RESEARCH ARTICLE

The ER membrane protein complex promotes biogenesis of sterol-related enzymes maintaining cholesterol homeostasis

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ABSTRACT

The eukaryotic endoplasmic reticulum (ER) membrane contains essential complexes that oversee protein biogenesis and lipid metabolism, impacting nearly all aspects of cell physiology. The ER membrane protein complex (EMC) is a newly described transmembrane domain (TMD) insertase linked with various phenotypes, but whose clients and cellular responsibilities remain incompletely understood. We report that EMC deficiency limits the cellular boundaries defining cholesterol tolerance, reflected by diminished viability with limiting or excessive extracellular cholesterol. Lipidomic and proteomic analyses revealed defective biogenesis and concomitant loss of the TMD-containing ER-resident enzymes sterol-O-acyltransferase 1 (SOAT1) and squalene synthase (SQS, also known as FDFT1), which serve strategic roles in the adaptation of cells to changes in cholesterol availability. Insertion of the weakly hydrophobic tail-anchor (TA) of SQS into the ER membrane by the EMC ensures sufficient flux through the sterol biosynthetic pathway while biogenesis of polytopic SOAT1 promoted by the EMC provides cells with the ability to store free cholesterol as inert cholesteryl esters. By facilitating insertion of TMDs that permit essential mammalian sterol-regulating enzymes to mature accurately, the EMC is an important biogenic determinant of cellular robustness to fluctuations in cholesterol availability.

This article has an associated First Person interview with the first author of the paper.

KEY WORDS: EMC, Endoplasmic reticulum, Squalene synthase, SOAT1, Cholesterol homeostasis

INTRODUCTION

The endoplasmic reticulum (ER) is a diverse organelle whose functions and physical contacts impact nearly all aspects of physiology. Its major roles in the cell include the biogenesis of nearly all secretory and membrane proteins, lipid biosynthesis, Ca2+ storage and the regulation of various metabolic pathways. The maintenance of ER homeostasis is therefore of central importance to overall cellular fitness, and identifying the requisite factors and pathways has been a major goal of contemporary cell biology. Accordingly, numerous genetic and biochemical screens have been performed to identify factors that affect ER homeostasis. Large-scale genetic interaction analyses in yeast have identified several ER-resident proteins and complexes with currently unknown functions (Jonikas et al., 2009; Schuldiner et al., 2005). One such complex is the ER membrane protein complex (EMC), an abundant, multi-subunit protein complex, present in all eukaryotic kingdoms (Wideman, 2015).

Yeast EMC was initially described as a stoichiometric complex of six subunits (EMC1–EMC6) whose individual disruption led to activation of the unfolded protein response (UPR) (Jonikas et al., 2009). EMC subunits were subsequently identified in mammalian cells as interactors of known ER-associated degradation (ERAD) components. In mammals, the EMC has ten subunits (EMC1–EMC10) (Christianson et al., 2012), with EMC7 and EMC10 later recognised as Sop4 and YDR056C in yeast (Wideman, 2015). Several studies have identified different EMC subunits in screens for modulators of functional surface expression of membrane proteins, such as the neuronal acetylcholine receptor in Caenorhabditis elegans (Richard et al., 2013; Satoh et al., 2015), rhodopsin in Drosophila and the ABC transporter Yor1 in yeast (Louie et al., 2012). EMC disruption has also been observed to affect phospholipid trafficking (Janer et al., 2016; Lahiri et al., 2014), autophagosome formation (EMC6, Li et al., 2013; Shen et al., 2016), neurological degeneration (EMC1, Harel et al., 2016), retinal dystrophy (EMC1, Abu-Safieh et al., 2013), SY40 egress from the ER (EMC1, Bagchi et al., 2016), and pathogenesis of flaviviruses including West Nile, Dengue and Zika (Le Sommer et al., 2012; Ma et al., 2015; Marceau et al., 2016; Savidis et al., 2016). The function(s) of the EMC linking these diverse phenotypes across various organisms remain an area of active investigation. In recent advances, the EMC was shown to be able to serve as an insertase for weakly hydrophobic transmembrane domains of tail-anchored (TA) proteins (Guna et al., 2018), modulate the co-translational expression of multi-pass membrane proteins with challenging TMDs (Shurtliff et al., 2018) and promote accuracy of G-protein-coupled receptor (GPCR) biogenesis through insertion of their first TMD (Chitwood et al., 2018). How the insertase activity of EMC underlies the range of phenotypes reported is not yet clear.

Here, we determine fundamental aspects of EMC assembly and architecture in mammalian cells. Leveraging these insights revealed that cells lacking the EMC are sensitive to extracellular cholesterol availability. By undertaking lipidomic analyses and quantitative proteomics, we identified lipid species and proteins whose
abundance was dependent on the EMC, including multiple factors intimately tied to cholesterol homeostatic maintenance. Biochemical and cell biological analyses demonstrated that the loss of these essential factors was due to premature degradation, implicating the EMC in assuring their correct biogenesis. We propose that robust maintenance of cholesterol homeostasis requires the insertase activity of the EMC for the optimal integration of essential biosynthetic and storage enzymes into the ER membrane. This function, and the immediate consequences for lipid and protein homeostasis, likely contribute to the diverse cellular and organismal phenotypes caused by loss of the EMC.

RESULTS

EMC integrity is maintained by a set of essential subunits

The mammalian EMC contains ten distinct subunits (Christianson et al., 2012) that differ extensively in both primary sequence and membrane topology (Fig. 1A). To rationally target the EMC in functional studies, we first sought to understand how each subunit contributes to the integrity of the mature complex. We monitored stability of the complex in response to subunit knockdown. All subunits of the EMC shown previously to co-purify (Guna et al., 2018), were observed to co-sediment as a single complex on sucrose gradients (Fig. S1A, fractions 7–9). Individually silencing EMC1, 2, 3, 5 or 6 by means of siRNAs or sgRNAs caused marked co-depletion of the remaining EMC subunits, whereas depletion of EMC4, 7, 9 or 10 was not notably disruptive (Fig. 1B; Fig. S1B,C). EMC8 knockdown reduced the levels of some subunits, but led to an increase in EMC9 (Fig. 1B, lane 9). The similarity of EMC8 and EMC9 (>40% amino acid identity) suggests that EMC9 might partially compensate for EMC8 loss. Although almost all EMC subunits were lost in EMC6 knockdowns, their corresponding mRNA levels were not significantly changed (Fig. S1D), suggesting that the remaining subunits are degraded post-translationally. As expected, any remaining EMC subunits in these knockdowns, their corresponding mRNA levels were not significantly changed (Fig. S1D), suggesting that the remaining subunits are degraded post-translationally. As expected, any remaining EMC subunits in these knockdown experiments showed altered sedimentation profiles (Fig. S1C), illustrating that the intact complex was disrupted.

As EMC integrity was most severely disrupted when EMC5 or EMC6 were depleted, we focused on these subunits for subsequent studies. Because of their importance to assembly, EMC5 and EMC6, along with EMC2 and EMC3 were designated as ‘core’ subunits of the EMC. We genetically modified U2OS Flp-In™ T-Rex™ cells by CRISPR/Cas-9 to generate knockouts of EMC5 (∆5) or EMC6 (∆6) (Fig. 1C; Fig. S1E–G, and see Materials and Methods), then stably reintroduced the respective genes into the single tetracycline-inducible FRT recombination locus. As with the knockdown experiments, most EMC subunits were at nearly undetectable levels in both the ∆EMC5 and ∆EMC6 cells (hereafter collectively referred to as ∆EMC). Doxycycline (DOX) induction restored the missing subunit and consequently the EMC to levels near normal (Fig. 1C). The finding that EMC5 expressed in ∆6 cells can be stabilised by including the proteasome inhibitor MG132 is evidence that co-depletion of EMC subunits occurs post-translationally (Fig. 1C, compare lanes 4 and 8). Furthermore, it suggests that EMC6 is the core subunit that initiates assembly of the EMC. These cell lines provided the means to systematically investigate the role(s) of EMC in mammalian cell physiology.

EMC deficiency sensitises cells to cholesterol excess and starvation

Disruptions to individual EMC subunits in both cell lines and model organisms have produced a wide range of seemingly unrelated phenotypes (Bagchi et al., 2016; Brockerhoff et al., 1997; Jonikas et al., 2009; Lahiri et al., 2014; Ma et al., 2015; Richard et al., 2013; Tang et al., 2017). Because the EMC6 knockout produced a near-complete loss of the complex, we screened ∆EMC cells for viability under a set of growth conditions. During this, we observed ∆EMC6 cell lines to be particularly sensitive to the amount of extracellular cholesterol available from growth media. EMC-deficient cells grew as well as wild-type (WT) cells under standard growth conditions (DGMEM plus 10% FCS), but showed significantly diminished viability in media supplemented with cholesterol delivered in complex with the binding agent methyl-β-

![Fig. 1. EMC5 and EMC6 are essential for EMC maturation.](Image)
cyclodextrin (Chol:MBCD) (Fig. 2A,B). Remarkably, limiting extracellular cholesterol availability also strongly reduced the viability of ΔEMC6 cells but not of WT cells. This cholesterol auxotroph phenotype was seen when the cells were grown in lipoprotein-depleted serum (LPDS, Fig. 2C) or with uncomplexed MBCD (Fig. S2A,B). Restoring EMC6 expression to ΔEMC6 cells (Δ6+6) reversed the sensitivity to cholesterol depletion, rescuing viability to WT levels (Fig. S2A-C) and ruling out effects unrelated to EMC loss. Importantly, we determined that a minimal non-toxic amount of Chol:MBCD (4 µM) restored viability to ΔEMC6 cells (Fig. 2D,E), confirming it is excess cholesterol that EMC-deficient cells are unable to tolerate.

