Palmitoylation is the Switch that Assigns Calnexin to Quality Control or ER Calcium Signaling

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Abstract

The palmitoylation of calnexin serves to enrich calnexin on the mitochondria-associated membrane (MAM). Given a lack of information on the significance of this finding, we have investigated how this endoplasmic reticulum (ER)-internal sorting signal affects the functions of calnexin. Our results demonstrate that palmitoylated calnexin interacts with sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) 2b and that this interaction determines ER calcium content and the regulation of ER-mitochondria calcium crosstalk. In contrast, non-palmitoylated calnexin interacts with the oxidoreductase ERp57 and performs its well-known function in quality control. Interestingly, our results also show that calnexin palmitoylation is an ER stress-dependent mechanism. Following a short term ER stress, calnexin quickly becomes less palmitoylated, which shifts its function from the regulation of calcium signaling towards chaperoning and quality control of known substrates. These changes also correlate with a preferential distribution of calnexin to the MAM under resting conditions or the rough ER and ER quality control compartment (ERQC) following ER stress. Our results have therefore identified the switch that assigns calnexin either to calcium signaling or to protein chaperoning.
Introduction

The main functions of the endoplasmic reticulum (ER) are the production of secretory and membrane proteins as well as lipids, and the storage of calcium. An integral requirement for these functions is the interaction of the ER with mitochondria, which occurs on the mitochondria associated membrane (MAM), a subdomain of the ER that makes close contacts between the ER and the mitochondria (Raturi and Simmen, 2012; Simmen et al., 2010). Here, the ER exchanges calcium with mitochondria via the ER calcium handling proteins inositol 1,4,5-triphosphate receptors (IP3R) and sarcoendoplasmic reticulum (SR) calcium transport ATPase (SERCA) (Rizzuto et al., 2009). Under resting conditions, calcium delivery from the ER to the mitochondrial matrix is needed to activate the mitochondrial enzyme pyruvate dehydrogenase (PDH), which drives the tricarboxylic acid (TCA) cycle (Cardenas et al., 2010). Given the critical role that mitochondrial metabolism plays for death and survival of the cell (Glancy and Balaban, 2012; Zhivotovsky and Orrenius, 2011), ER-mitochondria calcium exchange has to be regulated at the source. Various ER chaperones and oxidoreductases perform this function by dictating calcium channels and pumps to open or close dependent on ER redox and calcium homeostasis (Simmen et al., 2010). Examples are Ero1α that activates inositol 1,4,5 trisphosphate receptors (IP3Rs) (Li et al., 2009) and ERp44 that inhibits IP3Rs (Higo et al., 2005).

Calnexin is another key ER chaperone that binds to nascent glycoproteins (Rutkevich and Williams, 2011). This binding slows down client protein folding and prevents their aggregation via the retention of folding intermediates in the ER. Upon release from calnexin, glycoproteins may follow one of three pathways: they may fold rapidly and correctly, in which case they will be exported to the Golgi apparatus, or if they are not yet correctly folded they may be re-glycosylated and returned to the calnexin cycle (Ruddock and Molinari, 2006). Proteins that have been trapped in the calnexin cycle for a prolonged period are eventually subject to extensive mannose trimming by mannosidase I and ER-associated degradation enhancing α-mannosidase-like protein (EDEM), followed by retrotranslocation from the ER to the cytosol and degradation by the proteasome, a process known as ER-associated degradation (ERAD) (Lederkremer, 2009). All along, calnexin works in partnership with ERp57, an oxidoreductase that is
member of the protein disulfide isomerase (PDI) family (Coe and Michalak, 2010; Oliver et al., 1997; Zapun et al., 1998). Studies have shown that a certain subset of disulfide-bonded, heavily glycosylated proteins such as the low density lipoprotein receptor (LDLR) depend specifically on this partnership for efficient folding and subsequent trafficking through the secretory system (Jessop et al., 2007; Rutkevich and Williams, 2011).

Surprisingly, our laboratory and others have determined that calnexin is not only found on the rough ER, but is often enriched on the MAM (Hayashi and Su, 2007; Myhill et al., 2008; Wieckowski et al., 2009). This finding further reinforces the notion that calnexin is a central player for the regulation of ER calcium signaling, and is also consistent with the fact that calnexin interacts with the SERCA calcium pump (Roderick et al., 2000). Interestingly, calnexin’s role in calcium signaling could depend on ER homeostasis, since ER stress results in the relocation of calnexin towards the ER quality control compartment (ERQC) and its association with slowly folding proteins (Frenkel et al., 2004).

Therefore, we have examined whether the targeting of calnexin to different ER subdomains correlates with an ER stress-dependent role in ER calcium signaling towards mitochondria. We further examined whether such a role correlated with the extent of binding of calnexin to its known partners ERp57 and SERCA2b. Calnexin targeting to the MAM depends on a juxtamembrane palmitoylation motif (Lynes et al., 2012) that is implicated in mediating the interaction of calnexin with the translocon (Lakkaraju et al., 2012) and with lipid rafts on the plasma membrane as well (Ferrera et al., 2008). In addition to calnexin, palmitoylation is known to modify several ER proteins, including heme oxygenase-1 and ORAI1, but the consequences of this modification are currently unknown (Dowal et al., 2011; Kang et al., 2008). We had shown earlier that the interference with palmitoylation relocates heme oxygenase-1 (Lynes et al., 2012), but ER-associated palmitoylation might have multiple, diverse effects (Lakkaraju et al., 2012). To investigate the functional significance of palmitoylation-dependent targeting of calnexin, we characterized the functional consequences of calnexin’s palmitoylation state for the cell in terms of cellular calcium signaling and quality control of the known calnexin substrates LDLR and asialoglycoprotein receptor (ASGPR).
Results

