The inner nuclear membrane protein Sun1 mediates the anchorage of Nesprin-2 to the nuclear envelope

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Summary

Nesprins form a novel class of nuclear envelope-anchored spectrin-repeat proteins. We show that a direct association of their highly conserved C-terminal luminal domain with the inner nuclear membrane protein Sun1 mediates their nuclear envelope localisation. In Nesprin-1 and Nesprin-2 the conserved C-terminal amino acids PPPX are essential for the interaction with a C-terminal region in Sun1. In fact, Sun1 is required for the proper nuclear envelope localisation of Nesprin-2 as shown using dominant-negative mutants and by knockdown of Sun1 expression. Sun1 itself does not require functional A-type lamins for its localisation at the inner nuclear membrane in mammalian cells. Our findings propose a conserved nuclear anchorage mechanism between *Caenorhabditis elegans* and mammals and suggest a model in which Sun1 serves as a 'structural bridge' connecting the nuclear interior with the actin cytoskeleton.

Key words: Emerin, Enaptin, Lamin A/C, NUANCE, SUN domain, Syne

Introduction

Several lines of evidence suggest the presence of molecular links, which 'hard-wire' plasma membrane receptors, cytoskeletal components and nuclear scaffolds together, allowing the physical integration of the nucleus within a cell and the generation of nuclear signalling and/or mechanical responses to various intracellular and extracellular cues. Testimony of the existence of such connections are genetic data from a wide variety of species, which identify microtubules, microtubule-associated proteins, proteins containing giant spectrin repeats and SUN domain-containing molecules involved in nuclear anchorage and nuclear migration (Reinsch and Gönczy, 1998; Starr and Han, 2003; Patterson et al., 2004).

Even though terms such as 'nucleoskeleton' and the long-disputed 'nuclear matrix' have gained more significance and popularity, it is still not understood which molecular set-up is required to determine nuclear architecture and the linkage of the nucleus to cytoplasmic structures. The recently described ANC-1 of *C. elegans* (Starr and Han, 2002), MSP-300 of *Drosophila melanogaster* (Rosenberg-Hasson, 1996; Zhang et al., 2002) and the mammalian proteins Nesprin-1/Enaptin and Nesprin-2/NUANCE (Zhang et al., 2001; Zhang et al., 2002; Zhen et al., 2002; Padmakumar et al., 2004) form a novel family of proteins containing nuclear spectrin repeats that may organise and connect the nuclear membrane to the cytoskeleton as well as the nuclear lamina. These proteins are very large (>800 kDa) and are composed of an N-terminal α-actinin-type actin binding domain, a long rod containing spectrin repeats and a highly conserved C-terminal type II transmembrane domain (KASH domain) (Starr and Han, 2002), which anchors them in the nuclear envelope (NE), followed by a stretch of amino acids displaying strong homology to the C-terminus of the *Drosophila* Klarsicht protein (Mosley-Bishop et al., 1999). Nesprin genes are huge and complex, having the propensity to generate a wide variety of isoforms. These differ in length, domain composition, expression pattern and maybe also in their functional properties, and consequently a plethora of names is currently used for these proteins by various groups. Nesprin-1 isoforms have been described as CPG2, syne-1, myne-1 and Enaptin, whereas Nesprin-2 variants are also known as syne-2 and NUANCE (Apel et al., 2000; Mislow et al., 2002a; Zhang et al., 2001; Zhang et al., 2002; Zhen et al., 2002; Gough et al., 2003; Cottrell et al., 2004; Padmakumar et al., 2004).

Nesprins are widely expressed in a variety of tissues and cell types (Zhen et al., 2002; Padmakumar et al., 2004). At the subcellular level they were detected at the NE and also in the nucleus. In contrast to Nesprin-2, which localises predominantly to the nucleus and the NE, Nesprin-1 has a rather heterogeneous subcellular distribution and can also be detected at F-actin-rich structures (Zhang et al., 2001; Padmakumar et al., 2004). Consistent with their subcellular localisation, genetic studies involving their orthologues in lower eukaryotes suggest roles in the attachment of intracellular membrane compartments to the actin cytoskeleton. Mutations in *anc-1* of *C. elegans* disrupt the positioning of nuclei and mitochondria (Starr and Han, 2002). MSP-300 in *Drosophila* is required for embryonic muscle morphogenesis and the formation of embryonic muscle attachments (Rosenberg-Hasson et al., 1996).

Genetic studies in *C. elegans* have recently identified two inner nuclear membrane proteins, SUN1 and UNC-84, as structural links between the nucleoplasm and the cytoplasm. SUN1/Matefin (Fridkin et al., 2004) and UNC-84 are characterised by the presence of the SUN domain, which derived its name from proteins containing the Sad1p-UNC-84 homology domain (Hagan et al., 1995; Malone et al., 1999). SUN1 is essential for early embryonic and germ cell development (Fridkin et al., 2004) and required for the localisation of ZYG-12 to the NE. ZYG-12 functions in centrosomal attachment and the nuclear localisation of dynein (Malone et al., 2003). In contrast to UNC-84 (Lee et al., 2002), germ-line-specific SUN1 does not require Ce-lamin for its nuclear localisation (Fridkin et al., 2004). UNC-84 is required for proper nuclear migration and nuclear anchorage during worm development (Lee et al., 2002) and null alleles or missense mutations in UNC-84 also affect the nuclear envelope targeting of ANC-1 (Starr and Han, 2002). In mammals, two SUN domain-containing proteins have been described, although at least four unrelated members of the SUN domain family are present in the databases (J.G. and R.F., unpublished data) (Malone et al., 2003). The mammalian Sun1 protein is the real UNC-84 orthologue and was identified as an NE protein in a proteomic analysis of neuronal nuclear membranes (Dreger et al., 2001). Sun2 is transcribed from a separate gene and shows all properties of an inner nuclear membrane protein, with the SUN domain extending into the periplasmic space between the inner and outer nuclear membranes (Hodzic et al., 2004). Both Sun1 and Sun2 have also been identified in a comprehensive proteomic screen of inner nuclear membrane proteins in rat liver (Schirmer et al., 2003).

In this report, we identify and analyse the direct interaction between the mammalian inner nuclear membrane protein Sun1 and Nesprins, giant NE-cytoskeletal linker proteins. Our findings provide novel insights into the NE anchorage mechanism of Nesprins, demonstrating an evolutionarily conserved strategy from worms to mammals.

Materials and Methods

Tissue culture and transfection

COS7 and C3H/10T1/2 cells were grown as described (Padmakumar et al., 2004). HaCaT, HeLa cells and skin fibroblasts from wild-type and lamin A knockout mice (Sullivan et al., 1999) were routinely cultivated in DMEM supplemented with 10% fetal calf serum (Gibco Life Technologies) at 37°C in a humidified atmosphere containing 5% CO_2.

All the GFP fusion constructs were transfected in COS7 and C3H/10T1/2 cell lines by electroporation (200V, 950 μF; Gene Pulser Xcell, Bio-Rad). HaCaT cells were transfected by using the Amaxa nucleofector technology according to the manufacturer's instructions (Amaxa Biosystems). HeLa cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen).

Cloning strategies

All the GFP fusion proteins carry GFP at their N-terminus. The appropriate DNA fragments were amplified by PCR and cloned in either pGEMTeasy (Promega) or pCR-2.1-TOPO (Invitrogen) vectors before cloning them in the GFP, yeast two-hybrid or GST vectors. Nesprin-1 constructs were amplified from BC054456 IMAGE clone and Sun1 was amplified from IMAGE clone AAH48156. GFP-tmNesprin-1 (amino acids 8611-8749; AAN03486) and GFP-dnNesprin-1 (aa 8369-8749; AAN03486) were cloned into *Eco*RI/*Sal*I-cut EGFPC2 (Clontech) vector. GFP-tmNesprin-2 (aa 6833-6883; AAL33548) was cloned into the *Eco*RI/*Bam*HI site of EGFPC1 vector (Clontech). The full-length GFP-Sun1 (aa 1-913) was cloned into the *Eco*RI/*Bam*HI site of EGFPC2. GFP-Sun1ΔSUN (aa 1-720), GFP-Sun1N+1TM (aa 1-384) and GFP-Sun1-TM-C (aa 358-913) were cloned into the *Eco*RI/*Sal*I site of EGFPC2 vector. GFP-Sun1-N+2TM (aa 1-412), GFP-Sun1-TM-SD1,2 (aa 358-737), GFP-Sun1-TM-CΔSD2SUN (aa 358-632) and GFP-Sun1-CΔCC (358-491, 633-913) were cloned in EGFPC2 vector between the *Eco*RI and *Bam*HI sites. The KIAA0810 clone containing full-length human Sun1 was kindly provided by the KAZUSA research institute (Kikuno et al., 2004). The Gateway®-compatible destination vector pTB-RFB-EcoRV was created by insertion of the Gateway-transfer cassette into the *Eco*RV-site of pTRACER-EF1-Bsd (Invitrogen). Full-length Sun1 was amplified from the KIAA-vector by PCR. The amplicon was cloned into the pTOPO-D/Entry-vector (Invitrogen) to create a Gateway®-compatible Sun1 entry vector. The expression vector expressing human KIAA0810-Sun1 harbouring C-terminal V5, was created by LR-Clonase II-reaction using the Sun1 entry vector, pTB-RFB-EcoRV and the LR-enzyme mix according to manufacturer's instructions (Invitrogen). GFP-B1Δ2+ was a generous gift of Chris Hutchison (University of Durham, UK). The luminal domain of Nesprin-1 (LDN-1 residues 109-138, BE917568) was amplified from embryonic stem cell genomic DNA and cloned between *Eco*RI and *Sal*I sites in pGBKT7 (Clontech) and into pGEX4T-1 (Pharmacia) vectors. The luminal domain of Nesprin-2 (LDN-2, aa 6854-6883; AAL33548) was cloned into *Eco*RI and *Sal*I sites of pGEX-4T-1. The C-terminus of Sun1 (Sun1-C, aa 432-913), Sun1-CΔSUN (aa 432-737), Sun1-SUN (aa 738-913) was cloned into *Eco*RI/*Bam*HI-digested pGADT7 and pGBKT7 vectors (Clontech). Sun1-SD1 (aa 431-633) and Sun1-SD2 (aa 632-737) were cloned into *Eco*RI/*Bam*HI-cut pGADT7.

