CLSTN3B enhances lipid droplet function via endoplasmic reticulum contacts

Xing Zeng (✉ xing.zeng@utsouthwestern.edu)

UT Southwestern Medical Center

Chuanhai Zhang

UT Southwestern Medical Center

Goncalo Vale

The University of Texas Southwestern Medical Center <https://orcid.org/0000-0003-1104-0289>

Kaitlyn Eckert

UT Southwestern Medical Center

Jeffrey McDonald

University of Texas Southwestern Medical Center

Mei-Jung Lin

Department of Physiology

Mengchen Ye

UC Berkeley

Biological Sciences - Article

Keywords:

Posted Date: January 27th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1290351/v1>

License: This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Inter-organellar contact sites facilitate material exchanges between membrane-bound intracellular compartments and sustain the structural and functional integrity of organelles¹⁻³. The endoplasmic reticulum (ER)-lipid droplet (LD) contact sites contribute to LD biogenesis and lipid transfer between the ER and LDs⁴⁻¹¹. LDs in adipocytes are significantly larger than in non-adipocytes and crucial for energy storage and mobilization in response to body needs¹²⁻¹⁴. How adipocytes employ cell type-specific mechanisms to suppress unregulated lipolysis while remaining responsive to catecholamine-induced lipolysis is incompletely understood. Here we show that the mammalian adipocyte-specific protein Calsyntenin 3 β (CLSTN3B) works at the ER/LD interface to enhance LD functionality. We found that CLSTN3B harbors an N-terminal LD-targeting and a C-terminal ER transmembrane domain linked by an arginine-rich segment, promoting a tight association between the ER and LDs. CLSTN3B-deficient LDs have reduced surface phospholipids density and decreased binding of LD-targeting proteins, hence more prone to leakage of free fatty acids (FFA) and less responsive to catecholamine-induced lipolysis than wild-type (WT) LDs. Furthermore, *clstn3b* knockout (*clstn3b*^{-/-}) mice are more susceptible to high fat diet-induced metabolic complications than adiposity-matched WT mice, consistent with impaired LD functionality and compromised lipid-storing capacity of adipocytes. Our results demonstrate how CLSTN3B-mediated inter-organellar communication shapes LD functionality and translates into physiological significance at the animal level¹⁵

Full Text

Inter-organellar contact sites facilitate material exchanges between membrane-bound intracellular compartments and sustain the structural and functional integrity of organelles¹⁻³. The endoplasmic reticulum (ER)-lipid droplet (LD) contact sites contribute to LD biogenesis and lipid transfer between the ER and LDs⁴⁻¹¹. LDs in adipocytes are significantly larger than in non-adipocytes and crucial for energy storage and mobilization in response to body needs¹²⁻¹⁴. How adipocytes employ cell type-specific mechanisms to suppress unregulated lipolysis while remaining responsive to catecholamine-induced lipolysis is incompletely understood. Here we show that the mammalian adipocyte-specific protein Calsyntenin 3 β (CLSTN3B) works at the ER/LD interface to enhance LD functionality. We found that CLSTN3B harbors an N-terminal LD-targeting and a C-terminal ER transmembrane domain linked by an arginine-rich segment, promoting a tight association between the ER and LDs. CLSTN3B-deficient LDs have reduced surface phospholipids density and decreased binding of LD-targeting proteins, hence more prone to leakage of free fatty acids (FFA) and less responsive to catecholamine-induced lipolysis than wild-type (WT) LDs. Furthermore, *clstn3b* knockout (*clstn3b*^{-/-}) mice are more susceptible to high fat diet-induced metabolic complications than adiposity-matched WT mice, consistent with impaired LD functionality and compromised lipid-storing capacity of adipocytes. Our results demonstrate how CLSTN3B-mediated inter-organellar communication shapes LD functionality and translates into physiological significance at the animal level¹⁵.

CLSTN3B promotes ER/LD contact formation

We previously studied the subcellular localization of CLSTN3B in brown adipocytes ectopically expressing CLSTN3B-APEX2¹⁵. Whereas we observed clear APEX2 localization to the ER, we also noticed APEX2 localization to LD surfaces, with small LDs (diameter ~500 nm) being more completely encircled (Extended Data Fig. 1a). The morphology of the APEX2 signals suggests that they may reflect ER in contact with LDs. To see whether endogenous CLSTN3B has a similar localization pattern, we performed CLSTN3B immunostaining on freshly isolated primary brown adipocytes and observed colocalization of CLSTN3B with the ER marker KDEL on LDs (Fig. 1a), suggesting that CLSTN3B localizes to ER/LD contact sites. Interestingly, CLSTN3B signals were more prominent on small LDs than large ones (Fig. 1a), recapitulating the APEX2 observation. After norepinephrine (NE) treatment to induce large LDs breakdown into small LDs, we observed clear CLSTN3B and KDEL signals around the majority of LDs (Fig. 1a). In *clstn3b*^{-/-} brown adipocytes, we observed a complete loss of CLSTN3B signals and reduced KDEL signals on LDs (Fig. 1a), suggesting that CLSTN3B not only localizes to ER/LD contact sites but contributes to the formation of such structures. Conversely, transgenic overexpression of CLSTN3B strongly enhances ER encircling of LDs (Fig. 1a). To visualize CLSTN3B localization at the ultrastructural level, we performed immunogold staining of endogenous CLSTN3B in NE-pretreated primary brown adipocytes. We observed almost complete encircling of LDs by the ER and the presence of gold particles within the ER lumen around LDs (Fig. 1b). In contrast, LDs in NE-pretreated *clstn3b*^{-/-} brown adipocytes are poorly enwrapped by the ER and tend to deform compared with the WT LDs (Fig. 1c-d, Extended Data Fig. 1b). These observations show that CLSTN3B localizes to ER/LD contact sites in brown adipocytes and promotes ER/LD association.

We then examined CLSTN3B localization in white adipocytes, given that it is also significantly expressed in those cells¹⁵. Since it is difficult to detect CLSTN3B on large LDs by imaging, we performed CLSTN3B immunoblot on isolated LDs. Whereas we saw predominantly full-length CLSTN3B on small LDs from cold-acclimated mouse brown adipocytes, we detected CLSTN3B fragments of lower molecular weights than the full-length protein on large LDs from white adipocytes (Fig. 1e). Since the antibody recognizes the C-terminus of CLSTN3B, those fragments may represent C-terminal cleavage products. The same cleaved products were observed on large LDs from warm-acclimated brown adipocytes (Fig. 1e). Both fluorescence and EM images revealed that large LDs (diameters > ~2.5 μ m) in brown adipocytes are not as extensively associated with the ER as small LDs (Fig. 1a, Extended Data Fig. 1a, 1c). We have thus confirmed CLSTN3B localization to white adipocyte LDs and uncovered a correlation between LD size, extent of ER association and the form of CLSTN3B: small LDs tend to have full length CLSTN3B and more extensive ER enwrapping, whereas large LDs tend to have cleaved CLSTN3B and reduced ER association.

