THE EMBO JOURNAL

Manuscript EMBO-2010-73934

A classical NLS and the SUN domain contribute to the targeting of SUN2 to the inner nuclear membrane

Yagmur Turgay, Rosemarie Ungricht, Andrea Rothballer, Alexa Kiss, Gabor Csucs, Peter Horvath and Ulrike Kutay

Corresponding author: Ulrike Kutay, ETH Zürich

Review timeline:

Submission date:	12 February 2010
Editorial Decision:	11 March 2010
Revision received:	09 May 2010
Editorial Decision:	14 May 2010
Accepted:	14 May 2010

Transaction Report:

(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 11 March 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been seen by three referees whose comments to the authors are shown below. You will see that the referees are generally positive about your work and that they would support its ultimate publication in The EMBO Journal after appropriate revision. I would thus like to invite you to prepare a revised manuscript in which you need to address the issues raised by the referees in an adequate manner. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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 (Remarks to the Author):

The manuscript analyzes domains in SUN2 that are responsible for its targeting to the INM. Interestingly the authors identified a cNLS in the N-terminal aa 38-55, which binds Imp a and b in an RanGTP sensitive manner and is essential for nuclear import of an N-terminal fragment in in vitro nuclear transport assays in permeabilized cells and upon ectopic expression in cells. Lack of transport of a SUN2 fragment with mutated NLS as well as addition of the putative SUN2-NLS to cytoplasmic proteins convincingly demonstrated that the SUN2 N-terminus contains a bona fide functional cNLS domain. However full length SUN2 with mutated NLS localized to the INM nearly as efficiently as wild type controls (convincingly shown by a series of experiments including endogenous SUN2 downregulation and Digitonin versus Triton extraction). Interestingly the N-terminus also contained a COP binding Golgi retrieval signal that was important for efficient INM targeting. Finally, by producing chimeric proteins of SUN2 and SPAG4 the authors demonstrate that the SUN domain of SUN2 also contributes to INM targeting.

Overall this is a careful analysis of several domains in SUN2 that mediate its efficient targeting to the INM. In particular, the existence of a functional NLS motif in SUN2 is a novel and interesting finding, supporting and extending previous studies in yeast by the Blobel lab. This is the first report on an NLS-mediated INM targeting in mammalian cells.

The manuscript is well written and the experiments are in general well controlled and technically sound. There are only a few points that have to be addressed:

One general comment and concern is that cellular localization is determined by transient expression of ectopic SUN2 fragments. The authors should comment on how they controlled for expression levels of ectopic proteins. Leakage of integral INM proteins into the ER/Golgi is a general phenomenon upon overexpression.

Fig. 5: In view of the finding that a N-terminal SUN2 fragment containing the NLS, but missing the region between aa 92-109 is very inefficiently targeted to the INM, while the NLS was fully functional when fused to unrelated cytoplasmic protein, it would be interesting to test the functionality of the NLS in a fusion to an integral ER membrane protein. This experiment would also address whether the coatamer sequence is exclusively involved in retrieval from the Golgi, or whether it has some additional role in transport.

Fig. 7 is very complex and confusing. Please provide a schematic drawing to easily depict the different SPAG4 and SUN2 domains in the fusion proteins used. Furthermore, why is the SUN domain of SPAG4 not able to mediate INM targeting at least in chimeric proteins?

specific comments

Fig. 1A: the drawing of the construct SUN2 (159-260) and SUN2-delta-N158 do not fit together
Page 9, Fig. S2A: The differential permeabilization method is briefly mentioned here, while it is described in more detail on page 12. I recommend to provide more details on this assay, when it is first mentioned and refer to this in the later parts.

p10, Fig. 5A,B: accumulation of specific SUN2 fragments in the Golgi is an important finding. Data supporting this conclusion (colocalization with Golgi markers) should therefore be shown rather than simply refering to it as "data not shown".

p11, Fig.5C: There is significantly decreased binding of the NLS-mutant to COP proteins. The authors should comment on this.

Supp.Fig.3: The figure should also show the pre-bleach fluorescence. The recovery of SUN2-wt seems to be much faster than reported, e.g. in Oestlund JCS 2009, Nov 15.

Referee #2 (Remarks to the Author):

Turgay et al describe in their manuscript "A classical NLS and the SUN domain contribute to the targeting of SUN2 to the inner nuclear membrane" that for efficient localization of the integral membrane protein SUN2 to the inner nuclear membrane three determinants are required: a classical nuclear localization signal, which is found in many soluble nuclear proteins and recognized by the import receptors importin α and β, a basic sequence motif recognized by the COPI coatomer, which probably functions in retrieving escaped proteins from the Golgi apparatus, and the so called SUN domain, which is localized in the perinuclear space between outer and inner nuclear membrane and known to bind other nuclear membrane proteins.

