Functional association of Sun1 with nuclear pore complexes

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Introduction

The nuclear envelope (NE) is the selective barrier that defines the interface between the nucleus and the cytoplasm (Burke and Stewart, 2002; Gruenbaum et al., 2005). Because it mediates molecular trafficking between these two compartments, it plays an essential role in the maintenance of their biochemical identities. In addition to its transport function, the NE is also a key determinant of nuclear architecture, providing anchoring sites at the nuclear periphery for chromatin domains as well as for a variety of structural and regulatory molecules. A corresponding contribution to cytoplasmic structure has been described in which NE components may also influence cytoskeletal organization and mechanotransduction (Tzur et al., 2006; Worman and Gundersen, 2006).

The NE is composed of several structural elements, the most prominent of which are the inner nuclear membranes (INMs) and outer nuclear membranes of mammalian nuclear envelopes. Both immunofluorescence and immunoelectron microscopy reveal that Sun1 but not Sun2 is intimately associated with nuclear pore complexes (NPCs). Topological analyses indicate that Sun1 is a type II integral protein of the INM. Localization of Sun1 to the INM is defined by at least two discrete regions within its nucleoplasmic domain. However, association with NPCs is dependent on the synergy of both nucleoplasmic and luminal domains. Cells that are either depleted of Sun1 by RNA interference or that overexpress dominant-negative Sun1 fragments exhibit clustering of NPCs. The implication is that Sun1 represents an important determinant of NPC distribution across the nuclear surface.

Sun1 and 2 are A-type lamin-binding proteins that, in association with nesprins, form a link between the inner nuclear membranes (INMs) and outer nuclear membranes of mammalian nuclear envelopes. Both immunofluorescence and immunoelectron microscopy reveal that Sun1 but not Sun2 is intimately associated with nuclear pore complexes (NPCs). Topological analyses indicate that Sun1 is a type II integral protein of the INM. Localization of Sun1 to the INM is defined by at least two discrete regions within its nucleoplasmic domain. However, association with NPCs is dependent on the synergy of both nucleoplasmic and luminal domains. Cells that are either depleted of Sun1 by RNA interference or that overexpress dominant-negative Sun1 fragments exhibit clustering of NPCs. The implication is that Sun1 represents an important determinant of NPC distribution across the nuclear surface.
interact with nuclear or other NE components are retained and concentrated. Recent studies suggest that additional mechanisms may overlie this basic scheme. Ohba et al. (2004) showed that movement of integral proteins through the NPC membrane domain is energy dependent. Other studies suggest a role for the nuclear transport receptor adaptor karyopherin/importin-α in the transit of proteins to the INM (King et al., 2006; Saksena et al., 2006).

Recognition of ONM-specific membrane proteins raises the question of what prevents these proteins from escaping to the peripheral ER. In Caenorhabditis elegans, the localization of Anc-1, an ONM protein involved in actin-based nuclear positioning, requires Unc-84, an INM protein whose retention is lamin dependent (Lee et al., 2002; Starr and Han, 2002). These observations led to a model in which Unc-84 and Anc-1 interact across the PNS via their luminal domains, providing a mechanism for the tethering of ONM proteins.

In mammals, two large actin-binding proteins, nesprin 1 Giant (nesp1G; 1,000 kD) and nesprin 2 Giant (nesp2G; 800 kD), reside in the ONM (Apel et al., 2000; Zhang et al., 2001; Mislow et al., 2002; Zhen et al., 2002). The nesprins (also known as Syne 1 and 2) are related to both Anc-1 and a Drosophila melanogaster ONM protein, Klarsicht (Wolfe et al., 1998; Mosley-Bishop et al., 1999), in that they contain an ~50-amino acid C-terminal KASH (Klarsicht, Anc-1, Syne homology) domain consisting of a single transmembrane (TM) anchor and a short segment of ~30–40 residues that resides within the PNS. A third ONM KASH domain–containing protein, nesrin 3, interacts with plectin, which is a large (466 kD) cytolinker (Wilhelmsen et al., 2005).

Unc-84 contains an ~200-amino acid C-terminal region that shares homology with Sad1p, a Schizosaccharomyces pombe spindle pole body protein (Hagan and Yanagida, 1995). This sequence, which is known as the SUN (Sad1p, Unc-84) domain, resides within the PNS. The human genome encodes five SUN domain proteins. Two of these, Sun1 and 2, are lamin A–interacting proteins of the INM with topologies similar to that of Unc-84 (Hodzic et al., 2004; Crisp et al., 2006; Haque et al., 2006).

Both Sun1 and 2 cooperate in tethering nesp2G in the ONM (Padmakumar et al., 2005; Crisp et al., 2006; Haque et al., 2006; Hasan et al., 2006). This tethering involves interactions that span the PNS (Crisp et al., 2006), similar to that suggested for Unc84 and Anc-1. Unc84 also tethers Unc-83, another ONM KASH domain protein (McGee et al., 2006). Competition between nesprin 1 and 2 KASH domains (Zhang et al., 2007) suggests that nesp1G is similarly tethered. In this way, Sun1 and 2 function as links in a molecular chain that connects the actin cytoskeleton via nesprins to lamins and other nuclear components. We have termed this assembly the LINC (linker of nucleoskeleton and cytoskeleton) complex (Crisp et al., 2006). The fact that nesprin 3 binds plectin, a diverse cytolinker (Wilhelmsen et al., 2005), indicates that there may be multiple isoforms of the LINC complex responsible for integrating the nucleus with different components of the cytoskeleton.

