

# Lipase maturation factor 1 is required for endothelial lipase activity<sup>S</sup>

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**Abstract** Lipase maturation factor 1 (Lmf1) is an endoplasmic reticulum (ER) membrane protein involved in the posttranslational folding and/or assembly of lipoprotein lipase (LPL) and hepatic lipase (HL) into active enzymes. Mutations in Lmf1 are associated with diminished LPL and HL activities (“combined lipase deficiency”) and result in severe hypertriglyceridemia in mice as well as in human subjects. Here, we investigate whether endothelial lipase (EL) also requires Lmf1 to attain enzymatic activity. We demonstrate that cells harboring a (*cld*) loss-of-function mutation in the *Lmf1* gene are unable to generate active EL, but they regain this capacity after reconstitution with the *Lmf1* wild type. Furthermore, we show that cellular EL copurifies with Lmf1, indicating their physical interaction in the ER. Finally, we determined that post-heparin phospholipase activity in a patient with the LMF1<sup>W464X</sup> mutation is reduced by more than 95% compared with that in controls. Thus, our study indicates that EL is critically dependent on Lmf1 for its maturation in the ER and demonstrates that Lmf1 is a required factor for all three vascular lipases, LPL, HL, and EL.—Ben-Zeev, O., M. Hosseini, C-M. Lai, N. Ehrhardt, H. Wong, A. B. Cefalù, D. Noto, M. R. Averna, M. H. Doolittle, and M. Péterfy. Lipase maturation factor 1 is required for endothelial lipase activity. *J. Lipid Res.* 2011. 52: 1162–1169.

**Supplementary key words** combined lipase deficiency • endoplasmic reticulum • hepatic • metabolism • phospholipases

The vascular lipase family is composed of three evolutionarily related enzymes, lipoprotein lipase (LPL), hepatic lipase (HL), and endothelial lipase (EL) (1–3). Localized to the luminal face of tissue capillaries, lipases

hydrolyze two principal lipid substrates associated with lipoprotein particles, triglycerides (TGs), and phospholipids. The released fatty acids (FAs) resulting from lipase hydrolysis are taken up by subjacent tissue and used for energy storage (adipose), oxidation, and energy production (skeletal muscle and heart) and the synthesis of bioactive metabolites (variety of tissues).

LPL is principally a TG lipase involved in the metabolism of TG-rich lipoproteins (chylomicrons and very-low-density lipoproteins) in adipose and muscle and heart tissues (1). Its deficiency and overexpression have been linked to metabolic abnormalities such as hypertriglyceridemia, insulin resistance, and cardiomyopathy, indicating the critical role of this enzyme in TG metabolism (4–10). Unlike LPL, HL has comparable TG lipase and phospholipase activities and is involved in the hepatic metabolism of high-density lipoprotein (HDL) as well as apolipoprotein B (apoB)-containing lipoproteins (LpBs) (11, 12). Overexpression of HL reduces plasma levels of HDL and LpBs, whereas HL deficiency has the opposite effect (13–16). EL is predominantly a phospholipase affecting HDL metabolism, but it also shares a redundant role with HL in the metabolism of LpBs (11, 17). Indeed, modulation of EL activity in mice leads to changes in plasma HDL levels similar to those of HL (18–20), reflecting their related substrate specificities. Consistent with their multifaceted involvement in lipoprotein metabolism, LPL, HL, and EL are strongly associated with plasma lipid levels in the general population (21).

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Abbreviations: apoB, apolipoprotein B; *cld*, combined lipase deficiency gene; EL, endothelial lipase; ER, endoplasmic reticulum; Lmf1, lipase maturation factor 1; LpB, apoB-containing lipoproteins; TG, triglyceride.

