An integral protein of the inner nuclear membrane localizes to the mitotic spindle in mammalian cells

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Summary

Here, we characterize a transmembrane protein of the nuclear envelope that we name spindle-associated membrane protein 1 (Samp1). The protein is conserved in metazoa and fission yeast and is homologous to Net5 in rat and Ima1 in *Schizosaccharomyces pombe*. We show that, in human cells, the protein is a membrane-spanning polypeptide with an apparent molecular mass of 43 kDa. This is consistent with a predicted polypeptide of 392 amino acids that has five transmembrane segments and its C-terminus exposed to the nucleoplasm. During interphase, Samp1 was specifically distributed in the inner nuclear membrane. Post-transcriptional silencing of Samp1 expression resulted in separation of centrosomes from the nuclear envelope, indicating that it is functionally connected to the cytoskeleton. At the onset of mitosis, most of the protein dispersed out into the ER, as expected. However, during mitosis, a significant fraction of the protein specifically localized to the polar regions of the mitotic spindle. We demonstrate for the first time, in human cells, the existence of a membranous structure overlapping with the mitotic spindle. Interestingly, another integral inner nuclear membrane protein, emerin, was absent from the spindle-associated membranes. Thus, Samp1 defines a specific membrane domain associated with the mitotic spindle.

Key words: Inner nuclear membrane, Transmembrane proteins, Mitotic spindle, Mitosis, Cancer

Introduction

In eukaryotic cells, the nucleus containing the genetic information is separated from the cytoplasm by the nuclear envelope (NE) (Anderson and Hetzer, 2008). The NE consists of the outer nuclear membrane (ONM), the inner nuclear membrane (INM), the nuclear lamina and the nuclear pore complexes (NPCs). The NPCs are macromolecular complexes comprising about 30 different proteins termed nucleoporins (NUPs). The NPCs perforate the double nuclear membranes and are responsible for nucleocytoplasmic transport. The ONM is continuous with the rough endoplasmic reticulum (RER) and hence the perinuclear space (PNS) is continuous with the lumen of the ER. Although the ONM contains ER proteins and ribosomes, it also contains a unique set of proteins. The INM has been estimated to contain at least 80 unique transmembrane proteins according to proteomic analyses (Schirmer and Gerace, 2005), but only a few have been further characterized. The nuclear lamina underlies the INM and forms a meshwork of intermediate filaments, containing A- and B-type lamin proteins. Many INM proteins bind to the lamina and/or regulators of chromatin organization (Tsuchiya, 2008). Current research has also revealed 'protein bridges' stretching all the way from chromatin via the nuclear lamina, through the perinuclear space to the ONM and further to the cytoskeleton in the cytoplasm (Crisp and Burke, 2008; King et al., 2008). The NE has recently attracted increasing attention because mutations in lamins and some transmembrane INM proteins were found to give rise to a diverse group of human diseases, termed laminopathies (Worman and Courvalin, 2005). Surprisingly, these diseases affect a number of different tissues, such as muscle, adipose and neuronal tissue or cause premature aging, indicating that the NE has far more complex functions than simply being a barrier between the nucleoplasm and cytoplasm.

During cell division in metazoans, the NE undergoes disassembly, to provide room and accessibility for the mitotic machinery. The INM and ONM with their integral membrane proteins disperse out in the ER during prometaphase (Yang et al., 1997). The non-integral membrane proteins of the NE, including the nucleoporins, become solubilized and disassemble into individual proteins or distinct subcomplexes. Some of these proteins have unique functions and localization during mitosis (Belgareh et al., 2001; Joseph et al., 2004; Orjalo et al., 2006). The nuclear lamina also disperses throughout the cell during mitosis. Lamin A and lamin C behave as soluble proteins, whereas lamin B remains largely associated with the membrane (Gerace and Blobel, 1980).

During mitosis, a bipolar spindle is formed to separate duplicated DNA into daughter nuclei. The spindle consists of the spindle poles, the spindle microtubules, the kinetochores and the duplicated chromosomes. The spindle poles are defined by the centrosomes from which microtubule (MT) fibers extend. The MTs extend to the kinetochores, a specialized structure at centromeres. When all the kinetochores are properly attached to the spindle the mitotic machinery separates the sister chromatids. Kinetochores lacking properly attached MTs activate checkpoint proteins that halt mitosis until a functional bipolar spindle can form. Defects in mitotic spindle assembly can generate chromosomal instability and aneuploidy common in malignant cancers (Qi and Yu, 2006).

Many factors including molecular motors, regulators of microtubule dynamics, components of the NPC and the nucleocytoplasmic transport machinery associate and assist in spindle assembly and/or mechanical force generation required for bidirectional congression and segregation of chromatids. The presence of a matrix around the mitotic spindle has long since been proposed to serve as a scaffold for force generation and distribution during chromosome segregation (Johansen and Johansen, 2007). A distinct set of proteins has been suggested to be part of such a structure. However, the existence of a spindle matrix is still disputed because of difficulties in assessing evidence for functional mitotic roles for the assigned protein candidates (Travis, 2007). Here, we show that the previously uncharacterized mammalian NE protein Samp1 is specifically located in the INM during interphase and that during mitosis, a fraction of Samp1 molecules specifically localizes to the polar regions of the mitotic spindle. Our results show for the first time in fixed and live human cells, the existence of a specific membrane domain associated with mitotic spindles containing an integral membrane protein from the INM.

Results

A novel evolutionary conserved integral membrane protein of the nuclear envelope

We have investigated a protein previously identified in a proteomic analysis of rat liver nuclear envelopes referred to as NET5 (Schirmer and Gerace, 2005). A PSI-BLAST homology search shows that this protein is conserved throughout metazoa and down to *S. pombe* (Fig. 1A). Four diseases have been mapped to a chromosomal region overlapping the human *SAMP1* locus, 1p36.22: Volkmann-type congenital cataract, posterior polar cataract, suppression of anchorage and dystonia13 (OMIM data base; www.ncbi.nlm.nih.gov). The human homologous gene is predicted to encode a polypeptide of 392 amino acids with mainly α-helical secondary structure (accession no. NP_001010866). It has five predicted transmembrane segments (TMSs) and a highly conserved cytoplasmic/nucleoplasmic loop between TMS1 and TMS2 containing four characteristic CXXC motifs (Fig. 1B,C), which are predicted to be organized in two zinc fingers (http://pfam.sanger.ac.uk). Owing to its distribution during mitosis (see later) we have termed the human protein Samp1.

