Nuclear alignment in myotubes requires centrosome proteins recruited by nesprin-1

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Abstract

Myotubes are syncytial cells, generated by fusion of myoblasts. Among the numerous nuclei in myotubes of skeletal muscle fibres, the majority are equidistantly positioned at the periphery, except for clusters of multiple nuclei underneath the motor endplate. The correct positioning of nuclei is thought to be important for muscle function and requires nesprin-1, a protein of the nuclear envelope. Consistently, mice lacking functional nesprin-1 show defective nuclear positioning and mimic aspects of Emery-Dreifuss muscular dystrophy. In this study, we perform siRNA experiments in C2C12 myoblasts undergoing differentiation, demonstrating that the positioning of nuclei requires PCM-1, a protein of the centrosome that relocates to the nuclear envelope at the onset of differentiation, dependent on the presence of nesprin-1. PCM-1 itself is required for recruiting proteins of the dynein/dynactin complex and of kinesin motor complexes. This suggests that microtubule motors that are attached to the nuclear envelope support the movement of nuclei along microtubules, to ensure correct positioning in the myotube.
Introduction

Muscle dysfunctions in several diseases have been correlated with mutations in nuclear envelope genes, such as emerin, lamin A, and nesprins 1 and 2. In particular, mutations in these genes have been reported in Emery-Dreifuss muscular dystrophy, as well as ataxias and cardiomyopathies (Ellis, 2006; Gros-Louis et al., 2007; Wheeler et al., 2007; Zhang et al., 2007a; Attali et al., 2009; Puckelwartz et al., 2009, 2010). It has been speculated that these mutations may affect signalling pathways in muscle cells, but the exact role of nuclear envelope components in myopathies remains unclear. Whereas emerin and lamins are proteins that associate with inner nuclear membrane, the nesprins are transmembrane proteins that span a wide distance from the perinuclear space to the cytoplasm. In the perinuclear space, nesprins are anchored to proteins of the SUN family by their conserved C-terminal KASH domain, whereas in the cytoplasm, they are believed to interact with the cytoskeleton, via N-terminal calponin-homology or plectin-binding domains and spectrin repeats. At least four nesprin genes exist in humans and mice (nesprins 1, 2, 3, 4), of which nesprin-4 is specifically expressed only in secretory epithelia (Zhang et al., 2001; Wilhelmsen et al., 2005; Roux et al., 2009).

In muscle cells, nesprin-1 has been implicated in nuclear positioning in the syncytial cytoplasm and in clustering a subset of synaptic nuclei at the neuromuscular junction (Grady et al., 2005; Zhang et al., 2007b; Lei et al., 2009; Zhang et al., 2010). Consistently, nesprin-1 has been found particularly enriched on nuclei beneath the neuromuscular junction (Apel et al., 2000), besides fainter localization to non-synaptic nuclei. Interference with the function or the localization of nesprin-1 abolished correct myonuclear anchorage and led to respiratory failure and death in mice (Zhang et al., 2007b; Puckelwartz et al., 2009; Zhang et al., 2010). However, it remains unclear by what mechanism nesprin-1 mediates nuclear anchorage. In
various studies on non-muscular cells, nesprins have been implied to interact with the actin cytoskeleton (Starr and Han, 2002; Zhen et al., 2002; Padmakumar et al., 2004). In addition, nesprins and related proteins have been shown to interact with components of the microtubule network, such as kinesin, dynein, dynactin, and with the centrosome (Malone et al., 2003; Roux et al., 2009; Zhang et al., 2009; Zhou et al., 2009; Fridolfsson et al., 2010; Yu et al., 2011; Wilson and Holzbaur, 2012, 2015). Several groups reported that proteins of the centrosome, such as PCM-1, pericentrin and γ-tubulin, are re-located from the pericentriolar material to the nuclear envelope upon onset of myoblast differentiation, and that a significant amount of microtubules grow from the nuclear surface following this reorganisation (Tassin et al., 1985; Bugnard et al., 2005; Srsen et al., 2009; Fant et al., 2009). In this study, we show that nesprin-1 is essential for the relocalisation of centrosome proteins and components of microtubule motor complexes to the nuclear envelope in differentiating mouse myoblasts, and that nesprin-1 and the centrosome protein PCM-1 are needed for regular positioning of nuclei in these cells.
Results

Nesprin-1 expression correlates with the recruitment of pericentriolar proteins at the nuclear envelope.

To identify factors that recruit centrosome proteins to the surface of the nuclear envelope during myoblast differentiation, we investigated the potential role of proteins of the outer nuclear membrane. To date, the only known proteins specifically localized at the outer nuclear membrane are members of the KASH family; in mouse muscle cells these include nesprins 1, 2, and 3 (Hetzer, 2010).

We reinvestigated the localization of nesprins in C2C12 cells, since conflicting reports on the localization of nesprin-1 and 2 in skeletal muscle cells have been published (Apel et al., 2000; Zhang et al., 2001; Mislow et al., 2002; Zhang et al., 2005). Because nesprins exist in alternative splice variants, we performed immunofluorescence and western blot experiments with antibodies against the C-terminal KASH domain that is present in nuclear envelope-bound isoforms. In undifferentiated C2C12 myoblasts, nesprin-2 and nesprin-3 were expressed at substantial levels and localized to the nuclear envelope, whereas the expression of nesprin-1 was very weak (Fig. 1A, B). Upon differentiation into myotubes, nesprin-1 signal increased and accumulated at the nuclear envelope, while at the same time the centrosome protein pericentrin re-localized to the nuclear surface. The accumulation of nesprin-1 at the nuclear envelope correlated with the appearance of a 115kDa band on western blots, corresponding to the nesprin-1α isoform (Fig. 1B), which is the prominent isoform expressed in human muscle cells (Randles et al., 2010). Concomitantly, nesprin-2 signal disappeared from the nuclear envelope, while nesprin-3 remained (Fig. 1A). This pattern of nesprin localization at the nuclear envelope was confirmed using two different sets of antibodies.
against nesprins 1 and 2 (suppl. Fig. S1 A, B). Depending on the antibody used, nesprin-2-immunofluorescence in C2C12 myotubes was seen in intranuclear structures as previously described (Zhang et al., 2005), or diffuse in the cytoplasm (Fig. 1A, suppl. Fig. S1B). The nature of the intranuclear structures is unknown, but RNA silencing experiments suggest that these might represent unspecific targets of the antibody (Fig. S1B). Since immunoblotting against nesprin-2 failed to provide specific bands, we examined expression levels of nesprin-2 in comparison to those of nesprin-1, by reverse transcription from C2C12 RNA obtained prior and after differentiation, followed by PCR. Using PCR primers complementary to spectrin-repeat-containing sequences found in a large variety of possible splice variants of nesprin-1 or nesprin-2, we obtained strong signals for nesprin-1 only upon differentiation of C2C12 into myotubes, in contrast to nesprin-2, of which levels dropped in differentiating cells (Fig. 1C).