Yeast two-hybrid assay

Y190 yeast cells were co-transformed with the plasmids and transformants were selected on SD-Trp-Leu plates. Interaction was monitored by growth on plates containing selection media SD-Trp-Leu-His and 60 mM 3-amino 1,2,4-triazole. X-Gal assay was also performed to confirm the interaction. The protocols for performing the yeast transformation and X-gal assay are described in detail elsewhere (Yeast Protocols Handbook PT3024-1; Clontech).

Purification of GST fusion proteins and in vitro binding assays

Purification of GST fusion proteins and GST pull-down experiments were performed as described (Dreuillet et al., 2002). For the pull-down assay, COS7 cells were lysed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors (Roche). The 100,000 ***g*** supernatant of the lysate was incubated with equal amounts of GST fusions and the solutions were incubated at 4°C overnight with GST-sepharose beads on a roller. Samples were centrifuged and the pellets (washed three times with PBS) were analysed by SDS-PAGE and western blot analysis.

Antibodies and immunofluorescence microscopy

Sun1 polyclonal antibodies were produced against a peptide containing the first 14 amino acids of human Sun1 (MDFSRLHMYSPPQC; NP_079430) to create antiserum 281 (Eurogentec, Seraing, Belgium).

Western blotting and immunofluorescence (IF) studies were performed as described (Zhen et al., 2002; Gotzmann et al., 2000). The following antibodies were used: affinity-purified rabbit polyclonal anti-Nesprin-1/Enaptin (1:50 for IF), mouse monoclonal

anti-Nesprin-2/NUANCE mAb K20-478 (Zhen et al., 2002), unpurified rabbit polyclonal hSun1 281 (1:50 IF; 1:400 western blotting), rabbit polyclonal Nesprin-2 pAbK1 (1:50 IF, 1:1000 western blotting), mouse monoclonal anti-lamin A/C (1:50 IF; JoL2, Chemicon), mouse monoclonal anti-V5 (1:500 IF; 1:3000 western blotting; Invitrogen), polyclonal serum to LAP2α (1:5000; ImmuQuest) (Vlcek et al., 2002), guinea pig anti-LBR was a kind gift of Harald Hermann (1:250 IF) (Dreger et al., 2002), mouse monoclonal anti-LAP2β (1:5 IF) (Dechat et al., 1998) goat polyclonal anti-GST antibody (Amersham Biosciences), GFP-specific mAb K3-184-2 (Noegel et al., 2004). The secondary antibodies used were conjugated with Cy3 (Sigma), Alexa 488 or Alexa 568 (Molecular Probes, Leiden, The Netherlands), Texas Red (Jackson Laboratories, West-Grove, PA) and FITC (Sigma). Samples were analysed by wide-field fluorescence microscopy (DMR, Leica, Bensheim, Germany) or confocal laser-scanning microscopy (TCS-SP, Leica).

Subcellular fractionation

Subcellular fractionation was done essentially as described (Dechat et al., 1998; Gotzmann et al., 2000). In brief, cells were broken in hypotonic buffer using a Dounce homogeniser with a tight-fitting pestle. After addition of 8% sucrose, the soluble cytoplasmic and the insoluble nuclear fractions were separated by centrifugation at 2000 ***g*** for 15 minutes at 4°C. The nuclei-containing pellets were extracted in the same buffer supplemented with 1% Triton X-100, or 200 mM NaCl or combinations of both. Another extraction was performed using hypotonic buffer containing 7 M urea. The extracts were centrifuged at 15,000 ***g*** for 10 minutes and supernatants and pellets were analysed by western blotting.

siRNA knockdown of Sun1

The RNA interference-competent pSHAG-1 vector (containing a human U6 promoter fragment; –265 to +1), constructed using a pTOPO-ENTR/D backbone (Invitrogen, Carlsbad, USA), has been described recently (Paddison et al., 2002; http://katahdin.cshl.org:9331/RNAi_web/scripts/main2.pl). Oligonucleotides A and B, encoding a short hairpin RNA, were designed according to the RNAi-retriever protocol at http://katahdin.cshl.org:9331/homepage/portal/html/protocols/. For knocking down specifically human Sun1 the following oligonucleotides were used to create vectors pJG173 and pJG174, respectively: Oligo 173A, 5′-CTCGGACAGCATGCTGCAGTTGCTGCAGGAAGCTTGCTGCGGCGACTGCGGCATGTTGTCCGAGCGCTTTTTTT-3′; Oligo 173B, 5′-GATCAAAAAAAGCGCTCGGACAACATGCCGCAGTCGCCGCAGCAAGCTTCCTGCAGCAACTGCAGCATGCTGTCCGAGCG-3′; Oligo 174A, 5′-AGGACGTGACCTGCCTTGACACGTGGTTGAAGCTTGAGCCGCGTGTTAAGGCAGGTCACGTTCTCTGTTTTTT-3′; Oligo 174B, 5′-GATCAAAAAACAGAGAACGTGACCTGCCTTAACACGCGGCTCAAGCTTCAACCACGTGTCAAGGCAGGTCACGTCCTCG-3′. Annealed oligonucleotides were cloned into pSHAG-1 by insertion of resulting overhangs into the *Bse*RI/*Bam*HI-sites. The control vector pSHAG-FF1 encoding an shRNA targeting Firefly Luciferase was a kind gift of Greg Hannon (Cold Spring Harbor Laboratory, NY). The integrity of all vectors was verified by sequencing from both ends. RNAi-vectors targeting hSun1 were always used in combination for transient knockdown experiments. The cells were fixed 4 days after the transfection and examined by indirect immunofluorescence.

Results

Common mechanisms tether Nesprin-1 and Nesprin-2 at the NE

Nesprin-1 and Nesprin-2 localise to the NE by virtue of their conserved C-terminal domain, which includes a type II transmembrane domain followed by a stretch of 30 amino acids (luminal domain) extending into the perinuclear space, which is highly conserved in this family of nuclear proteins (Fig. 1A). In order to identify the sequences necessary for the NE localisation of Nesprins and elucidate the mechanism by which they are tethered at the NE, several GFP fusion proteins of their C-termini (Fig. 1B) were transiently expressed in various cell lines.

TmNesprin-1, representing the transmembrane domain and the periplasmic region of the protein localised to the NE in C3H/10T1/2 cells (Fig. 1C,E, arrow) and exerted a dominant-negative effect on the endogenous Nesprin-1 protein. Untransfected fibroblasts displayed strong Nesprin-1 staining at the NE (Fig. 1D,E, arrowhead), which disappeared in transfected cells (Fig. 1D,E, arrows). A similar effect was observed when COS7 cells expressed the corresponding tmNesprin-2 GFP fusion protein. Like tmNesprin-1, tmNesprin-2 localised to the NE (Fig. 1F, arrow) and displaced the endogenous Nesprin-2 (Fig. 1G,H, arrowheads) from the NE (Fig. 1G, arrow). As tmNesprin-2 did not contain regions upstream of the transmembrane domain as found in tmNesprin-1 (Fig. 1B), we concluded that the transmembrane domain and the luminal domains are sufficient to mediate NE targeting. The high homology between the C-termini of Nesprin-1 and Nesprin-2 further prompted us to investigate the effects of a dominant-negative Nesprin-1 GFP fragment on endogenous Nesprin-2. In these experiments we used the dnNesprin-1 GFP fusion (~75 kDa), which also harbours the two final spectrin repeats upstream to the transmembrane domain, thus resembling Nesprin-2α in its domain organisation (Zhang et al., 2001). The studies were performed in COS7 cells, which express Nesprin-2 strongly at the NE (Zhen et al., 2002). Similar to tmNesprin-1, dnNesprin-1 localised to the NE in COS7 cells (Fig. 1I,K, arrow) and displaced the endogenous Nesprin-2 (Fig. 1J, arrow). Experiments involving shorter versions of dnNesprin-1, such as tmNesprin-1 fusions, exhibited similar dominant-negative effects on Nesprin-2 (data not shown). To further study the potential significance of the highly conserved C-terminal proline residues (Fig. 1A, green bar) for the NE localisation of Nesprin-2, a GFP fusion protein tmNesprin-2ΔP lacking the PPPT-motif was generated. In contrast to tmNesprin-2 (Fig. 1F, arrow), this protein accumulated in the nuclear interior (Fig. 1L, arrowhead) and in ER-like structures (Fig. 1L, arrows) and did not affect the location of endogenous Nesprin-2 (Fig. 1M, arrowhead).