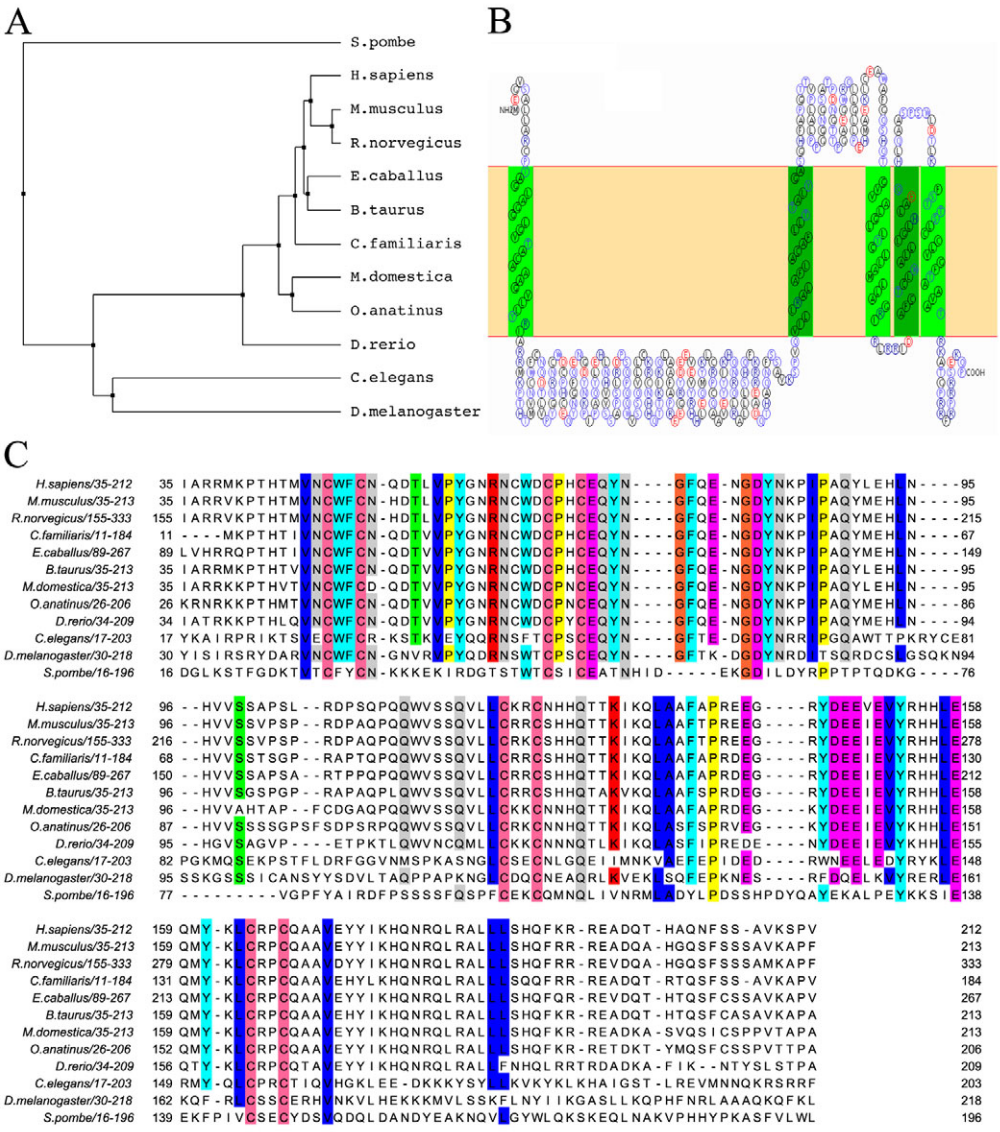

Fig. 1. Samp1 is a conserved integral membrane protein. (A) Phylogenetic tree of Samp1. The phylogenetic relationship between Samp1 homologs from eukaryotic species derived by using the software ClustalW. (B) Predicted membrane topology of human Samp1. Transmembrane segments and topology predicted using the TMHMM 2.0 software and drawn using SOSUI. (C) Multiple sequence alignment of the most conserved parts of the first nucleoplasmic loop of Samp1 from selected eukaryotic species. The alignment was generated using ClustalW. Conserved residues are highlighted in color if at least 80% of the sequences are conserved with respect to the presence of the following groups of amino acids: AILMV, blue; WFY, cyan; RK, red; DE, magenta; ST, green; P, yellow; C, pink; NQ, gray; and G, orange.

We raised antiserum against a peptide of 13 amino acids (residues 379-391) close to the C-terminus of Samp1. In western blot analysis of whole cell extracts of HeLa cells, the affinity-purified antiserum labeled a single band of 43 kDa (Fig. 2A), consistent with the predicted size of Samp1. In immunofluorescence microscopy, the antiserum specifically labeled the nuclear rim of HeLa cells (Fig. 2B). Overexpressed YFP-Samp1 distributed in a similar pattern (Fig. 2C). The results suggest that Samp1 is specifically located in the NE. Since post-transcriptional silencing using siRNAs directed against Samp1 resulted in disappearance of the 43 kDa antigen from the cell lysates (Fig. 2A), as well as disappearance of immunolabeling of the NE (Fig. 2B), we conclude that the antiserum is specific for Samp1.

To experimentally determine whether Samp1 was an integral membrane protein as predicted, we extracted crude nuclei from HeLa cells with 7 M urea or buffers containing Triton X-100 and different salt concentrations. Similarly to the transmembrane INM protein emerin, Samp1 was partially solubilized by extraction with Triton X-100 in high-salt buffer, but resisted Triton X-100 in low-salt buffer or 7M urea (Fig. 2D), strongly suggesting that Samp1 is an integral membrane protein.

