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Palmitoylated calnexin is a key component of the ribosome-translocon complex

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1st Editorial Decision 18 October 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, all three reviewers recognise the potential interest in your work, but all also raise significant concerns that would need to be addressed by a major revision of your work, before we could consider publication here.

Their reports are explicit, but I would just like to spell out what we see to be the major issues here:
- All three reviewers bring up concerns centred around the fact that you do not directly assay the effect of calnexin palmitoylation on its chaperone activity, but rather look indirectly at protein levels and/or secretion (ref 1 comments on fig 7; ref 2 point 5; ref 3 point 3). Addressing these various related concerns would be critical to better define how palmitoylation affects calnexin activity.
- Referee 2 in particular brings up important technical concerns, both with the siRNA data, and with the coIP experiments. Again, resolving these issues with the appropriate controls and additional experiments would be essential.

Given the overall interest expressed, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerey,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The chaperone function of calnexin is controlled by palmitoylation (EMBOJ-2011-79655)

The authors report that palmitoylation of the cytosolic domain of calnexin results in a conformational change, promotes association with the actin cytoskeleton and the ribosome-translocon complex and preferential localization in the rough ER/nuclear membrane. The authors identify DHHC6 as the ER palmitoyltransferase regulating calnexin modification. It is proposed, but not shown, that calnexin palmitoylation is essential for the binding of client proteins.

It is my opinion that the data shown in this manuscript are of interest for the EMBO J readership. I suggest, however, few modifications/control experiments that should be performed before considering this manuscript further.

Major:

Dynamic cycles of palmitoylation/de-palmitoylation may determine protein activity. In untreated cells, palmitate turnover on calnexin is very slow. Do the authors conclude that cycles of palmitoylation/de-palmitoylation are not regulating the function of calnexin? Does the absence of substrates, f.e. in cells treated with glucosidase inhibitors, affect the palm state of the protein or its sub-ER localization? Is it possible that only one of the two palm is cycling thereby activating/inactivating calnexin function to adapt it to cellular needs? The authors report that more than 90 % of endogenous calnexin is palm. However, DHHC up-regulation results in substantial incorporation of radiolabeled palm. Could this be an indication of rapid turnover of at least one palm? The authors should comment on these aspects.

In Figure 1C, transfection efficiency is normally quite low. Thus, most of the cells are probably not expressing the Sar mutant. The data shown in Figure 1C, therefore, do not prove that endogenous calnexin acylation exclusively occurs in the ER. I think that this panel should be removed. Calnexin is efficiently retained in the ER. In my opinion, this is sufficient to conclude that its acylation occurs at the ER membranes. Alternatively, this experiment should be replaced with a co-transfection experiment (calnexin-HA + Sar-GTP).

Figure 1D-I do not think that the experiment with CHX is conclusive. How long was the CHX treatment is not mentioned in the text. Can it be excluded that the CHX treatment depletes cells from a (short living?) thioesterase thereby stabilizing palmitoylated calnexin? The authors could discuss this.

Pages 7 and 16-Silencing of DHHC5 substantially decreases palm of endogenous calnexin. The fact that DHHC5 is mainly localized in the Golgi membranes does not exclude its presence in the ER

membranes. The inhibitory effect of its silencing on palm of endogenous calnexin is striking. As the authors write, it remains unclear why this is not reproduced for the recombinant calnexin. Understand this, goes probably beyond the scope of this work. However, based on the data shown here, I am not fully convinced that the authors can exclude a regulatory role for DHHC5 or can conclude that DHHC6 is the only palm enzyme involved in calnexin modification (Discussion), especially considering endogenous calnexin. It would actually be very interesting, in future work, to look at endogenous calnexin localization and substrate binding upon DHHC5 vs DHHC6 RNAi.

Pages 9-10, Figure 3-This figure is interesting. I do not think that the authors do comment it in sufficient details. To me, there seems to be a clear difference when calnexin is mono-palm at 502 vs 503. Could the presence/absence of palm at 503 (independent on the occupancy of 502) represent the elusive regulatory modification?

The data shown in Figure 3 (page 10, it should be Figure 4! And page 11, Figure S2 should be Figure S3!) would be much more convincing if co-localization experiments would have been performed (calnexin with an ER-resident protein that does not change its localization upon DHHC6 RNAi and calnexin and a nuclear envelope marker).

Do recombinant calnexins (wt, AC, CA, AA) have distinct localizations? Do they bind glycoproteins differently and/or does endogenous calnexin immunoisolated from untreated vs DHHC6 RNAi-treated cells bind glycoproteins differently? This would support the authors's claim that the palm of calnexin is essential for substrate binding (pages 2 and 5).

Page 12-Figure S2B should be Figure S3!

Page 12-The decrease is 40%, not 60.

Figure S4A, the authors should explain what is Pat6, and why it has been used here.

