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Static retention of the lumenal monotopic membrane protein torsinA in the endoplasmic reticulum

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 07 April 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. As you will see while referees 2 and 3 are more positive referee 1 is concerned that the conclusiveness and the completeness of the experimental evidence provided is not sufficient at this point to justify the conclusions drawn. On balance, it thus becomes clear that substantial revision will be required before the paper will be publishable. In particular, it will be indispensable to address both major points of referee 1, the major concern of referee 2 and points 1 and 2 of referee 3 in an adequate manner and to their full satisfaction. A final decision can only be made after revision.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses as well as on the final assessment by the referees.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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

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foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Please do not hesitate to get back to me at any time in case you would like to discuss any aspect of the revision further.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

This manuscript deals with the mechanism through which the lumenal protein torsinA is retained in the endoplasmic reticulum. The authors provide evidence that torsinA is a monotopic membrane protein that is statically retained in the ER through exclusion from ER exit sites. They show that the hydrophobic sequence at the N-terminus (NTD) has a helical structure, and is responsible for membrane association and ER retention. Based on mutational analysis, an asymmetric arrangement of small non-polar residues on one face of the helix is proposed to be important for membrane association.

The authors present a model in which monotopic membrane proteins partition away from regions of high membrane curvature/protein crowding, and are thus sorted out of ER exit sites, resulting in ER retention. Although this model is very attractive, data to support it are lacking, and I do not believe that the conclusions are justified. Overall, the data are of very high quality, and the experiments are well thought out and executed. If the authors can provide evidence to support their model, and show that this sorting mechanism is more widely applicable, then I think this paper would be suitable for publication in EMBO J. In the absence of this, the manuscript would be better suited to a more specialised journal. There are also a number of more minor points that need to be addressed.

Major points:

1. What evidence do the authors have to support their model that partitioning of the NTD out of ER exit sites underlies ER retention? Would predict that the Δ26-43 torsinA would enter ER exit sites/ERGIC following temp block - is this the case? In addition, should compare NTD of torsinA with other membrane interacting domains/TMDs to provide evidence for specific exclusion of the torsinA NTD from ER exit sites. The model would also predict that torsinA (but not Δ26-43 torsinA) would be enriched in membrane sheets - can the authors show this?

2. The experimental work is almost exclusively focussed on torsinA. What is the evidence that the sorting mechanism proposed also applies to other proteins? In order to support this being a general sorting mechanism, need to look at membrane association domains of other proteins proposed to be retained in this way.

Other comments:

1. Fig 2: Is Δ26-43 torsinA (lacking NTD) membrane associated? Some of the protein is secreted, but only a small proportion - what about the protein that is now apparently localised in the Golgi? The relationship between ER retention and membrane association is not clear and is essential for interpretation of the data.

2. Should show diffusion coefficient of torsinA-mGFP and Δ26-43 torsinA-GFP (compared to control transmembrane and lumenal proteins).

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3. Fig 3: Might predict that a hydrophobic domain such as residues 23-46 would be recognised by ER quality control system - this could explain retention of the mGFP fusions (the 1-25 fusion would become mGFP following signal sequence cleavage). Can the authors exclude this possibility?

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5. Fig 7:COX1 and erlin1 do seem to be excluded from ER exit sites. Need to show a control ER protein that is not statically retained is observed in ER sites under these conditions (not VSVG tsO45 upon temp drop as this represents a large wave of cargo - so concentration very easy to see).

Referee #2:

The mechanisms that prevent ER resident proteins from leaving this compartment are not fully understood. Signal-mediated retrieval seems to provide a back-up system, but the main mechanisms probably involve retention. This manuscript focuses on torsinA, a AAA+ ATPase localized to the ER lumen. The results document that a hydrophobic N-temrinal domain (NTD) in torsinA is necessary and sufficient for ER retention, and that the NTD associates with the inner surface of the ER membrane but does not span the membrane. This membrane-NTD association is crucial for ER retention, suggesting that ER retention of torsin A involves partitioning into specific membrane domains. Additional lumenal ER proteins may exhibit similar behavior. In sum, this study identifies a retention signal for a largely uncharacterized topological class of ER resident proteins, and provides insight into possible mechanisms of retention. I find the work to be thorough, carefully controlled, and scholarly. The data are of high quality and the results will be of broad interest to the cell biology community.