As alluded to in the previous paragraphs, the NE can influence cytoplasmic mechanics and the responses of cells to mechanical stress. Cells depleted of either A-type lamins or emerin, an INM protein, exhibit reduced cytoplasmic resilience and an inability to activate mechanosensitive genes (Broers et al., 2004; Lammertding et al., 2004, 2005, 2006). In humans, the loss or mutation of either A-type lamins or emerin is associated with several diseases (Muchir and Worman, 2004), including Emery-Dreifuss muscular dystrophy. It is not hard to imagine that the LINC complex might be the mediator of these effects given its proposed role in nucleocytoplasmic coupling.

Less clear is the extent and nature of the interactions of LINC complex components and how these might affect LINC complex function. In the case of nesprins 1 and 2 versus nesprin 3, there are obvious differences in terms of actin versus plectin association. At the INM, the situation with the Sun proteins is more ambiguous. We know that there is some degree of functional redundancy between Sun1 and 2 with respect to nesprin 2 tethering. Furthermore, we know that Sun1 and 2 can associate with lamin A but that this interaction is not required for their localization. In this study, we further explore the interactions of SUN proteins at the nuclear periphery. In doing so, we have been able to describe discrete regions within Sun1 that function both in localization to the INM and in oligomerization. Most importantly, we are able to demonstrate that Sun1 and 2 are segregated within the INM. Although Sun2 displays a roughly uniform distribution across the NE, Sun1 is concentrated at NPCs. Elimination of Sun1 or overexpression of Sun1 mutants leads to NPC clustering. The inference is that Sun1 but not Sun2 functions in maintenance of the uniform distribution of NPCs. It also follows that certain LINC complex isoforms may mediate the differential association of cytoskeletal elements with NPCs versus NPC-free regions of the NE.

## Results

Mammalian SUN proteins are encoded by at least five genes (Fig. 1). Of these, only Sun1 and 2 are widely expressed in somatic cells (Hodzic et al., 2004; Crisp et al., 2006). Sun3 (Crisp et al., 2006; Haque et al., 2006; Tzur et al., 2006), Sun4 (SPAG4; Shao et al., 1999; Hasan et al., 2006), and Sun5 (SPAG4Like; unpublished data) seem to be restricted largely to the testis. Each of the SUN proteins conforms to the same basic structure featuring an N-terminal domain followed by a block of hydrophobic amino acid residues, likely representing a TM domain, and a C-terminal SUN domain. The relationships between these proteins are displayed in Fig. 1. In the case of Sun1, the largest of the mammalian SUN proteins, the nucleoplasmic N-terminal domain is composed of 350–400 amino acid residues (Crisp et al., 2006). All of the sequence variants of Sun1 that arise through alternative splicing involve changes in this segment of the molecule (Fig. 1).

A prominent feature of Sun1 is the presence of four hydrophobic sequences, H1–H4, any one of which could potentially function as a TM domain. Previously, we showed that H1 at least does not span the INM (Crisp et al., 2006). This conclusion was based on a naturally occurring splice isoform that is missing sequences encoded by exons 6, 7, and 8 (Sun1Δ221–344; Δexons 6–8). This isoform lacks H1 yet displays appropriate NE localization and has the same topology within the INM as full-length Sun1. Nevertheless, as will be expanded upon,
although not a TM domain, H1 may still contribute to membrane association. Mouse Sun1 also contains a predicted C2H2 zinc finger. Several splice isoforms of mouse Sun1 have been identified that feature the loss of sequences encoded by exons 6–8, including H1. Corresponding GenBank/EMBL/DDBJ accession nos. are BAB29445 (Sun1∆6–8), AAT90501 (Sun1∆6), and BAC29339 (Sun1∆8). Four other mammalian SUN proteins are known. Sun2 is ubiquitously expressed and localizes to the INM. Sun3 (SUNC1), Sun4 (SPAG4), and Sun5 (SPAG4L; GenBank/EMBL/DDBJ accession no. NP_542406) appear to be expressed primarily in testis (unpublished data). When expressed in HeLa cells, Sun3 localizes to the NE (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1), whereas Sun4 (Hasan et al., 2006) and Sun5 (unpublished data) localize primarily to the ER.

Figure 1. The mammalian SUN protein family. Sun1 features four hydrophobic sequences, H1–H4, each of roughly 20 amino acid residues. Its membrane-spanning domain is contained within the H2–H4 region. The Sun1 N terminus, including H1, is nucleoplasmic. The C-terminal SUN domain resides in the PNS. Murine but not primate Sun1 contains a predicted C2H2 zinc finger. Several splice isoforms of mouse Sun1 have been identified that feature the loss of sequences encoded by exons 6–8, including H1. Corresponding GenBank/EMBL/DDBJ accession nos. are BAB29445 (Sun1∆6–8), AAT90501 (Sun1∆6), and BAC29339 (Sun1∆8). Four other mammalian SUN proteins are known. Sun2 is ubiquitously expressed and localizes to the INM. Sun3 (SUNC1), Sun4 (SPAG4), and Sun5 (SPAG4L; GenBank/EMBL/DDBJ accession no. NP_542406) appear to be expressed primarily in testis (unpublished data). When expressed in HeLa cells, Sun3 localizes to the NE (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1), whereas Sun4 (Hasan et al., 2006) and Sun5 (unpublished data) localize primarily to the ER.