Cholesterol homeostatic responses are intact in EMC-deficient cells

Failing to adapt to changes in exogenous cholesterol availability suggested that cholesterol homeostatic mechanisms might be compromised in ΔEMC cells. The ER-resident transcription factor sterol regulatory element-binding protein-2 (SREBP-2, also known as SREBF2) exerts homeostatic control over free cholesterol levels in cells by rapidly sensing cholesterol insufficiency. Relocation of SREBP-2 to the Golgi and release of its N-terminus [SREBP-2(N)] owing to the action of proteases, allows the active transcription factor to enter the nucleus and activate choleseterogenic genes (Adams et al., 2004; Brown et al., 2002). Surprisingly, the amount of SREBP-2(N) detected in nuclear fractions did not differ markedly between WT, Δ5 and Δ6 cell lines when grown in standard medium (Fig. 3A, compare lanes 2, 8 and 14), indicating that SREBP-2 did not sense any substantial disruption to free cholesterol levels in EMC-knockout cells. Cholesterol depletion with sub-lethal amounts of MBCD led to efficient SREBP-2 cleavage in each of the cell lines (Fig. 3A, compare lanes 4, 10 and 16), whereas addition of 25-hydroxycholesterol (25-HC), which promotes ER retention of SREBP-2, abolished even the small basal cleavage observed under normal conditions (Fig. 3A, compare lanes 6, 12 and 18). These findings indicate that SREBP-2 activity and responsiveness are normal in ΔEMC cells but not activated.

Transcription of representative SREBP-2 target genes, whose activation reinforces cholesterol biosynthesis and uptake to restore homeostasis, was slightly elevated in ΔEMC6 cells (Fig. 3B), but failed to reach statistical significance. Immunoblotting (Fig. 3C) and RNA-Seq analyses (Fig. S3) of ΔEMC cells supported these data, revealing no evidence of coordinated upregulation within genes of the cholesterol biosynthetic pathway at steady state. One exception was the elevation of squalene monooxygenase (SM, also known as SQLE; EC 1.14.14.17) at the protein (Fig. 3C) but not the mRNA level (Fig. 3B), which may be linked to sterol-dependent feedback controlling its degradation by the ubiquitin ligase MARCH6 (the equivalent of yeast Doa10) (Foresti et al., 2013; Gill et al., 2011). Cellular cholesterol was not differentially distributed in ΔEMC6 and WT cells (Fig. 3D). Furthermore, the transcriptional response of SREBP2-dependent genes [HMGCR, SM and SQS (also known as FDFT1)] to acute cholesterol depletion in ΔEMC6 cells was
EMC deficiency compromises cholesterol storage

During efforts to explore the basis of the ΔEMC phenotype, we performed whole-cell lipidomic analysis to identify potential points of dysregulation (Fig. 4A). We considered only lipid species that were consistently and significantly altered (P ≤ 0.05) in both ΔEMC5 and ΔEMC6 cells to minimise any clonal bias. The most striking effect was a ∼10-fold and ∼5-fold decrease in cholesteryl esters in both ΔEMC5 and ΔEMC6 cells (Fig. 4A,B), while most other lipid species (fatty acids, neutral lipids, sphingolipids etc.), including cholesterol, appeared relatively unaffected (Fig. S4A–J). Among those lipids affected by EMC loss, the changes in most did not reach statistical significance. Based on these findings, we focused on cholesteryl esters because they represent the principal form of stored cholesterol in mammalian cells (Chang et al., 2009) and are likely to be connected to the cholesterol sensitivity seen in ΔEMC cells.

SOAT1 expression is EMC-dependent

Cholesteryl esters are produced by sterol-O-acyltransferase/acyl-CoA-acyltransferase 1 (SOAT1, also known as ACAT1; EC 2.3.1.26), a homotetrameric ER-resident enzyme (Chang et al., 2009). SOAT1 uses long chain fatty acids to modify surplus free cholesterol for inert storage in lipid droplets (LDs) of extrahepatic tissues (Pol et al., 2014). In hepatic and gastrointestinal tissues, this role is comparably performed by the SOAT1 homolog (46% identity), SOAT2 (also known as ACAT2) (Oelkers et al., 1998). Cholesterol esterification by SOAT1 represents an important mechanism to control free cholesterol levels in cells and reduce the potential cytotoxicity associated with its accumulation (Feng et al., 2003; Warner et al., 1995). SOAT1 protein was markedly reduced in both EMC5 and EMC6 knockouts across multiple cell lines (Fig. 4C,D; Fig. S4K,L), while transcript levels did not differ significantly from WT (Fig. S4M). Importantly, SOAT1 returned to WT levels by restoring the EMC with the missing subunit (Δ5+EMC5, Δ6+EMC6; Fig. 4C,D).

To confirm the SOAT1 loss in EMC-deficient cells was post-transcriptional, we constructed a dual fluorescence reporter using GFP-(P2A)-RFP fused to SOAT1. When translated, a ribosome-skipping event induced by the P2A site releases the N-terminal GFP from RFP–SOAT1 at equimolar ratios (Fig. 4E; Fig. S4N). Conditions favouring RFP–SOAT1 destabilisation relative to GFP decrease the RFP:GFP ratio, in effect serving as a quantitative readout for protein stability (Guna et al., 2018). Transient expression of the SOAT1 reporter in EMC-deficient cells exhibited marked reduction of RFP:GFP ratios compared to expression in WT (Fig. 4F,G). Rescuing ΔEMC5 cells by expressing EMC5 (Δ5+EMC5) or treating with the proteasome inhibitor MG132 restored SOAT1 to levels near to those found in WT (Fig. 4F,G).
To assess whether SOAT1 destabilisation contributed to the heightened sensitivity of ΔEMC6 cells to elevated cholesterol (Fig. 2A), we treated WT and EMC-deficient cells with the SOAT1 inhibitor avasimibe (AVA) under conditions of high cholesterol load (+Chol:MBCD). WT cells were sensitive to cholesterol loading only in the presence of AVA, comparable to that observed for untreated ΔEMC6 cells (Fig. 4H). Of note, AVA further enhanced sensitivity of ΔEMC6 cells to Chol:MBCD-induced cell death, indicating that SOAT1 activity might be limiting but not entirely absent. These findings indicate a post-transcriptional defect in SOAT1 biogenesis coinciding with EMC deficiency that results in degradation and compromised esterification capacity.
Fig. 4. SOAT1 loss from EMC-deficient cells and attenuated cholesteryl ester formation. (A) Heat map representing the fold change of 117 lipid species in EMC5 (∆5 #5-4) and EMC6 (∆6 #3-9) deletion mutants relative to WT as identified by targeted metabolic analysis (n=2). Lipid species are arranged according to major structural class. The 14 lipid species significantly altered in both ∆5 and ∆6 cells (P≤0.05) are indicated (grey circles, black star). NL, neutral lipids; FA, fatty acids; AC, acyl carnitine; NAE, N-acyl ethanolamines; ST, sterols; PL, phospholipids; LPL, lyso phospholipids; SL, sphingolipids; NEL, neutral ether lipids; PLE, phospholipid ethers; LPLE, lysophospholipid ethers.

(B) Quantification of free cholesterol and cholesteryl esters in ∆5 and ∆6 cells relative to WT (dashed line). Means±s.e.m. are shown (n=4). ***P≤0.001, ****P≤0.0001 (Student’s t-test). (C,D) Western blots of whole-cell lysates (WCL) for ∆5 cells with or without the EMC5 expression vector (C) and ∆6 cells with or without the EMC6 expression vector (D) probed for SOAT1 and indicated EMC subunits. (E) Schematic representation of the dual reporter construct used in F-G. mRNA encoding GFP separated by a P2A sequence from RFP and FLAG-tagged SOAT1. Translation results in ribosome skipping and the generation of GFP and RFP-3xFLAG-SOAT1 at equimolar ratios. Differences in stability between both gene products gives rise to altered RFP:GFP ratios, serving as a sensitive readout for protein stability. (F) Fluorescently ratiometric GFP:GFP ratio in WT and ∆5 cells with or without the EMC5 expression vector and transiently expressing GFP-P2A-RFP-3xFLAG-SOAT1. At 24 h post transfection, cells were treated with MG132 (5 µg/ml) or DMSO (vehicle control) for 8 h and analysed by flow cytometry. EV, empty vector control. (G) Quantification of three independent experiments as performed in F. Means±s.e.m. are shown (n=3). ***P≤0.001 (Student’s t-test). (H) WT and ∆5 cells were exposed to Chol: MBCD (25, 37.5 and 50 µM, 20 h) with or without 10 µM avasimibe (A/Va, 20 h) and visualised by staining with Crystal Violet.

Various intracellular proteins exhibit EMC dependence

While loss of SOAT1 clarified the observed sensitivity to surplus extracellular cholesterol, it could not readily explain the cholesterol auxotrophy of ∆EMC cells. To investigate this, we used stable isotope labelling with amino acids in cell culture (SILAC) and quantitative proteomic analysis to compare WT and ∆EMC cells (Fig. S5A). By adopting stringent selection criteria that required reduction by ≥30% in two independent ∆EMC5 and ∆EMC6 cell lines, we aimed to mitigate any bias arising from clonal variation.

Analysis of total intracellular proteins identified 11 unique proteins whose signature peptides met these criteria (Fig. 5A; Fig. S5B,C). Of the proteins identified, SQR (EC 2.5.1.21), was the one consistently and most substantially downregulated across all four cell lines. This marked effect, together with its central position in the cholesterol biosynthesis pathway, led us to investigate SQR further.

SQS and cholesterol auxotrophy in EMC-deficient cells

SQS is an evolutionarily conserved ER-resident enzyme catalysing the first committed step in de novo sterol production (Beyta and Porter, 1976; Do et al., 2009). SQS reductively condenses two farnesyl-pyrophosphate (FPP) molecules to form the linear triterpene de novo sterol precursor 24-ethylcholesta-5,22-dien-3β-ol (Beyta and Porter, 1976; Do et al., 2009). SQS is a tail-anchored (TA) membrane protein that must be inserted post-translationally into the ER. TA proteins failing to insert in the ER membrane are typically degraded (Borgese and Fasana, 2011). The EMC was recently shown to be necessary and sufficient for SQS TMD insertion into the ER membrane (Guna et al., 2018). We confirmed this dependency by using an SQS variant with a C-terminal opsin tag that is able to accept N-linked oligosaccharides (Brambillasca et al., 2005) (Fig. 6C) and repeated the pulse-chase studies. In WT cells, SQS was stabilized in ∆EMC6 cells upon treatment with the VCP/p97 inhibitor NMS-873 (Fig. S6H), indicating that it was not degraded by the canonical ERAD pathways when the EMC was absent.