Taken together, these experiments underline the functional significance of the conserved proline-rich stretch of Nesprins for NE targeting. Moreover, the C-terminal domains act in a dominant-negative manner on the endogenous Nesprin proteins, displacing them from the NE. These findings also imply that the associations of the luminal domain of Nesprin-1 and Nesprin-2 in the perinuclear space involve identical binding partners conferring anchorage at the NE.

Sun1 binds to the luminal domain of both Nesprin-1 and Nesprin-2

A genetic interaction between ANC-1 and UNC-84 has been reported in *C. elegans*, although efforts to elucidate the direct molecular interaction failed (Starr and Han, 2002). A search of the mouse EST database for mammalian UNC-84 orthologues as potential Nesprin binding partners yielded two SUN

domain-containing proteins, Sun1 (accession number AAH48156, mouse chromosomal locus 5G.2) and Sun2 (AAT90499, residing on chromosome 15). Mouse Sun1 and Sun2 display 65% identity and 81% homology in their SUN domain and 47%/39% identity and 63%/59% similarity to the SUN domain of UNC-84, respectively. As Sun1 is more closely related to UNC-84 than Sun2, Sun1 was chosen for the current study. In addition, we used human Sun1, the closest human orthologue to UNC-84, displaying 48% identity and 64% similarity to the SUN domain of UNC-84. Human Sun1 was originally identified as KIAA0810 by the Kazusa DNA research institute (Kikuno et al., 2004; http://www.kazusa.or.jp/huge/). Mouse *Sun1* encodes a 100 kDa protein composed of 913 amino acids (Fig. 2A). It contains three putative transmembrane domains (aa 358-383, 386-407 and 413-431) located approximately in the middle of the protein, a predicted ZnF-C2H2 domain near the N-terminus, and two predicted coiled-coil domains in the C-terminus (aa 492-527 and 563-632). The last 175 residues are highly homologous to *C. elegans* UNC-84 and *S. pombe* Sad1 forming the evolutionarily conserved SUN domain. The domain structure of human Sun1 is identical to that of mouse, except that it lacks the proposed zinc-finger motif. The region between the transmembrane and the SUN domain was divided into subdomains SD1 and SD2 for functional tests. SD1 contains the two coiled-coil regions whereas SD2 does not display any known structural features (Fig. 2A).

In yeast two-hybrid assays we investigated the possible interaction between the luminal domain of Nesprin-1 and mouse Sun1. The last 30 amino acids (luminal domain) of mouse Nesprin-1 were fused in-frame to the binding domain of Gal4 and were tested for an interaction with five different C-terminal Sun1 fusion constructs with the activating domain of Gal4. These included Sun1-C (C-terminus of Sun1), Sun1-CΔSUN, which lacks the SUN domain, Sun1-SUN composed solely of the SUN domain, Sun1-SD1 and Sun1-SD2 (Fig. 2B). Co-transformation of the

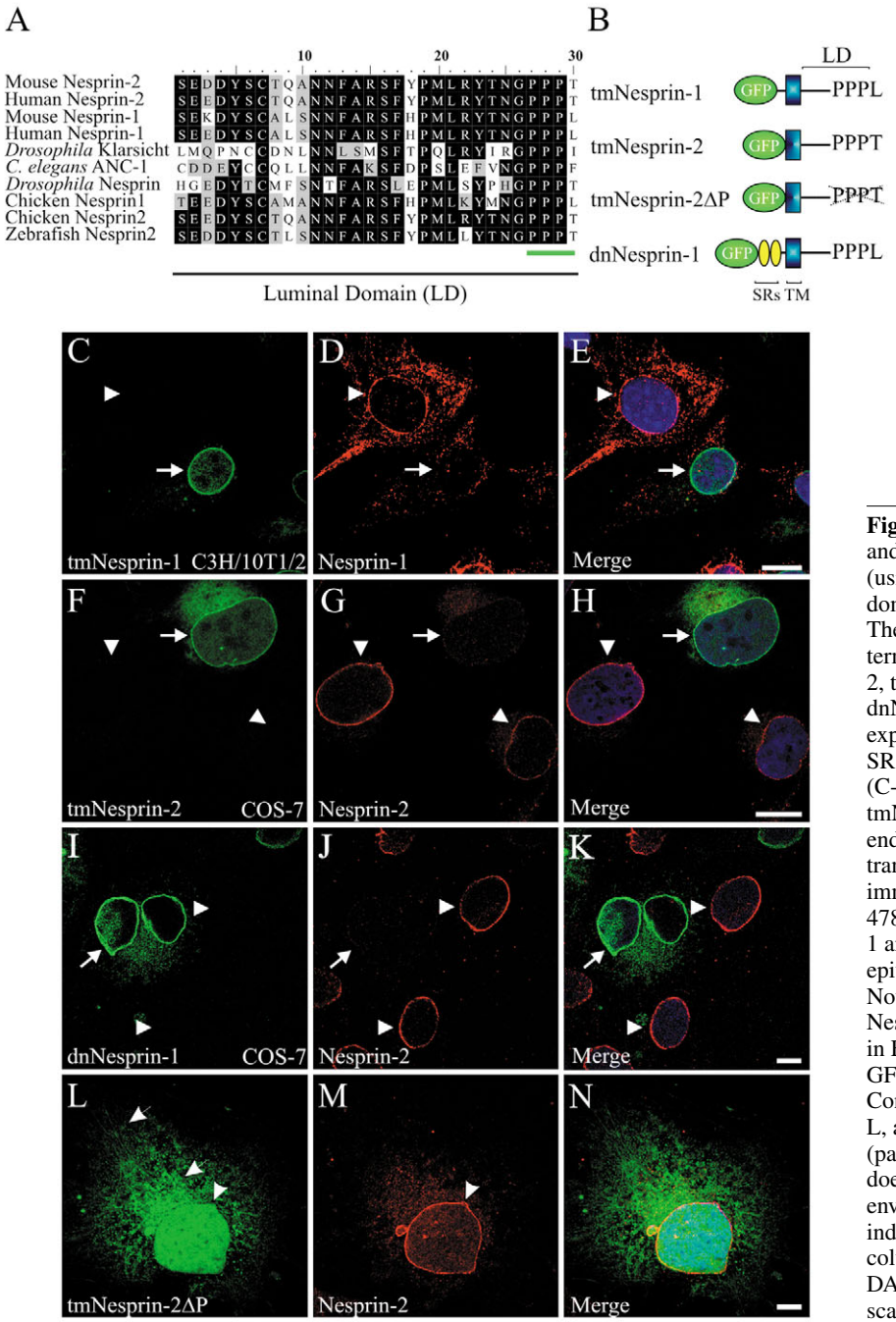

Fig. 1. The C-termini of Nesprins are conserved and sufficient for NE localisation. (A) Alignment (using MultiAlign) of the 30 amino acid luminal domains of various KASH-domain NE proteins. The green bar denotes the highly conserved C-terminal prolines. (B) The tmNesprin-1, tmNesprin-2, tmNesprin-2ΔP (lacks the last four aa) and dnNesprin-1 GFP fusion constructs used for the experiments shown in C-N. LD, luminal domain; SR, spectrin repeats; TM, transmembrane domain. (C-K) Dominant-negative effect of tmNesprin-1, tmNesprin-2 and dnNesprin-1 GFP fusions on the endogenous Nesprin proteins. Transiently transfected cells were fixed and subjected to immunofluorescence using the monoclonal K20-478 anti-Nesprin-2 and a rabbit polyclonal Nesprin-1 antibody. These antibodies did not recognise epitopes on the ectopically expressed polypeptides. Note the nuclear rim staining of endogenous Nesprin proteins in untransfected cells (arrowheads in E,H,K) and the absence of Nesprin staining in GFP-positive cells (arrows in E,H,K). (L-N) Confocal images demonstrate a cytoplasmic (panel L, arrows) and a diffuse nuclear staining pattern (panel L, arrowhead) for GFP-Nesprin-2ΔP, which does not affect endogenous Nesprin-2 at the nuclear envelope (arrowhead in M). The cell lines used are indicated in the lower right-hand corner of the first column of frames (C,F,I). DNA was stained with DAPI. Images were obtained by confocal laser-scanning microscopy. Bars, 10 μm.

plasmids into Y190 yeast cells followed by β-galactosidase assays revealed an interaction between Nesprin-1 and Sun1-C, whereas controls remained negative. Further experiments identified the SD2 of Sun1 (residues 632-737) as the primary Nesprin-1 binding site, whereas the SUN domain itself showed only a weak binding to Nesprin-1 in this assay. No interaction was found with SD1 (Fig. 2B).

