An Acyltransferase Controls the Generation of Hematopoietic and Endothelial Lineages in Zebrafish

Jing-Wei Xiong,* Qingming Yu,* Jiaojiao Zhang, John D. Mably

Abstract—Hematopoietic and endothelial cells develop from a common progenitor, the hemangioblast, or directly from mesodermal cells. The molecular pathway that regulates the specification of both cell lineages remains elusive. Here, we show that a lysocardiolipin acyltransferase, lycat, is critical for the establishment of both hematopoietic and endothelial lineages. We isolated lycat from the deletion interval of cloche, a zebrafish mutant that has dramatically reduced hematopoietic and endothelial cell lineages. Reduction of lycat mRNA levels in wild-type zebrafish embryos decreases both endothelial and hematopoietic lineages. lycat mRNA rescues blood lineages in zebrafish cloche mutant embryos. E165R and G166L mutations in the highly conserved catalytic domain in Lycat abolish its function in zebrafish hematopoiesis. Epistasis analysis supports that lycat acts upstream of scl and etsrp in zebrafish hemangioblast development. These data indicate that lycat is the earliest known player in the generation of both endothelial and hematopoietic lineages. (Circ Res. 2008;102:1057-1064.)

Key Words: acyltransferase ■ hemangioblast ■ hematopoiesis ■ vasculogenesis ■ zebrafish ■ cloche

It was proposed nearly a century ago that a common progenitor generates both the hematopoietic and endothelial lineages.1 This hypothesis was based on the observation that blood and endothelial cells develop within close proximity of each other in the extraembryonic yolk sac. Using in vitro mouse and human embryonic stem cell (ESC) differentiation assay, a blast colony–forming cell was characterized that clonally generates both endothelial and hematopoietic cells in the presence of vascular endothelial growth factor and bone morphogenetic protein 4.2–4 The blast colony–forming cell was later isolated in vivo from the posterior primitive streak of midgastrulation mouse embryos.5 A single-cell fate mapping in the zebrafish gastrula by uncaging fluorescent dextran suggests that hemangioblasts are interspersed with hematopoietic and endothelial progenitors in the ventral–lateral mesoderm.6 However, several studies in mice have shown that endothelial and hematopoietic lineages are independently derived from mesodermal cells.7,8

The zebrafish cloche (clo) mutant has significantly reduced endothelial and hematopoietic lineages, as well as no endocardium.9 cloche is thought to act at the level of thehemangioblast and represents the first single gene mutation that almost eliminates both endothelial and hematopoietic lineages. Epistasis analyses have shown that cloche acts upstream of zbp-89 (a Krüppel-like zinc finger–containing transcription factor), scl (a basic helix–loop–helix transcription factor), lmo2 (a LIM-containing protein), gata1 (GATA binding protein 1), fli1 (an ETS domain–containing protein), flk1 (fetal liver kinase 1), and etsrp (a ETS1-related protein) in hematopoietic and endothelial cell development in zebrafish.5–7 In addition, genome-wide microarray analyses have revealed that cloche specifically regulates a panel of hematopoietic and endothelial genes.18–20 All of these early studies have proved that cloche is the earliest gene acting in hemangioblast development. However, it remains unknown what is the molecular nature of the cloche gene.

Here, we have cloned lycat as a candidate gene for the cloche locus in zebrafish. The mouse homolog of Lycat is strongly expressed in the heart and is enriched in the Flk1+/Scl– and Flk1–Scl+ hemangioblast populations in embryoid bodies.21,22 We have previously reported that mouse Lycat gene plays essential roles in hemangioblast, hematopoietic, and endothelial lineage development using loss-of-function (siRNA knockdown) and gain-of-function (over- and ectopic expression) analyses in mouse ESC differentiation system.24 Both mouse and zebrafish lycat genes encode a transmembrane acyltransferase with a C-terminal endoplasmic reticulum localization signal. The mouse Lycat protein has acyltransferase enzymatic activities using lysocardiolipin as a substrate.22 Protein acyltransferase has emerged as an important player in regulating protein trafficking, sorting, and development.23,24 The porcupine (Porc) gene in fly was isolated and characterized as an endoplasmic reticulum–localized acyltransferase that is required for addi-

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tion of palmitate to Wnt3a and Wnt1 for producing fully functional Wnt proteins.25,26 Skinny hedgehog (Ski) was found to be another acyltransferase that is required for palmitoylation and biological activity of the Hedgehog.27 Therefore, some protein acyltransferases can be important signaling components regulating embryonic patterning and organogenesis. We therefore analyzed the zebrafish lyca gene function in hemangioblast, endothelial, and hematopoietic lineage development. Morpholino-mediated knockdown of zebrafish lyca results in the reduction of both endothelial and hematopoietic lineages and elimination of the endocardium, which is very similar to that in cloche. Microinjection of zebrafish lyca mRNA into cloche mutants partially rescues the cloche phenotype. Lyca acts upstream of several known hemangioblastic genes. Although lyca is deleted in the spontaneous cloche allele, we have not found causative mutations in the open reading frame of lyca from 2 ethynitrouracil-induced cloche alleles. This study suggests that lyca is the earliest known player in hematopoietic and endothelial (including endocardial) cell development, probably acting on the level of hemiogenasts.