In general, it is not well understood which signals are important to localize inner nuclear membrane proteins to their site of action. This is surprising especially in the light that in recent years not only the number of known inner nuclear membrane proteins increased enormously but also the functional diversity. Binding of some proteins to chromatin and/or lamin has been implicated for their targeting. In yeast, two inner nuclear membrane proteins were shown to require the import receptors importin α and βbut it remained unclear whether this is a common mechanism relevant for many proteins and also found in metazoan. The current manuscript suggests that this is indeed the case showing it for the first mammalian protein. Of equal importance, this study adds two more and unexpected determinants relevant for SUN2 localization suggesting that there might exist many and overlapping ways for inner nuclear membrane targeting.

The manuscript is of high importance to the fields of nuclear structure and function as well as membrane trafficking, but also interesting for a broader readership. The data are as usual from the Kutay lab of very high quality, well controlled and carefully analysed. The conclusions are supported by the data and add some novel and important aspects to the questions of targeting of membrane proteins. I happily recommend the manuscript for publication. Some minor points are indicated below.

1.) The statement that SUN2 fragments accumulate in the ER membrane system is misleading (first paragraph of the result section): SUN2 (ΔM158) and SUN2 (159-260) show rather Golgi localization as also later in the manuscript indicated by the authors.

2.) Why is the COPI binding to the SUN2 NLS mutant in figure 5c weaker as compared to the wt? The authors should at least comment this.

3.) There is a discrepancy in the description of the concentration of RANQ69L and transport receptors used in the pulldown experiments. Is it 2µM and 1µM, respectively, as stated in the Material and Method section or 2.5µM and 1.5µM, respectively, as stated in the figure legend?

4.) The parts of figure 4 B and C are reversed in the figure legend as compared to the text and the figure. Scale bars are missing in figure 4 and 5.

5.) A discussion whether the COPI binding motif is found in other inner nuclear membrane protein would be interesting. Similarly, is the SUN domain found in all members of the family and would it contribute to inner nuclear membrane localization?

6.) The labeling of the three argenine containing regions in the SUN2 alignment as INM? (Figure S1) is confusing and, also in the light of the experiments shown in Figure S5, misleading. Actually, I don't understand why the authors show these experiments: If it is to emphasize that for membrane proteins point mutations can cause misorientation that could lead to misinterpretations it would be a helpful warning for others, but the authors should comment this. If it is to explain other (contradicting?, published?) experiments the authors should refer to them. At the moment Figure S5 is only confusingly disconnected to the manuscript.

Referee #3 (Remarks to the Author):

This is a very well documented study in which the authors dissect out sorting motifs contained within Sun2, a mammalian inner nuclear membrane protein. Sun2, along with a second closely related INM protein, Sun1, functions to tether KASH domain proteins in the ONM.

The authors are able to conclude that there are multiple determinants within Sun2 that contribute to its localization within the INM. Other groups have provided evidence that at least some INM proteins contain a functional NLS that is recognized by Impa/b family members and which facilitate targeting to the INM. The authors demonstrate quite convincingly that Sun2 contains a bipartite NLS within its N-terminal nucleoplasmic domain and that this plays a role in Sun2 localization.

A second determinant contained within the N-terminal domain features an arginine cluster that interacts with components of COP1 coated vesicles. The implication based on a series of immunofluorescence experiments is that this represents an ER retrieval motif. This is borne out by the fact that recombinant Sun2, or deletion mutants thereof, become mislocalized to the Golgi apparatus. Finally the authors demonstrate that a luminal determinant also contributes to targeting. They speculate that interaction between the SUN domain and ONM KASH domain proteins my serve to stabilize Sun2 in the ONM.

On balance I feel that this is a significant piece of work that clarifies earlier ideas about the mechanisms of INM protein localization. It should therefore be suitable for publication in the EMBO J. There are a couple of issues that authors should address.

It is still not clear to me what role the lumenal domain plays in Sun2 localization. Based upon the findings with SPAG4(1-189)-SUN2(239-506) I am willing to go along with the authors' contention that KASH interactions may contribute to localization. The lack of INM localization of SPAG4(1-189)-SUN2(239-506) suggests to me that the coiled coil domain, in the absence of the SUN domain is unable to efficiently oligomerize with FL or endogenous Sun2. This should be very easy to address in pull-down experiments.