ER stress affects calnexin’s palmitoylation and localization within the ER

To test whether ER stress influences calnexin’s function in ER calcium signaling and chaperoning, we first examined calnexin’s localization following ER stress by biochemical fractionation. We decided to separate ER membranes into light and heavy membranes. Using this technique, we detected a shift of calnexin to light membranes that started at 1h tunicamycin treatment and increased up to 4h (Figure 1A). No such shift was observed for PDI or BAP31 (data not shown), two other ER proteins. To more precisely determine the origin and destination of calnexin during short term ER stress, we used a biochemical fractionation technique that we had developed to separate MAM markers from other ER markers (Myhill et al., 2008). HeLa cells were treated with two different ER stressors, thapsigargin and tunicamycin, for 4 hours, then homogenized and fractionated using Optiprep density gradient medium (Figure 1B, Supplemental Figures 1 and 2A). The general ER marker PDI and MAM-localized long-chain acyl-CoA synthetase 4 (FACL4) were used to control for the sedimentation of the MAM compartment. Under control conditions, close to 40% of calnexin co-fractionates with the MAM marker FACL4 and with the mitochondria at the bottom of the Optiprep gradient, as previously shown by our lab and others. However, after treatment with ER stressors, the association of calnexin, but not of PDI or FACL4 with MAM fractions was diminished to less than 20%. The relocation of calnexin did not lead to increased targeting to the plasma membrane (data not shown). Immunofluorescence microscopy similarly suggested reduced apposition between calnexin and mitochondria (Figure 1C). After 4 hours of treatment with tunicamycin a decrease in the overlap of the calnexin and mitotracker signals was observed (yellow signal, inset box) in HeLa cells labeled with antibodies against calnexin and PDI, as well as mitotracker. This result is consistent with a decrease in calnexin’s MAM localization. At first glance, the relocation of calnexin away from mitochondria during ER stress appears surprising, given earlier reports by us and others that this condition increases visible contacts between ER and mitochondria (Bravo et al., 2011; Csordas et al., 2006). At the same time, however, this movement of
calnexin away from the MAM suggests a specific function that is not due to known structural changes of the ER.

Next, we aimed to identify the mechanism that could lead to this relocation of calnexin. Since the phosphorylation state of calnexin is important for its localization within the ER and its binding to PACS-2 (Myhill et al., 2008), we tested the status of phosphorylation for each known site during an ER stress time course. Our results ruled out that a change in phosphorylation is behind the relocation of calnexin, since only long-term, but not short-term stress led to a reduction of calnexin phosphorylation on serine 565 that controls PACS-2 binding and serine 584 (Figure 2A) that controls the interaction of calnexin with ribosomes and SERCA2b (Chevet et al., 1999; Roderick et al., 2000). Another explanation for the change in calnexin’s localization during short-term ER stress could be that calnexin loses its palmitoylation, known to be responsible for its MAM targeting (Lynes et al., 2012). Thus, we used a click chemistry approach to compare the calnexin palmitoylation signal from untreated cells to that of cells treated with tunicamycin or DTT for 4h to induce a short term ER stress (Figure 2B, Supplemental Figure 2B). We observed that both ER stress situations indeed led to calnexin depalmitoylation. Therefore, contrary to long-term ER stress (Delom et al., 2007), short-term ER stress results in calnexin depalmitoylation, but not dephosphorylation, together with a loss of MAM enrichment.

Short-term ER stress affects calnexin’s interactions with SERCA2b

Next, we aimed to test if the relocation of calnexin within the ER that occurs in parallel with its depalmitoylation also affects its key interactors, because such an observation could give clues as to what palmitoylation of calnexin is necessary for. Since calnexin interacts with SERCA2b (Roderick et al., 2000), we decided to first test whether treatment with ER stressors also affects SERCA2b. Thus, we examined the distribution of SERCA2b on our Optiprep gradient as well as on heavy versus light membranes. While calnexin moved away from the MAM and heavy membranes of the ER, we could, however, not detect any movement of SERCA2b under these conditions (Figure 3A). This suggested that calnexin and SERCA2b undergo different fates during ER stress.
To test this hypothesis, we immunoprecipitated calnexin from control HeLa cells and HeLa cells treated with either DTT or tunicamycin for 4h. Under these ER stress conditions, we found that the interaction of calnexin with SERCA2b decreased significantly (Figure 3B). The ablation of the two cysteines of calnexin that are subject to palmitoylation (cysteines 503 and 504 in dog calnexin, CCAA) resulted in a similar reduction of the SERCA2b-calnexin interaction (Figure 3C). Calnexin phosphorylation on serine 584 had previously been implicated in the regulation of this interaction with SERCA2b (Roderick et al., 2000). However, we could not detect reduction of phosphorylation on this residue within the time frame that led to reduction of the SERCA2b-calnexin interaction. Consistent with these findings, we could also not detect reduced phosphorylation of calnexin in a mutant, where we had replaced two membrane-proximal cysteines with alanines (CCAA), resulting in non-palmitoylatable calnexin (Lynes et al., 2012) (Supplemental Figure 2C). Together, our results demonstrate that short term ER stress results in the depalmitoylation of calnexin, but not its dephosphorylation. Calnexin depalmitoylation subsequently results in reduced association of calnexin with SERCA2b.

**Calnexin palmitoylation allows for controlled ER-mitochondria calcium transfer**

Our results so far suggested that palmitoylated calnexin might regulate ER calcium signaling. If this were correct we would expect palmitoylated calnexin to regulate the flux of calcium between the ER and mitochondria via its role on SERCA (Roderick et al., 2000). Thus, we performed an extensive series of calcium assays using calnexin knockout mouse embryonic fibroblasts (MEFs) (Kraus et al., 2010) that we transfected with wild type and non-palmitoylatable calnexin. First, we inhibited SERCA calcium pumps with thapsigargin and measured the increase of cytosolic calcium with Fluo8. We were unable to detect any differences in the accumulation of cytosolic calcium with this assay, regardless of whether palmitoylated or non-palmitoylated calnexin was present or not (Figure 4A). No change in the accumulation of cytosolic calcium was observed using FURA-2 either (Supplemental Figure 3A). These results were consistent with earlier reports (Zuppini et al., 2002). However, we decided to measure ER calcium at the source using ER-targeted aequorin (Alvarez and Montero, 2002). Surprisingly, our
measurements showed about 80% more calcium in MEFs transfected with wild type calnexin, but not with calnexin that cannot be palmitoylated (Figure 4B). Since these cells did not show altered accumulation of cytosolic calcium from ER stores, our findings suggested that mitochondrial calcium uptake could be different as well in cells expressing calnexin compared to calnexin knockout MEFs (Arnaudeau et al., 2001; de Brito and Scorrano, 2008). Therefore, we tested whether mitochondria show differences in calcium uptake following the inhibition of ER calcium pumps. Indeed, we found that mitochondria in wild type MEFs take up significantly less calcium than knockout MEFs (Supplemental Figure 3B). Moreover, our results with the mitochondrial calcium indicator dye Rhod2 in the presence of thapsigargin show that the presence of wild type calnexin dampened mitochondrial calcium uptake following thapsigargin administration, when compared to calnexin knockout MEFs (Figure 4C). Importantly, this effect was not seen using non-palmitoylated calnexin. Since our results indicate that the presence of palmitoylated calnexin reduced the ability of mitochondria to import calcium from the ER, we next tested whether this was also true under the condition of ER stress, when cells normally increase mitochondrial calcium import from ER sources (Bravo et al., 2011; Csordas et al., 2006). Our results show that calnexin knockout MEFs were unable to boost the import of IP3R-released calcium into mitochondria after a 4h tunicamycin stress. However, the transfection of wild type calnexin, but not of non-palmitoylatable calnexin rescued this deficiency (Figure 4D), thus making calnexin knockout MEFs stress-responsive again. Together, our results suggested that calnexin must be palmitoylatable to control the activity of SERCA and thus the rate of calcium import into mitochondria.