Materials and Methods

Isolating lyca From the cloche Genetic Interval and Zebrafish Strains

The cloche alleles clo<sup>goa</sup> and clo<sup>h276</sup> have been previously described.28 A new ethynitrouracil-induced allele, clo<sup>goa</sup>, was also used, which was isolated in the laboratory of Mark C. Fishman in 2000. Transheterozygous zebrafish crosses from any 2 of the 3 gata1<sup>:GFP</sup> and Tg(flk1<sup>:GFP</sup>)29,30 (a gift from Dr Shuo Lin, General Hospital institutional guidelines. were raised and handled in accordance with the Massachusetts University of California, Los Angeles) were crossed with heterozygous Tg(flk1<sup>:GFP</sup>) embryos. Injected embryos were incubated at 28.5°C for examination of phenotypes or were fixed for in situ hybridization. The pictures were taken using a Leica MZ16 fluorescence microscope.

In situ Hybridization and Histology

In situ hybridization and histology were done using antisense lyca, flk1, flk1, etsp, scl, bmo2, and gata1 RNA probes.11–14,16

Reverse Transcription–PCR

Zebrafish embryos were pooled from Ctr-MO; clo mutant embryos from clo<sup>goa</sup>, clo<sup>h276</sup>, or clo<sup>goa</sup> heterozygous crosses; or lyca-MO3 morphant embryos at 18 or 30 hours postfertilization (hpf). Wild-type TL embryos were collected at different time points. Total RNA was isolated from embryos using TRizol reagents (Invitrogen). First-strand cDNA was synthesized using the SuperScript II RT system (Invitrogen). Semiquantitative PCR for zebrafish lyca, flk1, and β-actin were done with Qiagen Taq polymerases and quantitative PCR were done with TaqMan or SYBR Green probes using the 7000 Sequence Detection System (ABI Prism).

Site-Directed Mutagenesis and Rescue of gata1 Expression in Homozygous clo<sup>clom39</sup> Mutant Embryos

The wild-type lyca cDNA was used for site-directed mutagenesis. Mutations were created using the GeneEditor in vitro Site-Directed Mutagenesis System (Promega) following the instructions of the manufacturer. The E165R-G166L mutation was generated by a substitution of glutamic acid (E) to arginine (R) at amino acid 165 and glycine (G) to leucine (L) at amino acid 166. The following primers were used for making E165R-G166L: 5'-TGCAGCT-GCTGCTGTTCCTCCGTGCTGATCACCAGCTCA-3' (forward) and 5'-AGGGGACACGACAGCTGCACGGCTCTCT-3' (reverse). Capped mRNAs were synthesized from wild-type lyca cDNA and E165R-G166L cDNA templates using mMESSAGE mMACHINE SP6 and T7 Kits (Ambion). mRNA was injected into 1- or 2-cell-stage clo<sup>clom39</sup> or clo<sup>goa</sup>; Tg(gata1:GFP) embryos from heterozygous clo crosses. Injected embryos were incubated at 28.5°C and later fixed with 4% paraformaldehyde. gata1 expression in the injected embryos was examined by in situ hybridization. Embryos were subsequently genotyped by PCR with the marker Z1412 that is deleted in the clo<sup>clom39</sup> allele or with marker D, a CA repeat marker for clo<sup>goa</sup> allele (supplementary Figure 1). Marker D is as follows: forward primer, 5'-TCTGATGTCGCTGAG-3'; reverse primer, 5'-GCCGTGTGTTAACCCTA-3'. Marker Z1826 is outside of the deletion interval in the clo<sup>clom39</sup> allele, which was used as DNA quality control.

