

Requirements for the localization of nesprin-3 at the nuclear envelope and its interaction with plectin

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Summary

The outer nuclear membrane proteins nesprin-1 and nesprin-2 are retained at the nuclear envelope through an interaction of their klarsicht/ANC-1/syne homology (KASH) domain with Sun proteins present at the inner nuclear membrane. We investigated the requirements for the localization of nesprin-3 α at the outer nuclear membrane and show that the mechanism by which its localization is mediated is similar to that reported for the localization of nesprin-1 and nesprin-2: the last four amino acids of the nesprin-3 α KASH domain are essential for its interaction with Sun1 and Sun2. Moreover, deletion of these amino acids or knockdown of the Sun proteins results in a redistribution of nesprin-3 α away from the nuclear envelope and into the endoplasmic reticulum (ER), where it becomes colocalized with the cytoskeletal crosslinker protein plectin. Both nesprin-3 α and plectin can form

dimers, and dimerization of plectin is required for its interaction with nesprin-3 α at the nuclear envelope, which is mediated by its N-terminal actin-binding domain. Additionally, overexpression of the plectin actin-binding domain stabilizes the actin cytoskeleton and prevents the recruitment of endogenous plectin to the nuclear envelope. Our studies support a model in which the actin cytoskeleton influences the binding of plectin dimers to dimers of nesprin-3 α , which in turn are retained at the nuclear envelope through an interaction with Sun proteins.

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Introduction

The eukaryotic cell nucleus is surrounded by a nuclear envelope (NE), a double-layered membrane consisting of an outer nuclear membrane (ONM) and an inner nuclear membrane (INM) separated by a lumen called the periplasmic or perinuclear space (Gerace and Burke, 1988). The INM is characterized by a specific set of proteins, such as the lamin B receptor (LBR) and lamina-associated polypeptide 2 (LAP2) that can interact with lamins and/or chromatin structures (Dreger et al., 2001; Schirmer et al., 2003). By contrast, the ONM is continuous with the rough endoplasmic reticulum (RER) and shares most of its proteins with this compartment. The only proteins thus far identified as residing specifically at the ONM are members of the nesprin protein family (Padmakumar et al., 2004; Wilhelmsen et al., 2005; Zhang et al., 2001; Zhen et al., 2002).

The mammalian nesprin protein family initially comprised two members, nesprin-1 and -2, both of which have multiple isoforms (Padmakumar et al., 2004; Zhang et al., 2005). The full-length isoforms of these proteins are characterized by an N-terminal actin-binding domain (ABD), a series of spectrin repeats and a C-terminal KASH domain (Padmakumar et al., 2004; Zhen et al., 2002). Nesprin-1 and -2 are retained at the ONM by interactions between their KASH domains and Sun proteins present at the INM (Crisp et al., 2006; Haque et al., 2006; Padmakumar et al., 2005). Nesprin-1 and -2 can bind to

both Sun1 and Sun2, and the last four amino acids of the KASH domain (consensus sequence PPPX) are thought to be crucial for this interaction (Crisp et al., 2006; Padmakumar et al., 2005). Moreover, the binding of nesprin-2 to Sun proteins connects the ONM to the INM and assists in the maintenance of the integrity of the NE (Crisp et al., 2006).

The N-termini of nesprin-1 and -2 extend into the cytoplasm and can bind to actin filaments (Padmakumar et al., 2004; Zhen et al., 2002). Hence, nesprin-1 and -2 connect the ONM to the actin cytoskeleton and might play a role in nuclear migration or positioning. Sun1 and Sun2 extend their N-termini into the nucleoplasm, where they can interact with A- and B-type lamins (Crisp et al., 2006). However, the localization of Sun proteins at the NE does not depend on the binding to lamins (Crisp et al., 2006; Haque et al., 2006). The interaction of Sun proteins with both lamins and nesprin-1 and -2 establishes a connection between the cytoplasmic actin cytoskeleton and the nuclear intermediate filament system; this complex is referred to as the LINC complex (linker of nucleoskeleton and cytoskeleton). A similar complex exists in lower organisms and plays an important role in nuclear migration and positioning (McGee et al., 2006; Starr and Han, 2002; Starr and Han, 2003; Starr et al., 2001; Tzur et al., 2006; Wilhelmsen et al., 2006; Zhang et al., 2002).

We recently identified a third member of the nesprin protein family, nesprin-3 (Wilhelmsen et al., 2005). Similar to the other

nesprin proteins, nesprin-3 is characterized by a series of spectrin repeats and a C-terminal KASH domain. The protein is present at the ONM, and the KASH domain is both necessary and sufficient for its localization at that site. Furthermore, nesprin-3 has two isoforms, nesprin-3 α and nesprin-3 β , which differ in their N-termini. Although the structure of nesprin-3 is quite similar to that of nesprin-1 and -2, there are two main differences. Nesprin-3 is much smaller, ~116 kDa, and lacks an N-terminal ABD. The N-terminus of nesprin-3 α , but not that of nesprin-3 β , can interact with the cytoskeletal crosslinker protein plectin (Wilhelmsen et al., 2005).

Plectin is a multi-domain protein of the plakin family that is characterized by an N-terminal ABD, a plakin domain, a coiled-coil rod domain and a C-terminal segment containing plakin repeats (Elliott et al., 1997; McLean et al., 1996; Sonnenberg and Liem, 2007; Wiche, 1998). The protein can interact with intermediate filaments, actin stress fibers and microtubules (Foisner et al., 1988; Nikolic et al., 1996; Sanchez-Aparicio et al., 1997; Seifert et al., 1992) and mediates interactions between these three cytoskeletal systems (Seifert et al., 1992; Svitkina et al., 1996). Furthermore, plectin anchors intermediate filaments at cell-cell and cell-matrix junctional complexes, such as hemidesmosomes, desmosomes and focal contacts (Eger et al., 1997; Hieda et al., 1992; Seifert et al., 1992; Wiche, 1998). In this way, plectin influences the structural organization and integrity of the cytoskeleton and is thought to assist in the mechanical strengthening of cells (Andra et al., 1997; Wiche, 1989).

Plectin has a strong tendency for self-association (Foisner and Wiche, 1987) and forms parallel dimers by means of its coiled-coil rod domain (Foisner et al., 1991; Green et al., 1992; Uitto et al., 1996; Wiche, 1998; Wiche et al., 1991). Higher oligomeric states of plectin are also formed and might involve interactions of its globular end domains (Foisner and Wiche, 1987; Weitzer and Wiche, 1987). Indeed, it has been shown that the ABD of plectin can dimerize and cause the bundling or crosslinking of actin filaments (Fontao et al., 2001). A role for plectin in the regulation of actin dynamics has also been demonstrated: plectin is required for short-term rearrangements of the actin cytoskeleton in response to extracellular stimuli that activate the Rho, Rac and Cdc42 GTPase signaling cascades (Andra et al., 1998).

The interaction of plectin with nesprin-3 α is mediated by the plectin N-terminal ABD (Wilhelmsen et al., 2005). We previously suggested that nesprin-3 α and actin filaments compete for binding to the plectin ABD. This was supported by our observation that disruption of the actin network by latrunculin B led to an increased concentration of plectin at the NE. Moreover, this effect was dependent on the presence of nesprin-3 α , as shown by the siRNA-mediated knockdown of nesprin-3 (Wilhelmsen et al., 2005). As plectin binds to nesprin-3 α through its ABD and to intermediate filaments through its C-terminus, we hypothesized that the interaction between nesprin-3 α and plectin links the nucleus to the intermediate filament system.

In the present study, we further investigated the requirements necessary for the localization of nesprin-3 and plectin at the NE. We show that nesprin-3 α is retained at the NE by an interaction with Sun proteins and that the last four amino acids of its KASH domain (PPPX consensus sequence) are involved in this interaction. Furthermore, we demonstrate that nesprin-

3 α can form dimers and that the dimerization of plectin is required for the interaction with nesprin-3 α at the NE. In addition, overexpression of the plectin ABD stabilizes the actin cytoskeleton, which, in turn, influences the expression pattern of endogenous plectin.

Results

Nesprin-3 is retained at the NE in a manner similar to that retaining nesprin-1 and -2

To determine whether nesprin-3 is retained at the ONM by a mechanism similar to that retaining nesprin-1 and -2, we investigated the effect of overexpression of nesprin-1 and -2 on the localization of endogenous nesprin-3. It was demonstrated previously that overexpression of a GFP-tagged nesprin-1 construct, containing the two C-terminal spectrin repeats and the KASH domain (GFP-dnNesprin-1), had a dominant-negative effect on the amount of endogenous nesprin-2 localized at the NE (Libotte et al., 2005). A similar construct has also been described for nesprin-2 (GFP-dnNesprin-2) (Libotte et al., 2005). To investigate the effect of these constructs on the localization of nesprin-3, NIH3T3 cells were transiently transfected with constructs encoding GFP-dnNesprin-1 or GFP-dnNesprin-2 and stained to determine the levels of endogenous nesprin-3. As shown in Fig. 1A, the amount of endogenous nesprin-3 at the NE of transfected cells decreased in comparison with that in untransfected cells. Quantification of this effect shows a decrease in the average gray value of nesprin-3 at the NE from 170 ± 3 in untransfected cells to 80 ± 3 or 98 ± 3 in cells transfected with constructs encoding GFP-dnNesprin-1 or GFP-dnNesprin-2, respectively ($P < 0.001$) (Fig. 1B). This indicates that nesprin-3 is retained at the NE by a mechanism similar to that retaining nesprin-1 and -2.

Nesprin-3 α interacts with Sun proteins through its PPPX consensus sequence

Nesprin-1 and -2 are localized at the NE through an interaction with Sun proteins present at the INM (Crisp et al., 2006; Haque et al., 2006; Padmakumar et al., 2005). Moreover, the last four amino acids of the nesprin-2 KASH domain (consensus sequence PPPX) are essential for the interaction with Sun1 and the localization of nesprin-2 at the NE (Padmakumar et al., 2005). To investigate whether the last four amino acids of nesprin-3 α (PPPT) are likewise required for its retention at the NE through an interaction with the Sun proteins, a mutant of nesprin-3 lacking these amino acids, nesprin-3 $\alpha^{\Delta\text{PPPT}}$, was generated. PA-JEB keratinocytes stably expressing GFP-nesprin-3 α or GFP-nesprin-3 $\alpha^{\Delta\text{PPPT}}$ were stained for endogenous Sun1 or Sun2 and analyzed by confocal microscopy. GFP-nesprin-3 α colocalizes almost completely with both Sun1 and Sun2 (Fig. 2A), suggesting that it interacts with these proteins at the NE. By contrast, GFP-nesprin-3 $\alpha^{\Delta\text{PPPT}}$ is largely absent from the NE and instead distributes throughout the cytoplasm. Consequently, there is only limited colocalization of GFP-nesprin-3 $\alpha^{\Delta\text{PPPT}}$ with Sun1 and Sun2 (Fig. 2A). This indicates that the last four amino acids of nesprin-3 are indeed essential for its localization at the NE.

