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Nuclear envelope localization of human UNC84A does not require nuclear lamins

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Abstract The SUN proteins are a conserved family of proteins in eukaryotes. Human UNC84A (Sun1) is a homolog of *Caenorhabditis elegans* UNC-84, a protein involved in nuclear anchorage and migration. We have analyzed targeting of UNC84A to the nuclear envelope (NE) and show that the N-terminal 300 amino acids are crucial for efficient NE localization of UNC84A whereas the conserved C-terminal SUN domain is not required. Furthermore, we demonstrate by combining RNA interference with immunofluorescence and fluorescence recovery after photobleaching analysis that localization and anchoring of UNC84A is not dependent on the lamin proteins, in contrast to what had been observed for *C. elegans* UNC-84.

Keywords: SUN domain; Nuclear envelope; Lamin; Inner nuclear membrane; UNC-84; SPAG4

1. Introduction

An important feature of many eukaryotic cells is the capability to divide asymmetrically – a prerequisite for the generation of differentiated tissues. Nuclear migration and anchoring are important for asymmetric cell division. Both processes together help to position the nucleus at its appropriate location in the cell. This is established through complex linkage mechanisms connecting the nuclear envelope (NE) to the actin and microtubule cytoskeleton (reviewed in [1]).

Nuclear migration has been studied extensively in *C. elegans*. UNC-84 (uncoordinated) is an inner nuclear membrane (INM) protein shown to be involved in nuclear migration [2,3]. It consists of 1111 amino acids and contains one transmembrane domain located approximately in the middle of the protein. According to current models, *C. elegans* UNC-84 links the nucleus to the cytoskeleton by an interaction with UNC-83, a protein suggested to reside in the outer nuclear membrane (ONM) [4]. This interaction is proposed to be mediated by UNC-84's conserved C-terminal SUN domain (for *S*ad1p, *UN*C-84 homology) [4]. In the nucleus, UNC-84 is connected directly or indirectly to the nuclear lamina, since UNC-84 localization has been shown to be lamin-dependent in worms [5].

Four human proteins sharing UNC-84's conserved SUN domain can be identified by database searches, namely UNC84A, UNC84B, sperm associated antigen (SPAG4) and MGC33329. The homology of the four proteins is most prominent in their C-terminal SUN domain. It is, however, unclear at present what the functions of these proteins (including their SUN domains) are. A very recent study shows that UNC84A (Sun1) is localized at the NE and is important for the anchorage of Nesprin-2, a NE-associated spectrin-repeat protein [6]. Human UNC84B (Sun2) has been described as a type II transmembrane protein residing at the INM [7]. The third human SUN domain protein, SPAG4 has originally been identified as a sperm specific protein but is also expressed in a wide range of neoplastic tissues [8,9]. MGC33329 has not yet been characterized.

The mechanism of targeting membrane proteins to the INM is an area of active research (e.g. [10]). A model explaining the targeting of proteins to the INM is the "Diffusion–Retention" model. It suggests that proteins destined for the INM can freely diffuse from the endoplasmic reticulum (ER) via the nuclear pore membrane into the INM [11,12]. Whether or not these proteins are retained in the INM depends on their ability to engage into stable interactions with the nuclear lamina, chromatin, or both. Interestingly, no INM-specific signal sequences seem to be required for INM localization. Experimental evidence for the "Diffusion–Retention" model is derived from fluorescence recovery after photobleaching (FRAP) experiments. These demonstrated a high mobility of INM proteins as long as they reside in the ER, but a significantly decreased mobility as soon as they reach the INM, where they are retained [13,14].

Since function and localization properties of UNC84A (Sun1), the closest human homolog of *C. elegans* UNC84, are unclear, we set out to investigate these. Here we report a detailed analysis of the localization properties of human UNC84A. Moreover, we demonstrate that, in contrast to *C. elegans* UNC-84, localization and anchoring of human UNC84A is not dependent on the presence of lamins.