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The lipase maturation factor 1 (*Lmf1*) gene has been recently identified as the gene affected by the combined lipase deficiency (*clد*) mutation in the mouse (22). Homozygous *clد* mice develop severe hypertriglyceridemia and die shortly after birth due to complications arising from massive chylomicronemia (23). Although LPL deficiency is the principal cause of elevated plasma TG levels, HL activity is also diminished in *clد* mice. As mRNA and protein expression of LPL and HL in *clد* mice are unaffected, the lack of enzymatic activity is the result of lipase misfolding, causing aggregation and retention of the inactive lipase protein in the endoplasmic reticulum (ER). Thus, *Lmf1* is a critical factor in the posttranslational maturation of nascent lipase polypeptides into active enzymes (24). *Lmf1* is an ER membrane protein that has been shown to interact with LPL and HL through one of its loops extending into the ER lumen (25). Loss-of-function mutations (Y439X and W464X) have also been identified in the human ortholog of *Lmf1*, LMF1 (22, 26). Individuals homozygous for these mutations exhibit severe hypertriglyceridemia and diminished post-heparin LPL and HL activities, similar to combined lipase deficiency in mice.

An intriguing aspect of the *Lmf1* function is its specificity toward lipases. Whereas LPL and HL depend on *Lmf1* to attain catalytic activity, pancreatic lipase (PL), an evolutionarily related enzyme, is unaffected by the *clد* mutation (27). Additional members of the TG lipase superfamily have not been evaluated. An obvious candidate for *Lmf1* action is EL, which is the most closely related homolog of LPL. Thus, in the present study, we investigated the role of *Lmf1* in the expression of active EL. Our results suggest that EL activity is critically dependent on *Lmf1* function.

## MATERIALS AND METHODS

### Cell lines and transfection

Fibroblast cell lines derived from *clد* homozygous (*clد/clد*) and heterozygous (*clد/+*) mice have been described previously (27). Both the cells carrying the *clد* mutation and the HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin-streptomycin, sodium pyruvate, glutamine, and nonessential amino acids. Fibroblasts were transfected by electroporation using a Nucleofector device (Amaxa Biosystems) according to the manufacturer's instructions (program U-24, solution V). Electroporated cells were plated in collagen-coated 12 well plates. HEK293 cells were transfected with FuGENE6 transfection reagent (Roche) according to the manufacturer's instructions. Cells were harvested 24–48 h after transfection.

### Expression constructs

Human LPL, HL, PL, and EL cDNAs were subcloned into the pcDNA6 expression vector (Invitrogen) containing a C-terminal V5 epitope tag as described previously (28). For experiments using lipase affinity purification, a tandem affinity purification (TAP) tag was synthesized for in-frame integration into an *AgeI* site occurring just after the V5 epitope tag of pcDNA6 (29). After transfection, the resulting expressed LPL, HL, PL and EL proteins contained a C-terminal V5-TAP tag consisting of the V5 epitope followed by a single calmodulin-binding peptide domain,

a tobacco etch virus (TEV) protease site and ending in two adjacent IgG-binding domains derived from protein A (29). Mouse *Lmf1* cDNA was subcloned into the pcDNA3.1 expression vector (Invitrogen) containing an N-terminal c-Myc epitope tag (25).

### Affinity purification and Western blotting

Lipase affinity purification and immunoblotting were performed as described previously (25). Before Western blot analysis of EL in cell culture medium, samples were concentrated by heparin-Sepharose affinity purification in batch mode. Briefly, heparin-Sepharose slurry was added to aliquots of conditioned medium, incubated for 1 h, washed three times with 50 mM Tris-HCl, pH 7.5, and eluted with 2 M NaCl and boiling.

### Lipase assays

For lipase assays from cell extracts, lysates were prepared by sonication in 20 mM Tris-HCl buffer, pH 7.5, containing 0.2% deoxycholate and 10 U/ml heparin. The activity levels of LPL and HL were measured using the respective triolein substrates prepared by sonication (30). PL was assayed with the substrate used for HL in the presence of colipase (Sigma) as described previously (28). EL activity was determined using a phospholipase assay utilizing a glycerol-stabilized emulsion of cholesteryl oleate and phosphatidylcholine as described previously (31). Cellular protein was assayed using the bicinchoninic acid (BCA) reagent (Pierce). To determine secreted EL activity, cells were treated with 10 U/ml heparin before cell culture supernatants were harvested. For the estimation of EL activity in pre- and post-heparin plasma, the same phospholipid substrate was used in the presence and absence of 1 M NaCl; because EL activity is inhibited by high salt, salt-inhibited activities are reported (31). The University of Palermo and University of California, Los Angeles, Institutional Review Boards approved the study protocol. Written informed consent was given by all subjects.