To study CLSTN3B localization in heterologous systems, we ectopically expressed CLSTN3B in HEK293 and U2OS cells. When cells were induced to grow LDs by oleic acid (OA) treatment, CLSTN3B signals became encircling LDs and colocalized with ER markers, showing that those LDs were enwrapped by the ER (Fig. 1f, Extended Data Fig. 1d-f). In contrast, LDs formed in cells not expressing CLSTN3B generally

did not have ER associated with them (Fig. 1f). Electron microscopy revealed complete and tight encircling of LDs by the ER in CLSTN3B-expressing cells (Fig. 1g), similar to what was observed in brown adipocytes. To test whether CLSTN3B promotes ER/LD association under a cell-free condition, we incubated microsomes isolated from HEK293 cells expressing CLSTN3B-mCherry or an ER-targeting BFP reporter with LDs¹⁶. CLSTN3B-mCherry microsomes formed significantly more extensive contacts with LDs than the BFP control, recapitulating the *in vivo* situation (Fig. 1h-i). Taken together, we conclude that CLSTN3B promotes ER/LD contact formation in non-adipocytes and under a reconstituted condition.

CLSTN3B directly bridges LD and ER

To understand the sequence determinants underlying the localization of CLSTN3B, we analyzed its hydrophobicity plot¹⁷ and predicted its structure with AlphaFold¹⁸. The hydrophobicity plot revealed an extended N-terminal hydrophobic segment (approximately 1-198) and a transmembrane domain (approximately 248-269, identical with the transmembrane domain of CLSTN3), separated by an arginine-rich segment (Fig. 2a). Consistent with the hydrophobicity plot, AlphaFold predicted multiple long hydrophobic helices in the N-terminal region and a transmembrane domain in the C-terminal region (Extended Data Fig. 2). We therefore tested the localization of the N-terminal (1-198) and the C-terminal (199-357) fragments in HEK293 cells. The 1-198 fragment displayed unequivocal LD-localization (Fig. 2b), showing that the N-terminal hydrophobic segment is sufficient for LD-targeting. Importantly, LDs positive for 1-198 did not have the surrounding KDEL signals as observed for the full-length CLSTN3B (Fig. 2b), suggesting that the N-terminal fragment is unable to recruit the ER to LDs. The 199-357 fragment displayed exclusive ER localization without detectable LD localization (Fig. 2b), complementing the behavior of the N-terminal fragment. These data suggest that the N-terminal hydrophobic domain of CLSTN3B targets the LD whereas the C-terminal transmembrane domain is anchored at the ER.

To further probe the topology of CLSTN3B, we digested LDs from brown adipocytes with trypsin, separated the mixture into a buoyant LD and a pellet fraction containing dissociated ER fragments, and visualized the digestion products. The antibody detected two C-terminal cleavage products (Fig. 2c), likely being 134-357 (cleaved after R133, 25 kDa) and 167-357 (cleaved after K166, 21 kDa). Both fragments were partially partitioned into the pellet fraction (Fig. 2c), suggesting a reduced affinity for the LD resulting from the truncated hydrophobic domain. This finding supports that the C-terminus of CLSTN3B resides within the ER lumen so that the epitope is protected from trypsin digestion, also explaining the immunogold observation that the gold particles were exclusively found in the ER lumen (Fig. 1b). This topology puts the arginine-rich segment linking the LD-targeting domain and the ER transmembrane domain in the cytoplasmic space between the LD and the ER (Fig. 2e). Curiously, the arginine-rich segment seems resistant to trypsin digestion, possibly reflecting tight engagement of those arginine residues with negatively charged membrane phospholipids. Taken together, our findings showed that with an N-terminal LD-targeting domain and a C-terminal ER transmembrane domain combined in the same molecule, CLSTN3B directly bridges the LD and the ER.

The 25 kDa trypsin cleavage product resembles a natural cleavage product derived from endogenous CLSTN3B (C2 in Fig. 1e), suggesting a similar cleavage mechanism being employed *in vivo*. The corresponding cleavage site (R133) was predicted by AlphaFold to reside in a loop linking hydrophobic helices (Extended Data Fig. 2), thus may be exposed on the LD surface and susceptible to proteolysis. Such proteolysis generates C-terminal fragments with truncated LD-targeting domains, potentially representing a mechanism to promote ER/LD separation as LDs grow. To directly assess this idea, we examined the localization of a 131-357 fragment, mimicking the 25 kDa product. Although 131-357 retained some ability to promote ER/LD contact formation, the extent of LD enwrapping by the ER induced by this fragment was lower than the full-length protein (Fig. 2e-f), showing that truncating the N-terminal LD-targeting domain attenuates the ability of CLSTN3B to bridge the ER and LDs. This finding thus explains the reduced association of the ER with large LDs, given that large LDs predominantly harbor cleaved forms of CLSTN3B.

CLSTN3B promotes LD-protein binding

To investigate how CLSTN3B-mediated ER/LD contact formation affects LD function, we performed proteomics analysis of brown adipocyte LDs isolated from WT and *clstn3b*^{-/-} mice. As expected, we observed multiple significantly downregulated ER-associated proteins on the *clstn3b*^{-/-} LD compared with the WT (Fig. 3a-b and Supplementary Table), such as SEIPIN, BIP, VAPA, VAPB, and a panel of RAB proteins. Notably, multiple transport protein particle (TRAPP) complex subunits were found to be downregulated on the *clstn3b*^{-/-} LD (Fig. 3b, Supplementary Table). The TRAPP complex has been shown to exist on ER-derived vesicles, acts as the guanine nucleotide exchange factor (GEF) for RAB proteins to regulate membrane trafficking¹⁹, and implicated in LD homeostasis²⁰. Our data therefore suggest that the TRAPP complex and RAB proteins may coordinate with CLSTN3B in regulating LD function. Aside from ER-associated proteins, multiple LD-associated proteins were found to decrease on the *clstn3b*^{-/-} LDs, such as PLIN1, CIDEA, CIDEA, and CGI-58 (Fig. 3a).

We next examined whether similar changes in the levels of LD-associated proteins also occur on white adipocyte LDs. Indeed, we observed significant reductions in PLIN1 and CGI-58 associated with LDs from *clstn3b*^{-/-} white adipocytes compared with the WT (Fig. 3c). Taken together, our results showed that CLSTN3B enhances the targeting of LD-associated proteins in both brown and white adipocytes.