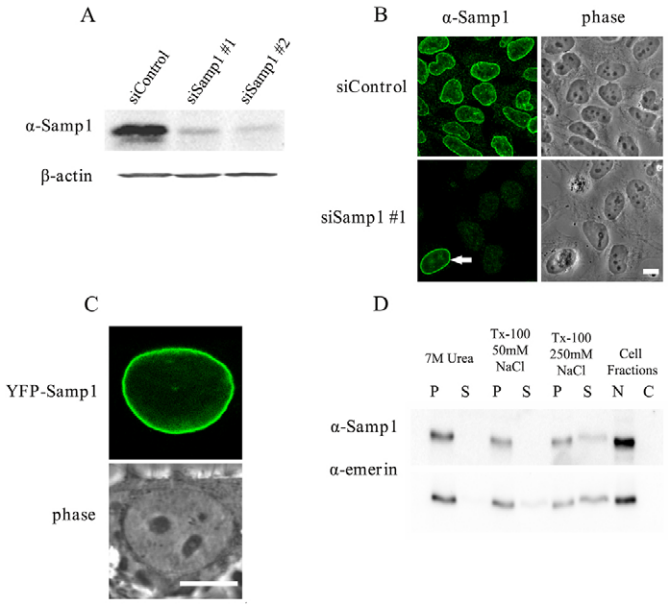

Fig. 2. Samp1 is an integral membrane protein of the nuclear envelope. (A) Western blot analysis of total proteins of HeLa cells treated for 96 hours with either unspecific control siRNA or siRNA #1 or siRNA #2 directed against Samp1. The anti-Samp1 antibodies recognized a major band with an apparent molecular mass of 43 kDa, which was almost completely reduced by siRNA-mediated silencing of Samp1. β-actin was used as loading control. (B) Immunofluorescence micrographs of HeLa cells labeled with anti-Samp1 antibodies and corresponding phase-contrast images. The cells were treated for 96 hours with either unspecific control siRNA or specific siRNA #1 specific for Samp1. Control cells displayed an intense and specific labeling along the nuclear rim. Cells treated with siRNA #1 were mixed with untreated HeLa cells before fixation (arrow) to illustrate the dramatic reduction in nuclear rim staining. Scale bar: 10 μm. (C) Fluorescence micrograph and corresponding phase-contrast image of a HeLa cell expressing YFP-Samp1. (D) HeLa cell nuclei were extracted with 7 M urea, Triton X-100 and 50 mM NaCl or Triton X-100 and 250 mM NaCl as indicated and centrifuged for 20 minutes at 100,000 × ***g***. The upper panel shows a western blot of SDS-PAGE-separated pellets (P) and supernatants (S) probed with anti-Samp1 antibody. Untreated nuclear (N) and cytoplasmic (C) fractions were also loaded. The bottom panel shows an image of the same blot stripped and reprobed with an antibody specific for emerin. Scale bar: 10 μm.

Samp1 is an inner nuclear transmembrane protein

To determine whether Samp1 is present on the ONM, INM, or both, we performed immunoelectron microscopy. The affinity-purified anti-peptide antibodies specific for Samp1 decorated the NE in Hs27 cells (Fig. 3A). Almost all (25 out of 27) gold particles were found at, or closer than 50 nm to, the INM, suggesting that the C-terminus of Samp1 is specifically located on the nucleoplasmic side of the INM. To confirm the immuno-EM study, we used immunofluorescence microscopy to study antibody accessibility in semi-intact HeLa cells. Cells were treated with low concentrations

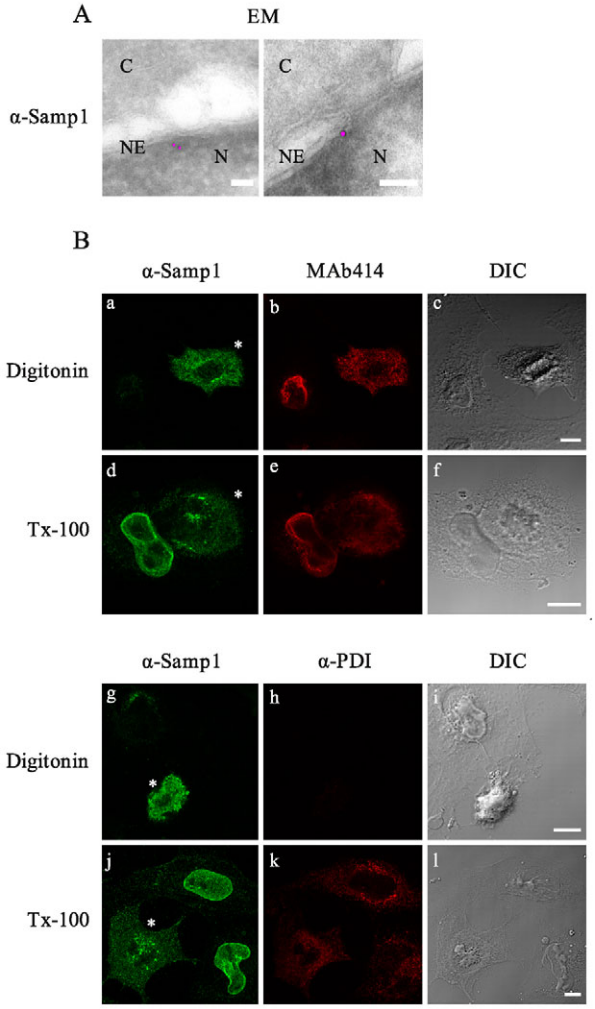

Fig. 3. Samp1 is specifically located at the INM in interphase. (A) Immunoelectron microscopy images showing immunogold particles decorating the nuclear envelope preferentially on the nucleoplasmic side. The immunogold particles are colored magenta. C, cytoplasm; N, nucleoplasm; NE, nuclear envelope. Scale bars: 50 nm. (B) Epitope accessibility assay. Cells treated with either digitonin (a-c,g-i) or Triton X-100 (d-f,j-l) were stained with α-Samp1 (a,d) and mAb414 antibodies (b,e), or α-Samp1 (g,j) and α-PDI antibodies (h,k). Each row shows a mitotic (*) and interphase cells. Note the positive staining of Samp1 in mitotic, but not in interphase cells permeabilized with digitonin (b,h). Anti-PDI antibodies were used as a control for intact intracellular membranes in mitotic or interphase cells after digitonin permeabilization. Corresponding DIC images are shown (c,f,i,l). Scale bars: 10 μm.

of digitonin, which selectively permeabilizes the plasma membrane but leaves intracellular membrane intact (Gerace et al., 1982). The C-terminus of Samp1 was not accessible in interphase cells treated with digitonin, because antibodies cannot pass through the nuclear envelope. However, in mitotic cells, when INM proteins diffuse out in the ER, the C-terminal epitope was accessible to antibodies after digitonin treatment (Fig. 3B). The results are in agreement with the immuno-EM data and demonstrate that Samp1 is specifically located in the INM. The results also support the predicted membrane topology with the C-terminus exposed to the nucleoplasm.