2. Materials and methods

2.1. Molecular cloning and transient transfection

The coding regions of human UNC84A (Sun1) and SPAG4 were amplified by PCR using HeLa cell cDNA as template. The PCR fragments were cloned into the *Bgl*II–*Eco*RI and *Bam*HI–*Eco*RI sites of pEGFPN3 (Clontech), respectively. Subclones were generated by

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Abbreviations: NE, Nuclear envelope; RNAi, RNA interference; FRAP, fluorescence recovery after photobleaching; INM, inner nuclear membrane; ER, endoplasmic reticulum; GFP, green fluorescent protein; LAP2β, lamina-associated polypeptide 2 beta

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PCR using the full-length clones as template. The green fluorescent protein-lamina-associated polypeptide 2 beta (GFP-LAP2β) clone was a gift of T.A. Rapoport (Boston, USA). HeLa cells were transfected using the FuGene transfection reagent (Roche). 24–48 h after transfection, cells were fixed in 1% paraformaldehyde for 10 min. To visualize GFP fusion proteins, coverslips were washed in PBS and mounted.

2.2. RNA interference, immunofluorescence, antibodies

Stable cell lines expressing UNC84A-GFP and GFP-LAP2β were transfected at 30% confluency with siRNAs specific for lamin A/C and lamin B1 using Oligofectamin (Invitrogen) [15,16]. After 72 h, indirect immunofluorescence was performed essentially as described [17]. The antibodies used were anti-lamin A/C (Novocastra), anti-lamin B1 (Zymed), anti-lamin B2 (LN43, abcam) and anti-mouse Texas-Red (Molecular Probes). The anti-β-tubulin antibody was from Sigma.

2.3. FRAP

Experiments were performed on a LSM510 confocal microscope equipped with an Ar 488 nm laser, a 500–550 nm bandpass filter and a 40×1.3 oil objective (Carl Zeiss). Regions of interest (ROI) were bleached, and subsequent fluorescence recovery was quantified over the ROI using the Zeiss LSM510 software.

3. Results

3.1. UNC84A localizes to the NE independently of its SUN domain

C. elegans UNC-84 as well as human UNC84B (Sun2) have been reported to localize to the NE [3,7]. The localization of human UNC84A was analyzed by expressing a GFP-tagged version in HeLa cells. As expected, the full-length protein (1–917) localized almost exclusively to the nuclear rim (Fig. 1). To address which parts of the coding region of UNC84A contribute to its nuclear rim localization, several deletion constructs were generated, each C-terminally fused to GFP (Fig. 1A). First, we deleted the conserved C-terminal SUN domain. This deletion mutant (1–723) localized exclusively to the nuclear rim, identical to the full-length protein (Fig. 1B).

3.2. Two different domains are required for proper localization of human UNC84A

According to the "Diffusion–Retention" model, INM proteins require interactions with the nuclear lamina or chromatin to be retained at the NE [12]. To identify putative nuclear retention domains within human UNC84A, a more extensive localization analysis of different deletion mutants was performed (Fig. 1B). Extending the C-terminal deletion to amino acid 500 (1–499) did not abolish nuclear rim localization of UNC84A, but the amount of protein found in membranes other than the NE (resembling ER and Golgi compartment) was significantly higher compared to the full-length protein. This finding suggests that the region between amino acids 500 and 723 of UNC84A weakly contributes to the localization of UNC84A to the NE.

Next, we examined the effect of N-terminal deletions. UNC84A-GFP lacking the first 200 amino acids (200–917) displayed nuclear rim localization and, in addition, localized outside the NE, in an ER/Golgi-like pattern, similar to the phenotype of the C-terminal deletion mutant (1–499). Combining this N-terminal deletion with the deletion of the SUN domain (200–723) showed the same phenotype. These findings argue for a second localization domain within the N terminus of UNC84A. The importance of both domains for proper