**Nesprin-1 but not nesprin-3 is needed for the recruitment of pericentriolar proteins to the nuclear envelope**

Because nesprin-2 was found absent from the nuclear envelope in myotubes, we tested whether nesprins 1 or 3 are involved in the recruitment of centrosome proteins to the nuclear envelope in differentiating myoblasts. We depleted up to 80% of nesprin-1α or nesprin-3 protein by siRNA treatment of C2C12 cells (Fig. 2A, B). As a consequence, the recruitment of pericentrin to the nuclear envelope was inhibited in nesprin-1-depleted cells that had fused into myotubes (Fig. 2C). Interestingly, numerous myotubes showed nesprin-1-positive as well as negative nuclei in the same cytoplasm. This was likely due to the fact that nesprin-1 depletion started already prior to fusion of C2C12 cells, enabling non-depleted cells to fuse with depleted cells. Thus, in heterogeneous myotubes, only nuclear envelopes with a high level of nesprin-1 also displayed enriched amounts of pericentrin (Fig. 2C, asterisk). Since pericentrin levels at the nuclear envelope varied between myotubes, we quantified the
immunofluorescence intensity within a perinuclear rim of approximately 0.5μm thickness. For these measurements, we analysed myotubes that contained both nesprin-1-positive and negative nuclei. Nuclei that were depleted of nesprin-1 showed only 37%±15 (n=20) of pericentrin intensity, as compared to control nuclei. The effects of nesprin-1 siRNA could be reproduced with dsRNAs against two different targeting sequences (see Materials and Methods), thus reducing the risk of unspecific off-target effects. In contrast to nesprin-1-depletion, the removal of nesprin-3 did not alter significantly the amounts of nuclear envelope-bound pericentrin (Fig. 2D). Quantification of pericentrin signal was performed in myotubes containing nesprin-3-depleted and undepleted nuclei (Fig. 2D, asterisk) in the same cytoplasm. Pericentrin levels on nesprin-3-depleted nuclear envelopes were 89%±20 (n=21). As a control, silencing of nesprin-2 was equally attempted (see Materials and Methods), but was difficult to monitor due to the absence of nesprin-2 immunofluorescence in C2C12 myotubes, or absence of specific nesprin-2 signal on immunoblots. Treatment with siRNA against nesprin-2 was without effect on the localization of centrosome proteins (Figure S1B). When investigating the fate of centrosome proteins other than pericentrin, we found that depletion of nesprin-1 also led to the reduction of PCM-1 at the nuclear envelope (Fig. 2E), γ-tubulin (Fig. 4A), and CDK5RAP2 (data not shown). None of these reductions were seen after depletion of nesprin-3 (Fig. 2F). We verified by immunoblotting that the depletion of nesprin-1 or nesprin-3 did not affect the overall protein levels of centrosome proteins, such as PCM-1 (Fig. 2 A, B). To test whether the anchoring of centrosome proteins to the nuclear envelope involved proteins of the Golgi apparatus or of the endoplasmic reticulum (ER), we tested the effects of the Golgi-disrupting drug Brefeldin A, and siRNA against the ER-resident chaperone calnexin (Fig. 2G, H). Although it is known that Golgi membranes accumulate around the nuclear surface upon differentiation of muscle cells (Tassin et al., 1985b) and that ER membranes are continuous with the outer nuclear membrane, none of the two seemed to
be essential for the anchorage of centrosome proteins, since neither Brefeldin A nor calnexin siRNA reduced the accumulation of pericentrin at the nuclear envelope (Fig. 2 G, H).

Because silencing of nesprin-1 expression prevented the accumulation of centrosome proteins at the nuclear envelope, we tested whether displacement of nesprins from the nuclear membrane caused a similar effect. Consistently, we found that high overexpression of the nesprin-1-KASH domain displaced endogenous nesprin-1 from the nuclear envelope in myotubes and prevented the relocalization of centrosome proteins to the nuclear envelope (Fig. 3A, B). This was also achieved by depletion of the SUN proteins 1 and 2, which localize to the inner nuclear membrane (Fig. 3C, D), and which are known to anchor nesprins to the nuclear envelope (Padmakumar et al., 2005; Crisp et al., 2006). To test the association between nesprin-1 and centrosome proteins biochemically, we performed immunoprecipitation of nesprin-1 from extracts of nuclear envelopes, prepared from C2C12 myotube nuclei (see ‘Materials and Methods’ and suppl. Fig. S1C for details). The centrosome protein PCM-1 was found to co-immunoprecipitate under these conditions, whereas γ-tubulin was absent from the precipitate (Fig. 3E). Consistently, immunoprecipitation of PCM-1 co-precipitated part of nesprin-1α in the reverse experiment (Fig. 3E).

To ensure that the depletion of nesprin-1 did not cause unspecific interference with centrosome protein re-localization by global disorganization of the nuclear envelope, we stained for nuclear pore complexes using monoclonal antibody 414 that recognizes a subset of nucleoporins (Davis and Blobel, 1986), and for nesprin-3. Both were still correctly localized in nesprin-1-depleted myotubes, indicating that the structure of the nuclear envelope was not globally affected (Fig. 3F; supplemental Fig. S1D).