These observations were further supported by biochemical assays where we used GST fusion proteins containing the luminal domain of Nesprin-1 and Nesprin-2 (yielding GST-LDN-1 and -2 respectively) to pull down the GFP-Sun1-C fusion protein, which lacks the transmembrane domains (GFP-Sun1-C, aa 432-913). Both GST fusion proteins were able to precipitate GFP-Sun1-C from COS7 cell lysates (Fig. 2D,E). A Nesprin-2 luminal domain deletion GST construct (GST-LDN-2ΔP) lacking the last four highly conserved amino acids (PPPT) (Fig. 2C) was generated and used for precipitation assays from COS7 lysates containing GFP-Sun1-C. In contrast to GST-LDN-1 and GST-LDN-2, no interaction with GFP-Sun1-C was observed for the GST-LDN-2ΔP fusion protein (Fig. 2E), which is consistent with the observation, that tmNesprin-2ΔP was unable to displace Nesprin-2 from the NE (Fig. 1L-N).

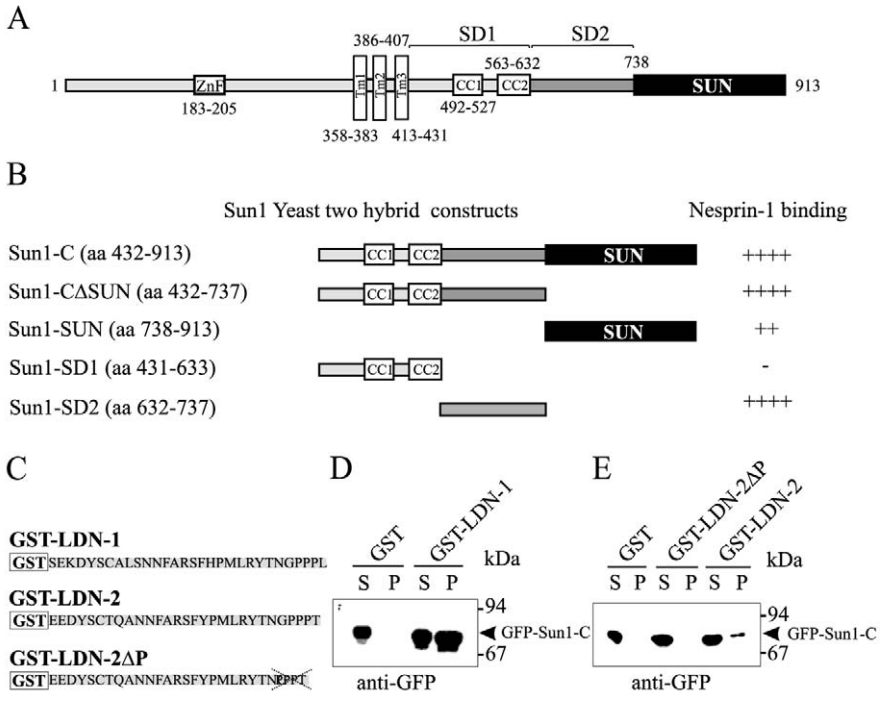

Fig. 2. The C-terminus of Sun1 associates directly with the luminal domains of Nesprin-1 and Nesprin-2. (A) Domain organisation of mouse Sun1. The domain locations as well as their amino acid positions are indicated according to the GenBank entry AAH48156. CC, coiled-coil domain; ZnF, zinc-finger domain; Tm, transmembrane domain. (B) Sun1 polypeptides corresponding to various Sun1 domains were fused to the Gal4 activating domain, whereas the Nesprin-1 luminal domain was fused to the Gal4 DNA-binding domain. The corresponding plasmids were co-transformed into yeast cells and the interactions were assessed by the filter lift β-galactosidase assay. ++++, strong; ++, weak; –, no blue colour development. (C) Schematic overview of the fusion proteins (GST-LDN-1, GST-LDN-2 and GST-LDN-2ΔP lacking the last 4 aa) used for the GST pull-down assay of COS7 cell homogenates expressing GFP-Sun1-C. LDN-1, luminal domain Nesprin-1; LDN-2, luminal domain Nesprin-2. (D,E) COS7 cell lysates expressing the C-terminal half of Sun1 (Sun1-C) were incubated with the immobilised GST-fusion proteins as indicated and GST for control. Unbound (S) and specifically bound (P) proteins were subjected to SDS-PAGE followed by western blot analysis using GFP-specific mAb K3-184-2.

Sun1 is a component of the nuclear envelope and the inner nuclear membrane

To determine whether mouse and human Sun1 are inner nuclear membrane proteins like UNC-84 in *C. elegans*, we performed a series of analyses using the polyclonal Sun1 antiserum 281 generated against a peptide derived from the N-terminus of human Sun1. Western blot analysis of HaCaT cell lysates using the unpurified Sun1 serum, detected a major 100 kDa band (Fig. 3A). Furthermore, the Sun1 antibody detected ectopically expressed full-length human Sun1 protein in cell lysates, which could be efficiently competed by increasing amounts of the peptide antigen (data not shown). To examine whether Sun1 interacts with Nesprin-2 in vivo, we performed immunoprecipitation studies. When the Nesprin-2 immunocomplexes (Fig. 3B, lane 4) were resolved by SDS-PAGE and subjected to silver staining we observed a faint 100 kDa band (Fig. 3B, lane 4, arrowhead), which was specifically recognised by the anti-Sun1 antibody (Fig. 3B, lane 4, right panel). These results indicate that Sun1 interacts with Nesprin-2 in vivo.

The Sun1 antiserum preferentially stained the NE where it colocalised with Nesprin-2 in HaCaT cells (Fig. 3C-E). In addition, the antibodies produced cytoplasmic background staining resulting most probably because we used Sun1 antiserum, which was not affinity-purified. The background staining also persisted after specific Sun1 knockdown (Fig. 7D,G and J) and therefore does not mirror a natural localisation for the endogenous Sun1 protein.

Furthermore stably expressed V5 epitope tagged human Sun1 colocalised with Lamin B receptor (LBR), a well-characterised inner nuclear membrane protein, in a number of cell lines, including HeLa (Fig. 3F-H), human Hek293, COS7 and SW-480 cells, murine NIH-3T3 fibroblasts and canine epithelial MDCK-cells (data not shown).

To test the association of human Sun1 with the NE at the biochemical level, nuclei of HeLa cells stably expressing Sun1 were extracted in buffers containing urea and non-ionic detergents. The distribution of human Sun1 in soluble (S) and insoluble (P) fractions was analysed by immunoblot analysis (Fig. 3I). The distribution of the well-characterised inner nuclear membrane protein LAP2β served as a control. Sun1 and LAP2β displayed similar properties, being completely resistant to extraction with high salt, chaotropic agents and detergent at low salt concentration (Fig. 3I). Only