I have only one substantive concern. After low temperature treatment, if a protein remains distributed through the entire ER, the authors conclude that the protein is "excluded" from ER exit sites. But the counterexamples are proteins such as VSVG that have specific ER export signals mediating COPII-dependent concentration. Is it clear that a bulk flow marker would behave differently than torsinA? In other words, does the low temperature behavior of torsinA merely reflect lack of a concentrative ER export signal rather than active exclusion from ER exit sites? To address this point, the authors could do a low temperature experiment with a construct such as ss-mGFP-KDEL, which should exit the ER by bulk flow.

Minor points:

1. In Fig 3A, please explain what the colors mean.

2. On p. 10, "Fig 3G" should read "Fig 3F".

3. Fig 4F needs an "F" label.

4. Fig 4A-C should be referenced in the text.

5. Fig 8i doesn't really fit with the data suggesting that ER exit of torsinA is inhibited. Perhaps the membrane composition of the transitional ER is different from that of the rough ER, and torsin A partitions selectively into the rough ER domain? That interpretation seems more consistent with the results presented here.

6. The term "intrinsic retention" in the title is ambiguous. How about "A retention signal blocks endoplasmic reticulum export of the lumenal monotopic membrane protein torsinA"?

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Referee #3:

This study investigates the mechanism of ER residence of TorsinA, a member of the AAA family of ATPases linked to early-onset torsion dystonia. TorsinA is a lumenal ER protein anchored to the membrane by an N-terminal hydrophobic domain (NTD), which previous studies had implicated in determining ER residence. Here the authors show that Torsin A is kept in the ER by static retention, i.e., by exclusion from ER exit sites, and that the NTD is both necessary and sufficient for this retention. By several converging lines of evidence they convincingly show that the NTD does not span the bilayer, thus defining Torsin A as a monotopic membrane protein. CD analysis suggests that the NTD may be in alpha helical conformation when in a lipid environment, and mutation of hydrophobic residues on one, but not on the other face of the putative alpha helix results in escape of the mutants from the ER and their secretion. The authors suggest that partitioning of membrane-associated non-spanning alpha helices into disordered, or flat domains of the ER may represent a general retention mechanism for monotopic ER lumenal proteins.

This is a very interesting study, which presents high quality data and introduces a novel concept in membrane traffic. The paper is clearly written and illustrated. Nevertheless a few points need clarification/correction before publication.

1) FRAP experiments: Figure 3D demonstrates >10 fold reduction of GFP diffusion when it is attached to torsinA's NTD. In Fig. 6D the diffusion of constructs with ineffective or secretion-inducing mutations in the NTD is analysed. In this analysis, E171Q-torsinA-mGFP is being used, thus the measurements made here cannot be compared with those of Fig. 3D, however, the increase in apparent D in the secretion mutants compared to those retained in the ER is quite modest in comparison to the data of Fig. 3D. Some important information is missing for a critical interpretation of the data, and should be supplied before publication. Needed are diffusion measurements for two additional constructs, i.e., E171Q-torsinA-mGFP with wild-type NTD (membrane-associated) and deleted NTD (soluble in the ER lumen). As an alternative (or in addition) mutant constructs of the 1-67mGFP could be produced and analysed.
Still referring to Fig. 6D, the meanings of the statistical comparisons shown in the panel are not clear to me: why are the mutations at positions 30 and 34 compared respectively with the ones at positions 28 and 31? One might as well have compared position 30 with position 31 and 34 with 28. Actually for four group comparisons an Anova test is required.