To determine the intracellular localization of GFP-nesprin-3 $\alpha^{\Delta\text{PPPT}}$, PA-JEB cells stably expressing this tagged nesprin mutant were stained for plectin and several cellular components. GFP-nesprin-3 $\alpha^{\Delta\text{PPPT}}$ no longer concentrates at

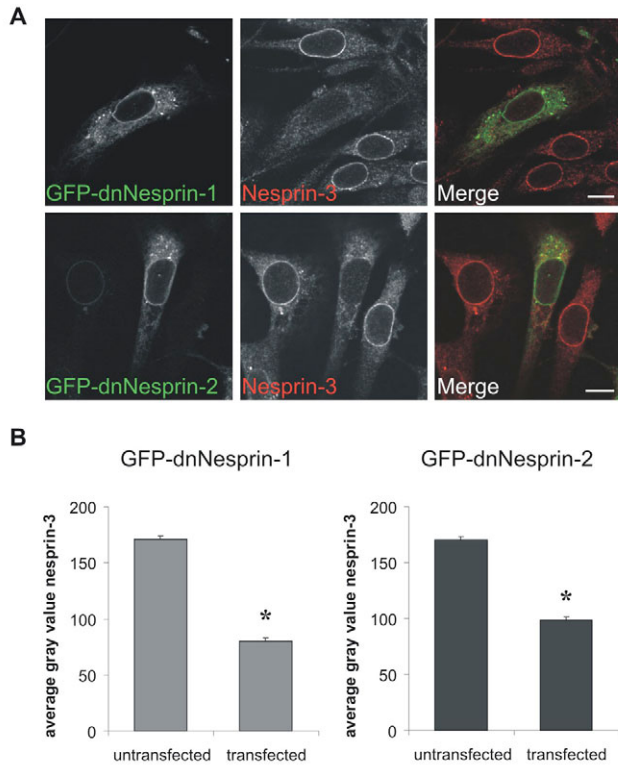


Fig. 1. Nesprin-3 is displaced from the NE upon overexpression of nesprin-1 and nesprin-2. (A) NIH3T3 cells were transiently transfected with constructs encoding GFP-dnNesprin-1 or GFP-dnNesprin-2, fixed in paraformaldehyde and stained for endogenous nesprin-3. Representative confocal images are shown. Bars, 10 μ m. (B) Quantitative analysis of the effect demonstrated in (A). The average gray value of endogenous nesprin-3 at the NE was measured for individual transfected and untransfected cells. Results are shown as the mean gray value \pm s.e.m. ($n=50$). *, $P<0.001$.

the NE but is primarily found on membrane structures in the cytoplasm, where it colocalizes with endogenous plectin (Fig. 2B). Furthermore, GFP-nesprin-3 Δ PPPT does not colocalize with markers for mitochondria (data not shown) or the Golgi complex (Fig. 2B) and only partially colocalizes with protein disulphide isomerase (PDI), a marker for the ER (Fig. 2B) (Luz and Lennarz, 1996). As the expression pattern of GFP-nesprin-3 Δ PPPT resembled that of PDI, a possible localization of GFP-nesprin-3 Δ PPPT at the ER was further investigated by electron microscopy. In PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 Δ PPPT, wild-type GFP-nesprin-3 α is mainly found at the ONM and to some extent at the ER (Fig. 2C). By contrast, although some GFP-nesprin-3 Δ PPPT still occurs at the ONM, the majority of the GFP-nesprin-3 Δ PPPT associates with the ER (Fig. 2C). Hence, deletion of the last four amino acids of nesprin-3 prevents its retention at the NE and leads to a dispersion of the protein over the ER.

The dispersion of GFP-nesprin-3 Δ PPPT over the ER might be caused by its inability to interact with Sun proteins. Therefore, we investigated, by co-immunoprecipitation, whether GFP-nesprin-3 Δ PPPT, in contrast to GFP-nesprin-3 α , is unable to interact with Sun proteins. GFP-nesprin-3 was precipitated from whole-cell lysates of PA-JEB cells stably

expressing either GFP-nesprin-3 α or GFP-nesprin-3 Δ PPPT and the precipitates were probed for the presence of GFP-nesprin-3, Sun proteins and lamins A and C. Sun1 and Sun2 were co-precipitated with GFP-nesprin-3 α but not with GFP-nesprin-3 Δ PPPT (Fig. 2D). This effect was specific for the Sun proteins, as LAP1 and LAP2, two other INM proteins, did not co-precipitate with either form of nesprin-3 α (data not shown). Furthermore, we were unable to co-precipitate lamin A and lamin C from cells expressing GFP-nesprin-3 α and GFP-nesprin-3 Δ PPPT (Fig. 2D). Taken together, these results indicate that nesprin-3 α interacts with Sun1 and Sun2 through its C-terminal four amino acids (PPPT).

Sun proteins are required for proper localization of nesprin-3 α at the NE

The localization of nesprin-3 Δ PPPT at the ER suggests that the interaction with Sun proteins is essential for the proper retention of nesprin-3 α at the NE. Sun proteins were previously reported to interact with lamin A (Crisp et al., 2006; Haque et al., 2006). To investigate whether Sun proteins and lamin A are indeed required for the localization of nesprin-3 α at the NE, we performed siRNA knockdown studies. PA-JEB cells stably expressing GFP-nesprin-3 α were mock transfected or transfected with siRNAs directed against the expression of lamin A/C (LmnA), Sun1, Sun2 or a combination of Sun1 and Sun2. Knockdown efficiency was tested by western blot (Fig. 3A) and the effect on the localization of GFP-nesprin-3 α was analyzed by immunofluorescence. The siRNA-mediated knockdown is specific as it has no effect on the expression of other INM proteins or lamin B (Fig. 3B). The decrease in the amount of lamins A and C has no, or only a limited, effect on the localization of Sun1 and Sun2 at the INM (data not shown). Similarly, knockdown of Sun2 or lamins A and C has no effect on the localization of GFP-nesprin-3 α at the ONM (Fig. 3B,C). However, knockdown of Sun1 or a combination of Sun1 and Sun2 leads to a decrease in the amount of GFP-nesprin-3 α at the NE of 27% and 40%, respectively ($P<0.001$) (Fig. 3B,C). This suggests that expression of Sun1, but not that of Sun2, is required for proper retention of nesprin-3 α at the ONM.

The fact that no effect of Sun2 knockdown was observed on the localization of GFP-nesprin-3 α could be attributable to an incomplete knockdown of Sun2. To investigate the interaction between Sun2 and nesprin-3 in more detail, PA-JEB cells stably expressing GFP-nesprin-3 α were transfected with a construct encoding the luminal domain of Sun2, encompassing the nesprin interaction site, fused to an ER retention signal. In untransfected cells, the localization of GFP-nesprin-3 α at the NE was normal (Fig. 3D). However, overexpression of the Sun2 luminal domain results in a relocalization of GFP-nesprin-3 α away from the NE and into the ER, where it becomes colocalized with the Sun2 luminal domain (Fig. 3D). Based on this finding, we conclude that nesprin-3 is not only retained at the NE through an interaction with Sun1 but also with Sun2.

The results thus far show that nesprin-3 is retained at the NE by the same mechanism as that responsible for the retention of nesprin-1 and -2. Specifically, nesprin-3 α interacts with both Sun1 and Sun2 through its PPPX consensus sequence. This interaction is required for the proper localization of nesprin-3 α at the NE as deletion of the consensus sequence, knockdown of the Sun1 proteins and overexpression of a Sun2 luminal-