CLSTN3B elevates LD phospholipids density

The reduced binding of LD-associated proteins to *clstn3b*^{-/-} LDs raises an intriguing question because those proteins target LD directly with their LD-binding motifs independent of ER/LD contact sites²¹. Many LD-associated proteins downregulated on *clstn3b*^{-/-} LDs bind phospholipids, such as PLIN1, SEIPIN, and CIDEA²²⁻²⁴. Hence their decreased LD-association may reflect a reduced surface phospholipids density on *clstn3b*^{-/-} LDs. To test this hypothesis, we isolated LDs from WT and *clstn3b*^{-/-} brown adipocytes, digested with proteinase K to remove contaminating membranous components, including the ER and mitochondria, extracted phospholipids with a three-phase extraction protocol²⁵, and quantitated

phospholipids with a phospholipidomics approach and a fluorometric assay (Fig. 4a, Extended Data Fig. 3a). To calculate surface phospholipids density, we first analyzed the volume and surface area distribution of a large population of WT and *clstn3b*^{-/-} LDs (Fig. 4b), measured triglycerides contents of each LD sample, calculated the total LD surface area, and divided by phospholipids levels. The phospholipidomics results showed reduced densities of all major classes of phospholipids detected (PC 29%, PE 20%, and PS 33% reduced) on *clstn3b*^{-/-} LDs compared with the WT (Fig. 4c, Supplementary Table 2), agreeing with the fluorometric assay results (Fig. 4d) (PC 21% and PE 28% reduced). To test whether the ability of CLSTN3B to increase LD surface phospholipids density can be recapitulated in a heterologous system, we repeated the same LD isolation (Extended data Fig.3b) and phospholipids measurement procedure with HEK293 cells. Consistent with the brown adipocyte result, LDs from HEK293 cells expressing CLSTN3B displayed 56% higher density of PC and 48% higher density of PE than non-expressing cells (Fig. 4e-f). Our results thus demonstrated that CLSTN3B increases LD surface phospholipids density in different cellular contexts, explaining enhanced binding of LD-associated proteins. Importantly, expressing neither the 1-198 nor the 198-357 fragment caused elevated LD surface phospholipids density, showing that ER/LD contact formation is essential for this effect (Extended Data Fig. 3c-d).

Phospholipids transfer has been shown to occur between ER and other organelles^{26,27}. We therefore hypothesize that CLSTN3B may facilitate phospholipids transfer between the ER and LDs. This process may be mediated by the arginine-rich segment linking the N-terminal LD-targeting and the C-terminal ER-transmembrane, as arginine-rich peptides can bring membranes into extreme proximity and induce phospholipids mixing and fusion^{28,29}. To test this idea, we purified microsomes labeled with propargyl-choline from HEK293 cells expressing CLSTN3B-mCherry or the BFP reporter, incubated with *clstn3b*^{-/-} LDs, digested bound microsomes, and visualized PC transferred to LDs by click chemistry³⁰ (Fig. 4g). We observed a significantly higher level of propargyl-choline on the surface of LDs incubated with CLSTN3B-mCherry microsomes than the BFP control (Fig. 4h). Omitting the copper catalyst yielded a barely detectable background (Fig. 4h), showing that the contribution from unclicked fluorophores adsorbed to LD surfaces was negligible. Our data thus support that CLSTN3B facilitates the ER-to-LD phospholipids transfer, potentially via the arginine-rich segment linking the LD-targeting and the ER-transmembrane domain (Fig. 4i).

CLSTN3B enhances LD functionality

An essential function of the adipocyte LD is to suppress basal lipolysis while promoting sensitivity to catecholamine-induced lipid mobilization³¹⁻³⁴. Given that multiple LD-associated proteins, including PLIN1, are downregulated on *clstn3b*^{-/-} LDs, we reasoned that CLSTN3B may be important for LD functionality. We therefore measured basal and NE-induced lipolysis in WT and *clstn3b*^{-/-} brown and white adipocytes. Both *clstn3b*^{-/-} brown and white adipocytes displayed elevated basal lipolysis, yet reduced NE-induced lipolysis compared with the WT cells (Fig. 5a), phenocopying *plin1*^{-/-} cells³¹. Similarly, *clstn3b*^{-/-}

mice displayed a significantly blunted lipolytic response to the β 3-adrenergic receptor agonist CL-316,243 compared with the WT mice (Fig. 5b), indicating impaired LD functionality.

We previously observed that *clstn3b*^{-/-} mice displayed insulin resistance as opposed to WT mice before body weight divergence on high fat diet¹⁵. We hypothesize that the underlying cause may be impaired lipid-storage capacity of adipocytes and increased FFA flux to extra-adipose organs. To test this idea, we analyzed metabolic phenotypes of adiposity-matched WT, *clstn3b*^{-/-}, and adipose-specific CLSTN3B transgenic (Tg) mice challenged with a high-fat diet to avoid the complication from differential obesity levels. We observed significantly elevated serum FFA, exacerbated liver steatosis and WAT inflammation in the *clstn3b*^{-/-} mice compared with the adiposity-matched WT mice, whereas the Tg mice displayed the opposite, all supporting that CLSTN3B promotes lipid-storage capacity of adipocytes (Fig. 5c-f). Furthermore, *Clstn3b*^{-/-} mice WAT exhibited upregulation of PPAR γ target genes (Fig. 5f), possibly reflecting increased PPAR γ agonism resulting from enhanced FFA production. This trend was reversed in Tg mice (Fig. 5f). Together, our mouse physiology data support the role of CLSTN3B in enhancing LD functionality by both suppressing basal lipolysis and enhancing sensitivity to catecholamine stimulation.

Discussion

Our results have elucidated how the mammalian adipocyte-specific protein CLSTN3B promotes ER/LD contact formation and replenishes LD surface phospholipids to suppress basal lipolysis and enhance catecholamine-stimulated lipolysis in both brown and white adipocytes. Hibernating mammals accumulate massive body fat in autumn and consume it during winter. The ability of CLSTN3B to facilitate energy storage and mobilization in response to body needs may have conferred them a survival advantage. In humans, the lipid-storing capacity of adipocytes is causally linked to insulin sensitivity and glucose homeostasis¹²⁻¹⁴. It would be interesting to study how the expression level or variants of human CLSTN3B affects susceptibility to metabolic diseases.

Our findings established CLSTN3B as a unique inter-organellar contact protein regulated by proteolysis. In adipocytes harboring large LDs, CLSTN3B predominantly exists in the cleaved forms on LDs. When large LDs break down into small ones, as in brown adipocytes under catecholamine stimulation, the full-length protein becomes the major form on LDs. Full-length CLSTN3B promotes formation of extensive ER/LD contacts around small LDs, whereas cleaved CLSTN3B loses part of the LD-targeting domain, resulting in less pronounced ER association with large LDs. By reducing ER/LD contacts, CLSTN3B cleavage may make LDs more accessible to cytosolic lipases and allow efficient lipid mobilization. Cleaved CLSTN3B seem sufficient in replenishing LD surface lipids, as demonstrated by the metabolic phenotypes of mice on high fat diet that are essentially driven by altered behaviors of white adipocytes with large LDs. The physiological significance of full length CLSTN3B, along with the extensive ER enwrapping of small LDs in cold-acclimated brown adipocytes, requires further study. One line of active investigation is focusing on the ER-anchored lipid metabolizing enzymes selectively expressed in brown adipocytes that are recruited to LDs by CLSTN3B as detected by our proteomics study.