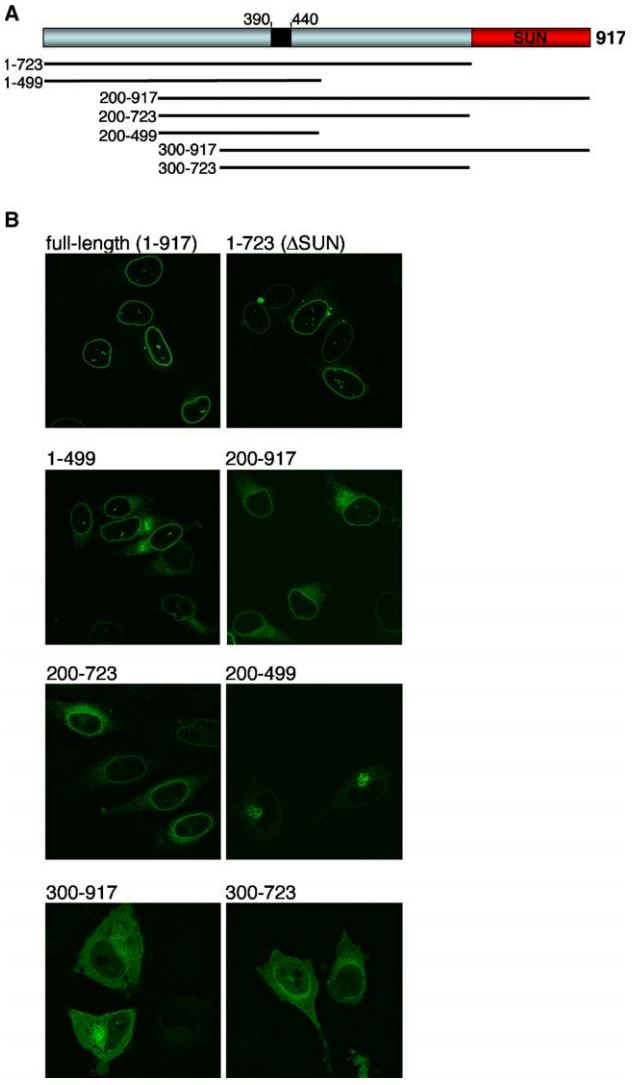

Fig. 1. Distinct regions in the N-terminal and C-terminal domains of human UNC84A contribute to NE localization. (A) Schematic representation of UNC84A depicting its conserved SUN domain (red) and hydrophobic regions comprising the transmembrane segment(s) (black). Black lines indicate the length of deletion constructs. (B) Transient transfection analysis of UNC84A-GFP deletion derivatives in HeLa cells. Pictures were obtained using confocal microscopy. Note that we analyzed the localization of these GFP fusion proteins in cells with both low and high GFP signal and found that localization was not severely altered by expression levels.

UNC84A targeting is underlined by the fact that the combined deletion of both domains (200–499) almost completely impaired NE localization. Instead, most of the protein was found in the Golgi.

The most striking effect on NE targeting was observed when the first 299 amino acids of UNC84A were deleted. This mutant (300–917) showed a very faint rim staining and most of the fusion protein was found in membranes outside the nuclear region, mostly within the ER and even at the plasma membrane. This demonstrates that the N-terminal domain of UNC84A is necessary for NE localization. As expected, additional deletion of the SUN domain (300–723) did not further contribute to mislocalization. Taken together, our data suggest that at least two distinct domains of UNC84A are required for exclusive nuclear rim localization. The N-terminal 299 amino

acids are most important for correct localization whereas a region preceding the SUN domain (amino acids 500–723) in the C-terminal part of the protein exhibits a minor contribution.

3.3. The N terminus of UNC84A can improve NE localization of a SPAG4 mutant

In order to confirm that the N-terminal part of UNC84A is indeed a functional 'retention' domain, we tested if this domain can confer NE targeting to a protein that does not show exclusive NE localization. As a reporter protein we chose SPAG4-GFP, which is localized to the ER and the NE after transient transfection (Fig. 2). When we removed the N-terminal part of SPAG4 (SPAG4 131–437) encompassing the region before the predicted transmembrane segment(s), the truncated SPAG4 protein mainly localized to the ER and only to a minor extent to the NE (Fig. 2B). When the first 300 amino acids of UNC84A were fused in-frame to the N terminus of the SPAG4 deletion mutant, the resulting chimeric protein (UNC84A (1–300)-SPAG4 (131–437)) displayed strong nuclear rim localization and some minor, residual ER localization. This phenotype is comparable to the localization of the UNC84A C-terminal deletion mutant (1–499, Fig. 1B) and demonstrates that the N terminus of UNC84A is sufficient to increase the fraction of SPAG4-GFP that is localized (or retained) at the NE.