Figure 2. Sun1 and 2 are segregated within the plane of the NE. Immunofluorescence microscopy of HeLa cells stably expressing mouse Sun1-GFP or human Sun2-GFP using antinucleoporin and anti-SUN protein antibodies. (A) Images of the nuclear surface reveal Sun1-GFP colocalization with Nup153. In contrast, the more diffuse Sun2-GFP is found in NPC-free regions. Endogenous Sun2 displays no colocalization with either NPCs labeled with the antinucleoporin antibody QE5 or with Sun1-GFP. The boxed areas in the top panels are magnified in the bottom three panels. (B) At late anaphase to early telophase, reforming nuclei exhibit a distinct distribution of NPC and NE components. Sun1-GFP localizes with Nup153 at the lateral margins of the mass of newly segregated chromatids and is absent from the Sun2-positive core region. These data suggest that Sun1 is closely associated with NPCs, a pattern that is established early in NE formation. Bars (A), 5 μm; (B) 4 μm.
tends to be excluded from the core region and instead concentrates on the lateral margins of the chromatid masses where NPC assembly is initiated.

Localization of Sun1 to the INM involves determinants in both N- and C-terminal domains (Padmakumar et al., 2005; Crisp et al., 2006; Haque et al., 2006; Hasan et al., 2006; Wang et al., 2006), although their relative contribution to localization and role in NPC association remain unknown. In addition, although previous studies (Crisp et al., 2006; Haque et al., 2006) have demonstrated that the TM domains of Sun1 are contained within the H2–H4 region, they provide an ambiguous view of the targeting properties of this segment of the molecule. We and others (Padmakumar et al., 2005; Crisp et al., 2006) had proposed that Sun1 might be a multispanning protein with three TM sequences corresponding to H2, H3, and H4. However, direct evidence to support such a model is lacking.

To better define Sun1 topology, we prepared a series of mutants containing different combinations of the H2, H3, and H4 hydrophobic sequences, all of which were found to confer some degree of membrane association. These mutants contained all or part of the N-terminal domain (residues 1–355) followed by one or more hydrophobic sequences and terminating with GFP (Fig. 4).

At the N and C termini of certain chimeras, we placed HA and myc epitope tags, respectively. These constructs were expressed by transfection in HeLa cells, which were subsequently treated with low concentrations of digitonin. Under these conditions,

Figure 3. **Sun1 but not Sun2 is closely associated with NPCs as revealed by immunoelectron microscopy.** (A) Views of NPC cross sections from tetracycline-induced HeLa cells expressing Sun1-GFP that reveal anti-GFP–associated gold particles close to NPCs. (B) Sections of uninduced cells display little or no gold labeling. (C) Images of NE cross sections from HeLa cells expressing Sun2-GFP reveal gold particles in the PNS but with no preferential association with NPCs. c, cytoplasm; n, nucleus. Arrowheads indicate gold particles. (D and E) Quantitative analysis of the distribution of gold particles from NE cross sections of HeLa cells expressing Sun1-GFP (D) or Sun2-GFP (E). The position of gold particles, which is defined by horizontal distance (from the NPC eightfold axis) and vertical distance (from the central plane of the NE), was measured in cross sectioned NES (as in A and C) and plotted in a single dot graphic. Micrographs are provided as a visual reference for the position of the gold particles. Histograms for the distribution of gold particles for horizontal and vertical distances are shown on adjacent panels. For both Sun1 and 2, gold particles were scored within 200-and 600-nm windows on either side of each NPC. The larger, more conservative window size still reveals an absolute eightfold higher labeling density of Sun1 over Sun2 within 120 nm of the NPC center, with a peak density at 66 nm. A total of 88 and 92 gold particles were scored for D and E, respectively. Because of the far broader distribution of Sun2-GFP, the scale in E is five times that in D. Bars, 100 nm.
the plasma membrane is permeabilized, but the ER and nuclear membranes remain intact. The permeabilized cells were then incubated with proteinase K. During the course of this incubation, Western blot analysis revealed that cytoplasmic (tubulin) and nuclear (lamin A/C and Nup153) proteins were degraded (proteinase K may enter the nucleus by degrading NPC proteins), whereas ER lumenal and PNS proteins such as protein disulfide isomerase (PDI) were protected (Fig. 4A). Permeabilization with TX-100 resulted in complete protein degradation. Nontransfected cells served as a negative control, whereas Sun1-GFP provided a positive control, with a 65–70-kD protected fragment. Western blot analysis was used to confirm the effectiveness of the digitonin permeabilization. Tubulin and lamins A/C were degraded after either digitonin or TX-100 permeabilization. In contrast, the ER lumenal protein PDI remained intact after digitonin permeabilization but was degraded after TX-100 treatment. Analyses focused on cells expressing sufficiently high levels of recombinant protein such that GFP fluorescence could be observed in both the NE and ER/cytoplasmic membranes. With digitonin permeabilization, both myc and HA epitopes were readily detected for HA-Sun1N380-GFP-myc and HA-Sun1N415-GFP-myc. In contrast, the HA but not C-terminal myc epitope tag was accessible for HA-Sun1N455-GFP-myc. In all cases, both myc and HA tags were accessible after TX-100 permeabilization. (C) Three more C-terminal GFP-tagged Sun1 constructs containing the first 220 residues of Sun1 fused to H3 or H3–H4 as well as full-length Sun1 lacking H2–H3 (Sun1N220H3-GFP, Sun1N220H34-GFP, and Sun1H23-GFP, respectively) were permeabilized as described in A and labeled with anti-GFP antibodies. With digitonin permeabilization, the presence of the H4 domain rendered the GFP moiety inaccessible to antibody. Collectively, these results indicate that H4 serves as Sun1’s sole TM domain. Bars, 4 μm.
immunofluorescence microscopy (the latter on permeabilized but not proteinase-treated cells; Fig. 4, A and B, respectively). In some experiments, we used specific antibodies to monitor the latency of the GFP moiety (Fig. 4 C).