We observed distinct effects of CLSTN3B on LD sizes in different contexts: *clstn3b*^{-/-} brown adipocyte LDs tend to be larger than WT LDs whereas LDs from CLSTN3B-expressing 293 cells are larger than non-expressing cells. The larger sizes of *clstn3b*^{-/-} LDs may reflect impaired sympathetic innervation of the *clstn3b*^{-/-} BAT and a tendency to fuse to alleviate reduced surface phospholipids density, as shown in cells deficient in PC synthesis³⁵. Nevertheless, the fusion of *clstn3b*^{-/-} LDs may be ultimately limited by the strong downregulation of CIDEA and CIDEA³⁶, because LDs from WT brown adipocytes differentiated *in vitro*, without the complication of sympathetic innervation, reach larger sizes than *clstn3b*^{-/-} cells. In contrast, LDs from CLSTN3B-expressing 293 cells are completely enwrapped by the ER, which may block the access of lipases and enhance the channeling of ER-synthesized TG to the LD, thus resulting in larger sizes.

The mechanism of CLSTN3B-mediated phospholipids transfer between the ER and the LD warrants further investigation. One possibility is that phospholipids exchange happens spontaneously when the two membranes are brought into extreme proximity by the arginine-rich segment, akin to TAT peptide-induced lipid mixing and membrane fusion^{28,29}. Alternatively, phospholipids transfer may require additional proteins localized to ER/LD contacts, with one candidate being VPS13C, a protein mediating inter-organellar phospholipids transfer⁶ and significantly downregulated on *clstn3b*^{-/-} LDs.

We previously reported that ablation of CLSTN3B impairs sympathetic nerve innervation of the BAT¹⁵. Our discovery of CLSTN3B's function on LDs suggests that chronic exposure to supraphysiological levels of FFA leaked from *clstn3b*^{-/-} brown adipocytes may contribute to sympathetic nerve atrophy. Interestingly, VPS13C also suppresses lipolysis in brown adipocytes and mutations in VPS13C cause Parkinson's Disease in humans^{37,38}. This intriguing parallelism suggests a potential connection between lipid metabolism and neuronal health and the *clstn3b*^{-/-} mice may represent a unique tool for gaining further insights.

References

- 1 Prinz, W. A., Toulmay, A. & Balla, T. The functional universe of membrane contact sites. *Nat Rev Mol Cell Bio* 21, 7-24, doi:10.1038/s41580-019-0180-9 PMID - 31732717 (2020).
- 2 Wu, H., Carvalho, P. & Voeltz, G. K. Here, there, and everywhere: The importance of ER membrane contact sites. *Science* 361, eaan5835, doi:10.1126/science.aan5835 PMID - 30072511 (2018).
- 3 Saheki, Y. & Camilli, P. D. Endoplasmic Reticulum—Plasma Membrane Contact Sites. *Annu Rev Biochem* 86, 1-26, doi:10.1146/annurev-biochem-061516-044932 PMID - 28301744 (2016).
- 4 Datta, S., Liu, Y., Hariri, H., Bowerman, J. & Henne, W. M. Cerebellar ataxia disease-associated Snx14 promotes lipid droplet growth at ER–droplet contacts Snx14 regulates ER–droplet contacts. *J Cell Biology* 218, 1335-1351, doi:10.1083/jcb.201808133 PMID - 30765438 (2019).

- 5 Du, X. et al. ORP5 localizes to ER–lipid droplet contacts and regulates the level of PI(4)P on lipid droplets. *J Cell Biol* 219, e201905162, doi:10.1083/jcb.201905162 PMID - 31653673 (2019).
- 6 Kumar, N. et al. VPS13A and VPS13C are lipid transport proteins differentially localized at ER contact sites. *J Cell Biol* 217, 3625-3639, doi:10.1083/jcb.201807019 PMID - 30093493 (2018).
- 7 Wang, H. et al. Seipin is required for converting nascent to mature lipid droplets. *eLife* 5, e16582, doi:10.7554/elife.16582 PMID - 27564575 (2016).
- 8 Xu, D. et al. Rab18 promotes lipid droplet (LD) growth by tethering the ER to LDs through SNARE and NRZ interactions. *J Cell Biol* 217, 975-995, doi:10.1083/jcb.201704184 PMID - 29367353 (2018).
- 9 Li, D. et al. The ER-Localized Protein DFCP1 Modulates ER-Lipid Droplet Contact Formation. *Cell Reports* 27, 343-358.e345, doi:10.1016/j.celrep.2019.03.025 PMID - 30970241 (2019).
- 10 Freyre, C. A. C., Rauher, P. C., Ejsing, C. S. & Klemm, R. W. MIGA2 Links Mitochondria, the ER, and Lipid Droplets and Promotes De Novo Lipogenesis in Adipocytes. *Mol Cell*, doi:10.1016/j.molcel.2019.09.011 PMID - 31628041 (2019).
- 11 Markgraf, Daniel F. et al. An ER Protein Functionally Couples Neutral Lipid Metabolism on Lipid Droplets to Membrane Lipid Synthesis in the ER. *Cell Reports* 6, 44-55, doi:10.1016/j.celrep.2013.11.046 PMID - 24373967 (2014).
- 12 Morigny, P., Boucher, J., Arner, P. & Langin, D. Lipid and glucose metabolism in white adipocytes: pathways, dysfunction and therapeutics. *Nat Rev Endocrinol* 17, 276-295, doi:10.1038/s41574-021-00471-8 PMID - 33627836 (2021).
- 13 Olzmann, J. A. & Carvalho, P. Dynamics and functions of lipid droplets. *Nat Rev Mol Cell Bio* 20, 137-155, doi:10.1038/s41580-018-0085-z PMID - 30523332 (2019).
- 14 Greenberg, A. S. et al. The role of lipid droplets in metabolic disease in rodents and humans. *J Clin Invest* 121, 2102-2110, doi:10.1172/jci46069 PMID - 21633178 (2011).
- 15 Zeng, X. et al. Innervation of thermogenic adipose tissue via a calsynenin 3 β –S100b axis. *Nature*, 1-7, doi:10.1038/s41586-019-1156-9 (2019).
- 16 Friedman, J. R. et al. ER Tubules Mark Sites of Mitochondrial Division. *Science* 334, 358-362, doi:10.1126/science.1207385 PMID - 21885730 (2011).
- 17 Rao, J. K. M. & Argos, P. A conformational preference parameter to predict helices in integral membrane proteins. *Biochimica Et Biophysica Acta Bba - Protein Struct Mol Enzym* 869, 197-214, doi:10.1016/0167-4838(86)90295-5 PMID - 2935194 (1986).