Results

Antisense lyca Morpholinos Reduce Endothelial and Hematopoietic Lineages

Embryos injected with any 1 of the 3 antisense morpholinos targeting the zebrafish lyca exon 2 splice donor site (lyca-MO2 or MO2), the exon 3 splice donor site (lyca-MO3 or MO3), and the translational start site (lyca-MO1 or MO1), respectively, significantly reduced blood cells and blood vessels and eliminated the endocardium (Figure 1, supplemental Figure II, and supplemental Table I). Embryos injected with the lyca-MO3 generated a mutant transcript retaining the fourth intron sequence, leading to out-of-frame translation after the amino acid G122 (Figure 1b, arrowhead MT; sequencing data not shown). Injection of lyca-MO3 morpholinos into the Tg(flk1:GFP) or the Tg(gata1:GFP) transgenic embryos dramatically reduced flk1- or gata1-expressing cells in the morphants, respectively (Figure 1h and 1k). Eighty-eight percent (132/150) of Tg(flk1:GFP) embryos microinjected with lyca-MO3 showed hematopoietic, endothelial, and endocardial defects (supplemental Table I). Coinjection of the zebrafish lyca-MO3 and mouse Lycat mRNA rescued the morpholino-induced reduction of flk1- and gata1-expressing cells in the injected embryos (Figure 1i).
and 1l; supplemental Table I), confirming that the morphant phenotype was caused by the knockdown of the zebrafish lycat gene. These data indicate that mouse Lycat is a functional ortholog of zebrafish lycat.

The zebrafish lycat-MO3 reduced the expression of scl in the intermediate cell mass in a dose-dependent manner (Figure 1m through 1p). Morphants injected with lycat-MO1 or MO1 (Figure 1s), lycat-MO2 or MO2 (Figure 1t), or lycat-MO3 or MO3 (not shown) showed significantly reduced fli1 expression in vessels. The fli1 expression in lycat-MO2 (Figure 1t) or lycat-MO3 morphants (not shown) is similar to that in homozygous clo embryo (Figure 1r), whereas very-low-level
flil expression remained in trunk vessels in lycat-MO1 morphants (Figure 1s). Lycat-MO2 morphant embryos have similar fli1 expression as that in cloche mutant embryos; therefore, the expression level of fli1 in Figure 1t represents experimental variation. The lycat-MO3 is the most potent one among the three antisense lycat morpholinos tested; therefore, lycat-MO3 is used for subsequent experiments. In summary, morpholino-mediated lycat knockdown results in a similar phenotype as that in cloche, suggesting that lycat is essential for hemangioblast, endothelial, and hematopoietic lineage development and that flk1, scl, gata1, and fli1 function downstream of lycat.

**Zebrafish and Mouse Lycat but Not an Enzyme-Defective Lycat Partially Rescues Hematopoiesis in cloche Mutant Embryos**

We found that both zebrafish and mouse Lycat mRNA partially rescued cloche embryos (Figure 2). In cloche mutant embryos from clo<sup>−/−</sup> Tg(gata1::GFP)<sup>−/−</sup> sibling crosses, the gata1::GFP expression was remarkably increased after injection with lycat mRNA (Figure 2c and 2d). Although the rescued cloche embryos lacked an intact circulation, they had significant number of blood cells, whereas uninjected cloche mutants had very few blood cells (data not shown). Using Tg(gata1::GFP) as a marker, the lycat mRNA injection was able to rescue 38.5% (35/91) of cloche mutant embryos (supplemental Table 1), genotyped with marker D (supplemental Figure 1a and data not shown). We failed to rescue the flk<sup>+</sup> and etsrp<sup>+</sup> endothelial cells in cloche embryos injected with lycat mRNA (not shown). These data are not entirely supportive of lycat as the cloche gene, although ectopic mRNA expression of a developmentally regulated gene does not always rescue its genetic mutant phenotype in zebrafish.

Recent studies have shown that acyltransferase from bacteria to mammals contains highly conserved catalytic motifs including H(X)4D and EGTR. The zebrafish Lycat protein contains both H(RTRL)D (amino acids 85 to 90) and EGTD (amino acids 165 to 168) motifs (supplemental Figure Ib, shadowed). We examined whether the EGTR motif in Lycat is essential for hematopoiesis in zebrafish. We generated a mutant Lycat with substitutions of both E165R and G166L (named E165R-G166L) by site-directed mutagenesis. We found that the E165R-G166L mutant lycat mRNA rescued hematopoietic gata1 expression only in 2% (2/89) of homozygous clom39 embryos, whereas the wild-type lycat mRNA rescued 47% (31/66) of cloche embryos (Figure 2g and 2j). Gata1-expressing homozygous cloche embryos were confirmed by genotyping with marker Z1412 deleted in the clom39 allele and a control marker Z1826 outside of the clo<sup>−</sup> deletion interval (not shown). These data establish that the catalytic activity of the Lycat protein is required for its function in hematopoietic development in zebrafish.

