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NgBR is necessary for cellular dolichol biosynthesis and protein N-glycosylation

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1st Editorial Decision 06 August 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. Let me first of all apologise for the delay in getting back to you with a decision. Unfortunately, we experienced difficulties in finding suitable and willing referees for this manuscript during the current summer holiday period.

Your manuscript has now finally been seen by three referees whose comments to the authors are shown below. As you will see while all three referees consider the study as interesting in principle they all feel that there is one key shortcoming that is central to the conclusiveness of the study and thus precludes publication here at this point. This caveat refers to the evidence provided for the conclusion that NgBR occurs in two different topologies within the ER membrane that have two different functional roles. Furthermore, the referees think that deeper insight into the interaction of NgBR and hCIT needs to be provided. Also, a number of other points are raised. Now, the problem really is that the major shortcoming affects key aspects of the experimental evidence provided, that further experimentation would be required to address these issues and that the outcome of such experiments cannot be predicted at this point. Given these uncertainties I see little choice but to come to the conclusion that we cannot offer to publish the manuscript at this point.

We would, however, not exclude the possibility to consider a new submission on the same topic should future studies allow you to strengthen the study considerably along the lines suggested by the reviewers and to provide considerably stronger data for two different NgBR topologies of different function as well as deeper understanding of its interaction with hCIT. I need to stress, however, that

if you wish to send a new manuscript this will be treated as a new submission rather than a revision and will be evaluated again at the editorial level and reviewed afresh involving our original referees if available at the time of resubmission, also with respect to the literature and the novelty of your findings at the time of resubmission. At this stage of analysis, though, I am sorry to have to disappoint you.

Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is an interesting paper that (i) introduces what appears to be an example of a membrane protein with two distinct membrane topologies and (ii) offers insights into the biosynthesis of dolichol, a key isoprenoid compound with a well documented role in glycosylation. The first point is not addressed in the detail that it deserves. However, at a basic level the results concerning the second point are clear - cis-isoprenyl transferase activity is clearly influenced by NgBR, and consequently there are discernible effects on the levels of dolichol-linbked oligosaccharides and N-glycosylated proteins. However, the approach taken for individual experiments is frequently circuitous. For example, the matter of alternate topologies cannot be convincingly addressed by deleting transmembrane domains; it would be better to introduce insertions (such as a specific protease cleavage site) whose orientation could be probed in intact microsomes. Also, the paper is poorly written, logically convoluted and unnecessarily long. Methods are inadequately described and figures are not properly explained. I illustrate these criticisms with some specific examples below:

1. Figure 1, as well as the paper in general, would benefit from a schematic illustration showing the topologies of the NgBR protein and its binding interactions with NPC2 and hCIT, presumably both in the context of the ER.

2. page 6, top. It is strange to report a discovery of conserved Asn residues in this way. Only two sequences (presumably mouse and human) are compared, so the extent of the conservation is unclear. Are there any other 'non-conserved' Asn residues that nevertheless constitute N-glycosylation sites? Which NgBR was used in this study? hNgBR or mNgBR? The effect of N->Q substitutions is not fully interpreted - site-selection by Stt3 isoforms may cause the N271Q substitution to eliminate glycosylation at both sites while the N144Q substitution causes loss of glycosylation at a single site.

3. The mannose uptake data are poorly described. The legend to Fig. 2B is not helpful and the experimental procedures indicate that the cells were lysed in RIPA buffer after metabolic labeling with [2-3H]mannose. Presumably the cells were first washed? What was the uptake at zero time of incubation (this would establish background). The data are presented as dpm/mg/min - this is hardly warranted based on a single 4 hr time point. Was uptake linear? Etc.

4. Fig. 3A: is this an immunoblot? Fig. 3B: What is Ad-NgBR? Given the impression of total knockdown of NgBR in Fig. 3A, and the eventual conclusion that NgBR is required for dolichol biosynthesis, why is there residual Man-P-Dol and Glc-P-Dol (Fig. 3C and 3D)? The face gel for these panels should be shown.

5. Page 9, line 4: the authors have chosen a curious pair of citations. The Kinoshita reference is irrelevant, and the Kornfeld reference could be replaced by the more relevant Schenk et al. review.

6. The authors begin a logical sequence of experiments on page 7 by stating the homology between the C-terminal portion of NgBR and cis-IPTases. This point is lost by the time they arrive at the conclusion on page 9 that NgBR potentiates cis-IPTase activity. This section could be better written, with the logic more clearly presented. The same point is mis-stated or lost at the start of the new section on page 9 where the authors try to connect the fact that NgBR has homology to cis-IPTase with the idea that it influences cis-IPTase activity. If it is homologous, it may have intrinsic activity. Homology has no bearing on whether it can influence a genuine, other cis-IPTase! All of this is confounded by a statement that appears one page later (page 10, middle) in which the authors' state that NgBR does not contain many of the residues needed for catalytic activity and IPP binding. In other words, the cis-IPTase homology idea is a red herring

7. Figure 6: Are the tagged hCIT proteins functional? What is WCL?

8. The last paragraph of the introduction needs to be written in a more illuminating way to be accessible to the readership of the journal.

Referee #2 (Remarks to the Author):

The study is impressive in that numerous approaches are utilized, and the overall experimental design usually includes the use of adequate controls. Taken together, the generated data strongly indicate that the Nogo-B receptor (NgBR) functions as an essential component of the Dol-P biosynthetic machinery. The authors conclude that NgBR interacts with the previously identified cis-isoprenyltransferase (cis-IPTase), thereby enhancing hCIT protein stability (which, in turn, promotes Dol-P production). However, despite these observations, the study stops short of yielding a sufficient amount of new mechanistic information to warrant publication at this time. In particular, a specific feature of their model needs to be more directly tested beyond the realms of their expression studies. I'm referring to their analysis of NgBR topology in the ER (which is central to the study) which is presently indirect, and therefore, not complete. Also, a few of the comments lack clarity and conclusions are speculative in that multiple alternate interpretations are valid. Finally, the immunolocalization study is incomplete, and I don't know that it is even needed to make their point.

-The introductory material provides a comprehensive background of the subject matter, and adequately stresses the importance of understanding the Dolichol monophosphate (Dol-P) biosynthetic pathway. However, because a general audience will eventually read the article, the authors should provide some more explanation as to why asparagine-linked glycosylation is important in cell biology.

-Figure 1 depicts the consequences of endo H treatment. A problem exists in that a longer gel is shown in panel C, than is shown in panel B. Panel C reveals the existence of a faster migrating band (even for the WT protein) that is absent in panel B. For this reason, it is nearly impossible to interpret the data.

-Again, in regard to the endo H experiments, more information about the entire amino acid sequence should be shown or stated in order to conclude that "these two sites [referring to N114 and N271] are the only mannose-linked residues in NgBR"? In addition, a longer gel is needed to convince the reader of that conclusion (as stated above). Also, why do the authors refer to "mannose-linked residues" rather than to "N-linked glycans"?

