LETTER

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Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity

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The endoplasmic reticulum (ER) is the main site of protein and lipid synthesis, membrane biogenesis, xenobiotic detoxification and cellular calcium storage, and perturbation of ER homeostasis leads to stress and the activation of the unfolded protein response[1]. Chronic activation of ER stress has been shown to have an important role in the development of insulin resistance and diabetes in obesity[2]. However, the mechanisms that lead to chronic ER stress in a metabolic context in general, and in obesity in particular, are not understood. Here we comparatively examined the proteomic and lipidomic landscape of hepatic ER purified from lean and obese mice to explore the mechanisms of chronic ER stress in obesity. We found suppression of protein but stimulation of lipid synthesis in the obese ER without significant alterations in chaperone content. Alterations in ER fatty acid and lipid composition result in the inhibition of sarco/endoplasmic reticulum calcium ATPase (SERCA) activity and ER stress. Correcting the obesity-induced alteration of ER phospholipid composition or hepatic *Serca* overexpression *in vivo* both reduced chronic ER stress and improved glucose homeostasis. Hence, we established that abnormal lipid and calcium metabolism are important contributors to hepatic ER stress in obesity.

It has been generally accepted that a surplus of nutrients and energy stimulates synthetic pathways and may lead to client overloading in the ER. However, it has not been demonstrated whether increased *de novo* protein synthesis and client loading into the ER and/or a diminished productivity of the ER in protein degradation or folding leads to ER stress in obesity. Intriguingly, dephosphorylation of eukaryotic translation initiation factor 2α (eIF2α) in the liver of high-fat-diet-fed mice reduced the ER stress response[3], indicating that additional mechanisms other than translational upregulation may also contribute to ER dysfunction in obesity. To address these mechanistic questions, we first fractionated ER from lean and obese liver tissues (Supplementary Fig. 1a, b) and then extracted ER proteins for comparative proteomic analysis to examine the status of this organelle in obesity. We identified a total of 2,021 unique proteins (Supplementary Table 1). Among them, 120 proteins were differentially regulated in obese hepatic ER samples (Supplementary Fig. 1c and Supplementary Table 2a, b). We independently validated the differential regulation when possible by immunoblot analyses and verified the fidelity of the system (Supplementary Fig. 1d). Gene ontology analysis identified the enrichment of metabolic enzymes—especially ones involved in lipid metabolism—in the obese ER proteome, whereas protein synthesis and transport functions were overrepresented among downregulated ER proteins (Fig. 1a). Consistently, we found that ER-associated protein synthesis was downregulated in the obese liver as demonstrated by polysome profiling (data not shown), whereas the expression of genes involved in *de novo* lipogenesis (*Fas*, *Scd1*, *Ces1d*, *Dgat2* and *Dak*) and phospholipid synthesis (*Pcyt1a* and *Pemt*) were broadly upregulated (Fig. 1b, c). We also observed upregulation of protein degradation pathways but did not find a broad change in the quantity of ER chaperones (Supplementary Fig. 2 and Supplementary Table 2a). Taken together, these data revealed a fundamental shift in hepatic ER function in obesity from protein to lipid synthesis and metabolism.