3.4. Lamins A/C and B1 are not required for UNC84A anchoring in human cells

In *C. elegans,* UNC-84 localization has been shown to be dependent on the presence of the nuclear lamina, which is composed of one single lamin isoform [5]. The number and complexity of nuclear lamins increased during evolution. Most differentiated mammalian cells contain three major lamins, namely lamins A/C, B1 and B2. Lamins A and C are encoded by a single gene and arise through alternative splicing (reviewed in [18]).

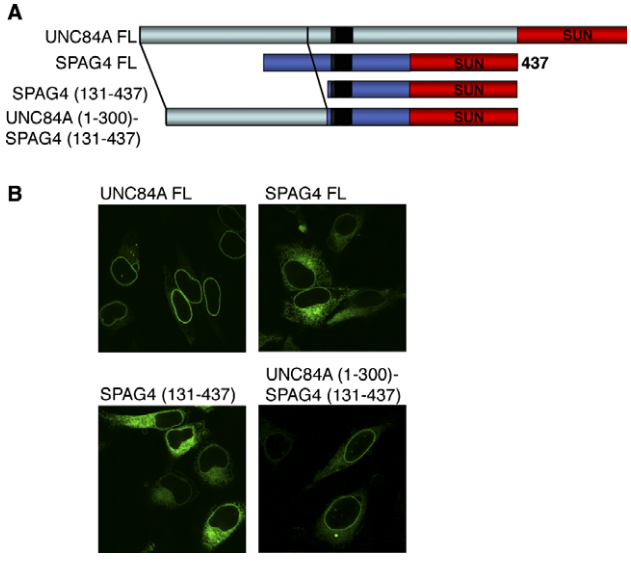

Fig. 2. The N-terminal 300 amino acids of UNC84A improve SPAG4 (131–437) localization to the NE. (A) Schematic representation of UNC84A, SPAG4, SPAG4 N-terminal deletion, and the UNC84A-SPAG4 chimerical protein fused C-terminally to GFP. The conserved SUN domain is indicated in red, putative transmembrane domain(s) in black. (B) Transient transfection analysis of GFP-fusion proteins. Pictures were obtained using confocal microscopy.

To test if lamins are important for the localization of UNC84A to the INM in human cells, the expression of lamins was downregulated by RNA interference (RNAi) using siRNAs specific for lamins A/C and B1. Three days after transfection of the siRNAs, the efficiency of lamin knockdown was examined by Western blotting using lamin A/C, lamin B1 and lamin B2 specific antibodies (Fig. 3A). Downregulation of both lamin A/C and lamin B1 was efficient. Interestingly, we observed that siRNAs specific for lamin B1 also affected lamin B2 levels. Cells transfected with siRNAs to lamin B1 or a mixture of siRNAs to lamins A/C and B1 were virtually devoid of lamin B2.

Having established that lamin depletion was efficient in HeLa cells, we next analyzed the effect of lamin knockdown on UNC84A localization in an UNC84A-GFP stable cell line. Strikingly, no difference in the localization pattern of