2) Table I, Fig. 7, text p. 15: The authors have included Erlin 1 and 2 as possible group II monotopic proteins, based on the compilation of Gilchrist et al., which included the two erlins as signal peptide bearing proteins. Actually, experimental evidence (Pearce et al., quoted by authors in the Table) indicates that neither of the two erlins has a cleaved signal sequence, and that the N-terminal hydrophobic sequence serves instead as a signal anchor. Furthermore, the erlins have no additional potential membrane-interacting hydrophobic sequences other than the one at their N-terminus. Thus, both sequence information and experimental results indicate that the erlins are bona fide type II transmembrane proteins. The two erlins should therefore be removed from the table and the IF analysis of erlin 1 in Fig. 7 should be deleted.

3) Western blot analysis of culture media (Methods, p. 25) - first two sentences of the section "Immunoprecipitation from culture medium": it is not clear whether in some experiments immunoprecipitation was performed from the medium of 16 h transfected cells, or whether in all experiments (not only in those with BFA), fresh medium was added at 16 h, so that we are always looking at the torsinA secreted in 6 h.

Minor points.

Title: to my knowledge, the expression "intrinsic retention" has not been used in the past to define retention mediated by exclusion from exit sites. For this phenomenon, the expression "static retention" has generally been used. If the authors feel that intrinsic retention is a better way of describing the exclusion process, they should explain why, somewhere in the Introduction or Discussion of the paper.

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Table I: The definition of the three groups should be given in a note under the table.

p. 15: the sentence "a receptor-mediated mechanism for restricting a protein to the ER does not currently exist" is a bit misleading, because, although strictly speaking a recycling protein is not restricted to the ER, in most people's minds a protein that at steady state is detectable only (or nearly only) in the ER would be considered to be ER restricted, yet the retrieval process is mediated by receptors. Therefore, the word "restricting" should be replaced by "excluding from exit sites". In addition "does not currently exist" should be replaced by "is currently considered unlikely".

1st Revision - authors' response 05 May 2011

We appreciate the helpful reviews of our manuscript, and attach a revised manuscript that addresses the questions and suggestions provided. Major additions include (a) additional temperature shift and FRAP experiments as requested for clarifying interpretation and (b) a new figure showing that both torsinA and the known lumenal monotopic protein COX1 partition preferentially into ER sheets (and not into ER tubules). Additional changes and responses are detailed below. We think these revisions have substantially strengthened the manuscript, and hope that it will now be acceptable for publication in the EMBO Journal.

Referee #1

This manuscript deals with the mechanism through which the lumenal protein torsinA is retained in the endoplasmic reticulum. The authors provide evidence that torsinA is a monotopic membrane protein that is statically retained in the ER through exclusion from ER exit sites. They show that the hydrophobic sequence at the N-terminus (NTD) has a helical structure, and is responsible for membrane association and ER retention. Based on mutational analysis, an asymmetric arrangement of small non-polar residues on one face of the helix is proposed to be important for membrane association.

The authors present a model in which monotopic membrane proteins partition away from regions of high membrane curvature/protein crowding, and are thus sorted out of ER exit sites, resulting in ER retention. Although this model is very attractive, data to support it are lacking, and I do not believe that the conclusions are justified. Overall, the data are of very high quality, and the experiments are well thought out and executed. If the authors can provide evidence to support their model, and show that this sorting mechanism is more widely applicable, then I think this paper would be suitable for publication in EMBO J. In the absence of this, the manuscript would be better suited to a more specialised journal. There are also a number of more minor points that need to be addressed.

Our responses to specific issues raised are below. Overall, we have carefully evaluated the relationship between our data, our conclusions, and the model we provide to make sure that the conclusions as presented are justified. We have changed a few sentences that might have been interpreted as overstatements, revised the abstract to better summarize the study for a general audience, and added data in response to specific concerns as below. We hope that the reviewer will now agree with us both that our conclusions are justified and that the paper will be of significant interest to the cell biology community.

Major points:

1. What evidence do the authors have to support their model that partitioning of the NTD out of ER exit sites underlies ER retention? Would predict that the Δ26-43 torsinA would enter ER exit sites/ERGIC following temp block - is this the case? In addition, should compare NTD of torsinA with other membrane interacting domains/TMDs to provide evidence for specific exclusion of the torsinA NTD from ER exit sites. The model would also predict that torsinA (but not Δ26-43 torsinA) would be enriched in membrane sheets - can the authors show this?