The results reveal that the N-terminal HA tag is always exposed to the cytoplasm or nucleoplasm. In contrast, the myc tag or GFP becomes latent (i.e., it resides within the ER lumen/PNS) whenever the H4 sequence is present within the chimera. No combination of H2 and H3 (either singly or together) would confer such latency. Conversely, neither H2 nor H3 could affect the orientation of H4 and, therefore, the latency of the myc tag or GFP. The only reasonable conclusion is that although H2 and, to a lesser extent, H3 may confer membrane association (Fig. 4 C), they do not cross the bilayer. Therefore, rather than being a multi-spanning protein as previously suggested, Sun1 would appear to be a type II membrane protein with a single TM domain represented by H4 (see Fig. 9).

With a better understanding of Sun1 topology, we next wished to identify NE and NPC retention domains. To this end, we generated an extensive family of chimeric Sun1 proteins. Because H4 appears to represent the sole TM domain, we sought to clarify the role of the other hydrophobic sequences. Sun1N355, which contains the H1 sequence but lacks H2 and H3, was found to localize to the NE (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1; Haque et al., 2006). This is in contrast to the nucleoplasmic localization of Sun1N355Δ221–343 (this corresponds to the exon 6–8 deletion; Fig. 5 A) or of Sun1N220 (Crisp et al., 2006), both of which lack any hydrophobic motif. When Sun1N355Δ221–343 was extended to include the H2 domain (Sun1N380Δ221–343), NE association was rescued (Fig. 5 A). Taking all of this data together, we can conclude that H1, H2, and, to a certain extent, H3 are each sufficient to confer membrane association. However, because the Sun1N220 region itself will accumulate readily within the nucleoplasm (Crisp et al., 2006), these experiments do not reveal whether any of the hydrophobic sequences themselves have intrinsic INM-targeting activity.

To address this question, we next examined the behavior of two additional H2-containing fusion proteins, which are both tagged at the N terminus with the myc epitope (Fig. 5 A). The first of these represented an N-terminal truncation lacking the initial 220 Sun1 residues but containing H1 and H2 (myc-Sun1 221–380), whereas the second was missing H1 in addition to the N-terminal 220 residues (myc-Sun1 261–380). The former localized to the NE and, to a lesser extent, to the peripheral ER. In contrast, the latter was primarily ER associated with little concentration in the NE. The implication of these results is that an NE localization motif is encoded by Sun1 residues 221–380. This region of the molecule must therefore share interactions with other nuclear or NE components.

We next examined whether the H2–H4 region alone has a role in NE targeting. When this sequence was fused to the N terminus of GFP, it localized predominantly to the Golgi apparatus and cell surface with little, if any, associated with the NE (Fig. 5 B). In contrast, a Sun1 N-terminal truncation consisting of the H2–H4 region followed by the Sun1 lumenal domain (H234Sun1L-GFP) localized efficiently to the NE (Fig S1; Padmakumar et al., 2005). However, we already know that a soluble form of the Sun1 luminal domain that is appropriately localized to the ER lumen and PNS is itself insufficient for NE targeting (SS-HA-Sun1L-KDEL; Crisp et al., 2006). There are at least two explanations for these results. The first is that the luminal domain does contain targeting information but that it is only functional when the domain is appropriately oriented or tethered to the ER or nuclear membranes. The second is that the H2–H4 hydrophobic region can direct localization to the INM but that this only occurs in the context of the Sun1 luminal domain. In other words, the Sun1 luminal domain can modify the behavior of the H2–H4 sequences. What we can rule out, however, is any suggestion that localization of H234Sun1L-GFP to the INM occurs by virtue of oligomerization with endogenous Sun1. Overexpression of H234Sun1L-GFP leads to the displacement of endogenous Sun1 from the NE while itself concentrating in the NE (Padmakumar et al., 2005; unpublished data). Additionally, depletion of Sun1 by RNAi has no effect on H234Sun1L-GFP localization (unpublished data).

To further address these issues, we replaced the H2–H4 hydrophobic region of both full-length Sun1 and H234Sun1L-GFP with the unrelated TM domain of Sun3 (to yield HA-Sun1(S3TM) and S3TMSun1L-GFP, respectively; Fig. 5 B). As shown in Fig. S2 A (available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1), when expressed in HeLa cells, HA-tagged Sun3 localizes to the NE but does not associate with NPCs. Sun3 contains a single predicted TM domain that resides between residues 46 and 65. As will become evident below (Fig. 5 B), this sequence contains no intrinsic NE-targeting activity. Translation of Sun3 in vitro in the presence of microsomes confirms that this sequence must function as a TM domain with a type II orientation (Fig. S2 B).