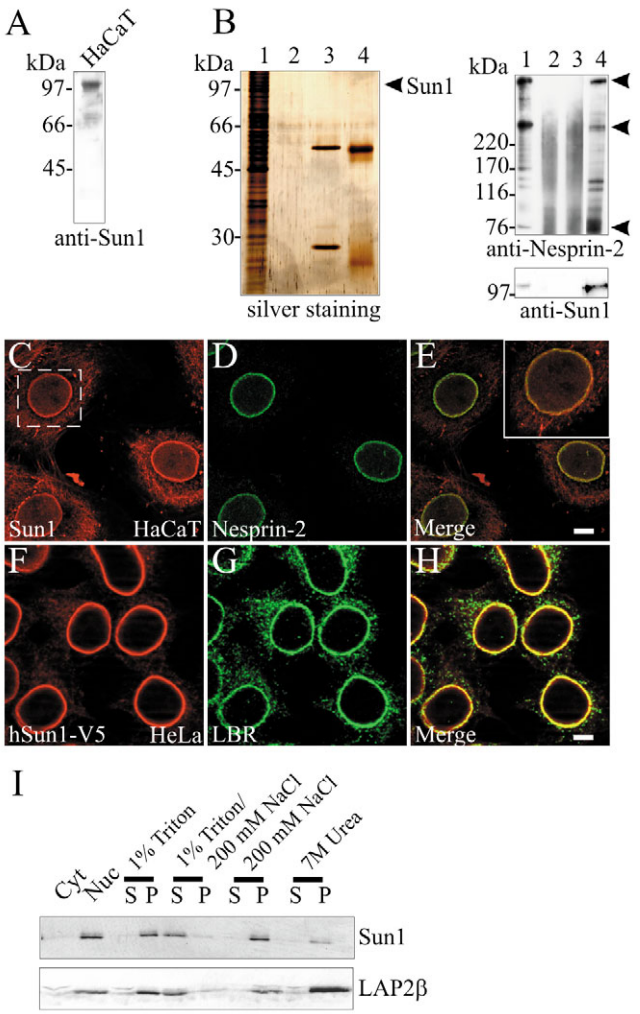

Fig. 3. Sun1 behaves like an integral inner nuclear membrane protein. (A) Western blotting analysis of HaCaT cell lysates using polyclonal Sun1-specific antibodies detects a major 100 kDa band. (B) Endogenous Sun1 protein co-immunoprecipitates with Nesprin-2. Immunocomplexes obtained from HaCaT cells with anti-Nesprin-2 (pAbK1) antibodies were analysed by SDS-PAGE and subjected to silver staining (left panel) or immunoblotting with anti-Nesprin-2 (mAb K20-478) and anti-Sun1 antibody (right panel). The major 800, ~400 and 75 kDa Nesprin-2 isoforms present in HaCaT cells are indicated by arrows (right panel). Lane 1, input lysate; lane 2, control precipitate with Protein A sepharose beads; lane 3, mock-IP control IgG antibody; lane 4, co-immunoprecipitate with anti-Nesprin-2 antibody pAb-K1. The bands observed in lane 4 represent signals obtained after short exposure whereas lanes 1-3 were obtained after prolonged ECL detection (30 minutes). Positions of molecular mass markers in kDa are shown on the left-hand side of the blots. (C-E) HaCaT cells were subjected to immunofluorescence using Sun1 (281) and Nesprin-2 antibodies (mAb K20-478), demonstrating the colocalisation of Sun1 with Nesprin-2 at the NE (E). The inset is a higher magnification of the dotted white box. (F-H) Ectopically expressed full-length human Sun1 (C-terminal V5-tag) is targeted to the nuclear envelope in HeLa cells, displaying strict colocalisation with the Lamin B receptor (LBR). Images were obtained using a confocal microscope. (I) Solubilisation properties of human Sun1 under various extraction conditions. Purified nuclei (Nuc) of HeLa cells, stably expressing V5-tagged human Sun1, were extracted in RIPA buffer containing urea, Triton X-100, salt or combinations thereof, as indicated. Soluble (S) and insoluble (P) fractions were analysed by western blotting. Cytosol (Cyt) served as a purity control. The same lysates were analysed for LAP2β, a known integral inner nuclear membrane protein. Bars, 7 μm.

treatment with detergent and medium salt concentrations (1% Triton X-100/200 mM NaCl) efficiently solubilised Sun1 and LAP2β. In accordance with these data KIAA0810 has been identified as a component of both the detergent- and chaotrope-resistant fractions in a proteomics screen (Dreger et al., 2001). More recently, Sun1 was exclusively detected in the salt- and sodium hydroxide-resistant fractions of a novel protocol to isolate unknown NE constituents (Schirmer et al., 2003).

In order to define the topology of Sun1 at the NE we performed digitonin permeabilisation of cells, which selectively disrupts the plasma membrane leaving the NE membranes intact (Adam et al., 1990), whereas Triton X-100 permeabilises all membranes. Antibodies to both Sun1 and lamin A/C stained the NE in Triton X-100-permeabilised COS7 cells (Fig. 4A-C, arrows). In addition, the antiserum strongly stained the nucleoplasm (Fig. 4A), whereas in HaCaT cells the staining was preferentially found at the NE (Fig. 3C). In digitonin-treated COS7 cells Sun1 and lamin A/C remain undetectable using these antibodies at the NE. Only the cytoplasmic staining of Sun1 antiserum 281 was still observed (Fig. 4D and F, arrowheads). Identical results were obtained for ectopically expressed human as well as mouse Sun1 (data not shown). Altogether, these data strongly suggest that Sun1 is an integral inner nuclear membrane protein.

Earlier studies on Nesprin-2 using digitonin permeabilisation assays suggested that Nesprin-2 is integrated into the outer nuclear membrane (Zhen et al., 2002). To investigate whether the highly homologous Nesprin-1 shares a similar localisation we analysed C3H/10T1/2 fibroblasts treated with Triton X-100 and digitonin. Unlike lamin A, Nesprin-1 staining could still be detected at the NE after selective digitonin permeabilisation indicating its presence at the outer nuclear membrane (Fig. 4J-L). However our findings do not exclude the possibility that Nesprin-1 also localises to the inner nuclear membrane. Collectively our findings suggest an asymmetric distribution at the nuclear membrane of the interaction partners Sun1 and Nesprins.

The N- and C-termini of Sun1 localise independently to the NE

The current sorting mechanism, which defines the localisation of NE transmembrane proteins during interphase is the 'diffusion-retention' model (Worman and Courvalin, 2000). According to this model NE proteins are co-translationally integrated into the ER followed by a lateral diffusion from the ER to the outer and inner nuclear membranes, interconnected by the nuclear pore complex. Proteins are then retained at the inner nuclear membrane owing to the presence of nuclear retention sequences allowing binding to nuclear proteins, chromatin or both.

To investigate the subcellular localisation and to determine the NE retention domains of mouse Sun1 we transiently transfected various Sun1-GFP fusion proteins (Fig. 5A) into COS7 cells. The expression and the appropriate molecular masses of the fusion proteins were confirmed by western blotting (data not

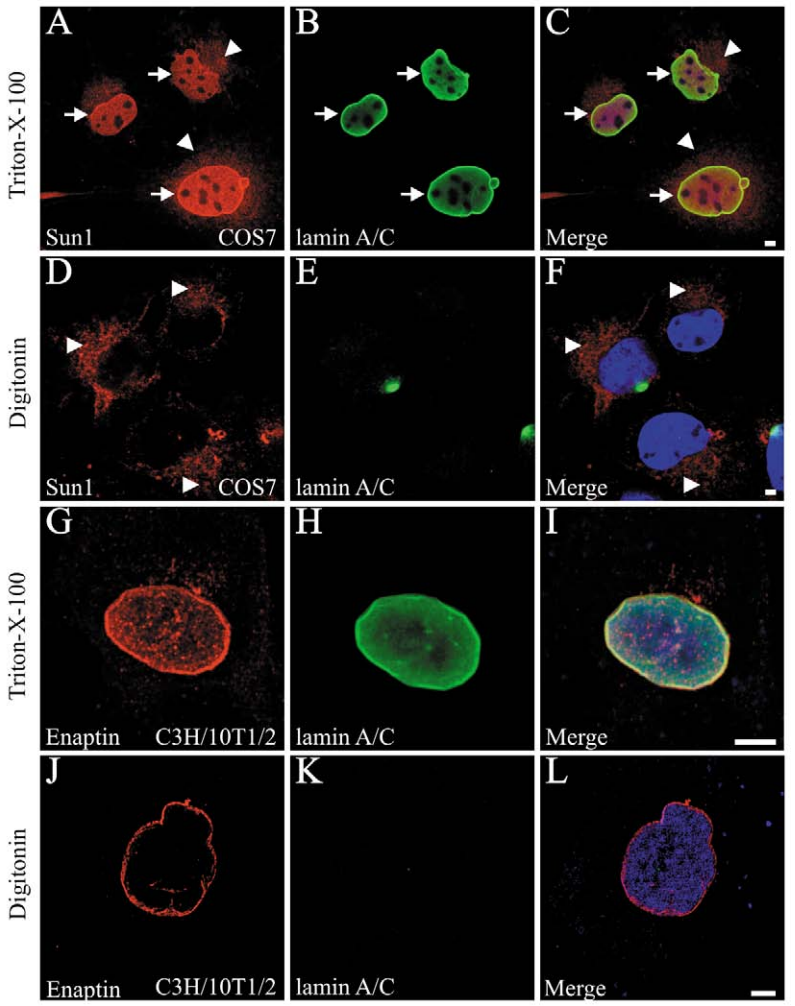

Fig. 4. Asymmetric distribution of Sun1 and Nesprin-1 at the nuclear membrane. (A-C) Triton X-100 treated COS7 cells subjected to immunofluorescence with Sun1 and lamin A/C antibodies, indicate the nuclear localisation of Sun1 (arrows). Non-specific staining of antibody 281 was observed in the cytoplasm (arrowheads; see also Fig. 3C). (D-F) In digitonin-treated COS7 cells only the non-specific staining remains (arrowheads) suggesting a localisation of Sun1 at the inner nuclear membrane. The integrity of the nuclear membrane is documented by the absence of lamin A/C staining (E). (G-I) In Triton X-100-permeabilised fibroblasts Nesprin-1 antibodies strongly stain the nucleus. (J-L) Nesprin-1 staining at the NE persists after digitonin treatment suggesting the presence of Nesprin-1 at the outer nuclear membrane. Note the absence of lamin A/C staining (K). DAPI was used to counterstain the nucleus. Confocal images are shown. Bars, 5 μm.

shown). Similar to its human orthologue (Fig. 3F) the full-length mouse Sun1 protein also localised to the NE (Fig. 5B). Deletion of the SUN domain (GFP-ΔSUN) did not affect targeting to the NE (Fig. 5C, arrow) showing that the SUN domain is not required for retention at the NE. Accumulation at the NE was also obtained with GFP fusion proteins containing the entire Sun1 N-terminus with a single (Sun1-N+1TM) or two transmembrane domains (Sun1-N+2TM) (Fig. 5D,E, arrows). Surprisingly, the C-terminus containing the three transmembrane domains (Sun1-TM-C) also localised to the NE in COS7 cells (Fig. 5F, arrows; Fig. 6A, arrow). Thus, the C-terminus of Sun1 is sufficient to confer NE targeting, however, much of the GFP fusion protein remained in the ER (Fig. 5F, arrowheads). Although more than 75% of the Sun1-N+1TM and Sun1-N+2TM fusions localised to the NE, only 58% of Sun1-TM-C displayed NE localisation. The accumulation of the proteins in the ER may reflect either the absence of important domains or result from the overexpression of improperly folded proteins. In summary, both the N- and C-termini of Sun1 localise independently to the NE, which is most likely facilitated by binding to different proteins in the nucleoplasm and the perinuclear space.