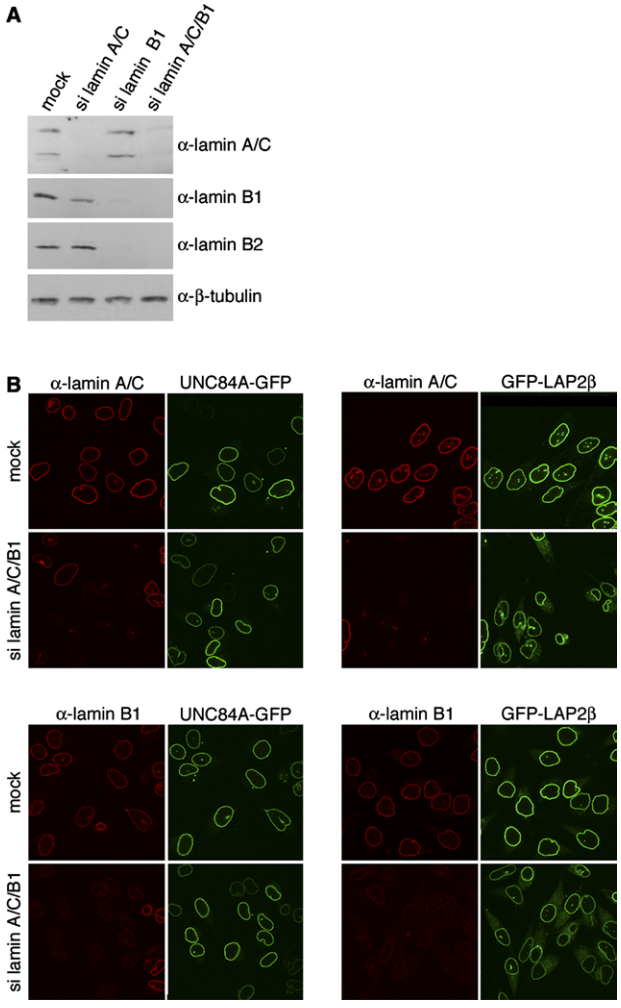

Fig. 3. Lamins are not required for the localization of UNC84A to the NE. (A) Depletion of lamins by RNAi is efficient. HeLa K cells were either mock-treated or transfected with siRNAs to lamin A/C, lamin B1 or a mixture of both. Cells were harvested after 72 h and subjected to Western blot analysis using lamin A/C, lamin B1, lamin B2 and β-tubulin antibodies. (B) Stable HeLa cell lines expressing UNC84A-GFP (green, left panels) or GFP-LAP2β (green, right panels) were either mock-treated or transfected with lamin A/C/B1 specific siRNAs. The downregulation of lamins was controlled by immunofluorescence using lamin A/C (top) or lamin B1 (bottom) specific antibodies and Texas Red labeled secondary antibodies. Pictures were taken by confocal microscopy.

UNC84A (Fig. 3B, left panels, green) was observed in cells treated with lamin A/C/B1-specific siRNAs when compared to control cells. Parallel immunofluorescence analysis using lamin A/C and B1 specific antibodies showed that the lamins had been efficiently depleted upon RNAi (Fig. 3B, left panels, red).

To make sure that the efficacy of lamin RNAi was sufficient to impair INM targeting of a lamin-dependent INM protein, we also analyzed LAP2β in a cell line stably expressing a GFP-LAP2β fusion. LAP2β is a B-type lamin associated protein of the INM and its localization is expected to be lamin-dependent (reviewed in [19]). Upon lamin A/C/B1- specific RNAi, the amount of GFP-LAP2β localized to the NE was reduced and LAP2β was instead also found in the ER network (Fig. 3B, right panels).

Taken together, these results indicate that in human cells lamins appear to be dispensable for UNC84A anchorage at the nuclear rim, while the localization of LAP2β was as expected lamin-dependent.

3.5. UNC84A lateral mobility is low at the nuclear rim

If lamins are indeed not needed for UNC84A localization and anchoring to the nuclear rim, then lamin knockdown by RNAi should not affect UNC84A mobility in the INM. To test this hypothesis, we compared UNC84A mobility in untreated and lamin-depleted cells by FRAP (Fig. 4). The analysis of the FRAP data revealed that UNC84A-GFP stably localized to the nuclear rim in untreated cells (Fig. 4, top panel). Importantly, upon RNAi to lamins, the lateral mobility of UNC84A-GFP was not increased and hence there was no recovery of the GFP signal in the bleached section of the nuclear rim over the time course of the experiment. In contrast, the relative mobility of GFP-LAP2β was significantly higher after knockdown of lamins as compared to the mock sample. Already after 200 s, a significant fraction of GFP-LAP2β had relocalized to the previously bleached region of the NE and, after 700 s, almost 70% of fluorescence recovery of GFP-LAP2β was reached in siRNA-treated samples (Fig. 4, lower