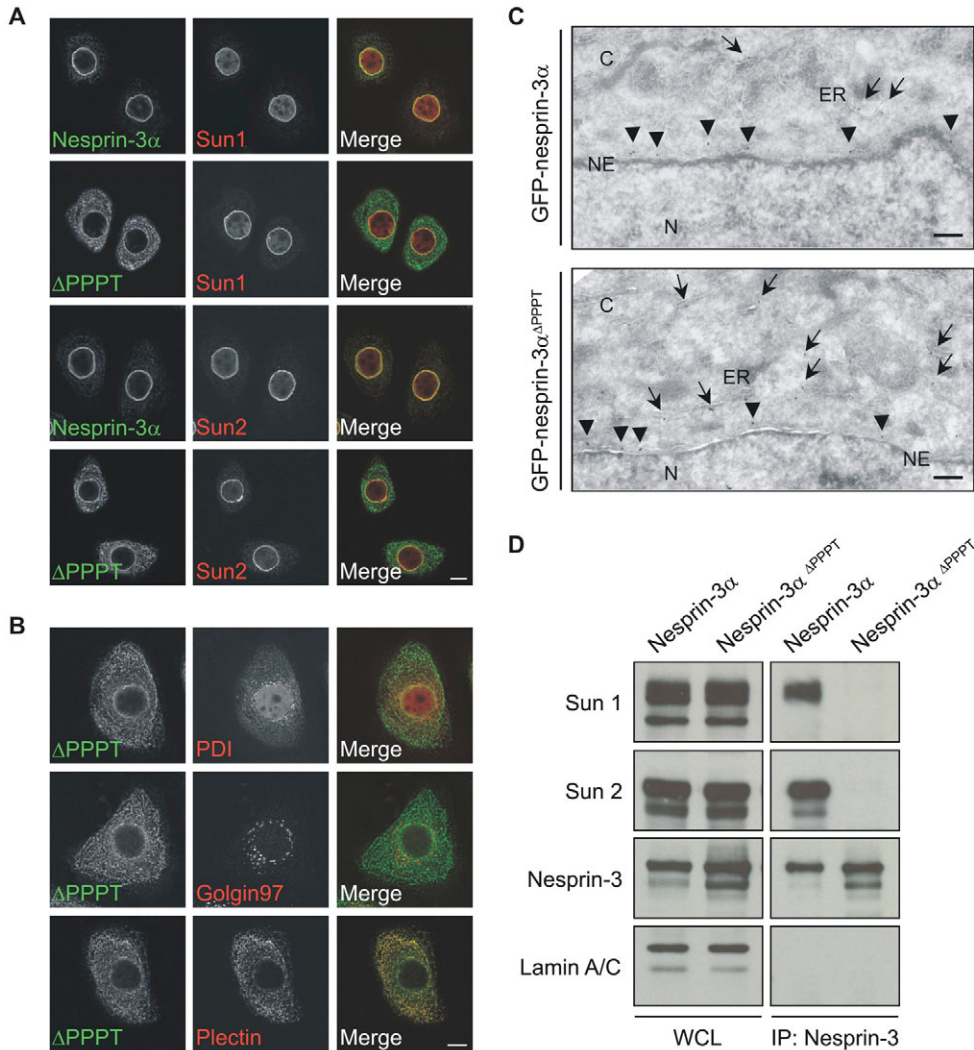


Fig. 2. Nesprin-3 α interacts with Sun proteins through its PPPX consensus sequence. (A) PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 $\alpha^{\Delta PPPT}$ were fixed in paraformaldehyde, stained for endogenous Sun1 and Sun2 and analyzed by confocal microscopy. Bar, 10 μ m. (B) PA-JEB cells stably expressing GFP-nesprin-3 $\alpha^{\Delta PPPT}$ were fixed in paraformaldehyde, stained for endogenous plectin (HD-121 antibody), the ER (PDI antibody) or the Golgi complex (Golgin97 antibody) and analyzed by confocal microscopy. Bar, 10 μ m. (C) Ultrathin sections of PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 $\alpha^{\Delta PPPT}$ were labeled with a pAb against GFP, followed by an incubation with 10 nm colloidal-gold-conjugated protein A. Nesprin-3 α associates with the ONM (arrowhead) and the ER (arrow). N, nucleus; C, cytoplasm; NE, nuclear envelope; ER, endoplasmic reticulum. Bars, 200 nm. (D) Whole-cell lysates (WCLs) from PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 $\alpha^{\Delta PPPT}$ were immunoprecipitated with the nesprin-3 mAb Nsp3. Immunoprecipitates (IPs) and WCLs were probed with antibodies directed against Sun1, Sun2, nesprin-3, and lamins A and C.

KDEL construct result in displacement of nesprin-3 α from the NE to the ER.

Nesprin-3 α can form dimers

Proteins containing spectrin repeats are thought to form dimers by means of an intermolecular interaction between their spectrin repeats (Djinovic-Carugo et al., 2002; Djinovic-Carugo et al., 1999; Imamura et al., 1988; Pascual et al., 1997; Yan et al., 1993). As nesprin-3 contains a series of spectrin repeats, the ability of nesprin-3 α to self-associate was investigated by co-immunoprecipitation. COS7 cells were transiently transfected with constructs encoding nesprin-3 α tagged with vesicular stomatitis virus (VSV) glycoprotein (VSV-nesprin-3 α) and hemagglutinin (HA)-tagged HA-nesprin-3 α , HA-plectin-1C ABD or HA- α -actinin ABD. The ABDs of plectin-1C and α -actinin served as a positive and a negative control, respectively, for nesprin-3 α binding. The HA precipitates were subsequently analyzed for the presence of VSV-nesprin-3 α . In support of our previous study (Wilhelmsen et al., 2005), the ABD of plectin-1C, but not that of α -actinin, could co-precipitate VSV-nesprin-3 α (Fig. 4). Furthermore, VSV-nesprin-3 α co-precipitates with HA-nesprin-3 α (Fig. 4), indicating that nesprin-3 α can self-associate. Moreover, a high-

molecular-mass variant of nesprin-3, double the size of nesprin-3 α , is occasionally observed in western blots prepared from non-denaturing gels (data not shown), independently showing that nesprin-3 α can exist as a dimer.

Plectin requires dimerization for its interaction with nesprin-3 α at the NE

We previously demonstrated that overexpression of nesprin-3 α , but not of nesprin-3 β , results in the recruitment of endogenous plectin to the NE (Wilhelmsen et al., 2005). This interaction is mediated by the plectin ABD and requires the presence of the first spectrin repeat in nesprin-3 α (Wilhelmsen et al., 2005). Like nesprin-3 α , plectin is also thought to form dimers, which is achieved by means of an intermolecular interaction of the rod domain (Foisner et al., 1991; Green et al., 1992; Wiche, 1998). To test whether dimerization of plectin is necessary for the interaction with nesprin-3 α at the NE, PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 β were transiently transfected with HA-tagged plectin constructs encoding full-length plectin or C-terminal deletion mutants that either lack (plectin 1-399 and 1-606) or contain (plectin 1-2532) the rod domain (Fig. 5A). The localization of these plectin proteins was subsequently analyzed by confocal

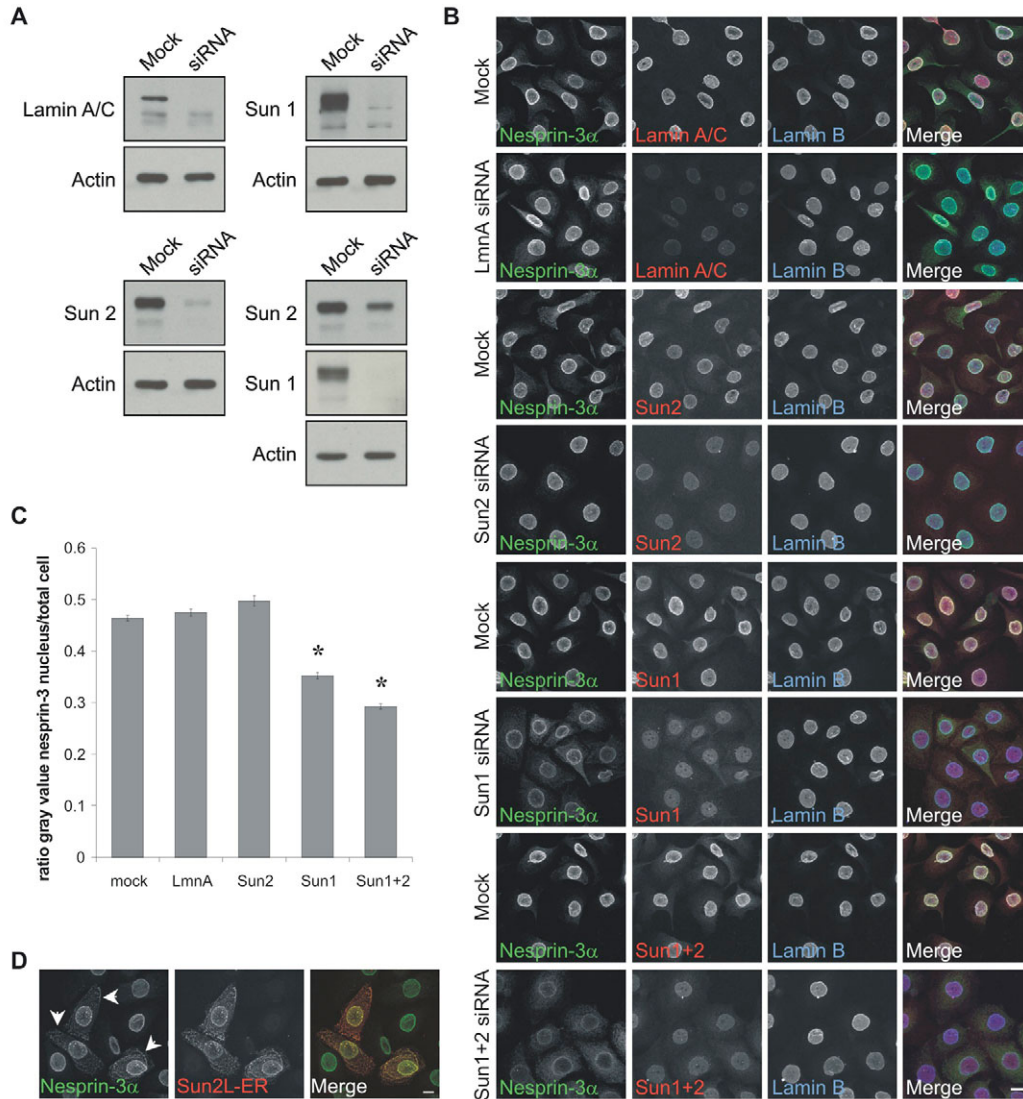


Fig. 3. Nesprin-3 α is dependent on Sun1 and Sun2 for its localization at the NE. (A) PA-JEB cells stably expressing GFP-nesprin-3 α were mock transfected or transfected with siRNA directed against lamin A/C (LmnA), Sun1, Sun2 or a combination of Sun1 and Sun2. 72 hours after transfection, cells were lysed and WCLs were analyzed for expression of the indicated proteins. (B) PA-JEB cells stably expressing GFP-nesprin-3 α were mock transfected or transfected with siRNAs directed against LmnA, Sun1, Sun2 or a combination of Sun1 and Sun2. 72 hours after transfection, cells were fixed in paraformaldehyde, stained for the indicated proteins and analyzed by confocal microscopy. Bar, 10 μ m. (C) Quantitative analysis of the effect shown in (B). The gray values of GFP-nesprin-3 α at the nucleus and the complete cell were determined for individual siRNA- and mock-transfected cells. Results are shown as the mean ratio of the gray value in the nucleus to the gray value in the total cell \pm s.e.m. ($n=50$). *, $P<0.001$. (D) PA-JEB cells stably expressing GFP-nesprin-3 α were transiently transfected with a Myc-tagged Sun2 luminal domain construct carrying an ER-retention signal (Sun2L-ER). After 72 hours, cells were fixed in paraformaldehyde and stained for Myc. Representative confocal images are shown. Arrows indicate a displacement of GFP-nesprin-3 α over the ER. Bar, 10 μ m.

microscopy. While full-length plectin and plectin 1-2532 colocalized with nesprin-3 α at the NE, plectin 1-339 and plectin 1-606 did not (Fig. 5B). As expected, none of the HA-tagged plectin proteins colocalized with nesprin-3 β at the NE (Fig. 5B). As only those proteins containing the rod domain were localized at the NE, the results suggest that only plectin dimers are efficiently recruited by nesprin-3 α .

To further confirm the essential role of the rod domain and the dimerization of plectin in the recruitment of plectin to the NE, we made use of the MD-EBS-1 cell line (Geerts et al., 1999; Koster et al., 2004). These cells were established from a

patient with muscular dystrophy-associated epidermolysis bullosa simplex (MD-EBS), who was homozygous for an 8-base-pair duplication in exon 31 of plectin (Smith et al., 1996). As a consequence, the MD-EBS-1 cells do not have full-length plectin, but express a variant that lacks the rod domain (Fig. 5C). By contrast, MD-EBS-2 cells completely lack expression of plectin (Fig. 5C). To investigate the influence of the rod domain on the recruitment of plectin to nesprin-3 α at the NE, PA-JEB cells expressing GFP-nesprin-3 α and MD-EBS-1 cells stably expressing either GFP-nesprin-3 α or GFP-nesprin-3 β were stained for plectin and analyzed by confocal microscopy.