Figures 5B and 5C-More than 90% of calnexin is palm (page 8) and palm is very stable. It is unclear to me why the over expression of DHHC6 should increase the association with the RTC (5B, 5C) or, quite dramatically (+50%), the association with actin (6E).

Page 12-Is it known whether the Ser653 calnexin mutant is also excluded from the nuclear envelope?

Page 13-Figure 7 should be Figure 6!

Page 13-Does Latrunculin A affect the localization of endogenous calnexin?

Figure 7 is not fully convincing. I could assume that calnexin inactivation may affect folding and secretion of a select population of glycoproteins. However, why should the silencing of calnexin specifically reduce the synthesis of glycoproteins is unclear. What about the synthesis of non-glycosylated proteins expressed in the ER?

The authors write, and their interesting model implies, that calnexin palm is crucial for efficient capture of nascent polypeptide chains (pages 2 and 5). It seems crucial to directly assess the capacity of calnexin (endogenous and/or recombinant) to bind substrates under the different experimental conditions.

Page 15, Figure 4F should be Figure 7E!

Minor:

Page 2, second line-Co-translationally
Page 3-Addition of N-glycans and formation of disulfide bonds occur both co- and post-translationally.
Page 4-The reference Deprez et al refers to the next sentence.
Page 4 + page 16-The sentence "How calnexin can capture nascent chains is unclear" should be deleted or modified. There is quite a lot of literature on that subject. I would rather explain that the

possible contribution of palmitoylation of the cytosolic tail of calnexin in the sub-localization of calnexin that may facilitate substrate binding has not been studied.
Page 5, line 4 from the bottom: dependent.
Page 6, Figure 1A-the control with hydroxylamine hydrochloride is not explained or mentioned in the text.
Page 8, third line 3-Transferrin.
Page 8, sixth line from the bottom, increase of the calnexin signal
Page 10, third line-we next included
Figure 5, the acronym OE, for over expressed, should be defined.
Page 18, third line from the bottom, domain
Page 18, last two lines, as it as is
Supplementary Figure 2D, the labeling of this figure (the +/- showing where DHHCs have been OE) is wrong.

Referee #2 (Remarks to the Author):

The paper from Lakkaraju et al reports follows on from previous studies showing that calnexin becomes palmitylated in mammalain cells. A characterisation of the lipid modification is carried out to identify the cysteine residues that are modified as well as identifying the specific palmytyltransferase carrying out the reaction. The paper then goes on to predict the consequence of palmitoylation on the conformation of calnexin, the subcellular localisation, interaction with the ribosome associated translocon, interactions with the actin cytoskeleton and the effect on gycoprotein folding. If the claims made by the authors are substantiated then this would indeed be an interesting paper, however, there are serious deficiencies in some of the experiments described and in their interpretation. These are listed below.

1. The abstract states that palmitylation triggers a conformation change in calnexin. No evidence is presented that proves this is the case. This is just a prediction based upon molecular dynamic simuations so should not be presented as a fact.
2. A significant portion of the paper describes experiments where proteins are depleted from cells by RNAi and then the effect of this depletion is assayed. The conclusion drawn is that the effect is due to the specific depletion, however, this is a dangerous assumption to make without the proper controls. For example the depletion of DHHC6 causes a change in cell morphology and localisation of calnexin away from the nuclear envelope. These consequences could easily be explained by off target effects of DHHC6 depletion or even the induction of an unfolded protein response. To prove this effect is really due to the DHHC6 depletion leading to a lack of palmitylation of calnexin one would need to show that the effect is reversed when an RNAi insensitive version of DHHC6 is co-expressed with the RNAi.
3. No mention is made in the paper of the known association of calnexin with mitochodrial associated membranes (MAMS). Disruption of this interaction by a lack of palmitylation could be an inportant aspect of calnexin function so should be addressed.
4. The assay for calnexin association with the RTC is problematic. It has been known since the early work of Ari Helenius' group that calnexin co-immunoprecipitaes with membrane proteins due to its inclusion in detergent/lipid micelles. It is therefore very difficult to interpret whether there is actually a protein/protein interaction in these co-immunoprecipitation studies. For client proteins this direct interaction can be confirmed by regulating the interaction following de/re-glucosylation. In addition anyone carrying out immunoprecipitation will know that actin is invariably a contaminant in these precipitates due to its cellular abundance and its ability to interact with proteins folowing cell lysis. Chemical crosslinking followed by very stringent immunoprecipitation may provide more conclusive proof that these proteins do actually interact in the cell. The association of calnexin with the ribosome has been suggested previously and is not a novel observation (The EMBO Journal (1999) 18, 3655 - 3666).
5. The final series of experiments states that they address the effect of calnexin palmitylation on the folding of proteins. However the assays used to not address folding directly, rather protein trafficking and secretion. Assays for folding would be easy to establish so either the authors change their claims to state that calnexin palmitylation affects the secretion of glycoproteins or they need to set-up some folding assays.
6. The tile of the paper states that the chaperone function of calnexin is controlled by palmitylation. In no part of the paper do the authors address a putative chaperone function. Is this distinct from its

functions as a lectin binding to monoglucosylated substrates?
7. There are several errors in the manuascript (too many to list). These range from the inaccurate figure numbering to errors in grammer and spelling. There are several instances of inappropriate terminology which does not help when reading. For example what is "the oligosaccharide tree" and what does "the public domain chaperone" mean?