On re-reading the literature it seems to me that the authors findings might actually be backed up by a previous study on Sun1 (Liu et al. (2007) JCB 178: 785-98). Although this does not identify sorting signals, the behaviour of certain Sun1 deletion constructs and fusion proteins bears a striking resemblance to what is described here. It certainly lends support to the author's contention that a conventional NLS might be acting in concert with an ER retrieval signal.

In summary, this is a useful study that highlights some new principles in INM protein sorting and should merit publication in EMBOJ following relatively minor modifications. On a trivial note, there are quite a few grammatical errors that need correcting.

1st Revision - authors' response 09 May 2010

Referee #1:

One general comment and concern is that cellular localization is determined by transient expression of ectopic SUN2 fragments. The authors should comment on how they controlled for expression levels of ectopic proteins. Leakage of integral INM proteins into the ER/Golgi is a general phenomenon upon overexpression.

We agree that this is a valid concern for INM proteins that solely rely on a retention mechanism for INM targeting. Unfortunately, overexpression cannot be avoided in this type of experiments.

Being aware of this phenomenon, we analyzed at the outset of our study whether mislocalization of ectopically expressed SUN2-GFP and mutants thereof to the ER would correlate with expression levels. In these experiments, we did not observe any correlation (now included in the Supplement, **new Figure S3**).

Second, throughout our studies, we always compared expression levels of different constructs by Western blotting ensuring that different fragments and mutants were expressed on average to a similar level (not shown). Third, when comparing the localization of individual GFP-tagged SUN2 fragments and mutants, we ensured to compare cells with a similarly low fluorescence intensity (GFP signal), which gives a direct measure for expression levels. By these three criteria, we ensured that our conclusions derived from comparing different SUN2 constructs are not influenced by overexpression.

Fig. 5: In view of the finding that a N-terminal SUN2 fragment containing the NLS, but missing the region between aa 92-109 is very inefficiently targeted to the INM, while the NLS was fully functional when fused to unrelated cytoplasmic protein, it would be interesting to test the functionality of the NLS in a fusion to an integral ER membrane protein. This experiment would also address whether the coatamer sequence is exclusively involved in retrieval from the Golgi, or whether it has some additional role in transport.

This is an excellent suggestion. We have therefore extended our analysis by producing chimera between N-terminal fragments of SUN2 and the ER-localized protein SPAG4. Our new data **(new Figure 7)** shows that a minimal fragment of SUN2 comprising amino acids 25 to 120 is able to enrich both full-length SPAG4 and an N-terminal membrane-bound part of SPAG4, SPAG4(1-189), at the INM. In these chimera, only the combined mutation of the NLS and the coatomer binding sequence compromised NE targeting, suggesting that the 4R motif might indeed contribute also more directly to INM targeting. Moreover, we also fused the N-terminal SUN2 fragment to the ER-localized form of cytochrome b5 and observed NE targeting **(new Figure S5)**.

Fig. 7 is very complex and confusing. Please provide a schematic drawing to easily depict the different SPAG4 and SUN2 domains in the fusion proteins used. Furthermore, why is the SUN domain of SPAG4 not able to mediate INM targeting at least in chimeric proteins?

Figure 7 is now Figure 8. To improve clarity, we have included a schematic drawing illustrating the assembly of the different fusion proteins in a new panel A to Figure 8.

It is indeed at first glance puzzling that the SUN domain of SPAG4 does not mediate INM targeting. However, it is consistent with the fact that we have so far been unable to detect an interaction between the SUN domain of SPAG4 and the C-terminal KASH domains of nesprin-1 and nesprin-2 (Rothballer and Kutay, unpublished).

specific comments

Fig. 1A: the drawing of the construct SUN2 (159-260) and SUN2-delta-N158 do not fit together

We have corrected this mistake.

Page 9, Fig. S2A: The differential permeabilization method is briefly mentioned here, while it is described in more detail on page 12. I recommend to provide more details on this assay, when it is first mentioned and refer to this in the later parts.

We have changed this as suggested.

p10, Fig. 5A,B: accumulation of specific SUN2 fragments in the Golgi is an important finding. Data supporting this conclusion (colocalization with Golgi markers) should therefore be shown rather than simply refering to it as "data not shown".

The colocalization with the Golgi marker protein giantin is now shown in the **new Figure S4**.

p11, Fig.5C: There is significantly decreased binding of the NLS-mutant to COP proteins. The authors should comment on this.