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complex activity is not compromised by EMC deficiency. We asked whether bypassing EMC-dependent insertion of SQS could rescue the viability of ΔEMC cells when depleted of extracellular cholesterol. But although ΔEMC6 cells were able to insert SQS-Sec61βTMD into the ER, this chimera could not restore viability following the depletion of extracellular cholesterol, perhaps indicating that the SQS TMD is essential for function or that additional EMC-dependent disruptions to sterol-related factors are involved downstream of SQS. These data demonstrate that the TMD of SQS directs its proper insertion by the EMC. Furthermore, insertion by the EMC is essential for SQS to become functional through appropriate ER membrane localisation that allows it to reach expression levels sufficient to provide robust sterol biosynthesis.

**DISCUSSION**

**Critical roles for subunits in EMC assembly**

Individual subunits of the EMC have been implicated in regulation of various proteins and processes, but how those activities are linked to the fully assembled, mature EMC oligomer had not been fully appreciated. In a systematic targeted knockdown screen for all 10 EMC subunits in multiple cell lines, we identified EMC6, EMC5 and EMC2 as essential for the intrinsic stability of other subunits within the EMC oligomer (Fig. 1). This observation implies first, that these ‘core subunits’ play a central role in EMC maturation. Secondly, disrupting individual core subunits confers loss of the entire EMC and any associated activity (Louie et al., 2012). Finally, roles attributed to most of the individual subunits, at least the essential ones, probably reflect activity of the fully assembled EMC. Robust phenotypes in model systems have been reported with the loss of individual EMC subunits (Richard et al., 2013; Satoh et al., 2015). Moreover, multiple siRNA and CRISPR/Cas9 loss-of-function screens for host factors supporting flavivirus infection/replication or cytotoxicity have identified more than one EMC subunit (Ma et al., 2015; Marceau et al., 2016; Savidis et al., 2016; Zhang et al., 2016). Among the strongest hits in these screens were EMC5 and EMC6, consistent with the essential roles in EMC maturation that we have defined.
or EMC10) did not markedly affect SQS maturation (Fig. S5J,K), suggesting that only selected subunits within the EMC oligomer contribute directly to inserting (or chaperoning) client TMDs. EMC activity could be derived from the coordinated contributions of multiple subunits or could be mediated by a single subunit, such as EMC3, which recent bioinformatic evidence has identified as a member of the Oxa1/Alb3/YidC family of membrane protein biogenesis factors (Anghel et al., 2017; Borowska et al., 2015). It is worthwhile noting that EMC4 depletion did not destabilise other subunits but did preclude maturation of SQS (Fig. S5J,K) and other TMD-containing proteins (Shurtleff et al., 2018), and also suppressed flavivirus infection (Savidis et al., 2016), reflecting an important role in EMC functionality. By systematically defining the approximate architecture of the EMC and interdependency of its subunits, previous and future studies of this complex can be interpreted with greater precision. Ultimately, a more detailed structure–function analysis of the mammalian EMC will be required to differentiate the distinct roles and contributions of each subunit.

**SQS and SOAT1 biogenesis require the EMC**

Until recently, the molecular mechanism and the physiological role(s) for the ubiquitously expressed and evolutionarily conserved EMC had not been fully elucidated. However, a growing body of work has increasingly consolidated that it has a direct involvement in the biogenesis of both TA and polytopic membrane proteins in the ER. Here, we provide evidence that this EMC activity is a determinant of the homeostatic boundaries for cholesteryl store within mammalian cells. EMC deficiency reduces cholesteryl ester stores but does not compromise the total free cholesterol in cells, nor does it trigger activation of SREBP2. However, the homeostatic challenges posed by growth in lipoprotein-depleted serum or exposure to high levels of extracellular cholesterol normally tolerated through robust sterol response mechanisms, become lethal without the EMC insertase activity. We traced the molecular basis of this striking phenotype in EMC-deficient cells to insufficient biogenesis and steady-state levels of at least two membrane-bound cholesterol homeostatic enzymes, SQS and SOAT1 (Fig. 7). Both SOAT1 and SQS were among proteins identified through quantitative proteomics and direct detection as strongly downregulated upon loss of the EMC. In contrast to the marked reduction observed for SQS (Fig. 5A,C–E), SOAT1 was not detected among the downregulated proteins by our proteomic analyses, perhaps due to an insufficient depth of coverage. Reductions in both SQS and SOAT1 protein levels were, however, reported in another proteomic analysis of EMC clients (Shurtleff et al., 2018), with SOAT1 also reduced in epithelial cells derived from EMC3-knockout mice (Tang et al., 2017).

SQS and SOAT1 differ significantly in their overall topology and mode of targeting to the ER membrane. SQS contains a single TMD at its C-terminus necessitating its insertion post-translationally into the ER membrane. SQS contains a single TMD at its C-terminus necessitating its insertion post-translationally into the ER membrane. SQS contains a single TMD at its C-terminus necessitating its insertion post-translationally into the ER membrane. SOAT1 contains nine (or more) TMDs (Guo et al., 2005) of varying hydrophobicity (Fig. S7A,B) and is inserted co-translationally by the Sec61 complex, potentially with assistance from the auxiliary translocon-associated protein (TRAP) complex (Nguyen et al., 2018). These are just two examples but it raises intriguing questions of how the EMC is able to equally promote the biogenesis of topologically diverse clients entering the ER through different pathways. In the case of SQS, the EMC is both necessary and sufficient for optimal insertion into the ER membrane, arising from its preference for TMDs of TA proteins with weak to moderate hydrophobicity (Guna et al., 2018). Without the EMC, SQS...
For the polytopic SOAT1, the precise step at which the EMC is required during its biogenesis is less clear. Reduction of endogenous SOAT1 protein, but not mRNA, and the ability to rescue exogenous SOAT1 by proteasome inhibition (Fig. 4F) is consistent with a co-translational role for the EMC. Such a co-translational role for the EMC during biogenesis of polytopic multi-pass proteins with TMDs enriched with charged and aromatic residues has been recently described (Shurtleff et al., 2018). Moreover, during the preparation of this manuscript, EMC involvement in polytopic GPCR biogenesis was reported and revealed its requirement for the accurate insertion of the β-adrenergic receptor’s first TMD into the ER membrane (Chitwood et al., 2018). Necessarily, both examples highlight cooperation with the Sec61 translocon during biogenesis of polytopic clients. Without the EMC, an unstable or suboptimal TMD encountered during membrane integration of the SOAT1 nascent chain could cause sufficient disruption to the folding programme and trigger degradation, potentially through ERAD (Shurtleff et al., 2018). Thus, we suspect that without the EMC, one (or more) SOAT1 TMDs may cause catastrophic folding failure during biogenesis, leading to premature degradation, loss of cholesterol esterification capacity and underlying cholesterol-induced cytotoxicity. The first crystal structure from the membrane-bound O-acyltransferase (MBOATs) superfamily, which includes SOAT1, has revealed a ring of 11 peripheral transmembrane helices in orientations that suggest synthesis and folding of this enzyme class may be complex (Ma et al., 2018). Further insight into the folding programme and structure of SOAT1 will be required to identify at which step the insertase activity of the EMC becomes critical.

**Setting the boundaries of cholesterol homeostasis**

Through its role in facilitating SQS and SOAT1 biogenesis, our data indicate that the insertase activity of the EMC serves as an important determinant of the boundaries of cholesterol homeostasis. Independently losing either SQS or SOAT1 activity is sufficient to invoke well-defined compensatory homeostatic responses, and so it was surprising that transcriptional feedback mechanisms (via SREBP-2) were not strongly activated in EMC-deficient cells (Fig. 3A,B; Fig. S3) nor were the levels of the key cholesterol-related factors LDLR and HMGCR elevated (Fig. 3B,D). The level of SM was inversely correlated with the EMC, but that could be attributable to multilayered post-translational regulation of SM stability via unsaturated fatty acids (Stevenson et al., 2014) and cholesterol (Gill et al., 2011). In order to maintain homeostasis with attenuated cholesterol biosynthesis and storage capabilities, EMC-deficient cells instead adapt, possibly by redistributing available cholesterol resources (Figs 3D, 4B, 7). This scenario may be uniquely created, at least in part, upon the coincidental loss of SQS and SOAT1. Without sufficient capacity to esterify and store cholesterol taken up (by LDLRs), we posit that it could become available to offset attenuated biosynthetic productivity rather than becoming cytotoxic. With homeostasis maintained, but at the cost of its cellular cholesterol reserves, the adapted state of EMC-deficient cells are precarious and at risk to cholesterol fluctuations that would otherwise be survivable. While the sensing and transcriptional response mechanisms for cholesterol change remain intact, their implementation is diminished because optimal expression of key responsive enzymes requires the EMC. Consequently, cell viability can only be maintained within a much narrower window of cholesterol availability.

It is worth noting that several other cholesterol-related proteins are also affected by EMC loss. Among them were ABCA3 (Tang et al., 2017) and TMEM97 [this study (Table S3) and Shurtleff et al., 2018].
both of which are membrane proteins linked with intracellular cholesterol trafficking. ABCA3 is a cholesterol-binding protein/transporter present in a population of late endosomes linked with cholesterol transport/handling across the plasma membrane (van der Kant et al., 2013). TMEM97 is the recently identified δ2 receptor (Alon et al., 2017) that binds to the intracellular cholesterol trafficking protein NPC1 (Niemann-Pick disease, type C1), reducing its abundance and in turn cholesterol transfer to the ER (Ebrahimim-Fakhari et al., 2016). Increasing cholesterol transfer from endosomes to the ER might be how EMC-deficient cells offset biosynthetic attenuation and still maintain apparent cholesterol homeostasis. Clearly, our study and others find that multiple enzymes related to cholesterol processing all share the common requirement of the EMC to facilitate their expression.

**Pleiotropic effects arising from EMC loss**

Our data suggest a strategy for how to trace complex phenotypes from EMC disruption (Ma et al., 2015; Richard et al., 2013; Satoh et al., 2015; Savidis et al., 2016; Zhang et al., 2016) to specific events at the ER, where the EMC resides and its activity is manifest. This will no doubt be a challenging task given the indirect disruptions to key regulatory or metabolic processes such as dysregulated cholesterol-dependent cellular pathways and to the cytosolic factors stabilised by EMC-dependent membrane proteins. Additionally, the increased FPP availability arising from insufficiency of SQS activity might also contribute to the phenotypes observed with EMC loss (Fig. SSF). For example, irreversibly modifying proteins with FPP (or GPPP) lipid anchors increases hydrophobicity, affects membrane association and modulates protein–protein interactions (Roskoski, 2003). Prenylated proteins play critical roles in cell signalling, growth, proliferation (i.e. Ras GTPases) and subcellular protein trafficking (i.e. Rab GTPases) (Wang and Casey, 2016). Elevated non-sterol isoprenoid production will also affect haem A, the polysaccharide carrier dolichol or the redox cofactor ubiquinone (coenzyme Q10) (Beytía and Porter, 1976; Haeupl et al., 2011), but the consequences for these processes may only become apparent when monitored directly.