Referee #3 (Remarks to the Author):

Review EMBOJ-2011-79655

The manuscript "The chaperone function of calnexin is controlled by palmitoylation" by Lakkaraju et al. shows that:
1) calnexin (cnx), a lectin chaperone of ER client glycoproteins, is palmitoylated at two conserved cysteine residues in its cytosolic tail;
2) palmitoylation of cnx is catalysed by DHHC6;
3) palmitoylation allows cnx to interact with the ribosome-translocon-complex via TRAPalpha and also with the actin cytoskeleton;
4) palmitoylation of cnx is required for its localization to rough ER and the nuclear envelope in particular;
5) palmitoylation of cnx is required for it to be an efficient chaperone.

The finding of cnx's palmitoylation is novel and the characterization of the role of palmitoylation for cnx function is thorough. No doubt, this work meets the quality standards of EMBO J. and will appeal to its broad readership.

However, the authors should clarify the following issues:

1) From the experiments it is evident that cnx is palmitoylated, using radioactive palmitate. It is not clear, however, whether other forms of acylation are possible or that cnx is decorated exclusively with palmitoyl chains.
The authors could check whether a mix or a selection of radioactive fatty acids other than palmitate yield any incorporation into cnx.

2) The authors show on the one hand that overexpression of DHHC6 leads to a substantial increase in palmitoylation of cnx, but on the other hand claim that at steady state over 90% of cnx is palmitoylated and that this modification is stable. The two claims are hard to rhyme, unless there is a kinetic explanation.
This issue could be solved by a pulse-chase with 35S labeling of cnx, monitoring palmitoyl incorporation into newly synthesized cnx, by immunoprecipitation of cnx and running the samples both on 1D and 2D gel electrophoresis, at several time points (e.g. 0, 15, 30, 60 & 120 min. after a 5-10 min pulse) for both control DHHC6 knock-down and DHHC6 over-expressing cells. While for the 1D gel the autoradiogram signal should remain constant, the signal for the 2D gel should diminish, as the cnx signal spreads out upon palmitoylation, according to the data shown in figure 2E. If palmitoylation is post-translational and if it takes quite a while before newly synthesized cnx is fully acylated, DHHC6 overexpression may speed up the process, which would solve the apparent discrepancy.

3) The authors demonstrate by co-immunoprecipitation that cnx interacts with TRAPalpha, Sec61alpha, the ribosome and also with actin, while cnx devoid of palmitoyl groups no longer does so. The conclusion is that cnx is a bona fide member of the ribosome-translocon complex (RTC). This interesting finding has profound implications on the interpretation of the remainder of the experiments.
For one, the finding that actin interacts to a lesser extent with non-palmitoylated cnx may be indirect, as actin may only hook up to the RTC once it is fully assembled (i.e. in conjunction with cnx). The interpretation of the results needs to be rewritten to acknowledge such a scenario.
Moreover, cnx function as a chaperone in the ER lumen may be unimpeded when it lacks palmitoyl moieties. The rationale for a decrease in secretory capacity or synthesis of glycoproteins may be solely due to the fact that the RTC is not functioning properly when it cannot team up with cnx and thereby reduces the flux of ER clients, but not the chaperone activity of cnx per se.

To address this question, the authors could exploit again a 35S pulse-chase followed by immuno-precipitation of cnx and follow the association of glycoproteins over time as originally established by Ou et al, 1993. Although the initial signal directly after the pulse may be lower, the kinetics of dissociation of newly synthesized glycoproteins over time may be identical. If so, the chaperone function of cnx is arguably unaltered, yet its crucial role as part of the RTC in guiding newly synthesized proteins into the ER lumen is disrupted once the palmitoyl moieties are absent.
In that scenario also the title should be changed, for instance into "Calnexin is palmitoylated to serve as key component of the ribosome-translocon complex"

Some minor issues deserve attention as well:

4) Could the effect of the DHHC5 knock-down be explained by an indirect effect? For instance, is also DHHC6 palmitoylated? Is DHHC5 responsible for that?

5) Could the authors discuss better why palmitoylation is key for localization of cnx to the nuclear envelope? What is their hypothesis?

6) Could the authors perhaps reorganize the text with care? Several sentences are grammatically incorrect or difficult to interpret.