Since efficient calcium transfer from the ER to mitochondria is a prerequisite for the mitochondrial membrane potential (Cardenas et al., 2010), we next tested the hypothesis that calnexin knockout cells could exhibit an abnormally high proton gradient across the mitochondrial membranes due to their high constitutive calcium transfer from the ER to mitochondria. By labeling calnexin knockout cells as well as their counterparts transfected with wild type calnexin and non-palmitoylatable calnexin with TMRM, we were indeed able to confirm this hypothesis by demonstrating that only wild type calnexin transfection resulted in a specific reduction of the mitochondrial membrane...
potential (Supplemental Figure 3C). This was also the case in the presence of KCl that equilibrates plasma membrane uptake of TMRM (data not shown). Throughout our experiments, we verified that expression of the two calnexin constructs was even (Figure 4E) and that our transfections did not result in the induction of the unfolded protein response (Supplemental Figure 3D). Therefore, ER-mitochondria calcium transfer is more efficient in the absence of calnexin, but the boosted calcium transfer observed under ER stress requires palmitoylated calnexin that increases the ER calcium content.

**Calnexin’s palmitoylation state regulates its interaction with ERp57**

In addition to its interaction with SERCA2b, calnexin interacts with the oxidoreductase ERp57 in the ER to mediate protein folding. This role of calnexin is particularly important for slow-folding substrates (Frenkel et al., 2004). Thus, we decided to also test whether calnexin’s interaction with ERp57 is similarly affected by ER stress conditions. First, we examined the distribution of calnexin and ERp57 by Optiprep gradient and fractionation of cellular membranes into heavy and light membranes. Contrary to SERCA2b (Figure 3A), the distribution of ERp57 closely matched the one by calnexin and both turned out to move from heavy to light membranes following an ER stress (Figure 5A), suggesting that these two proteins might share functions under ER stress. Indeed, we observed that when cells are treated with either DTT or tunicamycin for 4h, calnexin’s interactions with ERp57 increased (Figure 5B). ER stress could lead to the misfolding of calnexin itself, which could then trigger its association with other chaperones. To test this possibility, we expressed FLAG-tagged calnexin and mutant calnexin that cannot be palmitoylated. As shown in Figure 5C, the calnexin CCAA mutant that cannot be palmitoylated indeed interacted with ERp57 almost twice as well. Likewise, the binding of calnexin to ERp57 was about doubled in the presence of 2-bromopalmitate, an inhibitor of palmitoylation (Supplemental Figure 4A).

Our results suggested that the change of interaction between calnexin and ERp57 dependent on palmitoylation was of a functional nature. We therefore sought to identify a folding substrate that is particularly dependent on the calnexin/ERp57 folding pathway. For this purpose, we first chose the low density lipoprotein receptor (LDLR) that is known to form mixed disulfides with ERp57 and depend on calnexin folding (Jessop et
al., 2007). To determine whether LDLR maturation proceeds differently whether calnexin is palmitoylated or not, we tested this in the calnexin knockout MEFs that we transfected with wild type calnexin or the calnexin CCAA mutant. Here, we first determined that the expression level of the LDLR in lysates was unaltered (Figure 5D). Despite this however, the amount of LDLR on the cell surface increased with wild type calnexin, but decreased with CCAA calnexin (Figure 5D), suggesting non-palmitoylated calnexin is indeed more stringent in ER quality control retention than the at least partially palmitoylated wild type calnexin.

Efficient intracellular retention of LDLR by non-palmitoylated calnexin could indicate that this form of calnexin interacts with unfolded domains of proteins that await exit from the ER or alternatively ER-associated degradation (ERAD). Thus, we analyzed the interaction of calnexin with an ERAD substrate, the uncleaved precursor of asialoglycoprotein receptor (ASGPR) H2a, which is normally completely ER-retained (Kamhi-Nesher et al., 2001; Shenkman et al., 1997). CNX CCAA interacted much more robustly than CNX wild type with H2a (Figure 6A). CNX CCAA also caused a slight increase of total H2a, likely due to decreased targeting of this protein for ERAD. Pulse/chase analysis showed that dissociation of H2a from calnexin followed a different pattern when we transfected nonpalmitoylatable calnexin compared to wild type (Figure 6B). Whereas wild type calnexin dissociated efficiently with time from the ERAD substrate H2a, non-palmitoylatable CCAA calnexin reassociated and remained complexed to H2a for extended periods of time. For the pulse samples two bands can be seen for H2a precursor molecules, the lower one corresponding to an underglycosylated species (one of the glycosylation sites unoccupied). As we had shown before (Frenkel et al., 2004), the fully glycosylated species shifts progressively to a faster migration because of the trimming of mannose residues on its N-glycans, whereas the underglycosylated lower species is quickly degraded.

We next tested whether palmitoylation influences calnexin targeting to the pericentriolar ERQC, where calnexin and ERAD substrates like H2a accumulate upon inhibition of their degradation or under ER stress (Frenkel et al., 2004; Kamhi-Nesher et al., 2001; Kondratyev et al., 2007). Thus, we compared the localization of the non-palmitoylatable mutant with wild type calnexin. Whereas in untreated cells both proteins showed a
disperse ER pattern, upon short term proteasomal inhibition for 3 h, calnexin CCAA showed a more pronounced relocation to the juxtanuclear ERQC and colocalization with H2a linked to monomeric RFP (H2a-RFP), according to the Manders coefficient (Figure 6C-F). Together, our results demonstrate the preferential interaction of non-palmitoylated calnexin with ERAD substrates that extends to the ERQC, where dissociation from the misfolded glycoprotein would require the ability of calnexin to undergo palmitoylation.