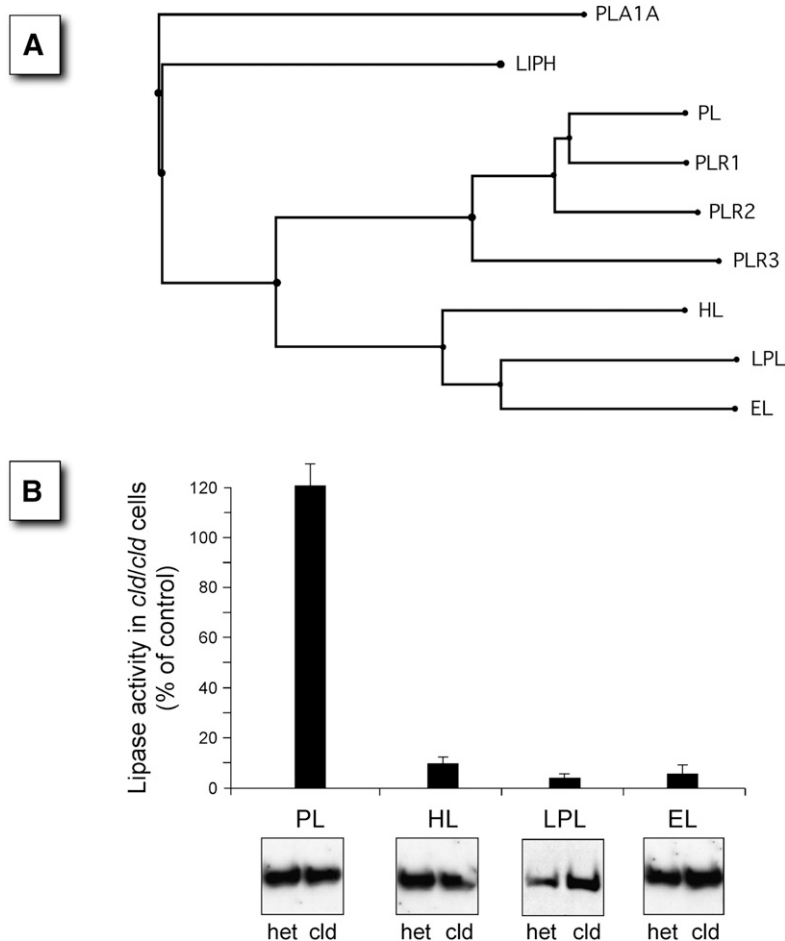
### Determination of plasma EL concentration

Post-heparin plasma samples were diluted 1:200 in 0.02 M PBS, and EL protein concentration was determined by ELISA (Uscn Life Science, Inc.) according to the manufacturer's instructions.

## RESULTS

### The *clد* mutation affects EL activity

We have previously demonstrated that the *clد* mutation abrogates the posttranslational maturation and activity of two related lipases, LPL and HL (32). However, the effect of the *clد* mutation on EL, a third lipase family member, has yet to be evaluated. To address the dependency on *Lmf1* function among various lipase family members, expression vectors encoding LPL, HL, EL, and a more distantly related lipase, PL (Fig. 1A), were transfected into fibroblasts harboring the recessive, loss-of-function *clد* mutation. After transfection, cells heterozygous (*clد/+*) and homozygous (*clد/clد*) for the mutation were assessed by measurements of lipase activities. As expected from previous studies (27, 32), the activity levels of LPL and HL, but not PL, were dramatically reduced in *clد/clد* cells (Fig. 1B). Importantly, we found that EL was as severely affected as LPL and HL, with only ~5% of the wild-type activity detected in homozygous mutant cells. These results suggest that the combined lipase deficiency phenotype associated