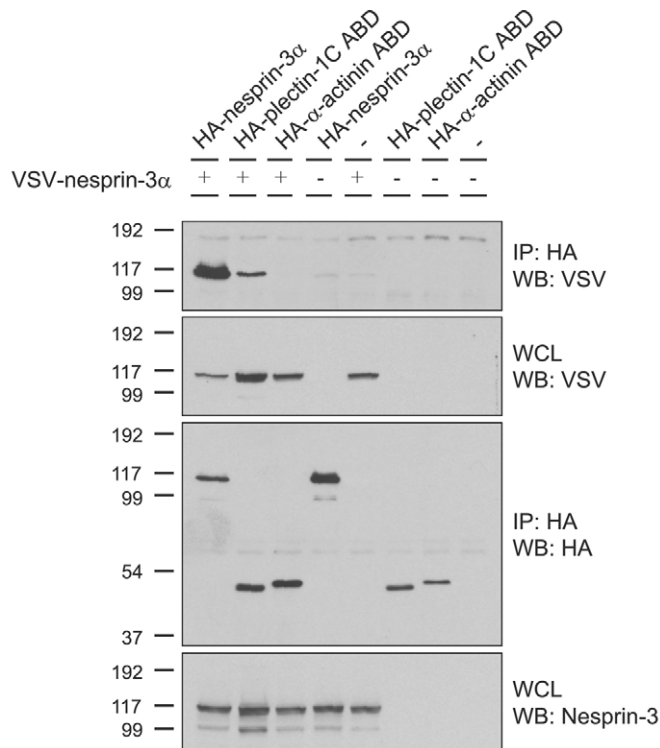


Fig. 4. Nesprin-3 α can form dimers. COS7 cells were transiently transfected with a construct encoding VSV-nesprin-3 α (lanes 1–3, 5) and expression constructs for HA-nesprin-3 α (lanes 1 and 4), HA-plectin 1C ABD (lanes 2 and 6) or HA- α -actinin ABD (lanes 3 and 7). The cells were lysed in RIPA buffer, and HA precipitates were probed for VSV glycoprotein (upper panel) and HA (third panel). WCLs were probed for the expression levels of VSV-nesprin-3 α (second panel) and total nesprin-3 (lower panel).

As the HD-121 plectin antibody applied in the other experiments recognizes the rod domain, this antibody could not be used for the MD-EBS-1 cell line (Okumura et al., 1999). We therefore used a plectin antibody (clone 31) that recognizes an epitope in the plakin repeats. However, this antibody gives a considerable background staining of the nuclei, which renders an accurate identification of plectin at the NE more difficult. As shown in Fig. 5D, PA-JEB cells that lack GFP-nesprin-3 α only show nuclear background staining, whereas PA-JEB cells expressing GFP-nesprin-3 α demonstrate an additional rim of plectin around the nucleus, which indicates that full-length plectin is recruited to the NE. By contrast, although nuclear background staining could still be observed in the MD-EBS-1 cells, rod-less plectin only very limitedly colocalizes with GFP-nesprin-3 α and is absent from the NE in cells expressing GFP-nesprin-3 β (Fig. 5D). This supports our notion that only plectin dimers can efficiently interact with nesprin-3 α at the NE.

Overexpression of the plectin ABD stabilizes the actin cytoskeleton and has a dominant-negative effect on the localization of endogenous plectin at the NE

Although the results in Fig. 4 indicate that the plectin ABD and nesprin-3 α interact at the NE, this does not occur upon transient overexpression of the plectin ABD in cells (Fig. 5B).

To investigate a potential effect of the plectin ABD on the localization of endogenous plectin, PA-JEB cells stably expressing GFP-nesprin-3 α were transiently transfected with constructs encoding HA-plectin ABD or HA-plectin ABD^{E95S}, a mutant of the plectin ABD that binds less strongly to nesprin-3 α (see supplementary material Fig. S1). The cells were subsequently stained for endogenous plectin. Interestingly, while untransfected cells clearly show staining of endogenous plectin at the NE, plectin is absent from the NE in transfected cells (Fig. 6A). This dominant-negative effect on the localization of endogenous plectin is observed, to a similar extent, with both the wild-type and the E95S plectin ABD (Fig. 6A). These results indicate that the dominant-negative effect is not caused through competition for nesprin-3 α binding between endogenous plectin and the plectin ABD.

As plectin has been implicated in the regulation of actin dynamics (Andra et al., 1997), we wondered whether the dominant-negative effect of the plectin ABD could be mediated through a stabilizing effect on the actin cytoskeleton. This was tested with cytochalasin D, a reagent known to disrupt actin filaments through the inhibition of actin polymerization. PA-JEB cells stably expressing GFP-nesprin-3 α were transiently transfected with a construct encoding the HA-plectin ABD, treated with cytochalasin D and stained for either F-actin or endogenous plectin. In untransfected cells, actin filaments are lost upon cytochalasin D treatment and endogenous plectin is found associated with nesprin-3 α at the NE (Fig. 6B). By contrast, when cells are transfected with a construct encoding the plectin ABD, actin fibers are still present after cytochalasin D treatment and endogenous plectin is found in the cytoplasm associated with them (Fig. 6B). This indicates that the plectin ABD stabilizes the actin cytoskeleton.

To confirm the effect of actin stabilization on the localization of endogenous plectin independent of the expression of the plectin ABD, we made use of the actin-stabilizing and polymerizing drug jasplakinolide. PA-JEB cells stably expressing GFP-nesprin-3 α were treated with jasplakinolide and stained for F-actin and endogenous plectin. In untreated cells, endogenous plectin is found associated with GFP-nesprin-3 α at the NE (Fig. 6C). Treatment with jasplakinolide results in the formation of peripheral actin aggregates that do not stain for phalloidin (Fig. 6C) as jasplakinolide competes with phalloidin for the same binding site (Bubb et al., 1994). Moreover, jasplakinolide causes a change in the distribution of endogenous plectin, away from the NE and into the peripheral actin aggregates (Fig. 6C). Hence, stabilization of F-actin by jasplakinolide influences the localization of endogenous plectin and results in a redistribution of plectin to the actin cytoskeleton. This observation supports the idea that the dominant-negative effect of the plectin ABD is caused by a stabilizing effect on F-actin structures.

Discussion

We have shown that nesprin-3 is retained at the ONM through an interaction of its PPPX consensus sequence with the INM proteins Sun1 and Sun2. This mechanism is identical to the one previously observed for nesprin-1 and -2 (Crisp et al., 2006; Padmakumar et al., 2005), indicating that all nesprin family members localize at the NE in a similar way. Furthermore, nesprin-3 α can form dimers, and dimerization of plectin is required for its interaction with nesprin-3 α at the NE. We also

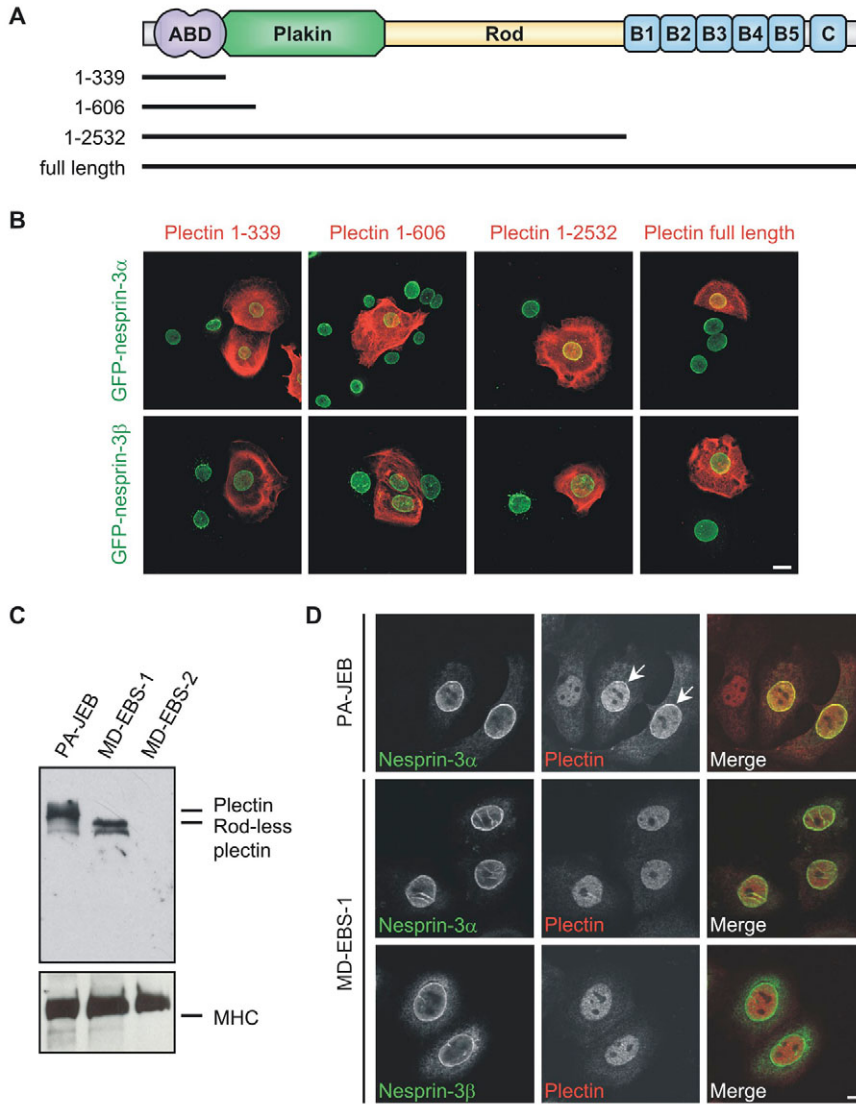


Fig. 5. Plectin dimers are required for the interaction with nesprin-3 α at the NE. (A) Schematic domain structure of a plectin molecule, containing an ABD (purple), a plakin domain (green), a rod domain (yellow) and a series of plakin repeats (blue). The length of the different plectin constructs used and their respective domains are indicated below. (B) PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 β were transiently transfected with HA-tagged constructs encoding plectin 1-399, plectin 1-606, plectin 1-2532 or full-length plectin. 48 hours after transfection, cells were fixed in paraformaldehyde, stained for HA and analyzed by confocal microscopy. Bar, 10 μ m. (C) PA-JEB, MD-EBS-1 and MD-EBS-2 keratinocytes were lysed and WCLs were analyzed by western blot for the expression of plectin (clone 31). Myosin heavy chain (MHC) served as a loading control. (D) PA-JEB cells expressing GFP-nesprin-3 α and MD-EBS-1 cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 β were fixed in paraformaldehyde, stained for plectin (clone 31) and analyzed by confocal microscopy. Arrows indicate the presence of plectin at the NE. Bar, 10 μ m.

demonstrated that overexpression of the plectin ABD results in stabilization of the actin cytoskeleton, which in turn influences the localization of endogenous plectin. This suggests that actin dynamics can regulate the interaction between plectin and nesprin-3 α .