We have repeatedly observed this decrease in binding of COPI to the NLS-mutant protein, but currently we do not understand the molecular explanation for this. With respect to its expression and solubility in E.coli, the mutant protein does not differ from the wild-type, suggesting that it is folded correctly. It could be that the NLS itself contributes to efficient COPI interaction. But clearly, the NLS motif cannot work autonomously because the 4A mutant is completely deficient in coatomer binding. As a contribution of the NLS to coatomer binding is very speculative at this point and not easy to test, we prefer not to start a lengthy discussion on this issue. Rather, we now mention this observation in the text, to read:

'When the 4R motif was mutated (2z-SUN2(1-158)4A), COPI association was lost (Fig. 5C). In contrast, 2z-SUN2(1-158) harbouring the NLS mutation still bound the coatomer complex, albeit with reduced efficiency when compared to the wild-type construct.'

Supp.Fig.3: The figure should also show the pre-bleach fluorescence. The recovery of SUN2-wt seems to be much faster than reported, e.g. in Oestlund JCS 2009, Nov 15.

We added a panel showing the pre-bleach fluorescence to Figure S6 (former S3).

Indeed, our recovery is faster than what has been observed by Oestlund et al.. Their and our FRAP experiments differ by at least two parameters. (1) Whereas Oestlund et al. used a GFP-SUN2 fusion, our FRAP analysis relied on a SUN2-GFP fusion protein. To test if the C-terminal GFP tag attached to the SUN domain might explain this difference, we have now also tested GFP-SUN2, but obtained identical recovery curves. (2) The second difference between both studies is the cell type. We have worked with HeLa cells. The Oestlund study used MEFs. We therefore suspect that the differences reflect cell type variations.

Referee #2:

The manuscript is of high importance to the fields of nuclear structure and function as well as membrane trafficking, but also interesting for a broader readership. The data are as usual from the Kutay lab of very high quality, well controlled and carefully analysed. The conclusions are supported by the data and add some novel and important aspects to the questions of targeting of membrane proteins. I happily recommend the manuscript for publication. Some minor points are indicated below.

1.) The statement that SUN2 fragments accumulate in the ER membrane system is misleading (first paragraph of the result section): SUN2 (ΔN158) and SUN2 (159-260) show rather Golgi localization as also later in the manuscript indicated by the authors.

We now explicitly mention the Golgi staining when describing the Figure.

2.) Why is the COPI binding to the SUN2 NLS mutant in figure 5c weaker as compared to the wt? The authors should at least comment this.

See response to reviewer 1.

3.) There is a discrepancy in the description of the concentration of RANQ69L and transport receptors used in the pulldown experiments. Is it 2μM and 1μM, respectively, as stated in the Material and Method section or 2.5μM and 1.5μM, respectively, as stated in the figure legend?

We apologize for the mistake. It is now corrected.

4.) The parts of figure 4 B and C are reversed in the figure legend as compared to the text and the figure. Scale bars are missing in figure 4 and 5.

Fixed.

5.) A discussion whether the COPI binding motif is found in other inner nuclear membrane protein would be interesting. Similarly, is the SUN domain found in all members of the family and would it contribute to inner nuclear membrane localization?

Indeed, when inspecting the sequence of other human INM proteins we found potential COPI binding motifs. We now refer to this in the discussion to read:

'Interestingly, when inspecting the sequences of other mammalian INM proteins, we could spot similar clusters of arginine residues in LBR (74-76), in emerin (44-46) and in LEM2 (130-132; 454-456), indicating that other INM proteins might also rely on the use of Golgi retrieval signals to support their efficient delivery to the INM.

For the comment on the SUN domain in different SUN family members, see answer to reviewer 1.

6.) The labeling of the three argenine containing regions in the SUN2 alignment as INM? (Figure S1) is confusing and, also in the light of the experiments shown in Figure S5, misleading. Actually, I don't understand why the authors show these experiments: If it is to emphasize that for membrane proteins point mutations can cause misorientation that could lead to misinterpretations it would be a helpful warning for others, but the authors should comment this. If it is to explain other (contradicting?, published?) experiments the authors should refer to them. At the moment Figure S5 is only confusingly disconnected to the manuscript.

We feel that Figure S5 (now Figure S8) adds important information to the manuscript, because there is a body of work from Sharon Braunagel and Max Summers on inner nuclear membrane sorting motifs in higher eukaryotic INM proteins. Our manuscript would be incomplete if we would not address the potential influence of such signals on INM targeting of SUN2. It is not intended to contradict any published work, but a matter of completeness. Further, the Figure adds an important observation with respect to NLS requirement, as for the SUN2(RR205/206AA) mutant, the NLS is shown to be pivotal for efficient NE localization (previous Figure S5B, now Figure S8B), further substantiating our conclusion on the importance of the NLS sequence for targeting membrane proteins to the INM.