-From the combination of results generated from the endoH study plus he NgBR TM deletion constructs, the authors conclude that the molecule exhibits multiple topogologies in the ER membrane. Although the multiple lines of experimentation support this notion, the dats are only circumstantial. For example, it is possible that the C-terminus is always oriented toward the lumen, but that an interacting protein is preventing the glycosylation of most of the molecules. Because their conclusion is central to the entire study, I suggest that a set of experiments be performed that will directly test this important prediction. One possibility would be to perform a protease protection analysis in which the plasma membrane of cells is selectively permeabilized, followed by incubation with an exogenously added protease. An altered sensitivity to this manipulation would verify the

existence of an altered membrane topology. This (or another direct technique) should be performed for the wildtype protein and each of the TM deletion constructs.

-For that same study, it is also important to remember that any data generated from their TM deletion constructs might reflect the consequences of protein misfolding. This possibility is not even mentioned. For that matter, how do they know that data generated from the mutated glycosylation sites does not reflect the consequences of protein misfolding? This too, should be mentioned.

-On page 10, the authors describe a sequence alignment of NgBR with characterized cis-IPTases, but fail to show the data, even as a supplemental figure. In the very next paragraph, they state that the C-terminal region of NgBR is conserved among several species. Either, this data should be shown or at least stated in a quantified manner. In other words, what degree of homology is detected? This information is important if it justifies the performance of subsequent experiments.

-On page 11, I appreciate that the authors mention the strong membrane morphology phenotypes visualized by electron microscopy, but a more detailed description is required. Just exactly what is the reader supposed to identify?

-On page 12, the authors show that NgBR-GFP and hCIT co-localize in both a reticular and punctate pattern of intracellular distribution (Figure 6D), and then use this as evidence to support the notion that the molecules interact within the lumen of the ER. The conclusions are premature because organelle markers must be utilized to identify the intracellular locations. Also, the punctate pattern might represent the Golgi complex (but this can't be established without the use of markers). If this is the case, then the two proteins might actually cycle between the ER and Golgi. The data, as shown, bring up more questions than answers. I suggest that the data either be removed, or a full study be performed.

Referee #3 (Remarks to the Author):

In this study, Harrison et al., demonstrate that NgBR, a protein previously shown to stabilize the cholesterol-binding protein NPC2, is also involved in dolichol biosynthesis. The authors show that in mammalian cells, NgBR interacts with and appears to stabilize cis-IPTase. Knockdown of NgBR decreased protein mannosylation, mannose-, glucose-, and G3Man9-linked dolichol lipids, isoprenyl transferase activity, and dolichol lipid levels. However, neither NgBR nor its yeast homolog Nus1, restored dolichol synthesis in a rer2 conditional knock-out strain. The authors therefore conclude that NgBR functions as a positive regulatory factor for cis-IPTase activithy. These findings add new insight into dolichol lipid biosynthesis and its complex mechanism of regulation in N-linked protein glycosylation.

An intriguing implication, that remains rather enigmatic, is the apparent dual role of the NgBR C-terminus in stabilizing NPC2 in the ER lumen and cis-IPTase in the cytosol. In order to explain these activities, the authors provide a vague hypothesis in which the C-terminus resides partly in the ER lumen and partly in the cytosol. This conclusion is based on inefficient glycosylation of two C-terminal N-linked consensus sites and the observation that removal of the second of three putative TMs has no effect on the glycosylation pattern. Unfortunately, no topological model is provided and it is difficult to envision one that would fit the data. It is equally conceivable that the C-terminus resides in the ER lumen and is simply inefficiently glycosylated. Additional studies are needed confirm the topology and justify these conclusions. Similarly, while removal of the NgBR C-terminus prevents co-immunoprecipitation with hCIT, this could occur for trivial reasons and does not confirm a direct role in binding. Homology of the NgBR C-terminal domain with cis-IPTase also remains unexplained. Some mechanistic understanding of how this domain might interact with its proposed binding partners would strengthen the study.

Minor concerns include:

1. If dolichol levels are undetectable in RNAi treated cells (Figure 4E), then what is the nature of the lipid linked oligosaccharides shown in Figure 2A and S1. While the inhibitory effects of siRNA on G3M9 are generally convincing, results for the M5 species are not (Fig 3A and Fig S1B)

2. Additional information is needed to interpret how free Asn-linked glycans were assessed (fig S1C-E). At least some description should be provided. I would recommend expanding the supplementary information to include more detailed methods.

3. Figure 4E is critical to establish a role of NgBR in dolichol synthesis, but the quality of the data is so poor that it is largely uninterpretable. A better gel with proper controls is needed.

4. Figure 5D nicely demonstrates that Nus1 and NgBR do not restore dolichol synthesis in the rer2 yeast strain. However contrary to the statement on page 11, the data do not show that Nus1 (or NgBR) activity requires rer2. These are two independent conclusions, given that all other experiments were carried out in mammalian cells. A similar analysis in yeast, using a Nus-1 conditional mutant (or knock out) supplemented with rer2, would probably resolve this issue.

5. Much of the discussion is poorly connected to the main point of the paper. For example, the connection between cholesterol homeostasis by NPC2 and cis-IPTase is not really addressed here. Emphasis on these two disparate functions leaves the reader wondering what is going on. The possibility that NgBR has multiple topologies is also a minor point that is poorly supported by the data. This topic would probably be best dealt with in a separate, definitive study. Similar concerns hold for the dolichol-dependent microdomains.

6. In Figure 3a, "LLO mixture" and "glc oligo mixture" should be better defined.

7. When referring to lane numbers in gels, it is helpful to number the lanes.

Resubmission 20 December 2010

Referee 1:

We thank the reviewer for the comments and insights regarding improvement of the manuscript. We have performed the suggested experiments and modified the text describing the methods and figures in an attempt to make the logic of the paper more easily accessible. We have addressed each point cited by the reviewer as found below.

1. Figure 1, as well as the paper in general, would benefit from a schematic illustration showing the topologies of the NgBR protein and its binding interactions with NPC2 and hCIT, presumably both in the context of the ER.

We agree that the first figure would be improved by the addition of a schematic outlining the topologies of NgBR, and we have included this model within Figure 1 and Figure 7.

2. page 6, top. It is strange to report a discovery of conserved Asn residues in this way. Only two sequences (presumably mouse and human) are compared, so the extent of the conservation is unclear. Are there any other 'non-conserved' Asn residues that nevertheless constitute N-glycosylation sites? Which NgBR was used in this study? hNgBR or mNgBR? The effect of N->Q substitutions is not fully interpreted - site-selection by Stt3 isoforms may cause the N271Q substitution to eliminate glycosylation at both sites while the N144Q substitution causes loss of glycosylation at a single site.