HA-Sun1(S3TM) behaved exactly like full-length Sun1 in that it concentrated in the NE (Fig. 5 B) in association with NPCs (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1). In contrast, S3TMSun1L-GFP displayed little or no NE localization and instead was found in the Golgi apparatus and at the cell surface (Fig. 5 B). Evidently, it is not retained in the nuclear membrane/ER system. Deletion of H2–H3 from H234Sun1L (H4Sun1L-GFP) also resulted in the loss of NE association (Fig. 5 B). The implication, then, is that H2–H3 encodes an NE localization function. If this is the case, attaching H2–H3 to the N terminus of S3TMSun1L-GFP should lead to its accumulation at the NE. Indeed, we do observe a partial restoration of NE localization (Fig. 5 B).

It is evident from these results that although the H2–H3 sequence promotes localization to the NE, its activity is strongly influenced by the Sun1 luminal domain. This is despite the fact that these regions of Sun1 reside on opposite sides of the INM. A possible explanation for this result is that the targeting activity of H2–H3 may be activated or enhanced by dimerization (or oligomerization), perhaps leading to increased avidity for nuclear or INM-associated binding partners. A prediction here is that the luminal domain of Sun1 should mediate dimerization (or oligomerization). This is borne out in transfection experiments in which full-length Sun1 was coexpressed in HeLa cells with a variety of epitope-tagged Sun1 deletion mutants (Fig. 6 A).
Immunoprecipitation analyses resulted in the efficient co-precipitation of full-length and mutant Sun1 only when the mutant form contained an intact lumenal domain. Further compelling evidence for lumenal domain–mediated oligomerization was provided by immunofluorescence observations of SS-HA-Sun1L-KDEL and S3TMSun1L. As described previously (Fig. 6 B), neither of these chimeric proteins concentrates to any great extent in the NE. However, the overexpression of full-length Sun1 will recruit both of these proteins to the NE. Collectively, these data clearly demonstrate that Sun1 homooligomerizes via lumenal domain interactions, most likely involving the predicted membrane proximal coiled coil.

Which Sun1 sequence elements are required for association with NPCs? Analysis of all of the Sun1 constructs that we have prepared revealed that apart from wild-type Sun1, only Sun1∆221–343 and Sun1(S3TM) associated with NPCs (Figs. 7 A and S3 A). Evidently, association with NPCs does not involve the H1 and H234 hydrophobic sequences acting in concert. To take these analyses further, we prepared a pair of chimeras in which we swapped the Sun1 and 2 lumenal domains. In neither case could we observe NPC association (Fig. S3 B). Instead, both recombinant proteins behaved like Sun2. Evidently, both nucleoplasmic and lumenal domains of Sun1 cooperate in conferring NPC association.

So far, we have shown that there are multiple determinants within the Sun1 nucleoplasmic domain that can confer localization to the INM. Hasan et al. (2006) used FRAP analysis to show that wild-type Sun1 is relatively immobile within the INM.
We performed similar analyses on a subset of our Sun1 deletion mutants that localize to the NE (Fig. 7 B). In all cases, these mutants display enhanced mobility relative to wild-type Sun1. Even deletion of the lumenal domain, which appears to contain no intrinsic targeting function but does promote oligomerization, leads to increased mobility within the INM. Thus, although Sun1 does contain multiple autonomous features involved in localization, stable localization to the NE requires that all be present. These findings are reminiscent of our conclusion that multiple features within the Sun1 molecule are required for NPC association.

What is the functional relevance of Sun1 association with NPCs? Proteomic studies provide no evidence that Sun1 is an intrinsic component of the NPC (Cronshaw et al., 2002). However, to determine whether Sun1 might contribute to NPC functionality, we examined nuclear transport in HeLa cells that had either been depleted of Sun1 by RNAi or that expressed Sun1 fragments, some of which resulted in a loss of endogenous NE-associated Sun1 (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200704108/DC1). To accomplish this, we took advantage of a GFP fusion protein bearing nuclear import and export signals (NLS-GFP–nuclear export sequence [NES]) and that shuttles between the nucleus and cytoplasm (Stade et al., 1997). We also used a hormone-inducible nuclear import substrate consisting of β-galactosidase fused to the glucocorticoid receptor (grβ; Bastos et al., 1996). Our results indicate that Sun1 has no substantial role in the nuclear transport of proteins, either import or export. Similarly, the distribution of poly A+ RNA revealed by in situ hybridization suggests that Sun1 makes little or no contribution to mRNA export (unpublished data).

However, Sun1 depletion was not without effect on pore complexes. We noticed that the loss of Sun1 was always associated with an altered distribution of NPCs (Fig. 8 A) as well as altered nuclear shape (Fig. 8 C). In wild-type cells, NPCs tend to be uniformly distributed across the nuclear surface. After Sun1 depletion, NPC aggregates or clusters could be observed leaving
NPC-free areas of varying sizes. This effect was Sun1 specific because the depletion of Sun2 left NPC distribution unchanged.

This effect of Sun1 depletion on NPC aggregation could be emulated by the overexpression of nucleoplasmic Sun1 deletion mutants in HeLa cells. The expression of these mutants often leads to a diminution in the amount of full-length Sun1 at the NE (and thus at NPCs). A quantitative analysis of NPC aggregation induced by both Sun1 depletion and Sun1 mutant expression is displayed in Fig. 8 B.