1st Revision - authors' response 13 December 2011

Reply to Referee #1:

1- *Dynamic cycles of palmitoylation/de-palmitoylation may determine protein activity. In untreated cells, palmitate turnover on calnexin is very slow. Do the authors conclude that cycles of palmitoylation/de-palmitoylation are not regulating the function of calnexin?*

Indeed under steady state condition, calnexin remains palmitoylated and functional. This point is now clarified in the text. The possibility of course exists that under a specific stimulus, depalmitoylation of calnexin is accelerated.

2- Does the absence of substrates, f.e. in cells treated with glucosidase inhibitors, affect the palm state of the protein or its sub-ER localization?

This is an interesting question that we investigated. The glucosidase inhibitor castanospermine had no effect on palmitoylation or depalmitoylation, nor with calnexin localization of its interaction with the RTC, as now mentioned in the text (page 19).

3- Is it possible that only one of the two palm is cycling thereby activating/inactivating calnexin function to adapt it to cellular needs?

The regulation of calnexin activity by palmitoylation is clearly an interesting and important question and the fact that it has two sites might indeed be a key element. Even though the two sites are probably not equivalent, we do not think that either one is cycling rapidly (within <30 min like Ras) under steady state conditions, since this would have led to a drop in the palmitate signal within the first hour. In the palmitate pulse-chase experiments that we have performed, only about 10% was lost in the first hour and less than 40% after 5 hours (Fig. 1C). We now mention in the discussion that the slow palmitate loss indicates that depalmitoylation is slow on both sites.
In a study that goes beyond the present manuscript we are addressing, through a combination of modeling and experiments, whether and why it is important to have 2 palmitoylation sites.

4- The authors report that more than 90 % of endogenous calnexin is palm. However, DHHC up-regulation results in substantial incorporation of radiolabeled palm. Could this be an indication of rapid turnover of at least one palm? The authors should comment on these aspects.

During our study, this was also a very puzzling point for us. And you are very right to ask us to

comment on this point. This is now done at the bottom of page 10.
Palmitate addition is faster than depalmitoylation, which is very slow, thus the palmitoylated form accumulates. We have also observed (this is not part of the manuscript) that in addition, WT calnexin is more stable than palmitoylation deficient. As a consequence, the vast majority of calnexin is palmitoylated at steady state, as indicated by the 2D gel analysis. This palmitoylated population is "silent" in our experiments where we monitor incorporation of radiolabeled palmitate, since the sites are already occupied. Thus when measuring palmitate incorporation, we are only monitoring the population with free sites. The speed at which these sites get modified, during the 2 hours radioactive pulse, depends on the amount of enzyme expressed in the cells. Thus upon enzyme over expression, more molecules/sites become modified by unit of time.

5- *In Figure 1C, transfection efficiency is normally quite low. Thus, most of the cells are probably not expressing the Sar mutant. The data shown in Figure 1C, therefore, do not prove that endogenous calnexin acylation exclusively occurs in the ER. I think that this panel should be removed.*
Calnexin is efficiently retained in the ER. In my opinion, this is sufficient to conclude that its acylation occurs at the ER membranes. Alternatively, this experiment should be replaced with a co-transfection experiment (calnexin-HA + Sar-GTP).

We agree with the reviewer and have removed the Sar1 overexpression experiment.

6- Figure 1D-I do not think that the experiment with CHX is conclusive. How long was the CHX treatment is not mentioned in the text. Can it be excluded that the CHX treatment depletes cells from a (short living?) thioesterase thereby stabilizing palmitoylated calnexin? The authors could discuss this.

The length of the CHX treatment is now mentioned in the text and the legend. CHX was added 1hr before the 2 hrs palmitate labeling. Depalmitoylation is very slow. Following the reviewers comment, we now describe this observation before the effect of CHX. During the 2 hrs palmitate pulse, very little depalmitoylation, less than 20% of the labeled population, occurs even in the absence of CHX. If the thioesterase is short-lived, than this depalmitoylation would indeed not occur in the presence of CHX, and would lead to a small increase in the measured palmitate incorporation. Considering the minor contribution of depalmitoylation however, the conclusion remains that while newly synthesis calnexin can rapidly get palmitoylated, the majority (>70%) becomes palmitoylated 1 hr or more after synthesis.

7- Pages 7 and 16- Silencing of DHHC5 substantially decreases palm of endogenous calnexin. The fact that DHHC5 is mainly localized in the Golgi membranes does not exclude its presence in the ER membranes.
The inhibitory effect of its silencing on palm of endogenous calnexin is striking. As the authors write, it remains unclear why this is not reproduced for the recombinant calnexin. Understand this, goes probably beyond the scope of this work. However, based on the data shown here, I am not fully convinced that the authors can exclude a regulatory role for DHHC5 or can conclude that DHHC6 is the only palm enzyme involved in calnexin modification (Discussion), especially considering endogenous calnexin. It would actually be very interesting, in future work, to look at endogenous calnexin localization and substrate binding upon DHHC5 vs DHHC6 RNAi.