**Fig. 1.** Members of the lipase gene superfamily that are affected by the *cld* mutation are shown. A: The phylogenetic tree of the lipase gene family shows a group of closely related members (HL, LPL, and EL) that form homodimers compared with the more distantly related PL, which is active as a monomer. The subunit structure of the remaining members is not known. All members of the family are secreted enzymes, and thus, all mature within the ER. The members include PLA1A, phospholipase A1 member A (Q53H76); LIPH, lipase member H (Q8WWY8); PL, pancreatic triacylglycerol lipase (P16233), and the three PL-related lipases, PLR1 (P54315), PLR2 (P54317), and PLR3 (Q17RR3); HL, hepatic triacylglycerol lipase (P11150); LPL, lipoprotein lipase (P06858); and EL, endothelial lipase (Q9Y5X9). B: The four members of the lipase gene family with known subunit structures were transfected into cells homozygous for the *cld* mutation (*cld/cld*), and expressed lipase activity was compared with that of control cells carrying only one copy of the *cld* allele (+/*cld*). Panels show representative Western blots of total cell lysates visualized using an antibody against a lipase-specific epitope tag.

with the *cld* mutation extends to a third member of the triglyceride lipase family, namely EL.

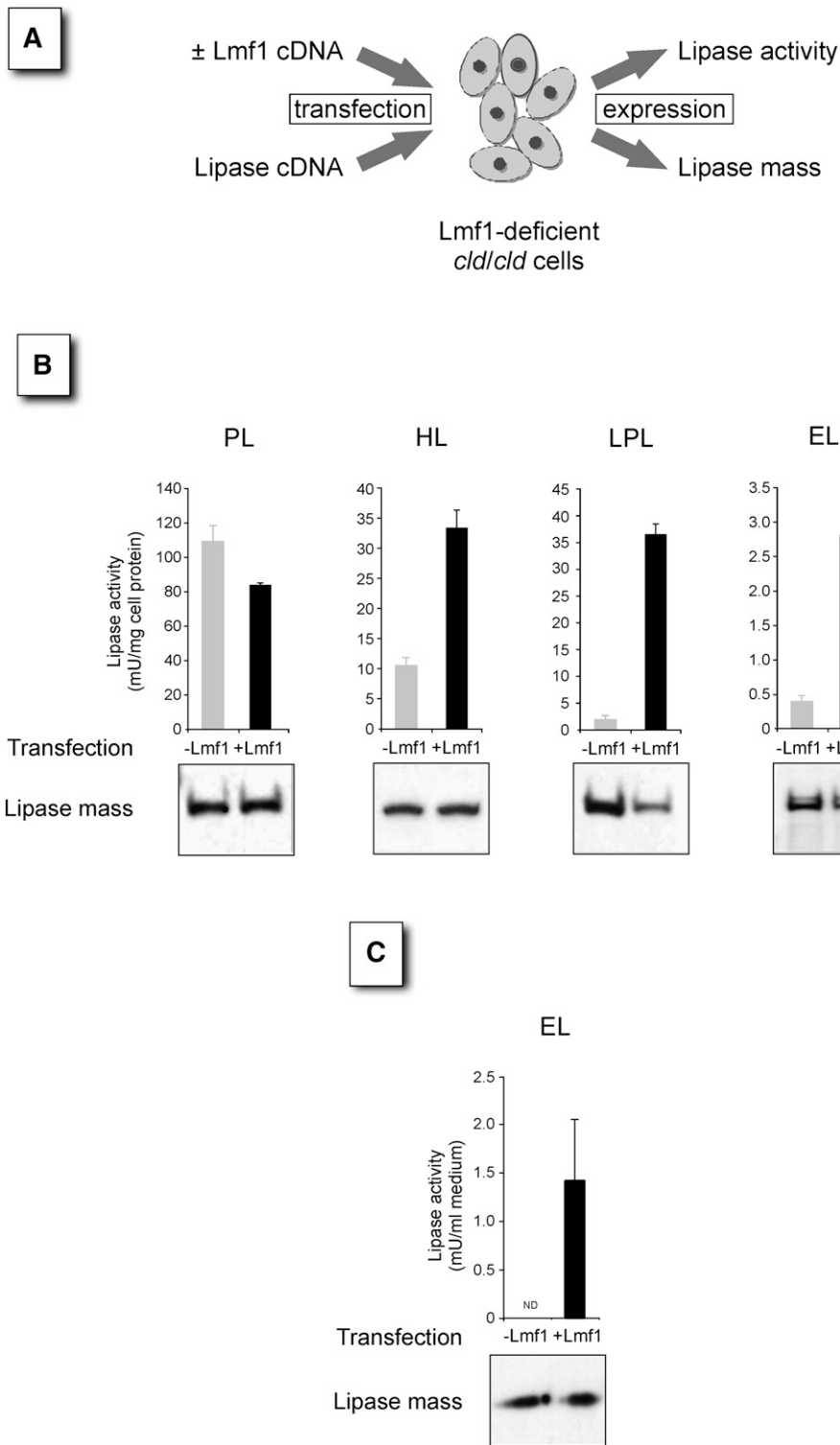
#### Lmf1 is required for the posttranslational maturation of EL