-As the reviewer suggests, we now include data showing that torsinA lacking its NTD behaves

similarly to the bulk-flow marker ER-GFP, both of which are readily detectable in ER exit sites (ER-GFP is shown in Fig. 1E and Δ26-43 torsinA in Fig. S1). This is in contrast to full-length torsinA which is excluded from ER exit sites (Fig. 1F). Both ER-GFP and Δ26-43 torsinA leave the ER by bulk flow rather than signal-facilitated forward transport, and as expected do not concentrate in the exit sites to the same level seen for VSV-G at 10°C. This data supports the model that NTD-dependent partitioning out of ER exit sites underlies retention of full-length torsinA in the ER.
-The concept of ER retention as a result of exclusion from ER exit sites is itself not new, and was elegantly put forward in a study from Borgese and collegues in 2008 (Ronchi *et al*. JCB *181*:105-118), who showed that a single TMD could mediate static retention in the ER when it was 17 residues but not 22 residues in length. Our work extends this concept to lumenal monotopic membrane proteins, generalizing the idea of partitioning as a mechanism for protein sorting in the early secretory pathway. We have added sentences clarifying this point. We note that we have also confirmed the results of this earlier study in the experiments in Fig. 5 which examine the behavior of NTD segments converted to TMDs by the introduction of hydrophobic residues: a TMD created by introducing 2 Leu residues into the NTD is retained in the ER, while that created by inserting 3 or 5 Leu residues is not.
-The concept that torsinA but not Δ26-43 torsinA should be enriched in ER membrane sheets (as opposed to in ER tubules) is an important prediction of our model. As shown in new Figs. 8 and S5, this is indeed the case. This is an exciting finding and adds torsinA and COX1 to the relatively short list of known ER sheet-preferring proteins. It also adds to the similarities between the partitioning-based sorting we describe and that previously described by Ronchi *et al*.

2. The experimental work is almost exclusively focussed on torsinA. What is the evidence that the sorting mechanism proposed also applies to other proteins? In order to support this being a general sorting mechanism, need to look at membrane association domains of other proteins proposed to be retained in this way.

-Our study uses the sorting of torsinA to uncover the need for new principles explaining the subcellular distribution and ER retention of lumenally oriented monotopic membrane proteins. We agree with the reviewer that it is important to generalize the models we propose to other proteins, and have added a few additional experiments and discussion of the well studied lumenal monotopic membrane protein COX1 to address this point. We use a 15°C temperature block to confirm that it, like torsinA, is statically retained in the ER and show that as might be expected it preferentially partitions into ER sheets. We cite an earlier study showing that the region of COX1 containing the membrane associating amphipathic helices expressed by itself remains in the ER (Li *et al*., 1998). Extending these experiments in the present context would be difficult because short segments of 4 amphipathic helices are involved in monotopic association of COX1 with the ER membrane (Picot *et al*., 1994). These experiments are beyond the scope of the present study. We do discuss the evidence that suggests that our model will extend to other lumenal monotopic proteins, and highlight the need for future studies in this area. Note that as suggested by reviewer #3, we removed the similar experiments with erlin-1 because of the likely possibility of its hydrophobic domain being a TMD and the protein thus being statically retained by the mechanism previously described by Borghese and colleagues.

Other comments:

1. Fig 2: Is Δ26-43 torsinA (lacking NTD) membrane associated? Some of the protein is secreted, but only a small proportion - what about the protein that is now apparently localised in the Golgi? The relationship between ER retention and membrane association is not clear and is essential for interpretation of the data.

The data supporting a relationship between the NTD, membrane association and ER retention include:

(a) the fact that the NTD is both necessary and sufficient for torsinA's hydrophobicity in Triton X-114 partitioning experiments (Fig. 3B).
(b) the fact that purified NTD fused to MBP associates directly with liposomes (Fig. 3F).
(c) the fact that deleting the NTD increases the diffusion coefficient of torsinA (Fig. 6E) and that selected mutations to the NTD lead to lesser but significant increases in the diffusion coefficient (Fig. 6F).
(d) the fact that deleting the NTD allows entry into the secretory pathway (as detected by

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overlap with ER exit sites and visual concentration in the Golgi similar to what is seen for other secreted proteins (Fig. S1)).