We have included a more thorough analysis of the N144 and N271 glycosylation sites (Figure 1A). There are no other ‘non-conserved’ N-X-Ser/Thr sites in NgBR that may serve as N-glycan acceptors in NgBR; this important distinction has been noted within the text. These studies of NgBR topology are focused on human NgBR; this has also been made more apparent within the figure legend and the text. While we agree that site selection by Stt3 isoforms is of interest in regards to the differential loss of EndoH sensitivity seen with mutation of N144 and N271, we think that a detailed analysis of the role of Stt3 in this process would constitute a separate study. However, this is an important point, and we now include this possibility as a point of discussion within the text.

3. The mannose uptake data are poorly described. The legend to Fig. 2B is not helpful and the experimental procedures indicate that the cells were lysed in RIPA buffer after metabolic labeling with [2- 3H]mannose. Presumably the cells were first washed? What was the uptake at zero time of incubation (this would establish background). The data are presented as dpm/mg/min - this is hardly warranted based on a single 4 hr time point. Was uptake linear? Etc.

We have included a more thorough description of the mannose uptake experiments in the methods section and within the figure legends. Media containing labeled mannose was removed and cells were washed twice with PBS prior to cell lysis. Preliminary experiments were performed to determine the rate of mannose uptake in these cells, and uptake was linear at the 4h timepoint used for analysis. Uptake at t=0 was minimal (0.04% of Ctrl RNAi-treated uptake at 4h timepoint). This information is now stated within the methods section and the corresponding figure legend.

4. Fig. 3A: is this an immunoblot? Fig. 3B: What is Ad-NgBR? Given the impression of total knockdown of NgBR in Fig. 3A, and the eventual conclusion that NgBR is required for dolichol biosynthesis, why is there residual Man-P-Dol and Glc-P-Dol (Fig. 3C and 3D)? The face gel for these panels should be shown.

Figure 3A is an immunoblot. We achieve an approximate 90% reduction in NgBR protein levels following 48h of siRNA treatment. The residual Man-P-Dol and Glc-P-Dol likely results from effects related to the remaining ~10% of NgBR that is refractory to siRNA treatment. Moreover, dolichol turns over slowly, with a half-life of 1-2d (Andersson, et al. FEBS Letters. 269:15-18), so we feel that the magnitude of lipid-linked oligosaccharide loss that we observe is reasonable. Due to this partial loss of LLOs, we think that scanner quantitation as a description of loss of Man-P-Dol and Glc-P-Dol is much more readily appreciated by the reader, rather than presentation of raw FACE gels. These quantitative data represent n=5 for the NS ctrl RNAi and n=6 for the NgBR RNAi samples. This information is now included within the methods section and the legend for the corresponding figure.

5. Page 9, line 4: the authors have chosen a curious pair of citations. The Kinoshita reference is irrelevant, and the Kornfeld reference could be replaced by the more relevant Schenk et al. review.

We have made the requested reference changes.

6. The authors begin a logical sequence of experiments on page 7 by stating the homology between the C-terminal portion of NgBR and cis-IPTases. This point is lost by the time they arrive at the conclusion on page 9 that NgBR potentiates cis-IPTase activity. This section could be better written, with the logic more clearly presented. The same point is mis-stated or lost at the start of the new section on page 9 where the authors try to connect the fact that NgBR has homology to cis-IPTase with the idea that it influences cis-IPTase activity. If it is homologous, it may have intrinsic activity. Homology has no bearing on ! All of this is confounded by a statement that appears one page later (page 10, middle) in which the authors' state that NgBR does not contain many of the residues needed for catalytic activity and IPP binding. In other words, the cis-IPTase homology idea is a red herring.

We have re-written the indicated sections of the text to impart a more clear and concise description of the logic behind the experimental design, as requested by the reviewer. These points are also now mentioned within the discussion section. Two pieces of evidence suggest that NgBR does not contain intrinsic cis-IPTase activity: 1) Miao et al. did not detect intrinsic catalytic activity associated with NgBR following immunoprecipitation (Miao, et al. Proc. Natl. Acad. Sci. 103: 10997-11002); 2) We do not observe a rescue of the rer2Δ growth nor glycosylation defects following NgBR expression. The lack of catalytic and IPP binding residues (through comparison with the bacterial cis-IPTase UPPS) does suggest that this lack of rescue of the *rer2* mutant is expected. The data which follows this observation strongly suggest that the mechanism by which NgBR is necessary for cis-IPTase activity is via its interaction with hCIT, promotion of hCIT stability, and potentiation of hCIT-dependent dolichol synthesis in the rer2Δ strain.

7. Figure 6: Are the tagged hCIT proteins functional? What is WCL?

The tagged hCIT proteins are functional as assessed by rescue of the rer2Δ phenotype (growth and

glycosylation defects – Figures 5B-C). WCL is 'whole cell lysate.' We have provided a better description of the methods within the appropriate figure legends.

8. The last paragraph of the introduction needs to be written in a more illuminating way to be accessible to the readership of the journal.

We have re-written part of the introduction to include background information regarding Asn-linked glycosylation in the hopes of making this section more accessible.

Referee 2:

We appreciate the reviewer's suggestions for improvement of the manuscript. We have incorporated experiments to address the reviewer's concerns and have modified the text to enhance its clarity. We have addressed each point cited by the reviewer as found below.

-The introductory material provides a comprehensive background of the subject matter, and adequately stresses the importance of understanding the Dolichol monophosphate (Dol-P) biosynthetic pathway. However, because a general audience will eventually read the article, the authors should provide some more explanation as to why asparagine-linked glycosylation is important in cell biology.

We have included a paragraph to outline the importance of Asn-linked glycosylation in cell biology as suggested by the reviewer.

-Figure 1 depicts the consequences of endo H treatment. A problem exists in that a longer gel is shown in panel C, than is shown in panel B. Panel C reveals the existence of a faster migrating band (even for the WT protein) that is absent in panel B. For this reason, it is nearly impossible to interpret the data.

We have repeated the experiments from the original panel B and panel C several times in multiple cell lines. The presence of the faster migrating bands is a phenomenon that is not seen consistently. For this reason, we have more confidence in the result depicted in panel 1B, and therefore include a new figure describing EndoH treatment of the NgBR TM mutants in panel C (now Figure S1A). We favor the hypothesis that the faster migrating bands seen in some experiments result from overexpression of the protein, and therefore vary with different levels of expression between experiments. Regardless of this varied expression, however, in every experiment we see two bands which migrate slower and are sensitive to EndoH treatment.

-Again, in regard to the endo H experiments, more information about the entire amino acid sequence should be shown or stated in order to conclude that "these two sites [referring to N114 and N271] are the only mannose-linked residues in NgBR"? In addition, a longer gel is needed to convince the reader of that conclusion (as stated above). Also, why do the authors refer to "mannose-linked residues" rather than to "N-linked glycans"?

No additional N-X-Ser/Thr residues exist in the NgBR sequence other than the N144 and N271 sites outlined in Figure 1. This distinction is now mentioned within the text.

