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GPIHBP1 and plasma triglyceride metabolism

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Abstract

GPIHBP1, a GPI-anchored protein in capillary endothelial cells, is crucial for the lipolytic processing of triglyceride-rich lipoproteins (TRLs). GPIHBP1 shuttles lipoprotein lipase (LPL) to its site of action in the capillary lumen and is essential for the margination of TRLs along capillaries—so that lipolytic processing can proceed. GPIHBP1 also reduces the unfolding of LPL's catalytic domain, thereby stabilizing LPL catalytic activity. Many different *GPIHBP1* mutations have been identified in patients with severe hypertriglyceridemia (chylomicronemia), the majority of which interfere with folding of the protein and abolish its capacity to bind and transport LPL. The discovery of GPIHBP1 has substantially revised our understanding of intravascular triglyceride metabolism but has also raised many new questions for future research.

Keywords

hypertriglyceridemia; chylomicronemia; lipoprotein lipase; lipid transport; endothelial cells; LU (Ly6-uPAR) family of proteins

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Conflict of interest

The authors have declared that no conflict of interest exists.

GPIHBP1 is a crucial protein in plasma triglyceride metabolism

The fact that triglycerides in the plasma are hydrolyzed by LPL along blood vessels was established more than 60 years ago [1, 2]. For most of that time, LPL was assumed to be attached to blood vessels by electrostatic interactions between positively-charged domains in LPL and the negatively-charged heparan sulfate proteoglycans (HSPGs) that line the surface of endothelial cells [3, 4]. This model was attractive because it seemed consistent with the observation that LPL can be released into the plasma with an injection of heparin. HSPGs were also thought to play a role in transporting LPL to the lumen of blood vessels. Finally, HSPGs in blood vessels were also thought to be important for binding triglyceride-rich lipoproteins (TRLs), allowing them to stop along blood vessels so that lipolysis could proceed [5, 6]. Over a period of decades, this HSPG-centric model for TRL metabolism was widely accepted, and the majority of experiments were devised and interpreted with this model in mind.

The first clue that the accepted model for TRL metabolism was incorrect came with the discovery of severe hypertriglyceridemia in mice lacking glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1 (GPIHBP1), a member of the “LU” protein family [LU: lymphocyte antigen 6–urokinase-type plasminogen activator receptor (uPAR)] [7, 8]. On a chow diet, the plasma triglyceride levels in *Gpihbp1*$^{-/-}$ mice were ~2000–5000 mg/dl, and on a high-fat diet they were as high as 35,000 mg/dl [8, 9]. The hypertriglyceridemia in *Gpihbp1*$^{-/-}$ mice was caused by defective processing of TRLs by LPL [8]. Beigneux and coworkers quickly discovered that GPIHBP1 binds LPL avidly and that GPIHBP1 is expressed on capillary endothelial cells [8]. These observations led them to speculate that GPIHBP1 could be the binding site for LPL and that it serves as a “platform for lipolysis” in capillaries [8]. Subsequent studies confirmed this concept and went on to show that GPIHBP1 actually transports LPL across endothelial cells to the lumen of capillaries [10], that GPIHBP1 is essential for TRL margination along capillaries [11], and that GPIHBP1 functions to stabilize the catalytic activity of LPL [12] (Figure 1, Key Figure).

GPIHBP1 structural domains

The four exons of *GPIHBP1* encode a polypeptide with four key features [7, 8, 13]. The first is an amino-terminal signal peptide, which is removed within the ER. The second is an acidic domain, with 21 of 26 amino acids in the human protein being aspartates or glutamates (Figure 2A) [8]. A similar acidic domain is found in the GPIHBP1 of all mammals, but the opossum “wins the prize.” The acidic domain in opossum GPIHBP1 contains 32 aspartates/glutamates in 39 consecutive residues, including a stretch of 23 consecutive aspartates. The third feature is a three-fingered LU domain, which contains 10 cysteines—all in a characteristic spacing pattern and all disulfide bonded (Figure 2A) [14, 15]. The fourth feature, located at the carboxyl terminus of the protein, is a signal sequence that triggers the addition of a GPI anchor; this region ends with a stretch of hydrophobic amino acids and is removed in the ER and replaced with a GPI anchor [16, 17]. In the Golgi, the sn-2 unsaturated fatty acyl chain of the glycosylphosphatidylinositol (GPI) anchor (in GPIHBP1 and all other GPI-anchored proteins) is removed and replaced with stearic acid.

That modification is critical for directing GPI-anchored proteins to cholesterol- and sphingomyelin-rich microdomains ("rafts") in the plasma membrane [17].

Once it was clear that GPIHBP1 bound LPL, GPIHBP1's acidic domain attracted scrutiny, simply because LPL was known to contain several positively-charged heparin-binding motifs. In early LPL–GPIHBP1 binding assays, GPIHBP1's acidic domain appeared to be quite important for LPL binding [18], but subsequent studies with more refined cell-based and cell-free binding assays revealed that the contribution of the acidic domain to LPL binding was small and that the LU domain was primarily responsible for LPL binding [19, 20]. Two other members of the triglyceride lipase protein family, hepatic lipase and endothelial lipase, contain positively charged heparin-binding domains but do not bind to GPIHBP1 [21].

The LU domain of GPIHBP1 contains an N-linked glycan (attached to N78 in the human sequence, Figure 2A). This N-linked glycan is important for efficient trafficking of GPIHBP1 to the cell surface [22]. Interestingly, GPIHBP1's acidic domain is also important for trafficking of the protein to the cell surface [18]. When the acidic domain is removed, the amount of GPIHBP1 that reaches the cell surface is markedly reduced [18].

GPIHBP1 expression in tissues

GPIHBP1 transcripts are detectable in nearly every peripheral tissue but are found in particularly high amounts in brown adipose tissue and heart—mirroring the high levels of *LPL* transcripts in those sites [8]. However, there are two tissues where levels of *GPIHBP1* and *LPL* transcripts differ. First, LPL is expressed in selected areas of the brain (*e.g.*, hippocampus) [23, 24], but the amount of GPIHBP1 in brain capillaries is low [8]. The low levels of GPIHBP1 in the brain parenchyma makes sense because neurons depend on glucose for fuel. However, the physiologic function of LPL in the brain—and whether the LPL in the brain plays a role in intravascular lipolysis—is unclear. Second, the levels of *GPIHBP1* transcripts in the lung are very high, while *LPL* transcripts are very low [25]. The GPIHBP1 in lung capillaries is functional in binding LPL. When bovine LPL is injected intravenously into a wild-type mouse, the injected LPL binds to GPIHBP1 on lung capillaries [11]. GPIHBP1 in lung capillaries appears to play a role in capturing LPL that escapes from peripheral tissues [25], but the physiologic importance of GPIHBP1 expression in the lung remains unclear because a complete deficiency of GPIHBP1 does not appear to elicit overt pulmonary abnormalities.

In the mouse, GPIHBP1 is present exclusively in capillaries and cannot be detected in larger blood vessels. Indeed, when a capillary expands in size by as little as ~50% (to become the smallest-possible venule), GPIHBP1 expression disappears [10]. The factors that regulate GPIHBP1 expression in capillary endothelial cells and prevent its expression in larger blood vessels are unknown and represent an important topic for future research.

GPIHBP1 transports LPL to the capillary lumen

The first clue that GPIHBP1 is required for the proper localization of LPL in tissues came from the discovery that LPL is released into the plasma quite rapidly in wild-type mice after

an intravenous injection of heparin, but it is released slowly in *Gpihbp1*$^{-/-}$ mice [26]. The rapid entry of LPL into the plasma of wild-type mice seemed consistent with the presence of LPL inside blood vessels, whereas the slow entry of LPL into the plasma of *Gpihbp1*$^{-/-}$ mice suggested that the LPL might be mislocalized [26]. Indeed, this proved to be the case. In the tissues of wild-type mice, nearly all of the LPL is bound to capillaries (Figures 1 and 3) [10, 27]. When GPIHBP1 is absent, the LPL in tissues is located within the interstitial spaces, with most "coating" myocytes and adipocytes (Figure 3) [10].