Sun1 associates with itself in vivo and the two coiled-coil domains are sufficient to target the C-terminus of Sun1 to the NE

In order to study the C-terminal Sun1 nuclear targeting sequences in more detail, three additional GFP constructs (Sun1-TM-SD1,2; Sun1-TM-CΔSD2SUN; Sun1-TM-CΔCC; Fig. 5A) were expressed in COS7 cells (Fig. 5G-I). The GFP fusion protein Sun1-TM-SD1,2 comprising SD1, SD2 and the three transmembrane domains displayed a NE localisation in 64% of transfected cells (Fig. 5G, arrow and Fig. 5J). As this polypeptide lacks the SUN domain, the SUN domain seems dispensable and not required to confer the NE localisation, as shown above for the GFP-ΔSUN mutant. To investigate whether the Nesprin binding domain SD2 in Sun1 is involved in the NE targeting of Sun1, we constructed a GFP fusion construct of the three transmembrane domains followed by SD1, composed of the two coiled-coil domains, but lacking SD2 (Sun1-TM-CΔSD2SUN). In 73% of transiently transfected COS7 cells we observed a clear NE association of the fusion protein (Fig. 5H, arrow and Fig. 5J), suggesting therefore the presence of a nuclear retention signal within SD1 of Sun1. Coiled-coils have traditionally been recognised as an oligomerisation unit in a large number of proteins (Burkhard et al., 2001). As SD1 is composed of two coiled-coil regions (CC1 and CC2, see Fig. 2A), it is possible that oligomerisation of the GFP-fusion protein with the endogenous Sun1 leads to retention at the nuclear envelope. This is supported by data from yeast two-hybrid experiments, which indicated an interaction of SD1 with itself (data not shown). To test the hypothesis that the NE localisation of N-terminally truncated Sun1 polypeptides is mediated by the coiled-coil region, we generated GFP-Sun-TM-CΔCC removing the two coiled-coil regions between amino acids 491-633, leaving the SUN as well as the transmembrane domains intact. Only 33% of transfected cells (Fig. 5J) displayed a NE localisation (Fig. 5I, arrows). In the majority of cases we observed substantial accumulation of the chimeric protein in the ER (arrowhead). Our data suggest that the coiled-coil region of Sun1 is important to mediate NE association, however the ability of the Sun1-TM-CΔCC fusion protein to localise to the NE is indicative of the existence of additional nuclear retention signal(s) in the C-

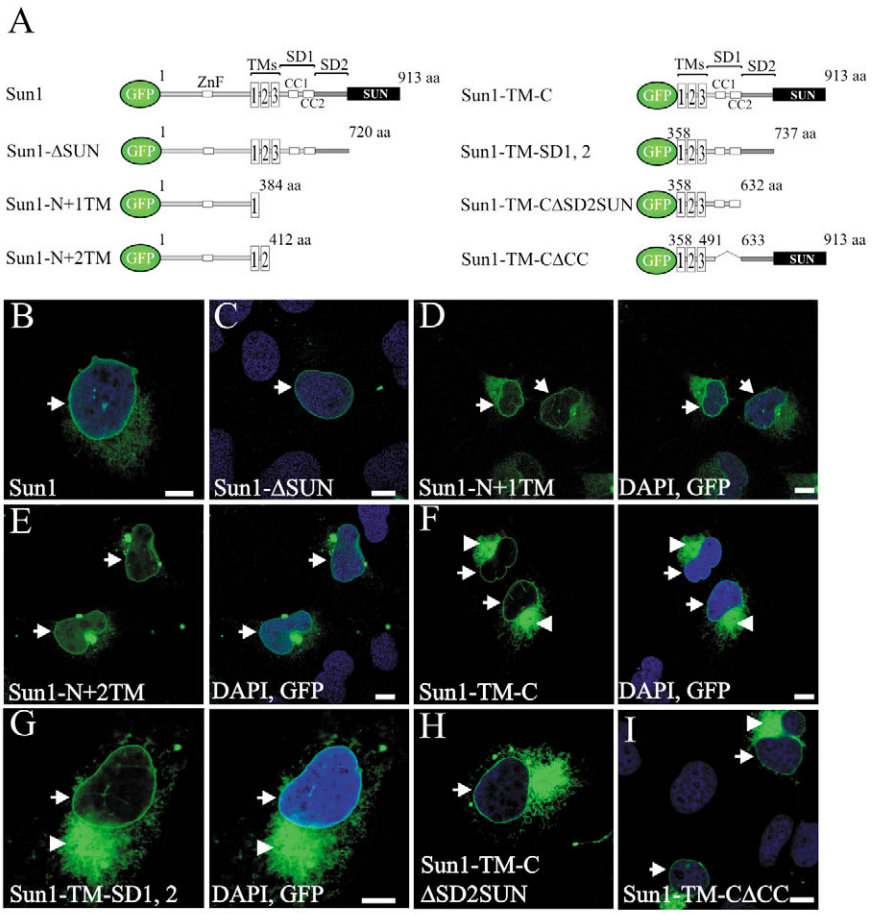

Fig. 5. Sun1 contains multiple, independent nuclear targeting signals. (A) Schematic representation of Sun1 GFP fusion constructs. Domain labelling is as in Fig. 2A. (B-I) Subcellular localisation of GFP Sun1 fusion proteins in COS7 cells observed by direct fluorescence confocal microscopy. Arrows indicate NE localisation whereas arrowheads indicate ER localisation. DAPI was used to counter-stain the nuclei. (J) Histogram representing a statistical evaluation (percentage of transfected cells) of the localisation profiles of the various SUN1-GFP fusions to the ER and the NE. Bars, 10 μm.

terminus of Sun1. Furthermore we cannot exclude the possibility that the Sun1 transmembrane domains themselves contain sorting signals and determine the NE localisation in a similar fashion to the lamin B receptor (Wozniak and Blobel, 1992; Smith and Blobel, 1993). The presence of multiple and independent nuclear retention signals across Sun1 is further supported by the fact that none of the fusion proteins localised as efficiently to the NE as the full-length Sun1 (Fig. 5J). However, its interaction with Nesprins is apparently not involved in retaining Sun 1 at the NE.

Sun1 affects the NE localisation of Nesprin-2

Immunofluorescence analyses of endogenous Sun1 using the Sun1 antiserum in COS7 cells transiently expressing Sun1-TM-C (which is not detected by the antiserum) revealed a displacement of endogenous Sun1 from the NE (Fig. 6B-C, arrow). Untransfected cells, however, displayed a proper NE localisation of Sun1 (Fig. 6B-C, arrowhead). Out of 200 transfected cells 82% showed significantly reduced NE staining of Sun1 (Fig. 6G). As Nesprin-2 associates with Sun1 we tested its localisation in Sun1-TM-C transfected cells as well (Fig. 6D-F). Similar to the endogenous Sun1, Nesprin-2 was displaced from the NE (Fig. 6E-F, arrow) in transfected cells, whereas in untransfected cells Nesprin-2 was properly localised at the NE (Fig. 6D-F, arrowhead). In a statistical analysis of 200 transfected cells Nesprin-2 was no longer found at the NE in 97% of cells (Fig. 6G). To further support the requirement of Sun1 for the localisation of Nesprin-2, we performed knockdown studies employing a mixture of two independently expressed siRNAs targeting the N-terminus of Sun1. Transient transfections of the RNAi-competent plasmids pJG173 and pJG174 in HeLa cells, which stably express V5-tagged hSun1 verified the efficacy of the Sun1 knockdown (Fig. 7A-C). Many transfected cells displayed a reduced anti-V5 hSun1 staining (asterisks), whereas the lamin A/C pattern appeared unaffected (Fig. 7B). Similar observations were made in transiently transfected Sun1 knockdown HaCAT cells (Fig. 7D-F). In keratinocytes where the nuclear Sun1 staining was absent (Fig. 7D; asterisks), lamin A/C staining was still observed at the nuclear envelope (Fig. 7E; asterisks), suggesting therefore that Sun1 is not essential for lamin A/C localisation. In sharp contrast, however, in Sun1 knockdown cells (Fig. 7G-L) the Nesprin-2 staining pattern was either very faint or absent using both N-terminally (Fig. 7H; asterisks) as well as C-terminally (Fig. 7K; asterisks) directed antibodies. In summary our data indicate that the proper localisation of Nesprin-2 at the NE requires Sun1.