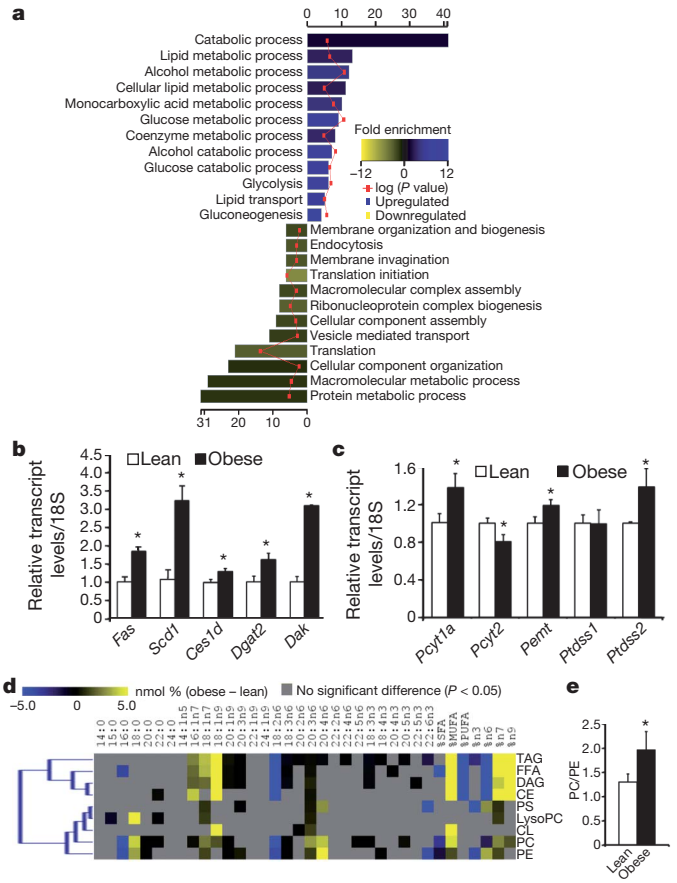

Figure 1 | Proteomic and lipidomic landscape of the lean and obese ER. **a**, Biological pathways associated with significantly regulated proteins in the obese ER proteome. Bar colours indicate the fold enrichment with significance values (negative log of *P* values) superimposed. **b**, **c**, Transcript levels of genes involved in lipid metabolism in the lean and obese mouse liver. **d**, Alterations of liver ER lipidome. Heatmap display of all significant ($P < 0.05$) alterations present between lean and obese ER lipidomes. The colour corresponds to differences in the relative abundance (nmol percentage) of each fatty acid among individual lipid groups detected in the lean and obese liver ER. **e**, The relative abundance of PC and PE in lean and obese liver ER samples. Values are mean ± s.e.m. ($n = 6$ for each group). $^*P < 0.05$, Student's *t*-test.

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The presence of chronic ER stress in obese liver (Supplementary Fig. 2) despite a reduction in ER-associated protein synthesis led us to postulate that the ER stress in obesity may not simply be invoked by protein overloading but also driven by compromised folding capacity, in which lipid metabolism may have a function[4]. For example, the ability of palmitate and cholesterol to induce ER stress in cultured cells correlates with their incorporation into the ER[5,6]. Therefore, we quantitatively determined all major lipid species and their fatty acid composition in ER samples isolated from lean and obese liver along with the diet consumed by these mice (Supplementary Fig. 3 and Supplementary Table 3). First, we found that the fatty acid composition of ER lipids in the lean mouse liver was distinct from corresponding dietary lipids, indicating the contribution of a basal level of *de novo* lipogenesis to the biogenesis of ER membranes *in vivo* (Supplementary Fig. 3a, b and Supplementary Table 3). Almost all ER-derived lipids were composed of significantly higher levels of saturated fatty acids (SFAs) whereas their polyunsaturated fatty acid (PUFA) content was much lower than those of corresponding dietary lipids, indicating that *de novo* synthesized SFAs are preferred over diet-derived PUFAs as the substrate for the synthesis of hepatic ER lipids. Second, the liver ER samples of lean and obese mice also had profoundly different compositions of fatty acids and lipids as illustrated by the clear separation of lean and obese ER lipidome in cluster analysis (Supplementary Fig. 3c). The obese ER was significantly enriched with monounsaturated fatty acids (MUFAs; Fig. 1d), a bona fide product of *de novo* lipogenesis, in liver. Third, the obese ER samples contained a higher level of phosphatidylcholine (PC) as compared to phosphatidylethanolamine (PE) (PC/PE = 1.97 versus 1.3, $P < 0.05$; Fig. 1e and Supplementary Table 3), two of the most abundant phospholipids on the ER membrane. The rise of the PC/PE ratio is probably caused by the upregulation of two key genes involved in PC synthesis and PE to PC conversion: choline-phosphate cytidylyltransferase A (*Pcyt1a*) and phosphatidylethanolamine *N*-methyltransferase (*Pemt*) (Fig. 1c and Supplementary Fig. 3a), and it is consistent with the essential role of PC for lipid packaging in the form of lipid droplets or lipoproteins, both of which are increased in obesity. In contrast, the PC/PE ratio in the lean hepatic ER was essentially identical as it is in the diet (Supplementary Table 3), indicating that the increase of PC/PE ratio in obesity is not due to food consumption, but the result of increased lipid synthesis in the obese liver.