Nesprin-1 and -2 are localized at the NE through a mechanism involving the INM proteins Sun1 and Sun2 (Crisp et al., 2006; Padmakumar et al., 2005). Overexpression of one of these two nesprin proteins influences the localization of the other and causes its displacement from the NE (Padmakumar et al., 2005; Zhang et al., 2007). We now extend these observations by demonstrating that nesprin-3 is also displaced from the NE upon overexpression of nesprin-1 or -2. Hence, identical protein interactions are responsible for the localization of all nesprin family members at the NE. Furthermore, the dominant-negative effect of overexpression of nesprin-1 or -2 indicates that the number of Sun docking sites in the periplasmic space is limited. This warrants a careful interpretation of nesprin overexpression studies as the observed effects will be influenced by the presence or absence of other nesprin proteins at the NE.

In this study, we demonstrate that nesprin-3 binds to Sun1 and Sun2 and that these proteins are required for its localization at the NE. Knockdown of Sun1 or overexpression of a Sun2 luminal-KDEL construct result in a reduced amount of GFP-nesprin-3 α at the NE. A similar observation was made previously for nesprin-2, which was found to be dependent on Sun1 for its localization at the NE (Crisp et al., 2006; Padmakumar et al., 2005). In addition, our results are in line with those of a previous study, showing that depletion of both Sun1 and Sun2 in HeLa cells causes a loss of full-length nesprin-2 from the NE (Crisp et al., 2006).

Sun1 can bind to lamin A and, more specifically, to the unprocessed form of lamin A (Crisp et al., 2006; Haque et al., 2006). However, Sun1 and Sun2 do not require functional A- or C-type lamins for their localization at the INM (Crisp et al., 2006; Hasan et al., 2006; Padmakumar et al., 2005). In addition, nesprin-1 binds to lamin A directly, and nesprin-2 is dependent on lamin A for its localization at the NE (Libotte et al., 2005; Mislow et al., 2002). However, these studies focused on smaller isoforms of nesprin-1 and -2 that are thought to reside at the INM instead of the ONM. In this study, we were

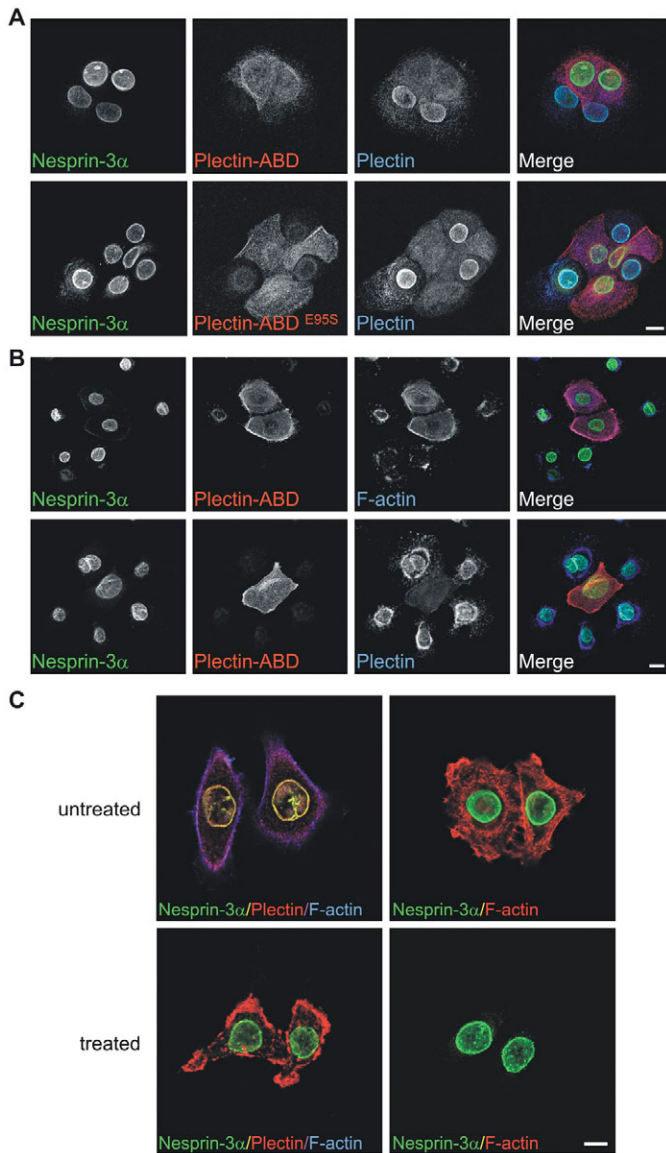


Fig. 6. Expression of the plectin ABD stabilizes the actin cytoskeleton and has a dominant-negative effect on the localization of endogenous plectin at the NE. (A) PA-JEB cells stably expressing GFP-nesprin-3 α were transiently transfected with constructs encoding HA-plectin ABD or HA-plectin ABD^{E95S}. 48 hours after transfection, cells were fixed in paraformaldehyde, stained for HA and endogenous plectin (HD-121 antibody) and analyzed by confocal microscopy. Bar, 10 μ m. (B) PA-JEB cells stably expressing GFP-nesprin-3 α were transiently transfected with a construct encoding the HA-plectin ABD. The cells were treated with cytochalasin D for 30 minutes, fixed in paraformaldehyde and stained for HA and either endogenous plectin (HD-121 antibody) or F-actin. Bar, 10 μ m. (C) PA-JEB cells stably expressing GFP-nesprin-3 α were treated with 100 nM jasplakinolide for two hours. Treated and untreated cells were fixed in paraformaldehyde and stained for F-actin and endogenous plectin (HD-121 antibody). Bar, 10 μ m.

unable to co-precipitate lamin A or lamin C with nesprin-3. This is not unexpected as, by an extensive analysis, no nesprin-3 isoforms were found in the nucleus or associated with the

INM. Hence, it is unlikely that nesprin-3 binds to lamin A or lamin C directly. However, this does not exclude the possibility of an indirect link with lamin A through the association with Sun proteins. This would require the formation of a protein complex containing nesprin-3, Sun proteins and lamin A that bridges the NE. However, although an interaction between lamin A and the Sun proteins might take place, lamin A and lamin C are not required for the localization of nesprin-3 α at the NE.

In the interaction between nesprin-3 α and the Sun1 and Sun2 proteins, the PPPX consensus sequence present in the KASH domain is involved. Similarly, the conserved C-terminal four amino acids of nesprin-2, PPPT, are essential for the interaction of nesprin-2 with Sun1 (Padmakumar et al., 2005). The requirement of the PPPX consensus sequence for the localization of nesprin-3 at the NE is illustrated by the observation that nesprin-3 α ^{ΔPPPT} does not concentrate at the NE but is found instead dispersed over the ER. However, there is still some residual nesprin-3 α ^{ΔPPPT} at the ONM. This can be explained in two ways. First, as the ONM is continuous with the RER, the nesprin-3 α ^{ΔPPPT} molecules that are not able to bind to Sun proteins on the INM, but are synthesized on ribosomes associated with either membrane, will become distributed equally over the RER and the ONM. Second, as nesprin-3 α can self-associate, GFP-nesprin-3 α ^{ΔPPPT} might form heterodimers with endogenous nesprin-3 α at the NE. As the murine and human nesprin-3 spectrin repeats are highly homologous, we can reasonably conclude that they might form heterodimers. However, only the KASH domain of the wild type nesprin-3 α in the dimer can bind to Sun proteins, and therefore their interaction with Sun proteins is likely to be weakened. Consequently, this should result in the amount of GFP-nesprin-3 α ^{ΔPPPT} retained at the ONM being reduced.

Nesprin-3 α can bind to the cytoskeletal crosslinker protein plectin. This interaction takes place between the N-terminal ABD of plectin and the first spectrin repeat of nesprin-3 α (Wilhelmsen et al., 2005). The finding that some C-terminally truncated plectin fragments containing the ABD do not colocalize with nesprin-3 α at first sight might seem to contradict the results from biochemical assays, in which nesprin-3 α co-precipitates with the plectin ABD. However, it should be understood that, in biochemical assays, no F-actin is present to which the plectin ABD can bind and that therefore, under these circumstances, all the ABDs of plectin are free to bind to nesprin-3 α . The plectin ABD can also bind to monomeric actin, but this binding is relatively weak and apparently does not have an impact on the binding of the plectin ABD to nesprin-3 α (Fontao et al., 2001). However, in cells, the plectin ABD can bind to F-actin. Moreover, in the co-immunoprecipitation experiments, the amount of the plectin ABD could well exceed the amount that can bind to F-actin, and therefore the remainder will be available to bind to nesprin-3 α . Interestingly, our data also indicate that the expression of the plectin ABD exerts a dominant-negative effect on the localization of endogenous plectin at the NE. This effect was not caused by direct competition with endogenous plectin for nesprin-3 α binding sites but by a stabilizing effect of the plectin ABD on the actin cytoskeleton. Stabilization of the actin cytoskeleton increases the amount of actin filaments, thereby creating more binding sites for endogenous plectin. As

a consequence, plectin is no longer found at the NE associated with nesprin-3 α . Taken together, these results support previous observations that plectin is involved in the regulation of the actin cytoskeleton (Andra et al., 1998; Fontao et al., 2001) and suggest that actin dynamics are likely to influence the interaction between plectin and nesprin-3 α at the NE.

