</sup> embryos. (B) Immunohistochemical analysis of fetal liver proliferation using an anti-MPM2 antibody shows that there is no decrease in the number of cells in mitosis (large brown-stained cells) in lcmt-1<sup>−/−</sup> fetal livers compared to WT fetal livers. The sections shown were counterstained with hematoxylin. (C) The graph shows the results of quantitation of Ki-67 positive cells in fetal livers from three independent matched (same litter) WT and lcmt-1<sup>−/−</sup> embryos. Results show the average ±S.D. (error bars) of Ki-67 positive, non-apoptotic cells in fetal livers from three independent matched (same litter) WT and lcmt-1<sup>−/−</sup> embryos, where at least three fields of ≥ 50 cells were analyzed in each experiment. There was no significant
difference between WT and *lcmt-1*/*- fetal livers as assayed by Student’s t test. (D) The graph shows results of quantitation of MPM-2 immunoreactivity in fetal livers. Results show the average ±S.D. (error bars) of MPM-2 immunoreactivity in fetal livers of three independent matched (same litter) WT and *lcmt-1*/*- embryos, where three separate fields per embryo were quantitated. There was no significant difference between WT control fetal livers and *lcmt-1*/*- fetal livers as assayed by Student’s t test.
Figure 7. Cellularity is greatly reduced in lcmt-1<sup>−/−</sup> fetal livers due at least in part to increased cell death. (A and B) Livers from E12.5 and E14.5 lcmt-1<sup>−/−</sup> embryos contain dramatically reduced numbers of cells. (A) Graph shows the average number of cells per fetal liver ±S.D. (error bars) at E12.5 and E14.5 for WT (+/+) and lcmt-1<sup>−/−</sup> (−/−) fetal livers. The results represent ten and fifteen independent experiments with matched WT and knockout fetal livers for the E12.5 and E14.5 stages, respectively. (B) Data from panel A graphed relative to the WT fetal livers to illustrate the percent decrease in cellularity. An 82% decrease in cellularity is seen for E12.5 lcmt-1<sup>−/−</sup> fetal livers and an 87% decrease for E14.5 lcmt-1<sup>−/−</sup> fetal livers. (C) Loss of LCMT-1 causes a great increase in the number of dead fetal liver cells at both E12.5 and E14.5. (D) Ter119<sup>+</sup> fetal liver cells are disproportionately decreased in lcmt-1<sup>−/−</sup> fetal livers, especially at E12.5. Data represent averages ±S.D. (error bars) from six independent experiments. (E) Ter119<sup>+</sup> fetal liver cell death is greatly increased in lcmt-1<sup>−/−</sup> fetal livers, especially at E12.5. Data represent averages ±S.D. (error bars) from six independent experiments.
Figure 8. *Lcmt-1* loss causes multi-lineage defects in fetal liver hematopoiesis and greatly reduces the number of competent hematopoietic progenitors in fetal livers. (A) Livers from *lcmt-1<sup>-/-</sup>* embryos exhibit reduced number of cells from multiple lineages. Graph represents an average ±S.D. (error bars) of cells with the indicated lineages for six pairs (littermates) of WT (+/+) and *lcmt-1<sup>-/-</sup>* (-/-) fetal livers as determined by cell counting and flow cytometry. (B) LCMT-1 loss greatly reduces the number of hematopoietic progenitor CFUs in E14.5 fetal livers. 2 x 10<sup>4</sup> nucleated cells from single cell suspensions...
of E14.5 WT (+/+) and lcmt-1-/- (-/-) fetal 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 in culture and are shown relative to WT. All were reduced in lcmt-1-/- fetal livers compared with WT littermates both at E14.5 and at E12.5 (not shown). Graphs represent an average ± S.D. (error bars) from three independent matched (same litter) WT and lcmt-1-/- analyses. Asterisks indicate significance versus WT control fetal livers as assayed by Student’s t test (BFU-E, p = 8.3 x 10^-4; CFU-GM, p = 0.019; CFU-GEMM, p = 0.0023; total, p = 0.012).

C) LCMT-1 loss greatly reduces the c-Kit+ Lin- Sca-1+ (KLS) cell population enriched in hematopoietic stem and progenitor cells. The graph shows the results of four independent experiments and expresses the amount of KLS cells as a percentage of total CD45.2 positive fetal liver cells. The result indicates that the KLS cell population is reduced more severely than the reduction in fetal liver cellularity in lcmt-1-/- embryos.

**Figure 9.**

Figure 9. *Lcmt-1* loss causes multi-lineage defects in hematopoietic repopulating function. 1.5 million CD45.2+ fetal liver cells from WT or *lcmt-1* KO E14.5 embryos were transplanted into lethally irradiated CD45.1+ BoyJ congenic recipients in a non-competitive transplantation experiment. Five WT and four knockout recipient mice were analyzed. Percent donor chimerism, total white blood cells (WBC), and absolute counts of donor-derived lineages in recipient mice at 5 months post-transplantation revealed multi-lineage defects with the most severe defects seen in B220+ B cells and CD4+ T cells. This result is representative of two independent experimental repeats.
Figure 10. CD45.2<sup>+</sup> lcmt-I<sup>−/−</sup> cells are completely outcompeted by CD45.1<sup>+</sup> congenic bone marrow cells in competitive transplantation. 0.1 million CD45.2<sup>+</sup> fetal liver cells from WT (WT) or lcmt-I<sup>−/−</sup> (KO) E14.5 embryos were transplanted into lethally irradiated CD45.1<sup>+</sup> BoyJ congenic recipients with 0.5 million competitor BoyJ BM cells in a competitive transplantation experiment. Six WT and four knockout out recipient mice were analyzed. (A) The representative flow cytometry data shown demonstrate that lcmt-I<sup>−/−</sup> cells failed to compete with the Boy J cells. (B) The graph shows averages ±S.D. (error bar) of WT and lcmt-I<sup>−/−</sup> CD45.2<sup>+</sup> donor cells in the recipient mice at 3 months post transplantation. The severe defect in lcmt-I<sup>−/−</sup> cells seen in this experiment was also seen in a second independent experiment (not shown) where a higher ratio of donor to competitor cells was used.
LCMT-1 Loss Causes Defects in Fetal Hematopoiesis

Figure 11. LCMT-1 coordinately regulates the methylation of the PP2A subfamily of protein phosphatases and thus their function in hematopoiesis and hematopoietic repopulating activity. Schematic shows a model of LCMT-1 function in hematopoiesis and hematopoietic repopulating function via methylation of the catalytic subunits of PP2A, PP4, and PP6 (PP2Ac, PP4c, and PP6c, respectively). Arrows with question marks indicate the need to determine the specific contributions of the methylated forms of each of these phosphatases in these functions, including the relevant methylation-dependent complex(es) and effects on HSCs. While it is not yet clear whether there are PP6 methylation-dependent complexes regulated by LCMT-1 (19), if they do not exist, methylation of PP6c still might regulate PP6 function via another mechanism. In this model, loss of LCMT-1 leads to dysfunction of all three PP2A subfamily phosphatases, while in normal cells regulation of LCMT-1 would coordinately regulate these phosphatases to modulate hematopoiesis and other cellular functions. Finally, based on this model, independent regulation of these phosphatases by LCMT-1 would likely require distinct colocalization or scaffolding of LCMT-1 with a particular PP2A subfamily phosphatase.
Global loss of Leucine Carboxyl Methyltransferase-1 causes severe defects in fetal liver hematopoiesis
Jocelyn A. Lee, Zhengqi Wang, Danielle Sambo, Kevin D. Bunting and David C. Pallas
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