The second clue that GPIHBP1 is important for LPL localization was uncovered by visualizing LPL in cross sections of capillaries. In capillaries of wild-type mice, LPL was found to partition equally between the basolateral and luminal plasma membranes, whereas in *Gpihbp1*$^{-/-}$ mice the LPL was absent from the capillary lumen and instead was found only on the outside of capillaries (presumably bound to HSPGs on or near the cell surface) [10]. Davies and coworkers pointed out that the mislocalization of LPL in *Gpihbp1*$^{-/-}$ mice likely explained the delayed entry of LPL into the plasma after an injection of heparin [10, 27].

The fact that the LPL in *Gpihbp1*$^{-/-}$ mice is attached to HSPGs within the interstitial spaces poses perplexing questions: Why isn't LPL in wild-type mice trapped by these very same HSPGs? Why does the LPL in wild-type mice "move past" HSPGs in the interstitial spaces and bind to GPIHBP1 on endothelial cells? Addressing these questions will require a better understanding of the dynamics of LPL interactions with HSPGs and GPIHBP1.

The absence of LPL in the capillary lumen in *Gpihbp1*$^{-/-}$ mice prompted cell culture experiments to determine if GPIHBP1 functions as a "trans-endothelial cell LPL transporter." Those studies revealed that GPIHBP1 was able to pick up LPL (or a GPIHBP1-specific monoclonal antibody) from one side of an endothelial cell monolayer and move it to the opposite side of the monolayer. GPIHBP1's transporter function was also evident in living mice. When a GPIHBP1-specific antibody was injected into skeletal muscle, the antibody promptly diffused within the interstitial spaces and surrounded myocytes. Within 30 min, however, the antibody can be detected on the luminal surface of capillaries [10]. Similarly, when a GPIHBP1-specific monoclonal antibody was injected intravenously into a wild-type mouse, it quickly bound to GPIHBP1 on the luminal surface of capillaries, but soon thereafter it could be detected on the basolateral plasma membrane of endothelial cells [27]. Electron microscopy (EM) studies revealed that LPL and GPIHBP1 move across endothelial cells in vesicles [27].

The *in vivo* studies with a GPIHBP1-specific monoclonal antibody showed that GPIHBP1 moves bidirectionally across endothelial cells—even though its function (as we understand it) is to move LPL only in one direction—to the capillary lumen. The movement of LPL from the lumen to the basolateral surface of endothelial cells seems inefficient, but that could be the price to be paid for using endothelial cell vesicles to transport LPL to the capillary lumen. GPIHBP1 appears to be a long-lived protein, continuously shuttling back and forth across capillaries for many hours and perhaps days [25]. With each appearance of GPIHBP1 at the basolateral face of capillaries, there is another opportunity to replenish LPL that may have been dislodged from GPIHBP1 and lost in the bloodstream. In addition, it is

possible that the shuttling of LPL to the basolateral surface of endothelial cells could be relevant to the regulation of LPL activity by ANGPTL4. Several groups have proposed that the subendothelial spaces could represent an important site for LPL regulation by ANGPTL4 [27, 28].

The fact that nearly all of the LPL in tissues is attached to GPIHBP1 on capillaries (Figures 3) is noteworthy. For years, those interested in LPL have focused on the biochemical properties of *free* LPL and *HSPG-bound* LPL rather than GPIHBP1-bound LPL. To understand the role of LPL in intravascular lipolysis, the field will ultimately need to define the effects of apo-AV, apo-CII, apo-CIII, and ANGPTL4 on *GPIHBP1-bound* LPL. Similarly, in order to understand the effects of various LPL polymorphisms (*e.g.,* the S447X polymorphism) on LPL activity, it will be important to study the effect of the polymorphism on GPIHBP1-bound LPL [29].

Phenotypic differences between *Gpihbp1*$^{-/-}$ and *Lpl*$^{-/-}$ mice

While adult *Gpihbp1*$^{-/-}$ mice have severe hypertriglyceridemia, the plasma triglyceride levels in newborn *Gpihbp1*$^{-/-}$ mice are only modestly elevated—despite the fact that newborn mice consume a high-fat milk diet [8]. In contrast, newborn *Lpl*$^{-/-}$ mice have plasma triglyceride levels of ~20,000 mg/dl and die within 24 h [30]. The distinct phenotypes of *Gpihbp1*$^{-/-}$ and *Lpl*$^{-/-}$ mice likely relate to the ability of suckling mice to produce LPL in the liver [8, 31]. Hepatic LPL is probably effective in processing TRLs—even in the absence of GPIHBP1—because the fenestrated capillaries of the liver would allow access of TRLs to LPL. Hepatic production of LPL also explains why the plasma triglyceride levels in adult *Gpihbp1*$^{-/-}$ mice fall when mice are placed on a western diet supplemented with large amounts of cholesterol [31]. In rodents, LPL expression in the liver is induced by dietary cholesterol [32].

The plasma triglyceride levels in ANGPTL4-deficient *Gpihbp1*$^{-/-}$ mice (*Gpihbp1*$^{-/-}$*Angptl4*$^{-/-}$) are much lower than in *Gpihbp1*$^{-/-}$ mice [11, 33]. The explanation for this observation is unclear and requires more study, but we suspect that it relates to the absence of the inhibitory effect of ANGPTL4 on the LPL produced by the liver. Immunohistochemistry studies have shown that LPL is not transported to the capillary lumen in *Gpihbp1*$^{-/-}$*Angptl4*$^{-/-}$ mice [11].

Consequences of LPL mislocalization in *Gpihbp1*$^{-/-}$ mice

The fact that LPL in *Gpihbp1*$^{-/-}$ mice cannot reach the lumen of capillaries in heart, skeletal muscle, and adipose tissue [10] provides a clear explanation for the severe hypertriglyceridemia in those mice. However, defective TRL processing does more than simply raise the levels of triglycerides in the plasma—it also changes tissue lipid metabolism. For example, defective TRL processing in *Gpihbp1*$^{-/-}$ mice results in more *de novo* lipogenesis in adipose tissue, leading to higher levels of 16:1 fatty acids relative to 18:2 and 18:3 fatty acids (an expected “signature” of increased *de novo* lipogenesis) [34]. The 16:1 to 18:2, 18:3 fatty acid ratio is reversed in the lipids of the liver, reflecting more hepatic uptake of dietary lipids when TRL processing is impaired in peripheral tissues. Consistent

with these findings, the expression of lipid biosynthetic genes in adipose tissue is higher in *Gpihbp1*$^{-/-}$ mice than in wild-type mice, while the reverse is the case in the liver [34].

Reduced delivery of lipid nutrients to parenchymal cells in *Gpihbp1*$^{-/-}$ mice was obvious when mice were examined by NanoSIMS, an imaging modality that creates high-resolution images of cells and tissues based on their isotope content [35, 36]. After delivering ^{13}C–labeled lipids to mice, one can visualize and quantify ^{13}C enrichment in the cells and tissues of mice with NanoSIMS imaging. For example, when ^{13}C–labeled fatty acids are administered to a wild-type mouse by gavage, they are incorporated into chylomicrons and subsequently can be detected in cytosolic lipid droplets of cardiomyocytes (Figure 4A) [35]. When ^{13}C–labeled fatty acids are administered to a *Gpihbp1*$^{-/-}$ mouse (where TRL processing is defective), ^{13}C enrichment in myocytes was minimal and a large fraction of the ^{13}C-lipids remained within TRLs in the capillary lumen (Figure 4A) [35].