-From the combination of results generated from the endoH study plus the NgBR TM deletion constructs, the authors conclude that the molecule exhibits multiple topogologies in the ER membrane. Although the multiple lines of experimentation support this notion, the data are only circumstantial. For example, it is possible that the C-terminus is always oriented toward the lumen, but that an interacting protein is preventing the glycosylation of most of the molecules. Because their conclusion is central to the entire study, I suggest that a set of experiments be performed that will directly test this important prediction. One possibility would be to perform a protease protection analysis in which the plasma membrane of cells is selectively permeabilized, followed by incubation with an exogenously added protease. An altered sensitivity to this manipulation would verify the existence of an altered membrane topology. This (or another direct technique) should be performed for the wildtype protein and each of the TM deletion constructs.

We agree that further analysis of NgBR topology is warranted. Our description of NgBR topology in

the context of this manuscript provides the rationale for studying the function of the NgBR conformer that exists with its C-terminus in the cytosol. The conclusion that is most central to this study is that a proportion of NgBR exists with its C-terminus in the cytosol. While we agree that a more detailed analysis of the mechanisms underlying the nature of NgBR topology determination is of interest, a thorough analysis of this process would constitute a separate study. We think that, in the context of this manuscript, the description of a cytosolic orientation of the NgBR C-terminus is sufficient to provide rationale for our clear description of an important role for NgBR in dolichol synthesis. These data will, we think, be useful to the scientific community in gaining a deeper understanding of dolichol metabolism and asparagine-linked glycosylation. We agree that a protease protection experiment is helpful in assessing the topology of NgBR and we have included this key experiment in the new Figure 1 (Figure 1C). A timecourse of trypsin treatment shows that a proportion of NgBR exists with its Cterminus in the cytosol, yet the glycosylated, protected NgBR species, remains relatively resistant to trypsin digestion in the presence of digitonin. These data are consistent with the model that a majority of the C tail is oriented towards the cytosol, which a minority of tail glycosylated in the lumen.

-For that same study, it is also important to remember that any data generated from their TM deletion constructs might reflect the consequences of protein misfolding. This possibility is not even mentioned. For that matter, how do they know that data generated from the mutated glycosylation sites does not reflect the consequences of protein misfolding? This too, should be mentioned.

We agree that mutations made in any protein can result in protein folding defects, and that this may confound interpretation. We agree with the reviewer that this should be mentioned within the text, and have made the necessary addition.

-On page 10, the authors describe a sequence alignment of NgBR with characterized cis-IPTases, but fail to show the data, even as a supplemental figure. In the very next paragraph, they state that the Cterminal region of NgBR is conserved among several species. Either, this data should be shown or at least stated in a quantified manner. In other words, what degree of homology is detected? This information is important if it justifies the performance of subsequent experiments.

We agree with the reviewer that a more thorough description of the homology would be helpful. We have included a sequence alignment of cis-IPTase proteins as a supplemental figure (Figure S4). This figure outlines the lack of catalytic and substrate binding residues in NgBR and its yeast ortholog NUS1 that are present in the other cis-IPTases. These residues are defined as such from structure/function studies of UPPS; references are included in the manuscript. We have also referenced the original description of NgBR (Miao et al, 2006) in which NgBR was determined to exhibit 49% similarity with ancestral cis-IPTases.

-On page 11, I appreciate that the authors mention the strong membrane morphology phenotypes visualized by electron microscopy, but a more detailed description is required. Just exactly what is the reader supposed to identify?

Arrows have been placed within the EM micrographs in the supplementary figure to better define the membrane morphology defects. These data are consistent with phenotypes seen in the yeast rer2Δ mutants that are deficient in dolichol synthesis (Sato, et al. Mol Cell Biol. 19:471-483). This clarification has been made within the text.

-On page 12, the authors show that NgBR-GFP and hCIT co-localize in both a reticular and punctate pattern of intracellular distribution (Figure 6D), and then use this as evidence to support the notion that the molecules interact within the lumen of the ER. The conclusions are premature because organelle markers must be utilized to identify the intracellular locations. Also, the punctate pattern might represent the Golgi complex (but this can't be established without the use of markers). If this is the case, then the two proteins might a ctually cycle between the ER and Golgi. The data, as shown, bring up more questions than answers. I suggest that the data either be removed, or a full study be performed.

We agree that the description of the intracellular localization in this manner is unnecessary for the conclusions reached in this study. We have removed these data as suggested by the reviewer.

Referee 3:

We thank the reviewer for the insightful comments that have improved the manuscript considerably. We have addressed each of the reviewer's concerns below.

An intriguing implication, that remains rather enigmatic, is the apparent dual role of the NgBR Cterminus in stabilizing NPC2 in the ER lumen and cis-IPTase in the cytosol. In order to explain these activities, the authors provide a vague hypothesis in which the C-terminus resides partly in the ER lumen and partly in the cytosol. This conclusion is based on inefficient glycosylation of two C-terminal N-linked consensus sites and the observation that removal of the second of three putative TMs has no effect on the glycosylation pattern. Unfortunately, no topological model is provided and it is difficult to envision one that would fit the data. It is equally conceivable that the C-terminus resides in the ER lumen and is simply inefficiently glycosylated. Additional studies are needed confirm the topology and justify these conclusions. Similarly, while removal of the NgBR C-terminus prevents co-immunoprecipitation with hCIT, this could occur for trivial reasons and does not confirm a direct role in binding. Homology of the NgBR C-terminal domain with cis-IPTase also remains unexplained. Some mechanistic understanding of how this domain might interact with its proposed binding partners would strengthen the study.

We agree with the reviewer that a topological model that outlines the putative membrane orientations of NgBR would improve the manuscript. We have added this model in Figure 1 and Figure 7. We have also included a protease protection experiment that shows the C-terminus of NgBR to be more sensitive to protease cleavage than Grp94 (an ER luminal chaperone), strongly suggesting that a proportion of the protein exists with its C-terminus in the cytosol (Figure 1C). We have also provided a more detailed description of the degree of homology between NgBR and other cis-IPTases as requested by the reviewer (Figure S4); this is outlined within the text.

1. If dolichol levels are undetectable in RNAi treated cells (Figure 4E), then what is the nature of the lipid linked oligosaccharides shown in Figure 2A and S1. While the inhibitory effects of siRNA on G3M9 are generally convincing, results for the M5 species are not (Fig 3A and Fig S1B).

Figure 4E represents de novo dolichol synthesis (cis-IPTase assay) on isolated membranes, whereas the data in Figure 2A and FigureS1 (Figure S2 in this revised version) represent steady-state measurements of lipid linked oligosaccharides in intact cells (which would be subject to rates of turnover). While we consistently see loss of the G3M9Gn2-PP-Dol (G3M9) species, the amounts and types of LLOs that accumulate are indeed variable as the reviewer notes. We think this is likely due to the slight variability of the amount of NgBR knockdown, and may reflect differences in the 'bottleneck' in the LLO pathway that is dependent on exactly how much Dol-P remains.

2. Additional information is needed to interpret how free Asn-linked glycans were assessed (fig S1C-E). At least some description should be provided. I would recommend expanding the supplementary information to include more detailed methods.