Altogether, our data suggest that nesprin-1 is specifically involved in anchoring centrosome proteins to the nuclear envelope during myoblast differentiation and myotube formation. To
test whether nesprin-1 is sufficient for the recruitment of centrosome proteins to the nuclear envelope, we expressed exogenous, mCherry-tagged nesprin-1α in undifferentiated C2C12 myoblasts, i.e. under conditions that show only minor amounts of endogenous nesprin-1 on the nuclear envelope. We observed that the tagged, exogenous nesprin-1α localized to the nuclear envelope even in undifferentiated C2C12 cells (Fig. 3G). Immunofluorescence of the centrosome protein PCM-1 revealed that 47%±1 of C2C12 cells that incorporated mCherry-nesprin-1α at the nuclear envelope also showed relocalization of PCM-1 (data from 4 different experiments, counting a total of 7163 cells). In these cells, PCM-1 was either entirely recruited to the nuclear surface (top row of Fig. 3G, left), or part of PCM-1 remained in the pool of cytoplasmic, pericentriolar satellites (2nd and 3rd row of Fig. 3G, left). PCM-1 accumulation at the nuclear envelope was not seen in non-transfected neighbouring cells. In control transfections, we expressed mCherry-tagged tubulin-α or mEmerald-tagged nesprin-3 (Fig. 3 H, I). Tagged alpha-tubulin incorporated into the microtubule network, but failed to relocalize PCM-1 to the nuclear envelope (only 2%±1 of a total of 1224 cells with nuclear envelope-bound PCM-1, in 3 different experiments). Likewise, tagged nesprin-3 localized to the nuclear envelope in addition to structures resembling the Golgi apparatus, but induced relocalization of PCM-1 only in 9%±3 of the transfected cells (n=124; Fig. 3H). Moreover, comparison of the ratio of fluorescence intensity of PCM-1 between the nuclear envelope (within a rim of 0.5µm thickness) and the neighbouring cytoplasm revealed that nesprin-1 expression increased PCM-1 immunofluorescence by a factor of 1.7±0.3 (n=5), whereas nesprin-3 expression led to an insignificant increase of 1.1±0.1. Altogether, this suggested that PCM-1 relocalization to the nuclear envelope upon nesprin-1α expression was specific. To exclude that those C2C12 cells displaying PCM-1 at the nuclear envelope had undergone premature differentiation in culture, we performed immunofluorescence of the differentiation marker myosin II heavy chain (Fig. 3J). We quantified that only 1% of mCherry-nesprin-1α-
expressing cells stained positively for myosin II heavy chain (in a total of 970 cells),
demonstrating that relocalization of PCM-1 to the nuclear envelope could be induced by
nesprin-1α, independent of a full differentiation program.

Abnormal nuclear positioning in myotubes depleted of nesprin-1 or PCM-1
Because the localization of the microtubule-nucleator γ-tubulin was reduced at nesprin-1-
depleted nuclear envelopes in myotubes (Fig. 4A, second column, right nucleus), we tested
whether the microtubule network in these cells was altered. Unlike control myotubes, nesprin-
1-depleted myotubes contained a large number of nuclei (~70%) that failed to re-grow
microtubules efficiently after recovery from nocodazole-induced depolymerisation (Fig. 4B),
suggesting that the absence of centrosome proteins from the nuclear surface prevented
microtubule nucleation. Surprisingly, in cell cultures that had not been treated for microtubule
depolymerisation, both control myotubes and nesprin-1-depleted myotubes displayed similar
microtubule network organization (Fig. 4C). To test whether the number of microtubules in
the vicinity of nuclei were reduced upon nesprin-1-depletion, we tracked individual
microtubules within a distance of ~1 μm to the nuclear surface, in image stacks of myotubes.
Optical sections of complete myotubes were grouped by performing sum projections of partial
stacks, allowing the tracking of individual microtubules over a large volume (Fig. 4D). We
counted approximately 600 microtubules close to each nucleus, for controls as well as for
nesprin-1-depleted nuclei (Fig. 4E). Long microtubules, oriented parallel to the axis of the
myotube, were usually seen curving around the surface of the nuclei in both conditions, with
only 12% ± 2 (control) to 14% ± 1 (nesprin-1-depletion) of microtubules oriented approximately
perpendicular to this axis (Fig. 4F). Analysis of deconvolved 3D data sets of microtubules
suggested that end-on contacts of microtubules with the nuclear envelope were rare, although
the exact origins and ends of microtubules were difficult to trace. To find out whether the
number of microtubules emanating from the nuclear surface was significant in myotubes in a steady state (with an established equilibrium of polymerized versus non-polymerized tubulin), we quantified the immunofluorescence signal intensity of microtubules in myotube segments containing nuclei (segment 1 in scheme Fig. 4G), in tangential segments at the periphery of nuclear envelopes (segment 2), and in areas free of nuclei (segment 3). Both segments 1&2, at or near nuclei, showed a microtubule intensity that was 10-20% higher as compared to nuclei-free cytoplasmic segments, raising the possibility that there was a small contribution of myotube nuclei to microtubule nucleation or microtubule anchoring. However, this percentage was not significantly altered in myotubes with nesprin-1-depleted nuclei (Fig. 4G), indicating that the small number of microtubules at the nuclear envelope did not require major amounts of nuclear envelope-bound centrosome proteins under equilibrium conditions, unlike microtubule re-growth after depolymerisation that is favoured only if many nucleation sites are instantly available (Fig. 4 A, B).