**Zebrafish lycat Acts Upstream of scl and etsrp to Specify the Hemangioblast**

To determine the role of lycat gene in zebrafish hemangioblast development, we examined the interactions of lycat with...
scl and etsrp in the lateral plate mesoderm (LPM) in early-somitogenesis-stage embryos. The flk1, etsrp, scl, lmo2, and gata1 genes were not expressed or significantly reduced in both anterior and posterior LPM in lycat-MO morphants (87% [47/54] have reduced flk1; 73% [38/52] have reduced etsrp; 96% [45/47] have reduced scl; 80% [32/40] have significantly reduced lmo2; and 95% [37/39] have reduced gata1; Figures 3b, 3l, 4b, 4g, and 4k). fli1 was not expressed.
in the anterior LPM in lycat-MO3 morphant embryo (49% [18/37]) (Figure 4h). The expression profile of these genes in lycat-MO3 morphants is very similar to that in homozygous clo embryos (data not shown). scl mRNA rescued flk1 (66%, 40/61), fli1 (88%, 44/50), etsrp (100%, 62/62), lmo2 (59%, 26/44), and gata1 (95%, 40/42) expression in lycat-MO3 morphants (Figures 3c, 3i, 3m, 4h, and 4i). In addition, overexpression of scl mRNA in lycat-MO3 morphants led to ectopic fli1 and lmo2 expression (Figures 3i and 4h) and increased the more lateral stripe of flk1- and etsrp-expression domains (Figure 3c and 3m, arrowheads) and expanded gata1-expression domain (Figure 4i). Similarly, overexpression of etsrp mRNA in MO3 morphants also rescued flk1 (69%, 24/35), fli1 (80%, 28/35), scl (78%, 25/32), lmo2 (53%, 16/30), and gata1 (82%, 23/28) expression, as well as led to ectopic flk1, fli1, scl, lmo2, gata1 expression (Figures 3d, 3j, 4c, 4i, and 4m). Therefore, lycat acts upstream of both scl and etsrp and is essential for fli1, flk1, etsrp, scl, lmo2, and gata1 expression during development of hemangioblasts, endothelial, and hematopoietic cells.

To further examine the relationship between lycat and etsrp, etsrp morpholinos were used to knockdown etsrp function in early somitogenesis embryos. In etsrp morphant (etsrp-mo) embryos, the flk1 expression was significantly reduced (93%, 28/30) in both anterior and posterior LPM (Figure 3e), fli1 was reduced in the anterior LPM (not shown), scl was reduced (79%, 45/57) in the anterior part of the posterior LPM (Figure 4d, arrowheads), and lmo2 and gata1 expressions were not affected (not shown). The etsrp morphant phenotypes could not be rescued by overexpression of lycat mRNA (Figures 3f and 4e) (100% [28/28] have reduced flk1 expression; 77% [20/26] have reduced scl expression). Together with the rescue of lycat-MO3 morphant phenotypes by etsrp mRNA (Figures 3d, 3j, 4c, 4i, and 4m), our data support that lycat acts upstream of etsrp in hemangioblast development and etsrp is critical for flk1-expressing angioblastic lineage and plays a role in generation of gata1- and lmo2-expressing hematopoietic lineages.

Antisense morpholino knockdown of scl was also applied to examine the scl and lycat relationship in hemangioblast development. scl morphant showed reduced gata1 expression (44%, 32/72) (Figure 4n) compared with that of control embryo (Figure 4j). This scl morphant phenotype could not be rescued by coexpression of lycat RNA (60%, 41/68) (Figure 4o). We have also carried out knockdown of both scl
isoforms (sclα and sclβ) as described\textsuperscript{31} and have found that lyCat RNA could not rescue gata1 expression in sclα and sclβ morphant embryos (data not shown). The lmo2 expression was neither affected in embryos injected with scl morpholinos nor embryos injected with both scl morpholino and lyCat RNA (not shown). Therefore, scl acts downstream of lyCat and is essential for the derivation of gata1-expressing hematopoietic lineages.

**Discussion**

In this study, we have isolated and characterized lyCat as an important hemangioblastic gene and a cloche candidate gene in zebrafish. LyCat is among the acyltransferase family members that have 3 putative transmembrane domains and a highly conserved acyltransferase domain (supplemental Figure Ib). Our loss-of-function (morpholino knockdown) and gain-of-function (ectopic RNA expression) analyses have firmly demonstrated the roles of lyCat in hemangioblast, endothelial (including the endocardium), and hematopoietic lineage development. This observation is further supported by our previous report on mouse lyCat function in these lineages during ESC differentiation.\textsuperscript{21} Mouse LyCat, or Alcat1, is also characterized as a lysocardiolipin acyltransferase that plays an important role in the cardiolin remodel pathway.\textsuperscript{22} However, our functional analyses of LyCat in zebrafish and ESC systems strongly suggest that LyCat may also act as a protein acyltransferase that regulates important hemangioblastic proteins. Identifying lyCat is a highly significant finding and will lead to novel molecular insights into the origin and formation of hemangioblasts. Second, lyCat is within the cloche genetic interval, and the lyCat morphant phenotype is remarkably similar to that of the cloche mutant. Therefore, lyCat remains as a strong candidate for the cloche locus. Although this report has not provided direct evidence for this, identification of lyCat and many close genetic markers related to the cloche locus certainly moves the field a step closer to ultimately revealing the molecular field of cloche.