GPIHBP1 and GPIHBP1-bound LPL are crucial for the margination of TRLs in capillaries

For TRL processing to occur, TRLs must stop along the luminal surface of capillaries. For years, the assumption was that TRLs stopped as a result of electrostatic interactions between TRL apolipoproteins and the HSPGs in the glycocalyx lining of blood vessels [3, 4, 37]. Loren Fong and coworkers challenged this idea and proposed that GPIHBP1 might be essential for TRL margination [11]. To explore this idea, TRLs from *Gpihbp1*$^{-/-}$ mice were labeled with the fluorescent dye Alexa555 and were injected, along with an Alexa488-labeled monoclonal antibody against GPIHBP1, into the tail vein of both wild-type and *Gpihbp1*$^{-/-}$ mice. After 30 sec, the mice were perfused with PBS, fixed *in situ*, and tissue biopsies were stained with an LPL antibody and prepared for microscopy. In wild-type mice, TRLs marginated along capillaries, colocalizing with GPIHBP1 and LPL, whereas in *Gpihbp1*$^{-/-}$ mice the margination of TRLs was nearly absent (Figure 5) [11]. The absence of TRL margination in *Gpihbp1*$^{-/-}$ mice was confirmed with other imaging modalities. First, transmission electron microscopy demonstrated many TRLs along heart capillaries of wild-type mice, but none were found in capillaries of *Gpihbp1*$^{-/-}$ mice [11]. Second, TRL margination was visualized by NanoSIMS imaging after injecting wild-type mice with ^{13}C-labeled TRLs. (Figure 4B). Third, studies with IRdye-labeled TRLs demonstrated that TRL margination was negligible in *Gpihbp1*$^{-/-}$ mice. In those studies, IRdye-TRLs were injected intravenously, and the amount of TRL margination was quantified in tissue sections with an infrared scanner [11]. The attractive feature of the “IRdye approach” is that TRL margination could be quantified in sections of the *entire heart*, thus avoiding sampling bias associated with the imaging of TRLs in a few high-powered fields [11].

While multiple imaging approaches demonstrated that GPIHBP1 is required for TRL margination in capillaries, a key question remained. Is GPIHBP1 crucial for margination, or is it GPIHBP1-bound LPL? Several lines of evidence suggest that GPIHBP1-bound LPL is important. First, TRL margination is virtually absent in capillaries of the lung, where GPIHBP1 is abundant but LPL expression is low [11]. However, after “loading up” the GPIHBP1 in lung capillaries with LPL (by administering an intravenous injection of LPL),

TRL margination along lung capillaries was robust. These studies supported the primacy of GPIHBP1-bound LPL in TRL margination. Another reason to suspect that GPIHBP1-bound LPL is important came from studies of *Gpihbp1*$^{-/-}$ mice carrying an endothelial cell–specific human LPL transgene [11]. The endothelial cell–derived human LPL significantly increased plasma LPL levels in *Gpihbp1*$^{-/-}$ mice and lowered plasma triglyceride levels by ~90%, but TRL margination along heart capillaries was negligible. Again, those studies pointed to the primacy of GPIHBP1-bound LPL for TRL margination. Studies in cultured cells implicated a tryptophan-rich region in the carboxyl terminus of LPL as having an important role in binding triglyceride-rich particles [11].

TRLs appear to marginate in “meadows” between tufts of glycocalyx

The involvement of GPIHBP1-bound LPL in TRL margination posed a conceptual problem. Because endothelial cells are thought to be covered by an HSPG-rich glycocalyx, it was difficult to imagine how TRLs in the bloodstream would interact with the GPIHBP1–LPL complex on the plasma membrane. To examine TRL margination in relation to the endothelial cell glycocalyx, unlabeled TRLs were injected into a wild-type mouse, and tissue sections were stained with a copper-containing dye that binds to the glycocalyx [11]. By EM, the glycocalyx covering endothelial cells in large veins was a thick and continuous “forest” (Figure 6). In capillaries, the glycocalyx was patchy, with tufts of glycocalyx interspersed between “meadows” where the glycocalyx was absent and the plasma membrane was exposed. TRLs appeared to marginate in the “meadows” (Figure 6) [11].

Dual-axis electron tomography studies suggested that some TRLs in heart capillaries are attached to membrane projections (or flaps) that extend from the plasma membrane of endothelial cells. The membrane projections are 6–7 nm thick and display the train-track morphology of a membrane bilayer. These membrane flaps, tentatively called “nanovilli,” were also identified in transcytotic vesicles of capillary endothelial cells and on the basolateral plasma membrane. Immunogold EM studies provided suggestive (but not definitive) evidence that “nanovilli” contain GPIHBP1. It seems possible that “nanovilli” play roles in both LPL transport across endothelial cells and TRL margination within the capillary lumen [11].

To decipher the function of GPIHBP1 in plasma triglyceride metabolism, imaging studies have been crucial. The discovery that LPL was mislocalized in *Gpihbp1*$^{-/-}$ tissues provided the impetus to GPIHBP1’s ability to transport LPL across endothelial cells (Figure 3). Similarly, insights into TRL margination depended on imaging (Figures 4–6). As we look to the future, imaging will remain crucial. For example, at the current time, no one understands how the lipid products of TRL processing move across endothelial cells towards parenchymal cells. We are optimistic that NanoSIMS imaging will yield insights into cellular mechanisms for lipid transport across endothelial cells—and that those insights will suggest new hypotheses for biochemical and genetic studies.

GPIHBP1–LPL interactions

Cell transfection experiments with GPIHBP1 expression vectors revealed that GPIHBP1's LU domain is crucial for LPL binding [18–20]. When GPIHBP1's LU domain was replaced with the LU domain from CD59, LPL binding was absent [18]. Also, mutating any of the 10 cysteines in GPIHBP1's LU domain abolished LPL binding, indicating that proper disulfide bond formation and the integrity of the three-fingered LU domain is essential for LPL binding [19]. Aside from the conserved cysteines, alanine-scanning mutagenesis uncovered 12 additional residues in GPIHBP1's LU domain that are required for LPL binding, and nine of those were within the central loop 2 defined by β-strands C and D in the LU domain (Figure 2B) [20].

Cell transfection studies have also helped to define LPL sequences required for GPIHBP1 binding. The carboxyl-terminal half of LPL (residues 298–448) is sufficient for specific binding of LPL to GPIHBP1 [38], and residues 400–435 appear to be particularly important [39]. Two *LPL* missense mutations, C418Y and E421K, first identified in patients with chylomicronemia [40, 41], abolish the capacity of LPL to bind to GPIHBP1 and do so without affecting binding to heparin or LPL catalytic activity [39].

The stoichiometry of LPL–GPIHBP1 binding is unclear and needs more study. LPL is generally thought to be a head-to-tail homodimer [42–44]; consequently, one might reasonably presume that LPL would have two binding sites for GPIHBP1. Thus far, however, no one has shown that this is the case. Addressing LPL–GPIHBP1 stoichiometry in a definitive fashion will likely require advanced imaging and biophysical approaches.

Recently, Mysling and coworkers [12] used purified preparations of LPL and GPIHBP1 to investigate LPL–GPIHBP1 interactions. LPL was purified from bovine milk, and soluble GPIHBP1 was purified from *Drosophila S2* cells [45]. LPL–GPIHBP1 interactions were investigated with several experimental approaches, including hydrogen–deuterium exchange/mass spectrometry (HDX-MS). HDX-MS experiments assess the propensity of amide hydrogens in the protein backbone to exchange with deuterium in the presence of heavy water (D_2O). When the solvent accessibility of a protein domain is unfettered, D_2O exchange is rapid, whereas deuterium uptake is slow when solvent access is limited by a tightly folded protein structure, a stable protein–protein interaction, and/or a stable hydrogen-bonding pattern. In the case of GPIHBP1, deuterium uptake into the acidic domain was extremely rapid, reflecting an absence of secondary structure in that domain (Figure 7). In contrast, deuterium exchange was low in residues 64–93 in GPIHBP1's LU domain, a highly ordered region with minimal accessibility to solvent. When GPIHBP1 was bound to LPL, additional sequences in GPIHBP1's LU domain (residues 104–128) were protected from deuterium uptake (Figure 7), implying that LPL binding protected those residues from solvent exposure [12]. Interestingly, LPL binding did not alter deuterium uptake in the acidic domain, suggesting that interactions between GPIHBP1's acidic domain and LPL are transient or that LPL binding to GPIHBP1 has little effect on the accessibility of the acidic domain to solvent.