We again fully agree and thank the reviewer for these interesting suggestions. We have modified the text, now mentioning that DHHC5 might modulate DHHC6 function, since DHHC6 is palmitoylated and might be a DHHC5 substrate (bottom of page 8 & top page 9)

8- Pages 9-10, Figure 3-This figure is interesting. I do not think that the authors do comment it in sufficient details. To me, there seems to be a clear difference when calnexin is mono-palm at 502 vs 503. Could the presence/absence of palm at 503 (independent on the occupancy of 502) represent the elusive regulatory modification?

This is a very interesting question, which we plan to address in the future. Position 503 could well be regulatory.

We now discuss the figure in a bit more detail (bottom of page 11), keeping in mind that this is a simulation and thus only predictions can be made.

9- The data shown in Figure 3 (page 10, it should be Figure 4! And page 11, Figure S2 should be Figure S3!) would be much more convincing if co-localization experiments would have been performed (calnexin with an ER-resident protein that does not change its localization upon DHHC6 RNAi and calnexin and a nuclear envelope marker).

We have now performed co-localization experiments of calnexin with a nucleoporin, and quantified the change in colocalization at the nuclear membrane upon silencing of DHHC6 (Fig. 4). We now also show that the presence of calnexin at the nuclear membrane is restored upon recomplementation with ectopically expressed DHHC6 cDNA.

10- Do recombinant calnexins (wt, AC, CA, AA) have distinct localizations?
This would clearly be a very nice experiment to do. Unfortunately, as observed for other ER proteins such as sec61ß, ectopic expression leads to expression through out the entire ER, even for the WT calnexin in DHHC6 silenced cells.

Do they bind glycoproteins differently and/or does endogenous calnexin immunoisolated from untreated vs DHHC6 RNAi-treated cells bind glycoproteins differently? This would support the authors's claim that the palm of calnexin is essential for substrate binding (pages 2 and 5).
This is an important point also raised by Reviewer III. We have now monitored substrate binding of WT and palmitoylation deficient calnexin and we find that substrate binding is completely lost for the mutant chaperone (Fig. 7F). This finding further demonstrates that palmitoylation is essential for substrate binding. It however does not mean that non-palmitoylated calnexin is unable to bind monoglocosylated substrates per se, just that in the cellular context this does not occur. Two possibilities can be envisioned: 1) cytosolic palmitoylation affects the lectin activity on the luminal side of the chaperone; 2) the lectin activity is not affected but when co-translational interaction with calnexin does not take place than post-translational interactions cannot occur. These two options are discussed in the manuscript.

Page 12-Figure S2B should be Figure S3!

Page 12-The decrease is 40%, not 60.

Figure S4A, the authors should explain what is Pat6, and why it has been used here.

These corrections have been made. We apologize for Pat6, this is the lab jargon for DHHC6: Palmitoyl Transferase.

11- Figures 5B and 5C-More than 90% of calnexin is palm (page 8) and palm is very stable. It is unclear to me why the over expression of DHHC6 should increase the association with the RTC (5B, 5C) or, quite dramatically (+50%), the association with actin (6E).

Historically we had performed the DHHC6 overexpression experiments before we realized that more than 90% of calnexin was palmitoylated. Now knowing this, there is no rational for overexpressing DHHC6 and we have therefore removed these experiments from the manuscript and should have earlier. Consistent with the fact that calnexin is almost 100% palmitoylated, DHHC6 overexpression had little effect. Except, as noted by the reviewer, on actin binding. We think that this is due to the fact that DHHC6 must have other ER substrates and thus this is partly indirect.

12- Page 12-Is it known whether the Ser653 calnexin mutant is also excluded from the nuclear envelope?
Again very interesting question. Unfortunately, due to the fact that ectopic expression does not reproduce endogenous localization we cannot provide an answer at present.

Page 13-Figure 7 should be Figure 6!
This was corrected. We apologize for the mislabeling through out.

13- Page 13-Does Latrunculin A affect the localization of endogenous calnexin?
Latrunculin A treatment severely affects cell morphology and thus modification of calnexin staining cannot be readily interpreted at this stage.

14- Figure 7 is not fully convincing. I could assume that calnexin inactivation may affect folding and secretion of a select population of glycoproteins. However, why should the silencing of calnexin specifically reduce the synthesis of glycoproteins is unclear. What about the synthesis of non-glycosylated proteins expressed in the ER?
We agree that synthesis should not be affected. Our wording was unclear and has been corrected. We did not specifically probe for synthesis, which we indeed assume to be unaffected. We monitored biogenesis, which includes synthesis and folding.

15- The authors write, and their interesting model implies, that calnexin palm is crucial for efficient capture of nascent polypeptide chains (pages 2 and 5). It seems crucial to directly assess the capacity of calnexin (endogenous and/or recombinant) to bind substrates under the different experimental conditions.
As mentioned above, we now show that palmitoylation deficient calnexin fails to bind its substrates (Fig. 7F), further supporting the proposed model.