The *cld* mutation in the *Lmf1* gene arose on a rare variant form of chromosome 17, called the *t*-haplotype (33). Due to several large inversions, the *t*-haplotype region is subject to recombination suppression with wild-type chromosomes and, as a consequence, has accumulated a variety of deleterious mutations during its evolutionary history (34). To investigate whether diminished EL activity in *cld/cld* cells is the consequence of the *cld* mutation affecting Lmf1 function or the result of other linked variants associated with the *t*-haplotype, we complemented *cld/cld* cells with wild-type (22). In these experiments, mutant *cld/cld* cells were cotransfected with the *Lmf1* wild type along with the various lipase expression constructs, and lipase activities were determined (Fig. 2A). As PL is not dependent on Lmf1 function, coexpression of wild-type Lmf1 did not increase the activity of this enzyme (Fig. 2B). In fact, PL activity is apparently reduced in the presence of Lmf1, an effect currently not fully understood. In contrast, Lmf1 expression elevated the activities of cell-associated HL, LPL, and EL several fold. The fold increases in LPL and EL activities are similar to those between the *cld* and heterozygous cells transfected with lipases only (Fig. 1B),

indicating that Lmf1 transfection restored the activity of these lipases to heterozygous levels. Furthermore, analysis of untransfected cells demonstrated that the low activity detected in the absence of Lmf1 (–Lmf1 in Fig. 2B) represents endogenous lipases and not exogenous EL (data not shown). Thus, EL activity is completely dependent on functional Lmf1 in our assay. Importantly, immunoblot analyses indicated that elevated lipase activities in Lmf1-expressing cells were not due to increased lipase protein mass but, rather, to higher specific activity (Fig. 2B). To determine the dependence of secreted EL activity on Lmf1, we also analyzed cell culture medium after heparin treatment. As shown in Fig. 2C, EL activity was undetectable in conditioned medium from Lmf1-deficient cells, but activity was rescued by Lmf1 expression. Interestingly, unlike LPL and HL (32), EL protein secretion was unaffected by the presence or absence of Lmf1 (Fig. 2C), indicating that inactive EL is readily secreted from cells carrying the *cld* mutation. In conclusion, our results demonstrate that, similar to LPL and HL, Lmf1 is critically required for the posttranslational processing of EL into active enzyme.

#### Lmf1 interacts with EL

To investigate whether EL dependency on Lmf1 function involves physical interaction between these two proteins, we performed affinity purification experiments.