Mysling and coworkers also examined the impact of GPIHBP1 binding on hydrogen–deuterium exchange in LPL [12]. Those studies revealed that GPIHBP1 protected a segment of LPL's carboxyl terminus (residues 402–419) from deuterium uptake. This protection was likely provided by GPIHBP1's LU domain because no such protection was evident with a synthetic peptide corresponding to GPIHBP1's acidic domain. The acidic domain peptide did reduce deuterium uptake in LPL residues 279–293, a region of LPL that contains a positively-charged heparin-binding motif [12]. Protein cross-linking experiments provided further support for a transient interaction site in that region [12].

Quantitative analyses of GPIHBP1–LPL binding

To dissect the contributions of GPIHBP1's LU and acidic domains to LPL binding, Mysling and coworkers used a surface plasmon resonance (SPR)–based assay [12]. In this assay, an LPL-specific monoclonal antibody was used to capture LPL in a defined orientation *via* its carboxyl-terminal domain (CTD) on a Biacore sensor chip. Next, the binding of full-length GPIHBP1 and a mutant GPIHBP1 lacking the acidic domain (GPIHBP1-Δacidic) to the immobilized LPL was analyzed. Both full-length GPIHBP1 and GPIHBP1-Δacidic bound tightly to the immobilized LPL, but the affinity of binding was higher for full-length GPIHBP1 (K_D = 25 nM *vs.* K_D = 91 nM for GPIHBP1-Δacidic). These differences were accounted for by differences in the association rate constants ("*on-rates*"); the dissociation rate constants ("*off-rates*") for the two GPIHBP1 proteins were similar. From these kinetic studies, it was proposed that one function of GPIHBP1's acidic domain is to facilitate, by electrostatic steering, a "first encounter complex" between LPL and GPIHBP1 [12].

Spontaneous unfolding of LPL's amino-terminal catalytic domain

Investigators who have studied intravascular lipolysis have long recognized that the catalytic activity of purified LPL declines rapidly at room temperature. The HDX-MS experiments by Mysling and coworkers provided new insights into the mechanism for this phenomenon [12]. When LPL was incubated with D_2O at room temperature, deuterium uptake into LPL's amino-terminal catalytic domain increased steadily over time, reflecting progressive unfolding of LPL's catalytic domain (*i.e.,* loss of secondary structure and increased exposure to D_2O). Protein unfolding was found in large portions of LPL's catalytic domain, including catalytic triad sequences. The unfolding of the catalytic domain was accompanied by a progressive loss in LPL activity (in assays using both triolein and soluble fluorescent substrates). Interestingly, LPL's carboxyl-terminal domain did not shown any signs of spontaneous unfolding by HDX-MS, implying that the secondary structure of that domain remained stable [12].

Binding of LPL to GPIHBP1 attenuates the unfolding of LPL's catalytic domain

To determine if GPIHBP1 might attenuate the spontaneous unfolding of LPL, Mysling and coworkers quantified deuterium uptake in LPL incubated at 25°C with full-length GPIHBP1, GPIHBP1-Δacidic, or a synthetic peptide corresponding to GPIHBP1's acidic domain [12]. The binding of full-length GPIHBP1 to LPL markedly reduced unfolding of LPL's catalytic

domain. GPIHBP1's acidic domain appeared to be important for this protective effect. First, incubation of LPL with the GPIHBP1 acidic domain peptide also reduced deuterium uptake into LPL's catalytic domain. Second, the ability of GPIHBP1-Δacidic to protect LPL from unfolding was less than that observed with full-length GPIHBP1.

In an earlier study, Sonnenburg and coworkers [33] reported that a soluble version of GPIHBP1 preserved the catalytic activity of immobilized LPL in the presence of ANGPTL4. In future studies, it will be interesting to determine if ANGPTL4 inactivates LPL by promoting the unfolding of LPL's catalytic domain, and if so whether that unfolding is inhibited by GPIHBP1 binding.

GPIHBP1 mutations cause chylomicronemia

A number of *GPIHBP1* mutations have been identified in patients with familial chylomicronemia [46–56] and have been tabulated in a recent review [55]. Most are missense mutations involving an LU domain cysteine or a residue close to a cysteine. For example, C65Y, C65S, C68Y, C68G, C68R, C83R, and C89F mutations have been encountered in patients with familial chylomicronemia (Figure 2B) [46–51, 55]. A S107C mutation, which introduces an unpaired cysteine into the LU domain, also causes chylomicronemia (Figure 2B) [52]. Q115P and T111P mutations, which introduce a proline adjacent to a conserved cysteine, have also been observed in chylomicronemia patients (Figure 2B) [53, 54]. Chylomicronemia has also been reported in association with a T80K mutation [56], which prevents N-linked glycosylation and would be expected to reduce trafficking of GPIHBP1 to the plasma membrane [22]. A G175R mutation has been reported to cause chylomicronemia [48]. This mutation is downstream from the LU domain and introduces a positively charged amino acid into the carboxyl-terminal hydrophobic signal peptide that is normally replaced by the GPI anchor. Thus, if GPI anchoring were to occur normally, residue 175 not be present in mature GPIHBP1. The G175R mutation was presumed to interfere with the addition of the GPI anchor [48], but this notion was not directly tested.

Two dysfunctional mutant *GPIHBP1* alleles are required to cause chylomicronemia. The plasma triglyceride levels in "GPIHBP1 homozygotes" are often >2,000 mg/dl (similar to levels in LPL-deficient patients), although some have had triglyceride levels less than 1000 mg/dl [46–54]. Many patients have had a history of pancreatitis. Heterozygous carriers of *GPIHBP1* mutations have no lipid abnormalities, implying that half-normal amounts of a functional GPIHBP1 are sufficient for normal intravascular lipolysis.

GPIHBP1 deficiency results in low levels of LPL in both pre- and post-heparin plasma. For example, in a "Q115P homozygote," post-heparin LPL levels were only ~10% of those in control subjects [53]. Pre-heparin LPL levels were also very low in "S107C homozygotes" [52]. The post-heparin LPL levels in "C65S/C68G compound heterozygotes" were only ~5% of those in control subjects [47].

When the LU domain mutations found in chylomicronemia patients are expressed in CHO cells, they invariably display little or no capacity to bind LPL [46, 47, 53]. In the case of the

cysteine mutants, Beigneux and coworkers initially suspected that the reduced LPL binding might result from reduced trafficking of GPIHBP1 to the surface of cells, but this was not the case [19]. Mutating a conserved cysteine in GPIHBP1 had little effect on the amount of GPIHBP1 reaching the cell surface, as judged by immunocytochemistry or by the amount of GPIHBP1 released from the cell surface with a phosphatidylinositol-specific phospholipase C. Thus, the reduced ability of GPIHBP1 cysteine mutants to bind LPL cannot be ascribed, at least in CHO cells, to reduced amounts of GPIHBP1 on the cell surface.

Many *GPIHBP1* missense mutations impair disulfide bond formation and result in the formation of GPIHBP1 dimers and multimers

Recent studies by Beigneux and collaborators provided a unifying concept for how the majority of mutations in GPIHBP1's LU domain impair LPL binding [52, 57]. In the case of the S107C mutation, introducing the unpaired cysteine into the LU domain resulted in inappropriate intermolecular disulfide bonds and the production of disulfide-linked GPIHBP1 dimers and multimers [52]. Subsequent studies revealed that the formation of GPIHBP1 dimers and multimers is a hallmark of many other GPIHBP1 proteins [57]. For example, when C65Y, C65S, and C68G GPIHBP1 mutants were expressed in CHO cells, GPIHBP1 trafficked to the cell surface but most of the protein was in the form of disulfide-linked dimers and multimers [57].

