

# Nesprin-3 augments peripheral nuclear localization of intermediate filaments in zebrafish

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## Summary

The outer nuclear membrane protein nesprin-3 binds the cytoskeletal linker protein plectin, which are proposed to anchor the intermediate filaments to the nuclear envelope. To investigate the function of nesprin-3 in vivo, we used the zebrafish as a vertebrate model system. Zebrafish nesprin-3 is expressed at the nuclear envelope of epidermal and skeletal muscle cells during development. Unexpectedly, loss of nesprin-3 did not affect embryonic development, viability or fertility. However, nesprin-3-deficient zebrafish embryos showed a reduced concentration of intermediate filaments around the nucleus. Additional analysis revealed the presence of two nesprin-3 isoforms in zebrafish, nesprin-3 $\alpha$  and nesprin-3 $\beta$ . Nesprin-3 $\beta$  is only expressed during early development and lacks seven amino acids in its first spectrin repeat that are crucial for plectin binding and recruitment to the nuclear envelope. These seven amino acids are highly conserved and we showed that residues R43 and L44 within this motif are required for plectin binding. Furthermore, several residues in the actin-binding domain of plectin that are crucial for binding to the integrin  $\beta$ 4 subunit are also important for the binding to nesprin-3 $\alpha$ , indicating partial overlapping binding sequences for nesprin-3 $\alpha$  and integrin  $\beta$ 4. All this shows that nesprin-3 is dispensable for normal development in zebrafish, but important for mediating the association of the intermediate filament system with the nucleus in vivo.

**Key words:** Intermediate filaments, Nesprin-3, Plectin, Zebrafish

## Introduction

The outer nuclear membrane (ONM) of the nuclear envelope (NE) is continuous with the rough endoplasmic reticulum and is characterized by the presence of members of the nesprin protein family. To date, four members have been identified in mammals, nesprin-1 (Padmakumar et al., 2004; Zhang et al., 2001), nesprin-2 (Zhang et al., 2001; Zhen et al., 2002), nesprin-3 (Wilhelmsen et al., 2005) and the recently identified nesprin-4 (Roux et al., 2009). All these proteins are characterized by multiple spectrin repeats (SRs) and a C-terminal Klarsicht/ANC-1/Syne homology (KASH) domain (Wilhelmsen et al., 2006; Zhang et al., 2002). The KASH domain comprises a transmembrane region followed by a short luminal domain that extends into the periplasmic space, where it interacts with the inner nuclear membrane (INM) proteins SUN1 and SUN2 (Crisp et al., 2006; Haque et al., 2006; Padmakumar et al., 2005). The SUN–nesprin interaction is essential for retaining nesprins at the ONM and for maintaining a proper NE architecture (Crisp et al., 2006). Inside the nucleus, SUN proteins interact through their N-terminal domain with the nuclear lamina (Crisp et al., 2006; Haque et al., 2006).

The nesprin family members differ from each other in the number of SRs and in their N-terminal domain. The giant isoforms of nesprin-1 and nesprin-2 have an N-terminal actin-binding domain (ABD) through which they connect the NE to the actin cytoskeleton (Padmakumar et al., 2004; Zhen et al., 2002). Nesprin-3 and nesprin-4 are much smaller in size and lack an N-terminal ABD. However, these proteins can link the cytoskeleton to the NE in an indirect manner. Nesprin-3 binds the cytoskeletal crosslinker protein

plectin, by which it can anchor the intermediate filaments (IFs) to the ONM (Wilhelmsen et al., 2005). Furthermore, nesprin-4 binds to the microtubule motor protein kinesin-1 (Roux et al., 2009), thereby establishing a link between the nucleus and microtubules. The nesprin–SUN complexes thus establish a connection between the cytoskeleton and the nuclear lamina; a complex referred to as the LINC complex (linker of nucleoskeleton and cytoskeleton) (Crisp et al., 2006; Stewart-Hutchinson et al., 2008).

The connection of the NE with the cytoskeleton suggests that nesprins have important functions in nuclear positioning and anchorage. Indeed, studies in *Caenorhabditis elegans* and *Drosophila melanogaster* showed nuclear anchorage defects upon loss of the nesprin-1 and nesprin-2 homologs ANC-1 and MSP-300, respectively (Starr and Han, 2002; Wilhelmsen et al., 2006; Yu et al., 2006). Furthermore, various nesprin-1 mutant mice are characterized by an abnormal clustering of skeletal muscle nuclei and the absence of synaptic nuclei from the neuromuscular junction (Puckelwartz et al., 2009; Zhang et al., 2010; Zhang, X. et al., 2007). Although nesprin-2 mutant mice have no overt abnormal phenotype (Lüke et al., 2008; Zhang, X. et al., 2007), mice in which the KASH domains of both nesprin-1 and nesprin-2 are deleted die shortly after birth due to respiratory failure (Zhang, X. et al., 2007), indicating a redundancy of their function.

Nesprin-3 is the only family member currently known to anchor the IF system to the NE. We have previously described the presence of two nesprin-3 isoforms in mice, nesprin-3 $\alpha$  and nesprin-3 $\beta$ , the latter not binding to plectin. These isoforms are produced as a result of alternative splicing (Wilhelmsen et al., 2005). Nesprin-3 $\alpha$

contains eight SRs and a C-terminal KASH domain, and the nesprin-3 $\beta$  isoform differs only in that it lacks the first SR. Because the first SR is essential for plectin binding, only nesprin-3 $\alpha$  can establish a connection with the IF system (Wilhelmsen et al., 2005). However, the function of nesprin-3 $\alpha$  in vivo is currently unknown.

Plectin is a cytoskeletal crosslinker protein of the plakin family that is highly versatile in its binding properties (Wiche, 1998). Similarly to other family members, plectin interconnects different components of the cytoskeleton and attaches them to junctional complexes at the plasma membrane (McLean et al., 1996; Sonnenberg and Liem, 2007). For example, plectin crosslinks the actin cytoskeleton with the IF system via its N-terminal ABD and C-terminal plakin repeats, respectively (Andrä et al., 1998; Garcia-Alvarez et al., 2003; Nikolic et al., 1996). In addition to its interaction with F-actin, the plectin ABD also mediates binding to the integrin  $\beta$ 4 subunit and nesprin-3 $\alpha$ , thereby connecting the IF system to both the plasma membrane and the NE (Geerts et al., 1999; Litjens et al., 2003; Wilhelmsen et al., 2005). Moreover, we have previously demonstrated that both nesprin-3 $\alpha$  and integrin  $\beta$ 4 compete with F-actin for plectin binding (Geerts et al., 1999; Ketema et al., 2007; Wilhelmsen et al., 2005). Deficiency of plectin in both patients and mice leads to skin fragility and muscular dystrophy, illustrating its essential role in maintaining cell integrity (Andrä et al., 1997; McLean et al., 1996).

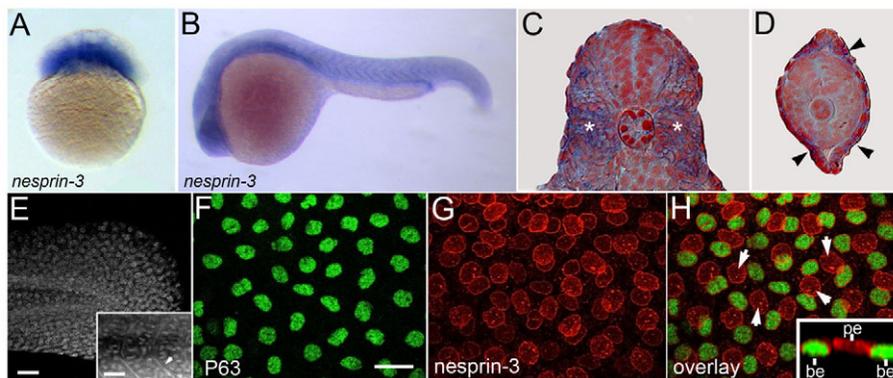
In the present study, we have investigated the function of nesprin-3 in vivo. Because nesprin-1, nesprin-2 and nesprin-3 are conserved in zebrafish (Simpson and Roberts, 2008; Tsujikawa et al., 2007), we have used this organism as a vertebrate model system to study the distribution of nesprin-3 during embryonic development. In addition, we have generated and analyzed nesprin-3-deficient zebrafish using TILLING (targeted induced local lesions in genomes), an approach for the identification of null mutations from a randomly *N*-ethyl-*N*-nitrosourea (ENU)-mutagenized zebrafish library (Wienholds and Plasterk, 2004).