**Fig. 2.** Reconstitution of Lmf1 expression in *cld/cld* cells rescues lipase activity. **A:** Schematic diagram shows the reconstitution assay. Lmf1-deficient cells are cotransfected with Lmf1 and lipase expression vectors. At 1–2 days after transfection, lipase activity and mass were assessed in cell lysates. **B:** Upper panels show cell-associated lipase activities ( $n = 3$ ) after cotransfection with Lmf1 (black bars) or empty vector (gray bars) and the respective lipases. Lower panels show representative Western blots of total cell lysates visualized using an antibody against a lipase-specific epitope tag. **C:** EL activity released into cell culture medium after heparin treatment of cells transfected with EL  $\pm$  Lmf1 is shown. Lower panel shows Western blot of cell culture medium. ND, not detectable.



Epitope-tagged Lmf1 and affinity-tagged lipase proteins were coexpressed in HEK293 cells. Lipase proteins were then affinity purified from cell extracts, with Lmf1 copurification assessed by immunodetection. As demonstrated previously (25), Lmf1 could be detected in association with LPL and HL but not with PL (Fig. 3). Furthermore, and consistent with its functional effect on EL, Lmf1 was readily detected together with affinity-purified EL, indicating that the proteins physically interact. To exclude the possibility that the interaction is due to abnormal lipase structure resulting from the C-terminal affinity tag, we confirmed that affinity-tagged EL was fully active (see supplementary Fig. I).

### LMF1 deficiency diminishes post-heparin phospholipase activity

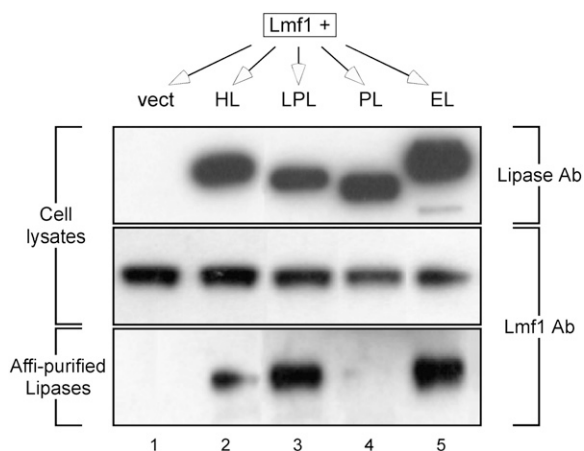
In order to evaluate whether Lmf1 is required for the expression of active EL in vivo, we initially attempted to analyze plasma from *clld* homozygous mice. However, as these mice die within 1–2 days after birth, we were unable to collect sufficient amounts of plasma for the reliable detection of EL activity. In contrast to mice, LMF1 deficiency in humans is not lethal, permitting the use of plasma from an LMF1-deficient patient we previously characterized (26). This individual carries a homozygous nonsense mutation in LMF1 (W464X) and exhibits diminished post-heparin activity levels for both LPL and HL associated with massive hypertriglyceridemia. Although a specific assay measuring the activity of EL has not been developed, it has been demonstrated that the majority of heparin-releasable plasma phospholipase activity can be attributed to EL in the mouse (19). Moreover, the activity of HL, another heparin-released phospholipase, can be discriminated

from that of EL based on their differential sensitivities to high salt concentrations (31). Thus, we hypothesized that if LMF1 deficiency caused diminished EL activity in vivo, it would result in reduced heparin-releasable and salt-inhibitable phospholipase activity in plasma of the patient carrying the W464X mutation. Indeed, the proband exhibited over 95% reduction in phospholipase activity compared with that of controls (Fig. 4). Furthermore, EL protein mass in post-heparin W464X plasma was similar to control levels (134.6 ng/ml vs. control median of 133.55 ng/ml). In conclusion, these data are consistent with those from our in vitro studies indicating that Lmf1 is required for EL to attain enzymatic activity.