Samp1 displays an unexpected distribution to the polar areas of the mitotic spindle

We investigated the distribution of endogenous Samp1 during the cell cycle. HeLa cells in different stages were fixed and stained using anti-Samp1 antibodies and antibodies specific to pericentrin to visualize centrosomes. Like many other nuclear membrane proteins, a fraction of Samp1 concentrated in an area of the NE around centrosomes in prophase and prometaphase when centrosomes move laterally over the NE to initiate nuclear envelope breakdown (NEBD) (Fig. 4a-d,e-h). In metaphase, the major part of Samp1 localized to the peripheral ER. However, a significant fraction remained concentrated around the spindle poles (Fig. 4i-l). This observation is surprising because INM proteins are normally displaced from the spindle area when the mitotic spindle assembles (Ellenberg et al., 1997). This peculiar localization pattern also occurred in anaphase (Fig. 4q-t). The fact that HeLa cells expressing YFP-Samp1 also displayed intense fluorescence in the polar regions of the mitotic spindle, confirms the immunostaining with anti-Samp1 antibodies (Fig. 4m-p). In telophase (Fig. 4u-x), accumulation of Samp1 could be detected in the reforming NE. Furthermore, we performed a time-lapse series to follow the redistribution of YFP-Samp1 during mitosis in live cells. In metaphase (Fig. 5a-b), YFP fluorescence was clearly detectable in the polar regions of the mitotic spindle and remained concentrated at the poles in anaphase A and B (Fig. 5c-f). In late telophase (Fig. 5i-j), YFP-Samp1 started to concentrate in the nuclear membrane surrounding the daughter nuclei. At this stage, the major portion of

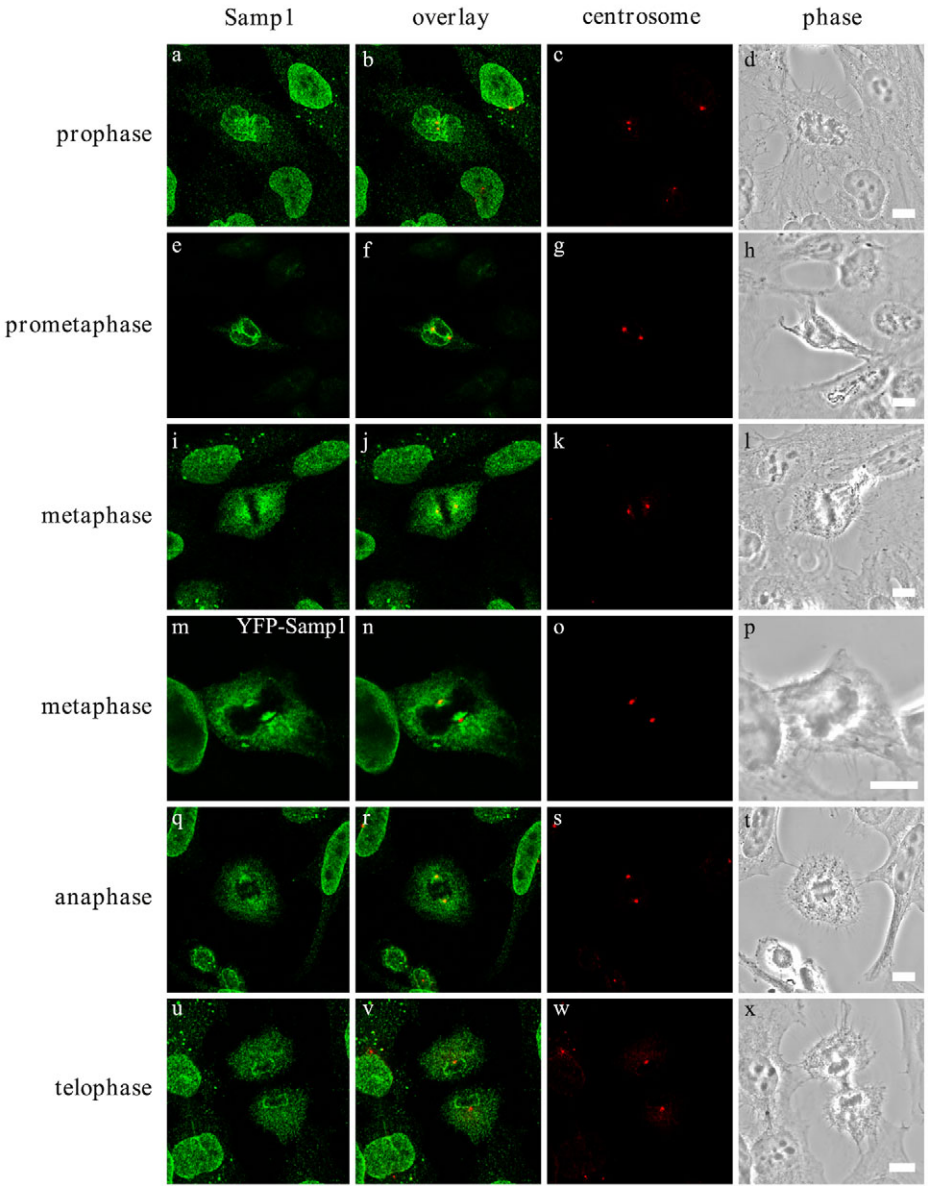

Fig. 4. Distribution of Samp1 in cells at different stages of the cell cycle. Immunofluorescence micrographs of fixed HeLa cells, double-labeled with anti-Samp1 (a,e,i,q,u) and anti-pericentrin antibodies (c,g,k,s,w) as a marker for the centrosomes. Corresponding overlays (b,f,j,r,v) and phase-contrast (d,h,l,t,x) images are shown. Images in m-p show a cell in metaphase expressing YFP-Samp1 and immunostained with anti-pericentrin. Samp1 accumulated close to centrosomes in prophase and prometaphase. In metaphase, a subfraction of Samp1 and YFP-Samp1 distributed in a subcentrosomal area close to the spindle poles. In telophase, Samp1 was recruited to the membrane of reforming nuclei. Scale bars: 10 μm.