Discussion

The results presented herein uncover a mechanistic description of the role of calnexin palmitoylation during ER stress. We demonstrate that reversible palmitoylation assigns calnexin to the interaction with SERCA2b and a role in the regulation of calcium transfer to mitochondria, whereas non-palmitoylated calnexin tends to interact with ERp57 to fulfill its role in protein folding and quality control (Figure 7). Our results therefore illustrate for the first time how palmitoylation can restrict calnexin to either role and propose palmitoylation as a key mechanism of ER stress signaling. Our observations identify palmitoylation as a quick signal that reassigns calnexin to specific tasks within the ER, unlike phosphorylation that determines calnexin interaction with the ribosome, and another ER chaperone, BAP31, in a less dynamic way. Changes in calnexin phosphorylation require significantly longer exposure to ER stress when compared to the changes in palmitoylation (Figure 2). Moreover, whereas the calnexin phosphorylation state results in intra-ER relocations mediated by the cytosolic sorting protein PACS-2 (Myhill et al., 2008), palmitoylation may regulate the calnexin localization by allowing (or not) its interaction with its effectors SERCA2b and ERp57, localized to sites of calcium signaling or protein folding, respectively. Interestingly, calnexin appears to follow a targeting pattern that is likely relatively unique, since ER and mitochondria move closer to each other during ER stress, suggesting an enrichment of ER proteins at the MAM or a tightening of ER-mitochondria contacts (Bravo et al., 2011; Csordas et al., 2006).

So far, the interaction of calnexin with its substrates has been explained from the substrate’s point of view, since calnexin binds exclusively monoglucosylated substrates
(Lederkremer, 2009). This property, together with the enzymatic action of UDPGlc:glycoprotein glucosyltransferase and glucosidases I and II leads to a cyclic interaction of folding substrates with calnexin until proper folding is reached or ERAD is triggered. Our new data now indicates that calnexin itself undergoes a palmitoylation-dependent cycle within the ER and shuttles back and forth from regulating calcium signaling at or close to the MAM and mediating protein folding and quality control at or close to the rER, including the targeting of unfolded or misfolded proteins to the ERQC (Leitman et al., 2012). Interestingly, these results could help explain the anti-stress function of 2-bromopalmitate (2BP) treatment that attenuates the induction of ER stress transcription factors and the progression of apoptosis (Baldwin et al., 2012). In this scenario, calnexin would become more efficient as a chaperone and would hence mitigate the effects of any additionally introduced ER stressor. Consistent with such an idea, we observed that the inhibition of palmitoylation with 2BP leads to a 2-fold increase in the binding of ERp57 with calnexin (Supplemental Figure 4A), increases the association with H2a and reduces the amount of mature LDLR on the surface (Supplemental Figure 4B). These findings, together with the decreased amount of surface LDLR with CCAA calnexin, suggest that palmitoylation facilitates the dissociation of calnexin from folding intermediates.

Accordingly, palmitoylated calnexin appears to be less important for protein folding, consistent with its demonstrated predominant localization to the MAM (Lynes et al., 2012). However, our results also reinforce the close relationship between ER folding assistants and the control of mitochondrial metabolism (Simmen et al., 2010). In our specific example, calnexin expression and reversible palmitoylation are required to allow the ER to properly signal via calcium towards mitochondria. It is apparently due to the presence of palmitoylated calnexin that the ER can signal a state of stress to mitochondria, likely to alleviate the accumulation of unfolded proteins within the ER (Bravo et al., 2011). However, this function is also critical for mitochondria metabolism, as suggested by the abnormally high mitochondria membrane potential in the absence of palmitoylated calnexin. Surprisingly, our results contradict previous reports that calnexin acts as an inhibitor of SERCA2b in Xenopus oocytes: direct measurement of ER calcium with aequorin suggests that at the basis of our effects is an increased activity of
SERCA2b in the presence of palmitoylatable calnexin, but not of the non-palmitoylated calnexin CCAA mutant (Figure 4B). A potential explanation for this discrepancy could lie in special properties of Xenopus oocytes or in the determination of calnexin’s influence on SERCA2b via the measurement of cytosolic calcium waves following calnexin over-expression used in the earlier study (Roderick et al., 2000) rather than direct measurements of ER calcium or assays of ER-mitochondria calcium cross-talk.

Despite this rather marked difference in ER calcium content, we have, however, not been able to detect differences in the induction of the unfolded protein response dependent on the presence or absence of calnexin (Supplemental Figure 3D). This is consistent with earlier findings on calnexin knockout cells (Coe et al., 2008). Rather, our findings confirm the paramount role the ER plays for the regulation of mitochondria metabolism as elegantly demonstrated by the Foskett lab (Cardenas et al., 2010).

While the enzyme that palmityolates calnexin has been identified as DHHC6 (Lakkaraju et al., 2012), our results predict that depalmitoylation enzymes of the thioesterase family are probably also important regulators of the ER stress response (Baekkeskov and Kanaani, 2009). However, since the APT inhibitor palmostatin B (Rusch et al., 2011) did not influence our observations (data not shown), it has to be yet unknown thioesterases that mediate calnexin shuttling between the MAM and the remainder of the ER. Given the demonstrated efficacy of interference with the ER stress response in various clinical applications (Wang and Kaufman, 2012), the interference with ER protein palmitoylation now provides a new avenue with new characteristics and opportunities.
Materials and Methods