Page 15, Figure 4F should be Figure 7E!
This was corrected

All the minor points have been addressed.

Answers to Referee #2

1. The abstract states that palmitylation triggers a conformation change in calnexin. No evidence is presented that proves this is the case. This is just a prediction based upon molecular dynamic simuations so should not be presented as a fact.
We have removed any mention on conformation from the abstract and text.

2. A significant portion of the paper describes experiments where proteins are depleted from cells by RNAi and then the effect of this depletion is assayed. The conclusion drawn is that the effect is due to the specific depletion, however, this is a dangerous assumption to make without the proper controls. For example the depletion of DHHC6 causes a change in cell morphology and localisation of calnexin away from the nuclear envelope. These consequences could easily be explained by off target effects of DHHC6 depletion or even the induction of an unfolded protein response.
To prove this effect is really due to the DHHC6 depletion leading to a lack of palmitylation of calnexin one would need to show that the effect is reversed when an RNAi insensitive version of DHHC6 is co-expressed with the RNAi.

The reviewer is absolutely correct that recomplementation experiments are important to rule out off target effects. The fact that our RNAi experiments were supported by the studies on mutant calnexin in the absence of any silencing tend to rule out the off target effect. We now confirm this showing that palmitoylation of calnexin is restored upon recomplementation of DHHC6 (Fig. S2C), as is its interaction with the RTC (Fig. 5BC) and its localization to the nuclear membrane (Fig 4C).
Whether silencing DHHC6 triggers UPR is an interesting point. We have performed a gene profiling analysis upon DHHC6 silencing and found that although slight changes in expression were observed for GADD45 (-1.35 fold), BiP (1.32 fold), XBP1 (1.4 fold), HERP (1.56 fold), the changes were very minor compared to what is observed upon UPR triggering, as described by the Glimcher group where changes of 4 to 27 fold were observed for these genes (Mol Cell Biol. 2003 23: 7448–7459). Also we did not observe significant XBP-1 splicing, nor an increase of the BiP protein levels.

3. No mention is made in the paper of the known association of calnexin with mitochodrial associated membranes (MAMS). Disruption of this interaction by a lack of palmitylation could be an important aspect of calnexin function so should be addressed.

This is clearly an interesting point that we had planned addressing in future studies. During the revision of the present manuscript a paper was however published in EMBO J showing that association of calnexin with MAMs requires its palmitoylation. These findings are now mentioned in the discussion.

4. The assay for calnexin association with the RTC is problematic. It has been known since the early work of Ari Helenius' group that calnexin co-immunoprecipitaes with membrane proteins due to its inclusion in detergent/lipid micelles. It is therefore very difficult to interpret whether there is actually a protein/protein interaction in these co-immunoprecipitation studies.

In principle we fully agree that without proper controls co-precipitation of membrane proteins could be due simply to the inclusion in the same detergent /lipid micelles. We however find that the interactions are lost upon TRAPα silencing, when point mutants of calnexin are analyzed or upon DHHC6 silencing. This important point is now discussed in the text.

For client proteins this direct interaction can be confirmed by regulating the interaction following de/re-glucosylation.
We now show that the interaction with client proteins does not occur for palmitoylation deficient calnexin.

In addition anyone carrying out immunoprecipitation will know that actin is invariably a contaminant in these precipitates due to its cellular abundance and its ability to interact with proteins following cell lysis. Chemical crosslinking followed by very stringent immunoprecipitation may provide more conclusive proof that these proteins do actually interact in the cell.

Indeed finding an interaction with actin might often be completely unspecific. We however find that the interaction is lost upon DHHC6 silencing or when expressing calnexin mutants, arguing against actin being a contaminant in these interactions. The role for actin is moreover supported by latrunculin treatment both on interaction with the RTC and in PrP folding. This important point is now discussed in the text.

The association of calnexin with the ribosome has been suggested previously and is not a novel observation (The EMBO Journal (1999) 18, 3655 - 3666).
We are well aware of this paper, which is mentioned multiple times in the manuscript and which led us to include the S563 mutant in our analysis, and we confirm the findings of the Bergeron group.

5. The final series of experiments states that they address the effect of calnexin palmitylation on the folding of proteins. However the assays used to not address folding directly, rather protein trafficking and secretion. Assays for folding would be easy to establish so either the authors change their claims to state that calnexin palmitylation affects the secretion of glycoproteins or they need to set-up some folding assays.
We have modified the text to clearly state that the assays monitor secretion, mentioning that secretion will not occur if folding in the ER is deficient.

6. The tile of the paper states that the chaperone function of calnexin is controlled by palmitylation. In no part of the paper do the authors address a putative chaperone function. Is this distinct from its functions as a lectin binding to monoglucosylated substrates?
We have now added experiments showing that palmitoylation deficient calnexin cannot bind its substrates (Fig. 7F). The function is indeed to bind mono-glucosylated substrates and thus protect them from aggregation/misfolding.