Deconvolving the contributions of individual EMC-dependent factors to complex phenotypes will require the uncoupling of function from protein biogenesis, a task that will be challenging until the entire range of EMC-dependent proteins can be identified. Our SILAC experiments, which identified SQS, provide an important step in this direction, but future studies will require greater depth to identify lower abundance proteins, such as SOAT1. It is worth mentioning that not all the downregulated proteins identified by SILAC from abundance proteins, such as SOAT1. It is worth mentioning that not future studies will require greater depth to identify lower range of EMC-dependent proteins can be identified. Our SILAC processes may only become apparent when monitored directly.

**Materials and methods**

**Plasmids and expression constructs**

Full-length EMC5 and EMC6 cDNAs were cloned into the pcdNA5/FRT/TO vector (Thermo Fisher Scientific, Waltham, MA) using the BamHI and XhoI restriction sites for stable integration into U2OS Flp-In™ T-Rex™ (or Flp-In™ T-Rex™ 293) cells. An EMC6 variant containing an N-terminal FLAG-HA tag (DYKDDDDKLDGGYPYDPVDYA) was generated by PCR and subcloned into pcdNA5/FRT/TO. To generate TMD insertion reporters, an HA tag (YPYDPVDYA) was appended via a short linker (GGTG) to the C-terminus of the catalytic domain of SQS (alcohol acyl transferase 1), fused through a linker (GS) to the predicted TMD (underlined) and flanking hydrophobic residues of either SQS (SRKHSIPVLSPVLMLALASWQYTLTLQ) or human Sec61β (SPGKLKVGPVVKVLLFLFARFLMLHGWGYKT), followed by an opsin tag (MNGTEGPNFYVSNNKTV) to yield HA:SQSopsin and HA:SQS-Sec61βTMDopsin respectively. Each was subcloned into the pcdNA5/FRT/TO vector. The SOAT-1 reporter (EGFP-P2A-RFP-3xFLAG-SOAT1) was generated by appending a sequence encoding EGFP-P2A-RFP to 3xFLAG-SOAT1 in pcdNA5/FRT/TO, as has been reported previously (Chitwood et al., 2018).

**Chemicals and compounds**

The following compounds were used: 25-hydroxycholesterol (25HC, Sigma-Aldrich, St. Louis, MO), DAPI (Sigma-Aldrich), aminoxyoxbin (Biotium, Fremont, CA), aniline hydrochloride (Sigma-Aldrich), asavisimibine (Sigma-Aldrich), cholesterol (Sigma-Aldrich), doxycycline (Sigma-Aldrich), filipin III (Santa Cruz Biotechnology, Dallas, TX), gel filtration markers kit (MWG1000, Sigma-Aldrich), GGTI 298 trifluoroacetate salt hydrate (Sigma-Aldrich), Hygromycin B Gold (InvivoGen, San Diego, CA), IAA (iodoacetamide, Sigma-Aldrich), lipoprotein-depleted fetal bovine serum (LPDS, Kalen Biomedical, Germantown, MD), LMNG (lauryl maltose neopentyl glycol, Anacron, Maumee, OH), methyl-β-cyclodextrin (MCBD, Sigma-Aldrich), MG132 (Merck Chemical Ltd, Darmstadt, Germany), N-ethylmaleamide (NEM) (Thermo Fisher Scientific), propidium iodide (Sigma-Aldrich), puromycin (Invigenov), SILAC Lys-8-Arg-10 kit (282986444, Silantes GmbH, Munich, Germany), sodium-meta-periodate (Sigma-Aldrich) and ZA (zaragozic acid A, Santa Cruz Biotechnology).

**Antibodies**

The following antibodies were used in this study: ACC1 (Cell Signaling, Danvers, MA, rabbit pAb, #4190), 1:1000; AlexaFluor R488 anti-mouse IgG (H+L) (Invitrogen, donkey pAb, #A21202), 1:4000 [immunoblotting (IB)], 1:400 [immunofluorescence (IF)]; AlexaFluor R488 anti-rabbit IgG (H +L) (Invitrogen, goat pAb, #A11008), 1:4000 (IB), 1:400 (IF); anti-mouse IgG-HRP (Santa Cruz Biotechnology, goat, #sc-2050), 1:10,000; EMC1 (rabbit pAb, kind gift of Enilza Espreafico, Sao Paulo, Brazil), 1:1000; EMC2 (Proteintech, Rosemont, IL, rabbit pAb, #25443-1-AP), 1:2000; EMC3 (Abcam, Cambridge, UK, rabbit pAb, #ab175537), 1:500; EMC4 (Abcam, rabbit pAb, #ab123719), 1:1000; EMC5/MMGT1 (Abcam, rabbit pAb, #ab174366), 1:1000; EMC6 (Abcam, rabbit pAb, #ab84902), 1:1000; EMC7 (Abgent, San Diego, CA, rabbit pAb, #AP11145c), 1:500; EMC8 (Abcam, rabbit pAb, #ab180065), 1:500; EMC9 (Abgent, rabbit pAb, #AP5632b), 1:500; EMC10 (Abcam, rabbit pAb, #ab180148), 1:1000; HA (purified from hybridoma, mouse mAb, clone 12CA5), 1:1000; histone H3 (Abcam, rabbit pAb, #ab1791), 1:1000; HMGCAR (Atlas Antibodies, Bromma, Sweden, mouse mAb, clone CL0260), 1:1000; Hsp70 (Santa Cruz Biotechnology, goat pAb, clone K-20, #sc-1600), 1:500; LDLR (R&D System, Minneapolis, MN, goat pAb, #AF2148), 1:1000; Rap1a (Santa Cruz Biotechnology, rabbit pAb, clone C-17, #sc-1482), 1:1000; SCDC1 (Abcam, mouse mAb, clone CD.E10, #ab19862), 1:1000; SM (Proteintech, rabbit pAb, #12544-1-AP), 1:1000; SOAT1 (Santa Cruz Biotechnology, mouse mAb, clone A-11, #sc-136959), 1:1000; SQS (Abcam, rabbit mAb, #ab109723), 1:2000; SQS (Abcam, rabbit mAb, #ab195046), 1:200 (IF), 1:2000 (WB); SREBP2 (R&D System, goat pAb, #AF7119), 1:1000; tubulin (Sigma-Aldrich, mouse mAb, #T5168/b-5-1-2), 1:1000; and ubiquitin (Cell Signalling Technologies, rabbit pAb, #39335), 1:1000.
Cell culture

U2OS Flp-In™ T-Rex™ [kind gift of Mads Gyrd-Hansen, Oxford, UK, described in Fiil et al., (2013)], Flp-In™ T-Rex™ 293 (Invitrogen) and Ewing sarcoma A673 cells (kind gift of Udo Oppermann, Oxford, UK, originating from the ATCC) were all maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco®, GlutaMAX®, Life Technologies) plus 10% v/v fetal calf serum (FCS, biosera, Kansas City, MO) and L-glutamine (2 mM, Life Technologies) in 5% CO₂ at 37°C. Stable expression cell lines generated by Flp recombinease-mediated integration were continuously cultured in DMEM with 10% FCS and 100 µg/ml hygromycin B (Hygromycin B Gold, InvivoGen). All cell lines were routinely tested for mycoplasma contamination.

Generation of stable Flp-In™ T-Rex™ cell lines

10⁶ cells per 6 cm tissue culture plate were co-transfected with the pcDNA™5/FRT/TO plasmid containing the gene of interest (3 µg) and Flp recombinease (pOG44, 1 µg) using Lipofectamine™ 2000 (Thermo Fisher Scientific). The following day, cells were trypsinised and seeded in 10 cm tissue culture plates to achieve 20–40% confluency at 48 h after transfection. Cells stably integrating the gene of interest were selected by supplementing growth medium with hygromycin B for 7–10 days at the following concentrations; 250 µg/ml (U2OS Flp-In™ T-Rex™) or 100 µg/ml (Flp-In™ T-Rex™ 293).

RNAi

To transiently silence expression of individual EMC subunits, the following ON-TARGETplus siRNA SmartPools (GE Healthcare/Dharmacon, Chicago, IL) (4-25 nmol) were used: EMC1 (LQ-014146-01-0002), EMC2 (LQ-010631-00-0002), EMC3 (LQ-010715-02-0002), EMC4 (LQ-021126-00-0002), EMC5/MMGT1 (LQ-018365-00-0002), EMC6 (LQ-014711-02-0002), EMC7 (LQ-021215-01-0002), EMC8 (LQ-032511-02-0002), EMC9 (LQ-017638-02-0002), EMC10 (LQ-018434-02-0002), non-targeting pool siRNA (D-001810-10-20). At 24 h after transfection, cells were expanded on 10 cm plates and incubated for an additional 48 h.

CRISPR/Cas9-mediated genomic editing

CRISPR/Cas9-mediated genomic editing was performed in accordance with previously described protocols (Ran et al., 2013). Cells were transfected with a pSpCas9(BB)-2A-Puro (PX459) plasmid (Addgene 62988) containing a sgRNA targeting EMC2, EMC5, EMC6 or SQR (2–3 sgRNAs/gene, Table S1). Cells were grown for 48 h and subsequently treated with puromycin (PURO, Invivogen, 1 µg/ml) for 72 h to select transfected clones. Single-cell clones were isolated from PURO-resistant cells by limiting dilution. Disruption to gene expression was confirmed by western blotting of whole cell lysates (described below) and/or by genomic sequencing (Fig. S1G).