Genetic studies in *Drosophila* and *Caenorhabditis elegans* have identified acyltransferases that are required for the generation of active morphogens, Wingless, and Hedgehog.\textsuperscript{26,27} Hedgehogs and Wnts are involved in hematopoiesis and vasculogenesis\textsuperscript{26,32,33} as well as many other important functions. Therefore, endoplasmic reticulum–associated, transmembrane acyltransferases are important for regulating embryonic patterning and organogenesis in different species. Zebrafish LyCat is predicted as an endoplasmic reticulum–localized transmembrane acyltransferase. Mouse LyCat has acyltransferase activity for Cardiolipin and may modify additional proteins.\textsuperscript{22} We have shown that mouse LyCat is enriched in the Fli1\textsuperscript{−}/Scl\textsuperscript{−} hemangioblasts and plays an important role in hematopoietic and endothelial cell development during ESC differentiation in vitro.\textsuperscript{31} It is possible that LyCat could act as a protein acyltransferase modifying 1 or several signaling components that control hematopoietic and endothelial specification in the early embryo. The identification of LyCat targets will reveal the mechanism of LyCat in this process. There is no consensus sequence for an acyltransferase target domain making it challenge to identify potential LyCat targets.\textsuperscript{34} Future studies are required to determine whether zebrafish LyCat directly modifies important known molecules, such as the Hedgehogs, Wnts, Etsrp, Scl, Runx1, and vascular endothelial growth factor pathway components.\textsuperscript{16,17,26,33} Another area of interest is to discover novel LyCat targets that influence hemangioblast development by using an unbiased genome-wide proteomics approach.\textsuperscript{35}

The zebrafish clo mutant has significantly reduced hemangioblastic and endothelial lineages.\textsuperscript{9} Gain-of-function analysis in zebrafish suggests that scl, lmo2, and etsrp are involved in specifying the hemangioblast from early lateral posterior mesoderm.\textsuperscript{11,14,16,17,36,37} However, morpholino knockdown or genetic mutant analyses of these genes have revealed that none of them is absolutely required for the formation of both endothelial and hematopoietic lineages in zebrafish embryos. Our data support that lyCat is required for flk1-, etsrp-, fli1-, gata1-, scl-, and lmo2-expressing cell formation (Figures 1, 3, and 4). To our knowledge, lyCat is the first hemangioblastic gene required for both endothelial and hematopoietic lineages in zebrafish embryos. Both scl and etsrp mRNA rescue lyCat morphant phenotype in endothelial and hematopoietic development (Figures 3 and 4), whereas lyCat mRNA fails to rescue etsrp or scl morphant phenotypes (Figures 3f, 4e, and 4o), supporting the notion that lyCat is upstream of both scl and etsrp. Our studies and others also support that etsrp, downstream to lyCat, is essential for flk1-expressing endothelial and gata1-expressing hematopoietic lineages, whereas scl is essential for generation of the gata1-expressing hematopoietic lineages and plays minor roles in flk1-expressing endothelial lineages (Figures 3 and 4 and data not shown).\textsuperscript{17}

The zebrafish lyCat zygotic expression pattern was unclear by RNA in situ hybridization probably because of very low levels of expression (data not shown). Zebrafish lyCat mRNA was detected in embryos from 1-cell-stage to 72 hpf by RT-PCR (data not shown). Using RNA in situ analysis with antisense lyCat probes, we did find transgenic lyCat mRNA expression in Tg(fli1:lyCat) embryos, in which lyCat is driven by the zebrafish fli1 promoter (data not shown), supporting that the antisense lyCat probes that we used were good and that the uncertainty in detecting endogenous lyCat mRNA may be attributable to its low level of expression (data not shown). Future studies need to fill this gap as well. We fully anticipate that deciphering LyCat targeting proteins and its expression pattern presumably in hemangioblast domains will lead to both molecular insights in hemangioblast development and therapeutic utility in regenerative medicine.

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Disclosures

None.