The desaturation of SFAs to MUFAs in the obese liver probably has a protective role in reducing lipotoxicity, whereas the decrease of PUFA content in the ER may limit its reducing capacity and contribute to ER stress[7]. However, a potential role of the PC/PE ratio in regulating ER homeostasis has not been studied before. Previous biochemical studies have shown that increasing PC content in the membrane inhibits the calcium transport activity of SERCA[5,8]. Consistently, we found that the addition of PC to liver-derived microsomes *in vitro* substantially inhibited SERCA activity (Fig. 2a). More importantly, overexpression of the PE to PC conversion enzyme *Pemt* in Hepa1-6 cells significantly inhibited microsomal SERCA activity, indicating that changes in the PC/PE balance in a cellular setting can significantly perturb SERCA function (Fig. 2b, c). As calcium has an important role in mediating chaperone function and protein folding in the ER, and given that SERCA is principally responsible for maintaining calcium homeostasis in this organelle, we postulated that the increased PC/PE ratio in the ER of obese liver might impair ER calcium retention and homeostasis *in vivo*, thereby contributing to protein misfolding and ER stress. In support of this possibility, we found that the calcium transport activity of microsomes prepared from the livers of obese mice was significantly lower than those isolated from lean animals (4.6 ± 0.2 versus 5.3 ± 0.3, $P = 0.046$; Fig. 2d), despite the fact that the SERCA protein level was modestly higher in the former, consistent with an inhibitory role of the PC/PE ratio on SERCA function.

Modest defects in SERCA activity have been implicated in the pathology of Darier's disease[9], and we found that a reduction in SERCA expression *in vivo* (Fig. 2e) and a concurrent reduction in its calcium transport activity (Fig. 2f) potently activated hepatic ER stress in lean mice as evidenced by IRE1α and eIF2α phosphorylation and changes in the expression of GRP78 and GRP94 (Fig. 2g, h). Therefore, there seems to be little redundancy in the function of SERCA beyond physiological fluctuations to maintain ER homeostasis, and the reduction in calcium transport activity could be a potential mechanism of hepatic ER stress in obesity.

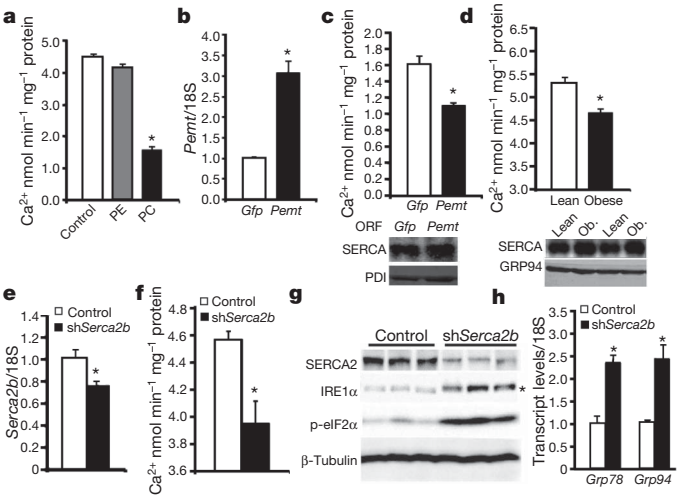

Figure 2 | An increased PC/PE ratio impairs SERCA activity and ER homeostasis. **a**, Calcium transport activity of microsomes loaded with PC and PE *in vitro*. **b**, **c**, Transcript levels of *Pemt* (**b**) and corresponding microsomal calcium transport activities (**c**) of Hepa1-6 cells expressing control (*Gfp*) or mouse *Pemt* open reading frames (ORFs). **d**, Calcium transport activity (top) and SERCA protein levels (bottom) of microsomes prepared from lean and obese mouse liver. **e**–**h**, Liver *Serca2b* transcript levels (**e**) and microsomal calcium transport activities (**f**), immunoblot (**g**) and quantitative RT–PCR (**h**) measurement of ER stress markers in the livers of lean mice expressing either *LacZ* (control) or *Serca2b* shRNAs. Asterisk in **g** denotes the phosphorylated IRE1α and in other panels denotes significant difference (*$P < 0.05$, $n = 4$) by Student's *t*-test. Values are mean ± s.e.m.