Crystal Violet staining

To measure cell density via Crystal Violet incorporation, cells were fixed in cold methanol:acetone (1:1) (10 min, 4°C). After removing the fixative, cells were stained with a solution of 10% ethanol plus 0.1% (w/v) Crystal Violet (10 min), rinsed with ddH₂O and air-dried. Representative micrographs were acquired by light microscopy and cell density per well was determined by imaging-based quantification of incorporated Crystal Violet dye by using an FLA-5100 laser scanner (Fujifilm Life Science, Singapore; 640 nm excitation wavelength, 25 µm resolution). The integrated pixel density of the resulting images was determined using the ImageJ software package.

Cell viability

Cell viability was determined by collecting both detached and attached cells. Attached cells were collected by incubation with PBS plus 10 mM EDTA (10 min, 37°C). Cells were pelleted and resuspended in ice-cold PBS plus 3 mM EDTA. Prior to their analysis by flow cytometry, propidium iodide (PI) was added to cells (1 µg/ml final concentration). During the entire procedure, cells were kept on ice. PI incorporation was determined using a FACS Canto (BD Biosciences, Franklin Lakes, NJ). Data were analysed using the FlowJo (FlowJo, LLC, Ashland, OR) software package.

RNA isolation, reverse transcription and qPCR

RNA was isolated from cells using the RNeasy Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer’s instructions. Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen). Transcript levels were measured in triplicate using the GoTaq® qPCR Master Mix (Promega, Madison, WI) and an RT-PCR cycler (Rotor Gene 6000, Qiagen) with the following conditions: holding step (2 min, 95°C), melting step (72–95°C at 1°C/step), with pre-melt conditioning (90 s at the first step), 60°C annealing temperature, 40 cycles. Primer pairs used in this study are listed in Table S2. Relative mRNA levels were calculated using the ΔΔCt method. Transcript levels of actin were used for normalisation. Statistical significance was calculated with a Student’s t-test.

Sample preparation for RNA-Seq analysis

All cell lines used for RNA isolation are cultured under normal, nutrient-replete conditions (10% FCS in DMEM, 2 mM glutamine). Per sample, 5–10⁶ cells were seeded onto 10 cm tissue culture plates and cells were collected after 24 h. Then, 3 ml TRI Reagent was directly added to the plates, and lysates were snap-frozen and stored at −80°C. Total RNA isolation was performed from 1 ml cell suspension: after adding 200 µl chloroform, the suspension was centrifuged (17,000 g, 5 min), rinsed with 80% ethanol and resuspended in 10.2 µl DEPC water. After DNase treatment (37°C, 30 min), 250 µl TRI Reagent was added and the chloroform extraction repeated as described above with adjusted volumes. The final RNA pellet was rinsed in 80% ethanol, dried and resuspended in 10 µl DEPC water.

RNA-Seq analysis

Paired-end read alignment was performed using the HISAT2 (v2.1) (Kim et al., 2015) read aligner in strand-specific (RF) mode and the hg19 human reference genome. DESeq2 (Love et al., 2014) integrated in the SeqMonk package (v1.4 Simon Andrews and Laura Biggins, Babraham Bioinformatics) was used was used to determine differential gene expression.

Flow cytometry

Cells cultured in the presence of DOX (100 ng/ml) were transfected with GFP-P2A-RFP-3xFLAG-SOAT1 (150 ng/well; 6-well plate) and incubated for 24 h before addition of MG132 (5 µg/ml) or DMSO (vehicle control) for 8 h. Flow cytometry was performed on a BD LSR Fortessa. 50,000 GFP-positive cells were collected and RFP:GFP ratios were determined using FlowJo (v. 10). For quantification (Fig. 4G), mean RFP and GFP fluorescence intensities were determined in GFP-positive cells. GFP:GFP fluorescence ratios were calculated for each sample and normalised to the ratio in WT cells.

Cholesterol starvation

Cells were seeded in 12-well tissue culture plates (10,000 cells/well) in DMEM plus 10% FCS. After 48 h, growth medium was replaced with DMEM containing 10% FCS and 4 mM MBCD (20 min, 37°C). Cells were rinsed three times with 1 ml PBS before addition of DMEM containing 5% FCS or 5% LPDS for 96 h and staining with Crystal Violet (see above). Alternately, cells were seeded in a 96-well plate at 10,000 cells/well and after 24 h, MBCD was diluted in media collected from each well and added back (0–5 mM final concentration). After 16 h, cells were either stained with Crystal Violet or measured for the percentage of cell death by determining the level of PI incorporation using flow cytometry (see above).

Preparation of Chol:MBCD complexes

Cholesterol (2.5 mM) was complexed with MBCD (25 mM) according to a protocol adapted from (Christian et al., 1997). Briefly, cholesterol powder (2.5 mM final) was added to an MBCD solution (25 mM). The resulting emulsion was vortexed and sonicated with a tip sonicator (1 min, 10 s intervals), followed by prolonged incubation (16 h, 37°C) under constant agitation. The solution was passed through a 0.45 µm PVDF syringe filter, aliquoted and stored at −20°C.
Whole-cell lipidomic analysis
U2OS Flp-In™ T-REX™ cells were seeded in 10 cm tissue culture plates (6×10^6 cells/plate). After ≥18 h, cells were rinsed, harvested in ice-cold PBS and the resulting cell pellets stored at −80°C. Whole-cell extracts were prepared and lipids quantified in accordance with previous reports (To et al., 2017).

SDS-PAGE and immunoblotting
Cells were mechanically collected in ice-cold PBS, centrifuged (1000 g, 5 min, 4°C), and the resulting cell pellets resuspended in RIPA buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% (v/v) SDS, 1% (v/v) sodium deoxycholate, 1% (v/v) NP-40, 2 mM EDTA, 1× complete™ Protease Inhibitor Cocktail (Roche), 1 mM PMSF]. Following incubation on ice (45 min), lysates were centrifuged to precipitate cellular debris (17,000 g, 20 min, 4°C). Protein concentrations were determined by performing a Bradford assay; 6× Laemmli buffer and ~20 mM DTT was added and lysates were denatured at 56°C (10 min). Protein samples were separated by SDS-PAGE and subsequently transferred to PVDF membranes (GE Healthcare) for immunoblotting. Membranes were blocked in PBST (PBS plus 0.1% Tween-20) containing 5% non-fat dry milk (60 min). Primary antibodies were incubated in PBST plus 2% BSA (1–3 h, 20°C or 16–24 h, 4°C). Membranes were incubated in PBST plus 5% non-fat dry milk containing either horseradish peroxidase (HRP)-conjugated or Alexa Fluor 488-conjugated secondary antibodies (1 h, 20°C) to allow detection of proteins by either ECL or fluorescence (described below).

Densitometric analysis of immunoblots
Individual protein levels were quantified by densitometry from western blots using target-specific primary antibodies and secondary antibodies conjugated to Alexa Fluor 488. Images were acquired using the ChemiDoc system (Bio-Rad) and bands quantified with the ImageJ software package. Only signal intensities below the saturation threshold [as determined by the Image Lab 5.1 software (Bio-Rad)] were used for quantification. The area under the curve for the integrated pixel intensities of each band was measured for proteins of interest and internal tubulin (TUB) controls. Band intensities were normalised to TUB derived from the same sample.

Affinity purification
Cells were collected as above and resuspended in lysis buffer (LB; 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM NEM, 1× complete™ Protease Inhibitor Cocktail) plus 1× Triton X-100 (v/v) or 1× LMNG (v/v) on ice (45 min). Detergent-soluble supernatants were isolated by centrifugation (17,000 g, 4°C, 20 min) and pre-cleared with Sepharose CL-4B (100 µl 50,000 slurry), (1–16 h, 4°C). Resulting lysates (0.3–1 mg) were used for affinity purification with HA-conjugated agarose beads (Roche) for 2 h (4°C). The resin was collected by centrifugation (1 min, 1000 g, 4°C), washed (2× bead volume) once with high-salt buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 5 mM EDTA, 2 mM NEM) and twice with LB. Bead-bound proteins were resuspended in 2× Laemmli buffer plus 20 mM DTT, heated (10 min, 56°C) and separated by SDS-PAGE.

SILAC-MS/MS
SILAC labelling and cell surface biotinylation
The PMP procedure was adapted from Weekes et al. (Weekes et al., 2012). Cells were maintained for ≥6 passages in media supplemented with isotopically labelled ‘light’ (K0/R0), ‘medium’ (K4/R6) or ‘heavy’ (K8/R10) amino acids. Cells were grown in 15 cm tissue culture plates, washed twice with ice-cold PBS (pH 6.7) and labelled with 1 mM sodium metaperiodate, 10 mM aniline and 100 mM aminoxy-biotin plus PBS (pH 6.7) under constant agitation in the dark (90, 4°C). The reaction was quenched by addition of 1 mM glyceral (30 min). The reaction mixture was removed and cells were washed with PBS (pH 7.4) plus 5% FCS followed by PBS (pH 7.4) containing 1 mM CaCl2 and 0.5 mM MgCl2.

Sample preparation for MS/MS
Cells were collected in ice-cold PBS using a cell strainer, centrifuged at 1500 g for 5 min and resuspended on ice in 300 µl of Triton X-100 lysis buffer [50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 5 mM EDTA, 2 mM NEM, 1 mM PMSF, 1× Roche Protease Inhibitor Cocktail] for 45 min. Cell debris was removed by centrifugation (25 min, 17,000 g, 4°C). Cell lysate from K0/R0, K4/R6 or K8/R10 cells were equally mixed based on the Bradford assay results. 77.8 µl of 50% streptavidin/agarose slurry was added per mg of whole-cell lysate and incubated for 90 min at 4°C. Supernatant and bead-bound material were isolated by centrifugation and processed separately. Beads were washed using Micro Bio-Spin Chromatography Columns (Bio-Rad, Cat. No. #732-6204). All washing and modification steps were performed with 600 µl buffer and centrifugation steps were performed at 1000 g for 1 min at 4°C. Beads were consecutively washed twice with Triton X-100 lysis buffer and with PBS/0.5% (w/v) SDS. Disulfide bonds were reduced by incubation with PBS/0.5% (w/v) SDS and 100 mM DTT at room temperature for 20 min. Subsequently, beads were washed twice with UC buffer (100 mM Tris-HCl pH 8.5, 6 M urea) and treated with UC buffer with 50 mM iodoacetamide at room temperature for 20 min. The samples were successively washed twice with UC buffer, 5 M NaCl, 100 mM Na2CO3, PBS and H2O. Peptides were obtained after overnight incubation with trypsin at 37°C. The samples were acidified to 1% final concentration of trifluoroacetic acid (TFA). The peptides were collected in new tubes by centrifugation in SpinX spin column (Sigma Aldrich) at 1000 g for 1 min and were desalted using a C18 Sep-Pak cartridges (Waters, Milford, MA) according to the manufacturer’s instructions. Briefly, each cartridge was conditioned with solution B (0.1% TFA in 65% CH3CN) and equilibrated with solution A (0.1% TFA in 98% H2O). Tryptic peptides were loaded and washed with solution A prior elution with solution B. The samples were dried in a vacuum concentrator and reresuspended in 20 µl solution A.