Because Sun1 may act as a tether for ONM nesprins, it is possible that NPC aggregation is a function of the loss of nesprins rather than a loss of Sun1. To determine whether this might be the case, we overexpressed a protein consisting of GFP fused to the KASH domain of either nesprin 1 or 2 (GFP-KASH1 or 2) in HeLa cells. Overexpression of GFP-KASH1 or 2 leads to the displacement of nesprins 1 and 2 from the NE (Zhang et al., 2007). Treatment of cells in this way was found to have no discernible effect on NPC distribution (unpublished data). These data suggest that Sun1 has a nesprin-independent role in maintenance of the uniform distribution of NPCs across the NE.

Discussion

Sun1 and 2 are of a pair of ubiquitous INM proteins that tether nesprins within the ONM. Nespr1G and nesp2G contain N-terminal actin-binding domains (Zhen et al., 2002; Padmakumar et al., 2004), whereas nesp3 binds plectin, a versatile cytolinker (Wilhelmsen et al., 2005). Thus, the SUN proteins represent links in a molecular chain that connects elements of the cytoskeleton to components within the nucleus. We have previously referred to translumenal Sun–nesprin pairs as LINC complexes (Crisp et al., 2006). Multiple LINC complex isoforms likely exist given the apparent redundancy of Sun1 and 2 in tethering nesprins. In addition, we can identify at least four or five splice isoforms of Sun1 alone, further increasing the LINC complex repertoire. The nesprins themselves (including nesp1G) are also represented by dozens of splice isoforms. Aside from nesp1G and nesp2G, the number of these that may be tethered by Sun proteins at the ONM remains unknown.

Previous studies indicated that KASH domain proteins play an important role in nuclear positioning in certain cell types (Mosley-Bishop et al., 1999; Starr et al., 2001; Starr and Han, 2002; Malone et al., 2003; Grady et al., 2005; Yu et al., 2006). However, the existence of links spanning the NE have far broader implications than mere nuclear location and present us with a mode (or modes) of nucleocytoplasmic coupling that may bypass NPCs. This notion is highlighted by biomechanical studies on Lmna-null fibroblasts, which exhibit impaired mechanotransduction and decreased viability under mechanical strain (Broers et al., 2004; Lammertding et al., 2004, 2005, 2006). Induction of the mechanosensitive genes iex-1 and egr-1 is attenuated, as is nuclear factor κB–regulated transcription in response to either cytokine or mechanical stimulation. Although nuclei in Lmna-null cells are both mechanically fragile and highly deformable, a surprising finding of Lammertding et al. (2004) is that these cells also feature reduced cytoplasmic resilience. Given that both Sun1 and 2 interact with A-type lamins, it is possible that the LINC complex might mediate mechanotransduction and the lamin-dependent changes in cytoplasmic organization.

Retention of Sun1 and 2 in the INM is independent of A-type lamins in some cell types (Padmakumar et al., 2005; Crisp et al., 2006; Haque et al., 2006; Hasan et al., 2006). This implies that there have to be other nuclear or NE components that interact with and retain the SUN proteins. Logically, based on our studies here, there have to be at least two discrete regions within Sun1 that are sufficient for INM localization. Evidence for this
can be seen in the differential effect of exogenous Sun1 and 2 on each other. Sun2 will not substantially displace Sun1 in HeLa cells. However, Sun1 can efficiently displace Sun2 from the INM (Crisp et al., 2006), presumably by competition for a common binding partner or anchor. Therefore, Sun1 likely has an additional binding partner that is not shared with Sun2. This is perhaps most obvious when considering that both of these proteins are segregated within the plane of the NE. Although Sun2 predominates in NPC-free regions, Sun1 is concentrated in the vicinity of NPCs, possibly forming a halo around each NPC. The mechanisms of interaction with NPCs remain unknown. However, it clearly requires contributions from both the nucleoplasmic and lumenal domains.

Our analyses suggest that there are at least two separate INM-targeting regions within the Sun1 nucleoplasmic domain. The first lies between residues 1–260 and includes the H1 hydrophobic sequence. The second is immediately downstream of the H1 sequence and includes the H2 and H3 hydrophobic sequences. The bulk of this second targeting region is absent in the Sun1Δ221–343 (i.e., the Δexon 6–8) splice isoform, although the H2 and H3 sequences are retained. Because Sun1Δ221–343 still colocalizes with NPCs, the bulk of this second targeting region cannot have an essential role in NPC association. The same is also true of the entire H234 region, which can be substituted by the Sun3 TM domain without affecting NPC association.

Although the H2–H3 hydrophobic sequence exhibits INM-targeting activity, it is only functional in the context of molecules containing the lumenal domain. The lumenal domain has no intrinsic targeting properties but does promote oligomerization, most likely based upon coiled-coil homodimers. We would suggest that manifestation of the INM localization function of H2–H3 requires dimerization/oligomerization, perhaps leading to increased avidity for an NE or nuclear binding partner.

The Sun1 TM domain is contained within the region of the molecule defined by the H2–H4 hydrophobic sequences. We and others had suggested that these might represent three TM domains (Padmakumar et al., 2005; Crisp et al., 2006). Our current studies suggest that H2 and H3 do not, in fact, span the INM, leaving H4 as the only TM sequence within the Sun1 molecule (Fig. 9 A). This view is reinforced by the existence of an apparent human Sun1 splice isoform (GenBank/EMBL/DDBJ accession no. NM_25154) lacking sequences encoded by exons 6–9 and missing H2. Our conclusion is that Sun1 has the topology of a type II membrane protein.