## Results

### Nesprin-3 is expressed at the NE of epidermal and skeletal muscle cells during zebrafish embryonic development

Nesprins are expressed in various tissues such as brain, skin, skeletal muscle and heart (Lüke et al., 2008; Wilhelmsen et al., 2005; Zhang et al., 2001). However, the expression profile and the function of nesprin-3 are still largely unknown. Because nesprin-3 is conserved in zebrafish, we decided to use zebrafish to study the function of nesprin-3 (Simpson and Roberts, 2008). To investigate the mRNA expression of *nesprin-3* during zebrafish embryonic development, we performed whole-mount in situ hybridization (ISH) with a digoxigenin-labeled antisense *nesprin-3* probe on wild-type embryos of various developmental stages. Maternal *nesprin-3* mRNA expression was observed during initial cellular division, before the start of zygotic transcription (Fig. 1A). At 24 hours post-fertilization (hpf), *nesprin-3* mRNA appeared to be expressed ubiquitously (Fig. 1B). Strongest *nesprin-3* expression was detected in skeletal muscle and the epidermis (Fig. 1C,D).

To study the distribution of nesprin-3 protein in zebrafish, we produced a rabbit polyclonal antibody directed against SR7 of zebrafish nesprin-3. Whole-mount immunohistochemistry on 3-day-old embryos revealed localization of nesprin-3 protein at the NE in epidermal (Fig. 1E) and skeletal muscle cells (Fig. 1E inset). In addition to the NE staining, we also observed staining at the myotendinous junctions and in the cytoplasm of skeletal muscle cells. However, because a similar staining pattern was found with the pre-immune serum (data not shown), we regarded this reaction as nonspecific. This nonspecific background staining hindered the study of nesprin-3 expression in skeletal muscle cells at most stages of development. Unlike in skeletal muscle cells, nesprin-3 expression at the NE of epidermal cells was observed during all stages of development [1–6 days post fertilization (dpf)]. The localization of nesprin-3 at the NE in epidermal cells was confirmed by double labeling with the basal epidermal marker p63 (Fig. 1F–H) (Lee and Kimelman, 2002).



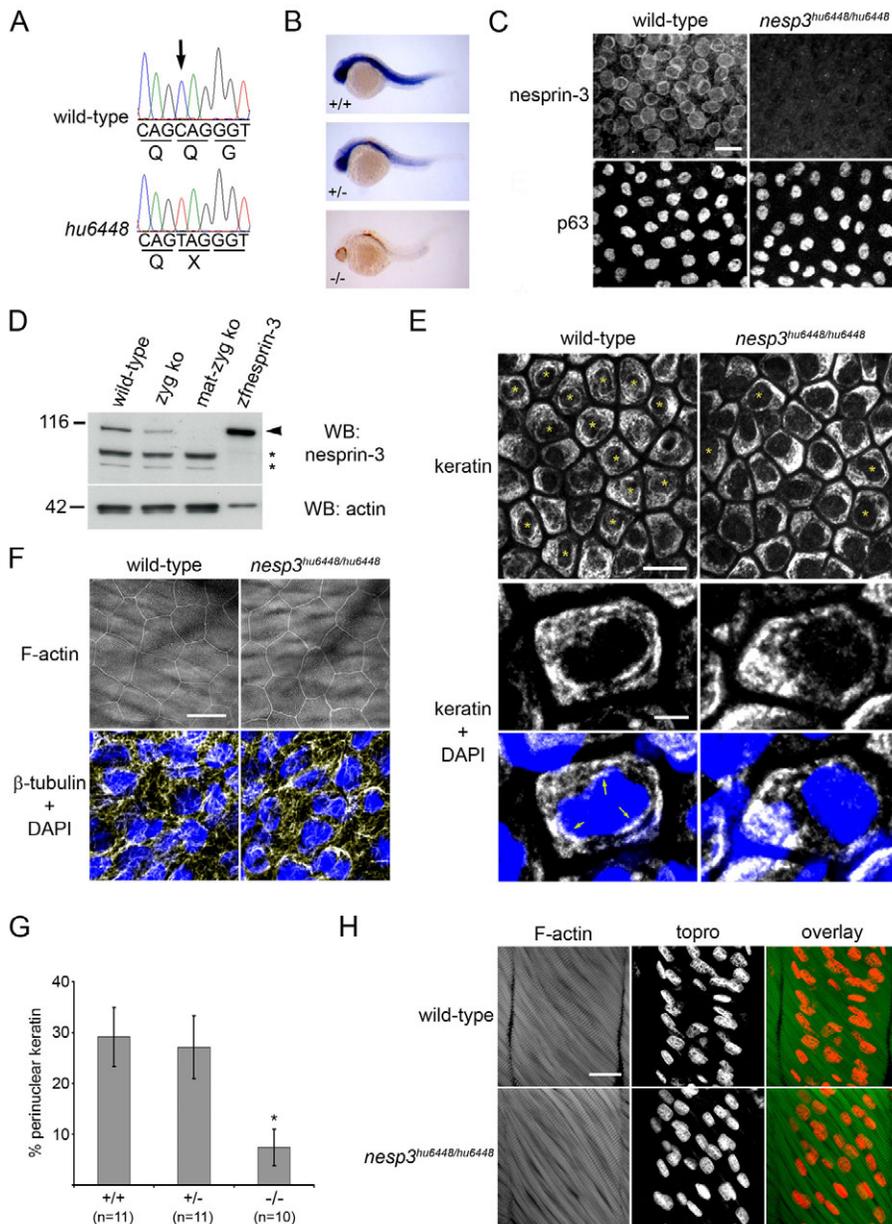
**Fig. 1. Nesprin-3 is expressed at the NE of epidermal and skeletal muscle cells during zebrafish embryonic development.** (A,B) Whole-mount ISH for *nesprin-3* mRNA expression at the 16-cell stage (A) and 1 dpf (B). (C,D) Semi-thin sections of 1-day-old embryos after ISH at the level of the trunk (C) and at the end of the tail (D). Asterisks indicate the *nesprin-3* mRNA expression in the skeletal muscle, and black arrowheads indicate the *nesprin-3* mRNA expression in the epidermis. Sections were counterstained with neutral red. (E) Whole-mount immunostaining with anti-nesprin-3 antibody on 3-day-old wild-type embryos in the tail-fin and skeletal muscle (inset). The arrow marks nonspecific (pre-immune) background staining at the myotendinous junctions. Scale bars: 50  $\mu$ m. (F–H) Co-staining with anti-p63 antibody (green, F) and anti-nesprin-3 antibody (red, G) in 3-day-old wild-type embryos shows nesprin-3 localization at the NE of basal epidermal cells and peridermal cells (H). White arrows indicate nesprin-3 localization at the NE of peridermal cells. The inset shows an xz-projection of two basal epidermal cells (be) with p63 staining in the nucleus (green) and nesprin-3 staining at the NE (red), whereas in the peridermal cell (pe) only nesprin-3 staining is observed at the NE (red). Scale bar: 20  $\mu$ m.