Targeting of Sun1 to the NE is independent of a functional lamin A/C network

In *C. elegans* UNC-84 localises to the NE in a lamin-dependent manner (Lee et al., 2002). To explore whether the UNC-84 orthologue Sun1 also depends on the lamin network we

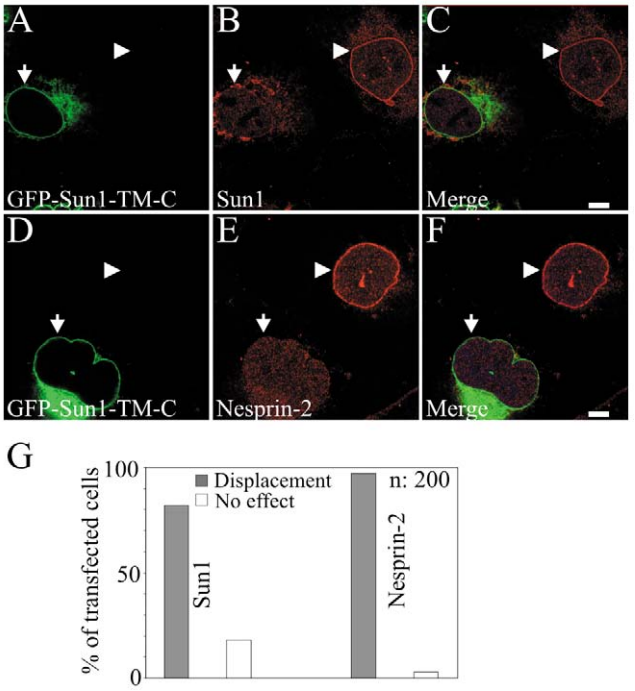

Fig. 6. GFP-Sun1-TM-C acts in a dominant-negative manner on endogenous Sun1 and Nesprin-2. COS7 cells expressing GFP-Sun1-TM-C were stained using specific antibodies to Sun1 (B,C) and Nesprin-2 (E,F). (A-F) Confocal images illustrating that GFP-Sun1-TM-C (transfected cells are indicated by arrows) interferes with the localisation of endogenous Sun1 (B and C, arrows) and Nesprin-2 (E and F, arrows). Note the differences in the Sun1 and Nesprin-2 staining pattern in transfected (arrows) versus untransfected (arrowheads) cells. (G) Histogram illustrating the displacement effects of the GFP-Sun1-TM-C fusion on the endogenous Sun1 and Nesprin-2 proteins. Data were obtained by evaluating 200 transfected cells. Bars, 7 μm.

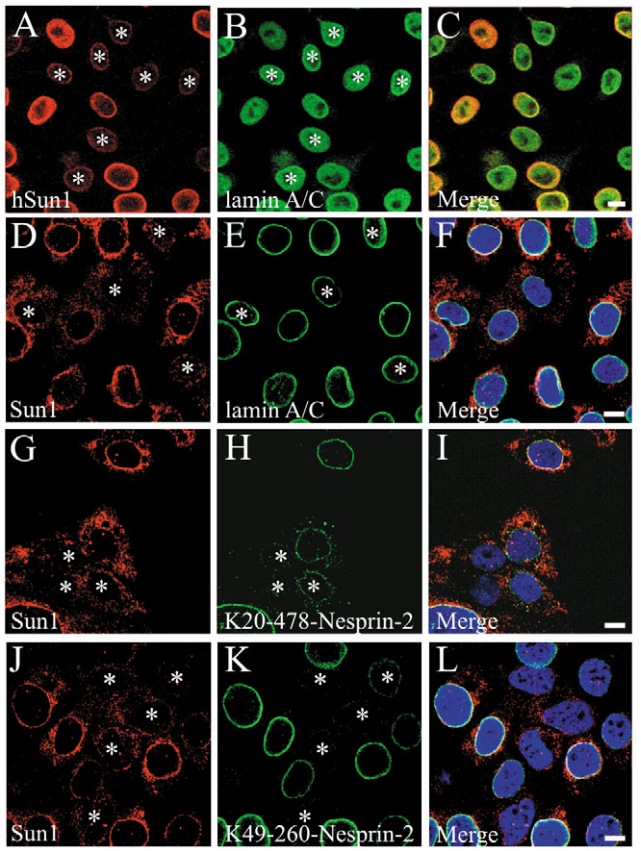

Fig. 7. Nesprin-2 localisation is affected in cells where Sun1 expression has been silenced by siRNA. (A-C) HeLa cells expressing stably V5-tagged hSun1 and HaCaT cells (D-L) were transiently transfected with a combination of plasmids (pJG173/174) encoding siRNAs targeting hSun1. The distribution of Sun1 (panel A, anti-V5; panels D,G,J, anti-Sun1 281 serum), lamin A/C (panels B and E) and Nesprin-2 (panel H, mAb K20-478; panel K, mAb K49-260) was investigated by indirect immunofluorescence in knockdown cells (indicated by asterisks). In Sun1 knockdown cells, the lamin A/C localisation remained unaltered (B and E), whereas Nesprin-2 staining was either absent or reduced. DNA was stained by DAPI. The images shown were taken by confocal microscopy and merged to visualise colocalisation (panels C,F,I,L). Bars, 10 μm.

transfected lamin A/C knockout fibroblasts (Sullivan et al., 1999) with the mouse Sun1 and Sun1-N+2TM GFP fusion proteins (Fig. 8A-C). In both wild-type and knockout fibroblasts the fusion proteins localised properly to the NE. Identical results were obtained with human V5 epitope-tagged Sun1 in lamin A/C knockout fibroblasts (Fig. 8D). To further substantiate our findings, we transiently transfected HeLa cells stably expressing human Sun1 with the *Xenopus* mutant GFP-B1Δ2+ (Fig. 8G-I). This fusion protein accumulates in intranuclear aggregates (Fig. 8H) and recruits endogenous lamins A/C, thus disturbing the functional organisation of the lamin A/C network (Dechat et al., 2000; Vaughan et al., 2001). Importantly, the expression of GFP-B1Δ2+ and loss of a functional A-type lamin network did not affect the NE localisation of Sun1 (Fig. 8G-I). Altogether, lamin A/C is not essential for the localisation of Sun1 at the NE. Based on UNC84 data, one could assume that B-type lamins may be required for retention of Sun1 at the inner nuclear membrane.

Discussion

Recent focus on NE composition and function has been primarily powered by the unexpected involvement of several NE components and associated proteins in human diseases (Burke et al., 2001; Gotzmann and Foisner, 2004). Understanding the pathology of all these diseases requires the identification and functional characterisation of all nuclear envelope constituents as a first step, as well as knowledge of the networking interactions that take place at the NE. Towards this end, using both biochemical as well as cell biological data we unravel the first link between an inner nuclear membrane protein (Sun1) and constituents of the outer and inner nuclear membranes (Nesprins).

Nesprins are targeted to the NE by binding to Sun1 through their conserved C-terminus

In an effort to study the nuclear localisation mechanism of the giant actin binding Nesprin-1 and Nesprin-2 proteins, we demonstrated that the evolutionarily conserved C-terminus of Nesprins is sufficient to target the proteins to the NE.

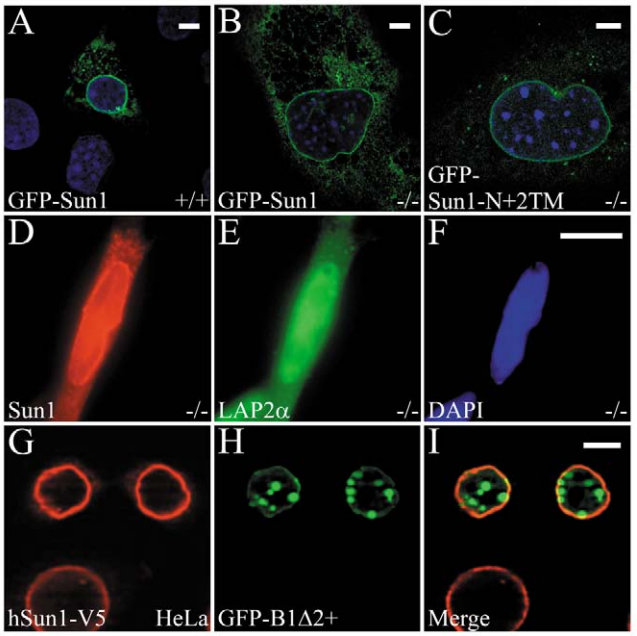

Fig. 8. Lamin A/C does not influence the NE localisation of Sun1. (A-C) Wild type (A) and lamin A/C knockout (B and C) mouse dermal fibroblasts were transfected with the mouse GFP-Sun1 and GFP-Sun1-N+2TM fusion proteins. Transiently transfected cells were processed for direct fluorescence microscopy. Note that both GFP fusion proteins localise to the nuclear envelope in the absence of lamin A/C (B and C). (D-F) Lamin A/C$^{-/-}$ fibroblasts were transfected with plasmid encoding V5-tagged human Sun1 and processed for immunofluorescence using antibodies to V5 and the LAP2α. (G-I) HeLa cells stably expressing human Sun1 (V5-tagged) were transiently transfected with a plasmid coding for the dominant negative GFP-lamin B1Δ2+ protein and stained for the V5 epitope. Images were obtained by confocal microscopy. Bars, 6 μm.