References

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Supplementary Materials

Zebrafish *lycat* was isolated as a candidate gene for *cloche*

We mapped *clo* close to the microsatellite markers Z17223, Z22194 and Z10362 on the telomere of Chromosome 13. *clo* is a spontaneous deletion allele missing about 0.26 cM between markers Z1826 and Z709 (Supplementary Fig.1a). We performed genetic fine mapping with 4173 homozygous *clo* mutants from heterozygous *clo* crosses. We found that *clo* was within 0.13 cM and 0.02 cM to two markers Z4252 and Z12051 on the centromere site, respectively, and that *clo* was within 0 cM to marker Z709. Z709 was then used to isolate YAC2 (35K05 from the Children’s Hospital Boston-YAC library) and BAC4 (109E22, Incyte-Zebrafish BAC from Dr. Peter de Jong) by PCR-based screens. YAC2 and BAC4 were shot-gun sequenced and assembled. Six microsatellite markers including Marker D in the telomere side of *clo* were isolated from the assembled sequence, which were found to be 0.01 cM to *clo* (Supplementary Fig. 1a). Therefore, the *clo* genetic interval was determined between Z12051 and Marker D. A 35 kb contig in the YAC2 assembly covered this *clo* interval, in which six genes were identified. Morpholino knocking-down *lycat* but not other five genes phenocopied the *clo* mutant (Fig. 1 and Supplementary Fig. 2). The zebrafish Lycat protein is 62% identical to the mouse homologue with conservation of the acyltransferase motif and the ER retention signal on the C-terminus.

Since sequencing of the *lycat* gene in two ENU-induced alleles, *clo* and *clo* has not revealed causative mutations in the *lycat* coding sequence (data not shown), we acknowledge that *lycat* may not be responsible for the *cloche* locus. Zebrafish *lycat* is embedded in highly repetitive sequences in a telomere region that
makes finding causal point mutations challenging. Because of this, we have not been able to obtain 100% of lycat genomic sequences. lycat mRNA levels are slightly down-regulated in clo\textsuperscript{m378} and clo\textsuperscript{v987b} embryos (data not shown). We have recently generated an anti-Lycat mouse antibody that could recognize recombinant zebrafish Lycat protein expressed in bacteria, but we have not had luck to detect endogenous Lycat protein in zebrafish embryos using Western blotting. Therefore, we are not able to evaluate Lycat protein levels in cloche mutant embryos at this time. We have isolated a 3.0 kb genomic DNA fragment, including sequences immediately upstream of the predicted start ATG codon, and we found that GFP driven by this promoter is expressed in ICM, nascent vasculature and blood cells during somitogenesis by a transient expression analysis (data not shown). The identification of conserved motifs in the 3.0 kb lycat promoter across zebrafish, fugu, mouse and humans will pinpoint the critical regulatory elements. We are currently trying transgenic rescue of cloche mutants with the lycat cDNA driven by the various portions of the promoter sequences. Subsequent sequence analysis will be needed to identify the point mutations in the regulatory elements. Therefore it remains to be determined how lycat is related to the cloche gene.

**Lycat morphant embryos have no endocardium and significantly reduced endothelial and hematopoietic cells and fail to form any lumenized vessels**

To address if there are any endothelial and endocardial cells in lycat-MO3 morphant embryos, we have done a systemic evaluation of the flk1\textsuperscript{+}-expressing cells in different stages of transgenic flk1:GFP embryos injected with lycat-MO3. We observed very few flk1\textsuperscript{+} cells in the posterior lateral plate mesoderm (LPM) of lycat-MO3
morphant embryos at 10S to 18S stages (Supplementary Fig. 2d; not shown) while many flk1+ cells in the anterior and posterior LPM of control transgenic embryos (Supplementary Fig. 2b; not shown). There were flk1+ endodermal cells that will contribute to the formation of pharyngeal in lycat-MO3 morphant embryos at 18S (Supplementary Fig. 2d), which is remarkably similar to that in cloche mutant embryos (data not shown)\(^1\). We observed increasing numbers of flk1+ cells and hematopoietic cells in the dorsal and posterior of the yolk extension in lycat-MO3 morphant embryos from 24 to 48 hpf (Fig. 1h; Supplementary Fig. 2f; Supplementary Fig. 3m). We found the flk1+ cells in MO3 morphants frequently started to migrate toward the anterior and dorsally at 48 hpf (Supplementary Fig. 2f), which are also observed, although less frequently, in cloche embryos at 48 to 72 hpf (not shown). This may reflect subtle different phenotypes between lycat morphants and cloche embryos, or alternatively reflect gradual loss of morpholino effects in lycat morphant embryos after 48 hours post injection. Furthermore, there was completely loss of the flk1+ endocardial cells in the heart of lycat-MO3 morphant embryos (Supplementary Fig. 2j), compared with those in control morphant embryos (Supplementary Fig. 2h). This was confirmed by hematoxylin and eosin staining in transverse sections, which we observed only a single layer of myocardial cells in the MO3 morphant heart at 48 hpf (Supplementary Fig. 2l), compared with both endocardial and myocardial layers in the control morphant heart (Supplementary Fig. 2k). These observations substantiate that lycat is required for the formation of endocardial, endothelial and hematopoietic cells in zebrafish.