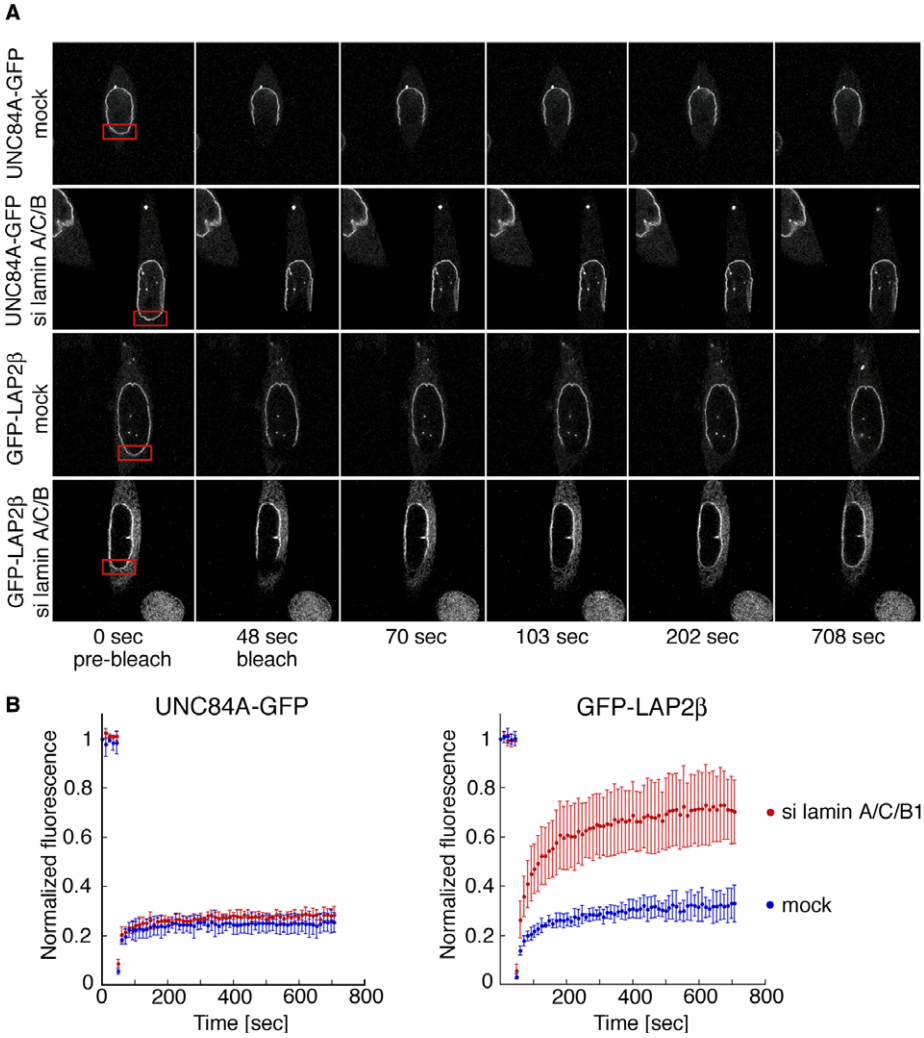

Fig. 4. Mobility of UNC84A is not affected by lamin depletion by RNAi. (A) Mobility of UNC84A-GFP or GFP-LAP2β were analyzed by FRAP. A time series of FRAP in the region of interest (red rectangle) is shown for UNC84A-GFP and GFP-LAP2β stable cell lines. Mock-treated and cells transfected with lamin A/C/B1 specific siRNAs are compared. Pictures were obtained by confocal microscopy. GFP fluorescence levels were equalized using the photomultiplier function. Note that lamins were efficiently depleted in 95% of the cells as analyzed by immunofluorescence. (B) Quantitation of the FRAP data reveals distinct recovery kinetics. At least 3 experiments were performed per day and stable cell line and used to deduce the standard deviation. The corresponding fluorescence loss in photobleaching data served as control for bleaching and moving of cells (not shown).

panel). Together, these findings correlate with the immunofluorescence data (Fig. 3B) and demonstrate that indeed UNC84A anchoring (and retention) does not require the presence of lamin proteins.