(e) We also note that the experiments looking at secretion are not pulse-chase studies and are intended merely to show which proteins are and which are not secreted.

We hope this clarifies what we think is a strong correlation between ER retention and membrane association.

2. Should show diffusion coefficient of torsinA-mGFP and Δ26-43 torsinA-GFP (compared to control transmembrane and lumenal proteins).

This has been added to Fig. 6E.

3. *Fig 3: Might predict that a hydrophobic domain such as residues 23-46 would be recognised by ER quality control system - this could explain retention of the mGFP fusions (the 1-25 fusion would become mGFP following signal sequence cleavage). Can the authors exclude this possibility?*

Immunoblots with several different anti-GFP antibodies detect only the 'full-length' fusion proteins in cells expressing the various NTD-mGFP fusions, indicating that significant cleavage to (secreted) mGFP is not occurring. One of these is the experiment shown in Fig. 2E.

4. Fig 6: Again the correlation between membrane association and ER retention is not clear. The difference in diffusion coefficient between the ER localised and the non-ER localised forms is very small. If L30R and L34R are not membrane associated, wouldn't a greater increase be expected (cf. Fig 3D)? What about comparing the different forms in BFA treated cells - then could rule out differences due to the proteins being localised in different compartments. Since this issue is critical for interpretation for the data, additional methods to look at membrane association should be used (eg. extraction with carbonate, mild detergent, liposome binding?).

- The important point in Fig. 6 is that there are statistically significant differences in the extent to which introducing Arg residues at different positions along the NTD affects torsinA's diffusion coefficient. We have now added (as also requested by reviewer #3) measurements of the FRAP of full-length and Δ26-43 torsinA-E171Q to Figure 6, clarifying the range of diffusion observed in the complete presence vs. absence of the NTD. Perhaps not surprisingly for a dynamic partitioning event, single point mutations that clearly have an effect on ER retention do not change the diffusion as dramatically as the complete deletion of the domain. This is all discussed in more detail in the text. We focus on experiments in living cells because this is the context in which the changes in sorting are observable.

5. Fig 7:COX1 and erlin1 do seem to be excluded from ER exit sites. Need to show a control ER protein that is not statically retained is observed in ER sites under these conditions (not VSVG tsO45 upon temp drop as this represents a large wave of cargo - so concentration very easy to see).

-We have added ER-GFP to Fig. 1, making it clear that a control protein known to exit the ER by bulk flow is detectable as colocalized with COPII at ER exit sites in our hands.

Referee #2

The mechanisms that prevent ER resident proteins from leaving this compartment are not fully understood. Signal-mediated retrieval seems to provide a back-up system, but the main mechanisms probably involve retention. This manuscript focuses on torsinA, a AAA+ ATPase localized to the ER lumen. The results document that a hydrophobic N-temrinal domain (NTD) in torsinA is necessary and sufficient for ER retention, and that the NTD associates with the inner surface of the ER membrane but does not span the membrane. This membrane-NTD association is crucial for ER retention, suggesting that ER retention of torsin A involves partitioning into specific membrane domains. Additional lumenal ER proteins may exhibit similar behavior. In sum, this study identifies a retention signal for a largely uncharacterized topological class of ER resident proteins, and provides insight into possible mechanisms of retention. I find the work to be thorough, carefully controlled, and scholarly. The data are of high quality and the results will be of broad interest to the cell biology community.

I have only one substantive concern. After low temperature treatment, if a protein remains

distributed through the entire ER, the authors conclude that the protein is "excluded" from ER exit sites. But the counterexamples are proteins such as VSVG that have specific ER export signals mediating COPII-dependent concentration. Is it clear that a bulk flow marker would behave differently than torsinA? In other words, does the low temperature behavior of torsinA merely reflect lack of a concentrative ER export signal rather than active exclusion from ER exit sites? To address this point, the authors could do a low temperature experiment with a construct such as ss-mGFP-KDEL, which should exit the ER by bulk flow.