Antibodies and Reagents

All chemicals were from Sigma (Oakville, ON) except Optiprep (Axis Shield, Norton, MA) and Lactacystin (EMD Millipore, Billerica, MA). Rainbow $^{14}$C-labeled methylated protein standards were obtained from GE Healthcare (Little Chalfont, Buckinghamshire, United Kingdom). Promix cell labeling mix ($^{35}$S)Met plus $^{35}$S)Cys, >1000 Ci/mmol was from PerkinElmer Life and Analytical Sciences (Boston, MA). Protein A-Sepharose was from Repligen (Needham, MA). The antibodies have been purchased as follows: mouse anti-ERp57 (StressMarq, Victoria, BC), mouse and rabbit anti-FLAG (Rockland, Gilbertsville, PA; Sigma, Oakville, ON), mouse anti-SERCA2b (EMD Millipore, Billerica, MA), mouse anti-tubulin (Sigma, St Louis, MO), rabbit anti-LDLR (Biovision, Milpitas, CA), goat anti-FACL4 (abcam, Cambridge, UK), mouse anti-PDI (Thermo-Pierce, Rockford, IL), mouse anti-complex 2 (Mitosciences, Eugene, OR), rabbit anticalnexin antibody (Lynes et al., 2012). The phospho-calnexin antibodies were from abcam (P563 dog, P583 human respectively) or generated by 21st Century Biochemicals (Marlboro, MA) on behalf of us (P534, P544 dog). Goat anti-mouse IgG conjugated to agarose was from Sigma. Goat anti-mouse/rabbit secondary fluorescent antibodies were from Life Technologies (Carlsbad, CA). The rabbit polyclonal anti-H2a carboxyterminal antibody was used in earlier studies (Tolchinsky et al., 1996). Goat anti-mouse IgG antibody conjugated to Cy2 was from Jackson Labs (West Grove, PA).

Cell culture and transfections

HeLa, mouse embryonic fibroblasts (MEFs) and human embryonic kidney (HEK) 293 cells were grown in DMEM plus 10% fetal calf serum (FCS) and NIH 3T3 cells in DMEM plus 10% new born calf serum. All cells were grown at 37°C under an atmosphere of 5% CO$_2$. HeLa cells were transiently transfected with Metafectene (Biontex, Martinsried, Germany). Transient transfection of NIH 3T3 cells was performed using an MP-100 Microporator (Digital Bio) according to the manufacturer's instructions.
Transient transfection of HEK 293 cells was done according to the calcium phosphate method. The experiments were performed 24-48 hours after transfection.

**Biotin labeling and pulldown of surface proteins**

Cells were washed twice with cold PBS with calcium and magnesium (PBS++) and then incubated with 0.3 mg/mL EZ Link Sulfo-NHS-LC-Biotin (Thermo-Pierce, Rockford, IL) in cold PBS++ for 30 minutes on ice at 4°C. The biotinylation reaction was then quenched for 5 minutes with 50 mM Glycine in PBS++, and rinsed twice with cold PBS. The cells were then lysed in mRIPA buffer with Complete Protease Inhibitors (Roche, Basel, Switzerland) and scraped into microcentrifuge tubes. Post-nuclear supernatants were obtained by centrifuging the lysates at 800 g for 5 minutes at 4°C. In parallel, lysates of non-biotinylated cells from each experimental condition were prepared as above. The biotinylated samples were then incubated at 4°C overnight on a rocker with 25 μL of 40% streptavidin-agarose beads (Sigma, St Louis, MO) prepared in PBS. The beads were washed once with PBS, and resuspended in Laemmli buffer, and heated at 100°C for 5 minutes. The lysates of the non-biotinylated cells were denatured in a similar manner. All samples were then analyzed by SDS-PAGE and Western blot.

**Detection of palmitoylation by click chemistry**

HeLa cells transfected for 48h with FLAG-Calnexin were treated with tunicamycin (10 μM) or DTT (2 mM) for 4 hours. Next, cells were labeled with 100 μM alkynyl-palmitate or palmitate conjugated to BSA for 3 hours. Cells were harvested in 0.1% SDS-RIPA buffer (containing CPI protease inhibitor, EDTA-free, Roche, Laval, QC) and calnexin was immunoprecipitated using the anti-FLAG antibody (Rockland). The click reaction was then carried out on the immunoprecipitated, labeled proteins by incubating them for 30 minutes at 37°C with 2mM TBTA, 50 mM CuSO₄, 50 mM TCEP and 2 mM Biotin-azide. Samples were then separated in duplicate by SDS-PAGE, and transferred to PVDF membranes which were washed with either 0.1 M Tris-HCl ph 7.0 or 0.1 M KOH. This alkali treatment removes fatty acids incorporated into proteins via thioester bonds but not via amide bonds and contributes to ensure the specificity of the signal. Palmitoylation was detected by probing both membranes with HRP-conjugated Neutravidin using ECL.
**Immunofluorescence microscopy**

HeLa cells were treated with tunicamycin (10 μM) or thapsigargin (1.5 μM) for 4 hours, and immunofluorescence microscopy was performed using the indicated primary antibodies, with the protocol described in (Gilady et al., 2010). LDLR surface binding was detected by binding of a 1:100 dilution of anti-LDLR antibody in DMEM, 10% FBS, 1% BSA. Intracellular FLAG-tagged calnexin was detected after permeabilization with 1% Triton X-100. The procedures employed with NIH 3T3 cells were described previously (Avezov et al., 2008; Kamhi-Nesher et al., 2001). Confocal microscopy was done on a Zeiss laser scanning confocal microscope (LSM 510; Carl Zeiss, Jena, Germany) as described before (Avezov et al., 2008). Colocalization analysis (Manders) was done using ImageJ and Imaris softwares.

**Co-immunoprecipitation experiments**

Cells were washed twice with PBS++ and incubated for 30 minutes at room temperature with 2 mM Dithiobis (succinimidyl proprionate) (Thermo Scientific, Rockford, IL) in PBS++ to crosslink protein-protein interactions. The cells were then washed twice more and incubated in 10 mM NH4Cl in PBS++ for 10 minutes to quench the crosslinking reaction. The cells were then washed a final time in PBS++ and harvested in CHAPS lysis buffer (1% CHAPS, 10 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA) containing Complete protease inhibitors (Roche, Basel, Switzerland). Post-nuclear supernatants were obtained by centrifuging the lysates for 5 minutes at 4°C at 800g, and were subsequently incubated with the indicated antibodies for one hour at 4°C on a rocker. Protein A Sepharose beads were then added and the lysates incubated for a further hour. The beads were then washed 3 times in CHAPS buffer and resuspended in Laemmli buffer and analyzed by SDS-PAGE and Western Blot. For immunoprecipitation from HEK 293 cells, cell lysis was done in 2% sodium cholate for 30 min on ice, and debris and nuclei were pelleted in a microfuge for 30 min at 4°C. The samples were immunoprecipitated with anti-FLAG and goat anti-mouse IgG-agarose overnight, followed by washes and elution by boiling with sample buffer containing β-mercaptoethanol.
**Metabolic labeling**

Subconfluent (90%) cell monolayers in 100-mm dishes were labeled with $[^{35}\text{S}]\text{Cys}$. Co-immunoprecipitation with anti-calnexin antibody was performed as described before (Frenkel et al., 2004). Briefly, after metabolic labeling, cells were lysed in HBS buffer pH 7.5, containing 2% sodium cholate, cell lysates were immunoprecipitated with anti-FLAG antibody, boiled in 1% SDS, then diluted with 10 volumes of 1% Triton X-100, 0.5% sodium deoxycholate in HBS and reimmunoprecipitated with anti-H2a antibody. SDS-PAGE gels were analyzed by fluorography using 20% 2,5-diphenyloxazole and were exposed to Biomax MS film using a transcreen-LE from Kodak (Vancouver, BC). Quantitation was performed in a Fujifilm FLA 5100 phosphorimager (Japan).