**Positioning of myotube nuclei, dependent on nesprin-1 and PCM-1**

The most notable change in myotubes, depleted of nesprin-1, was an abnormal positioning of nuclei within the syncytial cytoplasm (Fig. 5A): whereas 76% of control myotubes showed nuclei aligned evenly along the long axis of the cell, only 21% of nesprin-1-depleted myotubes contained aligned nuclei (Fig. 5A, graph). Moreover, the spacing between nuclei was reduced and multiple nuclei often concentrated into small packages (Fig. 5A). To determine whether centrosome proteins mediated any aspect of nesprin-1-dependent nuclear positioning, we depleted PCM-1, since this has previously been characterized as a protein necessary for the recruitment of many other centrosome components (Dammermann and Merdes, 2002), also including binding of pericentrin to the nuclear envelope (suppl. Fig. S1E; in the reverse experiment, i.e. depletion of pericentrin, PCM-1 was not altered). As compared
to nesprin-1-depletion, a similar although weaker effect on nuclear positioning was seen after depletion of PCM-1, yielding 55% of cells with aligned nuclei (Fig. 5A, graph). The depletion led to removal of 74% of PCM-1 in cultures overall (Fig. 5B, quantification of immunoblot), but for the quantification of nuclear positioning, only myotubes lacking PCM-1 at the nuclear envelope were counted. We verified that depletion of PCM-1 did not affect the overall protein levels of nesprin-1 (Fig. 5B). Moreover, we verified by immunofluorescence that nuclear envelopes depleted of PCM-1 still retained nesprin-1 (Fig. 5B, right).

To understand the potential mechanisms that caused misalignment of nuclei in myotubes depleted of nesprin-1 or PCM-1, we investigated proteins that are known to mediate microtubule-dependent transport. Treatment of differentiated C2C12 cultures with erythro-9-[3-(2-hydroxynonyl)]adenine (EHNA), an ATPase inhibitor that has been shown to interfere with dynein function (Bouchard et al., 1981; Penningroth et al., 1982), led to inhibition of nuclear alignment (Fig. 5A graph). The effect of the dynein inhibitor ciliobrevin A was equally tested, but ciliobrevin A was found to prevent significantly the fusion of myoblasts into myotubes (data not shown). Moreover, the protein p150/glued, a component of the dynactin complex that has been implicated in dynein activation, as well the kinesin light chain 1 (KLC1) were found to localize to the nuclear envelope in control myotubes (Fig. 5C, D). Depletion of nesprin-1, but also of PCM-1 prevented the localization of both p150 and KLC1 at the nuclear envelope (Fig. 5C, D). On the other hand, depolymerization of the microtubule network in myotubes did not remove p150 from the nuclear envelope (Cadot et al., 2012; Fig. 5E), suggesting that motor complex-associated proteins are bound to the nuclear surface, dependent on nesprin-1 and PCM-1, but independent of microtubules.
Discussion

In the present study we employ a cell culture model for muscle differentiation, to show that centrosome proteins are anchored to the nuclear envelope by nesprin-1, and that this interaction is necessary for the recruitment of motor proteins that position nuclei in syncytial myotubes. Our findings are consistent with earlier studies, in which nesprin-related KASH domain proteins have been implicated in nuclear migration and positioning (Starr, 2007), such as UNC-83 in *C. elegans* (Starr et al., 2001), and MSP-300 and Klarsicht in *Drosophila* photoreceptor cells and in striated muscle (Mosley-Bishop et al., 1999; Patterson et al., 2004; Elhanany-Tamir et al., 2012). In mammals, the KASH domain of nesprins is required for nuclear positioning in neurons and in muscle cells, as elimination of KASH-encoding exons or displacement of endogenous nesprins by overexpressed KASH results in defective nuclear migration, localization and anchorage (Grady et al., 2005; Zhang et al., 2007b; Zhang et al., 2009; Zhang et al., 2010; Wilson and Holzbaur, 2012, 2015). Nuclear movement has previously been suggested to be mediated by a physical interaction between nesprins or related proteins such as ZYG-12 and the centrosome, which is at the centre of a radial microtubule network (Malone et al., 2003; Zhang et al., 2009). In early differentiated myotubes, the centrosome no longer exists as a microtubule-organizing centre, but its constituent proteins are re-localized all around the surface of the nucleus (Tassin et al., 1985; Bugnard et al., 2005; Srsen et al., 2009). We show that this depends on the differentiation-specific expression of the nesprin-1α isoform, and that other nesprins are not involved in this process. We did not find nesprin-2 at nuclear membranes of newly differentiated C2C12 cells, although splice variants of nesprin-2 have previously been identified in skeletal muscle (Duong et al., 2014; Zhang et al., 2005). It is possible that nuclear and non-nuclear forms of nesprin-2 are expressed at more advanced stages of muscular differentiation, e.g. upon sarcomere formation, that are not mimicked under our experimental conditions (Zhang et al.,
The only other nesprin at the nuclear envelope at early differentiation, nesprin-3, has a cytoplasmic domain that is largely dissimilar from nesprin-1α (only 8% of sequence identity outside its KASH domain), and does not recruit centrosome proteins (Fig. 2D, F). The detection of premature PCM-1-relocalization to the nuclear envelope prior to differentiation, upon mCherry-nesprin-1α expression, led us to conclude that nesprin-1α is a receptor for centrosome proteins at the nuclear envelope. It is possible that additional proteins are involved for efficient anchoring, since we only obtained partial recruitment of PCM-1. Another possibility would be that differentiation-specific posttranslational modifications of nesprin-1α are needed for a strong interaction with centrosome proteins, and that these modifications are missing in our transfected cells, at the myoblast stage.