7. There are several errors in the manuascript (too many to list). These range from the inaccurate figure numbering to errors in grammer and spelling. There are several instances of inappropriate terminology which does not help when reading. For example what is "the oligosaccharide tree" and what does "the public domain chaperone" mean?

We apologize for the many errors and have done our best to remove possibly all. We have changed the terminology.

Answers to Referee #3

1) From the experiments it is evident that cnx is palmitoylated, using radioactive palmitate. It is not clear, however, whether other forms of acylation are possible or that cnx is decorated exclusively with palmitoyl chains.
The authors could check whether a mix or a selection of radioactive fatty acids other than palmitate yield any incorporation into cnx.

This is a very interesting general question related to palmitoylation of membrane proteins and we will plan such experiments for the future. We now specified in the introduction that S-acylation is not restricted to the attachment of C16 and may involve addition of other chains. It has indeed been shown for viral proteins that cysteines in the continuation of the transmembrane domain (as is the case for calnexin) receive a stearate (C18) while more distal cysteines receive a palmitate. In most of the text, "palmitoylation" was replaced by "S-acylation" except when 3H-palmitate incorporation was concerned.

2) The authors show on the one hand that overexpression of DHHC6 leads to a substantial increase in palmitoylation of cnx, but on the other hand claim that at steady state over 90% of cnx is palmitoylated and that this modification is stable. The two claims are hard to rhyme, unless there is a kinetic explanation.
This issue could be solved by a pulse-chase with 35S labeling of cnx, monitoring palmitoyl incorporation into newly synthesized cnx, by immunoprecipitation of cnx and running the samples both on 1D and 2D gel electrophoresis, at several time points (e.g. 0, 15, 30, 60 & 120 min. after a 5-10 min pulse) for both control DHHC6 knock-down and DHHC6 over-expressing cells. While for the 1D gel the autoradiogram signal should remain constant, the signal for the 2D gel should diminish, as the cnx signal spreads out upon palmitoylation, according to the data shown in figure 2E. If palmitoylation is post-translational and if it takes quite a while before newly synthesized cnx is fully acylated, DHHC6 overexpression may speed up the process, which would solve the apparent discrepancy.

This important point was also raised by reviewer 1 and it was initially quite a puzzle for us as well. We should have explained this point in the original manuscript and now have on page 9, following the 2D gel analysis.
The reviewer is exactly right, palmitoylation is mostly post-translational as shown in figure 1C and the effect is kinetic. As shown by the 2D gels, the bulk of calnexin in the cell is acylated and probably dual acylated. The calnexin molecules in which the cysteines are S-acylated are silent in our 3H-palmitate incorporation assays.
We thus monitor the amount of palmitate that is incorporated, during the 2hr-labeling period, into the calnexin population that has free sites. The amount of palmitate that gets incorporated per unit of time depends of course on the size of the pool of calnexin that has free sites but also on the amount of enzyme expressed by the cell.
Upon DHHC6 overexpression, the amount of palmitoylation that is incorporated into calnexin increases during 2hrs is higher than for control cells due to the fact that the speed at which calnexin acquires the acyl chain is increased as shown in the figure for the reviewer (not included here).

3) The authors demonstrate by co-immunoprecipitation that cnx interacts with TRAPalpha, Sec61alpha, the ribosome and also with actin, while cnx devoid of palmitoyl groups no longer does so. The conclusion is that cnx is a bona fide member of the ribosome-translocon complex (RTC). This interesting finding has profound implications on the interpretation of the remainder of the experiments.
For one, the finding that actin interacts to a lesser extent with non-palmitoylated cnx may be indirect, as actin may only hook up to the RTC once it is fully assembled (i.e. in conjunction with cnx). The interpretation of the results needs to be rewritten to acknowledge such a scenario.

We agree with the reviewer and actually addressed this point experimentally while the manuscript was being reviewed. We find that if the ribosomes are stripped of the ER by a short puromycin treatment, then calnexin does not interact with actin or with the RTC anymore. Thus as the reviewer

suggested, actin only hooks on when the full supercomplex is assembled. It subsequently stabilizes the complex since latrunculin A treatment leads to supercomplex disassembly. This new data has been added to Fig. 6EF.

Moreover, cnx function as a chaperone in the ER lumen may be unimpeded when it lacks palmitoyl moieties. The rationale for a decrease in secretory capacity or synthesis of glycoproteins may be solely due to the fact that the RTC is not functioning properly when it cannot team up with cnx and thereby reduces the flux of ER clients, but not the chaperone activity of cnx per se.

To address this question, the authors could exploit again a 35S pulse-chase followed by immuno-precipitation of cnx and follow the association of glycoproteins over time as originally established by Ou et al, 1993. Although the initial signal directly after the pulse may be lower, the kinetics of dissociation of newly synthesized glycoproteins over time may be identical. If so, the chaperone function of cnx is arguably unaltered, yet its crucial role as part of the RTC in guiding newly synthesized proteins into the ER lumen is disrupted once the palmitoyl moieties are absent.