Total protein precipitates were treated with N-glycanase, neutral and acidic glycans were separated by ion exchange, followed by FACE analysis of the free glycans (this information is now provided within the methods section as well as in the corresponding supplemental figure legend). We think analysis of the entire glycan pool provides a better representation of the effects on the pathway rather than studying specific, selected glycoproteins.

3. Figure 4E is critical to establish a role of NgBR in dolichol synthesis, but the quality of the data is so poor that it is largely uninterpretable. A better gel with proper controls is needed.

The specific activity of the mammalian enzyme is significantly lower than that of the yeast enzyme (see Shridas, et al., Biochem Biophys Res Comm 312:1349-1356). This is apparent when comparing the data from Figure 4E with that of Figure 5D and Figure 7C in our study. We have tried numerous times to improve the qualitative aspect of this RP-TLC, however, we have thus far been unable to sufficiently improve the product yield to an extent that would allow for an improved image. It is possible that a significant amount of product is lost during the dephosphorylation procedure prior to running the products on RP-TLC (described in the methods). We think, though, that the essential nature of NgBR in the synthesis of cis-IPTase products is quite clear from the analysis in Figure 4E.

Long-chain cis-IPTase products are seen in the Ctrl RNAi-treated cells, whereas these products are undetectable in the NgBR RNAi-treated cells.

4. Figure 5D nicely demonstrates that Nus1 and NgBR do not restore dolichol synthesis in the rer2 yeast strain. However contrary to the statement on page 11, the data do not show that Nus1 (or NgBR) activity requires rer2. These are two independent conclusions, given that all other experiments were carried out in mammalian cells. A similar analysis in yeast, using a Nus-1 conditional mutant (or knock out) supplemented with rer2, would probably resolve this issue.

This is a speculative comment, and it has been removed from the discussion. In addition, we have purchased 2 independent *nus 1* damp strains that do not work as advertised.

5. Much of the discussion is poorly connected to the main point of the paper. For example, the connection between cholesterol homeostasis by NPC2 and cis-IPTase is not really addressed here. Emphasis on these two disparate functions leaves the reader wondering what is going on. The possibility that NgBR has multiple topologies is also a minor point that is poorly supported by the data. This topic would probably be best dealt with in a separate, definitive study. Similar concerns hold for the dolichol-dependent microdomains.

We agree that the discussion could be made more succinct. We have modified portions of the discussion to reflect the reviewer's concerns. We removed a passage discussing NPC2 and cholesterol homeostasis. We have also removed several sentences regarding dolichol-dependent microdomains. We have edited the section of the discussion that proposes the NgBR multiple topology hypothesis in accord with the reviewer's suggestion that this area be focused on in a separate, more definitive study.

6. In Figure 3a, "LLO mixture" and "glc oligo mixture" should be better defined.

LLO stands for 'lipid-linked oligosaccharide.' 'Glc oligo mixture' is a mixture of glucose polymers available from Prozyme. These terms are now better defined within the figure legend.

7. When referring to lane numbers in gels, it is helpful to number the lanes.

For consistency, we no longer refer to the lanes by numbers within the text.

2nd Editorial Decision 31 January 2011

Thank you for sending us your revised manuscript. After some delay due to the past Christmas holiday season our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referees 1 and 3 think that there are a number of remaining issues that need to be addressed before we can ultimately accept your manuscript (see below). I would therefore like to ask you to deal with the issues raised in an amended version of the manuscript.

We are looking forward to receiving your re-revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The paper remains poorly written and frustratingly un-quantitative. Many experimental details are

missing and the figures are poorly described. The key results are poorly weighted such that, for example, the mixed membrane topology of NgBR does not even feature in the abstract. I have made a series of detailed comments on the first half of the article. The authors are advised to attend carefully to detail before re-submitting a substantially re-written revised manuscript.

INTRODUCTION

Page 3, paragraph 1, line 8: 'that' should be replaced with 'and'
Page 3, paragraph 2, lines 7-8: the etymology of dolichol should be removed (this is not a review article!)
Page 3, paragraph 2, line 9: examples of chain lengths should be removed (this information is essentially provided on the previous line and not relevant to the paper)
Page 3, paragraph 2, lines 9-10: 'Asn-linked protein glycosylation' should be replaced with 'protein N-glycosylation' to make it consistent with other examples in the same sentence
Page 3, paragraph 2, line 11: the word 'linked' should be removed
Page 4, paragraph 1, line 3: surely 'lipid-linked oligosaccharide' should be replaced with 'dolichol-linked oligosaccharide' as the paper is about dolichol and there is no doubt that the lipid in question is dolichol?
Page 4, paragraph 1, lines 11-12: the term 'protease-sensitive manner' should be removed. Why not simply say 'protein-dependent manner? Or acknowledge directly that there are specific flippases?
Page 4, paragraph 1, line 15: typo - 'en' should be 'on'
Page 5, paragraph 1, line 3: a sentence should be added to explain how polyprenyl diphosphates are converted to dolichol as dolichol biosynthesis is the subject of the paragraph
Page 4, paragraph 2, line 1: the sentence should be modified to include a qualifier: ... Nogo-B Receptor, an ER membrane protein, as a binding partner ....
Page 4, paragraph 2, lines 2-3: this sentence makes poor sense. Having identified NgBR as a binding partner for Nogo-B why screen for binding partners? There is no prior reference to the cis-IPTase homology domain of NgBR - this appears in the next paragraph.
Page 4, paragraph 2, line 4: the sentence should be modified to state ... a lysosomal protein that is ...
Page 6, paragraph 2: the second sentence should be removed
Page 6, paragraph 2, line 5: 'the' should be removed in .... and the regulating ....

FIGURE 1 AND ASSOCIATED RESULTS

Panel B uses COS-7 cells whereas panel C uses HeLa cells - it is unclear why these different cells were used. Presumably the blots are done with anti-HA antibodies in both panels, but this is not stated. What is the point of the Hsp90 blot in panel B? The Grp94 blot in panel C is presumably to control for ER membrane intactness, but this is not mentioned anywhere.
Line 11 of the first paragraph of the Results (page 7) does not make sense; it should be re-written '.... that the C-terminus in at least a portion of NgBR molecules is present .....'
Page 8, line 4: this sentence should be moved to the end of the paragraph

Page 9, paragraph 2, line 9: what is the percent homology? Over a stretch of how many amino acids? Which cis-IPTases were used for comparison? The paper would be clearer if - at this stage - the authors simply stated the facts of the homology between NgBR and cis-IPTases, noted that key catalytic and IPP binding residues are not present in NgBR, and that no activity can be expected from this domain consistent with the inability of the protein to rescue rer2-delta cells. This should replace the first two sentences of paragraph 2 (page 9) where the reader is led to believe that the topological orientation of the NgBR c-terminus has to do with its cis-IPTase activity.