How do centrosome proteins affect nuclear positioning in myotubes? Whereas in non-muscular cells forces can be transmitted to the nucleus by the radial, centrosomally anchored microtubule network due to the physical link between the centrosome and the nucleus, a different mechanism is likely active in myotubes: we suggest that the re-localized centrosome proteins all around the nuclear envelope anchor microtubule-dependent motors of the kinesin family and of dynein, allowing nuclei to move laterally along microtubules and to position along the length of the myotube. The dependence of myonuclear distribution on microtubules has originally been inferred from pharmacological experiments and from correlative studies of nuclear position and the microtubule network (Englander and Rubin, 1987; Bruusgaard et al., 2006), and has more recently been shown by Cadot et al. (2012) and Metzger et al. (2012). Initial formation of microtubule filaments in myotubes may involve microtubule-nucleating activity on the nuclear surface (Tassin et al., 1985; Bugnard et al., 2005; Fant et al., 2009). However, microtubules in our early differentiated C2C12 cultures were mostly seen in a parallel orientation along the length of the cell, with long microtubules running past nuclei instead of attaching end-on to their nuclear surface (Fig. 4C, E). Later in the differentiation
process, the microtubule network is further remodelled into an orthogonal grid-like organization, with a large fraction of microtubules growing off Golgi elements (Oddoux et al., 2013). It is possible that upon formation of a microtubule network in myotubes, release of microtubule-ends from their initial nucleation sites or generation of new distant nucleation sites occurs, as seen in many non-muscular cell systems undergoing differentiation (Mogensen, 1999; Musa et al., 2003; Stiess et al., 2010). This would explain loss of microtubule end-binding at the nuclear surface, as seen in our study. Moreover, previously reported microtubule nucleation from nuclear envelopes in myotubes may have been experimentally enhanced by depolymerization of the microtubule network: under these conditions, the concentration of free tubulin is high, and re-nucleation can occur from sites that may not specifically nucleate microtubules under physiological conditions (Mitchison and Kirschner, 1985).

The concept of nuclear positioning by gliding along microtubules is supported by previous studies demonstrating an interaction of kinesin and dynein/dynactin motor complexes with mammalian nesprins 1, 2, and 4, and with the related KASH-domain proteins ZYG-12 and UNC-83 in C. elegans (Malone et al., 2003; Roux et al., 2009; Zhang et al., 2009; Fridolfsson et al., 2010; Yu et al., 2011; Wilson and Holzbaur, 2012, 2015). In myotubes, we propose that this interaction is indirect, mediated by nuclear envelope-bound centrosome proteins, because the depletion of the centrosome protein PCM-1 induces loss of dynactin and kinesin subunits from the nuclear surface. Since PCM-1 is necessary for the targeting and assembly of a variety of proteins of the pericentriolar material (Dammermann and Merdes, 2002), anchoring of motor complexes and nuclear alignment may occur through other centrosome proteins such as pericentrin and Cep135, both known to bind directly to dynein and dynactin polypeptides (Purohit et al., 1999; Uetake et al., 2004). Consistently, the centrosome protein pericentrin was lost from the nuclear envelope when PCM-1 was depleted (suppl. Fig. S1C). Besides
contributing to nuclear positioning, the accumulation of centrosome proteins at the nuclear envelope may serve additional roles in muscular cell types, since a similar accumulation has been found in mono-nucleate mouse cardiomyocytes (Zebrowski et al., 2015). It has been speculated that in mammalian cardiomyocytes, re-localization of centrosome proteins helps maintaining the post-mitotic cell cycle state (Zebrowski et al., 2015).

In skeletal muscle, the ordered positioning of nuclei should be important for correct muscle function, since abnormal positioning has been found in mouse models in which the KASH domain of nesprin-1 was deleted, and which mimicked aspects of Emery-Dreifuss muscular dystrophy (Zhang et al., 2007b; Puckelwartz et al., 2009; Zhang et al., 2010). Interestingly, point mutations in the nesprin-1 gene have been found in a subgroup of patients suffering from Emery-Dreifuss muscular dystrophy (Zhang et al., 2007a). Since defective nuclear positioning is reproduced in our cell culture model, and shown to involve PCM-1 in addition to nesprin-1, it will be important to test in the future whether the localization of centrosome proteins and microtubule motors at the nuclear envelope is affected in any way in Emery-Dreifuss muscular dystrophy, and whether this is involved in pathogenetic mechanisms.
Materials and Methods

Cell culture

C2C12 mouse myoblasts (American Type Culture Collection) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) + Glutamax-I (Invitrogen) supplemented with 20% fetal bovine serum (FBS) and 0.5% chicken embryonic extract. Cells were induced to differentiate by switching to differentiation medium (DMEM + Glutamax-I supplemented with 0.5% FBS, 5µg/mL insulin, 5µg/ml transferrin).

siRNA transfection

siRNAs were designed to target the following sequences: CCAGGGTGAAAGAAGCTAAA and GCTCCTGCTGCTGCTTATT for nesprin-1, AGCCACAGAACTCCAAAT for nesprin-2, GCTACGTAGAATCATCACA for nesprin-3, TGAGCTTCGTGATTCTCAG for PCM-1 (Dammermann and Merdes, 2001), CGTCGGATGCTCTGGATTT and CAGGTGCCTTCGAAATATT for SUN-1, CCGCTGCTCTGAGACTTTT and GCCCTTGCTGCAGACTTTT for SUN-2, and AAGCATCATGCCATCTCTGCT for calnexin. Control experiments were done using the scrambled siRNA sequence UUCUCCGAACGUGUCACGU (Qiagen). C2C12 were seeded at 80,000 cells/cm², and induced to differentiate 4h later. Transfection was performed after 24h in differentiation medium, with Lipofectamine RNAiMAX (Invitrogen). The final concentrations of most siRNAs were 25-30 nM, except for PCM-1, for which a final concentration of 500 nM was necessary to achieve efficient depletion. For nesprin-1 depletion, cells differentiated for 2-3 days were transfected twice with HiPerfect reagent (Qiagen) and 150 nM siRNA, at 48h intervals. For Western blot analysis, cells were recovered by scraping, washed in PBS, and subsequently washed in ‘wash buffer’ (50mM Tris, pH 7.5, 150mM NaCl, and 2mM EDTA). Cells were resuspended in wash buffer containing 0.5% Triton X-100, 1mM DTT and a
cocktail of protease inhibitors (Complete/EDTA free, Roche). Cells were finally lysed by sonication. 70µg of extracts were subjected to SDS-PAGE.