We have added the suggested experiment, showing that ss-ERmGFP (bulk flow GFP) is present in areas positive for COPII (Fig. 1E). This differs from both torsinA which is excluded from these areas and from VSVG which is heavily concentrated in them. Note that colocalization between ssGFP and COPII containing exit sites is also seen at 37°C, as expected for a protein that is (unlike misfolded VSVG) also secreted under normal conditions. The same is true for Δ26-43 torsinA (Fig. S1).

Minor points:
1. In Fig 3A, please explain what the colors mean. Done.

2. On p. 10, "Fig 3G" should read "Fig 3F". Fixed.

3. Fig 4F needs an "F" label. Fixed.

4. Fig 4A-C should be referenced in the text. Fixed.

5. Fig 8i doesn't really fit with the data suggesting that ER exit of torsinA is inhibited. Perhaps the membrane composition of the transitional ER is different from that of the rough ER, and torsin A partitions selectively into the rough ER domain? That interpretation seems more consistent with the results presented here.

Our drawing (now Fig 9) is meant to schematize two potentially complementary factors that could contribute to the selective partitioning of torsinA into ER sheets and away from ER exit sites. In our edited drawing, panel i refers to possible differences in the membrane composition of the transitional ER vs. the ER sheets in which torsinA is localized. Panel ii is intended to emphasize the potential impact of membrane curvature on partitioning of lumenal monotopic ER membrane proteins such as torsinA. Obviously other factors may also contribute to the observed partitioning, and the model is not meant to exclude these considerations. We have clarified these issues in the discussion.

6. The term "intrinsic retention" in the title is ambiguous. How about "A retention signal blocks endoplasmic reticulum export of the lumenal monotopic membrane protein torsinA"?

We agree with the reviewer that “intrinsic retention” could be confusing and have removed this from the title. We prefer not call the NTD a ‘retention signal’ because this implies the presence of a specific sequence responsible for retention. The properties we describe are more general in nature and can apply to any monotopic membrane interacting domain. We have therefore adjusted the title to be: “Static retention of the lumenal monotopic membrane protein torsinA in the endoplasmic reticulum”.

Referee #3

This study investigates the mechanism of ER residence of TorsinA, a member of the AAA family of ATPases linked to early-onset torsion dystonia. TorsinA is a lumenal ER protein anchored to the membrane by an N-terminal hydrophobic domain (NTD), which previous studies had implicated in determining ER residence. Here the authors show that Torsin A is kept in the ER by static retention, i.e., by exclusion from ER exit sites, and that the NTD is both necessary and sufficient for this retention. By several converging lines of evidence they convincingly show that the NTD does not span the bilayer, thus defining Torsin A as a monotopic membrane protein. CD analysis suggests that the NTD may be in alpha helical conformation when in a lipid environment, and mutation of hydrophobic residues on one, but not on the other face of the putative alpha helix results in escape of the mutants from the ER and their secretion. The authors suggest that

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partitioning of membrane-associated non-spanning alpha helices into disordered, or flat domains of the ER may represent a general retention mechanism for monotopic ER lumenal proteins.

This is a very interesting study, which presents high quality data and introduces a novel concept in membrane traffic. The paper is clearly written and illustrated. Nevertheless a few points need clarification/correction before publication.

1) FRAP experiments: Figure 3D demonstrates >10 fold reduction of GFP diffusion when it is attached to torsinA's NTD. In Fig. 6D the diffusion of constructs with ineffective or secretion-inducing mutations in the NTD is analysed. In this analysis, E171Q-torsinA-mGFP is being used, thus the measurements made here cannot be compared with those of Fig. 3D, however, the increase in apparent D in the secretion mutants compared to those retained in the ER is quite modest in comparison to the data of Fig. 3D. Some important information is missing for a critical interpretation of the data, and should be supplied before publication. Needed are diffusion measurements for two additional constructs, i.e., E171Q-torsinA-mGFP with wild-type NTD (membrane-associated) and deleted NTD (soluble in the ER lumen). As an alternative (or in addition) mutant constructs of the 1-67mGFP could be produced and analysed.
Still referring to Fig. 6D, the meanings of the statistical comparisons shown in the panel are not clear to me: why are the mutations at positions 30 and 34 compared respectively with the ones at positions 28 and 31? One might as well have compared position 30 with position 31 and 34 with 28. Actually for four group comparisons an Anova test is required.