Although Sun1 has only a single membrane-spanning domain (H4), the three other hydrophobic sequences, H1, H2, and H3, can confer membrane association. Nucleoplasmic
domain constructs that contain at least one of the three all become associated with the INM, whereas their absence leads to nucleoplasmic localization. It remains unclear whether these hydrophobic sequences interact directly with the INM lipid bilayer or whether association is mediated by other INM proteins. The former would appear more likely because regardless of expression level, H1-, H2-, or H3-containing proteins always appear membrane associated. The interaction of extended hydrophobic sequences such as H2–H3 with the lipid bilayer is not without precedent. For instance, the tubular ER protein reticulon 4 contains a 30–40-residue hydrophobic sequence that forms a hairpin, which dips into the cytoplasmic face of the ER lipid bilayer without crossing it (Voeltz et al., 2006).

The segregation of Sun1 and 2 within the plane of the NE and the association of Sun1 with NPCs is quite striking. Could Sun1 be an NPC component? The complement of mammalian NPC subunits identified by Cronshaw et al. (2002) using proteomic approaches does not include Sun1. However, the same is also true of the authentic vertebrate NPC membrane protein Ndc1 (Stavru et al., 2006). Perhaps Sun1’s additional associations with the nuclear lamina and possibly chromatin limits its coextraction with NPC proteins. Regardless, we can find no evidence that Sun1 contributes to nucleocytoplasmic transport, and, consequently, we feel that Sun1 is unlikely to represent an intrinsic NPC component. Instead, a more reasonable scenario is that Sun1 is associated with the NPC periphery and may define a novel microdomain within the nuclear membranes, which, in turn, could blur the boundary between NPCs and the bulk of the NE (Fig. 9 B). The presence of Sun1 and, by implication, nesprins at NPCs could provide a basis for older ultrastructural observations that cytoskeletal elements, particularly intermediate filaments, frequently seem to contact pore complexes (Goldman et al., 1985).

The distribution of NPCs across the NE is not random. Rather, they are arrayed in a uniform (although not regular) fashion that is constrained by a minimum NPC separation (Maul, 1977). We have observed that the depletion of Sun1 (but not Sun2) or overexpression of truncated forms of Sun1 lead to the formation of NPC aggregates or clusters. This suggests that Sun1 has a role in the maintenance of uniform NPC distribution across the nuclear surface. In mammalian cells, NPCs are largely immobile and maintain their relative positions over many hours (Dagile et al., 2001; Rabut et al., 2004). The implication is that NPC clusters in Sun1-depleted cells may arise during de novo NPC assembly as well as during postmitotic reassembly.

A-type lamins are also determinants of NPC distribution because Lmna-null mouse embryonic fibroblasts (MEFs) frequently feature clustered or aggregated NPCs (Sullivan et al., 1999). Furthermore, Maeshima et al. (2006) have shown that A-type lamins strongly influence the distribution of NPCs and pore-free regions of the NE. It is important to bear in mind, however, that cells that normally lack A-type lamins (early embryonic cells, for instance) do not display obviously clustered NPCs. It follows that there must be additional mechanisms to define NPC distribution that predominate in certain cell types. Such mechanisms might potentially involve B-type lamins (Maeshima et al., 2006).

Because Sun1 interacts with lamin A via the N220 region of its N-terminal domain (Crisp et al., 2006), it could function as an adaptor between the nuclear lamina and the NPC (Fig. 9). Of more significance is our observation that Sun1 has a preference for farnesylated pre–lamin A. Given that pre–lamin A exists only transiently in normal cells, this raises the possibility that Sun1 might function in the targeting and assembly of newly synthesized lamin A at the nuclear face of the INM. If this is the case, given the localization of Sun1, NPCs could actually function as nucleation sites of A-type lamina assembly.

Figure 9. Sun1 topology and interactions. (A) Sun1 is envisaged as forming homodimers via interactions involving the membrane-proximal coiled coil within its C-terminal lumenal domain. Nucleoplasmic domain interactions may also contribute to homodimer formation. Sun1 functions as a tether for ONM nesprin proteins. Nesprins 1 and 2 provide links to the actin cytoskeleton, whereas nesprin 3 binds plectin, a versatile cytolinker. (B) Sun1 is associated with NPCs, and, in addition, its nucleoplasmic domain displays preferential binding to newly synthesized pre–lamin A. Therefore, Sun1 may provide a link between NPCs and the A-type lamins. In this way, the Sun1-mediated nucleation of A-type lamina assembly may occur at NPCs.
Ultimately, this may help define the distribution of NPCs. Our next goal will be to test this notion by determining whether there is a spatial relationship between A-type lamina assembly and NPCs. Regardless of the outcome of these studies, it is becoming increasingly clear that there are complex networks of interactions at the nuclear periphery involving NPCs, INM and ONM proteins, and nuclear lamins. These interactions appear to define not only the organization of the NE but also determine cytoskeletal mechanics and perhaps mediate signaling between the nucleus and cytoplasm.

Materials and methods

Cell culture and transfections

HeLa cells and MEFs, both Lmna+/− and Lmna−/− (Sullivan et al., 1999), were maintained in 7.5% CO2 and at 37°C in DME (Invitrogen) plus 10% FBS (HyClone), 10% penicillin/streptomycin (Invitrogen), and 2 mM glutamine. Plasmid DNA was introduced into HeLa cells and MEFs by using the Polyfect reagent as described previously (Crisp et al., 2006) with or without the LipofectAMINE 2000 reagent (Invitrogen). To transfect a 3.5-cm2 tissue culture of cells with LipofectAMINE 2000, 6 μl of transfection reagent or 2 μg of plasmid DNA were each added to separate 100-μl vol of Optimem (Invitrogen) and were combined and incubated at RT for 20 min. Subsequently, the cell medium was replaced with serum-free DME and the 200-μl transfection mix added dropwise and was incubated at 37°C for 1 h, after which time the medium was replaced with DME/10% FCS. Cells were analyzed 1–2 d later.