To address the concern of the reviewer, we have extended the legend to Figure S1, better describing the putative INM sorting motifs and why we tested altogether three of them. Moreover, we have revised and shortened the discussion of Figure S8 in the manuscript. Last, we have corrected two slight mistakes in the labeling of two pictures in Figure S8 which might have contributed to confusion.

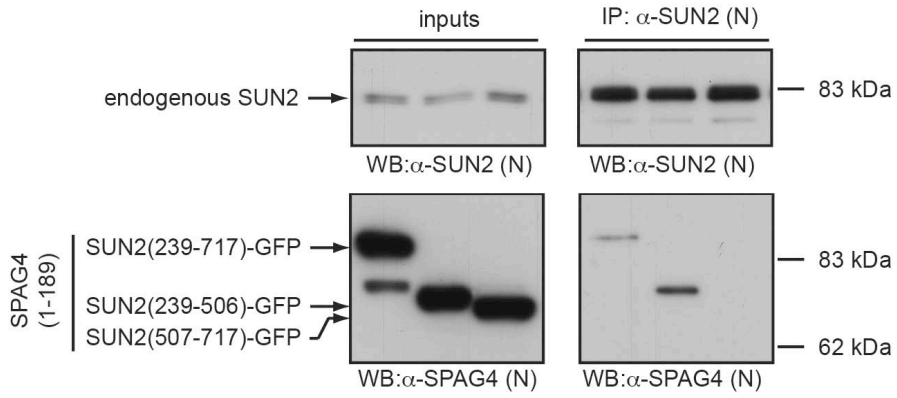
Referee #3 (Remarks to the Author):

On balance I feel that this is a significant piece of work that clarifies earlier ideas about the mechanisms of INM protein localization. It should therefore be suitable for publication in the EMBO J. There are a couple of issues that authors should address.

It is still not clear to me what role the lumenal domain plays in Sun2 localization. Based upon the findings with SPAG4(1-189)-SUN2(239-506) I am willing to go along with the authors' contention that KASH interactions may contribute to localization. The lack of INM localization of SPAG4(1-189)-SUN2(239-506) suggests to me that the coiled coil domain, in the absence of the SUN domain is unable to efficiently oligomerize with FL or endogenous Sun2. This should be very easy to address in pull-down experiments.

We have performed the requested pull-down experiment (see Reviewer3.tif). The experiment shows an immunoprecipitation of endogenous SUN2 from cells expressing SPAG4 chimeras. As expected, all SPAG4(1-189) fusion constructs comprising the coiled-coil domain of SUN2, i.e. fusion constructs of SPAG4 with the entire lumenal part of SUN2 (aa 239-717) or only the coiled-coil domain of SUN2 (aa 239-506) can oligomerize with endogenous SUN2, whereas the fusion with the isolated SUN domain (aa 507-717) cannot.

These data are fully consistent with the coiled-coil domain of SUN2 being the main determinant for dimerization. Still, as noted by the reviewer, SPAG4(1-189)-SUN2(239-506) does not efficiently localize to the INM. This is best explained by the fact that the ectopically expressed constructs only associate with endogenous SUN2 to a minor extent (see Figure). This is consistent with the results presented in Figure S2, demonstrating that siRNA-mediated depletion of SUN2 slightly stimulates NE localization of the ectopically expressed wild-type or NLS-mut SUN2.



On re-reading the literature it seems to me that the authors findings might actually be backed up by a previous study on Sun1 (Liu et al. (2007) JCB 178: 785-98). Although this does not identify sorting signals, the behaviour of certain Sun1 deletion constructs and fusion proteins bears a striking resemblance to what is described here. It certainly lends support to the author's contention that a conventional NLS might be acting in concert with an ER retrieval signal.

We agree.

In summary, this is a useful study that highlights some new principles in INM protein sorting and should merit publication in EMBOJ following relatively minor modifications. On a trivial note, there are quite a few grammatical errors that need correcting.

We have thoroughly revised the text.

2nd Editorial Decision 14 May 2010

Thank you for sending us your revised manuscript. Our original referee 1 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner. The paper will now be publishable in The EMBO Journal and 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 (Remarks to the Author):

The revised manuscripts addresses most of the concerns raised by the reviewers.
Importantly it at least addresses the issue of overexpression and includes control experiments in a new supplementary figure.

Chimeras between SUN2 and the ER protein SPAG4 were included in the analysis, revealing a more direct role of the coatamere sequence in nuclear transport.
Other issues concerning clarity of data presentation and clarifications in the text have been addressed.