However, a layer of nesprin-3-positive cells overlying the cells double-labeled with p63 and nesprin-3 was also observed (Fig. 1H inset). Because the zebrafish embryonic epidermis contains a basal and a peridermal cell layer at this stage of development (Le Guellec et al., 2004), we conclude that nesprin-3 is expressed in both epidermal cell layers. Furthermore, in a heterogeneous epidermal cell population derived from 21-day-old larvae, we observed nesprin-3 expression at the NE (data not shown). This suggests that nesprin-3 also resides at the NE of epidermal cells during later stages of development.

### Complete loss of nesprin-3 does not affect embryonic development, viability or fertility in zebrafish

To study the function of nesprin-3 in vivo, we screened for mutant alleles in zebrafish. From a library of ENU-mutagenized zebrafish we isolated a *nesprin-3* mutant allele, hu6448. The hu6448 allele

carries a cysteine to threonine mutation in exon 4 of *nesprin-3* (ENSDARG0000023237), leading to the introduction of a premature stop codon at position 195 (Fig. 2A). Because a premature stop mutation often affects the stability of mRNA by a process referred to as nonsense-mediated decay, we investigated the *nesprin-3* mRNA expression in *nesprin-3* mutant embryos. Whole-mount ISH was performed on a batch of embryos obtained from a cross between two heterozygous parents. This revealed that *nesprin-3* mRNA is absent in homozygous *nesprin-3* mutant embryos, whereas reduced *nesprin-3* mRNA expression levels were observed in heterozygous embryos as compared to those in wild-type siblings (Fig. 2B). In addition, whole-mount immunohistochemistry analysis was performed to determine whether nesprin-3 protein is present in the homozygous *nesprin-3* mutant embryos. Indeed, nesprin-3 protein was absent from epidermal cells in 3-day-old homozygous *nesprin-3* mutant



**Fig. 2. Loss of nesprin-3 in zebrafish results in dissociation of the IF system from the nucleus in basal epidermal cells.** (A) Sequence chromatograms of wild-type (upper panel) and *nesprin-3*<sup>hu6448</sup> mutant (lower panel) *nesprin-3* cDNA showing the cysteine to threonine mutation (black arrow) that induces a premature stop codon (X). (B) Whole-mount ISH for *nesprin-3* mRNA expression in homozygous wild-type (upper panel), heterozygous (middle panel) and homozygous mutant embryos (lower panel) at 24 hpf. (C) Co-staining using anti-nesprin-3 antibody (upper panels) and anti-p63 antibody (lower panels) in wild-type sibling (left panels) and *nesprin-3*<sup>hu6448/hu6448</sup> mutant embryos (right panels) at 3 dpf. (D) Western blot (WB) analysis for zebrafish nesprin-3 on lysates from a batch of wild-type embryos, a batch of embryos from a cross between two heterozygous carriers (zyg ko) and a batch of maternal-zygotic mutant embryos (mat-zyg ko). Lysates were prepared at 8 hpf. As a control we used a lysate of COS7 cells overexpressing zebrafish nesprin-3α (zfnesprin-3). Arrowhead indicates the size of zebrafish nesprin-3. Asterisks mark two nonspecific bands. Actin levels served as a loading control. (E) Whole-mount immunostaining using anti-pan keratin antibody in wild-type sibling (left panels) and *nesprin-3*<sup>hu6448/hu6448</sup> mutant embryos (right panels) at 5 dpf. Asterisks mark basal epidermal cells in which keratin filament organization around the nucleus can be observed. The nuclei were visualized by counterstaining with DAPI (lower panels). (F) 5-day-old wild-type and *nesprin-3*<sup>hu6448/hu6448</sup> mutant embryos were stained with phalloidin to visualize the F-actin cytoskeleton and immunostained using an anti-β-tubulin antibody to visualize the microtubule system. The nuclei were counterstained with DAPI. (G) Quantification of the percentage of basal epidermal cells in which we observed keratin filament organization around the nucleus in wild-type sibling (+/+), heterozygous sibling (+/-) and *nesprin-3*<sup>hu6448/hu6448</sup> mutant embryos (-/-). *n* represents the number of embryos analyzed. Results are shown as the mean ± s.d. \**P*<0.05. (H) 3-day-old wild-type and *nesprin-3*<sup>hu6448/hu6448</sup> mutant embryos were stained with phalloidin and counterstained with TO-PRO-3 iodide to visualize the positioning of nuclei in skeletal muscle cells. Scale bars: 20 μm; 10 μm (E, lower panels).

embryos (Fig. 2C). Together, this shows that the hu6448 allele is a complete *nesprin-3* null mutant.

The homozygous *nesprin-3* mutant embryos showed no obvious defects during embryonic development and were fertile. Because *nesprin-3* mRNA is expressed maternally (Fig. 1A), it is possible that the amount of *nesprin-3* protein produced from this mRNA is sufficient to carry the embryo through early stages of development. To investigate the contribution of maternal *nesprin-3* mRNA, we generated maternal-zygotic *nesprin-3* mutant embryos from a cross between two homozygous *nesprin-3* mutant parents. Western blot analysis demonstrated that the *nesprin-3* protein was absent in the maternal-zygotic mutant embryos, whereas a 50% reduction in *nesprin-3* protein level, compared to homozygous wild-type, was observed for a batch derived from two heterozygous parents (Fig. 2D). The maternal-zygotic *nesprin-3* mutant embryos also developed normally and were fertile, thereby ruling out the possibility that maternally derived *nesprin-3* compensates for a lack of *nesprin-3* in the developing knockout embryos.

#### Association of the IF system with the NE is reduced in *nesprin-3*-deficient zebrafish embryos

Previous *in vitro* studies have suggested that *nesprin-3 $\alpha$* , by binding to plectin, connects the IF system with the NE (Wilhelmsen et al., 2005). We predicted that, in epidermal cells of *nesprin-3* mutant embryos, the connection between the nucleus and IF system was disrupted, which could lead to potential disturbances in the organization of the IF system in the proximity of the nucleus. Because *nesprin-3* is present at the NE of epidermal cells during zebrafish development, we studied the structure of keratin filaments in basal epidermal cells of 5-day-old wild-type sibling and *nesprin-3* mutant embryos. Using whole-mount immunohistochemistry with an anti-pan keratin antibody, we observed cytoplasmic keratin filaments in all epidermal cells of wild-type embryos. Furthermore, in many cells a dense filamentous keratin network around the nucleus could be distinguished (Fig. 2E). In *nesprin-3* mutant embryos, this organization of keratin filaments around the nucleus was reduced, whereas the cytoplasmic keratin organization appeared unaffected (Fig. 2E). Organization of other cytoskeletal components such as actin filaments and microtubules was unaffected by the loss of *nesprin-3* in epidermal cells (Fig. 2F). We quantified the percentage of epidermal cells in which a dense filamentous keratin network was observed around the nucleus in homozygous wild-type, heterozygous and homozygous *nesprin-3* mutant embryos. In homozygous wild-type and heterozygous embryos, keratin filaments were found around the nucleus in approximately 29% and 27% of the cells, respectively. By contrast, the percentage of epidermal cells with perinuclear keratin was only 7% in *nesprin-3* mutant embryos ( $P < 0.05$ ) (Fig. 2G). Hence, *nesprin-3* has no effect on the overall organization of the IF system, but is important for the association of the IF cytoskeleton with the nucleus in epidermal cells *in vivo*.