- 18 Jumper, J. et al. Highly accurate protein structure prediction with AlphaFold. *Nature* 596, 583-589, doi:10.1038/s41586-021-03819-2 PMID - 34265844 (2021).
- 19 Barrowman, J., Bhandari, D., Reinisch, K. & Ferro-Novick, S. TRAPP complexes in membrane traffic: convergence through a common Rab. *Nat Rev Mol Cell Bio* 11, 759-763, doi:10.1038/nrm2999 PMID - 20966969 (2010).
- 20 Li, C. et al. COPI–TRAPP II activates Rab18 and regulates its lipid droplet association. *Embo J* 36, 441-457, doi:10.15252/embj.201694866 PMID - 28003315 (2017).
- 21 Kory, N., Farese, R. V. & Walther, T. C. Targeting Fat: Mechanisms of Protein Localization to Lipid Droplets. *Trends in Cell Biology* 26, 535-546, doi:10.1016/j.tcb.2016.02.007 PMID - 26995697 (2016).
- 22 Barneda, D. et al. The brown adipocyte protein CIDEA promotes lipid droplet fusion via a phosphatidic acid-binding amphipathic helix. *eLife* 4, doi:10.7554/eLife.07485 PMID - 26609809 (2015).
- 23 Yan, R. et al. Human SEIPIN Binds Anionic Phospholipids. *Dev Cell* 47, 248-256.e244, doi:10.1016/j.devcel.2018.09.010 PMID - 30293840 (2018).
- 24 Hansen, J. S., Maré, S. d., Jones, H. A., Göransson, O. & Lindkvist-Petersson, K. Visualization of lipid directed dynamics of perilipin 1 in human primary adipocytes. *Sci Rep-uk* 7, 15011, doi:10.1038/s41598-017-15059-4 PMID - 29118433 (2017).
- 25 Vale, G. et al. Three-phase liquid extraction: a simple and fast method for lipidomic workflows[S]. *J Lipid Res* 60, 694-706, doi:10.1194/jlr.d090795 PMID - 30610084 (2019).
- 26 Lahiri, S. et al. A Conserved Endoplasmic Reticulum Membrane Protein Complex (EMC) Facilitates Phospholipid Transfer from the ER to Mitochondria. *Plos Biol* 12, e1001969, doi:10.1371/journal.pbio.1001969 PMID - 25313861 (2014).
- 27 Raychaudhuri, S. & Prinz, W. A. Nonvesicular phospholipid transfer between peroxisomes and the endoplasmic reticulum. *Proceedings of the National Academy of Sciences* 105, 15785-15790, doi:10.1073/pnas.0808321105 PMID - 18836080 (2008).
- 28 Allolio, C. et al. Arginine-rich cell-penetrating peptides induce membrane multilamellarity and subsequently enter via formation of a fusion pore. *P Natl Acad Sci Usa* 115, 11923-11928, doi:10.1073/pnas.1811520115 PMID - 30397112 (2018).
- 29 Yang, S.-T., Zaitseva, E., Chernomordik, L. V. & Melikov, K. Cell-Penetrating Peptide Induces Leaky Fusion of Liposomes Containing Late Endosome-Specific Anionic Lipid. *Biophys J* 99, 2525-2533, doi:10.1016/j.bpj.2010.08.029 PMID - 20959093 (2010).
- 30 Jao, C. Y., Roth, M., Welti, R. & Salic, A. Metabolic labeling and direct imaging of choline phospholipids in vivo. *Proceedings of the National Academy of Sciences* 106, 15332-15337,

doi:10.1073/pnas.0907864106 PMID - 19706413 (2009).

- 31 Tansey, J. T. et al. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. *Proceedings of the National Academy of Sciences* 98, 6494-6499, doi:10.1073/pnas.101042998 PMID - 11371650 (2001).
- 32 Sztalryd, C. et al. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J Cell Biology* 161, 1093-1103, doi:10.1083/jcb.200210169 PMID - 12810697 (2003).
- 33 Lass, A. et al. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metab* 3, 309-319, doi:10.1016/j.cmet.2006.03.005 PMID - 16679289 (2006).
- 34 Granneman, J. G., Moore, H.-P. H., Krishnamoorthy, R. & Rathod, M. Perilipin Controls Lipolysis by Regulating the Interactions of AB-hydrolase Containing 5 (Abhd5) and Adipose Triglyceride Lipase (Atgl)*. *J Biol Chem* 284, 34538-34544, doi:10.1074/jbc.m109.068478 PMID - 19850935 (2009).
- 35 Krahmer, N. et al. Phosphatidylcholine Synthesis for Lipid Droplet Expansion Is Mediated by Localized Activation of CTP:Phosphocholine Cytidylyltransferase. *Cell Metab* 14, 504-515, doi:10.1016/j.cmet.2011.07.013 PMID - 21982710 (2011).
- 36 Wu, L. et al. Cidea controls lipid droplet fusion and lipid storage in brown and white adipose tissue. *Science China Life Sciences* 57, 107-116, doi:10.1007/s11427-013-4585-y PMID - 24369348 (2014).
- 37 Lesage, S. et al. Loss of VPS13C Function in Autosomal-Recessive Parkinsonism Causes Mitochondrial Dysfunction and Increases PINK1/Parkin-Dependent Mitophagy. *Am J Hum Genetics* 98, 500-513, doi:10.1016/j.ajhg.2016.01.014 PMID - 26942284 (2016).
- 38 Ramseyer, V. D., Kimler, V. A. & Granneman, J. G. Vacuolar protein sorting 13C is a novel lipid droplet protein that inhibits lipolysis in brown adipocytes. *Mol Metab* 7, 57-70, doi:10.1016/j.molmet.2017.10.014 PMID - 29175050 (2018).

Methods

Mouse strains. The *clstn3b*^{-/-} and the adipose-specific *clstn3b* transgenic mice were described in a previous publication¹⁵. All mice were maintained under a 12 hr light/12 hr dark cycle at constant temperature (23°C) unless otherwise specified with free access to food and water. All animal studies were approved by and in full compliance with the ethical regulation of the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. Male mice of 8-12 weeks of age were used for physiological experiments unless stated otherwise. Sample size was chosen based on literature and pilot experiment results to ensure statistical significance could be reached. Randomization was not performed because mice were grouped based on genotype.