Furthermore, we showed that the overexpression of C-terminal Nesprin peptides caused a dominant-negative effect on the distribution of endogenous Nesprins provoking their displacement from the NE. These results not only suggest that their NE retention mechanism may be the same, but in addition C-terminal isoforms may have regulatory functions. Temporary and spatially controlled expression of Nesprins-1α, β and Nesprin-2α-γ may allow a modulation of Nesprin-based contacts to the actin cytoskeleton, as their presence would result in the reduction or absence of the large ABD-containing isoforms from the NE.

Based on data from *C. elegans* demonstrating a genetic interaction between *C. elegans* ANC-1 and UNC-84 (Starr and Han, 2002), we studied the NE anchorage of Nesprins by the UNC-84 orthologue in higher eukaryotes. Yeast two-hybrid and GST pull-down experiments demonstrated that a region termed SD2, composed of amino acids 632-737 of Sun1 does indeed interact in vivo and in vitro with both Nesprin-1 and Nesprin-2. In *C. elegans*, missense mutations in or near the SUN domain of UNC-84 probably disrupt the capacity of SD2 to associate with ANC-1. Our yeast two-hybrid data suggest, however, that the SUN domain is not the main Nesprin interaction domain. It may well be that these particular SUN domain mutations affect the proper folding of the protein resulting in non-functional C-terminal domains in the perinuclear space. In addition, the SUN domain may have different functions in *C. elegans* compared to higher eukaryotes. In fact, in *C. elegans* the SUN domain of UNC-84 is directly involved in the NE recruitment of UNC-83 a nuclear transmembrane protein, which is essential for proper nuclear migration (Starr et al., 2001; Lee et al., 2002). To date no orthologue of UNC-83 is known in higher eukaryotes. Irrespective of SUN domain function in various organisms, its conservation in evolution implies that Sun1 exhibits additional functions besides the tethering of Nesprins to the NE.

Sun1 is an inner nuclear membrane protein

By performing digitonin experiments we demonstrated the presence of Sun1 in the inner membrane of the NE. Our results are in accordance with a nuclear envelope proteomics approach, which identified Sun1 as an integral membrane protein of the inner nuclear membrane (Dreger et al., 2001). Similar findings were also obtained for its paralogue Sun2, also a type II transmembrane domain protein (Hodzic et al., 2004). Moreover, experiments involving proteinase K protection assays and digitonin experiments with Sun2 suggested the presence of the C-terminus in the perinuclear space whereas the N-terminus points to the nucleoplasm thus allowing an interaction with the nuclear lamina (Hodzic et al., 2004). Because of the overall similarity between Sun1 and Sun2 we assume a similar topology for the Sun1 full-length protein. This arrangement is required to allow the interaction of the SD2 domain with the luminal domain of Nesprins.

The NE localisation of Sun1 at the NE does not depend on lamin A/C

The proper localisation of full-length or the N-terminal half of Sun1 in lamin A/C knockout fibroblasts demonstrated that lamin A/C is not required for the NE localisation of Sun1. Moreover, disruption of a functional lamin A/C system had no detectable effects on the proper localisation of Sun1. The *C. elegans* UNC-84 requires the B-type Ce-lamin for its envelope localisation (Lee et al., 2002). Thus localisation of mammalian Sun1 at the NE may also depend on B-type lamins. In contrast, both Nesprin-1 and Nesprin-2 localisation at the NE depend on lamin A/C, and NE-targeting of human Sun2 also requires a functional lamin A/C network (Libotte et al., 2005) (our unpublished data). Therefore, those findings suggest that additional proteins are implicated in the tethering of Nesprins at the NE. Whether additional SUN domain-containing proteins in addition to Sun1 are involved in those associations warrants further investigation.

Sun1 connects through the Nesprin proteins the nucleus to the cytoskeleton

Our studies support a model whereby Nesprin-1 and Nesprin-2 are anchored at the nuclear envelope through a Sun1-mediated interaction (Fig. 9). Although the N-terminus of Sun1 may provide a link to the nuclear lamina, its C-terminal subdomains are implicated in intramolecular (SD1) and intermolecular (SD2 and SUN) perinuclear space interactions. Whereas SD2 was identified as the Nesprin binding domain we failed to assign a specific function to the evolutionarily

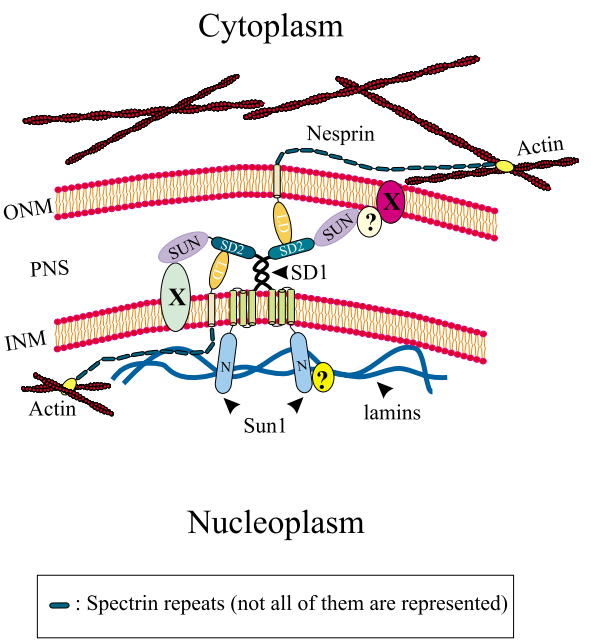

Fig. 9. Model illustrating the interactions of Sun1 with Nesprins at the nuclear envelope. Unknown nuclear envelope proteins and interactions are indicated by X and ?, respectively. To reduce complexity a homotypic dimerisation of Sun1 via the coiled-coil regions is postulated, although other coiled-coil-containing proteins might form heterotypic complexes with Sun1. INM, inner nuclear membrane; LD, luminal domain; N, N-terminal domain; ONM, outer nuclear membrane; PNS, perinuclear space.

conserved SUN domain. Additional studies will be needed to elucidate its biological significance. Unlike many other NE proteins such as lamins, emerin and Sun1, Nesprins are present on both sides of the NE. Their presence at the inner nuclear membrane, is substantiated in particular by electron microscopy studies and by the physical association of Nesprins with the inner nuclear membrane proteins lamin A/C and emerin (Mislow et al., 2002b; Libotte et al., 2005; Zhang et al., 2005). The absence therefore of Nesprin-2 staining in Sun1-silenced cells strongly suggests that Sun1 recruits and integrates both outer as well as inner nuclear membrane Nesprin-2 pools through their luminal domains (Fig. 9). Such a scenario is substantiated by the ability of Sun1 to oligomerise and by the fact that Nesprin-2 appears as clusters along both sides of the nuclear membrane in HaCaT cells (Libotte et al., 2005). At the moment however, it is not clear how such a structural crossbridging is established at the molecular level. Are identical domains such as SD2 in Sun1 implicated in those associations? If so, is the SD2 domain flexible enough to allow such interactions? Or, do different Sun1 domains recruit topologically different Nesprin populations? Alternatively, it is also possible that additional proteins that are recruited to the perinuclear space by Sun1 are implicated in those associations. Independent of the molecular details, such models appear very attractive because they grant a mechanism by which SUN-domain proteins might provide a linkage to the nuclear lamina by binding to B-type lamins, generating a continuity between the nuclear interior and cytosolic compartments.

Furthermore, this linkage may also account for the close proximity of the two NE membranes. Recent evidence implicates TorsinA in connecting the NE to the cytoskeleton. Torsin A an AAA+ ATPase of the ER localises to the NE when mutated in the neurological human disorder early-onset torsion dystonia (Goodchild and Dauer, 2004; Naismith et al., 2004; Gerace, 2004). TorsinA mutants affect several aspects of NE structure, including its morphology, perinuclear spacing and nuclear pore distribution (Naismith et al., 2004). Whether TorsinA modulates the Sun1/Nesprin interactions needs to be seen. In any case, molecular interactions governing and regulating the connections between the nucleoskeleton and cytoskeleton seem to be highly complex and we may have encountered only the tip of the iceberg. Nuclear envelope proteomic approaches suggest the presence of 67 novel or uncharacterised nuclear membrane proteins (Schirmer et al., 2003), which might potentially be involved in these linker complexes.

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