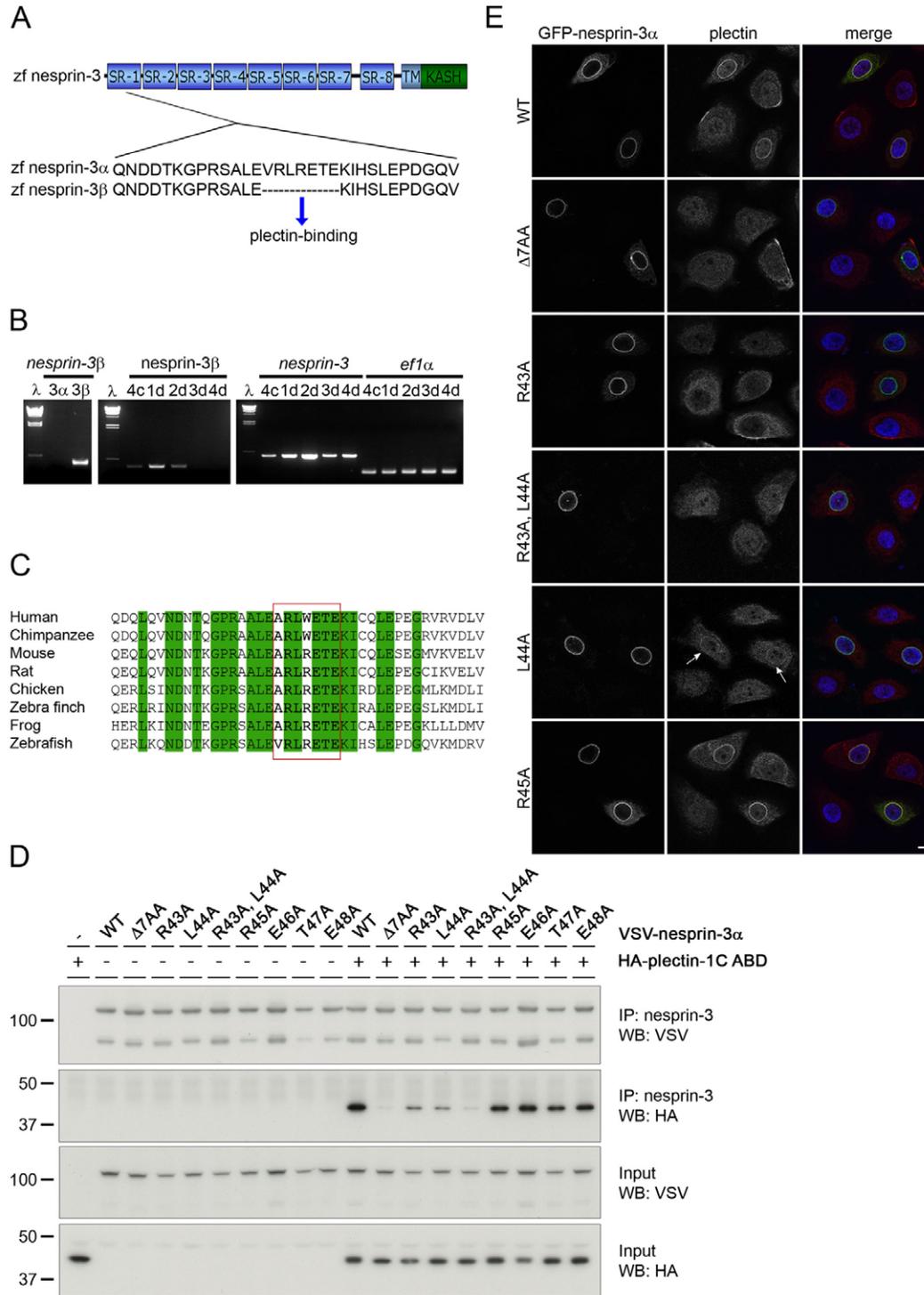
It has previously been demonstrated that *nesprin-1* is involved in nuclear positioning and anchorage in skeletal muscle cells (Puckelwartz et al., 2009; Zhang et al., 2010; Zhang, X. et al., 2007). Because *nesprin-3* is expressed in skeletal muscle and because loss of this protein affects the connection between the nucleus and the IF system in epidermal cells, we wondered whether loss of *nesprin-3* influences the positioning and anchorage of nuclei in multinucleated skeletal muscle cells. To investigate this, we stained 3-day-old embryos from a cross between two heterozygous parents for F-actin and the nuclear marker TO-PRO-3 and examined

the position of the nuclei by confocal microscopy. This revealed no significant alterations in the positioning of nuclei in skeletal muscle cells of *nesprin-3* mutant embryos as compared with wild-type siblings (Fig. 2H). Furthermore, we observed no motility defects in *nesprin-3* mutant embryos or adult zebrafish. In summary, the data presented here indicate that *nesprin-3* is dispensable for normal embryonic development in zebrafish, but is important for the efficient localization of IFs at the nuclear periphery in epidermal cells.

#### Zebrafish express two *nesprin-3* isoforms: *nesprin-3 $\alpha$* and the plectin-binding-deficient *nesprin-3 $\beta$*

Two *nesprin-3* isoforms have been identified in mice, *nesprin-3 $\alpha$*  and *nesprin-3 $\beta$* , the latter not binding to plectin (Wilhelmsen et al., 2005). In murine *nesprin-3 $\beta$* , the first SR important for plectin binding is absent due to alternative splicing of the *nesprin-3* transcript (Wilhelmsen et al., 2005). To examine the presence of *nesprin-3* isoforms in zebrafish, we prepared full-length zebrafish *nesprin-3* cDNA from 2-day-old zebrafish embryos. Sequencing of several cDNA clones revealed the presence of two *nesprin-3* transcripts: *nesprin-3 $\alpha$*  (3300 base pairs) and *nesprin-3 $\beta$*  (3279 base pairs). Zebrafish *nesprin-3 $\alpha$*  encodes 1100 amino acids, including eight SRs and a C-terminal KASH domain (Fig. 3A). It is highly homologous to mouse *nesprin-3 $\alpha$*  except for an additional stretch of 125 amino acids located between SR7 and SR8. Due to the presence of a cryptic splice donor site within exon 2, an alternative transcript can be generated that lacks 21 nucleotides. Translation of this *nesprin-3 $\beta$*  transcript resulted in a deletion of seven amino acids (VRLRETE) from the first SR, thereby producing the *nesprin-3 $\beta$*  protein (Fig. 3A). To examine the expression of *nesprin-3 $\beta$*  mRNA, we performed a PCR on cDNA from various stages of development (4-cell stage, 1–4 dpf). By using *nesprin-3 $\beta$* -specific primers, we showed that *nesprin-3 $\beta$*  is expressed during the early stages of development (until 2 dpf), but not at later stages (Fig. 3B). By contrast, when primers recognizing both the *nesprin-3 $\alpha$*  and *nesprin-3 $\beta$*  transcripts were used, *nesprin-3* mRNA was also detected during later stages of development (Fig. 3B). These data indicate that *nesprin-3 $\alpha$*  is the main isoform expressed during the later stages of zebrafish development.

Mouse *nesprin-3 $\alpha$*  binds plectin via its first SR, but the exact binding site on *nesprin-3 $\alpha$*  is not known. Because the seven amino acid motif (VRLRETE) deleted in zebrafish *nesprin-3 $\beta$*  is highly conserved among species (Fig. 3C), we investigated whether this motif is crucial for plectin binding. We therefore deleted the corresponding motif (ARLRETE) from mouse *nesprin-3 $\alpha$*  (*nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup>*) and tested whether plectin was bound by co-immunoprecipitation. COS7 cells were transiently transfected with constructs encoding hemagglutinin (HA)-tagged plectin-1C ABD and either vesicular stomatitis virus (VSV)-tagged *nesprin-3 $\alpha$*  wild-type or *nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup>*. *Nesprin-3* precipitates were subsequently analyzed for the presence of HA-pectin-1C ABD. Whereas the plectin ABD co-immunoprecipitated with wild-type *nesprin-3 $\alpha$* , co-precipitation of the plectin ABD was hardly detectable in *nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup>* precipitates (Fig. 3D). Because overexpression of *nesprin-3 $\alpha$*  leads to the recruitment of endogenous plectin to the NE (Ketema et al., 2007; Wilhelmsen et al., 2005), we investigated the influence of deletion of the seven amino acid motif on plectin recruitment. Upon transient transfection of PA-JEB keratinocytes with a construct encoding GFP-*nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup>*, no plectin was recruited to the NE (Fig. 3E). These results indicate that the seven amino acid motif in the first SR of *nesprin-3 $\alpha$*  is crucial for plectin