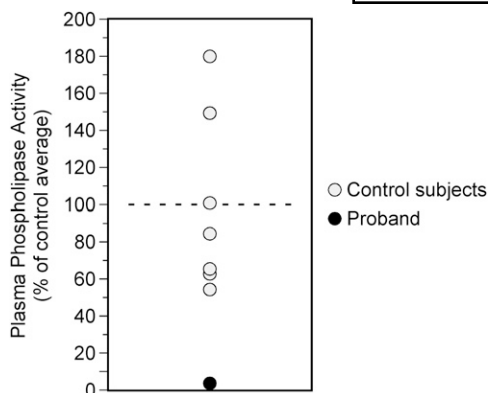
### DISCUSSION

We report in this study that in addition to LPL and HL, EL also requires Lmf1 to attain enzymatic activity. Multiple lines of evidence support this conclusion, including the inability of Lmf1-deficient cells to produce active EL, physical interaction between EL and Lmf1, and the virtual absence of post-heparin phospholipase activity in an LMF1-deficient patient. Thus, we extend the “combined lipase deficiency” phenotype resulting from the lack of functional Lmf1 to include a third member of the lipase gene family, EL.

The identification of three lipases dependent on Lmf1 suggests a shared posttranslational maturation pathway for these proteins. An obvious structural similarity between LPL (35), HL (36), and EL (37) is that their activity depends on homodimerization, an assembly step that takes place in the ER where Lmf1 resides. While these three lipases are thought to have a crystal structure similar to that of PL, only PL is active as a monomer (38), and only this lipase is independent of Lmf1 function (27). Thus, we propose that Lmf1 is involved in the assembly and/or stabilization of lipase homodimers in the ER. Several previous studies are consistent with this hypothesis. First, assembly of LPL monomers into active dimers takes hours in vitro (39) but only minutes in the ER (40), suggesting that this process is likely to be aided by a chaperone(s), such as Lmf1. Likewise, lipase homodimers rapidly dissociate to misfolded monomers in vitro unless stabilized by binding to factors such as heparin (39). In contrast, the LPL dimer is highly stable in the ER lumen, suggesting that an ER-specific stabilizing factor prevents such dissociation in vivo (40, 41). Second, in Lmf1-deficient cells, the amount of LPL homodimers is severely diminished with a concomitant increase in LPL aggregates (27). This aggregation is unlikely to be due to defective folding of the monomer, as LPL is properly glycosylated in these cells, indicating that early steps of maturation facilitated by the calnexin cycle are unaffected (27). Instead, the formation of aggregates likely reflects the accumulation of monomers due to inefficient homodimer assembly and/or stabilization in the absence of Lmf1. Indeed, the LPL monomer has been demonstrated to be prone to aggregation, probably due to exposure of a hydrophobic surface area that is shielded in the assembled homodimer (39, 42). Finally, phylogenetic



**Fig. 3.** HL, LPL, and EL, but not PL, copurify with the Lmf1 protein. HEK293 cells were cotransfected with Lmf1 and each of four affinity-tagged lipase constructs (HL, LPL, PL, and EL), including an empty vector control (vect). The top and middle panels represent Western blots of total cell lysates visualized using antibody (Ab) to either lipase-specific (V5) or Lmf1-specific (*c-myc*) epitope tags. Each expressed lipase construct was then affinity (affi) purified from total cell lysates under mild conditions that favor retention of protein-protein interactions. The bottom panel represents a Western blot of the affinity-purified lipases probed using antibody directed to the Lmf1-specific epitope tag.



**Fig. 4.** LMF1 deficiency abrogates plasma phospholipase activity. Heparin-released salt-sensitive phospholipase activity in a patient carrying the homozygous LMF1<sup>W464X</sup> mutation (closed symbol) is expressed as a percentage of mean of control subjects (open symbols).

analysis of lipases indicates that PL diverged from an ancestral lipase prior to the evolutionary emergence of LPL, HL, and EL, which share a more recent root (43). Similar to PL, bacterial and fungal lipases also function as monomers (44), suggesting that the homodimeric subunit structure is a more recent evolutionary development. Interestingly, the evolutionary emergence of Lmf1 homologs coincides with that of homodimeric lipases (M. Doolittle, unpublished data), raising the possibility that Lmf1 coevolved with the LPL/HL/EL branch of lipases to facilitate attainment of their unique structure. Further mechanistic studies will be required to address this hypothesis.