Generation of tetracycline-inducible stable cell lines

A HeLa cell line stably expressing a tetracycline repressor protein from a pcDNA6/TR plasmid (Trex-HeLa; Invitrogen) was transiently transfected with pcDNA4/TO plasmid (Invitrogen) containing murine Sun1GFP or human Sun2GFP. After transfection, cells were selected with 200 μg/ml KOSAc, 20 mM Hepes, pH 7.4, and 2 mM MgCl2 for 45 min. The pellet was permeabilized with 0.1% TX-100 in PBS on ice for 15 min (Adam et al., 1990). The cells were then fixed with 3% PFA in PBS for 10 min followed by permeabilization with the primary polyclonal anti-GFP antibody ab290 (Abcam) for 1 h. After washing three times with 0.1% BSA in PBS, the cells were incubated with a secondary anti-rabbit IgG antibody conjugated to 10-nm gold particles (Ted Pella Inc.) for 1 h followed by washing three times in PBS. Cells were then fixed and prepared for embedding/thin section electron microscopy (Rollenhagen et al., 2003).

In situ proteinase K digestion

HeLa cells were transfected in triplicate for each construct. After transfection (24 h), the cells were incubated in Meta/Cys-free media for 45 min followed by incubation in medium containing 50 μCi [35S]Met/Cys (MP Biomedicals) for 1 h. After two rinses with ice-cold PBS, one well was incubated in 4 μg/ml proteinase K (Sigma-Alrich) in KHM buffer (110 mM KOAc, 20 mM Hepes, pH 7.4, and 2 mM MgCl2) for 45 min. Another well was permeabilized with 24 μM of ice-cold digitonin in KHM for 15 min followed by 4 μg/ml proteinase K digestion in KHM for 45 min. The third well was incubated with 4 μg/ml proteinase K for 45 min in 0.5% TX-100/KHM. Subsequently, PMSF was added to all wells to a final concentration of 40 μg/ml. The first two wells were gently washed in KHM buffer with 40 μg/ml PMSF again to remove excess proteinase K. Cells were lysed in 0.4% SDS, 2% TX-100, 400 mM NaCl, 50 mM Tris-HCl, pH 7.4, 40 μg/ml PMSF, 1 mM DTT, plus 2 μg/ml pepstatin A and 1 μg/ml leupeptin and passed through a 23-gauge needle five times before centrifugation for 10 min at 16,000 g. Soluble proteins were immunoprecipitated with rabbit anti-myC or GFP by protein A-Sepharose. After three washes, proteins were incubated with sample buffer before separation by SDS-PAGE. Gels were stained with Coomassie brilliant blue and incubated with Amplify (GE Healthcare) for 20 min before drying. Autoradiographs were obtained from dried gels. In parallel, a nontransfected cell lysate was analyzed by Western blotting to validate the permeabilization and digestion conditions.

In vitro transfections and proteinase K digestions were performed as described previously (Crisp et al., 2006).

Nuclear transport assays

To observe nuclear export, an NES-GFP-NLS (NES-MGGKKRKV) construct was transfected into HeLa cells either alone or with other expression vectors. CRM1-dependent nuclear export was assayed with an NES-GFP-NLS (NES-MGGKKRKV) 24 h after transfection. Cells were then transfected with a glucocorticoid receptor linked to a membrane (G4; Macintosh) running IPLab Spectrum software (Scanalytics). Images were collected using a confocal microscope [LSM 510; Carl Zeiss Microimaging, Inc.] with a 63×1.4 NA objective [Carl Zeiss Microimaging, Inc.]. GFP was excited with the 488-nm line of an Ar laser, and GFP emission was monitored using a 505-nm longpass filter. Cells were maintained at 37°C using an incubator (ASI Air Stream; Newtek). In transfected cells, a rectangular region typically 2–4 μm in height was bleached in three iterations using a 488-nm laser line at 100% laser power. Cells were monitored at 5-s intervals for up to 300 s. Data were analyzed as described previously (Dundl et al., 2002; Phair et al., 2004) to take into account bleaching during the imaging phase. Recovery values are means ± SD from at least five cells from at least two independent experiments.

Immunoelectron microscopy

Immunoelectron microscopy of Sun1 was performed by preembedding and labeling of the tetracycline-inducible cell line expressing Sun1-GFP. After fluorescence examination to verify GFP expression, cells were trypsinized and pelleted by centrifugation. The pellet was permeabilized with 0.1% TX-100 for 1 min in PBS and fixed with 3% PFA in PBS for 10 min followed by three washes with PBS. The fixed cells were then incubated with 2% BSA in PBS for 10 min followed by incubation with the primary polyclonal anti-GFP antibody ab290 (Abcam) for 1 h. After washing three times with 0.1% BSA in PBS, the cells were incubated with a secondary anti-rabbit IgG antibody conjugated to 10-nm gold particles (Ted Pella Inc.) for 1 h followed by washing three times in PBS. Cells were then fixed and prepared for embedding/thin section electron microscopy (Rollenhagen et al., 2003).

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