Nesprin-3: a versatile connector between the nucleus and the cytoskeleton

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Abstract
The cytoskeleton is connected to the nuclear interior by LINC (linker of nucleoskeleton and cytoskeleton) complexes located in the nuclear envelope. These complexes consist of SUN proteins and nesprins present in the inner and outer nuclear membrane respectively. Whereas SUN proteins can bind the nuclear lamina, members of the nesprin protein family connect the nucleus to different components of the cytoskeleton. Nesprin-1 and -2 can establish a direct link with actin filaments, whereas nesprin-4 associates indirectly with microtubules through its interaction with kinesin-1. Nesprin-3 is the only family member known that can link the nuclear envelope to intermediate filaments. This indirect interaction is mediated by the binding of nesprin-3 to the cytoskeletal linker protein plectin. Furthermore, nesprin-3 can connect the nucleus to microtubules by its interactions with BPAG1 (bullous pemphigoid antigen 1) and MACF (microtubule–actin cross-linking factor). In contrast with the active roles that nesprin-1, -2 and -4 have in actin- and microtubule-dependent nuclear positioning, the role of nesprin-3 is likely to be more passive. We suggest that it helps to stabilize the anchorage of the nucleus within the cytoplasm and maintain the structural integrity and shape of the nucleus.

Introduction
In eukaryotic cells, the nucleus is surrounded by an NE (nuclear envelope), consisting of an inner and an outer nuclear membrane [1]. The nucleus is mechanically coupled to the cytoskeleton by members of the nesprin protein family that reside in the outer nuclear membrane. Together with SUN proteins of the inner nuclear membrane, these proteins form LINC (linker of nucleoskeleton and cytoskeleton) complexes that connect the nucleoskeleton with the cytoskeleton. They play a key role in nuclear positioning and centrosome attachment to the nucleus [2–4].

Of the mammalian nesprin protein family, four members have been identified to date: nesprin-1–4 [5–9]. Nesprins are characterized by the presence of a C-terminal KASH (Klarsicht/ANC-1/SYNE homology) domain that consists of a transmembrane region and approximately 30 amino acids extended into the lumen of the NE, the perinuclear space. Furthermore, these proteins have multiple spectrin repeats in their N-terminal region. The giant isoforms of nesprin-1 and -2 are highly homologous with one another and differ from other nesprins by their enormous size (∼976 and ∼764 kDa respectively). Additionally, nesprin-1 and -2 contain an N-terminal ABD (actin-binding domain) that connects the NE with the actin cytoskeleton. Linkage of nesprin-3 and -4 to the cytoskeleton is indirect. Nesprin-4 binds the motor protein kinesin-1 and thus connects the NE with microtubules [7], whereas nesprin-3 mediates interaction with IFs (intermediate filaments) through its binding to plectin [6]. In the present review, we focus on nesprin-3 and discuss its binding partners, expression pattern and potential function.

Domain structure and binding partners of nesprin-3
Nesprin-3, also known as NET (nuclear envelope transmembrane protein) 53 [10], is a highly conserved member of the nesprin family in vertebrates [11]. In both mice and zebrafish, the nesprin-3 gene encodes two protein isoforms, nesprin-3α and nesprin-3β [6,12]. Nesprin-3α consists of eight spectrin repeats followed by a C-terminal KASH domain that mediates the type II insertion of this protein into the outer nuclear membrane (Figure 1). The nesprin-3β isoform is the result of alternative mRNA splicing and, in the case of mouse nesprin-3β, the use of an alternative transcription initiation site [6,12]. Compared with the nesprin-3α isoform, mouse nesprin-3β lacks the complete first spectrin repeat, whereas zebrafish nesprin-3β only lacks a conserved stretch of seven amino acids in the first repeat (Figure 1).