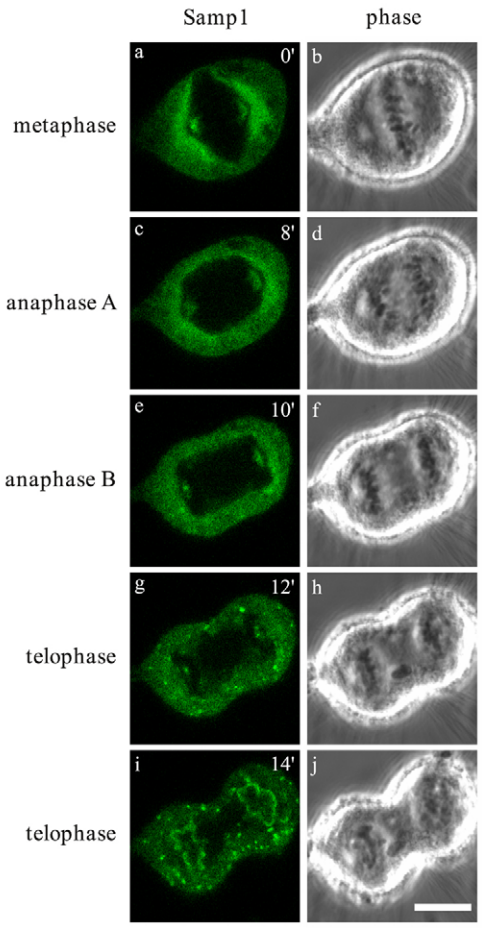

Fig. 5. A subfraction of YFP-Samp1 localizes to the polar regions of the mitotic spindle in live cells. A time-lapse series of a mitotic HeLa cell expressing YFP-Samp1. Selected fluorescence and corresponding phase-contrast images are shown. In metaphase and anaphase (a-f, 0-10 minutes), a subfraction of YFP-Samp1displayed intense fluorescence at the polar regions of the mitotic spindle. In late telophase (i-j, 14 minutes), YFP-Samp1 was recruited back to the NE surrounding the two daughter nuclei. Time in minutes from start is denoted in the upper right corner. Scale bar: 10 µm.

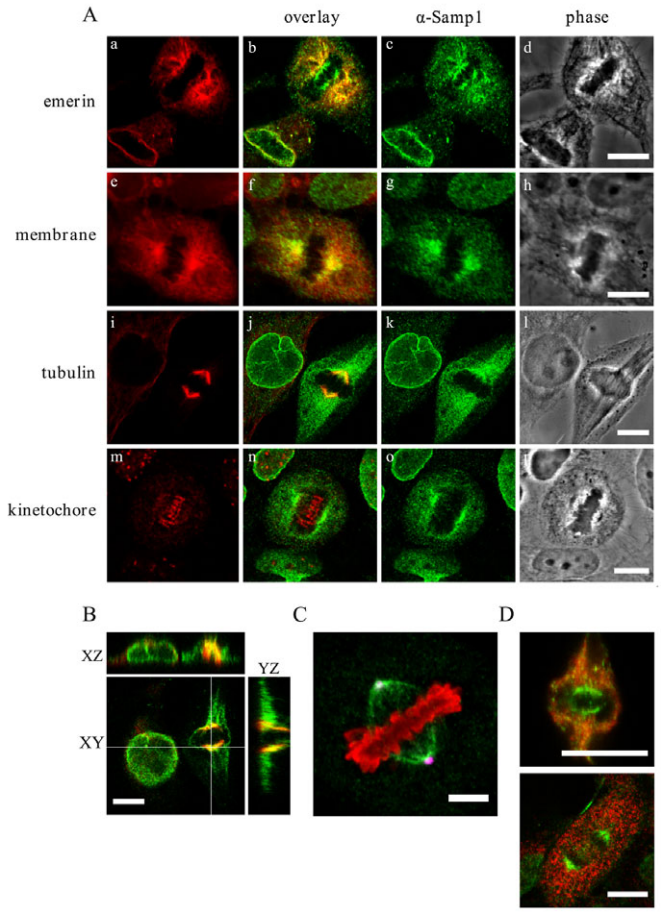

Fig. 6. Characterization of a membrane domain associated with the mitotic spindle. (A) Fluorescence micrographs and corresponding phase-contrast images of HeLa cells double-labeled with anti-Samp1 and anti-emerin antibodies (a-d), membrane dye $DiOC_6$ (e-h), anti-tubulin (i-l) or anti-CENPA (m-p). Note the colocalization of Samp1 and tubulin in the polar part of the spindle, devoid of emerin. (B) 3D projection of a *z*-series of confocal images of a HeLa cell in metaphase double-labeled with anti-Samp1 (green) and anti-tubulin (red) antibodies. (C) Multicolor fluorescence image of a metaphase HeLa cell treated with Samp1-targeted siRNA labeled with antibodies against Samp1 (green), phosphohistone 3 (red) and pericentrin (magenta). (D) Top, fluorescence micrograph of a neuroblastoma cell in metaphase double-labeled with antibodies against Samp1 (green) and emerin (red). Bottom, fluorescence micrograph of a MDCK cell in anaphase double-labeled with anti-Samp1 (green) and anti-protein disulfide isomerase (red) antibodies. Scale bars: 10 µm.

Samp1 still remained in the ER, indicating that Samp1 is recruited at a rather late stage in postmitotic nuclear envelope formation.

We decided to analyze the unusual distribution of Samp1 in further detail. It has previously been shown that a fraction of the integral INM protein emerin localizes in the vicinity of centrosomes during mitosis (Dabauvalle et al., 1999). We therefore wanted to compare the localization of emerin and Samp1 in mitotic cells, to see whether they are part of the same structure in metaphase. Samp1 colocalized with emerin in the ER and close to the centrosomes, but in addition, also occupied the subcentrosomal area between spindle poles closer to the center of the cell (Fig. 6Aa-d). Emerin was completely excluded from this zone. However, the area did contain membranes, as shown by colocalization with $DiOC_6$, which is a marker for intracellular membranes (Terasaki, 1989; Yang et al., 1997) (Fig. 6Ae-h). The subcentrosomal population of Samp1 also colocalized with tubulin in the polar region of the spindle (Fig. 6Ai-l,B) but not with anti-CENPA staining of kinetochores (Fig. 6Am-p). Therefore, Samp1-containing membranes are specifically associated with the mitotic spindle and constitute a specific subdomain differing in protein composition form bulk mitotic ER membranes. Strikingly, after eliminating the majority of Samp1 by post-trancriptional silencing, the remaining Samp1 molecules specifically distributed in the spindle-associated membrane domain rather than in the ER (Fig. 6C). The apparent affinity of Samp1 for this membrane domain suggests that Samp1 might have an important function in processes associated with the mitotic spindle. Samp1 also displayed a similar distribution in the polar regions of the spindle (Fig. 6D) in mitotic neuroblastoma and MDCK cells, showing that the spindle-associated membrane domain is a general phenomenon and not unique for HeLa cells.