FIGURE 2

Inexplicably, Figure 2 uses yet another cell type from those used in Figure 1.
The authors use panel A to conclude that NgBR influences [3H]mannose incorporation into proteins. However, the [3H] counts in panel A correspond to whole cells lysed in RIPA buffer: this includes proteins, lipids, and cytosolic metabolites generated from [3H]mannose.
Panel B does a subtraction of [3H]counts in the lysate versus media to calculate [3H]mannose uptake. The error in such measurements is likely to be huge. One correct way to do this is to spin the cells through a density medium (typically an oil) into perchloric acid - this effectively separates the

culture medium from the cells and disrupts the cells so that metabolites can be analyzed.
Panel C: how was the NgBR blot done? Was an epitope tag antibody used? There is no sample corresponding to the control RNAi. What is the point of showing lanes where no [3H]mannose was used? If the [3H]mannose incorporation into proteins is reduced to the extent suggested in this panel, it may be a good idea to check for surface expression of GPI-anchored proteins.

FIGURE 3

The rebuttal to the comments on Figure 3B (loss of NgBR by blot analysis in Fig. 2C, yet significant residual Man-P-Dol and Glc-P-Dol) is unconvincing. If dolichol turns over slowly as indicated in the rebuttal, then there should be only a muted effect on [3H]mannose incorporation into proteins etc. However, from the FACE gel in Fig. 3A, there would appear to be no lipid-linked oligosaccharides in the NgBR-silenced cells. There must be a more sensitive way to reconcile the difference in Oligo-PP-Dol vs Man-P-Dol and Glc-P-Dol levels.

FIGURE 4

Panel B: The amount of cDNA transfected cannot be used as a convincing measure of the amount of NgBR expressed. Why not use an HA standard and try to quantitate the NgBR blot?
Panel D: Three data points cannot be used to calculate an unique Km value, and certainly not to 4 significant figures!

Referee #2 (Remarks to the Author):

The results of this study (which utilizes numerous experimental approaches and a comprehensive experimental design) are presented in a mature study that provides new insight into the complexities associated with dolichol lipid biosynthesis. The novel observation is very timely, especially when one considers the re-discovered relevance of post-translational modifications (including glycosylation) in regulating numerous aspects of cell biology and modulating disease pathogenesis. The generated data are well-controlled. Also, the text, figures, and schematics cooperate to adequately describe the mechanism by which the Nogo-B receptor (NgBR) functions as an essential component of the dolichol biosynthetic machinery, including the existence of alternative protein topologies.

Referee #3 (Remarks to the Author):

This is a markedly improved revision of a prior manuscript demonstrating that the Nogo-B receptor, NgBR plays a previously unappreciated role in N-linked glycosylation by stimulating Dolicol-PP biosynthesis in the ER membrane. The study now is better organized, more focused and includes additional data to support the conclusions. The authors use a wide variety of assays to show that protein mannosylation, dolicol levels, formation of dolicol-linked oligosaccharides, hCIT activity in microsomes, and hCIT stability are all affected by NgBR manipulations. The main finding that NgBR is necessary but not sufficient for hCIT activity is well supported by convincing data. Whether or not NgBR exists in two topological is still not entirely established, but this no longer detracts from the major focus of the paper. Overall, the results here provide a convincing argument for a novel role of NgBR in dolichol biosynthesis.

My only major concern is that there is no direct demonstration that NgBR knockdown has any effect on N-linked glycosylation. The mannose transfer experiments shown in Figure 2 do not demonstrate transfer GlcNac2Man9Glc3 core sugars to substrate. In fact the only substrate examine was CPY in yeast rer2Δ cells where the defect was not corrected by NgBR complementation. Thus the critical link with N-linked glycosylation needs to be established unequivocally. This could easily be done with a simple IB of known glycoproteins in NgBR knock-down cells. Indeed, it is surprising that such experiments were not included, given the title of the paper.

Minor concerns include the following.

Proteolysis data in figure 1 showing that NgBR is more susceptible to trypsin than Grp94 in semi permeabilized cells supports the proposal that a portion of the protein resides in the cytosol. However, glycosylated species are also trypsin sensitive (albeit less so) at 10, 15 and 20 minutes time points, suggesting that digitonin also render the ER accessible to protease to some extent.

Data in Figure 4D is insufficient to derive meaningful Km values. Only three data points are present and the curve for RNAi treated samples bears no relationship to them. To the author's credit though, the marked decrease in [14C]-IPP incorporation is very striking.

Decrease in hCIT levels following NgBR shRNA treatment, although modest, is an important finding that should be moved to a main figure (not supplementary). A control (IB) should also be included to ensure that the differences are not due to loading artifact.

Figure S5 is not convincing and should be removed.

Relevance of the model shown in Figure 7D is not evident from the study. While I can see why such a conclusion is attractive, there is simply no evidence that hCIT stimulation is mediated by the NgBR topological isoform with its C-terminus in the cytosol. The observed effects could just as well be mediated by the TM2-3 loop or indirect interactions with TM spans or lumenal regions of accessory proteins.

1st Revision - authors' response 10 March 2011

Response to Reviewer 1:

We thank the reviewer for the helpful criticism. We are concerned with the continued frustration with the text and have taken into account each point raised in the reviewer's critique. Please find below the modifications we have made in accordance with the reviewer's suggestions.

INTRODUCTION

Page 3, paragraph 1, line 8: 'that' should be replaced with 'and' - OK
Page 3, paragraph 2, lines 7-8: the etymology of dolichol should be removed (this is not a review article!) - OK
Page 3, paragraph 2, line 9: examples of chain lengths should be removed (this information is essentially provided on the previous line and not relevant to the paper)
Page 3, paragraph 2, lines 9-10: 'Asn-linked protein glycosylation' should be replaced with 'protein N-glycosylation' to make it consistent with other examples in the same sentence-OK
Page 3, paragraph 2, line 11: the word 'linked' should be removed OK
Page 4, paragraph 1, line 3: surely 'lipid-linked oligosaccharide' should be replaced with 'dolichol-linked oligosaccharide' as the paper is about dolichol and there is no doubt that the lipid in question is dolichol?

We prefer to be consistent throughout the manuscript with use of the term 'lipid-linked oligosaccharide' since our experiments have not yet addressed the possibility that the deficiencies in *cis*-IPTase activity we are studying have any secondary effects on polyprenol reductase.

Page 4, paragraph 1, lines 11-12: the term 'protease-sensitive manner' should be removed. Why not simply say 'protein-dependent manner? Or acknowledge directly that there are specific flippases? OK
Page 4, paragraph 1, line 15: typo - 'en' should be 'on' OK
Page 5, paragraph 1, line 3: a sentence should be added to explain how polyprenyl diphosphates are converted to dolichol as dolichol biosynthesis is the subject of the paragraph OK, this is now included.
Page 4, paragraph 2, line 1: the sentence should be modified to include a qualifier: ... Nogo-B Receptor, an ER membrane protein, as a binding partner .... OK

Page 4, paragraph 2, lines 2-3: this sentence makes poor sense. Having identified NgBR as a binding partner for Nogo-B why screen for binding partners?