Binding partners identified for nesprin-3 are the SUN proteins and plectin. SUN proteins are situated in the inner nuclear membrane with their N-termini facing the nucleoplasm [13,14]. They are characterized by the presence of a conserved SUN domain at their C-terminus that extends into the lumen of the NE and binds the KASH domain of nesprin family members [14,15] (Figure 2). Interaction with SUN proteins is required to retain nesprin molecules...
Nesprin-3 isoforms and binding partners

The domain organization of the nesprin-3α isoform is conserved in mice and zebrafish, whereas the nesprin-3β isoform is different. The area in the grey rectangle is enlarged below the nesprin-3 isoforms and indicates the difference between nesprin-3α and nesprin-3β in zebrafish. The two residues in blue are conserved among species and are essential for binding to the plectin ABD. The different isoforms of BPAG1 that bind nesprin-3α, BPAG1α and BPAG1β, are indicated. BPAG1α3 is unable to bind nesprin-3α. For simplicity, only the domains shared by two MACF isoforms are shown. The ABDs of the different nesprin-3α-interacting partners are all composed of two CH domains. IF-BD, IF-binding domain; MT, microtubule; MT-BD, microtubule-binding domain; PBD, plectin-binding domain.

Nesprin-3 can bind to SUN1, SUN2 and SUN3 and these interactions require the last four amino acids (PPPT) of the nesprin-3 KASH domain [15–17]. SUN proteins can also interact with lamin A [2,18], establishing a nesprin–SUN protein bridge between the nuclear lamina and the cytoplasm (Figure 2).

The cytoplasmic N-terminus of nesprin-3 interacts with plectin, a member of the plakin family of cytoskeletal linker proteins [19]. Interestingly, nesprin-3 was initially identified as a binding partner of the plectin ABD by yeast two-hybrid analysis [6]. Two residues in the first spectrin repeat of nesprin-3α are essential for this interaction [12] (Figure 1). As nesprin-3β lacks these residues, this isoform is unable to interact with plectin. Plectin is a highly versatile molecule that can cross-link the actin cytoskeleton with IFs via its N-terminal ABD and C-terminal plakin repeats respectively [20] (Figure 1). Binding of F-actin (filamentous actin) and nesprin-3α to the ABD of plectin are considered to be mutually exclusive events due to overlap within their binding sites [12]. As a consequence, the interaction between nesprin-3α and plectin can only facilitate NE linkage to the IF system and not to F-actin (Figure 2). The plectin gene (PLEC1) encodes multiple isoforms that differ in their N-termini and are differentially expressed in tissues [21]. Nesprin-3α interacts with the ABD of several plectin isoforms, such as plectin-1A and plectin-1C [6], suggesting that the alternative sequences preceding the ABD in the different plectin isoforms do not influence the binding of plectin to nesprin-3α.

The plectin ABD consists of two CH (calponin homology) domains that together form a functional actin-binding motif (Figure 1). This domain is not unique to plectin and is also present in other cytoskeletal linker proteins, nesprin-1 and nesprin-2 [5,8,19]. Nesprin-3α not only interacts with the plectin ABD, but also binds to the ABD of the neuronal and muscular isoforms of BPAG1 (bullous pemphigoid antigen 1), BPAG1α and BPAG1β [6,22] (Figure 1). BPAG1α3, a variant of BPAG1α that lacks the first CH domain, cannot bind to nesprin-3α [22,23]. Consistent with the binding ability to nesprin-3α, the residues critical for binding of the plectin ABD to nesprin-3α are located in the first CH domain and are conserved in BPAG1 [12,24].

Whereas plectin contains a C-terminal IF-binding region, BPAG1α and BPAG1β have a C-terminal microtubule-binding domain [19] (Figure 1). From this, one could postulate that nesprin-3α mediates a link between the NE and microtubules (Figure 2). Such a link could also occur through interaction of nesprin-3α with the ABD of yet another microtubule-binding plakin: MACF (microtubule–actin...
Figure 2 | Schematic representation of the interactions of nesprin-3 in cells
Nesprin-3α can link the NE to IFs or microtubules via its interactions with the cytoskeletal linker proteins plectin, BPAG1 and MACF. Whereas plectin can also cross-link IFs with F-actin, BPAG1 and MACF can interconnect the microtubule and actin cytoskeleton. A close-up of the NE shows the interaction between nesprin-3 and SUN proteins, which is thought to take place between dimers of both proteins [2,16]. Additionally, nesprin-3 can interact with the luminal proteins torsinA and torsinB, which assume a hexameric configuration. INM, inner nuclear membrane; MT, microtubules, NPC, nuclear pore complex; ONM, outer nuclear membrane.