**Immunoprecipitation experiments**

Differentiated C2C12 cells, scraped from petri dishes, were centrifuged in 10mM HEPES, pH 8.0, 1.5mM MgCl₂, 1mM DTT, 1mM PMSF, followed by lysis with 20mM Tris-HCl, pH 7.0, 150mM NaCl, 1% NP-40, 10% glycerol, 5mM EDTA, and a protease inhibitor cocktail (“Complete/EDTA free”, Roche). After sonication and centrifugation for 30 min at 16,100g, 4°C, resulting nuclear pellets were extracted with 20mM Tris-HCl, pH 7.0, 400mM NaCl, 1% octyl beta-D-glucopyranoside, 5mM MgCl₂, 1mM DTT, and protease inhibitors. Protein extracts were diluted to a final concentration of 1mg/ml, in buffer containing 200mM NaCl and 0.5% octyl beta-D-glucopyranoside, and samples of 1ml were incubated with rabbit anti-nesprin-1 or rabbit anti-PCM-1 for several hours at 4°C. Immunoprecipitation was performed with 50µl magnetic protein A beads (Biorad) per sample, followed by rinsing four times in the same buffer, and elution in gel loading buffer containing 2.3% SDS.

**Pharmacological treatments**

Microtubules were depolymerized by treatment with 20µM nocodazole for 40 min at 37°C, and for 40 min on ice. Cells were rinsed once with PBS and twice with growth medium, before re-polymerisation was induced in growth medium at 37°C. Dynein ATPase activity was inhibited using erythro-9-[3-(2-hydroxynonyl)] adenine (EHNA) at 0.1mM for 24-48h. The Golgi apparatus was disassembled by treatment with 5µg/ml Brefeldin A for 90 minutes.
Molecular cloning and transfection

The mouse nesprin-1 cDNA IRAKp961K22116Q was obtained from “Deutsches Ressourcenzentrum für Genomforschung”. A plasmid encoding mEmerald-nesprin-3 was obtained from Addgene (#54203), originally provided by Dr. Michael Davidson, Florida State University, Tallahassee. Nesprin-1α or the nesprin-1 KASH domain were amplified by PCR using the forward primers AAAAAAGAATTCATGGTGGTGGTGGCAGAGGACTTG or AAAAAAGAATTCTCCCCGTTCCTGACCCCCAGGCC, respectively, in combination with the reverse primer TTTTTTGGTACCTCAGAGTGAGGAGGACCGTT. The resulting DNA was digested with EcoRI and KpnI, and cloned into the respective sites of pmCherry-C2 or pEGFP-C2 (Clontech). Transfection of myoblasts was performed using an Amaxa Nucleofector system according to the manufacturer’s protocol (KitV, LonzaBio). Cells were induced to differentiate at 4h to 6h after transfection. For quantitative PCR experiments, total RNA was isolated from C2C12 myoblasts and myotubes using an RNeasy Mini Plus kit (Qiagen). Equal amounts of RNA (1 μg) were added to a reverse transcriptase reaction mix (Superscript II Reverse Transcriptase, Invitrogen) with random primers. The resulting cDNA was subjected to quantitative PCR using a Biorad C1000 thermal cycler, coupled to the CFX96 real-time system, using the SSoFast Evagreen Supermix (Biorad) for 40 cycles. Primers were used for nesprin-1 (forward: cttctgttcggagatctc, reverse: aggtgagtccaataagcagca), nesprin-2 (forward: atgtcaccagcccagagg, reverse: gacgagcctactcaatctt), as well as for two housekeeping genes, hypoxanthine guanine phosphoribosyl transferase (HGPRT1, forward: TCCTCTCAGAGCGCTTTT, reverse: CCTGGTTCATCATCGCTAATC), and ribosomal protein S16 (Rps16, forward: AGGAGCGATTTTGGGTGGTGG, reverse: GCTACCAGGGCCTTTGAGATGG).

Calibration curves were performed on each myoblast and myotube cDNA, to measure the efficiency of the primer pairs used. Corresponding reaction mix containing RNA without
reverse transcriptase was used as a negative control for the qPCR of each sample. Levels of nesprin-1 and nesprin-2 cDNA were expressed relative to those of Rps16 and HGPRT1, using the Biorad CFX Manager software.

**Immunofluorescence microscopy**

C2C12 cells grown on coverslips were fixed in methanol at −20°C and processed for immunofluorescence following standard protocols. For staining of γ-tubulin, microtubules, and dynactin complexes, cells were pre-permeabilized in PEM buffer (80mM PIPES, 5mM EGTA, 2mM MgCl₂, pH 6.8) containing 0.5% Triton X-100 (30s-5 min) or 0.05% saponine (5-15 min). The coverslips were rinsed with PEM containing 4% polyethylene glycol and fixed with 4% paraformaldehyde in PEM for an initial period of 5 min, followed by a second fixation step with paraformaldehyde in carbonate buffer (50mM, pH 10) for 10min. Antibody labelling was performed according to standard protocols.

**Antibodies**

The following antibodies were used: rabbit anti-nesprin-1 (Abcam), mouse anti-nesprin-1 MANNESAL(7A12) (Randles et al., 2010), rabbit anti-nesprin-2 (raised against mouse nesprin-2, amino acids 1465 to 1707 of clone NP_001005510), rabbit anti-nesprin-2, #4 (Zhang et al., 2007b), mouse mAb NSP-3 anti-nesprin-3 (Ketema et al., 2007), mouse anti-α-tubulin clone B-5-2-1 (Sigma), rabbit anti-calnexin (Stressgen Bioreagents), mouse anti-γ-tubulin TU-30 (Exbio), mouse anti-sarcomeric myosin MF20 (DSHB), mouse anti-nuclear pore complex proteins, clone 414 (Abcam), mouse anti-p150[glued] (BD Transduction Laboratories), mouse anti-pericentrin (BD Transduction Laboratories), rabbit anti-pericentrin (Covance), anti-KLC1 H75 (Santa Cruz), rabbit and mouse anti-PCM-1 (Dammermann and Merdes, 2002), rabbit anti-SUN-1 and SUN-2 (raised against peptides NH2-CHKLEPVFDSPRMSR-CONH2 and...
NH2-CLGRFTYDQEGLS-NH2 for SUN-1, and NH2-CVFKDSPLR-LKRS-NH2 and NH2-CGTFAYDQEPIQT-NH2 for SUN-2, respectively; Covalab). We used Alexa 488 and 568 secondary antibodies (Molecular Probes) for immunofluorescence and peroxidase-conjugated antibodies (Jackson ImmunoResearch Laboratories) for immunoblotting, followed by ECL.
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References