To address if the trace amounts of endothelial cells around the yolk extension can form functional vessels, we examined endothelial genes expression in vessels of ctrl and
MO3 morphant embryos with tie1 and etsrp (a-j) and by flk1:GFP (k, l, n, o). We found that both tie1 and etsrp were reduced in MO3 morphants (b, d), and tie1$^+$ and etsrp$^+$ endothelial cells in MO3 morphants (f, h, j) failed to form lumenized dorsal aorta and axial veins as those in ctrl embryos (e, g, i) at 20 and 48 hpf. Similarly, the flk1$^+$ cells in MO3 morphant (o) failed to form lumenized dorsal aorta and axial vein as those in ctrl (n) embryos at 48 hpf. These observations support that lycat is also required for vascular endothelial cell differentiation in addition to its critical role in the generation of both endothelial and hematopoietic lineages.
**Supplementary Table 1. Statistics of lycat morphants, rescued morphants and clo embryos by lycat RNA.**

a. The Ctr-MO was dissolved in egg water at 0.5 mM and was injected into 1- to 2-cell Tg(flk1:GFP) embryos. None of the 65 injected embryos exhibit the cloche phenotype at 36 hpf and 48 hpf. This represents one of five independent experiments.

b. The zebrafish lycat morpholino MO3 was dissolved in egg water at 0.3 mM and was injected into 1- to 2-cell Tg(flk1:GFP) embryos. 132 out of 150 injected embryos showed a clear cloche phenotype with a lack of flk1+ endocardium, blood vessels and visible blood cells at 36 hpf and 48 hpf. 18 embryos showed mild defects in blood vessels and blood cells but had flk1 expression in the endocardium, which were scored as wild-type phenotype. This represents one of five independent experiments.

c. Zebrafish lycat MO3 (0.3 mM) and lycat RNA (0.3 mg/ml) were co-injected into 1- to 2-cell flk1:GFP embryos. 36 out of 69 injected embryos (52%) had no clo phenotype at 36 hpf. This represents one of three independent experiments.

d. Zebrafish lycat RNA (0.3 mg/ml) was injected into embryos from clo<sup>v6087b/+;gata1:GFP<sup>tg</sup>/<sup>tg</sup> sibling cross, and 35 out of 91 (38.5%) genotypic homozygous cloche embryos showed a wild-type phenotype. Genotyping was done by PCR using marker D (Supplementary Fig. 1a). This represents one of three independent experiments.
## Supplementary Table 1

<table>
<thead>
<tr>
<th></th>
<th>clo (phenotype)</th>
<th>Wild-type (phenotype)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>a. 0.5mM Ctr-MO</strong></td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td><strong>b. 0.3mM MO3</strong></td>
<td>132</td>
<td>18</td>
</tr>
<tr>
<td><strong>c. 0.3mM MO3+ 0.3mg/ml Lycat RNA</strong></td>
<td>33</td>
<td>36</td>
</tr>
<tr>
<td><strong>d. 0.3 mg/ml zebrafish lycat RNA</strong></td>
<td>56</td>
<td>35</td>
</tr>
</tbody>
</table>
Supplementary Figure 1. A zebrafish lycat gene was isolated from the clofv087b genetic interval. a, a genetic and physical map of the cloche locus. cloche was mapped on the telomere of chromosome 13 close to the microsatellite markers Z17223, Z22194 and Z10362 using bulked segregant analysis. Fine mapping was done with 4173 clofv087b mutants. The clofv087b interval was defined between Z12051 and a microsatellite marker D derived from BAC109E22 (Incyte-Zebrafish BAC by Dr. Peter de Jong). The clom39 allele is a spontaneous mutation, and was subsequently discovered to have a deletion during the course of cloche mapping. Physical walking was done using PCR-based screens for zebrafish genomic clones, and the ends of BAC, PAC or YAC clones were rescued and sequenced. BAC4 (BAC109E22 from Incyte-Zebrafish BAC by Dr. Peter de Jong) and YAC2 (YAC2=35K05 from the CHB-YAC library) were identified to cover the clofv087b interval. BAC4 and YAC2 were subjected to shot-gun sequencing, and the sequence was assembled using the Phred/Phrap/Consed programs. We assembled several contigs, and found a single contig with 35 Kb in length that covers the critical clo interval, and derived microsatellite markers from CA repeats in the assembled sequences. Bioinformatic tools including Genscan and BLAST were used to identify exons, introns and genes in this contig. Six zebrafish genes including Peas (Accession number NM_057161), PACS1a (NM_018026), CGI-127 (AF172940), LYCAT/ALCAT1 (NM_182551), LBH (NM_030915) and hole (BC004221) were found (1a). The clom39 deletion spans about 0.26 cM between Z1826 and the T7 end of BAC4. Zebrafish lycat gene contains seven exons and spans about 13,000 bp in length. b, The zebrafish lycat cDNA we isolated is identical to the full-length cDNA clone deposited in the GeneBank (BC066444) that contains 2452 nucleotides and encodes a 388-amino acid protein (1b).
**Lycat** has a conserved acyltransferase domain (Italian bold) with highly conserved catalytic sites including $\text{H(X)4D}$ and EGTR (shadowed), three putative transmembrane domains (underlined) and a putative endoplasmic reticulum retention signal (underlined bold).