Nesprin-3 expression pattern
Nesprin-3 expression analysis of mouse tissues by Western blotting demonstrated its presence in all of them, albeit at various levels [6]. Furthermore, nesprin-3 was found to be an integral NE protein of rodent liver and rat skeletal muscle [10,31]. As yet, these studies do not provide information on the actual cell types that contribute to the expression of nesprin-3 in tissues. However, results from studies with cell lines suggested that the protein is expressed in many different cell types, including MEFs, macrophages, T-cell hybridomas and different squamous cell carcinoma cells [6]. Additionally, nesprin-3 was found to be present in human leucocytes [32]. In zebrafish embryos, nesprin-3 was primarily expressed in epidermal and skeletal muscular tissue [12].

Differentiation of cells of the human HL-60/S4 myeloid leukaemic line towards macrophages led to an induction of nesprin-3 expression [33]. Similarly, the expression of plectin and vimentin was also up-regulated upon differentiation. In contrast, expression of these proteins was either low or absent in undifferentiated cells or cells differentiated towards granulocytes [33]. Differentiation-dependent regulation of the expression of nesprin-3 has also been observed in C2C12 myoblasts. Nesprin-3 is strongly expressed in undifferentiated myoblasts, but expression decreases as these cells differentiate into myotubes ([34] and M. Ketema and A. Sonnenberg, unpublished work).

In most cell types, the expression of nesprin-3 is confined to, and homogeneously distributed over, the NE [16,35,36]. However, there are some exceptions. In round and elongating spermatids, nesprin-3 was primarily observed at both the posterior pole and the anterior side of the sperm head [17]. When spermatogenesis progresses, the localization of nesprin-3 becomes restricted to the anterior pole of the sperm head, where it is suggested to form a non-nuclear complex at the acrosomal membrane system with SUN1η, an isoform of SUN1 [17]. Although plectin expression is reported in spermatids [37], it is unclear whether this non-nuclear LINC complex mediates attachment of the sperm head to the IF system. Another example of non-nuclear nesprin-3 localization is found in pre-adipocytes, in which nesprin-3 was primarily associated with ER membranes [38]. Interestingly, upon induction of in vitro
adipogenesis, nesprin-3 was transiently localized at the NE [38]. It will be interesting to investigate whether the change in nesprin-3 localization is dependent on the expression of SUN proteins and whether a similar redistribution is also observed for other nesprin family members. The dynamics of nesprin-3 localization and expression during differentiation need further investigation.

**Function of nesprin-3**

Despite the functional conservation between zebrafish and mouse nesprin-3, nesprin-3-deficient zebrafish are viable and fertile, and display no obvious abnormal phenotype [12]. The only defect observed to date is a partial loss of the association of keratin filaments with the nuclear perimeter in epidermal cells [12]. Abnormalities were also absent following (partial) disruption of the nesprin-2 gene ([Syne]-2) [39,40]. However, on closer inspection, several defects have now been observed. These include defects in nuclear migration in neuronal precursor and photoreceptor cells during cortical and retinal development, respectively [39,41,42]. In fact, these abnormalities are found in tissues with low or absent expression of nesprin-1, supporting a redundancy in function between nesprin-1 and -2. More detailed analyses have to be performed to investigate whether, as in mice with disrupted Syne-2, the loss of nesprin-3 in zebrafish is associated with subtle defects.