We decided to investigate the molecular basis behind the association of Samp1 with the mitotic spindle. Samp1 distribution in the spindle area was evident in cells treated with cytochalasin

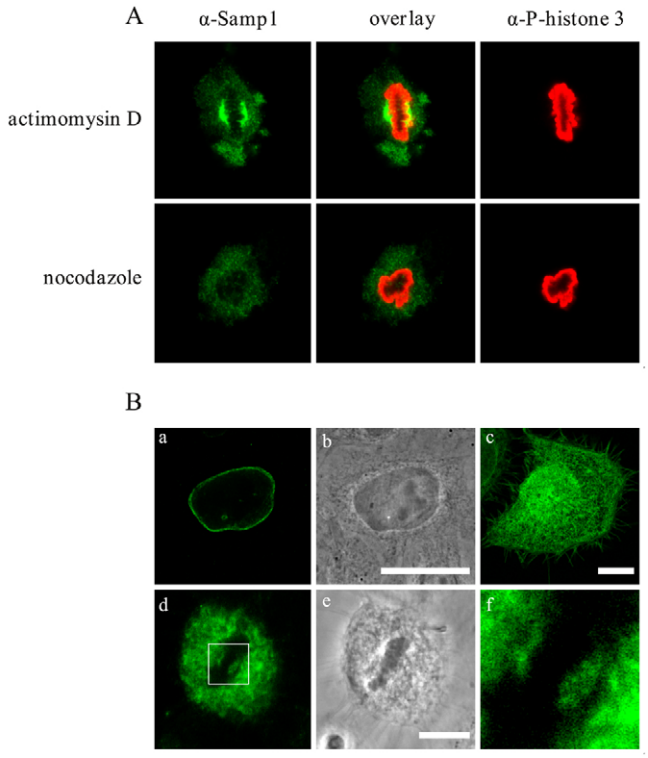

Fig. 7. Spindle association of Samp1 depends on its large conserved loop and the microtubule cytoskeleton. (A) Immunofluorescence micrographs of mitotic HeLa cells treated with cytochalasin D or nocodazole, as indicated, and double-labeled with anti-Samp1 (green) and anti-phosphohistone 3 (red) antibodies. (B) HeLa cells expressing chimeric CD8-Samp1 (a,b,d,e) or CD8-GFP (c) immunostained using anti-CD8 antibodies. In interphase, CD8-Samp1 specifically distributed in the nuclear rim (a) whereas CD8-GFP distributed in cytoplasmic membranes and in the plasma membrane (c). In metaphase, CD8-Samp1 distributed in the ER and in the mitotic spindle area (framed in d) is shown enlarged and enhanced in f. Scale bars: 10 µm.

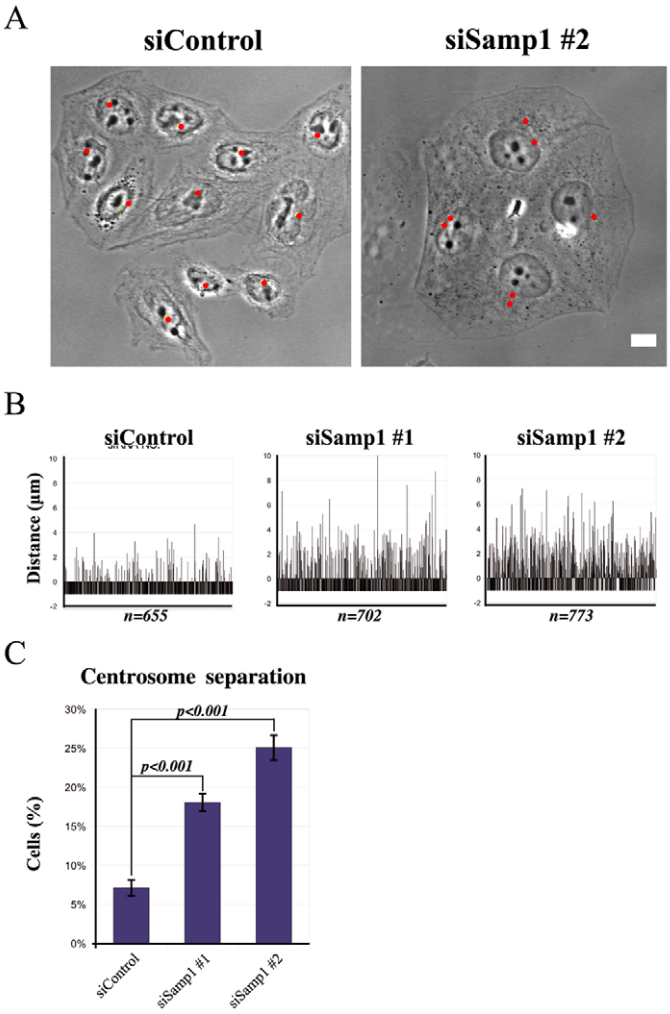

Fig. 8. Separation of centrosomes from the nuclear envelope after post-trancriptional silencing of Samp1 expression. (A) Immunofluorescence using anti-pericentrin antibodies (red) and phase-contrast overlay images of HeLa cells treated with control or Samp1-directed siRNA for 96 hours (as indicated). Note the increased distance between centrosomes (marked by red dots) and the NE. Scale bar: 10 µm. (B) Measurement of centrosome-NE distance (*y*-axis) in individual cells. Centrosomes located above or below the nuclear equator were given the value –1; *n*, number of cells. (C) Mean percentage of cells (± s.d.) with a centrosome-NE distance >1.24 µm. Statistical significance (*P*) was calculated using the two-proportion z-test.

D, but not those treated with nocodazole (Fig. 7A), suggesting that location in the spindle area depends on microtubules, but not on the actin cytoskeleton. To define the sorting determinants of Samp1, we designed a fusion protein between the plasma membrane protein CD8 and Samp1. The resulting CD8-Samp1 fusion protein containing the N-terminus and transmembrane segment of CD8 (residues 1-204) and the conserved large loop of Samp1 (residues 35-210) specifically located to the nuclear rim in interphase (Fig. 7Ba). In mitotic cells, the CD8-Samp1 fusion protein distributed in the ER and in the spindle-associated membrane domain (Fig. 7Bd,f), just like endogenous Samp1, showing that amino acids 35-210 of the loop of Samp1 are sufficient for targeting to the INM, as well as spindle association.

A functional connection between Samp1 and centrosomes

To determine any role that Samp1 might have in the cell, we studied phenotypic changes in cells after post-transcriptional silencing of Samp1 expression using siRNA. The most obvious effect was an increased distance of centrosomes from the NE in interphase (Fig. 8A). In control cells, the mean centrosome-NE distance was 0.62 µm measured in the equatorial plane of the nucleus. Only 7% of control cells displayed a centrosome-NE distance of >1.24 µm (twice the mean distance) (Fig. 8C). After silencing of Samp1 expression using two independent Samp1-directed siRNA molecules, the fractions of cells with a centrosome-NE distance >1.24 µm were 18% and 25%, respectively (Fig. 8C). The significant increase in centrosome-NE distance indicates a functional connection between Samp1 and centrosomes.