In this study, we were able to show that nesprin-3 α is capable of dimerization. This was not unexpected as spectrin repeats have previously been shown to mediate protein-protein interactions as well as dimerization (Djinovic-Carugo et al., 2002; Djinovic-Carugo et al., 1999; Imamura et al., 1988; Pascual et al., 1997; Yan et al., 1993). Furthermore, one of the nesprin-1 isoforms was shown to self-associate. Mislow et al. showed, by yeast-two-hybrid studies and blot overlay assays, that nesprin-1 α can self-associate through an interaction between the third and fifth spectrin repeats (Mislow et al., 2002). Based on the known anti-parallel dimerization of other proteins containing spectrin repeats (Djinovic-Carugo et al., 1999; Imamura et al., 1988; Pascual et al., 1997; Yan et al., 1993), it has been suggested that the N-terminus of one nesprin-1 α molecule can interact with the C-terminus of another nesprin-1 α molecule. This would result in the intermolecular anti-parallel dimerization of nesprin-1 α (Mislow et al., 2002). Although the actual spectrin repeats involved in the dimerization of nesprin-3 α await further identification, these previous observations suggest that dimerization of nesprin-3 α might also be anti-parallel.

Previous studies have shown that plectin can form dimers in solution (Foisner and Wiche, 1987; Weitzer and Wiche, 1987; Wiche, 1998). In fact, the dimerization of plectin is thought to be mediated by its coiled-coil rod domain (Foisner et al., 1991; Green et al., 1992; Uitto et al., 1996; Wiche, 1998; Wiche et al., 1991). Our results indicate that plectin requires dimerization for its interaction with nesprin-3 α at the NE. Shorter N-terminal plectin fragments, capable of binding nesprin-3 α in co-immunoprecipitation studies, were not found at the NE in cells overexpressing nesprin-3 α . Only plectin constructs containing the rod domain were found at the NE, indicating that the rod domain is essential for localization of plectin at the NE. Additionally, GFP-nesprin-3 α stably expressed in MD-EBS-1 cells, which only express a rod-less variant of plectin, was unable to efficiently recruit this plectin isoform to the NE. From these results, we conclude that only plectin dimers can efficiently interact with nesprin-3 α at the NE.

In contrast to PA-JEB cells, which do not express the integrin β 4 subunit, MD-EBS keratinocytes show normal expression of β 4. Like its binding to nesprin-3 α , plectin binds to β 4 through its N-terminal ABD (Geerts et al., 1999). Hence, the absence of rod-less plectin from the NE in MD-EBS-1 cells overexpressing GFP-nesprin-3 α might be a consequence of a competition for plectin binding between nesprin-3 α and integrin β 4. However, we do not consider this a likely explanation as we previously demonstrated that even if GFP-nesprin-3 α is overexpressed in PA-JEB- β 4 cells (i.e. PA-JEB cells reconstituted with β 4) full-length plectin is still recruited to the NE (Wilhelmsen et al., 2005).

Using pull-down assays, we have previously shown that the ABD of MACF is also capable of interacting with nesprin-3 α (Wilhelmsen et al., 2005). However, we were unable to show that overexpression of nesprin-3 α causes a redistribution of

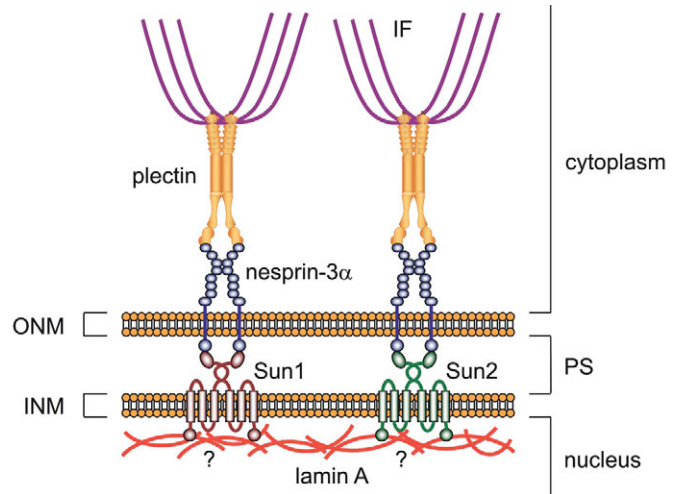


Fig. 7. Model illustrating the nesprin-3 LINC complex. Nesprin-3 α dimers (blue) are retained at the ONM through an interaction with Sun1 (brown) or Sun2 (green) protein dimers. The binding of nesprin-3 α to dimers of plectin (yellow) connects the nucleus to the intermediate filament system. A potential interaction of Sun proteins with lamin A (red), indicated by a question mark, establishes an indirect link between the cytoskeleton and the nucleoskeleton: the nesprin-3 LINC complex. ONM, outer nuclear membrane; INM, inner nuclear membrane; PS; periplasmic space; IF, intermediate filaments.

MACF to the NE, unlike the effect on plectin. Interestingly, MACF does not contain a coiled-coil rod domain but, instead, has a spectrin-based central domain. It is therefore tempting to speculate that the inability of nesprin-3 α to recruit MACF to the NE is due to an inability of this protein to form parallel dimers. This hypothesis will require further investigation.

In addition to nesprin-3 α and plectin, Sun proteins are also thought to form dimers. Padmakumar and colleagues have mentioned yeast-two-hybrid data indicating that a region of Sun1 containing two coiled-coil domains is capable of self-interaction, suggesting that Sun1 forms dimers or oligomers (Padmakumar et al., 2005). Furthermore, it has recently been shown that Sun2 can form homodimers as well as forming heterodimers with Sun1 (Wang et al., 2006). Together with the dimerization of plectin and nesprin-3 α , this implicates a model in which homo- or heterodimers of Sun proteins interact with dimers of nesprin-3 α , which in turn can bind to dimers of plectin (Fig. 7). As plectin binds to nesprin-3 α by its N-terminal ABD, the C-terminus is free to interact with intermediate filaments in the cytoplasm. By contrast, Sun proteins are thought to interact with nuclear lamins (Crisp et al., 2006; Haque et al., 2006). Taken together, this suggests that intermediate filaments in the cytoplasm are indirectly linked to the nuclear lamina (Fig. 7). Hence, the LINC complex not only connects the nucleoskeleton with the actin cytoskeleton but also might connect it to the intermediate filament system.

Materials and Methods

Constructs

GFP-tagged dominant-negative nesprin-1 and -2 constructs were kindly provided by Iakowos Karakasisoglou (University of Cologne, Germany) (Libotte et al., 2005). The luminal domain of Sun2 that includes the whole C-terminal region downstream of the transmembrane domain was amplified by PCR and cloned in the *Sall*-*NotI*

restriction sites of pShooter/Myc/ER (Invitrogen). This cloning resulted in the expression of a recombinant protein fused to an N-terminal signal sequence and a C-terminal Myc epitope, followed by a KDEL sequence for protein retention within the ER lumen. The HA-tagged plectin constructs and the constructs encoding VSV-nesprin-3 α , VSV-nesprin-3 β and HA- α -actinin ABD have been described previously (Geerts et al., 1999; Litjens et al., 2003; Litjens et al., 2005; Wilhelmnsen et al., 2005). Nesprin-3 α was subcloned into pcDNA3-HA, a derivative of the eukaryotic expression vector pcDNA3 (Invitrogen) that contains an extra sequence 5' of the multiple cloning site encoding the HA tag, by first inserting the C-terminal part using *Bam*HI-*Xba*I restriction sites. The N-terminal part was subsequently inserted using a *Bam*HI digest of nesprin-3 α from pcDNA3-VSV. The VSV-nesprin-3 α^{Δ PPPT construct was made by inserting a PCR fragment containing the deletion [using primers 5'-GCTGGACTCGAGACGCTG-3' (forward) and 5'-GGTCCTCTAGACTAGCCATTGTACCG-3' (reverse)] into the *Xho*I-*Xba*I restriction sites of pcDNA3-VSV-nesprin-3 α . Nesprin-3 α^{Δ PPPT was subcloned into pEGFP-C3 (Clontech laboratories) using *Hind*III-*Xba*I sites. The *Eco*47III-*Xba*I sites of this construct were used to obtain a GFP-nesprin-3 α^{Δ PPPT fragment for cloning into the *Swa*I-*Sna*BI sites of pLZRS-IRES-zeo. All constructs were verified by sequencing, and protein expression and size were confirmed by western blotting.

Antibodies

The preparations of the rabbit polyclonal antibodies (pAbs) against nesprin-3 and Sun2 have been described previously (Hodczik et al., 2004; Wilhelmnsen et al., 2005). A murine nesprin-3 monoclonal antibody (mAb) Nsp3 was made by immunization of BALB/c mice with the GST fusion protein encoding the seventh spectrin repeat of nesprin-3 α (Wilhelmnsen et al., 2005). After five injections, the mice were sacrificed and isolated splenic lymphocytes were fused with Sp2/0 mouse myeloma cells. Hybridoma supernatants were collected and screened for antibodies by ELISA and western blot. Selected hybridomas were cloned three times by limiting dilution. The rabbit pAb directed against human Sun1 was kindly provided by Sue Shackleton (University of Leicester, UK). The mouse mAb HD-121 against plectin/HD1 was provided by Katsushi Owaribe (University of Nagoya, Japan) (Hieda et al., 1992). The mAb against lamins A and C (131C3) (MUBio Products BV) was a gift from Frans Ramaekers (University of Maastricht, The Netherlands). The rabbit pAbs directed against PDI and GFP were kindly provided by Hidde Ploegh (Whitehead Institute, Cambridge, MA) and Jacques Neeffjes (The Netherlands Cancer Inst., Amsterdam, The Netherlands), respectively. Myc-tagged proteins were detected with the mAb 9E10. The mAb 12CA5 and pAb Y-11 recognizing HA were purchased from Santa Cruz Biotechnology. The mAb directed against actin was purchased from Chemicon International. The anti-VSV glycoprotein mAb PSD4 was obtained from Sigma, the anti-Golgin-97 mAb CDF4 from Molecular Probes, the anti-plectin mAb clone 31 from BD Biosciences Pharmingen and the anti-lamin B goat pAb M-20 from Santa Cruz Biotechnology. Goat anti-rabbit and goat anti-mouse TexasRed-conjugated antibodies were purchased from Invitrogen. Goat anti-mouse and donkey anti-goat Cy5-conjugated antibodies were obtained from Jackson ImmunoResearch. Alexa Fluor 568 phalloidin and BODIPY 650/660 phalloidin were purchased from Molecular Probes. The donkey anti-rabbit and goat anti-mouse horseradish peroxidase-conjugated antibodies were obtained from GE Healthcare.