We carried out two different but complementary approaches to correct aberrant lipid metabolism induced SERCA dysfunction and examined the effects on ER homeostasis in the obese liver. If the alteration in PC/PE ratio seen in obese liver is a significant contributor to ER stress, correction of this ratio to lean levels by reducing *Pemt* expression should improve calcium transport defects and produce beneficial effects on hepatic ER stress and metabolism. Using an adenovirally expressed short hairpin RNA (shRNA), we were able to achieve ~50–70% suppression of the *Pemt* transcript in obese liver (Supplementary Fig. 4a). As postulated, suppression of *Pemt* led to a decrease of PC content from ~39% to ~33%, which was compensated by an ~7% increase of PE content from ~17% to 24% (Supplementary Table 4). As a result, the PC/PE ratio is reduced to 1.3 (equivalent to the lean ratio), as compared to 2.0 detected in the ER of the obese liver (Fig. 3a). The reduction of the PC/PE ratio was accompanied by a significant improvement in the calcium transport activity of the ER prepared from the *Pemt*-knockdown obese mice (Fig. 3b). As the improvement of calcium transport function occurred with few and minor changes in the overall fatty acid composition of ER (Supplementary Fig. 4b, c and Supplementary Table 5), our results confirmed the rise in PC/PE ratio as an inhibitory factor of SERCA activity in obesity. More importantly, hepatic ER stress indicators including the phosphorylation of IRE1α and eIF2α as well as the expression of C/EBP homologous protein (CHOP), homocysteine-inducible, ER stress-inducible protein (HERP) and Der1-like domain family member 2 (DERL2) were all reduced upon suppression of *Pemt* in obese mice (Fig. 3c, d and Supplementary Fig. 4d). Relief of chronic ER stress in leptin-deficient ($Lep^{-/-}$) mice has been associated with improvement of hepatic steatosis and glucose homeostasis[10,11].

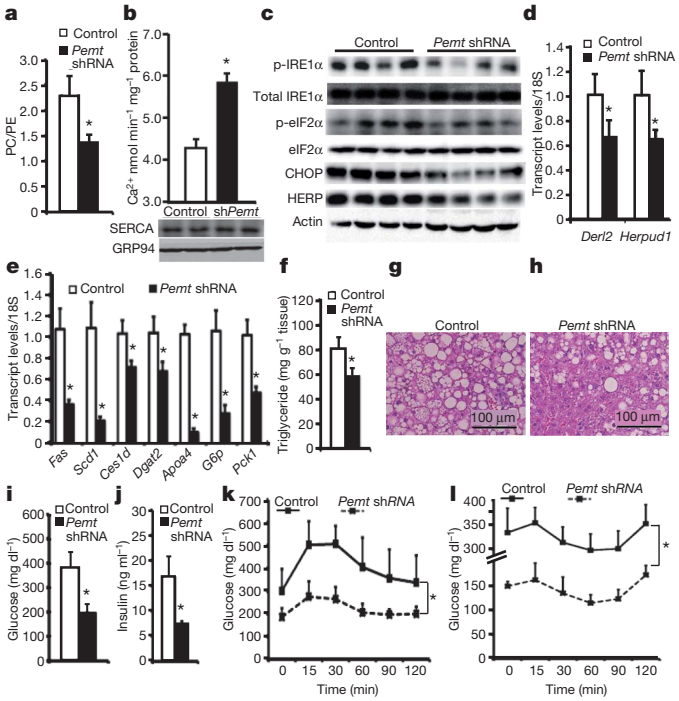

Figure 3 | Suppression of liver *Pemt* expression corrects the ER PC/PE ratio, relieves ER stress and improves systemic glucose homeostasis in obesity. **a**, **b**, PC/PE ratio (**a**) and calcium transport activity (**b**) of liver ER from $Lep^{-/-}$ mice expressing *LacZ* (control) or *Pemt* shRNAs. **c**, **d**, Immunoblot (**c**) and quantitative PCR (**d**) measurement of ER stress markers in the liver. **e**–**h**, Expression of hepatic lipogenesis and gluconeogenesis genes (**e**), triglyceride content (**f**) and haematoxylin & eosin staining (**g** and **h**) of liver samples. **i**, **j**, Plasma glucose (**i**) and insulin (**j**) levels in control and *Pemt* shRNA-treated $Lep^{-/-}$ mice after 6 h food withdrawal. **k**, **l**, Plasma glucose levels of control and *Pemt* shRNA-treated $Lep^{-/-}$ mice after intraperitoneal administration of either 1 g kg^{-1} of glucose (**k**) or 1 IU kg^{-1} of insulin (**l**). All data are mean ± s.e.m. ($n = 4$ for **a**–**e**, $n = 6$ for **f**–**l**), $^*P < 0.05$ (one-way ANOVA for data presented in **k** and **l**, and Student's *t*-test for others).

