Supplementary Discussion

LD-targeted proteins can be divided into two classes. Class I (or ERTOLD) proteins feature hydrophobic hairpins that allow them to diffuse from the ER bilayer onto the LD monolayer, while class II (or CYTOLD) proteins contain amphipathic helices that facilitate their direct recruitment from the cytoplasm to the LD[1,2]. Our data suggest that CLSTN3β should be categorized as a class I protein: its N-terminal LD-targeting domain does not have clear amphipathic character and is predicted to fold into a series of three hydrophobic hairpin-like structures. Our analysis of CLSTN3β truncation mutants supports this model empirically, as each individual hairpin is sufficient for LD targeting on its own but targeting efficiency markedly improves when multiple hairpins are combined. Thus, with its ER-anchored TM domain and three LD-targeting hairpins, the CLSTN3β protein seems uniquely optimized for ER-LD contact formation. Structural analysis of CLSTN3β will be essential to more precisely define the conformation of this novel LD-targeting domain.

With unbiased proteomics, we identified the CIDE proteins as binding partners of CLSTN3β that contribute to its effects on LD size. CLSTN3β binds to the C-terminal region of CIDEs and impairs their ability to facilitate lipid transfer between LDs. Consistent with these findings, the effect of CLSTN3β overexpression on LD morphology mirrors that of CIDE depletion – both manipulations lead to small LD accumulation and increased triglyceride breakdown[3–12]. In particular, CLSTN3β overexpression mimics mutations in the C-terminal region of CIDEs that have been shown to block lipid transfer without affecting LD localization or LD-LD docking[9,13]. To the best of our knowledge, CLSTN3β is the first example of a protein that selectively inhibits CIDE function. Thus, our findings at least partially explain the distinct LD morphologies of brown/beige and white adipocytes, all of which express high levels of CIDE proteins.

Brown adipocyte LDs are presumed to be an important source of free fatty acids (FFAs) for adipose thermogenesis, at least during the acute phase of cold exposure. However, recent studies have demonstrated that brown adipocytes can shift to alternative fuel sources when intracellular lipids are not available. Multiple groups have reported that the absence of lipolytic machinery, or LDs altogether, in brown adipocytes does not result in hypothermia during acute cold exposure[14–16]. Interestingly, mice defective in LD utilization are able to compensate through a variety of mechanisms, including enhanced uptake of circulating FFAs, increased utilization of alternative fuel sources (e.g. glucose), and even storage of glycogen in BAT. These provocative findings highlight the extent to which loss of "normal" BAT thermogenesis can be offset by diverse compensatory mechanisms. Nevertheless, while brown adipocyte LDs may not be essential for maintaining euthermia, the fact that their absence triggers such dramatic reprogramming of BAT metabolism suggests that they do play an important role in adipose thermogenesis under physiological conditions. Accordingly, we found that AdC3KO mice acclimated normally to the cold despite having substantially larger LDs in BAT, presumably because of compensatory mechanisms such as increased carbohydrate utilization. Indeed, ^{18}F-FDG PET/CT analysis revealed that AdC3KO BAT took up substantially more glucose than WT BAT during acute cold exposure. However, if mice were acclimated to thermoneutrality prior to acute cold challenge – thereby minimizing the need for compensatory pathway engagement – CLSTN3β knockout mice showed a defective thermogenic response.

While this study was in progress, it was reported that CLSTN3β functions as a chaperone for the neurotrophic factor S100B and promotes sympathetic innervation of thermogenic adipose tissue[17]. Since the *Clstn3* locus also encodes a brain-specific plasma membrane protein involved in synapse organization[18–22], this is an attractive model for CLSTN3β function – one that was also considered in this study. We were able to visualize sympathetic innervation of whole BAT lobes from WT and AdC3KO mice by modifying the published AdipoClear protocol, which has previously been used to image WAT[23–25]. We cannot fully exclude the possibility that our methods were insufficiently sensitive to detect an effect of CLSTN3β on adipose innervation, but our data did not reveal that gain or loss of CLSTN3β function affected sympathetic innervation or adrenergic signaling. Rather, we found that acute expression of CLSTN3β in BAT was still able to promote multilocularity and reduce lipid deposition in adult mice housed at thermoneutrality, where adrenergic tone is minimal. Our proposed physiologic role for CLSTN3β in LD dynamics is also consistent with the observation that *Clstn3b* remains highly expressed and transcriptionally regulated in adult mice even though adipose innervation is thought to be largely established during development[26]. Notably, CIDEA and CIDEC were previously found to be the most and seventh-most downregulated proteins in CLSTN3β knockout BAT, respectively[17].

We did observe a reduction in S100B protein levels in CLSTN3β KO BAT under certain conditions (Extended Data Fig. 8I); however, our data suggest that this is a secondary effect of CLSTN3β deletion *in vivo*, not a direct effect of CLSTN3β on S100B stability or secretion (Extended Data Fig. 8J). The principal function of CLSTN3β seems unlikely to be to serve as an obligate chaperone for S100B because S100B is expressed in a different set of cell types than is CLSTN3β, and ER-localized CLSTN3β is targeted for ubiquitination and degradation via ERAD. Furthermore, the adipocyte-selective N-terminal component of the CLSTN3β protein is oriented towards the cytoplasmic face of the ER and robustly associates with LDs via a hairpin-like domain. These structural features do not seem to be optimized for a chaperone function. Importantly, our studies do not exclude the possibility that S100B acts, on its own, as an adipocyte-derived neurotrophic factor.

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