Discussion

We have characterized a conserved protein of the nuclear envelope in human cells, termed Samp1. Samp1 is homologous to Ima1, the recently characterized INM protein from *S. pombe* (King et al., 2008). Consistently, we show that Samp1 is a membrane-spanning protein specifically located in the INM in human cells. During mitosis, most Samp1 is dispersed in the ER, as expected. Surprisingly, a fraction of Samp1 concentrated to the polar regions of the mitotic spindle, colocalizing with tubulin and lipid membrane marker. At the same time, these spindle-associated membranes lacked another INM protein, emerin, previously shown to enrich

in regions adjacent to mitotic spindle (Dabauvalle et al., 1999). The ER marker protein disulfide isomerase was also excluded from this membrane domain. These results demonstrate the existence of a distinct membrane subdomain associated with the mitotic spindle, which has a unique protein composition manifested by Samp1. This peculiar distribution of Samp1 occurred in metaphase and anaphase; however, in telophase, Samp1 was recruited back to the reforming NEs of the daughter nuclei.

Our discovery of a new distinct membrane-containing domain in the vicinity of the spindle appears to be similar to the fibrillar-granular matrix containing lamin B3 that was observed submerging the mitotic spindles assembled in vitro on protein kinase Aurora A beads incubated with *Xenopus* extracts (Tsai et al., 2006). However, the spindles assembled in vitro were more elongated. A specific distribution of lamin B in the spindle area of mammalian cells has not yet been reported.

Several studies have highlighted the need for a spindle matrix to explain the mechanism behind force generation during chromosome segregation (and for recruitment of motor proteins) and the field was recently reviewed by Johansen and Johansen (Johansen and Johansen, 2007). A matrix-like structure located in the central spindle area has been observed in syncytial *Drosophila* embryos. This structure has been shown to contain four proteins: skeletor, chromator, EAST and megator (Qi et al., 2005; Qi et al., 2004; Rath et al., 2004; Walker et al., 2000). Except for megator, which is homologous to Tpr, a vertebrate NPC protein, none of the others appears to have homologues in higher eukaryotes. In addition, an integral membrane protein of the ER, Axs, was reported to have an important role in meiotic spindle formation. However, Axs appeared to be absent from the internal spindle area, but rather distributed in a sheath-like layer enclosing the meiotic spindle in *Drosophila* (Kramer and Hawley, 2003). Recently titin, a gigantic elastic protein normally involved in muscle contraction was identified in the spindle area of *Caenorhabditis elegans* cells (Zastrow et al., 2006) and in insect spermatocytes (Fabian et al., 2007).

Components of the nuclear envelope have previously been identified in the mitotic spindle and even assigned important roles in spindle formation and function (Belgareh et al., 2001; Harel and Forbes, 2004; Joseph et al., 2004; Orjalo et al., 2006). Some components of the NPC, for example RanBP2 and Nup107, are involved in assembly of the NPC and in nucleocytoplasmic transport in interphase. However, during mitosis, a fraction of these proteins distributes along MTs and at kinetochores, where they have fundamentally important roles in spindle assembly and function. Our data highlight the presence of an integral membrane protein from the NE in the spindle area.

The observed separation of centrosomes from the nuclear envelope after silencing of Samp1 expression is especially interesting in light of the recently published paper on the homologous protein of Samp1 in *S. pombe* Ima1 (King et al., 2008). In fission yeast, the NE does not disassemble during mitosis, and the spindle pole body constitutes the MTOC. Ima1 binds to heterochromatic regions of the centromeres at a specialized region of the INM in the vicinity of the spindle pole body called MAS (MTOC-attached site). In this way, Ima1 is part of an interface between chromatin and the microtubule cytoskeleton at the spindle pole body. During interphase of mammalian cells, the centrosome (the functional equivalent of the spindle pole body) and its associated microtubules are inaccessible to the nuclear compartment. As it is situated in the INM, Samp1 has potential access to heterochromatin, but any interactions with centrosomes and/or microtubules would have to be indirect. It is tempting to speculate that human Samp1 (in analogy with Ima1) could be involved in a mechanosensory transduction network between the cytoskeleton and the nuclear interior. Only after NE breakdown, which takes place in prometaphase in human cells, would Samp1 be allowed to associate more directly with microtubules of the mitotic spindle.

Materials and Methods

Computer-assisted analysis

The phylogenetic relationship between Samp1 homologs from eukaryotic species was derived by aligning the conserved nucleoplasmic loop of Samp1 using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/). Membrane topology was predicted using TMHMM 2.0 software (http://www.cbs.dtu.dk/services/TMHMM/).

Antibodies

Anti-Samp1 antibodies were raised in rabbit (Agrisera, Vännäs, Sweden) against a 13 amino acid peptide corresponding to residues 379-391 (CGPRRFRPRRSEKQ) (Innovagen, Lund, Sweden). Antisera were affinity-purified against the antigenic peptide immobilized to Sulfolink beads by using standard protocols (Pierce, Rockford, IL). Primary antibodies used for immunofluorescence are as follows. Samp1, produced as described above, dilution 1:6000; Pericentrin, rabbit polyclonal from Abcam (ab4448), dilution 1:1000; Phospho-Histone H3 (Ser10), mouse monoclonal from Cell Signaling (no. 9706), dilution 1:150; Tubulin, mouse monoclonal from Santa Cruz Biotechnology (sc-8035), dilution 1:50; Emerin, mouse monoclonal from Santa Cruz Biotechnology (sc-25284), dilution 1:500; GFP, rabbit polyclonal from Invitrogen, dilution 1:1000; CENPA, mouse monoclonal from abcam (ab13939), dilution 1:500; PDI (phospho disulfide isomerase), mouse monoclonal from BioSite (SPA-891), dilution 1:1000; Nups mAb414, mouse monoclonal from BioSite (MMS-120P), dilution 1:5000; CD8 antibodies, mouse monoclonal MCA1226 (Serotec) was used at 1:100 dilution. As secondary antibodies for immunofluorescence we used Alexa Fluor 488-, Alexa Fluor 568- and Alexa Fluor 647-labeled donkey anti-mouse and anti-rabbit antibodies, diluted 1:5000 (Invitrogen). Primary antibodies for western blot were Samp1, which was produced as described above, dilution 1:6000; β-actin, mouse monoclonal from Sigma, dilution 1:5000; Emerin, mouse monoclonal from Santa Cruz Biotechnology (sc-25284), dilution 1:500. As secondary antibodies for western blot we used horseradish peroxidase (HRP)-coupled anti-mouse or anti-rabbit antibodies from donkey (GE Healthcare, Chalfont St Giles, UK) diluted 1:2000.