Sample preparation of cytoplasmic fractions
The cytoplasmic fraction was obtained from the supernatant after affinity purification of biotinylated proteins. Proteins were enriched by chloroform/methanol precipitation and resuspended in 6 M urea, 0.1 M Tris-HCl pH 7.8. The solution was diluted 1:6 with H2O and proteins were digested using a 1:50 ratio (trypsin:protein). Tryptic peptides were purified as described above.

Peptide analysis by UHPLC-coupled mass spectrometry
Peptide analysis was performed by ultra-high performance liquid chromatography ( Dionex Ultimate 3000 UHPLC, Thermo Fisher Scientific) coupled with tandem mass spectrometry (Q Exactive, Thermo Fisher Scientific) on a PepMap RSLC column (C18, 2 µm, 100 A, 75 µm x 50 cm). Peptides were eluted on a gradient starting with 2% buffer B (0.1% TFA and 5% DMSO in CH3CN) at 3 min to reach 35% by 123 min at a flow rate of 250 nL/min. Data acquisition was performed at a resolution of 70,000 full-width half maximum at mass/charge of 400 with lockmass enabled (445.120025 m/z), top 15 precursor ion selection and dynamic exclusion of 27 s, and fragmentation was performed in higher-energy C-trap dissociation (HCD) mode with a normalised collision energy of 28.

Data processing
Raw MS files were processed by using MaxQuant v. 1.5.1.2 (Cox and Mann, 2008). The data was searched using the reviewed human database downloaded from Uniprot in April 2015. The search was performed using the following settings: two missed cleavages were allowed and the peptide ion tolerance was adjusted to 0.05 Da. Variable modifications included N-terminal acetylation, methionine oxidation and deamidation (NQ). Carbamidomethyl cysteine was defined as a fixed modification. The false discovery rate for peptides and proteins was set at 0.01 against a reversed decoy database. For protein quantification, unique and razor peptides were used with a minimum ratio count of 1. Re-quantify was enabled. Data generated by MaxQuant was further analysed using Perseus v. 1.5.0.31 (Tyanova et al., 2016). Reverse peptides, proteins only identified by site and potential contaminants were removed from the dataset. Normalised ratios for H-L and M-L were log2 transformed. Intensities were log10 transformed. Significance B values were calculated with Perseus. Proteins with a decrease in abundance of at least 30% were classified as downregulated. Proteins commonly downregulated by at least 30% in multiple cell lines were identified by using Perseus. Venn diagrams were generated with Adobe Illustrator CS6.
35S-methionine/cysteine pulse-chase
Pulse-chase assays of U2OS Flp-In™ T-Rex™ cells (WT/Δ6) were carried out according to a previously described protocol (Christianson et al., 2008). Briefly, cells were starved in methionine/cysteine-free DMEM (Lonza) plus 10% dialyzed FCS (10 min) and metabolically labelled by adding 35S-methionine/cysteine [Met/Cys, EXPRESS35S35S Protein Labelling Mix (PerkinElmer); 150 µCi/10 plate] for 10 min. The medium was removed, then cells were rinsed with PBS and incubated in DMEM plus 10% FCS and methionine/cysteine (50 mM each) for the indicated times. Cells were collected and resuspended in L-Pplus 1% Triton X-100 and post-nuclear fractions pre-cleared overnight using unconjugated Sepharose beads. SQS was immunoprecipitated from the detergent-soluble fraction using 70 µl of 50% protein G resin (Roche) slurry and 2 µl of anti-SQS antibody (ab195046, see above). Immunoprecipitated material was resuspended in 2 × Laemmli buffer plus 20 mM DTT and separated by SDS-PAGE, and the radiolabelled proteins visualised and quantified using a phosphoimager and the QuantityOne and Image Lab software packages (Bio-Rad). Relative protein levels were plotted as a function of time and protein half-lives \( t_{1/2} = \frac{\ln(2)}{\tau} \) were calculated using non-linear regression (exponential one-phase decay).

Velocity sedimentation gradients
Whole-cell lysates were prepared in LB plus 1% (v/v) LMNG, and 0.5–1 mg post-nuclear detergent-soluble lysate were applied to continuous sucrose gradients (5–15%) generated using a GradientMaster 108 R (Biocomp; parameters: 5–15% sucrose, short, S1/1; \( t=2:50\) min; angle=81.5, speed=15 rpm). Sucrose was dissolved in a physiological salt solution (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 5 mM EDTA, 1 mM PMSF) plus 1% LMNG [36,000 rpm (OptimaTM L-100 XP, SX41 rotor; Beckman Coulter, Brea, CA), 16 h, 4°C]. A total of 13 fractions were collected and proteins concentrated by incubation with 0.61 M trichloracetic acid for 1% LMNG [36,000 rpm (OptimaTM L-100 XP, SX41 rotor; Beckman Coulter, Brea, CA), 16 h, 4°C]. The original short read data used for RNA-seq analysis can be found in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra, accession SRP149865). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcarra et al., 2018) partner DB-PRIDE (http://pride.expasy.org/) with the dataset identifier PXD006603.

Statistical analysis
Statistical significance was calculated using Student’s t-test (Holm-Sidak method for multiple comparisons) and defined as \( P<0.05 \).

Bioinformatic analysis
Primary amino acid sequences for human EMCI-10 and human SQS were obtained from UniProt (http://www.uniprot.org/), with common motifs annotated using Pfam (http://pfam.xfam.org/) (Finn et al., 2016), TMDs predicted by TOPCONS (http://topcons.net/) (Tsirigos et al., 2015) and N-linked glycosylation sites predicted by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Acknowledgements
We would like to thank the following individuals. For technical assistance, Skirmantas Kriaucionis (RNA-Seq analysis), Marketa Tomkova, Pijus Brazauskas (genomic sequencing) and Mark Shipman (microscopy), and for careful reading and commenting on the manuscript by Mary Muers, Liz Miller, Peter Espenshade, Mads Gyrd-Hansen, James Ollzmann and members of the Christianson Lab. We thank Ta-Yuan Chang for helpful discussions.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: N.V., J.C.C.; Methodology: N.V., J.C.C.; Validation: N.V., J.C.C.; Formal analysis: N.V., M.T., S.M.L., J.C.C.; Investigation: N.V., M.T., S.M.L., D.K.N., J.C.C.; Resources: S.J., D.K.N., R.S.H., J.C.C.; Data curation: N.V., J.C.C.; Writing - original draft: N.V., J.C.C.; Writing - review & editing: N.V., J.C.C.; Supervision: D.K.N., R.S.H., B.M.K., J.C.C.; Project administration: J.C.C.; Funding acquisition: J.C.C.

Funding
J.C.C. is supported by a grant from the UK Medical Research Council (MR/L001209/1) and by the Ludwig Institute for Cancer Research. DKN is supported by a grant from the National Institutes of Health (NIH; R01-CA172667). R.S.H. is supported by the UK Medical Research Council (MC_UP_A022_1007). Work in the B.M.K. lab was funded by the John Fell Fund, University of Oxford (133/075) and the Wellcome Trust (097813/Z/11/Z), Deposited in PMC for immediate release.

Data availability
The original short read data used for RNA-seq analysis can be found in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra, accession SRP149865). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcarra et al., 2018) partner repository (https://www.ebi.ac.uk/pride/archive/) with the dataset identifier PXD006603.

Supplementary information
Supplementary information available online at http://jcs.biologists.org/lookup/doi/10.1242/jcs.223453.supplemental

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Figure S1. Contribution of individual subunits to EMC biogenesis.

(A) Lysates of U2OS Flp-In™ TRex™ cells solubilized in LMNG were separated by velocity sedimentation on a 5 – 15% sucrose gradient and collected as 13 fractions, revealing a single peak between 300 - 400 kDa (fractions 7-9, blue). Non-specific bands are denoted (*).

(B) Flp-In™ TRex™ 293 cells transfected with siRNAs targeting EMC1 – EMC10 or a non-targeting control (NTC) were collected after 72 h. WCL was separated by SDS-PAGE and resulting western blots probed with the indicated antibodies to EMC subunits.
(C) Comparison of EMC sedimentation as in (A) from LMNG lysates of WT and EMC2-depleted cells. The mature EMC (fractions 7-9, blue) and putative assembly intermediates (fractions 4-6, red) are indicated and reflected by changes to sedimentation of EMC1/5/6/10.

(D) Transcript levels of non-targeted EMC subunits determined by qRT-PCR from U2OS Flp-In™ TRex™ cells depleted of EMC6 relative to non-targeting control (NTC)-treated cells as described in Figure 1B. Means ± S.D. (n = 3) are shown and significance determined by Students t-test: *p ≤ 0.05, ***p ≤ 0.001.

(E – G) Knockout of EMC5 and EMC6 by CRISPR/Cas9.

Western blots of WCL from selected U2OS Flp-In™ TRex™ clones knocked out for EMC5 (E, Δ5) or EMC6 (F, Δ6) by CRISPR/Cas9 using different sgRNAs and probed with antibodies to the indicated EMC subunits and tubulin (TUB). Colored circles indicate clone identity throughout the study. (G) Genomic sequencing of individual Δ5, Δ6 and ΔSQS clones used in this study.
Figure S2. Cholesterol depletion induces cell death in EMC-deficient cells.

(A) WT and ΔEMC6 (Δ6) cells reconstituted with an empty vector control (WT + EV, Δ6 + EV) or EMC6 (Δ6 + 6) were treated with MBCD (4, 5 mM, 16 h) and stained with crystal violet. Scale bar = 100 µm.

(B) Quantification of propidium iodide (PI) incorporation into untreated and MBCD-exposed cells (5 mM, 16 h) as measured by flow cytometry. Means ± S.D. of PI positive cells are shown (n = 5) and significance determined by Students t-test: ****p ≤ 0.0001.