**Fig. 3. R43 and L44 in the first SR of nesprin-3 are essential for plectin binding.** (A) Representation of the zebrafish nesprin-3α and nesprin-3β isoforms. The zebrafish nesprin-3β isoform misses seven amino acids in the first SR. TM, transmembrane domain. (B) As a control for their specificity, nesprin-3β primers were tested on a *nesprin-3α* cDNA construct (3α) and a *nesprin-3β* cDNA construct (3β). RT-PCR on cDNA from wild-type embryos of various stages of development: 4-cell (4c), 1 dpf (1d), 2 dpf (2d), 3 dpf (3d) and 4 dpf (4d) with the nesprin-3β-specific primers, primers for total nesprin-3 and primers for *ef1α*. (C) Alignment of the nesprin-3 protein sequence containing the seven amino acid motif in several species. Residues indicated in green are conserved in all species. The amino acids present in the seven amino acid motif are enclosed by a red box. (D) COS7 cells were transiently transfected with constructs encoding HA-plectin-1C ABD, VSV-mouse-nesprin-3α<sup>WT</sup>, VSV-mouse-nesprin-3α<sup>Δ7AA</sup> or point mutants of VSV-mouse-nesprin-3α. Cells were lysed and both input samples and nesprin-3 precipitates (IP) were probed for VSV glycoprotein and HA. WB, western blot; WT, wild-type. (E) PA-JEB keratinocytes were transiently transfected with GFP-mouse-nesprin-3α<sup>WT</sup>, GFP-mouse-nesprin-3α<sup>Δ7AA</sup> or point mutants of GFP-mouse-nesprin-3α. Cells were fixed in PFA and stained for endogenous plectin. Merged images also show counterstaining of the nuclei with TO-PRO-3 iodide. Arrows indicate the recruitment of endogenous plectin to the NE in cells expressing GFP-mouse-nesprin-3α<sup>L44A</sup>. Scale bar: 10 μm.

binding. Moreover, they also suggest that the nesprin-3 $\beta$  isoform of zebrafish does not bind plectin.

### Arginine 43 and leucine 44 of mouse nesprin-3 $\alpha$ are essential for plectin binding and recruitment to the NE

To identify the actual residues involved in plectin binding, the amino acids comprising the seven amino acid motif in mouse nesprin-3 $\alpha$  were mutated to alanine. These point mutants of nesprin-3 $\alpha$  were subsequently tested for their ability to bind the plectin ABD in co-immunoprecipitation experiments. Upon co-transfection in COS7 cells, lesser amounts of the plectin ABD were co-precipitated with nesprin-3 $\alpha$ <sup>R43A</sup> and nesprin-3 $\alpha$ <sup>L44A</sup> than with the other point mutants of the seven amino acid motif (Fig. 3D). When the R43A and L44A mutations were combined, co-immunoprecipitation of the plectin ABD was reduced to the same extent as observed for the nesprin-3 $\alpha$ <sup>A7AA</sup> mutant (Fig. 3D). To confirm these observations, the mutants were subsequently tested for their ability to recruit endogenous plectin to the NE. Upon transfection of the different GFP-tagged nesprin-3 $\alpha$  mutants in PA-JEB keratinocytes, plectin recruitment to the NE was abrogated for the R43A and the R43A-L44A double mutant of nesprin-3 $\alpha$  (Fig. 3E). The L44A mutation severely diminished plectin recruitment, but did not completely abrogate it. By contrast, other mutations in the seven amino acid motif had no effect on the recruitment of endogenous plectin to the NE (Fig. 3E; data not shown).

All this shows that residues R43 and L44 present in the first SR of mouse nesprin-3 $\alpha$  are crucial for plectin binding and recruitment to the NE, thereby establishing an indirect link between the nucleus and the IF system.

### Binding sites on the plectin ABD for nesprin-3 $\alpha$ and the integrin $\beta$ 4 subunit partially overlap

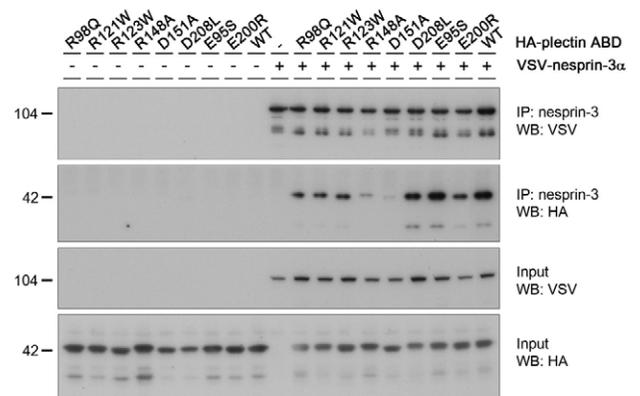
Besides its interaction with nesprin-3 $\alpha$ , the plectin ABD can also interact with F-actin and the integrin  $\beta$ 4 subunit (Geerts et al., 1999; Litjens et al., 2003). The crucial residues in the ABD that mediate the interaction with integrin  $\beta$ 4 were previously identified and found to overlap with those important for binding to actin (de Pereda et al., 2009; Litjens et al., 2003; Litjens et al., 2005). Moreover, two arginine residues in integrin  $\beta$ 4, R1225 and R1281, are of crucial importance for the interaction with the plectin ABD (Koster et al., 2001). Because binding of nesprin-3 $\alpha$  to the plectin ABD is also mediated via an arginine residue, we determined whether the binding sites for nesprin-3 $\alpha$  and integrin  $\beta$ 4 on the plectin ABD overlap with each other.

We initially used a yeast two-hybrid assay to test the interaction of several point mutants of the plectin-1C ABD with either nesprin-3 $\alpha$  or part of the cytoplasmic domain of the integrin  $\beta$ 4 subunit ( $\beta$ 4<sup>1115-1436</sup>). The point mutants were selected on the basis of their reported effects on the interaction with integrin  $\beta$ 4 (de Pereda et al., 2009; Litjens et al., 2005). Most of these mutations abolished integrin  $\beta$ 4 binding; only the E200R and D208L mutants did not. As shown in Fig. 4A, the binding profiles of nesprin-3 $\alpha$  and integrin  $\beta$ 4 partially overlap. Both nesprin-3 $\alpha$  and integrin  $\beta$ 4 bound wild-type plectin ABD and the E200R and D208L point mutants (Fig. 4A). Binding was not detected when residues E95, R148 and D151 of the plectin ABD had been mutated (Fig. 4A). However, there were also differences in plectin binding between integrin  $\beta$ 4 and nesprin-3 $\alpha$ . Whereas R98Q, R121W and R123W reduced binding to integrin  $\beta$ 4, they did not influence the interaction with nesprin-3 $\alpha$  (Fig. 4A).

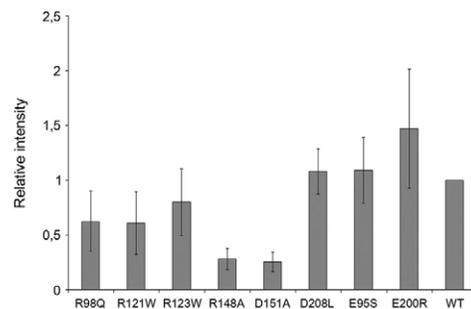
A

GAL4(BD)	GAL4(AD)	
	$\beta$ 4 <sup>1115-1436</sup>	nesprin-3 $\alpha$
WT	++	++
E95S	-	-
R98Q	-	++
R121W	-	++
R123W	-	++
R148A	-	-
D151A	-	-
E200R	++	++
D208L	++	++

B



C



### Fig. 4. Partial overlapping binding sites for nesprin-3 $\alpha$ and the integrin $\beta$ 4 subunit on the plectin ABD. (A) Binding of plectin-1C ABD point mutants to $\beta$ 4<sup>1115-1436</sup> and nesprin-3 $\alpha$ in yeast two-hybrid assays. Plating efficiencies on selective SC-LTHA plates are expressed relative to those on nonselective SC-LT plates of the same transformation. ++, >40%; +, 10–40%; and -, <10%.