Cell culture

NIH3T3 and COS7 cells were grown in DMEM (GIBCO Life Technologies) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 U/ml streptomycin. PA-JEB, PA-JEB-GFP-nesprin-3 α , PA-JEB-GFP-nesprin-3 α^{Δ PPPT, PA-JEB-GFP-nesprin-3 β , MD-EBS-GFP-nesprin-3 α and MD-EBS-GFP-nesprin-3 β keratinocytes were grown in keratinocyte serum-free medium (GIBCO Life Technologies) supplemented with 50 μ g/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin and 100 U/ml streptomycin. MD-EBS keratinocytes stably expressing GFP-nesprin-3 α or GFP-nesprin-3 β have been described previously (Wilhelmnsen et al., 2005). Stable integration of GFP-nesprin-3 α^{Δ PPPT was performed as described previously (Sterk et al., 2000). Briefly, retrovirus carrying the GFP-nesprin-3 α^{Δ PPPT construct was produced by calcium phosphate-mediated transfection of amphotropic Phoenix packaging cells. PA-JEB cells were infected with the recombinant virus by the DOTAP method (Boehringer), selected on Zeocin (Invitrogen) and sorted for the expression of GFP-nesprin-3 α^{Δ PPPT by FACS. PA-JEB cells stably expressing GFP-nesprin-3 α were treated with 0.5 μ g/ml cytochalasin D (Sigma-Aldrich) for 30 minutes at 37°C in a humidified atmosphere containing 5% CO₂. Jasplakinolide (Molecular Probes) was used at a concentration of 100 nM for 2 hours under similar conditions. Cells were fixed immediately after treatment and processed for immunofluorescence.

Transfection and siRNA

NIH3T3 cells were transiently transfected with Effectene transfection reagent (Qiagen) according to the instructions provided by the manufacturer. Cells were analyzed 24–48 hours after transfection. Transient transfection of COS7 cells was achieved using the DEAE-dextran method. Cells were lysed after 48–72 hours of transfection. Transfection of PA-JEB cells stably expressing GFP-nesprin-3 α was mediated by Lipofectin (Invitrogen). PA-JEB cells stably expressing GFP-nesprin-

3 α were depleted of Sun1, Sun2 or lamin A and lamin C using appropriate SmartPool oligonucleotide duplexes (Dharmacon). Cells were transfected with siRNAs using DharmaFect1 transfection reagent (Dharmacon) according to the manufacturer's instructions. Analysis took place after at least 72 hours of culture.

Cell lysis and immunoprecipitation

Cells grown to confluency in 10 cm tissue-culture dishes were lysed in 1 ml radioimmunoprecipitation (RIPA) buffer [10 mM sodium phosphate, pH 7, 150 mM NaCl, 1% Nonidet P40, 1% DOC, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 100 μ M sodium vanadate and protease inhibitor cocktail (Sigma-Aldrich)]. Lysates were cleared by centrifugation at 20,000 g in a microcentrifuge at 4°C for 45 minutes. Nesprin-3 proteins were immunoprecipitated by incubation of cell lysates with 100 μ l supernatant containing the nesprin-3 mAb Nsp3 for 1 hour on ice, followed by a subsequent incubation with 100 μ l of a 10% slurry of γ -bind-Sepharose (GE Healthcare) with rocking for 3 hours at 4°C. For immunoprecipitation with anti-HA, cell lysates were incubated by rocking for 2 hours at 4°C with 100 μ l of a 10% slurry of anti-HA mAb prebound to γ -bind-Sepharose. Immunoprecipitates were washed four times with lysis buffer, boiled for 5 minutes in SDS-sample buffer (50 mM Tris, pH 6.8, 10% glycerol, 1% SDS, 0.1% β -mercaptoethanol and 0.025% bromophenol blue) and resolved by SDS-PAGE.

Immunofluorescence

Cells grown on glass coverslips were fixed in 1–3% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. After blocking with 2% BSA in PBS, the cells were incubated with the primary antibody for 1 hour at room temperature. Cells were washed three times with PBS and incubated for 45 minutes at room temperature with the secondary antibody. After three washes with PBS, coverslips were mounted in Mowiol-DAPCO and viewed under a confocal laser scanning microscope (model TCS NT or SP-2 AOBIS; Leica). Data was analyzed using Adobe Photoshop and ImageJ software. Quantification, based on maximal projection of z-stacks, was achieved by determining the gray value of nesprin-3 at the NE and relating it to the total gray value of nesprin-3 in individual cells.

Electron microscopy

PA-JEB cells stably expressing GFP-nesprin-3 α or GFP-nesprin-3 α^{Δ PPPT were fixed for 2 hours in a mixture of 2% paraformaldehyde and 0.2% glutaraldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM MgCl₂, 10 mM EGTA, pH 6.9) and processed for ultrathin cryosectioning, as described previously (Calafat et al., 1997). The sections were incubated with a pAb against GFP for 60 minutes, followed by an incubation with 10 nm colloidal-gold-labeled protein A for 30 minutes. After immunolabeling, the cryosections were embedded in a mixture of methyl cellulose and uranyl acetate and examined with a FEI Tecnai 12G2 electron microscope.

Statistical analysis

The results are shown as the mean \pm s.e.m. Comparisons were made using the Student's *t* test, assuming equal variances. Statistical significance was defined as *P* < 0.001.

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References

- Andra, K., Lassmann, H., Bittner, R., Shorny, S., Fassler, R., Propst, F. and Wiche, G. (1997). Targeted inactivation of plectin reveals essential function in maintaining the integrity of skin, muscle, and heart cytoarchitecture. *Genes Dev.* **11**, 3143–3156.
- Andra, K., Nikolic, B., Stocher, M., Drenckhahn, D. and Wiche, G. (1998). Not just scaffolding: plectin regulates actin dynamics in cultured cells. *Genes Dev.* **12**, 3442–3451.
- Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L. and Korn, E. D. (1994). Jasplakinolide, a cytotoxic natural product, induces actin polymerization and competitively inhibits the binding of phalloidin to F-actin. *J. Biol. Chem.* **269**, 14869–14871.
- Calafat, J., Janssen, H., Stahle-Backdahl, M., Zuurbier, A. E., Knol, E. F. and Egesten, A. (1997). Human monocytes and neutrophils store transforming growth factor- α in a subpopulation of cytoplasmic granules. *Blood* **90**, 1255–1266.
- Crisp, M., Liu, Q., Roux, K., Rattner, J. B., Shanahan, C., Burke, B., Stahl, P. D. and Hodczik, D. (2006). Coupling of the nucleus and cytoplasm: role of the LINC complex. *J. Cell Biol.* **172**, 41–53.
- Djinovic-Carugo, K., Young, P., Gautel, M. and Saraste, M. (1999). Structure of the alpha-actinin rod: molecular basis for cross-linking of actin filaments. *Cell* **98**, 537–546.