(C) Cells depleted of cholesterol by MBCD (4 mM, 20 min) were switched to FCS (5%) or LPDS (5%) containing growth media (6 d) and visualized by staining with crystal violet. Scale bar = 100 µm.
Figure S3. RNA-Seq analysis of genes involved in cholesterol biosynthesis.

Heat map of log2 transformed, normalised transcript levels of genes coding for proteins involved in cholesterol biosynthesis. U2OS WT, ∆EMC5 (∆5, clones #5-4 and #6-14) and ∆EMC6 (∆6, clones #1-1 and #3-9) cells were collected at steady-state. Data from three independent experiments (n = 3) are shown. HMGCS1/2, 3-Hydroxy-3-Methylglutaryl-CoA Synthase 1/2; HMGCR, 3-Hydroxy-3-Methylglutaryl-CoA Reductase; MVK, Mevalonate Kinase; PMVK, Phosphomevalonate Kinase; MVD, Mevalonate Diphosphate Decarboxylase; IDI1/2, Isopentenyl-Diphosphate Delta Isomerase 1/2; FDPS, Farnesyl Diphosphate Synthase; GGPS1, Geranylgeranyl Diphosphate Synthase 1; FDFT1, Farnesyl-Diphosphate Farnesyltransferase 1; SQLE, Squalene Epoxidase; LSS, Lanosterol Synthase; CYP51A1, Cytochrome P450 Family 51 Subfamily A Member 1; LBR, Lamin B Receptor; MSMO1, Methylsterol Monooxygenase 1; NSDHL, NAD(P) Dependent Steroid Dehydrogenase-Like; HSD17B7, Hydroxysteroid 17-Beta Dehydrogenase 7; DHCR7, 7-Dehydrocholesterol Reductase; DHCR24, 24-Dehydrocholesterol Reductase; ACAT2, Acetyl-CoA Acetyltransferase 2.
Figure S4. Relative abundance of major lipid species in EMC-deficient cells.

Lipid abundance in WT (black), ΔEMC5 (Δ5 #5-4) (dark grey) and ΔEMC6 (Δ6 #3-9) cells (light grey) was measured by LC-MS/MS. Means ± S.E.M. (n = 5) and significance (Students t-test: *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.001, ****p ≤ 0.0001) are shown for: (A) neutral lipids, (B) neutral ether lipids, (C) fatty acids, (D) phospholipids, (E) sphingolipids, (F) phospholipid ethers, (G) lysophospholipid ethers, (H) acyl carnitine, (I) lysophospholipids, and (J) N-acyl ethanolamines.
(K - L) Reduced SOAT1 expression in EMC-deficient cell lines.

Western blots probed for endogenous SOAT1, EMC5 and EMC6 from EMC5 (Δ5) and EMC6 knockout (Δ6) clones generated from U2OS Flp-In™ TRex™ (K) or A673 cells (L).

(M) Relative SOAT1 mRNA levels in WT, EMC5/6 knockout cell lines reconstituted with an empty vector control (Δ5 + EV, Δ6 + EV) or EMC5/6 (Δ5 + 5, Δ6 + 6). Mean ± S.D. (n = 3) and significance are shown, Students t-test: *p ≤ 0.05, **p ≤ 0.01.

(N) Expression of GFP-P2A-RFP-3xFLAG-SOAT1 reporter in U2OS Flp-In™ TRex + EV cells. Cells were treated as described in Figure 4F and whole-cell lysates separated by SDS-PAGE.
Figure S5. SQS biogenesis is EMC-dependent.

(A - C) Changes to intracellular protein levels accompanying EMC loss.

(A) Workflow for triple-label SILAC-MS/MS. Two individual U2OS Flip-In™ TRex™ cell lines deficient in either EMC5 (∆5 #5-4, ∆5 #6-14) or EMC6 (∆6 #1-1, ∆6 #3-9) were labeled with medium (R6/K4, M) or heavy (R10/K8, H) amino acids. Parental wild-type (WT) cells remained unlabeled (R0/K0, L). Intracellular fractions were enriched following affinity depletion of the cell surface sialoglycoproteome by aminooxy-biotin modification and streptavidin agarose. Signature peptides were identified by MS/MS and their relative abundance was determined by calculating M/L and H/L ratios. Scatter plots of all identified proteins in EMC5 knockout cells (∆5 #5-4, ∆5 #6-14) (B) and EMC6 knockout cells (∆6 #1-1, ∆6 #3-9) (C).
Normalized H/L and M/L ratios were converted to a log2 scale and plotted against one another. Dashed lines demarcate the log2 = 0.5 threshold (corresponding to ≥30% change). Identified proteins are represented as single points, proteins decreased by ≥30% are annotated with gene names, and SQS is highlighted in red. Proteins not consistently reduced by ≥30% in all cell lines are shown in brackets.

(D – F) Cholesterol auxotrophy results from loss of SQS activity.

(D) Acute depletion of cholesterol from WT or ΔSQS cells by treating with MBCD (4, 5 mM, 16 h) and staining with crystal violet. Where indicated, WT cells were treated with zaragozic acid (ZA, 100 µM, 16 h) along with MBCD. Scale bar = 100 µm.

(E) Growth of ΔSQS cells in media containing 5% FCS or 5% LPDS ± Chol:MBCD (0, 0.5, 4 µM, 96 h) followed by staining with crystal violet. Scale bar = 100 µm.

(F) Western blots of WCL from WT, Δ6 ± EMC6 cells induced with DOX (1 ng/ml, 24 h), probed for unprenylated Rap1a. Where indicated, cells were treated with GGTL 298 (25 µM, 4.5 h) or zaragozic acid A (ZA, 10 µM, 4 h). SQS and EMC6 are shown for comparison. TUB serves as a loading control.

(G - K) SQS expression is compromised in EMC-deficient cells.

(G) Immunofluorescence images of endogenous SQS in WT and EMC-deficient cells collected by confocal microscopy. Scale bar = 10 µm.

(H) Western blot analysis of A673 single-cell EMC5 (left) or EMC6 knockout clones (right) generated by CRISPR/Cas9 and different sgRNAs. EMC subunits and SQS are shown.

(I) Western blot analysis of Flp-In™ TRex™ 293 WT and single-cell clones for CRISPR/Cas9-mediated knockout of EMC6. EMC6 and SQS are shown.

(J) siRNA-mediated knockdown of EMC subunits (72 h) in U2OS Flp-In™ TRex™ cells with resulting western blots probed for SQS, EMC4 and tubulin (TUB).

(K) Quantification of SQS from (J) by densitometry using Alexa 488 quantification and normalized to NTC (dashed line) are shown. Means ± S.D. (n = 3) are depicted and significance was determined by Students t-test: **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.
Figure S6. Loss of SQS and SOAT1 expression are not interdependent.

(A) SOAT1 western blot of lysates from WT, Δ6+EMC6 or EV and ΔSQS cells. Reconstitution was induced with DOX (10 ng/ml, 24 h). Hsp70 serves as a loading control.

(B) WT cells treated with zaragozic acid (ZA, 1 µM) for the indicated times with western blots of WCLs probed with antibodies to SOAT1, Rap1a (unprenylated) and SM.

(C) WT and ΔSQS cells were supplemented with Chol:MBCD (25, 37.5, 50 µM, 20 h) and stained with crystal violet. Scale bar = 100 µm.

(D) Quantification of three independent experiments performed as in (C). Means ± S.D. are shown.

(E) WT cells treated with avasimibe (AVA, 1 µM) for the indicated times with western blots of WCLs probed with antibodies to SQS and SM.

(F) WT cells grown in 5% FCS or 5% LPDS were treated with AVA (5 µM, 96 h) and visualized by crystal violet staining. Scale bar = 100 µm.

(G) Relative SQS transcript levels in Δ5 and Δ6 cells reconstituted with the missing subunit (Δ5 + 5, Δ6 + 6) or an empty vector control (Δ5 + EV, Δ6 + EV). Expression of EMC5/6 was induced with DOX (1 ng/ml, 24 h). mRNA levels were determined by qPCR and normalized to WT control (dashed line). Means and S.D. are shown (n = 3).

(H - I) Insertion of the SQS tail anchor is EMC-dependent.
(H) \(^{35}\text{S}\)-Met/Cys radiolabeled cells (WT, Δ6) were chased (2, 4 h) ± MG132 (10 μg/ml, 4 h) or NMS-873 (10 μM, 4 h). A representative IP of endogenous SQS is shown. Unspecific bands (*) are indicated.

(I) Pulse-chase assay performed as in Figure 6D with cells expressing either HA:SQS\textsubscript{opsin} or an HA:SQS\textsubscript{opsin} whose TMD was replaced with the TMD of Sec61β (HA:SQS-Sec61βTMD\textsubscript{opsin}).
Figure S7. SOAT1 topology and in silico prediction of TMD hydrophobicity.

(A) Schematic representation of SOAT1 topology as reported previously (Guo et al., 2007).