Plates were scored after 5 and 10 days of growth. (B) COS7 cells were transiently transfected with constructs encoding VSV-mouse-nesprin-3 $\alpha$ , wild-type HA-plectin-1C ABD or point mutants of HA-plectin-1C ABD. Cells were lysed and both input samples and nesprin-3 precipitates (IP) were probed for VSV glycoprotein and HA. WB, Western blot; WT, wild-type. (C) Quantitative analysis of the effect demonstrated in (B). Intensities are corrected for nesprin-3 precipitation and normalized to the value for wild-type HA-plectin-1C ABD. Results are shown as the mean  $\pm$  s.d. of three different experiments per mutant.

To confirm the role of these residues in the binding of the plectin ABD to nesprin-3 $\alpha$ , the point mutants were tested for their interaction with nesprin-3 $\alpha$  in a co-immunoprecipitation experiment. VSV-tagged nesprin-3 $\alpha$  was coexpressed in COS7 cells together with HA-tagged wild-type plectin ABD or its point mutants. As expected, wild-type plectin ABD as well as its R98Q, R121W, R123W, E200R and D208L point mutants co-precipitated with nesprin-3 $\alpha$  (Fig. 4B,C). Similar to the results of the yeast two-hybrid assay, co-precipitation of the R148A and D151A

mutants of the plectin ABD was strongly reduced. Interestingly, although the E95S mutation affected nesprin-3 $\alpha$  binding in the yeast two-hybrid assay, mutation of E95 had no effect on the binding to nesprin-3 $\alpha$  in the co-immunoprecipitation experiment (Fig. 4B,C).

The yeast two-hybrid and co-immunoprecipitation data demonstrate that residues R148 and D151 of the plectin ABD are required for both nesprin-3 $\alpha$  and integrin  $\beta$ 4 binding, thereby indicating that the binding sites for these two proteins on the plectin ABD partially overlap (Fig. 5).

## Discussion

In this study, we have characterized the localization of nesprin-3 in zebrafish and studied the consequences of its deficiency. Although nesprin-3 is expressed at the NE of epidermal and skeletal muscle cells during zebrafish development, nesprin-3-deficient zebrafish are viable, fertile and have no defects in their motility. Detailed analyses of *nesprin-3* null mutant embryos revealed reduced localization of the IF system at the NE in epidermal cells. In addition, we showed that two residues (R43 and L44) in the first SR of nesprin-3 $\alpha$ , which are lacking in nesprin-3 $\beta$ , are crucial for the recruitment of plectin to the NE and facilitate the connection between the NE and the IF system. This is the first *in vivo* model in which it could be demonstrated that the loss of nesprin-3 is associated with reduced anchorage of IFs to the NE.

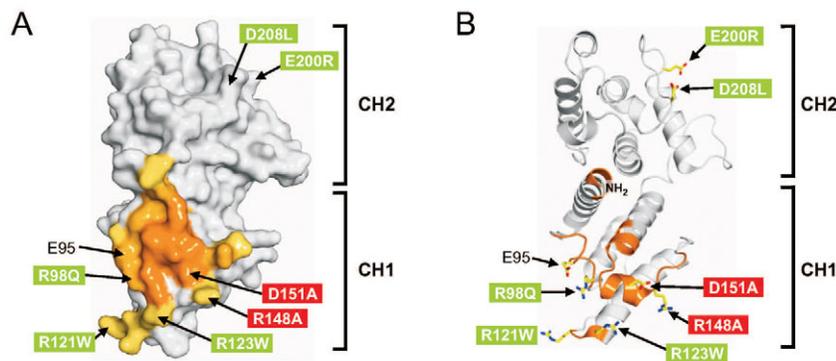
Despite the fact that the association of the IF system with the NE was reduced in nesprin-3-deficient zebrafish, they unexpectedly did not display an overt abnormal phenotype. This result surprised us because: first, nesprin-3 $\alpha$  is highly conserved and, as in mice, there is an isoform in zebrafish (nesprin-3 $\beta$ ) that does not bind plectin; second, nesprin-3 $\alpha$  and nesprin-3 $\beta$  are differentially expressed during zebrafish development; and third, nesprin-3 $\alpha$  is the only nesprin family member currently known to mediate a connection of the NE with the IF system.

Although nesprin-3 $\alpha$  is the main isoform expressed during later stages of development, *nesprin-3 $\beta$*  mRNA is expressed predominantly during the early stages. This suggests that the connection of the NE with the IF system needs to be prevented in certain cell types during the early stages of zebrafish development. For example, during skeletal muscle differentiation in the first days

of zebrafish development, single myocytes need to fuse in order to form a multinucleated skeletal muscle fiber (Ochi and Westerfield, 2007). During this process, a complex reorganization of the cytoskeleton is needed to stabilize and anchor multiple nuclei in differentiating skeletal muscle cells (Peckham, 2008). However, because nuclear positioning and anchorage appear normal in muscles of zebrafish lacking the two nesprin-3 isoforms, it seems unlikely that the differential expression of the two isoforms has much influence on the connection of the NE with the IF system. Notably, in a recent study of mutant mice lacking the C-terminal SR and KASH domain of nesprin-1, nuclear positioning and anchorage were disturbed (Zhang et al., 2010). Moreover, several missense mutations in nesprin-1 and nesprin-2 have been associated with the pathogenesis of Emery–Dreifuss muscular dystrophy (Zhang, Q. et al., 2007). Thus, in contrast to the role of nesprin-1 and nesprin-2 in normal muscle physiology, that of nesprin-3 seems to be limited.

It has previously been shown that disruption of a linkage similar to that of plectin and nesprin-3 $\alpha$ , namely that between plectin and the integrin  $\alpha$ 6 $\beta$ 4 at the plasma membrane, results in severe defects in cellular stability and integrity (Koster et al., 2001; Nakano et al., 2001; Pulkkinen et al., 1998). However, these defects were evident only in cells that have undergone severe mechanical stress. Cellular stress was induced in our zebrafish developmental model by raising *nesprin-3* mutant embryos in viscous medium. Interestingly, induction of mechanical stress produced no obvious defects in epidermal or skeletal muscle cells (data not shown). Although this finding argues against a role of nesprin-3 $\alpha$  in maintaining cellular stability and integrity, it cannot be excluded that the strength of the mechanical stress applied in this experiment was insufficient to reveal such a role of nesprin-3. Furthermore, it should be understood that the binding of the IF cytoskeleton to the NE was not completely lost, suggesting the existence of alternative mechanisms to anchor IFs to the NE. One possible example to support this idea is that the IF cytoskeleton can be connected indirectly to the NE through interactions between plectin and F-actin, which in turn bind nesprin-1 and nesprin-2.