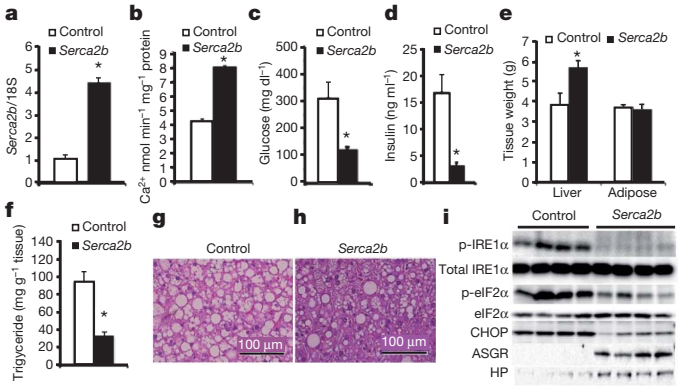

Figure 4 | Exogenous *Serca* expression alleviates ER stress and improves systemic glucose homeostasis. **a**, **b**, Liver *Serca2b* transcript levels (**a**) and microsomal calcium transport activities (**b**) of control or *Serca2b*-overexpressing obese mice. **c**–**e**, Plasma glucose (**c**), plasma insulin levels (**d**) and tissue weights (**e**) of $Lep^{-/-}$ mice as in panel **a**. **f**–**i**, Triglyceride content (**f**), haematoxylin & eosin staining (**g**, **h**) and immunoblot analyses (**i**) of ER stress markers (IRE1α and eIF2α phosphorylation, and CHOP) and secretory proteins (ASGR and HP) in the obese liver expressing *Serca2b* compared to controls. All values are mean ± s.e.m. ($n = 4$ for **a** and **b**, $n = 6$ for **c**–**h**). $^*P < 0.05$ (Student's *t*-test).

Consistently, genes involved in hepatic lipogenesis (*Fas*, *Scd1*, *Ces1d*, *Dgat2*) and lipoprotein synthesis (*Apoa4*) were significantly downregulated in the obese liver after suppression of *Pemt* (Fig. 3e). As a result, these mice exhibited a significant reduction in hepatic steatosis and liver triglyceride content (Fig. 3f–h). Genes involved in glucose production (*G6pc*, *Pck1*) in the liver were significantly downregulated (Fig. 3e), and there were also significant reductions in both hyperglycaemia and hyperinsulinaemia in obese mice after the suppression of hepatic *Pemt* expression (Fig. 3i, j). Glucose and insulin tolerance tests revealed significantly enhanced glucose disposal after *Pemt* suppression (Fig. 3k, l). A similar phenotype is also observed upon suppression of hepatic *Pemt* in high-fat-diet-induced obesity, with reduced ER stress and improved glucose homeostasis (Supplementary Fig. 5). These data are consistent with the phenotype seen in *Pemt*-deficient mice, which exhibit protection against diet-induced insulin resistance and atherosclerosis[12]. Therefore, correcting the PC/PE ratio of the ER can significantly improve calcium transport defects, reduce ER stress and improve metabolism, supporting the hypothesis that changes in lipid metabolism contribute to SERCA dysfunction, ER stress and hyperglycaemia in both genetic- and diet-induced models of obesity.