(B) In silico prediction of SOAT1 TMD hydrophobicity using the Kyte & Doolittle (K-D) method and calculated for the TMD only (light green) or using a sliding window (WINDOW = 5 aa, dark green) by ProtScale (https://web.expasy.org/protscale/). The calculated K-D value of the SQS TMD is indicated (dashed line). SOAT1 contains several TMDs whose K-D values are lower (TM4, 5) or comparable (TM2, 7, 9) to that of the SQS TMD.
### Table S1. gRNA sequences

| gRNA               | target gene | sequence                          | used to generate                  |
|--------------------|-------------|-----------------------------------|-----------------------------------|
| NV EMC2 2 (854)    | EMC2        | CACAGAGTCAAGCGATTAACAGG           | U2OS Flp-In™ TRex™: Δ2 #2-6       |
| JC hMMGT 1 (410)   | EMC5        | GCATCATGGCGCCGTCGCTGTGG           | U2OS Flp-In™ TRex™: Δ5 #5-9       |
|                    |             |                                   | A673: Δ5 #1-4                      |
|                    |             |                                   |                                   |
| JC hMMGT 2 (411)   | EMC5        | CGCAGCGGAAAAGGCGGCGTGCGG         | U2OS Flp-In™ TRex™: Δ5 #6-14       |
|                    |             |                                   | A673: Δ5 #2-4                      |
| JC hMMGT 3 (412)   | EMC5        | CACTGCCAATAGATGTAAGTTGG          | A673: Δ5 #3-17                     |
| JC hEMC6 1 (401B)  | EMC6        | GCCGCCTCCTGATGAACGGCGG           |                                   |
|                    |             |                                   |                                   |
| JC hEMC6 2 (402)   | EMC6        | CCGAGGTCGGGCAATAATCCAGG          | Flp-In™ TRex™ 293: Δ6 #2-23       |
| JC hEMC6 3 (403)   | EMC6        | GAACGGCGGCCCTTCCCGCTTGG         |                                   |
|                    |             |                                   |                                   |
| NV FDFT1 1 (855)   | SQS         | GCCGACATTTGCGGGAGAATGGG          | U2OS Flp-In™ TRex™: ΔSQS #3-15     |
| Primer  | Sequence (5'-3')                  |
|---------|-----------------------------------|
| Actin forward | GAGGCACTCTTTCCAGCCTT             |
| Actin reverse  | AAGGTAGTTCGGTGGAGTCC            |
| EMC1 forward  | GGAATGTCTTGCACAGCTA             |
| EMC1 reverse  | AGATCCTTTTCGAACCCAGTA           |
| EMC2 forward  | TCAAACACACTGCTGGCAA             |
| EMC2 reverse  | AAGTTGATGGGAGTCTTCTT            |
| EMC3 forward  | GCCAAAGTAATGCCAGTAC             |
| EMC3 reverse  | TCGAAGTGAGGTCTTGGGC             |
| EMC4 forward  | TTCCGACACTTCAAGTGTAG            |
| EMC4 reverse  | GTCTCCCACACTGAACGCC             |
| EMC5 forward  | CTGCACATGGATCGGGAAATG           |
| EMC5 reverse  | GCGGAAAGTACTCGAGACCA            |
| EMC6 forward  | GCCGCCGCTCTGGATTATT             |
| EMC6 reverse  | GGAGGCGAGCTCAGGGAGAGA           |
| EMC7 forward  | CTACATGAGACGGGAAATG             |
| EMC7 reverse  | GCTGTAGATTTGCCAGATG             |
| EMC8 forward  | CTGCAGCTCTGATGCTAGAC            |
| EMC8 reverse  | CCATCTGTTTCTCTAGGTGCT           |
| EMC9 forward  | TCAACCAGGTGATGTGTGG             |
| EMC9 reverse  | GACCTTAGTTCAAGGACGGAC           |
| EMC10 forward | TAGATCGATGACAGTGCAA             |
| EMC10 reverse | GCAGGGAAAAAGGAGAGGAGGAC         |
| HMGCR forward | GGACCCTTTGGCTTAGAGAA           |
| HMGCR reverse | CCACCAAGACCTATTGGCTCTG         |
| SM forward    | CTATGCAGAGCCCAATGGCAAGT         |
| SM reverse    | ACAACAGTCAGTGAGCATGGAGT         |
| SQS forward   | GGCAAGCGGAAGGTGGCAAGT           |
| SQS reverse   | CTGGTCTGATTGGATATCTTGGTAGCAA   |
| SOAT1 forward | GATGAAGGAGGAGGCTGGTC            |
| SOAT1 reverse | GGAAGCTGGGTGGCAGTTGTAT          |
| Majority Protein IDs | Protein IDs | Also detectable in EMC6 KOs vs WT? | Ratio M\_EMC5KO\#6-14 / L\_WT normalized | Ratio H\_EMC6KO\#5-4 / L\_WT normalized |
|---------------------|-------------|-----------------------------------|------------------------------------------|------------------------------------------|
| BAP31               | sp\[P51572\]BAP31\_HUMAN | Y                                 | 0.45265                                  | 0.30205                                  |
| BGAL                | sp\[P16278\]BGAL\_HUMAN   | N                                 | 0.6871                                   | 0.60476                                  |
| CERU                | sp\[P00450\]CERU\_HUMAN   | Y                                 | 0.5315                                   | 0.2684                                   |
| DHL                 | sp\[Q00821\]DHL\_HUMAN    | Y                                 | 0.67075                                  | 0.64772                                  |
| DHX9                | sp\[Q00821\]DHX9\_HUMAN   | Y                                 | 0.67481                                  | 0.57474                                  |
| EMC2                | sp\[Q00821\]EMC2\_HUMAN   | N                                 | 0.45852                                  | 0.12459                                  |
| GGH                 | sp\[Q00821\]GGH\_HUMAN    | Y                                 | 0.64391                                  | 0.6523                                   |
| GNS                 | sp\[P15586\]GNS\_HUMAN    | N                                 | 0.52858                                  | 0.31681                                  |
| HM13                | sp\[Q8TCT9\]HM13\_HUMAN   | Y                                 | 0.56249                                  | 0.69006                                  |
| HNRDL               | sp\[Q14979\]HNRDL\_HUMAN  | Y                                 | 0.539                                    | 0.68775                                  |
| IFG1                | sp\[Q00821\]IFG1\_HUMAN   | Y                                 | 0.35945                                  | 0.4535                                   |
| K1C18               | sp\[P00821\]K1C18\_HUMAN  | Y                                 | 0.67854                                  | 0.50497                                  |
| NSF                 | sp\[Q00821\]NSF\_HUMAN    | Y                                 | 0.5315                                   | 0.57474                                  |
| PTN1                | sp\[P18031\]PTN1\_HUMAN  | Y                                 | 0.40108                                  | 0.55274                                  |
| RISC                | sp\[Q00821\]RISC\_HUMAN   | N                                 | 0.54377                                  | 0.57032                                  |
| RL35                | sp\[P42766\]RL35\_HUMAN   | Y                                 | 0.38315                                  | 0.58226                                  |
| TTHY                | sp\[Q5U7I5\]TTHY\_PANTR   | N                                 | 0.2013                                   | 0.015316                                  |

| Majority Protein IDs | Protein IDs | Also detectable in EMC5 KOs vs WT? | Ratio M\_EMC6KO\#3-9 / L\_WT normalized | Ratio H\_EMC5KO\#1-1 / L\_WT normalized |
|---------------------|-------------|-----------------------------------|------------------------------------------|------------------------------------------|
| 4F2                 | sp\[P08195\]4F2\_HUMAN | Y                                 | 0.58931                                  | 0.5315                                   |
| AAAT                | sp\[Q15758\]AAAT\_HUMAN | Y                                 | 0.62953                                  | 0.38724                                  |
| APOC3               | sp\[P02656\]APOC3\_HUMAN| N                                 | 0.037958                                 | 0.034339                                  |
| Cата                | sp\[P00420\]Cата\_HUMAN | Y                                 | 0.64265                                  | 0.49207                                  |
| CD9                 | sp\[P42766\]CD9\_HUMAN  | Y                                 | 0.33267                                  | 0.63726                                  |
| CYB5                | sp\[P00167\]CYB5\_HUMAN | Y                                 | 0.46222                                  | 0.58704                                  |
| CYC                 | sp\[Q99999\]CYC\_HUMAN | Y                                 | 0.46059                                  | 0.58108                                  |
| DDX47               | sp\[Q9HOS4\]DXD47\_HUMAN| N                                 | 0.44401                                  | 0.57464                                  |
| EMC1                | sp\[Q00821\]EMC1\_HUMAN | N                                 | 0.51103                                  | 0.41148                                  |
| FADS1               | sp\[P02656\]FADS1\_HUMAN| N                                 | 0.037958                                 | 0.034339                                  |
| FADS2               | sp\[Q00821\]FADS2\_HUMAN| N                                 | 0.3055                                   | 0.65086                                  |
| FAM3C               | sp\[Q00821\]FAM3C\_HUMAN| N                                 | 0.13015                                  | 0.2693                                   |
| FKBP8               | sp\[Q15758\]FKBP8\_HUMAN| N                                 | 0.48616                                  | 0.37428                                  |
| H1X                 | sp\[P02656\]H1X\_HUMAN  | Y                                 | 0.23814                                  | 0.68005                                  |
| H4                  | sp\[Q15758\]H4\_HUMAN   | Y                                 | 0.15312                                  | 0.40361                                  |
| KAD2                | sp\[P42766\]KAD2\_HUMAN | Y                                 | 0.25973                                  | 0.69152                                  |
| LAMB3               | sp\[Q00821\]LAMB3\_HUMAN| N                                 | 0.59107                                  | 0.27541                                  |
| LMNB1               | sp\[Q00821\]LMNB1\_HUMAN| N                                 | 0.61728                                  | 0.70252                                  |
| LTOR1               | sp\[Q15274\]LTOR1\_HUMAN| N                                 | 0.66855                                  | 0.65367                                  |
| NADC                | sp\[Q15274\]NADC\_HUMAN | N                                 | 0.55962                                  | 0.56464                                  |
| P5CR1               | sp\[P02656\]P5CR1\_HUMAN| N                                 | 0.037958                                 | 0.034339                                  |
| PGRC2               | sp\[Q15274\]PGRC2\_HUMAN| Y                                 | 0.54622                                  | 0.63447                                  |
| ROA0                | sp\[Q99999\]ROA0\_HUMAN | Y                                 | 0.64327                                  | 0.6254                                   |
| ROAA                | sp\[Q15274\]ROAA\_HUMAN | Y                                 | 0.50912                                  | 0.6254                                   |
| RT09                | sp\[Q15274\]RT09\_HUMAN | Y                                 | 0.62019                                  | 0.6254                                   |
| RT23                | sp\[Q15274\]RT23\_HUMAN | Y                                 | 0.63568                                  | 0.68099                                  |
| SARNP               | sp\[P02656\]SARNP\_HUMAN| N                                 | 0.46258                                  | 0.693                                    |
| SHIP1               | sp\[Q15274\]SHIP1\_HUMAN| Y                                 | 0.037958                                 | 0.034339                                  |
| SUMF2               | sp\[Q15274\]SUMF2\_HUMAN| Y                                 | 0.49067                                  | 0.69058                                  |
| THIM                | sp\[P02656\]THIM\_HUMAN | Y                                 | 0.56182                                  | 0.70657                                  |
| TMM97               | sp\[Q5U7I5\]TMM97\_HUMAN| N                                 | 0.35083                                  | 0.46437                                  |