**Reverse transcriptase polymerase chain reaction to assay the unfolded protein response (UPR)**

Calnexin knockout cells transfected as indicated were treated with 1.5 μM thapsigargin for 16h or left as controls. Total RNA was isolated using TRIzol (Life Technologies, Carlsbad, CA) and amplified with primers for Xbp-1 (CCTTGTGGTTGAGAACCCAGG and CTAGAGGCTTGGTGTATAC) and GAPDH, respectively (AACTTTGGCATTGTGGAAGG and ACACATTGGGGGTAGGAACA). PCR products were separated on 7.5% and 1% acrylamide gels, respectively.

**Plasmid-based ER and Mitochondria Calcium Measurements**

ER calcium was measured with pHSVerAEQ (gift from Antonio Cuadrado, Madrid, Spain). Cell were transfected with this plasmid (and constructs expressing calnexin as indicated) and processed for measurements 24h following transfection as published (Alvarez and Montero, 2002). Following the depletion of ER calcium with 0.5 mM EGTA and 1μM 2,5-di-tert-butyl-benzohydroquinone (Sigma), aequorin was reconstituted with 10μM coelenterazine hcp (Sigma). After leaving the cells for 1h at RT in the dark, cells were perfused with medium containing 1mM calcium. Luminescence from this condition and total luminescence following digitonin permeabilization was assayed on a Lumat luminometer (Berthold, Bad Wildbad, Germany). For mitochondrial calcium, mitochondrially targeted R-GECO1 was used (Zhao et al., 2011). Calnexin wild
type and knockout MEFs were transfected with a plasmid encoding mitochondrial R-GECO1, whose fluorescence was measured on a Olympus Fluoview FV1000 microscope and quantified using the FV10-ASW 3.1 software (Olympus, Richmond Hill, ON). Fluorescence measurements were initialized after establishment of a baseline, followed by the addition of 1μM thapsigargin.

**Construction of mitochondria-targeted R-GECO1 plasmid**

To construct a plasmid expressing mitochondria-targeted R-GECO1, the gene for R-GECO1 in pTorPE\(^1\) was used as a template. PCR was carried out by using primers: ‘GCaMP_FW_BamHI_mito’ (sequence: GAGGATCCAAACCATGGTCGACTCATCAGTC) and ‘GCaMP_RV_HindIII’ (sequence: CGCAAGCTTCTACTTCGCTGTCATCATTTGTAC). PCR products were purified using 1% agarose gel (Agarose S, Nippon Gene Co.) electrophoresis. DNA was extracted from the appropriate gel slice using the GeneJet DNA Purification Kit (Thermo Scientific). The PCR product was then digested with BamHI and HindIII (Thermo Scientific), repurified as described above, and ligated into the the CMV-mito-GEM-GECO1\(^1\) vector previously digested with the same restriction enzymes. The resulting ligation products were transformed into DH10B E. coli by electroporation, and the transformed E. coli were grown on agar plates (with 400 μg/ml ampicillin) overnight. E. coli colonies on agar plates were then picked up and cultured in LB media (with 100 μg/ml ampicillin, 250 rpm shaking) for 12 to 16 hours. DNA plasmid purification was performed using the GeneJET Plasmid Miniprep Kit (Thermo Scientific), and the purified mitochondria-targeting R-GECO1 DNA plasmids were verified by sequencing at the University of Alberta Molecular Biology Services Unit.

**Flow Cytometry**

Cells were loaded with either 1 μM Fluo8 (AAT Bioquest, Sunnyvale, CA) 1 μM Rhod2 (Life Technologies, Carlsbad, CA) or 20 nM TMRM (Sigma, St Louis, MO) and incubated in DMEM (Life Technologies, Carlsbad, CA) for 30 minutes at 37°C. Cells were then harvested in HEPES Buffered Saline (0.1% glucose, 0.1% BSA) and subjected to flow cytometry using a FACS Scan cytometer (BD Biosciences, Mississauga, ON).
before treatment and 10 (50 μM histamine) or 20 seconds (1.5 μM thapsigargin) after drug administration.

**Optiprep Gradient Fractionations**

HeLa cells were treated with the indicated stressors and then washed twice in PBS++ and collected in mitochondria homogenization buffer (10mM HEPES pH 7.4, 250 mM sucrose, 1mM EDTA, 1mM EGTA). The cell suspension was passed 15 times through an 18 μM clearance ball bearing homogenizer (Isobiotech, Heidelberg, Germany). The cells were subsequently centrifuged for 10 minutes at 800xg at 4°C, to pellet nuclei and unbroken cells. The supernatant was layered over a 10%-30% continuous gradient of Optiprep density gradient medium (Axis-Shield, Norton, MA), and was centrifuged in an SW55 Ti rotor (Beckman Coulter, Mississauga, ON) for 3 hours at 32700 rpm. Fractions were taken from the top of the gradient and analysed by SDS-PAGE and Western Blot.

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Figure Legends

Figure 1: ER stress affects calnexin’s ER-internal distribution. A. Heavy and light membrane fractionation following ER stress. Homogenized HeLa cell lysates were separated into heavy and light membranes following a 1h or 4h treatment with 10 μM Tunicamycin (Tuni). Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for PDI, calnexin and mitochondrial complex 2. The graph shows the distribution time course for PDI and calnexin. B. Optiprep fractionation following ER stress. Homogenized HeLa cell lysates were separated via Optiprep following a 4h treatment with 10 μM Tunicamycin (Tuni) into 6 fractions. Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for PDI (pan-ER) and FAACL4 (MAM), as well as calnexin. The graph shows the calnexin amount in the MAM fraction (6) from 3 independent experiments (Statistics: P=0.035). C. Calnexin signal proximal to mitochondria depends on ER stress. Control HeLa cells and cells treated with 10 μM Tunicamycin were processed for immunofluorescence microscopy as described and analyzed for the signals of calnexin, PDI and mitochondria (mitotracker). The merged images (calnexin = green, PDI = blue, mitochondria = red) are shown on the right with a zoomed area of just calnexin and mitotracker signals. Manders coefficients: 0.36 for CNX wild type, 0.30 for CNX CCAA (from 14 cells).