Reagents. Reagents: FFA fluorometric assay kit (Cayman, item no.700310); Lipofectamine 3000 (Invitrogen, L3000-015); Norepinephrine (Sigma, A9512); HCS LipidTOX™ Deep Red Neutral Lipid Stain (Invitrogen™, H34477); HCS LipidTOX™ Green Neutral Lipid Stain (Invitrogen™, H34477); Trypsin Platinum (Promega, VA9000); Protease K (Sigma, P2308); Oleic acid (Sigma, O1383); BSA (Sigma, A7030); FFA-free BSA (Millipore, Code82-002-4); Trypsin inhibitor (Worthington, LS003571); Protease inhibitor cocktail (100X) (Thermo scientific, 1861279); Free glycerol reagent (Sigma, F6428-40ML); Propargyl choline (Cayman, 25870); BTAA (Click chemistry tools, 1236-100); CalFluor 647 Azide(Click chemistry tools, 1272-1); Phosphatidylcholine Assay Kit (Colorimetric/Fluorometric) (Abcam ab83377); Phosphatidylethanolamine Assay Kit (Fluorometric) (Sigma, MAK361).

Antibody: CLSTN3B antibody was described previously¹⁵; KDEL (Abcam, ab184819); PLIN1 (Abcam, ab3526); CGI-58 (Abcam, ab183739); UCP1(Abcam, ab209483); PDI (Cell signaling, C81H6); CS (Abcam, ab129095).

Constructs: PCDNA3.1-*clstn3b* was described previously¹⁵; *clstn3b*-mCherry, *clstn3b*(1-131)-mCherry, *clstn3b*(1-198)-mCherry, *clstn3b*(131-357)-mCherry, and *clstn3b*(199-357)-mCherry were synthesized by Gene Universal Inc. (Newark DE 19713); BFP ER-reporter was purchased from Addgene (49150).

Electron microscopy. Primary adipocytes were isolated as previously described and allowed to attach to MatTek glass bottom grid dishes (P35G-1.5-14-CGRD)¹⁵. The cells were treated with 1 μ M norepinephrine overnight to induce the formation of small LDs when specified. Cells were then fixed with 4% paraformaldehyde + 0.1% glutaraldehyde in PBS containing 7.5% sucrose for 30 min at RT, blocked with 50 mM glycine for 15 min, permeabilized with 0.25% saponin for 30 min. Cells were then incubated with the CLSTN3B antibody at 4°C overnight and then with the fluoronanogold secondary antibody (Nanoprobes 7403-1) for 2 h at RT. Images were taken on a Zeiss 900 confocal microscope to identify the coordinates of the cells for EM analysis. The cells were further gold-enhanced for 2.5 min using a gold enhancement kit (Nanoprobes). After washing with water and 0.1M cacodylate buffer, samples were fixed with 1% OsO₄ and 0.8% potassium ferricyanide in 0.1M cacodylate buffer for 1h, stained en bloc with 2% aqueous uranyl acetate, dehydrated with increasing concentration of ethanol, and embedded in Epon. Blocks were sectioned with a diamond knife (Diatome) on a Leica Ultracut 7 ultramicrotome (Leica Microsystems) and collected onto copper grids. Images were acquired on a JOEL 1400 Plus transmission electron microscope equipped with a LaB6 source using a voltage of 120 kV, and the images were captured by an AMT BIOSPRINT 16M-ActiveVu mid mound CCD camera.

Fixed cell imaging. Primary adipocytes were isolated as previously described and attached to glass coverslips pre-coated with laminin and poly-D-lysine¹⁵. HEK293 cells and U2OS cells were plated onto glass coverslips and treated with 400 μ M oleic acid for 24 hr to induce LD formation. Images were taken on a ZEISS 900 confocal microscope with Airyscan.

Image analysis. To analyze isolated lipid droplets, images were imported to Imaris (Bitplane). Individual LDs were rendered by the "Surface" function and surface area and volume were measured. The rendering

parameters were kept the same between treatments in each experiment. To analyze LD-associated phosphatidylcholine (PC) or microsomes in vitro, images were imported to ImageJ (NIH). Individual LDs were selected by the "Analyze particle" function, and a larger ROI was generated for each LD via the "Dilate" function to encompass surrounding PC or microsomes. PC or microsome signal intensity was measured and plotted.

LD isolation and digestion. For BAT LD, BAT was dissected, minced into tiny pieces with a spring scissor and transferred to a motorized homogenizer in HES buffer (20mM HEPES + 1mM EDTA + 250mM Sucrose). The homogenate was filtered with double-layer gauze and centrifuged at 2000 g for 5 min. The infranant was removed with a syringe and the buoyant LD fraction was transferred with a wide-opening tip into 5 ml tubes and washed with HES buffer 2 times. The LD was then transferred to Ultra-Clear ultracentrifuge tubes (Beckman-Coulter), adjusted to a final concentration of 20% sucrose, and overlaid by 5% sucrose/HE and HE (20mM HEPES + 1mM EDTA). The gradient was centrifuged at 16,000 g for 10 min at 4°C. The buoyant LD was then collected.

For HEK293 LD, 15-cm dishes of HEK293 cells were grown to 90% of confluence and treated with 400 μ M oleic acid in growth medium for 24 h. Cells were scraped and collected in 2 mL of lysis buffer (25 mM Tris-HCl, pH 7.4, 100 mM KCl, 1 mM EDTA, 5 mM EGTA, and protease inhibitor cocktail), and lysed by passing through a needle (27-gauge). The lysates were then centrifuged at 1,500 g for 5 min. The supernatants were adjusted to 2.5 mL final volume containing 20% sucrose and transferred into a 10 mL polycarbonate ultracentrifuge tube, and overlaid sequentially with 2.5 mL of 10% sucrose, 2.5 mL of 4.2% sucrose, and 2.5 mL of lysis buffer. The gradient was centrifuged at 150,000 g for 1 hr at 4°C. The buoyant LD was then collected.

For topology determination, LDs isolated from BAT were incubated with trypsin (50 μ g/ml, Promega) for 30 min, followed by centrifuging LD fractions through the aforementioned sucrose gradient at 210,000g for 1 hr. The buoyant LD fraction and the pellet fraction were collected separately.

To remove the LD-bound organelles, the LD fraction was digested with 1 mg/mL proteinase K for 5-15 min at 37°C and 1mM PMSF was added to inactivate proteinase K. The mixture was centrifuged through the aforementioned sucrose gradient at 210,000g for 1 hr. The buoyant LD was then collected.

Proteomics. LD was isolated from mouse BAT as described above without protease digestion. The suspension was mixed with 10x volume of acetone and incubated at -20°C overnight for delipidation and protein precipitation. The mixture was centrifuged at 12,000 g for 5 min. The pellets were washed with acetone and dried by heating at 60°C for at least 15 min. Pellets were then washed with 20% TCA to remove excess sucrose, washed with acetone, and dried. The pellets were dissolved in 50 mM triethylammonium bicarbonate (TEAB, PH=8), 5% SDS at 60°C with shaking for 30 min. Protein concentration was determined with a BCA method. Proteomics analysis was performed following a previously described procedure³⁹.