Identification of NgBR resulted from a screen for binding partners of Nogo-B. We later screened for other proteins that bind NgBR. We have modified this paragraph in an attempt to avoid any confusion.

There is no prior reference to the cis-IPTase homology domain of NgBR - this appears in the next paragraph.

We have removed prior introduction of the *cis*-IPTase homology domain and instead mention it only in the latter paragraph to avoid confusion.

Page 4, paragraph 2, line 4: the sentence should be modified to state ... a lysosomal protein that is ... OK
Page 6, paragraph 2: the second sentence should be removed

We would prefer to keep this sentence in the introduction, as it speaks to the relevance of the identification of NgBR as a component of *cis*-IPTase activity.

Page 6, paragraph 2, line 5: 'the' should be removed in .... and the regulating .... OK

FIGURE 1 AND ASSOCIATED RESULTS

Panel B uses COS-7 cells whereas panel C uses HeLa cells - it is unclear why these different cells were used. Presumably the blots are done with anti-HA antibodies in both panels, but this is not stated.

The blots for NgBR are with anti-HA. This is now stated within the accompanying figure legend.

What is the point of the Hsp90 blot in panel B?

The experiments uses whole cell lysates and the Hsp90 blot documents equal loading of protein in each lane. It also serves as a control, since it is not glycosylated.

The Grp94 blot in panel C is presumably to control for ER membrane intactness, but this is not mentioned anywhere.

We chose to use HeLa cells for the limited proteolysis experiments for two reasons: 1) We have experience in using these cells to perform limited proteolysis experiments; 2) The Grp94 antibody has higher affinity for human Grp94 than monkey Grp94. The reviewer is correct that Grp94 was used to control for permeabilization of the ER. We mention this use of Grp94 within the text; nevertheless, we have also now added this description of the purpose of Grp94 within the legend for Figure 1.

Line 11 of the first paragraph of the Results (page 7) does not make sense; it should be re-written '.... that the C-terminus in at least a portion of NgBR molecules is present .....'

We have made the above changes as requested by the reviewer.

Page 8, line 4: this sentence should be moved to the end of the paragraph.

We have modified the sentence in question: 'It is also important to note that a large fraction of NgBR exists with its C-terminus oriented towards the cytosol as shown by limited proteolysis experiments.'

Page 9, paragraph 2, line 9: what is the percent homology? Over a stretch of how many amino acids? Which cis-IPTases were used for comparison? The paper would be clearer if - at this stage - the authors simply stated the facts of the homology between NgBR and cis-IPTases, noted that key catalytic and IPP binding residues are not present in NgBR, and that no activity can be expected

from this domain consistent with the inability of the protein to rescue rer2-delta cells. This should replace the first two sentences of paragraph 2 (page 9) where the reader is led to believe that the topological orientation of the NgBR c-terminus has to do with its cis-IPTase activity.

The C-terminal 158 amino acids of NgBR exhibit 44% sequence similarity with the *M. luteus cis*-IPTase UPPS. We have modified the paragraph in question with this information. On page 12, paragraph 2, we note the absence of some of the catalytic, IPP and FPP binding residues in comparison with other *cis*-IPTases (Figure S4). The absence of these residues does not, however, preclude the possibility that NgBR might exhibit catalytic function in a manner distinct from UPPS. We think it would be premature within the context of the manuscript as presented to include a description of the rer2Δ experiment prior to our description of the necessity of NgBR for N-linked glycosylation, LLO biosynthesis, and *cis*-IPTase activity. We do agree with the reviewer that an explicit statement regarding the percent homology is appropriate at the suggested point in the manuscript, and we have made this correction as requested.

FIGURE 2

Inexplicably, Figure 2 uses yet another cell type from those used in Figure 1.

We used EA.hy926 cells due to the reproducible knockdown of NgBR expression that we have characterized previously (Harrison, et al. 2009).

The authors use panel A to conclude that NgBR influences [3H]mannose incorporation into proteins. However, the [3H] counts in panel A correspond to whole cells lysed in RIPA buffer: this includes proteins, lipids, and cytosolic metabolites generated from [3H]mannose.

Panel A in Figure 2 reports [3H] mannose incorporated into TCA precipitable protein. The lystates were precipitated with TCA (10% final) and pellets extensively washed with TCA. This information was present within the methods, however, was not stated within the corresponding figure legend. We have now placed a more explicit description of the methods within the legend for Figure 2.

Panel B does a subtraction of [3H]counts in the lysate versus media to calculate [3H]mannose uptake. The error in such measurements is likely to be huge. One correct way to do this is to spin the cells through a density medium (typically an oil) into perchloric acid - this effectively separates the culture medium from the cells and disrupts the cells so that metabolites can be analyzed.

The main of this experiment was to test if the loss of NgBR reduced [3H] mannose uptake and it did not. While we agree with the reviewer that other means to measure mannose uptake exist, we are confident that our approach is sufficient to address significant differences between the two experimental conditions. The inherent error in the approach we have taken is apparently negligible (as our data allowed a degree of significance of $p<0.001$ between the control and treated cells).

Panel C: how was the NgBR blot done? Was an epitope tag antibody used? There is no sample corresponding to the control RNAi. What is the point of showing lanes where no [3H]mannose was used? If the [3H]mannose incorporation into proteins is reduced to the extent suggested in this panel, it may be a good idea to check for surface expression of GPI-anchored proteins.

To better describe panel C, we have included the following text within the corresponding figure legend: 'An NgBR polyclonal antibody was used to detect endogenous NgBR expression whereas anti-β-actin was used to assess equal loading of lysates.'
The first 3 lanes of this gel correspond to control RNAi treatments. This distinction is now present within Figure 2C. Lanes without 3H-mannose are included to rule out any potential artifacts of chemiluminescence from sample preparation.

FIGURE 3

The rebuttal to the comments on Figure 3B (loss of NgBR by blot analysis in Fig. 2C, yet significant

residual Man-P-Dol and Glc-P-Dol) is unconvincing. If dolichol turns over slowly as indicated in the rebuttal, then there should be only a muted effect on [3H]mannose incorporation into proteins etc. However, from the FACE gel in Fig. 3A, there would appear to be no lipid-linked oligosaccharides in the NgBR-silenced cells. There must be a more sensitive way to reconcile the difference in Oligo-PP-Dol vs Man-P-Dol and Glc-P-Dol levels.