We thank the reviewer for this suggestion and we have performed this experiment comparing WT calnexin with palmitoylation deficient calnexin and we find that palmitoylation deficient calnexin is unable to bind any substrate, the signal being very similar to that of WT calnexin in the presence of the glucosidase inhibitor castanospermine. Also silencing of DHHC6 leads to a strong decrease in the binding of endogenous calnexin to substrates.
We discuss that the following scenarios are possible: 1) palmitoylation deficient calnexin is unable to bind monoglucosylated proteins, 2) if calnexin can not bind its substrate as it emerges from the translocon, then if can never capture it. We cannot distinguish between these to possibilities in the cellular context. Distinguishing these to possibilities would require setting up an in vitro or semi in vitro system where we could provide calnexin, palmitoylated or not, with unfolded monoglucosylated substrates. It is not clear where this experiment can actually be done. We therefore mention the two options in the discussion.

In that scenario also the title should be changed, for instance into "Calnexin is palmitoylated to serve as key component of the ribosome-translocon complex"

We have changed the title as suggested since we did not show that the ability of calnexin to bind monoglucosylated substrates is per se affected by palmitoylation. Actually I would be somewhat surprised if it did affect this function. In any case, in the cellular context, non-palmitoylated calnexin cannot “perform” its function.

Minor issues

4) Could the effect of the DHHC5 knock-down be explained by an indirect effect? For instance, is also DHHC6 palmitoylated? Is DHHC5 responsible for that?
Indeed the effect of DHHC5 silencing is probably indirect. Also DHHC6 is palmitoylated (Gorleku et al, 2011) and DHHC6 palmitoylation could indeed be mediated by DHHC5. This possibility is now mentioned in the text.

5) Could the authors discuss better why palmitoylation is key for localization of cnx to the nuclear envelope? What is their hypothesis?
At present we do not know why exactly palmitoylation affects localization. We do know that it is not due to interaction with the RTC complex since silencing TRAPα does not affect calnexin localization. Our hypothesis is that the lipid/protein composition of the nuclear membrane/ER sheet is different from that of the ER tubules and that palmitoylated calnexin preferentially partitions in the former. This is now clarified in the text.

6) Could the authors perhaps reorganize the text with care? Several sentences are grammatically incorrect or difficult to interpret.
The text has been extensively edited to correct grammar and clarify the meaning.

2nd Editorial Decision 22 December 2011

Thank you for sending us your revised manuscript. Our three original referees have now seen it again, and you will be pleased to learn that they now all support publication in The EMBO Journal.

Prior to acceptance, there are a number of editorial issues that need further attention:

* You may wish to look into the minor points put forward by referee 1, and include the suggested corrections.

* Please include an author contribution section and a conflict of interest statement into the main body of the manuscript text after the acknowledgements section.

* Could you please clarify the number of independent repeats in the legends of figures S2 and S3?

* With respect to the title, I do like the content of referee 3's version, but I agree that the language could still be smoothened. How about: 'Palmitoylated calnexin is a key component of the ribosome-translocon complex"?

Thank you very much again for considering our journal for publication of your work. I am looking forward to your amended manuscript files.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The authors convincingly addressed most of my concerns/suggestions.

The major improvement of the new submission are data shown in figure 7F (whose left panel is however mislabelled). The authors should also add MW markers.

Page 21, Ohno et al talk about 12 and not 17 DHHC in the secretory pathway.

Referee #2 (Remarks to the Author):

The paper is now suitable for publication without any further corrections.

Referee #3 (Remarks to the Author):

The authors have presented in a clear and convincing manner that calnexin is S-acylated and that this modification allows the protein to become part of the ribosome-translocon complex, which in turn is essential for glycoprotein clients to enter into the ER lumen and fold. These findings are fundamental to understanding glycoprotein biogenesis and will appeal to the broad readership of EMBO Journal.

To my opinion all issues raised by the reviewers have been addressed in an adequate manner. I recommend that the manuscript be published with no further modifications at the editor's earliest convenience

2nd Revision - authors' response 02 January 2012

Please find enclosed the corrected version of our manuscript EMBOJ-2011-79655R.
The following requested changes have been made:

- an author contribution section and a conflict of interest statement has been included in the main body of the manuscript text after the acknowledgements section.

- the number of independent repeats is now mentioned in the legends of figures S2 and S3

- the title has been modified according to your suggestion: Palmitoylated calnexin is a key component of the ribosome-translocon complex
- The labeling in Fig. 7 has been corrected and the MW markers have been added.

- Page 21, we now mention that Ohno et al found 12 DHHC in the ER and that our personal observations suggest there are 16.

I thank you for the smooth reviewing process. I don't know if you keep a database of your reviewers. If you do, I think that reviewers I and III really did an excellent job. Their questions were really good, they were very fair. It was a pleasure to address their comments.