We have added new experiments to Fig. 6 (new Fig. 6E). These measurements (comparing the diffusive behavior of E171Q torsinA in the presence vs. absence of the NTD) clarify the data presented in Fig. 6F by setting the upper and lower limits for diffusion in this system. The important point of the data remains that there are statistically significant (now correctly analyzed, see Figure legend) differences between the two groups of mutant proteins.

2) Table I, Fig. 7, text p. 15: The authors have included Erlin 1 and 2 as possible group II monotopic proteins, based on the compilation of Gilchrist et al., which included the two erlins as signal peptide bearing proteins. Actually, experimental evidence (Pearce et al., quoted by authors in the Table) indicates that neither of the two erlins has a cleaved signal sequence, and that the N-terminal hydrophobic sequence serves instead as a signal anchor. Furthermore, the erlins have no additional potential membrane-interacting hydrophobic sequences other than the one at their N-terminus. Thus, both sequence information and experimental results indicate that the erlins are bona fide type II transmembrane proteins. The two erlins should therefore be removed from the table and the IF analysis of erlin 1 in Fig. 7 should be deleted.

We agree that this is a likely possibility and have removed the data on erlin proteins from the manuscript. We note that their exclusion from ER exit sites (reported in the original submission of our manuscript) supports the working paradigm in this paper, whether they are transmembrane domain proteins statically retained in the ER by exclusion from exit sites or similarly excluded lumenal monotopic proteins.

3) Western blot analysis of culture media (Methods, p. 25) - first two sentences of the section "Immunoprecipitation from culture medium": it is not clear whether in some experiments immunoprecipitation was performed from the medium of 16 h transfected cells, or whether in all experiments (not only in those with BFA), fresh medium was added at 16 h, so that we are always looking at the torsinA secreted in 6 h.

Methods have been clarified. In each experiment, secreted protein was collected for the same amount of time. In the BFA experiment, both control and BFA treated samples received new medium at the same time and we are looking in each case at the torsinA secreted in 6 hrs.

Minor points.

Title: to my knowledge, the expression "intrinsic retention" has not been used in the past to define retention mediated by exclusion from exit sites. For this phenomenon, the expression "static retention" has generally been used. If the authors feel that intrinsic retention is a better way of describing the exclusion process, they should explain why, somewhere in the Introduction or Discussion of the paper.

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As also requested by reviewer #2, we have deleted the phrase intrinsic retention. (We had intended this as a way of stating that the determinants of ER retention are entirely encoded within the protein sequence, but obviously this was confusing). We now use static retention in the revised title.

Table I: The definition of the three groups should be given in a note under the table.

Done.

p. 15: the sentence "a receptor-mediated mechanism for restricting a protein to the ER does not currently exist" is a bit misleading, because, although strictly speaking a recycling protein is not restricted to the ER, in most people's minds a protein that at steady state is detectable only (or nearly only) in the ER would be considered to be ER restricted, yet the retrieval process is mediated by receptors. Therefore, the word "restricting" should be replaced by "excluding from exit sites". In addition "does not currently exist" should be replaced by "is currently considered unlikely".

Changes have been made.

2nd Editorial Decision 01 June 2011

Thank you for sending us your revised manuscript. Our original referees have now seen it again. In general, the referees are now positive about publication of your paper. Still, referee 1 thinks that there is one remaining issue that needs to be addressed (see below) before we can ultimately accept your manuscript. Referee 2 has one minor suggestion. I would therefore like to ask you to address these issues. Furthermore, I would like to ask you to include an author contribution section into the main body of the manuscript text according to our updated guidelines.