Phospholipids quantitation. LD was isolated from BAT or HEK293 cells and digested with proteinase K following the procedure described above. The collected LD fractions (100-200 μ L suspension in HES buffer) were transferred into round bottom glass tubes and extracted with a mixture of 1 mL hexane, 1 mL methyl acetate, 0.75 mL acetonitrile and 1 mL water as previously described²⁵. The extracts were vortexed for 5 s and centrifuge at 2,671g for 5 min to partition into 3 phases. The upper and middle phases were collected into separate glass tubes and dried under N₂. Phospholipids in the middle phase were measured with the Phosphatidylcholine Assay Kit (Colorimetric/Fluorometric) (Abcam ab83377) and Phosphatidylethanolamine Assay Kit (Fluorometric) (Sigma, MAK361) following the manufacturer's instructions. TG in the upper phase was dissolved in 350 μ L ethanolic KOH (2 part EtOH and 1 part 30% KOH) and incubated overnight at 55°C for complete hydrolysis. The volume was then brought to 1200 μ L with H₂O: EtOH (1:1) and vortexed to mix. Two hundred μ L was transferred to a new tube, mixed with 215 μ L 1M MgCl₂ and vortexed. The mixture was incubated on ice for 10 min and centrifuged at 13,000g for 5 min. Glycerol content in the supernatant was determined with the Free Glycerol Reagent (Sigma, F6428) following the manufacturer's instructions.

For phospholipidomics analysis, a 50 μ L aliquot of LD suspension was transferred to fresh glass tubes for liquid-liquid extraction (LLE). LLE were performed at room temperature (including centrifugation) to maintain consistent solubility and phase separation. For the three-phase lipid extractions (3PLE)²⁵, 1 mL of hexanes, 1 mL of methyl acetate, 0.75 mL of acetonitrile, and 1 mL of water were added to the glass tube containing the sample. The mixture was vortexed, then centrifuged at 2,671 $\times$ g for 5 min, resulting in separation of three distinct liquid phases. The middle organic phase (polar lipid content), was collected in a separate glass tube with a Pasteur pipette and spiked with 20 μ L of a 1:5 diluted SPLASH Lipidomix standard mixture. The samples were dried under N₂ air flow and resuspended in 400 μ L of hexane. Lipids were analyzed by LC-MS/MS using a SCIEX QTRAP 6500+ (SCIEX, Framingham, MA) equipped with a Shimadzu LC-30AD (Shimadzu, Columbia, MD) high-performance liquid chromatography (HPLC) system and a 150 $\times$ 2.1 mm, 5 μ m Supelco Ascentis silica column (Supelco, Bellefonte, PA). Samples were injected at a flow rate of 0.3 mL/min at 2.5% solvent B (methyl tert-butyl ether) and 97.5% Solvent A (hexane). Solvent B was increased to 5% over 3 min and then to 60% over 6 min. Solvent B was decreased to 0% during 30 sec while Solvent C (90:10 (v/v) isopropanol-water) was set at 20% and increased to 40% during the following 11 min. Solvent C is increased to 44% over 6 min and then to 60% over 50 sec. The system was held at 60% solvent C for 1 min prior to re-equilibration at 2.5% of solvent B for 5 min at a 1.2 mL/min flow rate. Solvent D [95:5 (v/v) acetonitrile-water with 10 mM Ammonium acetate] was infused post-column at 0.03 ml/min. Column oven temperature was 25°C. Data was acquired in positive and negative ionization mode using multiple reaction monitoring (MRM). The LC-MS/MS data was analyzed using MultiQuant software (SCIEX). The identified lipid species were normalized to its corresponding internal standard. All solvents used were either HPLC or LC/MS grade and purchased from Sigma-Aldrich (St Louis, MO, USA). Splash Lipidomix standards were purchased from Avanti (Alabaster, AL, USA). All lipid extractions were performed in 16 $\times$ 100mm glass tubes with PTFE-lined caps (Fisher Scientific, Pittsburg, PA, USA). Glass Pasteur pipettes and solvent-resistant plasticware pipette tips (Mettler-Toledo, Columbus, OH, USA) were used to minimize leaching of polymers and plasticizers.

To calculate LD surface phospholipids density, we divided phospholipids abundance as measured by the fluorometric kit or phospholipidomics by total LD surface area of each sample. To calculate total LD surface area, we divided total TG content of each sample by the mean LD volume to derive total LD number, which is then multiplied by the mean LD surface area. Mean LD volume and surface area were determined by analysis of LD images as described above.

***In vitro* reconstitution of CLSTN3B-mediated ER/LD contact formation and phospholipids transfer.** To reconstitute CLSTN3B-mediated ER/LD contact formation, HEK293 cells were transfected with the *clstn3b*-mCherry or the BFP-ER reporter and cultured for 48 hr. Then cells were washed with warm PBS twice, scraped off, and lysed by passing through a 27G needle 10 times. The lysates were centrifuged at 10,000 g for 10 min to remove nuclei and mitochondria. The supernatants were centrifuged through the aforementioned sucrose gradient at 210,000 g for 1 hr. The microsome pellets were suspended in the assay buffer (25 mM HEPES, 150 mM NaCl, 10 mM MgCl₂, 1 mM CaCl₂, 1 mM ATP, 0.5 mM GTP). LDs were harvested from and a parallel batch of HEK293 cells treated with 400 μ M oleic acid in growth medium for 24 hr as described above. LDs and microsome suspension were then mixed and incubated at 37°C overnight. The mixture was stained with HCS LipidTOX™ Green Neutral Lipid Stain (1:200) for 15 min and imaged on a ZEISS 900 confocal microscope. To reconstitute CLSTN3B-mediated phospholipids transfer, HEK293 cells were transfected with the *clstn3b*-mCherry or the BFP-ER reporter construct for 24 hr and then treated with 100 μ M propargyl-choline for 12-24 hr. Microsome suspension was prepared as described above. LDs isolated from *clstn3b*^{-/-} mouse BAT was incubated with the microsome suspension at 37°C for 3 hr. The mixture was then centrifuged at 100 g for 5 min. The buoyant LD fraction was collected and incubated with the click reaction solution (50 μ M Calfluor647 azide, BTAA-CuSO₄ complex (50 mM CuSO₄, BTAA/CuSO₄ 6:1, mol/mol) and 2.5 mM sodium ascorbate) for 1 hr at RT. The LDs were then digested with 1 mg/mL protease K for 5 min, treated with 1 mM PMSF for 5 min, and centrifuged at 100 g for 5 min. The buoyant LD fraction was gently washed with PBS two times, stained with HCS LipidTOX™ Green Neutral Lipid Stain (1:200) for 15 min. Images were then taken on a ZEISS LSM 900 confocal microscope.

Lipolysis assay. Lipolysis assay on freshly isolated primary white and brown adipocytes were performed as previously described⁴⁰. To measure the whole-body response to CL-316,243, mice were put under isoflurane anesthesia and i.p. injected with CL-316,243 at 1 mg/kg. Blood samples were collected from tails at 0 and 10 min. Serum FFA levels were measured with the Free fatty acid fluorometric assay kit (Cayman, 700310) following the manufacturer's instructions.