Markedly increased amounts of GPIHBP1 dimers and multimers is relevant to the pathogenesis of chylomicronemia because *only* GPIHBP1 monomers have the capacity to bind LPL [52, 57, 58]. The inability of disulfide-linked GPIHBP1 dimers and multimers to bind LPL was evident in immunoprecipitation studies (Figure 8). Beigneux and coworkers took advantage of the fact that *Drosophila* cells, when transfected with a plasmid encoding wild-type GPIHBP1, secrete GPIHBP1 monomers as well as disulfide-linked dimers and multimers (Figure 8). The production of dimers and multimers was likely a consequence of defective disulfide bond formation in the setting of protein overexpression. The GPIHBP1 monomers, dimers, and multimers were then mixed with human LPL and incubated with agarose beads coated with an LPL-specific monoclonal antibody. As expected, LPL bound to GPIHBP1, and that complex was captured by the antibody-coated beads. When the GPIHBP1 bound to the beads was examined under nonreducing conditions, only GPIHBP1 monomers were present—even though large amounts of dimers and multimers were present in the starting material (Figure 8). Thus, only monomeric GPIHBP1 has the capacity to bind LPL.

An increased propensity for dimerization and multimerization occurs with most "non-cysteine" GPIHBP1 mutants that abolish LPL binding (*e.g.*, Y66A, L71A, I93A, T104A, T105A, H106L, S107A, T108R, V126A) [57]. However, there was one exception. Changing W109 to other amino acid residues abolished LPL binding but was associated with a *reduced* propensity for protein dimerization/multimerization [57]. W109 mutations likely interfere with LPL binding in a more direct fashion—probably by disrupting the GPIHBP1–LPL binding interface. In support of this idea, tryptophans are known to be overrepresented in protein–protein binding interfaces [59–61], and W109 is one of only a handful of residues in

the LU domain (aside from the conserved cysteines) that is perfectly conserved in mammalian evolution. Also, the HDX-MS studies showed that W109 is located in a region of GPIHBP1 that is protected from deuterium uptake when GPIHBP1 is bound to LPL [12].

GPIHBP1 is apparently absent in lower vertebrates

LPL as well as other key molecules in intravascular triglyceride metabolism (*e.g.*, apo-CII, apo-AV, ANGPTL4, CD36) are found in both mammals and "lower vertebrates" (*e.g.*, birds, amphibians, reptiles, fish). However, GPIHBP1—the protein that is so important for LPL transport, TRL margination, and LPL activity in mammals—is seemingly absent in other vertebrates [13]. Lower vertebrates have LU family members, but none appears closely related to mammalian GPIHBP1, and none has an acidic domain [13]. The seeming absence of GPIHBP1 in other vertebrates poses obvious questions. In other vertebrates, does LPL reach the capillary lumen? Or might a large fraction of the LPL remain in the interstitial spaces? In other vertebrates, does an unrelated protein shuttle LPL across endothelial cells? Investigating these questions is important because the answers could yield unexpected insights into mechanisms for TRL processing in mammals.

Concluding remarks

The role of LPL in plasma triglyceride metabolism has been recognized for decades, but the discovery of GPIHBP1 has substantially changed textbook descriptions of intravascular TRL processing (Figure 1). We now know that GPIHBP1 shuttles LPL to the capillary lumen and that a deficiency of GPIHBP1 mislocalizes LPL to the interstitial spaces. It is now clear that GPIHBP1 (not HSPGs) is the binding site for LPL in capillaries and that GPIHBP1-bound LPL is required for the margination of TRLs—so that TRL processing can proceed. We also know that GPIHBP1 protects LPL from unfolding and loss of catalytic activity. Finally, we now recognize that GPIHBP1 deficiency is a cause of severe, lifelong chylomicronemia. Most of the disease-causing mutations in *GPIHBP1* disrupt disulfide bond formation and promote the formation of GPIHBP1 dimers and multimers, which have no capacity to bind LPL. These discoveries, together with evidence that elevated plasma triglyceride levels increase the risk for coronary heart disease [62–65], have led to an awakening of interest in plasma triglyceride metabolism.

Continuing efforts to understand intravascular triglyceride metabolism are important. The discovery of GPIHBP1 has set the stage for more focused research efforts on mechanisms for intravascular lipolysis. The field needs to define the properties of GPIHBP1-bound LPL, understand LPL–GPIHBP1 stoichiometry, and investigate mechanisms by which GPIHBP1 protects LPL from unfolding. Also, we need to understand why GPIHBP1 is expressed in capillaries but not in larger blood vessels. We also need to understand mechanisms for triglyceride metabolism in lower vertebrates, where GPIHBP1 is apparently absent. Another issue that needs more work is whether GPIHBP1 might bind and transport additional proteins across capillaries—aside from LPL. Finally, the field must investigate the cellular mechanisms by which the lipid products of TRLs move across endothelial cells towards parenchymal cells.

Acknowledgments

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Glossary

Familial chylomicronemia
A severe form of hypertriglyceridemia that is often caused by mutations in *GPIHBP1, LPL, APOC2,* or *APOA5.*

Glycosylphosphatidylinositol-anchored high density lipoprotein–binding protein 1 (GPIHBP1)
The lipoprotein lipase transporter in capillary endothelial cells.

GPIHBP1's acidic domain
A stretch of amino acids at the amino terminus of GPIHBP1 that is highly enriched in glutamates and aspartates.

Lipoprotein lipase (LPL)
A triglyceride hydrolase that hydrolyzes triglycerides in triglyceride-rich lipoproteins.

Lymphocyte antigen 6–Urokinase-type plasminogen activator receptor (LU) proteins
A family of proteins containing 8 or 10 cysteines, all arranged in a characteristic spacing pattern and all disulfide-bonded so as to form a three-fingered structural motif. GPIHBP1, Urokinase-type plasminogen activator receptor (uPAR), CD59, and SLURP1 are particularly well-characterized members of the LU protein family.

Triglyceride-rich lipoproteins (TRLs)
TRLs include chylomicrons (secreted by intestinal enterocytes) and very low density lipoproteins (VLDL, secreted by hepatocytes).

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TRENDS BOX

- GPIHBP1 is required for proper localization of LPL in capillaries.
- GPIHBP1 shuttles LPL from the interstitial spaces to the capillary lumen, where the enzyme hydrolyzes triglycerides in triglyceride-rich lipoproteins (TRLs).
- In GPIHBP1 knockout mice, LPL does not reach the capillary lumen, resulting in impaired processing of TRLs, markedly increased plasma triglyceride levels, reduced delivery of lipid nutrients to parenchymal cells, increased *de novo* lipogenesis in adipose tissue, and increased uptake of TRL lipids by the liver.
- *GPIHBP1* missense mutations in humans cause severe hypertriglyceridemia (chylomicronemia). The majority of these mutations interfere with the proper formation of disulfide bonds, resulting in the production of GPIHBP1 dimers and multimers with no capacity to bind LPL.