I am looking forward to receiving your amended manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

The additional experiments and text have substantially improved the manuscript. However, there remains an important point that I do not believe has been adequately addressed. A key prediction of the model is that deletion of the NTD would result in torsinA entering ER exit sites, and this was a major question raised in my original review. However, the new data presented in Fig S1 (showing distribution of Δ26-43 torA-GFP relative to ER exit site marker) are not convincing. The colocalisation of Δ26-43 torA-GFP with Sec31A should be quantified and compared to full length torA-GFP and ER-GFP (as in Fig 1G), and the data presented in Fig 1.

Minor point: Fig 7 title: remove reference to erlin-1

Referee #2:

The authors have addressed the previous concerns, and in my opinion the revised manuscript is an impressive story that is suitable for publication.

I have only one minor request for clarification about the new data showing that torsinA is preferentially localized to ER sheets rather than tubules. On pp. 16-17 the authors state: "Because of the inherent curvature of the tubules, we would expect proteins in the lumenal leaflet to distribute preferentially into the sheets rather than the tubules." The reasoning behind this statement should be explained briefly at this point, and should be developed more fully in the Discussion.

The idea favored by the authors is that cytosolically oriented proteins such as COPII or reticulons promote membrane curvature and thereby squeeze the lumenal leaflet, making it unfavorable for torsinA to partition into the curved region of the membrane. But what if lipids redistribute between the leaflets to relieve crowding? Moreover, curving the membrane incurs an energy penalty, so one could argue that partitioning of torsinA into a curved region would be favored because it would reduce curvature and thereby relieve membrane stress. By the same token, partitioning of torsinA into flat sheets might tend to promote energetically unfavorable concave curvature.

Alternatively, as briefly mentioned at the end of the Discussion, perhaps oligomeric torsinA is wide and flat, and therefore cannot insert easily into a curved membrane. In this case the relevant issue is not the crowding of the lipids, but rather the rigidity of the oligomeric protein. It will be helpful if these various perspectives can be discussed in a somewhat more systematic way.

Referee #3:

The revised version of the manuscript by Vander Heyden et al., is considerably improved and addresses all my concerns as well as those of the other two reviewers.

2nd Revision - authors' response 05 June 2011

Thank you for the positive reviews of our paper and remaining helpful suggestions for improvement. We have revised the paper to add the data requested by Referee 1 (added to Fig 2) and clarify the issue raised by Referee 2, as detailed below. We include an author contribution section in the manuscript text (on the cover page). If this is not where it belongs, please feel free to move it to appropriate location. We will look forward to hearing from you, and thank you for the helpful handling of our paper.

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The prediction that deletion of the NTD will result in torsinA entering ER exit sites is indeed a key prediction of our model that we test and show to be the case. We have added the requested data and quantitation (colocalization of Δ26-43 torA-mGFP – both wild-type and E171Q - relative to ER exit site marker). We have placed this data in Fig 2 F-H (rather than Fig 1) because the NTD is first identified and discussed in Fig 2. The data and quantification in Fig 2 F-H can be directly compared with that of full-length torA-GFP and ER-GFP in Fig 1, as stated in the text.

Minor point: Fig 7 title: remove reference to erlin-1

Done.

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We appreciate the referee's comments and have modified our presentation of the membrane curvature issue. In the results section (pp. 16-17, heading "Preferential partitioning of lumenal monotopic membrane proteins into ER sheets") we added a few sentences and references to explain why we think that monotopic membrane proteins would prefer sheets. In the discussion section, we made parallel changes, and added specific consideration of how the rigidity of the torsinA oligomer might influence its partitioning. These changes clarify our presentation, and we thank the referee for the suggestions.

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3rd Editorial Decision 15 June 2011

Thank you for sending us your re-revised manuscript. Our original referee 1 is now happy with it, and the paper will be publishable in The EMBO Journal. You will receive a formal acceptance letter shortly.

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1

The authors have quantified the colocalisation between the ER exit site marker and torsinA as requested. This shows deletion of the NTD results in increased localisation of torsinA in ER exit sites.

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