4. Discussion

Mouse Unc-84A/Sun1 protein, a potential INM protein with a predicted type II membrane topology, has first been picked up in the proteomic analysis of NE membranes [20]. We have studied human UNC84A. Like its *C. elegans* homolog, human UNC84A localizes to the NE. We identified two distinct domains that contribute to the targeting of UNC84A to the nuclear rim. The first domain lies within the N-terminal part of UNC84A (1–300) whereas the second (500–723), less important localization domain, is located in the C-terminal part, preceding the SUN domain (Fig. 1). Deletion of either of these targeting domains resulted in a partial mislocalization of UNC84A to membranes other than the NE. While our paper was in preparation, a comprehensive study on the Sun1-dependent localization of Nesprin-2 to the NE was published [6]. This study demonstrated the importance of the same two domains of mouse Sun1 for its localization to the NE [6].

The conserved part of all UNC-84 homologs is the SUN domain. The SUN domain of UNC84A, however, is not required for NE localization (Fig. 1 and [6]). Likewise, the SUN domain of UNC84B (Sun2) has recently been shown to be dispensable for UNC84B retention at the INM [7]. Together, these findings support the model that the SUN domain serves as a protein–protein interaction motif which is important for SUN protein function, e.g., linking other partners to the NE, rather than serving as a retention domain. Indeed, two proteins in *C. elegans*, namely UNC-83 and ANC-1 (a Nesprin homolog) [21], have been shown to require the presence of the SUN domain in UNC-84 for their proper localization to the outer nuclear membrane. A common function for the SUN domains of the different SUN family members is not yet known but one could speculate that SUN domains might be generally used for connecting the inner and outer NE by providing a perinuclear tethering device.

In *C. elegans*, proper UNC-84 localization requires the nuclear lamina [5]. In contrast, another *C. elegans* SUN protein, Matefin, localizes to the NE independently of lamin [22]. Recently, mouse Sun1 has also been shown to localize correctly in the absence of lamin A and C [6]. In this latter study, it could, however, not be excluded that lamin B was sufficient for Sun1 retention at the INM [6]. Our data demonstrate that human UNC84A depends neither on lamin A/C nor lamin B1 (and likely lamin B2) for INM retention, since simultaneous knockdown of these lamins by RNAi did not affect UNC84A localization to the NE (Fig. 3). Furthermore, mobility measurements by FRAP analysis confirmed these results (Fig. 4).

The question arises how human UNC84A is retained in the INM if not via the lamin proteins? Three different possibilities seem plausible. First, UNC84A could bind to DNA directly. This would require a DNA binding domain. Indeed, mouse Sun1 contains a Zn-finger motif within its N-terminal domain [6]. Whether or not this Zn-finger binds to DNA has not been addressed so far. Remarkably, it is this N-terminal domain, which was found to be crucial for mouse Sun1 localization [6]. However, even though the overall domain organization of mouse and human UNC84A is similar, human UNC84A does not contain this Zn-finger motif. Therefore, the Zn-finger motif cannot have a conserved role in UNC84A targeting. It needs to be experimentally addressed in future, if the different UNC84A isoforms can bind DNA directly. The second possibility is anchoring via an interaction with chromatin. One example for such an anchoring mechanism is the lamin B receptor (LBR). LBR forms a complex with histones H3/H4 and heterochromatin protein 1 (HP1) [23]. The third possibility is retention of UNC84A by an interaction with another protein of the INM or the NPC. The future characterization of candidate proteins, for instance by RNAi, will hopefully reveal how UNC84A is retained in the NE.

The molecular functions of UNC84A and the other three SUN proteins in mammalian cells still remain to be defined. We have shown that UNC84A is not very mobile in the NE – a useful feature for a component possibly involved in nuclear anchorage and migration.

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