Figure 2: Analysis of calnexin phosphorylation and palmitoylation during an ER stress time course. A. Calnexin phosphorylation of its three sites is individually altered during an ER stress time course. HeLa cells treated for the indicated times with 10 μM Tunicamycin were snap-lysed with sample buffer and analyzed via Western blot using phospho-specific antibodies against the three known sites serine 555, 565, 584 (dog nomenclature). B. Calnexin palmitoylation is reduced during ER stress. HeLa cells were incubated for 4h with 10 μM Tunicamycin and then processed for click chemistry as described (Lynes et al., 2012).
Figure 3: ER stress and palmitoylation affect calnexin’s interaction with SERCA2b.
A. Optiprep and membrane fractionation following ER stress. Homogenized HeLa cell lysates were separated via Optiprep following a 4h treatment with 10 μM Tunicamycin (Tuni) into 6 fractions. Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for SERCA2b. Homogenized HeLa cell lysates were separated into heavy and light membranes following a 4h treatment with 10 μM Tunicamycin (Tuni). Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for calnexin and SERCA2b. The graph shows the calnexin and SERCA2b amounts in the heavy membrane fraction from 3 independent experiments (Statistics: P=0.013 for calnexin, P=0.395 for SERCA2b).
B. Calnexin-SERCA2b co-immunoprecipitation following ER stress. HeLa cells were treated for 4 h with either 2 mM Dithiothreitol (DTT) or 10 μM Tunicamycin (Tuni). DSP-crosslinked lysates (5% inputs) and calnexin immunoprecipitates were analyzed for calnexin and co-immunoprecipitating SERCA2b. The graph shows results from 3 independent experiments (Statistics: P=0.0073 for DTT, P<0.001 for Tunicamycin).
C. Calnexin/calnexin palmitoylation mutant (CCAA)-SERCA2b co-immunoprecipitation. HeLa cells were transfected with FLAG-tagged wild type or CCAA calnexin. DSP-crosslinked lysates (5% inputs) and FLAG immunoprecipitates were analyzed for FLAG-tagged calnexin and co-immunoprecipitating SERCA2b. The graph shows results from 3 independent experiments (Statistics: P=0.006).

Figure 4: Palmitoylated calnexin regulates ER calcium signaling. A. Measurement of cytosolic calcium following thapsigargin-mediated ER calcium release. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were loaded with Fluo8. Cells were then treated with 1.5 μM thapsigargin and probe fluorescence was recorded before and after thapsigargin treatment by flow cytometry (Statistics: P=0.8419 for calnexin wild type, P=0.8816 for calnexin CCAA).
B. Measurement of ER calcium content. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were co-transfected with a plasmid encoding ER-targeted aequorin. Luminescence was plotted from three independent experiments following the protocol outlined in Materials and Methods.
P=0.08  C. Measurement of mitochondrial calcium following thapsigargin-mediated ER calcium release. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were loaded with Rhod2. Cells were then treated with 1.5 μM thapsigargin and probe fluorescence was recorded before and after thapsigargin treatment by flow cytometry (Statistics: P=0.02 for calnexin wild type, P=0.8583 for calnexin CCAA). D. Measurement of mitochondrial calcium following histamine-mediated ER calcium release. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were loaded with Rhod2. Cells were then treated with 50 μM histamine and probe fluorescence was recorded before and after histamine treatment by flow cytometry (Statistics: P=0.2581 for calnexin knockout, P<0.001 for calnexin wild type, P=0.088 for calnexin CCAA). E. Calnexin expression levels of representative cells from flow cytometry experiments. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were lysed and processed for Western blot using the FLAG antibody.

Figure 5: ER stress and palmitoylation affect calnexin’s interaction with ERp57. A. Optiprep and membrane fractionation following ER stress. Homogenized HeLa cell lysates were separated via Optiprep following a 4h treatment with 10 μM Tunicamycin (Tuni) into 6 fractions. Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for ERp57, PDI and calnexin. Homogenized HeLa cell lysates were separated into heavy and light membranes following a 4h treatment with 10 μM Tunicamycin (Tuni). Membrane fractions were analyzed as indicated by SDS-PAGE and Western Blot for ERp57, PDI and calnexin. The graph shows the ERp57, PDI and calnexin amounts in the heavy membrane fraction from 3 independent experiments (Statistics: P=0.056 for ERp57, P=0.088 for PDI, P=0.013 for calnexin). B. Calnexin-ERp57 co-immunoprecipitation following ER stress. HeLa cells were treated for 4 h with either 2 mM Dithiothreitol (DTT) or 10 μM Tunicamycin (Tuni). DSP-crosslinked lysates (5% inputs) and calnexin immunoprecipitates were analyzed for calnexin and co-immunoprecipitating ERp57. C. Calnexin/calnexin palmitoylation mutant (CCAA)-ERp57 co-immunoprecipitation. HeLa cells were transfected with FLAG-tagged wild type or CCAA calnexin. DSP-crosslinked lysates (5% inputs) and FLAG
immunoprecipitates were analyzed for FLAG-tagged calnexin and co-immunoprecipitating ERp57. The graph shows results from 3 independent experiments (Statistics: P=0.003). D. LDLR surface biotinylation. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were lysed and processed for Western blot using the LDLR antibody. In parallel, the same set of cells was processed for surface biotinylation as described in Materials & Methods and probed for biotinylated surface LDLR. The bar graph shows the means of three independent experiments (P=0.07). Cells were also processed for LDLR surface binding, followed by permeabilization and detection of intracellular transfected FLAG-tagged calnexin as shown at the bottom.