Real-time qPCR analysis. The following primers were used for qPCR analysis of gene expression. Cd36-fwd, GGACATTGAGATTCTTTCTCTG, rev, GCAAAGGCATTGGCTGGAAGAAC; clstn3b-fwd, CTCCGCAGGAACAGCAGCCC, rev, AGGATAACCATAAGCACCAG; tnfrsf11b-fwd, GGTGCCTATGTCTCAGCCTCTT, rev, GCCATAGAACTGATGAGAGGGAG; ccl2-fwd, GCTACAAGAGGATCACCAGCAG, rev, GTCTGGACCCATTCTTCTTGG; adgre1-fwd, CGTGTGTTGGTGGCACTGTGA, rev, CCACATCAGTGTTCCAGGAGAC; lipe-fwd, TTGGGGAGCTCCAGTCGGA, rev, TCGTGCGTAAATCCATGCTGT; fabp4-fwd, ACACCGAGATTTCTTCAAACCTG, rev, CCATCTAGGGTTATGATGCTCTTCA; L19-fwd,

GGTCTGGTTGGATCCCAATG, rev, CCCATCCTTGATCAGCTTCCT; lpl-fwd, GGGAGTTTGGCTCCAGAGTTT, rev, TGTGTCTTCAGGGGTCCTTAG; cfd-fwd, CATGCTCGGCCCTACATGG, rev, CACAGAGTCGTCATCCGTCAC.

Statistics and Reproducibility.

All data shown are mean $\pm$ SEM. Statistical significance was calculated by unpaired Student's two-sided t-test for comparisons between two groups and ANOVA for comparisons between three groups. All experiments have been successfully repeated with similar results for at least three times.

Additional Reference for methods

39 Cui, M. et al. Nrf1 promotes heart regeneration and repair by regulating proteostasis and redox balance. Nat Commun 12, 5270, doi:10.1038/s41467-021-25653-w PMID - 34489413 (2021).

40 Chen, Y. et al. Crosstalk between KCNK3-Mediated Ion Current and Adrenergic Signaling Regulates Adipose Thermogenesis and Obesity. Cell 171, 836-848.e813, doi:10.1016/j.cell.2017.09.015 PMID - 28988768 (2017).

Declarations

Acknowledgements

We thank Andrew Lemoff and the Proteomics core at UTSW for the proteomics analysis; the Molecular Pathology core at UTSW for the histology analysis; the EM core at UTSW for EM sample preparation; Xiaowei Chen and Xinxuan Xu for advice on Click Chemistry experiments; Xiaowei Chen and Xiaolei Su for critical reading of the manuscript; Xing Zeng is a Rita C. and William P. Clements, Jr. Scholar in Biomedical Research. This study was supported by the Endowed Scholars in Medical Science Program at UTSW and Cancer Prevention and Research Institute of Texas grant RR200084 to X.Z.

Author contributions

X.Z. conceived the project. C.Z., X.Z., and M.L. designed and performed experiments. G.V., K.E., and J.M. performed phospholipidomics analysis. M.Y. performed imaging analysis. C.Z. and X.Z. interpreted the data and wrote the manuscript with discussion and contributions from all authors.

Competing interests

The authors declare no competing interests.

Correspondence and requests for materials should be addressed to X.Z.

Figures

Figure 1

Figure 1

CLSTN3B localizes to ER/LD contact sites

a, Fluorescence microscopic analysis of CLSTN3B and KDEL localization in primary brown adipocytes. Scale bar: 5 μ m. b, c, Electron microscopic analysis of CLSTN3B localization (b) and ER/LD contacts (c) in primary brown adipocytes. Arrows denote gold particles labeling CLSTN3B. Scale bar: 200 nm. d, Quantitation of the extent of ER enwrapping of LD in brown adipocytes (n=10 LDs). e, Western blot analysis of CLSTN3B on LDs isolated from brown and white adipose tissue. FL, full length; C1, C2, C-terminal cleavage fragments. f, Fluorescence microscopic analysis of CLSTN3B and KDEL localization in HEK293 cells. g, Electron microscopic analysis of ER/LD morphology in HEK293 cells -/+ CLSTN3B expression. Scale bar: 400 nm. h, Fluorescence microscopic analysis of CLSTN3B-mediated microsome/LD contacts formed *in vitro*. i, Quantitation of the LD-associated microsome Area (n=980 LDs). Scale bar: 5 μ m.

Figure 2

Figure 2

CLSTN3B bridges the LD and the ER

a, Hydrophobicity plot of CLSTN3B. b, Fluorescence microscopic analysis of CLSTN3B 1-198 and 199-357 localization in HEK293 cells. Scale bar: 5 μ m. c, Western blot analysis of trypsin digested CLSTN3B. d, Topology of CLSTN3B. e, Fluorescence microscopic analysis of CLSTN3B 131-357 localization in HEK293 cells. Scale bar: 5 μ m. f, 3D-reconstruction of full-length (FL) CLSTN3B and 131-357 enwrapping of LD.

Figure 3

Figure 3

CLSTN3B promotes LD-protein binding

a, Proteomics of WT and *clstn3b*^{-/-} LD (n=4 mice). b, Pathway enrichment analysis of (a). c, Western blot analysis of LD protein levels in WT and *clstn3b*^{-/-} white adipocytes.

Figure 4

Figure 4

CLSTN3B promotes phospholipids transfer from ER to LD

a, Schematics of LD surface phospholipids density quantitation. b, c, d, Volume and surface area distribution (b), phospholipidomics (c), and fluorometric phospholipids (d) analysis of WT and *clstn3b*^{-/-} brown adipocyte LDs (n=5 mice). e, f, Volume and surface area distribution (e), and fluorometric phospholipids analysis (f) of HEK293 cell LDs +/- CLSTN3B expression (n=3 replicates). g, h, Schematics (g), images and quantitation (h) of *in vitro* reconstitution of ER to LD phospholipids transfer (n=14 LDs). Scale bar: 2 μ m. i, Model of CLSTN3B-mediated ER to LD phospholipids transfer.

Figure 5

Figure 5

CLSTN3B enhances LD functionality

a, Lipolysis assay of WT and *clstn3b*^{-/-} brown and white adipocytes (n=3 replicates). b, Serum FFA of WT and *clstn3b*^{-/-} mice injected with CL (n=7 mice). c, Serum FFA of adiposity-matched WT, *clstn3b*^{-/-} and CLSTN3B transgenic mice on high fat diet (n=7 mice). d, e, f, Liver steatosis (d), WAT inflammation (e), and WAT gene expression analysis (f) of adiposity-matched WT, *clstn3b*^{-/-} and CLSTN3B transgenic mice on high fat diet (n=6 mice). Scale bar: 100 μm.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalTable.xlsx](#)
- [ZhangetalExtendedDataFigures.pdf](#)
- [SupplementalLegends.docx](#)