We respectfully differ on this point, and actually conclude that the results of Figures 2A, 2C, and 3 are in excellent agreement, and are fully consistent with the remainder of the study. While the 3H-mannose/TCA precipitation of proteins used in 2A for mannose incorporation could in principle detect many mannosyl conjugates, the fact that tunicamycin eliminated about 90% of the readout shows that in practice, this assay is highly selective for 3H-mannose in total LLOs and/or N-glycans. At least for the LLO portion, by labeling continuously for 4 hrs this essentially becomes a steady-state method. Incorporation of 3H-mannose was reduced by RNAi to ~45% of control. The other completely independent steady-state method we used, FACE analysis, showed ~25% of the mature LLO G3M9Gn2 levels after NgBR RNAi. In contrast to what the reviewer points out, in the images we submitted this residual amount of LLO was easily seen in the FACE gel in 3A, and easily quantified. These substantial, but not complete losses of 3H-mannose labeled material and G3M9Gn2-LLO in two independent assays are therefore in agreement with the remaining amounts of MPD and GPD, which were also both ~40% in 3C-D. We also point out in Fig S2 a loss of about 30% of total N-glycans. While by design all these experiments measure different things, and cannot be expected to give exactly equivalent quantitative data, they do all lead to similiar conclusions - substantial but not complete loss of dolichol-derived material.

The question of turnover is consequently straight-forward. We agree with the reviewer that the RNAi was highly effective, eliminating most of the NgBR protein. And while dolichyl-P molecules turn over slowly, they do turn over at an appreciable rate - estimates for half-life have been between 24-48h (Andersson, et al. 1990. FEBS Letters; 269(1):15-8). So, under these conditions we would expect much but not all dolichol to be eliminated, and we feel that our measurements for LLOs, MPD, GPD, and N-glycans are therefore quite reasonable.

FIGURE 4

Panel B: The amount of cDNA transfected cannot be used as a convincing measure of the amount of NgBR expressed. Why not use an HA standard and try to quantitate the NgBR blot?

The amounts in Figure 4B are intended as relative values. In response to transiently increased levels of NgBR expression, cis-IPTase activity is enhanced. We have removed the specific amounts of NgBR transfected and replaced these values with a symbol for increasing amounts of NgBR expression (relative to control).

Panel D: Three data points cannot be used to calculate a unique Km value, and certainly not to 4 significant figures!

We agree and the Km values have been removed from Figure 4D.

Response to Reviewer 2:

We thank the reviewer for the helpful criticism and appreciate the reviewer seeing the novelty of our findings.

Response to Reviewer 3:

We thank the reviewer for the helpful criticism and appreciate the reviewer seeing the novelty of our findings. Please find below the modifications (in bold) we have made in accordance with the reviewer's suggestions.

My only concern is that there is no direct demonstration that NgBR knockdown has any effect on N-linked glycosylation. The mannose transfer experiments shown in Figure 2 do not demonstrate transfer GlcNac2Man9Glc3 core sugars to substrate. In fact the only substrate examine was CPY in yeast rer2; cells where the defect was not corrected by NgBR complementation. Thus the critical link with N-linked glycosylation needs to be established unequivocally. This could easily be done with a simple IB of known glycoproteins in NgBR knock-down cells.

In the original Figure S2, we reported results with total cell-associated neutral and charged N-glycans. Each were decreased about 30% by RNAi, and rescued with expression of an RNAi resistant NgBR construct. We feel that the total glycan approach is superior to IP of selected glycoproteins, which could potentially give results unrepresentative of the greater glycoprotein pool.

Minor concerns include the following:

Proteolysis data in figure 1 showing that NgBR is more susceptible to trypsin than Grp94 in semi permeabilized cells supports the proposal that a portion of the protein resides in the cytosol. However, glycosylated species are also trypsin sensitive (albeit less so) at 10, 15 and 20 minutes time points, suggesting that digitonin also render the ER accessible to protease to some extent.

We agree. We surmise that two possibilities may account for this differential sensitivity of glycosylated species. The most likely explanation, as suggested by the reviewer, is that digitonin treatment at these later time points results in a slight permeabilization of the ER bilayer that renders the luminal conformer of NgBR susceptible to proteolysis. Some degree of differential permeabilization through use of digitonin still exists, however, especially when one compares loss of these glycosylated species in the 10min digitonin alone vs. digitonin plus triton X-100 treatments. The differential sensitivity of the ER luminal Grp94 also correlates with effective use of digitonin as a selective permeabilization agent.
Another slightly more complicated, yet plausible, scenario might involve an oligomeric species of NgBR, in which the luminal conformer is rendered unstable by proteolysis of the cytosolic conformer. We have preliminary evidence suggesting that NgBR may exist in an oligomeric state, and we are therefore considering this second hypothesis at the moment.

Data in Figure 4D is insufficient to derive meaningful Km values. Only three data points are present and the curve for RNAi treated samples bears no relationship to them. To the author's credit though, the marked decrease in [14C]-IPP incorporation is very striking.

We have removed the Km values from Figure 4D in accord with the reviewer's suggestion.

Decrease in hCIT levels following NgBR shRNA treatment, although modest, is an important finding that should be moved to a main figure (not supplementary). A control (IB) should also be included to ensure that the differences are not due to loading artifact.

We have moved the original Figure S6 to the main text. It is now placed as Figure 7C. Hsp90 in this IB serves as both a control for fractionation (enriched in the cytosolic fraction) and as a loading control.

Figure S5 is not convincing and should be removed.

We have removed Figure S5 in accord with the reviewer's suggestion.

Relevance of the model shown in Figure 7D is not evident from the study. While I can see why such a conclusion is attractive, there is simply no evidence that hCIT stimulation is mediated by the NgBR topological isoform with its C-terminus in the cytosol. The observed effects could just as well be mediated by the TM2-3 loop or indirect interactions with TM spans or lumenal regions of accessory proteins.

Whereas we agree with the reviewer regarding the precise nature of the NgBR-hCIT interaction, we respectfully suggest that the evidence does suffice to support the model in Figure 7D (now Figure 7E). Our observation that NgBR is necessary but not sufficient for *cis*-IPTase activity coupled with data presented in Figure 6 showing the C-terminus to be necessary for coIP with hCIT strongly advocates for the proposed model. To symbolize the other possible interpretations of these data, we have included an arrow and question mark between NgBR and hCIT on the cytosolic leaflet of the ER bilayer within the revised Figure 7E.

3rd Editorial Decision 04 April 2011

Thank you for sending us your (re-)revised manuscript. Our original referees 1 and 3 have now seen it again, and you will be pleased to learn that they now support publication.

Before this will happen, there are a number of editorial issues that need further attention. According to our updated guidelines I need to ask you to include an author contribution section into the manuscript text. Furthermore, please add the statistical details for figure 4D. Finally, in the figure legend of figure 3 (statistical details) you refer to figure 3E. Yet, figure 3 contains panels A to D only. Please let us have a suitably amended manuscript text file as soon as possible via email. We will upload it for you, and I will then formally accept the manuscript.

Thank you very much again for considering our journal for publication of your work.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

The paper is improved, although the authors limited their revisions to the text that I commented on in the previous review and did not restructure the entire manuscript using my suggestions as examples. There remain redundancies and inadequacies in the presentation, but this now becomes an issue of style. The core data of the paper, i.e., that NgBR is a cis-IPTase cofactor and that it adopts 2 membrane topologies are novel and of general interest.

Referee #3

no remarks to the author