Nesprins physically integrate the cytoskeleton with the nucleoskeleton through interactions with SUN proteins localized at the INM, which in turn bind lamins (Crisp et al., 2006; Haque et



**Fig. 5. Comparison of the binding sites for nesprin-3 $\alpha$  and the integrin  $\beta$ 4 subunit on the structure of the plectin ABD.** (A) Surface representation of the ABD of plectin (PDB code 3F7P), which consists of two calponin homology domains (CH1 and CH2). The integrin  $\beta$ 4-binding surface is located in the CH1 and is colored in orange (darker for residues at the core of the interface). The labels indicate the point mutations that were analyzed and are colored indicating whether they compromised binding to nesprin-3 $\alpha$  (red) or not (green). R148 and D151 lay adjacent in the 3D structure and form part of the integrin  $\beta$ 4-binding site. (B) Ribbon representation of the ABD in the same orientation as in A. For clarity, only the side chains of the residues analyzed are shown. The molecular representations were created with the program Pymol (DeLano, 2002).

al., 2006; Ketema et al., 2007). It has been suggested that nesprins play an important role in the transmission of mechanical signals from the cytoskeleton into the nucleus (Jaalouk and Lammerding, 2009; Wang et al., 2009). Notably, these signals might cause changes in the organization of lamins that affect their interaction with chromatin and DNA, thereby influencing gene transcription (Andres and Gonzalez, 2009). Moreover, because both the IF and actin cytoskeleton are anchored at the plasma membrane via integrins and dystroglycan, stress to which these cell surface receptors are submitted might lead to a direct transmission of mechanical signals into the nucleus via these protein networks (Geerts et al., 1999; Legate et al., 2009; Reznicek et al., 1998; Reznicek et al., 2007). However, the importance of this mechanical connection for the regulation of gene transcription and the role of nesprin-3 in this process await further investigation.

The identification of zebrafish nesprin-3 $\beta$  enabled us to further investigate the crucial residues in mouse nesprin-3 $\alpha$  that mediate plectin binding. We have identified two residues (R43 and L44) in the first SR of nesprin-3 $\alpha$  that are essential for efficient plectin binding. Both residues are conserved in other species, suggesting that the mechanism responsible for plectin binding by nesprin-3 $\alpha$  is similar in these species. The fact that one of the residues is an arginine is of interest because arginines have also been identified as crucial residues in the binding of the integrin  $\beta$ 4 subunit to the plectin ABD (Koster et al., 2001). Moreover, as for the binding of the plectin ABD to integrin  $\beta$ 4, we found that residue D151 in the plectin ABD is important for binding to nesprin-3 $\alpha$ . In the interaction of plectin with integrin  $\beta$ 4, D151 forms a salt bridge with R1225 (de Pereda et al., 2009). Hence, it is tempting to speculate that this same residue also forms a salt bridge with R43 on nesprin-3 $\alpha$ .

The finding that D151 and R148 on the plectin ABD are important for its binding to both integrin  $\beta$ 4 and nesprin-3 suggests that the binding sites for these two proteins on the plectin ABD overlap (Fig. 5). They, however, are not identical because several other residues important for binding to integrin  $\beta$ 4 are not involved in nesprin-3 binding. We have previously shown that both integrin  $\beta$ 4 and nesprin-3 $\alpha$  compete with F-actin for binding to plectin (Geerts et al., 1999; Ketema et al., 2007). The overlap in nesprin-3 $\alpha$  and integrin  $\beta$ 4 binding sites reported in this study suggests that F-actin, nesprin-3 $\alpha$  and the integrin  $\beta$ 4 subunit all compete with each other for binding to plectin.

The binding of wild-type and mutant plectin ABD to nesprin-3 $\alpha$  was determined in two independent assays, i.e., a yeast two-hybrid assay and a pull-down experiment. The results obtained with the two assays are in good agreement, except for the interaction of the E95S mutant with nesprin-3 $\alpha$ . This interaction was readily detected in the co-precipitation experiment, but was not observed in yeast. Differences in the ratio between the two interacting partners and/or the influence of the GAL4 binding domain on the structure of the E95S mutant might account for the discrepancy between the two assays. An alternative explanation is that, by replacing the glutamic acid with serine, we introduced a phosphorylation site for a kinase present in yeast. Phosphorylation of the mutant ABD might have altered its conformation such that it can no longer interact with nesprin-3 $\alpha$ . The exact explanation for this discrepancy, however, is unclear and requires further investigation.

In summary, this is the first study in which the loss of nesprin-3 in an animal model has been described and we have demonstrated that nesprin-3 is dispensable for zebrafish embryonic development.

Nevertheless, nesprin-3 $\alpha$  contributes to the establishment of a chain of proteins that connects the nucleus with the IF system in epidermal cells. Further study of this and other animal models yet to be developed should provide more detailed information on the function of nesprin-3 at the cellular level.

## Materials and Methods

### Fish strains and screening F1 ENU-mutation library

Embryos and adult fish were raised and maintained under standard laboratory conditions. An ENU-induced mutation library was screened for mutations in exon 4 of the *nesprin-3* gene as described (Wienholds and Plasterk, 2004). The mutant allele (*nesprin-3<sup>hu644b</sup>*) was outcrossed against the TL strain.

### Constructs

Full-length zebrafish *nesprin-3 $\alpha$*  was produced from cDNA with the following primers: forward 5'-CCATCGATACCATGACCCAGCAGGAGCAGCATG-3', reverse 5'-GCTCTAGATTAAGTGGGTGGAGACCCTCG-3', and cloned into pCS2+ using *Clal* and *XbaI* restriction sites. The *nesprin-3* fragment for probe syntheses was produced with the following primers: forward 5'-CGTCTGTATGCTGACTGGAG-3', reverse 5'-AGAGAAAGTGTCTTCGACCAG-3', and cloned into the pGEM-T Easy vector. The following primers were used to PCR *nesprin-3 $\beta$* : forward 5'-AGCACTGGAGAAAATCCACA-3', reverse 5'-TCCTGTACGGTTGAACAGAG-3'; *nesprin-3*: forward 5'-AAGAGCTGACTCTCCAGCAC-3', reverse 5'-GGAAGGATCTGCCTTTCAC-3'; and *efl $\alpha$* : forward 5'-GTGCTGTGCTGATTGTTGCT-3', reverse 5'-TGTATGCGCTGACTTCTTGTG-3'. The GST-zebrafish-nesprin-3-SR7 construct was produced by amplifying the coding region for SR7 from pCS2+-zebrafish-nesprin-3 $\alpha$  using the following primers: forward 5'-CCGGAATCCCGAGAGCATGAGAGCTTTC-3' and reverse 5'-GATAAGAATGCGGCCGAGTGGTCTGAAGAAGCCTG-3'. The PCR product was cloned into pGEX-4T-2 (GE Healthcare) using the *EcoRI* and *NotI* restriction sites.

The GAL4 fusion plasmids used in this study are depicted in Fig. 4A.  $\beta$ 4<sup>1115-1436</sup> and nesprin-3 $\alpha$  in pACT2 and plectin-1C ABD in pAS2.1 have been described previously (Geerts et al., 1999; Schaapveld et al., 1998; Wilhelmsen et al., 2005). The various plectin point mutants were generated by the PCR overlap extension method or created previously (Litjens et al., 2005). The constructs encoding VSV-mouse-nesprin-3 $\alpha$ , HA-plectin-1C ABD<sup>WT</sup>, HA-plectin-1C ABD<sup>E95S</sup>, HA-plectin-1C ABD<sup>R98Q</sup> and HA-plectin-1C ABD<sup>R123W</sup> have been described before (Geerts et al., 1999; Wilhelmsen et al., 2005). The other plectin-1C ABD point mutants were amplified from pAS2.1 and subcloned into pcDNA3-HA. The seven amino acid deletion was introduced into pcDNA3-2 $\times$ VSV-mouse-nesprin-3 $\alpha$  by PCR of the plasmid using the following primers: forward 5'-AAAATCTGCCAGCTGGAGTCTG-3' and reverse 5'-CTCCAGGCCGCTCGGGGC-3'. The PCR product was subsequently ligated to obtain the VSV-mouse-nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup> construct. Point mutants of VSV-mouse-nesprin-3 $\alpha$  were generated either by the PCR overlap extension method or using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mouse nesprin-3 $\alpha$ , nesprin-3 $\alpha$  <sup>$\Delta$ 7AA</sup> and the nesprin-3 $\alpha$  point mutants were subcloned from pcDNA3-2 $\times$ VSV into mGFP-C1 by first inserting the 3' part using the *BamHI*-*XbaI* restriction sites. The 5' parts were subsequently inserted using a *BamHI* digest of the nesprin-3 variants.