We then carried out overexpression of hepatic *Serca in vivo* to overcome the partial inhibition of SERCA activity by PC (Fig. 4a). Indeed, exogenous SERCA expression in the liver of $Lep^{-/-}$ mice improved the calcium import activity of the ER (Fig. 4b), restored euglycaemia and normoinsulinaemia within a few days, and markedly improved glucose tolerance (Fig. 4c, d and Supplementary Fig. 6). Upon *Serca* expression, the liver showed an increase in size but a marked reduction of lipid infiltration (Fig. 4e–h) and suppression of IRE1α and eIF2α phosphorylation, along with a significant reduction in CHOP levels (Fig. 4i). In these liver samples, there was also a marked increase in two secretory proteins that were otherwise diminished in obesity: asialoglycoprotein receptor (ASGR) and haptoglobin (HP) (Fig. 4i). As the folding and maturation of ASGR is most sensitive to perturbations of calcium homeostasis in the ER[13], our results indicate that exogenously increased SERCA expression restored calcium homeostasis and relieved at least some aspects of chronic ER stress in the obese liver. Taken together, these data reinforce the hypothesis that lipid-driven alterations and ER calcium homeostasis are important contributors to hepatic ER stress in obesity.

The chronic activation of ER stress markers has been observed in a variety of experimental obese models as well as in obese humans[14]. Furthermore, treatment of obese mice and humans with chemical chaperones results in increased insulin sensitivity[10,15]. Our systematic, compositional and functional characterization of hepatic ER landscape from lean and obese mice revealed a diametrically opposite regulation of ER functions regarding protein and lipid metabolism and revealed mechanisms giving rise to ER stress. In particular, an increase in the PC/PE ratio in the ER, driven by the upregulation of *de novo* lipogenesis in obesity, was linked to SERCA dysfunction and chronic ER stress *in vivo*. During the review of this manuscript, a study reported downregulation of the SERCA protein level in obese liver[16], which was not evident in our analysis and seemed to have resulted from the choice of methodology in ER protein preparations (Supplementary Fig. 7). Nevertheless, other mechanisms such as oxidative and inflammatory changes associated with obesity can also perturb ER homeostasis by affecting ER calcium fluxes[17–19] and will be important to study in the future.

The identification of a lipid-driven calcium transport dysfunction and ER stress provides a fundamental framework for understanding the pathogenesis of hepatic lipid metabolism and chronic ER stress in obesity. First, excessive food intake inevitably stimulates lipogenesis for energy storage, and PC is the preferred phospholipid coat of lipid droplets and lipoproteins[20]. Therefore, there is a biological need for the synthesis of more PC for packaging and storing the products of hepatic lipogenesis. Second, *de novo* fatty acid synthesis in the obese liver produces ample amounts of MUFA, which is effectively incorporated into PC but not PE, which further distorts the PC/PE ratio and impairs ER function. The resulting ER stress facilitates the secretion of excessive lipids from the liver without ameliorating hyperinsulinaemia-induced lipogenesis[21], and thus hepatosteatosis and ER stress ensue. As a result,

relieving ER stress in obesity may ultimately depend on breaking this 'lipogenesis–ER-stress–lipogenesis' vicious cycle and restoring ER folding capacity. Therefore, we suggest that genetic, chemical or dietary interventions that modulate hepatic phospholipid synthesis and/or ER calcium homeostasis function might represent a new set of therapeutic opportunities for common chronic diseases associated with ER stress, such as obesity, insulin resistance and type 2 diabetes.

METHODS SUMMARY

Male leptin-deficient ($Lep^{-/-}$) and wild-type littermates in the C57BL/6J background were either bred in-house or purchased from the Jackson Laboratory (strain B6.V-Lep^{ob}/J, stock number 000632). Transduction of adenoviruses (serotype 5, Ad5) for the expression of open reading frames (ORFs) or shRNAs was carried out between 10–11 weeks after birth, and all mice were killed between 12–13 weeks of age, unless noted otherwise. ER fractionation for proteomic and lipidomic analysis were carried out as previously described[22]. Calcium transport experiments were performed as previously described[23], with some modifications. Quantitative RT–PCR, western blot analysis, histology and *in vivo* animal experiments were carried out as previously described[10,24]. Oligonucleotide sequences used in this study are listed in Supplementary Table 6. Detailed experimental procedures and protocols are described in the Supplementary Material.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions S.F. designed, performed experiments, analysed and interpreted the results and wrote the manuscript; L.Y. and P.L. performed some animal experiments; O.H., L.D., W.H. and X.L. performed statistical and bioinformatic analysis of the proteomic data; S.W.M quantified the lipid composition of ER and analysed the data; A.R.I. analysed the protein composition of ER; G.S.H. generated the hypothesis, designed the project, analysed and interpreted the data and wrote the manuscript.

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