Outstanding Questions Box

- What are the properties of GPIHBP1-bound LPL? Most of our understanding of LPL has come from studies with purified LPL, but most of the LPL in tissues is bound to GPIHBP1. How do apo-CII, apo-CIII, and apo-AV affect the activity of GPIHBP1- bound LPL?
- The stoichiometry of LPL GPIHBP1 binding needs to be defined. LPL is thought to be a homodimer and would be expected to have two GPIHBP1 binding sites. Thus far, however, no one has assessed the stoichiometry of LPL GPIHBP1 binding.
- Does GPIHBP1 transport other proteins (aside from LPL) across capillaries? Does GPIHBP1 interact with other lipid raft proteins in capillary endothelial cells?
- Is GPIHBP1-bound LPL protected from inactivation by ANGPTL4 and ANGPTL3? If so, which GPIHBP1 domains are important for the protective effect?
- What factors control GPIHBP1 expression? Why is GPIHBP1 expressed in capillaries but not in larger blood vessels? Is GPIHBP1 expression in capillaries activated by a paracrine factor from parenchymal cells? Why is GPIHBP1 expression low in capillaries of the brain?
- A better understanding of LPL movement in the subendothelial spaces is needed. Following secretion from myocytes and adipocytes, LPL likely encounters HSPGs that coat the surface of those cells. What mechanisms account for the movement of LPL from those HSPGs binding sites to GPIHBP1 on capillaries?
- How do the fatty acid products of TRL processing move across endothelial cells? Do they simply diffuse broadly or move across endothelial cells in vesicles?
- GPIHBP1 is seemingly absent in "lower vertebrates." Does LPL reach the capillary lumen in those species? Does an unrelated protein transport LPL across capillary endothelial cells in those species?

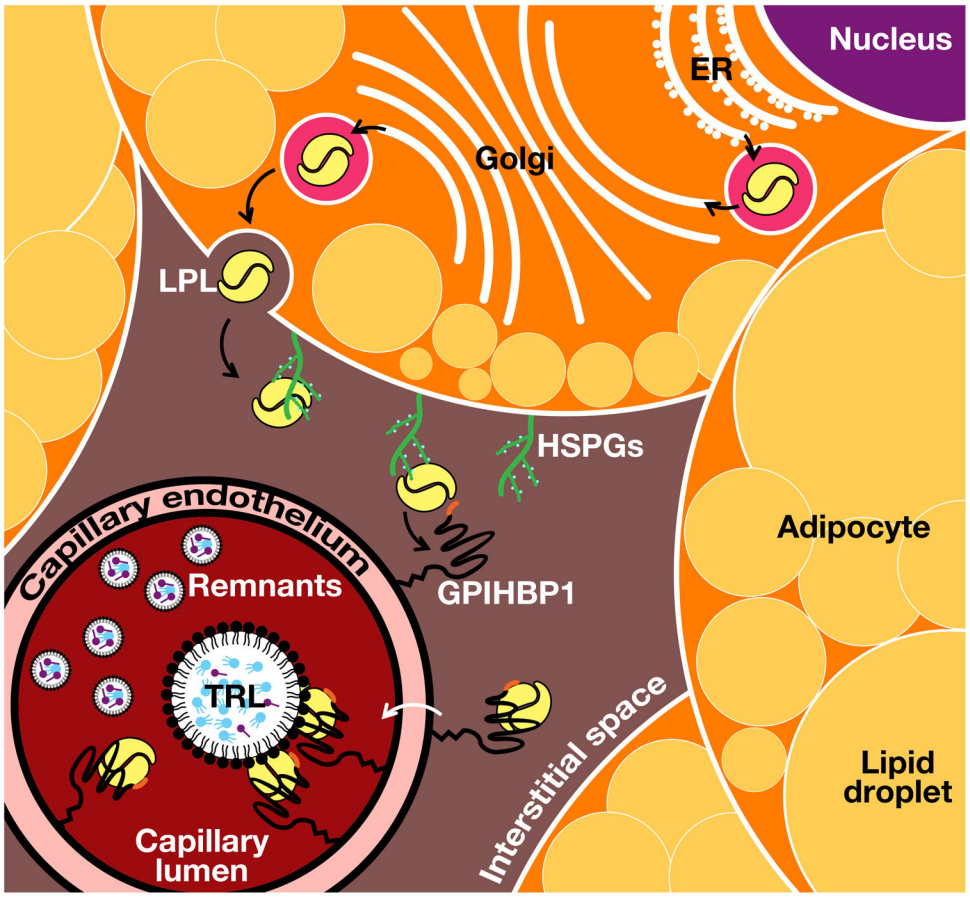

Figure 1 Key Figure. Role of GPIHBP1 in plasma triglyceride metabolism

Lipoprotein lipase (LPL) is synthesized by parenchymal cells (for example adipocytes, as depicted here) and secreted into the interstitial spaces. After secretion, LPL is first captured by heparan sulfate proteoglycans (HSPGs) but then moves quickly to GPIHBP1 on the surface of capillary endothelial cells. GPIHBP1's cysteine-rich LU domain is primarily responsible for high-affinity binding of LPL, but the amino-terminal acidic domain (orange) likely plays an accessory role in capturing and binding LPL. The acidic domain also stabilizes LPL activity. GPIHBP1-bound LPL is then transported within vesicles across endothelial cells to the capillary lumen. The GPIHBP1–LPL complex is responsible for the margination of triglyceride-rich lipoproteins (TRLs) in the bloodstream, allowing triglyceride hydrolysis by LPL to proceed. Following LPL-mediated triglyceride hydrolysis, the lipoprotein particles (now called *Remnants*) are released back into the bloodstream.

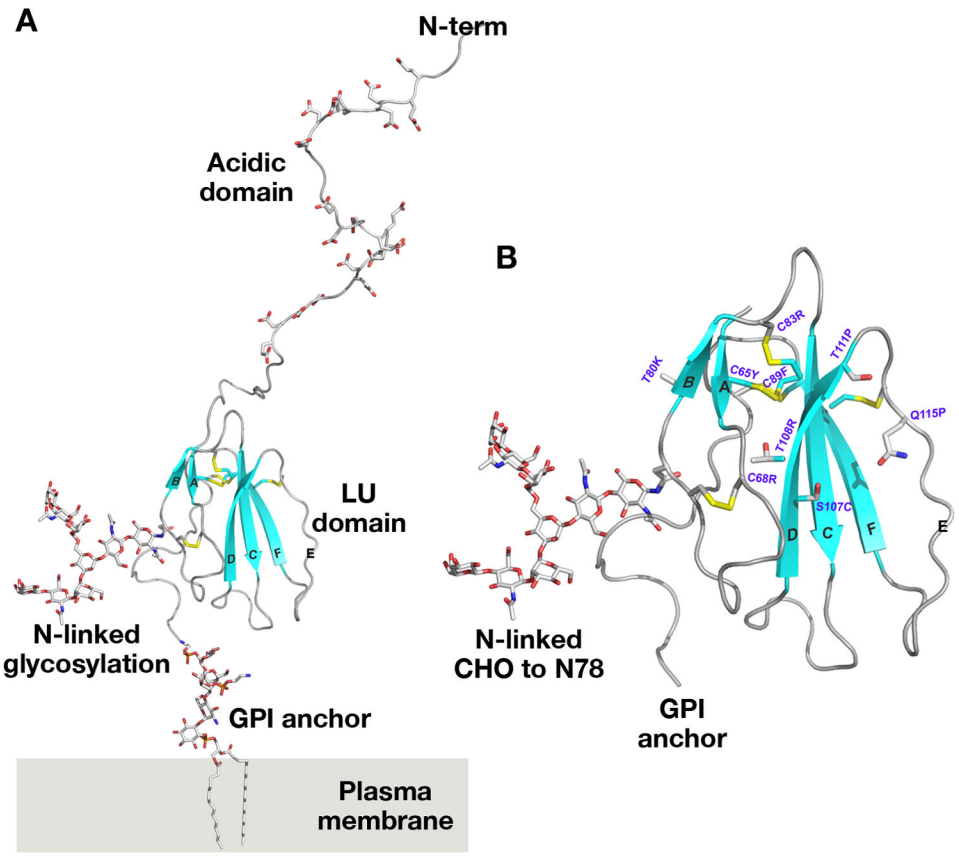

Figure 2. Structural features of GPIHBP1 and locations of clinically significant *GPIHBP1* mutations