Figure 6. Involvement of non-palmitoylated calnexin in glycoprotein quality control. A. Increased interaction of an ERAD substrate with CNX CCAA as compared to CNX WT. HEK-293 cells were cotransfected with a vector encoding for an ERAD substrate glycoprotein, H2a and FLAG-tagged CNX wt or CNX CCAA. The cells were lysed in 2% sodium cholate (Methods) and 10% of the lysates were run on 10% SDS-PAGE and immunoblotted with anti-H2a antibody (bottom panel). The rest of the lysates were immunoprecipitated with mouse anti-FLAG and goat anti-mouse IgG-agarose, subjected to 10% SDS-PAGE and immunoblotted with anti-H2a (top panel) or with anti-FLAG (middle panel). Relative amounts of H2a co-immunoprecipitated with anti-FLAG were plotted relative to the total amounts. B. Slower dissociation of H2a from non-palmitoylated CNX. HEK 293 cells were transfected as in (A). Two days post-transfection the cells were pulse-labeled for 20 min with [35S]-cys and chased for the indicated times in the presence of the proteasome inhibitor MG-132. After the pulse (0 h chase) or the chase periods, the cells were lysed, H2a was immunoprecipitated from 15% of the cell lysates (right panels) and the remainders were immunoprecipitated with anti-FLAG antibody followed by elution and re-immunoprecipitation with anti-H2a, as described in Methods (left panels). Control antibody was used instead of anti-FLAG in a control sample (Cont.). All immunoprecipitates were separated in 12% SDS-PAGE followed by phosphorimaging. For the pulse samples, two bands can be seen for H2a precursor molecules, the lower ones corresponding to underglycosylated species (one of
the glycosylation sites unoccupied). The fully glycosylated species shifts progressively to a faster migration because of the trimming of mannose residues on its N-glycans. The graph shows percent of H2a coimmunoprecipitated with CNX WT and CNX CCAA normalized to total amounts (IP anti-H2a), after chase relative to the pulse, from phosphorimager quantitations of the gels, average of three independent experiments ± SE. C-F. Increased ERAD substrate colocalization at the juxtanuclear ERQC, with CNX CCAA as compared to CNX WT upon proteasomal inhibition. Plasmids encoding for H2a linked to monomeric RFP (H2a-RFP) and FLAG-tagged CNX wt (C, D) or CNX CCAA (E, F) were cotransfected in NIH 3T3 cells. One day after transfection cells were incubated for 3h in the absence (C, E) or presence of 25 μM Lac (D, F), fixed, permeabilized and incubated with mouse anti-FLAG and Cy2-conjugated goat-anti-mouse IgG. The samples were analyzed in an LSM confocal microscope. Representative confocal optical slices are shown. Bar=10μm. Manders coefficients: 0.77 for CNX wild type, 0.88 for CNX CCAA (from 21 cells).

Figure 7: Model for the calnexin shuttling between calcium signaling and quality control. Palmitoylated calnexin interacts with SERCA on the MAM to regulate ER calcium signaling and to a lesser extent on the rER with the translocon. Non-palmitoylated calnexin interacts with ERp57 on the rER to mediate protein folding and quality control. Additionally, non-palmitoylated calnexin can interact with ERAD substrates on the ERQC. Palmitoylation redirects chaperoning calnexin back to its role in calcium signaling.

Supplemental Figure 1: Full gels for Figure 1B. Representations of the control gels seen in Figure 1B for calnexin, FACL4, and PDI. Molecular weight markers are shown on the left and arrows indicate the bands of interest.

Supplemental Figure 2: Additional analysis of calnexin palmitoylation and phosphorylation. A. Optiprep fractionation following thapsigargin treatment. Homogenized HeLa cell lysates were separated via Optiprep following a 4h treatment with 1.5 μM Thapsigargin into 6 fractions. Membrane fractions were analyzed as
indicated by SDS-PAGE and Western Blot for PDI (pan-ER), SERCA2b and FACL4 (MAM), as well as calnexin. **B.** Calnexin palmitoylation is reduced during DTT stress. HeLa cells were incubated for 4h with 5 mM DTT and then processed for click chemistry as described (Lynes et al., 2012). **C.** Neither calnexin palmitoylation nor the phosphorylation status of serines 554 and 564 influence serine 583 phosphorylation. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type CCAA, M3 (S554, 564→A), or M4 (S554, 564→D) calnexin were lysed and processed for Western blot using the phospho-serine 583 antibody.

**Supplemental Figure 3: Influence of calnexin presence on mitochondria calcium import, mitochondria membrane potential and ER ability to trigger the unfolded protein response.** **A.** Measurement of cytosolic calcium following thapsigargin-mediated ER calcium release. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were loaded with FURA-2. Cells were then treated with 1.5 μM thapsigargin and probe fluorescence was recorded before and after thapsigargin treatment by flow cytometry. The increases of fluorescence for the three conditions were not statistically different from each other. **B.** Plasmid-based measurement of mitochondrial calcium content following thapsigargin. Calnexin wildtype and knockout (ko) MEFs were transfected with a plasmid encoding mitochondria-targeted R-GECO-1. The increase in relative fluorescence units was assayed from three independent experiments following the protocol outlined in Materials and Methods. A representative curve for wild type and knockout cells is shown on the right. **C.** Measurement of mitochondrial membrane potential. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were loaded with TMRM and probe fluorescence was recorded by flow cytometry (Statistics: P=0.001 for calnexin wild type, P=0.4135 for calnexin CCAA). **D.** Xbp-1 splicing measured by reverse transcriptase PCR. Calnexin knockout (ko) MEFs and ko cells transfected with FLAG-tagged wild type or CCAA calnexin were treated with 1.5 μM thapsigargin for 16h, followed by analysis of the Xbp-1 mRNA. PCR products were separated on a 7.5% acrylamide gel for Xbp-1 and a 1% acrylamide gel for GAPDH.
Supplemental Figure 4: 2BP promotes the calnexin chaperone activity. A. Calnexin-ERp57 co-immunoprecipitation following 2BP treatment. HeLa cells were treated for 4 h with 100 μM 2BP. DSP-crosslinked lysates (5% inputs) and calnexin immunoprecipitates were analyzed for calnexin and co-immunoprecipitating ERp57. P=0.07. B. LDLR surface biotinylation following 2BP treatment. HeLa cells were lysed and processed for Western blot using the LDLR antibody. In parallel, the same set of cells was processed for surface biotinylation and probed for biotinylated surface LDLR.

References


Figure 1, Lynes et al.
Figure 2, Lynes et al.
Figure 3, Lynes et al.
Figure 4, Lynes et al.
Figure 5, Lynes et al.
Figure 6, Lynes et al.
Lynes et al., Figure 7