As suggested above, nesprin-3 might link the NE not only to IFs, but also to microtubules via BPAG1a and BPAG1b or MACF. A link to microtubules has been demonstrated previously for nesprin-4 [7]. Furthermore, nesprin-1 and -2 cannot only connect the NE to the actin cytoskeleton, but also to the molecular motor proteins dynein and kinesin [41–44]. Both proteins play an important role in the bidirectional movements of the nucleus when it migrates along microtubules. In contrast, the connection of nesprin-3 via BPAG1a and BPAG1b or MACF to microtubules is likely to be more important after completion of nuclear migration when the conditions become more static. It therefore seems unlikely that nesprin-3 and the other nesprin family members are redundant in their function to mediate binding to microtubules. However, a functional redundancy among members of the nesprin family could arise through the fact that the three cytoskeletal systems are connected to each other by members of the plakin family of cytoskeletal cross-linkers [19] (Figure 2). Hence, although a loss of nesprin-3 will weaken the link between the NE and the IF system, this link will be largely kept intact through connections between IFs and actin microfilaments, which in turn are connected to the NE via nesprin-1 and -2.

The cytoskeleton is not only attached to the NE, but also connected to the plasma membrane at cell–matrix adhesions such as focal adhesions and hemidesmosomes [19]. We have proposed previously that an indirect protein link exists between the plasma membrane (via integrin α6β4 and plectin), IFs and the NE (via plectin and nesprin-3α) [6]. This protein link might be a direct route for sensing mechanical stress, ultimately leading to altered gene expression [45]. The function of LINC complexes in the maintenance of mechanical integrity was investigated by transfection of fibroblasts with dominant-negative KASH constructs [15,36]. As SUN proteins are a limiting factor in the recruitment of nesprins to the NE, overexpression of a KASH construct will lead to a displacement of nesprins from the NE. This results in functional disruption of all endogenous LINC complexes. Cytoskeletal organization in the perinuclear area was indeed affected following overexpression of dominant-negative KASH constructs. The close association of the vimentin network with the NE was partially lost, and actin stress fibres were fragmented in appearance around the nucleus [36]. Furthermore, the intracellular force transmission between the cytoskeleton and the nucleus was disturbed [36], resulting in a reduced mechanical stiffness of the cells [15]. These effects are reminiscent of previously reported observations in Lmna−/− MEFs, in which loss of lamin A/C reduced mechanical stiffness [46–48]. Absence of lamin A/C also resulted in a redistribution of nesprin-3 from the NE to the ER and disrupted attachment of cytoskeletal components to the NE [35,49]. The similarities in phenotype between LINC complex disruption and loss of lamin A/C support further the theory that nesprins and lamin A are part of the same complex.

Although LINC complexes are clearly involved in maintenance of mechanical integrity, it is currently unknown whether this function is dependent on nesprin-3 or primarily mediated by other members of the nesprin family. The simultaneous loss of nesprin-3 and vimentin from the nuclear perimeter in Lmna−/− cells and cells transfected with dominant-negative KASH constructs is in accordance with a nesprin-3- and plectin-mediated interaction between the NE and the IF system. However, Lombardi et al. [36] also demonstrated that the ectopic expression of mini-nesprin-2 giant, a short fusion protein consisting of the ABD and KASH domain regions of nesprin-2, enhanced the intracellular force transmission between the cytoskeleton and the nucleus. This suggests that a connection with the actin cytoskeleton, as mediated via nesprin-1 and -2, plays a more prominent role in the establishment of mechanical integrity than the attachment of other cytoskeletal components to the NE. Furthermore, silencing of nesprin-2 was also found to affect the organization of keratin bundles in the nuclear perimeter [43], suggesting that the localization of IFs at the NE might not be solely dependent on nesprin-3. Hence, further studies are needed to identify the actual nesprin family members required for the maintenance of cellular stiffness.

**Future perspectives**

Studies regarding nesprin-1 and -2 have demonstrated tissue-specific and differentiation-dependent expression patterns [39,50]. Phenotypic observations in knockout mice were primarily made in tissues in which these nesprin family members are differentially expressed [39,41,42]. Hence,
References


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Further work aimed at identifying nesprin-3 function should include a comparison of its cell and tissue distribution with that of the other nesprins. This information should provide insight as to which tissues are affected by loss of nesprin-3 in animal models. Subsequent mechanical studies with nesprin-3-knockout cells will clarify whether nesprin-3 has a role in force transmission to the nucleus or whether its attachment to the IF system is primarily responsible for the strength and stability of the nucleus.

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