(A) Molecular model of GPIHBP1. GPIHBP1 has an intrinsically disordered amino-terminal acidic domain, a highly ordered LU domain, and a glycosylphosphatidylinositol (GPI) anchor that tethers the protein to the plasma membrane. The acidic amino acids (aspartate, glutamate) within the acidic domain are highlighted by red sticks; the β-sheets in the LU domain are colored cyan and are numbered A–F as described previously [66]; disulfide bonds in the LU domain are highlighted with yellow sticks. Also shown is a single bi-antennary N-linked glycan, which in human GPIHBP1 is attached to N78 in the LU domain. Image generated with PyMOL; modified from [12]. **(B)** Diagram showing selected *GPIHBP1* missense mutations causing chylomicronemia. Many of the disease-causing mutations eliminate conserved cysteines in the LU domain (*e.g.,* C65Y, C68R, C83R, C89F); introduce a new cysteine into the LU domain (*e.g.,* S107C); or change a residue close to a conserved cysteine (*e.g.,* T108R, T111P, Q115P). All of these mutations impair disulfide bond formation, resulting in the formation of GPIHBP1 dimers and multimers [57]. Also shown is a T80K mutation, which eliminates an N-linked glycosylation site in the LU domain and would be expected to reduce GPIHBP1 trafficking to the plasma membrane. The model of GPIHBP1's LU domain was created by PyMOL as described [12].

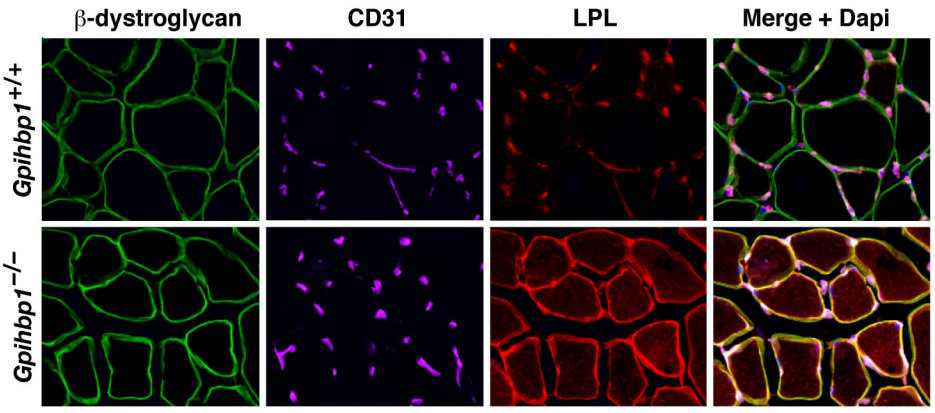

Figure 3. Confocal micrograph showing LPL in capillaries in the skeletal muscle of a wild-type mouse (*Gpihbp1*$^{+/+}$), while the LPL is mislocalized to the interstitial spaces in a *Gpihbp1* knockout mouse (*Gpihbp1*$^{-/-}$)

Images show β-dystroglycan (a marker of skeletal myocytes) (green), CD31 (a marker of endothelial cells) (purple), and LPL (red) in the skeletal muscle from a *Gpihbp1*$^{+/+}$ mouse and a *Gpihbp1*$^{-/-}$ mouse. The LPL was largely bound to capillaries in the *Gpihbp1*$^{+/+}$ mouse but was mislocalized to the interstitial spaces around myocytes in the *Gpihbp1*$^{-/-}$ mouse, presumably bound to heparan sulfate proteoglycans (HSPGs). Reproduced with permission [10].

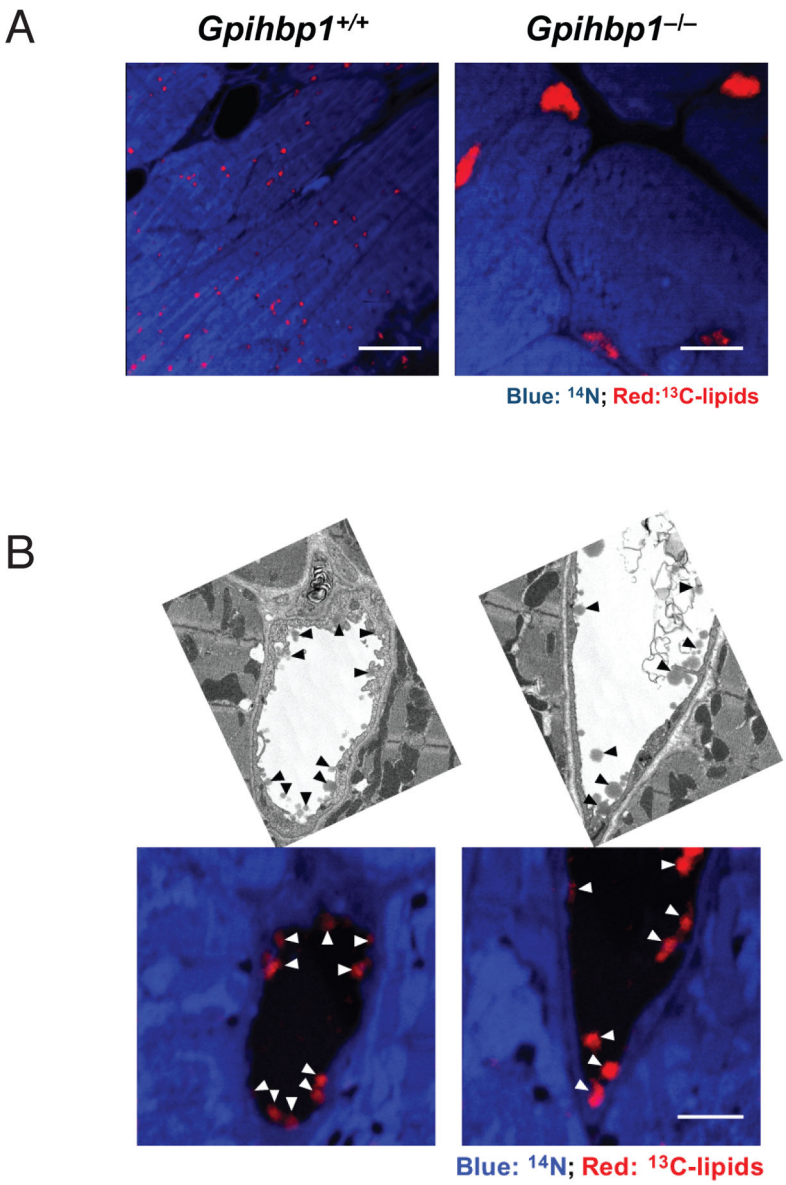

Figure 4. Visualizing triglyceride metabolism with NanoSIMS imaging

(A) Documenting the delivery of TRL-derived lipids to cytosolic fat droplets in cardiac myocytes. A wild-type mouse and a *Gpihbp1* knockout mouse (*Gpihbp1*$^{-/-}$) were given ^{13}C-fatty acids by gavage. The ^{13}C-lipids were incorporated into chylomicrons, which then entered the circulation. In the wild-type mouse, the chylomicrons were processed by LPL in capillaries, providing ^{13}C lipids to the heart. As illustrated by the NanoSIMS images, ^{13}C enrichment was high in the cytosolic fat droplets (arrowheads) in cardiomyocytes of the wild-type mouse (note the red droplets in myocytes). In the *Gpihbp1*$^{-/-}$ mouse, the delivery of ^{13}C lipids to myocytes was reduced, and most of the ^{13}C remained in chylomicrons within the capillary lumen (L). Scale bar, 6 μm. (B) Visualizing the margination of ^{13}C-labeled TRLs along heart capillaries. ^{13}C-labeled TRLs were injected into a wild-type mice, and tissues were collected 15 min later for imaging. Top panels, backscattered electron

images demonstrating margination of TRLs (arrowheads) along the capillary endothelium in the heart. Lower panels, $^{13}C/^{12}C$ NanoSIMS images on the same tissue sections showing that the TRLs (arrowheads) are enriched in ^{13}C. Scale bar, 2 μm. In the NanoSIMS images, the ^{14}N signal (depicting cellular morphology) is shown in blue; the $^{13}C/^{12}C$ signal, which ranges from 1.1 to 2.2%, is red.