Studies of naturally occurring populations (21, 45, 46) and experimental models (4, 16, 19) have demonstrated a central role for secreted lipases in plasma lipid metabolism. These lipases regulate lipoprotein metabolism through their bifunctional lipolytic activities (i.e., triacylglycerol and phospholipid lipase) and interactions with multiple classes of lipoprotein particles. Although the metabolic consequences of deficiencies (4, 16, 19) and overexpression (10, 14, 18) of individual lipases have been extensively studied, metabolic interactions among the lipases remain poorly characterized. Considering the redundant enzymatic activities and substrate specificities and the distinct, yet overlapping expression patterns of lipases, such interactions may have important contributions to dyslipidemia and atherosclerotic cardiovascular disease. For example, an association analysis of LPL, HL, and EL variants revealed genetic interaction in the determination of plasma TG levels in a normal population (47). To investigate the interaction between HL and EL directly, Brown et al. (17) recently generated HL/EL double-knock-out mice representing the first engineered mouse model of combined lipase deficiency. These mice exhibit phenotypes that neither of the single-lipase knock-outs do, including increased neonatal lethality and the accumulation of small LDL particles, which revealed a redundant role for HL and EL in the metabolism of apoB-containing lipoproteins. LMF1 deficiency represents a unique metabolic scenario where the activities of three lipases are simultaneously

diminished and offers further insights into lipase interactions. For example, hypertriglyceridemia is typically associated with low HDL levels, a consequence of enhanced clearance resulting from the lipolytic actions of HL, and perhaps EL, on TG-enriched HDL particles (48). Yet, despite severely elevated TG levels due to LPL deficiency, HDL-cholesterol in the LMF1<sup>W464X</sup> patient is within normal range (26), likely reflecting reduced activities of HL and EL. Although the lack of HDL-lowering effect is predictable based on known activities of the individual lipases, more detailed characterization of the combined lipase deficiency phenotype will no doubt uncover further insights about lipase interactions and their effects on lipoprotein metabolism.

In addition to plasma lipoprotein remodeling, lipases are also critical components in tissue lipid homeostasis. Through the liberation of FA from lipoprotein-bound glycerolipids, these enzymes provide substrates for energy storage and generation in adipose and muscle, respectively (49), and the synthesis of FA-derived signaling molecules involved in transcriptional regulation in various tissues (50). All of these processes are likely to be affected by the lack of active lipases in LMF1 deficiency. Adipose tissue may offer an interesting illustration of the metabolic effects of combined lipase deficiency. Under normal circumstances, LPL-mediated TG hydrolysis is the main source of FA for adipose TG synthesis and storage (51). Nonetheless, adipose tissue mass is largely unaffected by LPL deficiency both in human subjects (52, 53) and animal models (5, 54, 55), which has been attributed to adaptations in adipocyte metabolism. First, de novo synthesis of FAs is increased in the absence of LPL activity (5, 56). Furthermore, EL expression is substantially upregulated in LPL-deficient adipose tissue, providing an alternative pathway for FA uptake (57). However, the latter compensatory mechanism is expected to be ineffectual in LMF1 deficiency due to impaired posttranslational maturation of EL. Thus, lipid homeostasis is likely to be more severely affected in LMF1-deficient than LPL-deficient adipose tissue, especially in humans where lipogenic capacity is lower than in mice (58). Consistent with this hypothesis, an LMF1-deficient human subject exhibits lipodystrophy (22). Similar to adipose tissue, reciprocal regulation of lipase expression also occurs in other tissues. Namely, LPL and HL are upregulated in EL-deficient muscle and liver, respectively, further highlighting the functional redundancy and interrelatedness of lipase family members (20). It is therefore likely that LMF1 deficiency in these tissues leads to metabolic defects not observed in the absence of individual lipases. Testing of this hypothesis awaits the generation of viable adult and tissue-specific Lmf1-deficient mouse models. **■**

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