- Djinovic-Carugo, K., Gautel, M., Ylanne, J. and Young, P. (2002). The spectrin repeat: a structural platform for cytoskeletal protein assemblies. *FEBS Lett.* **513**, 119-123.
- Dreger, M., Bengtsson, L., Schoneberg, T., Otto, H. and Hucho, F. (2001). Nuclear envelope proteomics: novel integral membrane proteins of the inner nuclear membrane. *Proc. Natl. Acad. Sci. USA* **98**, 11943-11948.
- Eger, A., Stockinger, A., Wiche, G. and Foisner, R. (1997). Polarisation-dependent association of plectin with desmoplakin and the lateral submembrane skeleton in MDCK cells. *J. Cell Sci.* **110**, 1307-1316.
- Elliott, C. E., Becker, B., Oehler, S., Castanon, M. J., Hauptmann, R. and Wiche, G. (1997). Plectin transcript diversity: identification and tissue distribution of variants with distinct first coding exons and rodless isoforms. *Genomics* **42**, 115-125.
- Foisner, R. and Wiche, G. (1987). Structure and hydrodynamic properties of plectin molecules. *J. Mol. Biol.* **198**, 515-531.
- Foisner, R., Leichtfried, F. E., Herrmann, H., Small, J. V., Lawson, D. and Wiche, G. (1988). Cytoskeleton-associated plectin: in situ localization, in vitro reconstitution, and binding to immobilized intermediate filament proteins. *J. Cell Biol.* **106**, 723-733.
- Foisner, R., Feldman, B., Sander, L. and Wiche, G. (1991). Monoclonal antibody mapping of structural and functional plectin epitopes. *J. Cell Biol.* **112**, 397-405.
- Fontao, L., Geerts, D., Kuikman, I., Koster, J., Kramer, D. and Sonnenberg, A. (2001). The interaction of plectin with actin: evidence for cross-linking of actin filaments by dimerization of the actin-binding domain of plectin. *J. Cell Sci.* **114**, 2065-2076.
- Geerts, D., Fontao, L., Nievers, M. G., Schaapveld, R. Q., Purkis, P. E., Wheeler, G. N., Lane, E. B., Leigh, I. M. and Sonnenberg, A. (1999). Binding of integrin $\alpha 6 \beta 4$ to plectin prevents plectin association with F-actin but does not interfere with intermediate filament binding. *J. Cell Biol.* **147**, 417-434.
- Gerace, L. and Burke, B. (1988). Functional organization of the nuclear envelope. *Annu. Rev. Cell Biol.* **4**, 335-374.
- Green, K. J., Virata, M. L., Elgart, G. W., Stanley, J. R. and Parry, D. A. (1992). Comparative structural analysis of desmoplakin, bullous pemphigoid antigen and plectin: members of a new gene family involved in organization of intermediate filaments. *Int. J. Biol. Macromol.* **14**, 145-153.
- Haque, F., Lloyd, D. J., Smallwood, D. T., Dent, C. L., Shanahan, C. M., Fry, A. M., Trembath, R. C. and Shackleton, S. (2006). SUN1 interacts with nuclear lamin A and cytoplasmic nesprins to provide a physical connection between the nuclear lamina and the cytoskeleton. *Mol. Cell Biol.* **26**, 3738-3751.
- Hasan, S., Guttinger, S., Muhlhäusser, P., Anderegg, F., Burgler, S. and Kutay, U. (2006). Nuclear envelope localization of human UNC84A does not require nuclear lamins. *FEBS Lett.* **580**, 1263-1268.
- Hieda, Y., Nishizawa, Y., Uematsu, J. and Owaribe, K. (1992). Identification of a new hemidesmosomal protein, HD1: a major, high molecular mass component of isolated hemidesmosomes. *J. Cell Biol.* **116**, 1497-1506.
- Hodzic, D. M., Yeater, D. B., Bengtsson, L., Otto, H. and Stahl, P. D. (2004). Sun2 is a novel mammalian inner nuclear membrane protein. *J. Biol. Chem.* **279**, 25805-25812.
- Imamura, M., Endo, T., Kuroda, M., Tanaka, T. and Masaki, T. (1988). Substructure and higher structure of chicken smooth muscle alpha-actinin molecule. *J. Biol. Chem.* **263**, 7800-7805.
- Koster, J., van Wilpe, S., Kuikman, I., Litjens, S. H. and Sonnenberg, A. (2004). Role of binding of plectin to the integrin $\beta 4$ subunit in the assembly of hemidesmosomes. *Mol. Biol. Cell* **15**, 1211-1223.
- Libotte, T., Zaim, H., Abraham, S., Padmakumar, V. C., Schneider, M., Lu, W., Munck, M., Hutchison, C., Wehnert, M., Fahrenkrog, B. et al. (2005). Lamin A/C-dependent localization of Nesprin-2, a giant scaffold at the nuclear envelope. *Mol. Biol. Cell* **16**, 3411-3424.
- Litjens, S. H., Koster, J., Kuikman, I., van Wilpe, S., de Pereda, J. M. and Sonnenberg, A. (2003). Specificity of binding of the plectin actin-binding domain to $\beta 4$ integrin. *Mol. Biol. Cell* **14**, 4039-4050.
- Litjens, S. H., Wilhelmson, K., de Pereda, J. M., Perrakis, A. and Sonnenberg, A. (2005). Modeling and experimental validation of the binary complex of the plectin actin-binding domain and the first pair of fibronectin type III (FNIII) domains of the $\beta 4$ integrin. *J. Biol. Chem.* **280**, 22270-22277.
- Luz, J. M. and Lennarz, W. J. (1996). Protein disulfide isomerase: a multifunctional protein of the endoplasmic reticulum. *EXS* **77**, 97-117.
- McGee, M. D., Rillo, R., Anderson, A. S. and Starr, D. A. (2006). UNC-83 IS a KASH protein required for nuclear migration and is recruited to the outer nuclear membrane by a physical interaction with the SUN protein UNC-84. *Mol. Biol. Cell* **17**, 1790-1801.
- McLean, W. H., Pulkkinen, L., Smith, F. J., Rugg, E. L., Lane, E. B., Bullrich, F., Burgeson, R. E., Amano, S., Hudson, D. L., Owaribe, K. et al. (1996). Loss of plectin causes epidermolysis bullosa with muscular dystrophy: cDNA cloning and genomic organization. *Genes Dev.* **10**, 1724-1735.
- Mislow, J. M., Holaska, J. M., Kim, M. S., Lee, K. K., Segura-Totten, M., Wilson, K. L. and McNally, E. M. (2002). Nesprin-1alpha self-associates and binds directly to emerin and lamin A in vitro. *FEBS Lett.* **525**, 135-140.
- Nikolic, B., MacNulty, E., Mir, B. and Wiche, G. (1996). Basic amino acid residue cluster within nuclear targeting sequence motif is essential for cytoplasmic plectin-ventral network junctions. *J. Cell Biol.* **134**, 1455-1467.
- Okumura, M., Uematsu, J., Hirako, Y., Nishizawa, Y., Shimizu, H., Kido, N. and Owaribe, K. (1999). Identification of the hemidesmosomal 500 kDa protein (HD1) as plectin. *J. Biochem.* **126**, 1144-1150.
- Padmakumar, V. C., Abraham, S., Braune, S., Noegel, A. A., Tunggal, B., Karakesisoglou, I. and Korenbaum, E. (2004). Enaptin, a giant actin-binding protein, is an element of the nuclear membrane and the actin cytoskeleton. *Exp. Cell Res.* **295**, 330-339.
- Padmakumar, V. C., Libotte, T., Lu, W., Zaim, H., Abraham, S., Noegel, A. A., Gotzmann, J., Foisner, R. and Karakesisoglou, I. (2005). The inner nuclear membrane protein Sun1 mediates the anchorage of Nesprin-2 to the nuclear envelope. *J. Cell Sci.* **118**, 3419-3430.
- Pascual, J., Pfuhl, M., Walther, D., Saraste, M. and Nilges, M. (1997). Solution structure of the spectrin repeat: a left-handed antiparallel triple-helical coiled-coil. *J. Mol. Biol.* **273**, 740-751.
- Sanchez-Aparicio, P., Martinez de Velasco, A. M., Niessen, C. M., Borradori, L., Kuikman, I., Hulsman, E. H., Fassler, R., Owaribe, K. and Sonnenberg, A. (1997). The subcellular distribution of the high molecular mass protein, HD1, is determined by the cytoplasmic domain of the integrin $\beta 4$ subunit. *J. Cell Sci.* **110**, 169-178.
- Schirmer, E. C., Florens, L., Guan, T., Yates, J. R., 3rd and Gerace, L. (2003). Nuclear membrane proteins with potential disease links found by subtractive proteomics. *Science* **301**, 1380-1382.
- Seifert, G. J., Lawson, D. and Wiche, G. (1992). Immunolocalization of the intermediate filament-associated protein plectin at focal contacts and actin stress fibers. *Eur. J. Cell Biol.* **59**, 138-147.
- Smith, F. J., Eady, R. A., Leigh, I. M., McMillan, J. R., Rugg, E. L., Kelsell, D. P., Bryant, S. P., Spurr, N. K., Geddes, J. F., Kirtschig, G. et al. (1996). Plectin deficiency results in muscular dystrophy with epidermolysis bullosa. *Nat. Genet.* **13**, 450-457.
- Sonnenberg, A. and Liem, R. K. (2007). Plakins in development and disease. *Exp. Cell Res.* **313**, 2189-2203.
- Starr, D. A. and Han, M. (2002). Role of ANC-1 in tethering nuclei to the actin cytoskeleton. *Science* **298**, 406-409.
- Starr, D. A. and Han, M. (2003). ANChors away: an actin based mechanism of nuclear positioning. *J. Cell Sci.* **116**, 211-216.
- Starr, D. A., Hermann, G. J., Malone, C. J., Fixsen, W., Priess, J. R., Horvitz, H. R. and Han, M. (2001). Unc-83 encodes a novel component of the nuclear envelope and is essential for proper nuclear migration. *Development* **128**, 5039-5050.
- Sterk, L. M., Geuijen, C. A., Oomen, L. C., Calafat, J., Janssen, H. and Sonnenberg, A. (2000). The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin $\alpha 6 \beta 4$ and may regulate the spatial organization of hemidesmosomes. *J. Cell Biol.* **149**, 969-982.
- Svitkina, T. M., Verkhovskiy, A. B. and Borisy, G. G. (1996). Plectin sidearms mediate interaction of intermediate filaments with microtubules and other components of the cytoskeleton. *J. Cell Biol.* **135**, 991-1007.
- Tzur, Y. B., Wilson, K. L. and Gruenbaum, Y. (2006). SUN-domain proteins: 'Velcro' that links the nucleoskeleton to the cytoskeleton. *Nat. Rev. Mol. Cell Biol.* **7**, 782-788.
- Uitto, J., Pulkkinen, L., Smith, F. J. and McLean, W. H. (1996). Plectin and human genetic disorders of the skin and muscle. The paradigm of epidermolysis bullosa with muscular dystrophy. *Exp. Dermatol.* **5**, 237-246.
- Wang, Q., Du, X., Cai, Z. and Greene, M. I. (2006). Characterization of the structures involved in localization of the SUN proteins to the nuclear envelope and the centrosome. *DNA Cell Biol.* **25**, 554-562.
- Weitzer, G. and Wiche, G. (1987). Plectin from bovine lenses. Chemical properties, structural analysis and initial identification of interaction partners. *Eur. J. Biochem.* **169**, 41-52.
- Wiche, G. (1989). Plectin: general overview and appraisal of its potential role as a subunit protein of the cytomatrix. *Crit. Rev. Biochem. Mol. Biol.* **24**, 41-67.
- Wiche, G. (1998). Role of plectin in cytoskeleton organization and dynamics. *J. Cell Sci.* **111**, 2477-2486.
- Wiche, G., Becker, B., Lubber, K., Weitzer, G., Castanon, M. J., Hauptmann, R., Stratowa, C. and Stewart, M. (1991). Cloning and sequencing of rat plectin indicates a 466-kD polypeptide chain with a three-domain structure based on a central alpha-helical coiled coil. *J. Cell Biol.* **114**, 83-99.
- Wilhelmson, K., Litjens, S. H., Kuikman, I., Tshimbalanga, N., Janssen, H., van den Bout, I., Raymond, K. and Sonnenberg, A. (2005). Nesprin-3, a novel outer nuclear membrane protein, associates with the cytoskeletal linker protein plectin. *J. Cell Biol.* **171**, 799-810.
- Wilhelmson, K., Ketema, M., Truong, H. and Sonnenberg, A. (2006). KASH-domain proteins in nuclear migration, anchorage and other processes. *J. Cell Sci.* **119**, 5021-5029.
- Yan, Y., Winograd, E., Viel, A., Cronin, T., Harrison, S. C. and Branton, D. (1993). Crystal structure of the repetitive segments of spectrin. *Science* **262**, 2027-2030.
- Zhang, Q., Skepper, J. N., Yang, F., Davies, J. D., Hegyi, L., Roberts, R. G., Weissberg, P. L., Ellis, J. A. and Shanahan, C. M. (2001). Nesprins: a novel family of spectrin-repeat-containing proteins that localize to the nuclear membrane in multiple tissues. *J. Cell Sci.* **114**, 4485-4498.
- Zhang, Q., Ragnauth, C., Greener, M. J., Shanahan, C. M. and Roberts, R. G. (2002). The nesprins are giant actin-binding proteins, orthologous to *Drosophila melanogaster* muscle protein MSP-300. *Genomics* **80**, 473-481.
- Zhang, Q., Ragnauth, C. D., Skepper, J. N., Worth, N. F., Warren, D. T., Roberts, R. G., Weissberg, P. L., Ellis, J. A. and Shanahan, C. M. (2005). Nesprin-2 is a multi-isomeric protein that binds lamin and emerin at the nuclear envelope and forms a subcellular network in skeletal muscle. *J. Cell Sci.* **118**, 673-687.
- Zhang, X., Xu, R., Zhu, B., Yang, X., Ding, X., Duan, S., Xu, T., Zhuang, Y. and Han, M. (2007). Syne-1 and Syne-2 play crucial roles in myonuclear anchorage and motor neuron innervation. *Development* **134**, 901-908.
- Zhen, Y. Y., Libotte, T., Munck, M., Noegel, A. A. and Korenbaum, E. (2002). NUANCE, a giant protein connecting the nucleus and actin cytoskeleton. *J. Cell Sci.* **115**, 3207-3222.