Figures

**Figure 1:**

**Localization of nesprins during C2C12 differentiation.** (A) Immunolocalization of nesprin-1 (rabbit-anti-nesprin-1), nesprin-2, nesprin-3 (green), and pericentrin (red) in proliferating myoblasts and in differentiated myotubes. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI, blue). Bar, 10µm. (B) Immunoblots of cell lysates from C2C12 myoblasts (myobl.) and myotubes, stained with rabbit anti-nesprin-1, mouse anti-nesprin-1 (monoclonal “MANNES1A”) and mouse anti-nesprin-3 antibodies. As a loading control, alpha-tubulin was probed. The position of protein molecular weight markers for both nesprins 1 and 3 is indicated on the left. (C) Quantitative PCR of nesprin-1 and nesprin-2 cDNA, obtained by reverse transcription of total RNA from C2C12 myoblasts and myotubes. Levels of nesprin-1 and nesprin-2 (mean ±SD, n=2) were displayed relative to those obtained by qPCR of housekeeping cDNAs (ribosomal protein S16 and hypoxanthine guanine...
phosphoribosyl transferase), calculated by Biorad CFX Manager software. Nesprin-1 levels obtained from myotubes were set to “1” for normalization.
Figure 2:

Nesprin-1 is essential for the recruitment of centrosome proteins to the nuclear envelope in differentiated C2C12 cells. (A, B) Immunoblots of cell lysates from C2C12 myotubes, treated with control RNA, or with siRNA against nesprin-1 or nesprin-3. Blots were probed with antibodies against nesprin-1, nesprin-3, PCM-1, or alpha-tubulin, respectively. (C, D) Pericentrin localization (red) in control C2C12 myotubes (top row), and in (C) nesprin-1-depleted or (D) nesprin-3-depleted myotubes. Green, nesprin-1 or nesprin-3, respectively; blue, DNA. The asterisks in the bottom rows of (C, D) indicate nuclei of non-depleted myoblasts that have fused into myotubes with nesprin-1-depleted or nesprin-3-depleted myoblasts, respectively. (E, F) PCM-1 localization (red) in control or nesprin-1 or nesprin-3-depleted myotubes. Green, nesprin-1 or nesprin-3, respectively. (G) C2C12 myotubes, controls (top row), or treated with 5μg/ml Brefeldin A for 90 minutes (BFA, bottom row). Immunofluorescence of Golgi protein GM130 (green), and pericentrin (red). (H) Localization of pericentrin (red) in myotubes treated with control RNA or siRNA against calnexin (green). Bar, 10μm.
**Figure 3:**

**SUN/KASH proteins are required for the recruitment of centrosome protein PCM-1 to the nuclear envelope.**

(A) Localization of endogenous nesprin-1 (red) in C2C12 myotubes overexpressing (top) control EGFP, or (bottom) the EGFP-tagged nesprin-1 KASH domain (green). (B) Localization of endogenous PCM-1 in C2C12 myotubes, expressing varying levels of the EGFP-nesprin-1 KASH domain. Nuclei were stained with DAPI (blue). (C) Immunoblots of lysates of C2C12 myoblasts (myobl.) and myotubes, stained with antibodies against SUN1 and SUN2, as well as immunoblots of lysates treated with control RNA, or with siRNA against SUN1 or SUN2, probed with the respective antibodies. Immunoblots of corresponding cell lysates, probed with antibody against alpha-tubulin ($\alpha$-tub.), are shown as loading controls. The position of protein molecular weight markers for both immunoblots of SUN1 and SUN2 is indicated on the right of the second column. (D) Localization of pericentrin (red) in myotubes depleted simultaneously of SUN1 and SUN2 (immunofluorescence of SUN2 in green). (E) Immunoprecipitation of nesprin-1 (left column) or PCM-1 (right column) from nuclear envelope preparations from C2C12 cells (see ‘Materials and Methods’ and suppl. Fig. S1C). For comparison, total C2C12 lysates, as well as the eluates from magnetic beads without antibody, or from magnetic beads coupled with control IgG, were loaded. Immunoprecipitations were performed from OGP/NaCl fractions, as depicted in lanes 4 of suppl. Fig. S1C. Immunoblots were probed with antibodies against PCM-1, nesprin-1, and $\gamma$-tubulin. The loading control shows a Ponceau-stained region of the Western blots, depicting the IgG heavy chains used for immunoprecipitation. (F) Localization of nuclear pore complexes, detected with monoclonal antibody 414 (NPC, red) in myotubes treated with control RNA or siRNA against nesprin-1 (green). (G) Expression of mCherry-nesprin-1$\alpha$ (red) in C2C12 myoblasts, prior to differentiation. Arrowheads point to areas of the nuclear envelope with massive PCM-1 accumulation (green in the merged image). (H, I)
Expression of mCherry-tubulin-α (red), or mEmerald-nesprin-3 (red), as controls, respectively. Immunofluorescence of PCM-1 is shown in green. (J) Expression of mCherry-nesprin-1α (red); immunofluorescence of the differentiation marker myosin II heavy chain (green). Bar, 10µm.
Figure 4:

Nesprin-1-depletion does not alter microtubule organization in myotubes. (A) Left column: localization of nesprin-1 (green) and γ-tubulin (red) in a control myotube. Right column: nesprin-1 (green) and γ-tubulin (red) in a myotube formed with a non-depleted (left) and nesprin-1-depleted nucleus (right). (B) Regrowth of microtubules (red), at 3 min after recovery from depolymerization, in control or nesprin-1-depleted C2C12 cells. Green:
nesprin-1. Graph: quantification of the microtubule repolymerization activity at the nuclear envelope in myotubes (nuclear envelopes with visible microtubule stubs ≥ 2μm length). n=100. Errors bars correspond to mean ± 2xSEM (confidence interval at 95%). (C) Immunofluorescence of microtubules in control or nesprin-1-depleted C2C12 myotubes. Negative-contrast-images of single, deconvolved optical sections of an image stack are shown, in addition to merged images, depicting microtubules in green, nesprin-1 in red, and nuclei in blue. (D) Enlarged perinuclear region of a control myotube, showing sum projections of microtubules from three consecutive partial stacks of 1.2μm thickness (each obtained from 6 sections at 0.2μm distance; position of sections are indicated in each image). The track of a selected single microtubule is indicated by arrowheads. On the right, a merged image is shown in three colours (#14-19: blue, #20-25: green, #26-31: red). (E) Individual microtubules were tracked manually, in complete image stacks of myotubes (n=5 for controls or nesprin-1 siRNA), reconstructed from partial stacks as shown in (D). The number of microtubules running within a distance of ~1μm to the nuclear surface were counted. (F) Microtubules tracked in (E) were analysed for their orientation. The percentages of microtubules running parallel to the long axis of the myotubes are shown. (G) The immunofluorescence signal intensities of microtubules were determined in sum projections from entire image stacks of myotubes, in myotube segments containing nuclei (segment 1 in scheme), in tangential segments at the periphery of nuclear envelopes (segment 2), and in areas free of nuclei (segment 3). N=5 for controls or nesprin-1 siRNA. Each value obtained for segment 1 or 2 was divided through the value of a neighbouring segment 3. The resulting values equalled percentages of segments 3 (defined as 100%). Bars in (B) and (D), 10μm.
Figure 5:

Nuclear positioning in myotubes, dependent on nesprin-1 and PCM-1, both involved in recruiting microtubule motor proteins to the nuclear envelope. (A) Alignment of nuclei along the axis of myotubes (dotted line) in control, nesprin-1 or PCM-1-depleted C2C12 cells. Immunofluorescence of nesprin-1 and PCM-1 is shown. Nuclei were stained with DAPI.
(DNA). Graph showing % of myotubes with correctly aligned nuclei along the axis of the cell. From the left: control, nesprin-1-depleted, PCM-1-depleted, and EHNA-treated C2C12 cells. N=300 cells for each condition, error bars correspond to mean ± 2xSEM (confidence interval at 95%). (B) Left: PCM-1-depleted C2C12 myotubes (siRNA PCM-1), as well as control myotubes were analysed by immunoblotting, using antibodies against PCM-1, nesprin-1, or alpha-tubulin, respectively. Right: PCM-1-depleted myotubes were analysed by immunofluorescence, using antibodies against PCM-1 (red) and nesprin-1 (green). Nuclei were stained with DAPI (blue). A myotube is shown with a PCM-1-depleted and an undepleted nucleus side by side. (C) Localization of p150glued in control, nesprin-1 and PCM-1-depleted C2C12 myotubes. Green: nesprin-1 or PCM-1, respectively; red: p150glued; blue, DNA. (D) Localization of kinesin light chain-1 (KLC1) in control, nesprin-1 and PCM-1-depleted myotubes. Due to incompatibility of multiple rabbit antibodies for co-staining of KLC1, nesprin-1, and PCM-1, the depletion of nesprin-1 and PCM-1 were verified indirectly, by immunofluorescence staining with mouse antibody against pericentrin (green) that was absent from the nuclear envelope under these conditions (see supplemental Fig. S1E). Red: KLC1; blue: DNA. (E) p150glued localization (red) in control cells, and in cells after depolymerization of microtubules (green) by nocodazole treatment. Nuclei were stained with DAPI (blue). Bar, 10µm.
Supplemental Material

Figure S1
**Supplemental figure S1:**

(A, B) Localization of nesprin-1 and nesprin-2 in C2C12 myoblasts and myotubes, using (A) mouse anti-nesprin-1 MANNESA(7A12) (green), and (B) rabbit anti-nesprin-2, #4 (left, green), or rabbit anti-nesprin-2, AA1465-1707 (right, green). Immunofluorescence of pericentrin is shown in red. RNA silencing against nesprin-2 was performed, lowering the perinuclear immunofluorescence signal of nesprin-2 in undifferentiated myoblasts (arrowhead in column ‘siRNA nesprin 2’), without affecting pericentrin relocation to the nuclear envelope in myotubes (large cell, highlighted by dashed lines). The intranuclear immunofluorescence in myotubes, as obtained with nesprin-2 antibody #4, remained after siRNA treatment, suggesting an unspecific reactivity. The nuclear envelope staining in myoblasts, as well as the cytoplasmic immunofluorescence obtained with antibody against nesprin-2 AA1465-1707 was lowered by siRNA treatment (B, right column, green signal), suggesting that both are specific. (C) Fractionation of C2C12 myotubes, analysed by immunoblotting. Volume equivalents are shown of total lysate (1st lane), supernatant after lysis in 1% NP-40, followed by centrifugation (2nd lane), pellets after NP-40 extraction (3rd lane). Three volume equivalents are shown of the NP-40 pellets lysed in 1% octyl beta-D-glucopyranoside + 400 mM NaCl, followed by centrifugation (sup. OGP/NaCl, 4th lane), as well as of the final pellets after OGP/NaCl extraction (5th lane). Immunoblots were probed with antibodies against PCM-1, nesprin-1, lamin A, alpha-tubulin, and histone H3. (D) Localization of nesprin-1 (green) and nesprin-3 (red) in myotubes treated with control RNA, or with siRNA against nesprin-1, or with siRNA against nesprin-3. (E) Localization of pericentrin (green) and PCM-1 (red) in myotubes treated (top) with siRNA against PCM-1, or (bottom) with siRNA against pericentrin. Arrows indicate PCM-1-depleted or pericentrin-depleted nuclei. Nuclei were stained with DAPI (blue). Bar, 10µm.