Western blot

Cells were scraped and pelleted by centrifugation (1200 × ***g***), resuspended in sample buffer, boiled for 5 minutes and sonicated. Equal amounts of cellular protein were loaded on a 10% SDS-polyacrylamide gel. SDS-PAGE separated proteins were transferred onto a nitrocellulose filter, blocked with 5% milk in PBS-T, incubated with primary antibodies for 1 hour, washed four times for 15 minutes with blocking buffer and then incubated with secondary antibodies for 1 hour. After additional washing in PBS-T, filters were subjected to ECL detection (GE Healthcare) and analyzed using the Luminescent Image Analyzer, LAS1000plus system.

Extraction

Crude nuclear preparations were obtained by incubating cells in ice-cold hypotonic buffer (10 mM HEPES pH 7.4, 5 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT) and homogenizing with a tight-fitting pestle. The soluble cytoplasmic fraction was separated from the insoluble nuclear fraction by centrifugation at 2000 × ***g*** for 20 minutes at 4°C. Nuclear fractions were extracted in buffer supplemented with 1% Triton X-100, 50 mM NaCl, 250 mM NaCl or 7 M urea or combinations of NaCl and Triton X-100. Extraction samples were centrifuged at 100,000 × ***g*** for 20 minutes. Analysis of supernatant and pellet fractions were performed by western blot. The INM protein emerin was used as a control.

Immunoelectron microscopy

Cells were fixed in 3% paraformaldehyde in 0.1 M phosphate buffer, washed and centrifuged to a pellet and embedded in 10% gelatin. Samples were then infiltrated into 2.3 M of sucrose and frozen in liquid nitrogen. Sectioning was performed at –95°C. Immunolabeling procedure was performed as follows: grids were placed directly on drops of 0.15 M NaCl containing 20 mM glycine followed by incubation in 2% BSA (Sigma fraction V) and 2% gelatin (IGSS quality, Amersham Biosciences, Amersham, UK) in 0.1 M phosphate buffer to block non-specific binding. Sections were then incubated with the primary antibody diluted 1:200 in 0.1 M of phosphate buffer containing 0.1% BSA and 0.1% gelatin overnight in a humidified chamber at room temperature. The sections were thoroughly washed in the same buffer and bound antibodies were detected with protein A coated with 10 nm gold (Biocell, BB International, Cardiff, UK) at a final dilution of 1:50. Sections were rinsed in buffer and fixed in 2% glutaraldehyde and contrasted with 0.1% uranyl acetate and embedded in 2% methylcellulose and examined in a Tecnai 10 (FEI company, Eindhoven, The Netherlands) at 80 kV. Digital images were taken by a Megaview III camera (Soft Imaging System, Münster, Germany) (Qinyang et al., 2004).

Cloning

To fuse the genes encoding Samp1 and YFP, the cDNA encoding full-length Samp1 (IMAGE clone ID: 30357476, ATCC-10699381) was used as a template for amplification. The forward primers 5′-CTCTAAGCTTATGGAGGGAGTGAG-CGCGCTGCTGGC-3′ and 5′-CTCTAAGCTTTGGAGGGAGTGAGCGCGCT-GCTGGC-3′ containing a *Hin*dIII restriction site, and reverse primers 5′-TCCGGATCCGATGGCTGCTTCTCTGACCTTCGGGGGCC-3′ and 5′-TCCGG-ATCCTTATGGCTGCTTCTCTGACCTTCGGGGCC-3′ containing a *Bam*HI restriction site. After digestion with *Hin*dIII and *Bam*HI (Fermentas), the fragments were ligated into pEYFP-N1 and pEYFP-C1 (BD Biosciences), respectively.

Cell culture, plasmid transfection and siRNA transfection

HeLa (ATCC no. CCL-2), Hs27 (CRL-1634), MDCK (CCL-34) and neuroblastoma SHSY-5Y (CRL-2266) cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. For transiently expression of YFP-Samp1, the FuGENE® HD Transfection Reagent was used (Roche). For knockdown by RNAi, HeLa cells were transfected with a final concentration of 12 nM siRNAs [Ambion, sense sequences: #1 Ambion IDnr 284971 (5′-GGAAGUGUUGACAGUGUGAtt-3′), #2 (5′-GCGGCUGUGGAGUACUACAtt-3′)] using HiPerfect (Qiagen). For control experiments, scrambled siRNA (Invitrogen) was used.

Immunofluorescence

For immunofluorescence, cells were grown on glass coverslips, carefully washed once in PBS, fixed on ice in 3.7% paraformaldehyde in PBS for 20 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. Cells were then blocked in PBS containing 0.1% Tween-20 (PBS-T) and 2% BSA. The samples were then incubated for 1 hour with primary antibodies in blocking buffer, washed with blocking buffer three times, and incubated with secondary antibodies in blocking buffer for 1 hour. After additional washes in PBS-T, the coverslips were mounted in Fluoromount-G (SouthernBiotech). For membrane staining, cells were incubated in PBS containing 10 μg/ml $DiOC_6$ for 10 seconds and then washed twice before mounting. For mitotic digitonin INM localization determination assay, cells grown on coverslips were selectively permeabilized using digitonin (40 μg/ml in TB; 20 mM HEPES, 110 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT, and 1 mM EDTA at pH 7.3) for 5 minutes on ice, fixed and stained as above, omitting permeabilization and Tween-20.

Confocal laser-scanning microscopy (CLSM)

Imaging was performed on a Leica TCS-SP laser scanning confocal microscopy (Leica, Heidelberg, Germany) with a 63× 1.32 NA oil-immersion objective using a 488 nm 20 mW Ar laser line, a solid-state 561 nm, 10 mW laser and a 633 nm, 10 mW He/Ne laser line. Emission spectra were collected between 500-550 nm (Alexa Fluor 488 and YFP), 570-650 nm (Alexa Fluor 568) and 645-700 nm (Alexa Fluor 647). The laser lines scanned sequentially. Images were processed using ImageJ to linearly adjust 8-bit intensity range and merged channels.

Measurements of centrosome to nuclear envelope distance

The distance between centrosomes and the nuclear envelope were measured on phase-contrast overlays on confocal immunofluorescence images of siRNA-treated cells stained with anti-pericentrin. Only interphase cells that appeared normal were analyzed. Centrosomes were classified as ‘separated’ if the centrosome-NE distance exceeded 1.24 μm and as ‘associated’ if the centrosome-NE distance was 1.24 μm or less. Differences in the percentage of cells with separated and associated centrosomes were evaluated using the two-proportion *z*-test.

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