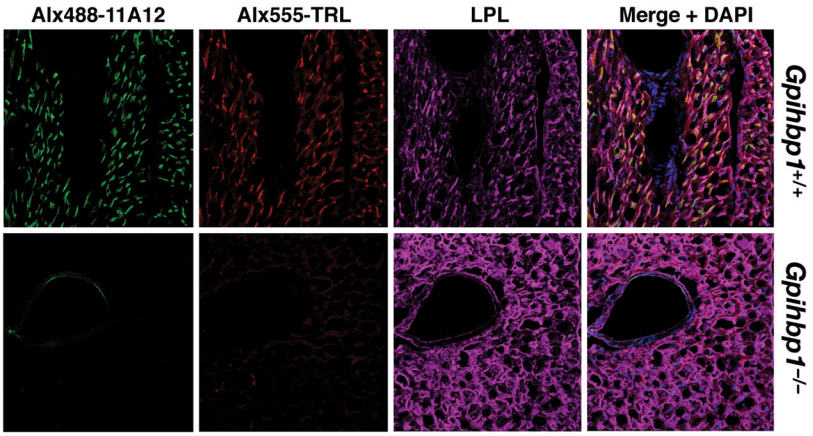

Figure 5. Margination of triglyceride-rich lipoproteins (TRLs) along capillaries depends on GPIHBP1

Confocal images to assess the binding of an Alexa488-labeled anti-GPIHBP1 monoclonal antibody (11A12, green) and Alexa555-labeled chylomicrons (red) after they were injected into a wild-type (*Gpihbp1*$^{+/+}$) mouse and a *Gpihbp1*$^{-/-}$ mouse. Fixed sections were stained with a mouse LPL–specific antibody (magenta) and DAPI (blue). In *Gpihbp1*$^{-/-}$ mice, TRL margination was virtually absent, and LPL was mislocalized within the interstitial spaces. Reproduced with permission [11].

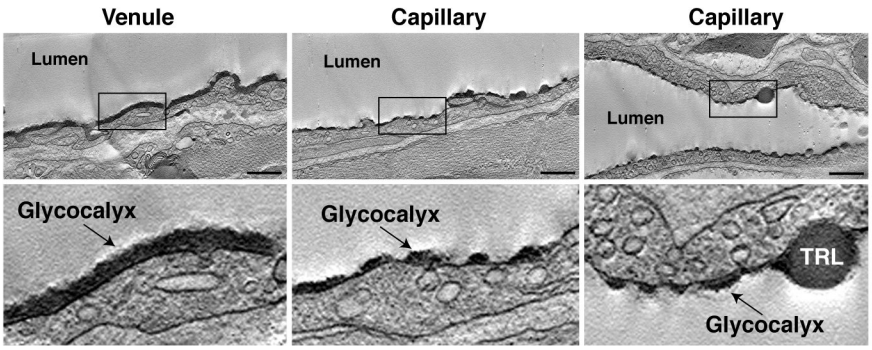

Figure 6. Alcian blue–stained electron micrographs, revealing a patchy distribution of glycocalyx in capillaries

Unlabeled triglyceride-rich lipoproteins (TRLs) were injected into a wild-type mouse. After 30 sec, the mice were perfused with PBS and then perfusion-fixed with glutaraldehyde containing Alcian blue (to stain the glycocalyx). Higher-magnification images of the boxed areas are shown in the lower panels. In heart capillaries of wild-type mice, TRLs appeared to bind to “meadows” inbetween patches of glycocalyx. Scale bar, 800 nm. Reproduced with permission [11].

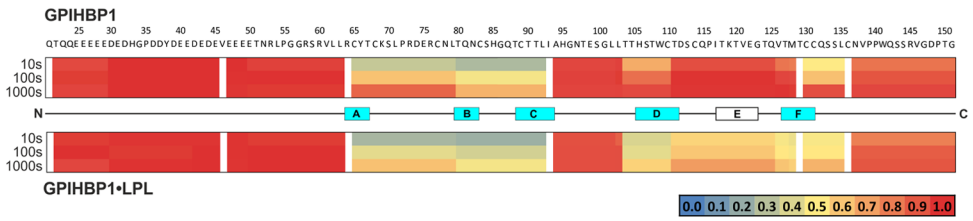

Figure 7. Deuterium uptake in free GPIHBP1 and LPL-occupied GPIHBP1

Deuterium uptake was assessed after 10-, 100-, and 1000-sec incubations of proteins with heavy water (D_2O). Relative amounts of deuterium uptake are depicted according to a color code (blue, no deuterium uptake; red, complete deuterium uptake). In the absence of LPL, some regions of GPIHBP1's LU domain were protected from deuterium uptake (residues 64–93). When GPIHBP1 was bound to LPL, additional regions of the LU domain (residues 104–128) were protected from deuterium uptake. Amino acid numbering is according to the entire GPIHBP1 coding sequence, including the amino-terminal signal peptide. Mature GPIHBP1 starts at residue 21. Reproduced with permission [12].

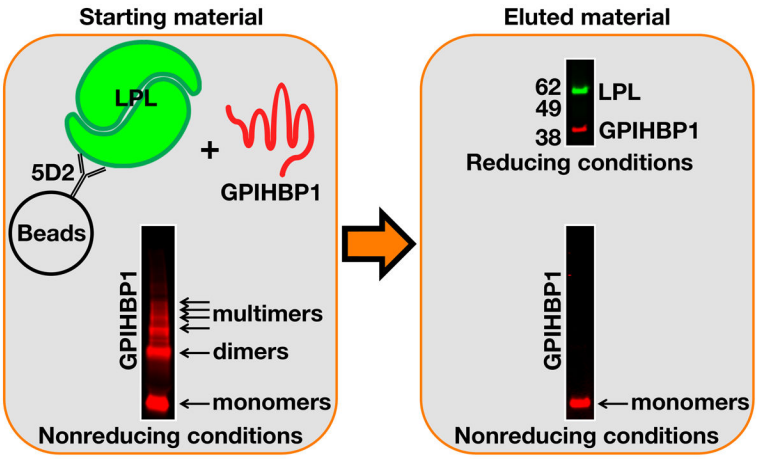

Figure 8. Only GPIHBP1 monomers, and not dimers or multimers, are capable of binding LPL

A secreted version of wild-type GPIHBP1 was expressed in *Drosophila* S2 cells; these cells secrete GPIHBP1 monomers as well as disulfide-linked dimers and multimers. The GPIHBP1 was incubated with V5-tagged human LPL along with agarose beads that had been coated with the LPL-specific monoclonal antibody 5D2. After incubating the mixture for 1 h at 4 °C, the beads were washed. Next, the beads were incubated with SDS sample buffer, which releases any LPL captured by the antibody along with any GPIHBP1 that was bound to the LPL. Shown on the left is a western blot on the starting GPIHBP1 preparation, using an IRdye680-labeled monoclonal antibody against GPIHBP1 (11A12). That GPIHBP1 preparation, which was electrophoresed under nonreducing conditions, contained large amounts of GPIHBP1 monomers, dimers, and multimers. Shown on the right are western blots on the proteins released from the antibody-coated beads. On the top is a western blot performed under reducing conditions using an IRdye800-V5 antibody (green) and an IRdye680-labeled antibody 11A12 (red). As expected, the 5D2-coated beads pulled down LPL as well as LPL-bound GPIHBP1. Shown on the bottom is a western blot under nonreducing conditions with IRdye680-labeled antibody 11A12; this blot shows that only GPIHBP1 monomers bound to LPL.