**Supplementary Figure 2.** Lycat-MO3 eliminates the endocardium and reduces endothelial cells in transgenic flk1:GFP embryos. (a-j) The anterior to the left and the posterior to the right; (k, l) dorsal to the upper and ventral to the lower. a-d, bright-field (a) and dark-field (b) imaging of Ctr-MO-injected embryo at 18S (18-somite stage), and bright-field (c) and dark-field (d) imaging of MO3-injected embryo at 18S. White arrowheads point to the anterior and posterior LPM; red arrowheads point to the flk1$^+$ endodermal cells (en). e-f, lateral view of the posterior trunk of Ctr-MO (e) and MO3 (f) -injected embryo at 48 hpf. a, the aorta; v, the axial vein; and isv, intersegmental vessels. g-j, lateral view of the anterior part of Ctr-MO (g, bright-field; h, dark-field) and MO3 (i, bright-field; j, dark-field) –injected embryos at 48 hpf. hrt, the heart. (k, l) Hematoxylin and eosin staining of transverse sections of Ctr-MO (k) and MO3 (l) –injected embryos at 48 hpf. ec, the endocardium; and mc, the myocardium.

**Supplementary Figure 3.** Lycat MO3 morphant embryos fail to form lumenized vessels. a-d, and k-m: the anterior to the left and dorsal on the top; e-j, transverse JB4 sections cross the yolk extension as shown in panel a with the dorsal on the top; and n-o, transverse vibrotome sections cross the yolk extension as shown in panel k and optical sections of flk1:GFP taken by LSM5 confocal microscope, with the dorsal on the top.
b: in situ analysis of *tie1* in control (ctrl) (a) and MO3 morphant (mo3) (b) embryos at 20 hpf; c-d: in situ analysis of *etsrp* in ctrl (c) and MO3 morphant (d) embryo at 20 hpf; e-h: transverse sections of *tie1* stained ctrl embryo at 20 hpf (e), MO3 morphant embryo at 20 hpf (f), ctrl embryo at 48 hpf (g) and MO3 morphant embryo at 48 hpf (h); i-j: transverse sections of *etsrp* stained ctrl (i) and MO3 morphant (j) embryos at 20 hpf; k-j: dark-field images of ctrl (k) and MO3 morphant (l) flk1:GFP embryos at 48 hpf; m, blood cells (bl) observed at the posterior to the yolk extension in MO3 morphant embryo at 48 hpf; and n-o: transverse optical sections of ctrl (n) and MO3 morphant (o) flk1:GFP embryos at 48 hpf. nc, notochord; ao, dorsal aorta; av, axial vein; y, yolk.
Supplementary Figure 1.

a.

b.  

MVSPRGVCFLLFLLLGSVGFSVFLGPLLMLMLLSPSRYRWITDRIVATWLTLTPLVALLELVLGVKVVVTGDGFIPGERSVIIMNHRTRLDWMFLWCCLLRYSLRQEIKICLKAALKSVPGFGWAMQVASFIFIQRWEDDRTHMSNMLQYFCRIREPVQLLFLFPEGTDLTENTRASSDFAEKNGLQKYEYVHLPRTTGFTFIVDTLRGDNLDAVHDITVAYPQNIPOTERHLLAGVFPREIHFHVQRFTVASCAPAAGLQAWCQERWRKERRLQRFYETVPRRFADAPAVGVCVREPCQSGQCVCVPRCKSEGRVRIILVASSLLYWSVFITAACASLCLCPPAQFYFLFMVVFFLCQQRFTEGGELMALACHRYWSRRSA**DKQE**
Supplementary Figure 2.
Supplementary Figure 3.
**Supplementary Movie 1:**

This movie records, at 125 frames per second, the heart beating and blood circulation of live wild-type, cloche mutant and lycat-MO3 morphant embryos at 48 hours post fertilization. The lycat-MO3 morphant heart is remarkably similar to the cloche mutant heart, and they both have a single layer of myocardium and weaker contractility than that in the wild-type heart.

**Reference:**