All PCR fragments were generated using the proofreading *Pwo* DNA polymerase (Roche). Plasmids were verified by sequencing, and protein expression, and size was confirmed by western blotting.

### Yeast two-hybrid assay

Yeast strain *S. cerevisiae* PJ69-4A (a gift from Philip James, University of Wisconsin, Madison, WI), which contains the genetic markers W1-901, L2-3, H3-200, gal4 $\Delta$ , gal80 $\Delta$ , LYS2::GAL1-HIS3, GAL2-ADE2 (James et al., 1996), was used as the host for the two-hybrid assay. The use of PJ69-4A was essentially as described previously (Geerts et al., 1999; Schaapveld et al., 1998). Cells were spread out on plates containing yeast synthetic complete medium lacking leucine and tryptophan (SC-LT plates) or leucine, tryptophan, histidine and adenine (SC-LTHA plates). Plates were scored after 5 and 10 days of growth at 30°C.

### Zebrafish nesprin-3 antibody production

The GST-zebrafish-nesprin-3-SR7 construct was transformed in Rosetta cells. Fusion protein expression was induced by 100  $\mu$ M isopropyl thio  $\beta$ -D-galactoside (IPTG) at 18°C overnight. Bacteria were lysed by sonication in resuspension buffer [50 mM Tris pH 8.0, 100 mM NaCl, 0.05%  $\beta$ -mercaptoethanol and protease inhibitor cocktail (Sigma)]. Brij-35 was added to a final concentration of 0.02% and insoluble material was removed by centrifugation. GST fusion proteins were purified by binding to glutathione Sepharose 4B (GE Healthcare). The beads were washed four times with wash buffer [50 mM Tris pH 8.0, 100 mM NaCl, 0.05%  $\beta$ -mercaptoethanol, 0.02% Brij-35 and protease inhibitor cocktail (Sigma)] and fusion proteins were eluted from the glutathione Sepharose beads using 10 mM reduced glutathione in 50 mM Tris pH 8.0. Rabbit antiserum against zebrafish nesprin-3 was obtained by immunization with the recombinant GST-SR7 protein. The rabbit was immunized four times with 100  $\mu$ g fusion protein per immunization before the antiserum was obtained.

### Antibodies

The rabbit polyclonal antibody (pAb) against mouse nesprin-3 has been described previously (Wilhelmsen et al., 2005). The following other primary antibodies were used: anti-VSV glycoprotein monoclonal antibody (mAb) (P5D4; Sigma), anti-HA mAb (12CA5; Santa Cruz Biotechnology), anti-plectin mAb (clone 31; BD Transduction Laboratories), anti-actin mAb (Chemicon International), anti-p63 mAb (1:100; 4A4; Santa Cruz Biotechnology), anti- $\beta$ -tubulin mAb (1:100; N357; Amersham Life Science) and Ks pan 1–8 mAb (1:10; Progen Biotechnik). The following secondary antibodies were used: goat-anti-mouse FITC (Rockland Immunochemicals), goat-anti-mouse Texas Red (Invitrogen), goat-anti-rabbit Texas Red (Invitrogen), goat-anti-mouse horseradish peroxidase (GE Healthcare) and donkey-anti-rabbit horseradish peroxidase (GE Healthcare). Alexa Fluor 488 phalloidin and TO-PRO-3 iodide 642/661 were obtained from Invitrogen, and DAPI was purchased from Sigma-Aldrich.

### In situ hybridization

Embryos were fixed overnight in 4% paraformaldehyde (PFA) in PBS at 4°C, washed in PBS and stored in methanol at –20°C. Whole mount ISH was performed as described previously (Thisse et al., 1993). The RNA probe was produced according to standard protocols.

### Plastic sectioning

Embryos stained by whole-mount ISH were transferred from benzyl benzoate/benzyl alcohol to 100% methanol, incubated for 10 minutes, washed twice with 100% ethanol for 10 minutes and incubated overnight in 100% Technovit 8100 infiltration solution (Kulzer) at 4°C. Next, embryos were embedded overnight in Technovit 8100 embedding medium (Kulzer) at 4°C. Sections of 7  $\mu$ m thickness were cut with a Reichert-Jung 2050 microtome, stretched on water and mounted on glass slides. Sections were dried overnight. Counterstaining was done using 0.05% neutral red for 12 seconds, followed by extensive washing with water. Sections were preserved with Pertex and mounted under a coverslip.

### Cell culture and transfection

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 keratinocytes were grown in keratinocyte serum-free medium (GIBCO Life Technologies) supplemented with 50  $\mu$ g/ml bovine pituitary extract, 5 ng/ml EGF, 100 U/ml penicillin and 100 U/ml streptomycin. PA-JEB cells were transiently transfected with Lipofectin (Invitrogen) according to the instructions provided by the manufacturer. Transient transfection of COS7 cells was achieved using the DEAE-dextran method. All cells were analyzed 24–48 hours after transfection.

### Immunofluorescence

Cells grown on glass coverslips were fixed in PBS containing 1% PFA for 15 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 1.5 hours at room temperature. Cells were washed three times with PBS and incubated for 1 hour at room temperature with the secondary antibody. After three washes with PBS, coverslips were mounted in Mowiol-DAPCO. Embryos were fixed overnight in PBS containing 2% PFA at 4°C, washed with 0.3% Triton X-100 in PBS, blocked with 1% BSA and 0.3% Triton X-100 in PBS, and incubated with the primary antibody. After overnight incubation at 4°C, embryos were washed for 3 hours with 0.3% Triton X-100 in PBS, blocked with 1% BSA and 0.3% Triton X-100 in PBS for 1 hour, and incubated with the secondary antibody for 3 hours. Embryos stained for keratin or  $\beta$ -tubulin were fixed in Dent's fixative (80% methanol, 20% DMSO). Embryos and cells were viewed under a confocal laser scanning microscope (model SP-2 AOBIS; Leica).

### Lysis and immunoprecipitation

Protein extracts were prepared from 100 devalked embryos (8 hpf) in radioimmunoprecipitation (RIPA) buffer (2  $\mu$ l/embryo). RIPA lysis buffer contained 10 mM sodium phosphate pH 7, 150 mM NaCl, 1% Nonidet P40 (NP40), 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 100  $\mu$ M sodium vanadate and protease inhibitor cocktail (Sigma). COS7 cells grown to confluency in 10-cm tissue culture dishes were lysed in 1 ml lysis buffer consisting of M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) and NP40 lysis buffer [1% NP40, 150 mM NaCl, 50 mM Tris pH 8.0 and protease inhibitor cocktail (Sigma)] in a ratio of 9:1. Lysates were cleared by centrifugation at 20,000 g in a microcentrifuge at 4°C for 60 minutes. Nesprin-3 proteins were precipitated by incubation of the cell lysates with 1.5  $\mu$ l rabbit-anti-mouse-nesprin-3 pAb for 1 hour on ice, followed by a subsequent incubation with 100  $\mu$ l of a 10% slurry of Protein A Sepharose (Amersham Biosciences) for 2.5 hours at 4°C while being steadily inverted. Immunoprecipitates were washed four times with Tris-buffered saline, boiled for 5 minutes in SDS sample buffer (50 mM Tris pH 6.8, 10% glycerol, 1% SDS, 0.5%  $\beta$ -mercaptoethanol and 0.025% bromophenol blue) and resolved by SDS-PAGE.

### Quantification and statistics

Perinuclear keratin was quantified by determining the percentage of cells with NE-associated keratin staining per embryo. A minimum of 36 cells per embryo in at least two microscopic fields were analyzed in a blind fashion. Western blot quantification was performed with Adobe Photoshop software. All data are represented as the mean  $\pm$  s.d. Comparisons were carried out with the one-way Anova and statistical significance was assumed for  $P < 0.05$ .

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