Nesprin-2 is a multi-isomeric protein that binds lamin and emerin at the nuclear envelope and forms a subcellular network in skeletal muscle

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Summary

Nesprin-2 is a multi-isomeric, modular protein composed of variable numbers of spectrin-repeats linked to a C-terminal transmembrane domain and/or to N-terminal paired calponin homology (CH) domains. The smaller isoforms of nesprin-2 co-localize with and bind lamin A and emerin at the inner nuclear envelope (NE). In SW-13 cells, which lack lamin A/C, nesprin-2 epitopes and emerin were both mislocalized and formed aggregates in the endoplasmic reticulum (ER). The larger isoforms and other CH-domain-containing isoforms co-localize with heterochromatin within the nucleus and are also present at the outer NE and in multiple cytoplasmic compartments. Nesprin-2 isoforms relocalize during in vitro muscle differentiation of C2C12 myoblasts to the sarcomere of myotubes. Immunogold electron microscopy using antibodies specific for three different epitopes detected nesprin-2 isoforms at multiple locations including intranuclear foci, both membranes of the NE, mitochondria, sarcomeric structures and plasma membrane foci. In adult skeletal muscle, confocal immunolocalization studies demonstrated that nesprin-2 epitopes were present at the Z-line and were also associated with the sarcoplasmic reticulum (SR) in close apposition to SERCA2. These data suggest that nesprin-2 isoforms form a linking network between organelles and the actin cytoskeleton and thus may be important for maintaining sub-cellular spatial organisation. Moreover, its association at the NE with lamin and emerin, the genes mutated in Emery-Dreifuss muscular dystrophy, suggests a mechanism to explain how disruption of the NE leads to muscle dysfunction.

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Key words: Nuclear envelope, Lamin, Actin, Muscle, Sarcoplasmic reticulum

Introduction

In search of vascular smooth muscle cell (VSMC) differentiation markers, we previously identified two ubiquitously expressed genes encoding members of a novel family of nuclear membrane-associated proteins, nesprin-1 (isoforms of which are also known as syne-1/myne-1) and nesprin-2 (isoforms of which are also known as syne-2/NUANCE). Their general structure is that of type II integral membrane proteins comprising a long N-terminal cyto- or nucleo-plasmic domain, composed of multiple spectrin repeats that are predicted to form a dystrophin-like rod domain, linked to a C-terminal transmembrane domain (Zhang et al., 2001). Nesprin transcripts undergo extensive alternative initiation and splicing to generate multiple isoforms and initially we identified five nesprin-1/-2 isoforms, which differed in the length of their rod domains. Transient transfections of enhanced green fluorescent protein (EGFP)-fusion constructs of these nesprin-1 and -2 isoforms into human VSMCs and mouse C2C12 myoblasts demonstrated their localization to the nuclear envelope (NE). A C-terminal-60 residue membrane-spanning region, designated the KLS domain, was homologous to the C-terminus of the *Drosophila* Klarischt protein. The KLS domain was essential for the NE localization of both nesprin-1 and nesprin-2 isoforms (Zhang et al., 2001).

The NE consists of an inner and outer nuclear membrane joined at nuclear pores (Gerace and Burke, 1988). The outer membrane is connected to the peripheral ER, whereas the inner membrane is supported by the lamina, a network of intermediate filament proteins, composed of lamins and lamin associated proteins, including the lamin B receptor (LBR), lamina-associated polypeptides 1 and 2 (LAP1 and LAP2), emerin and MAN1 (Lin et al., 2000; Stuurman et al., 1998). Emerin, LAP2 and MAN1 contain a domain termed the LEM (LAP2, Emerin, MAN1) which binds to BAF (barrier to autointegration factor), an essential protein involved in nuclear assembly, chromatin structure and gene expression (reviewed by Segura-Totten and Wilson, 2004). Nesprin-1 also has an interrupted LEM domain, however it is non-functional for BAF

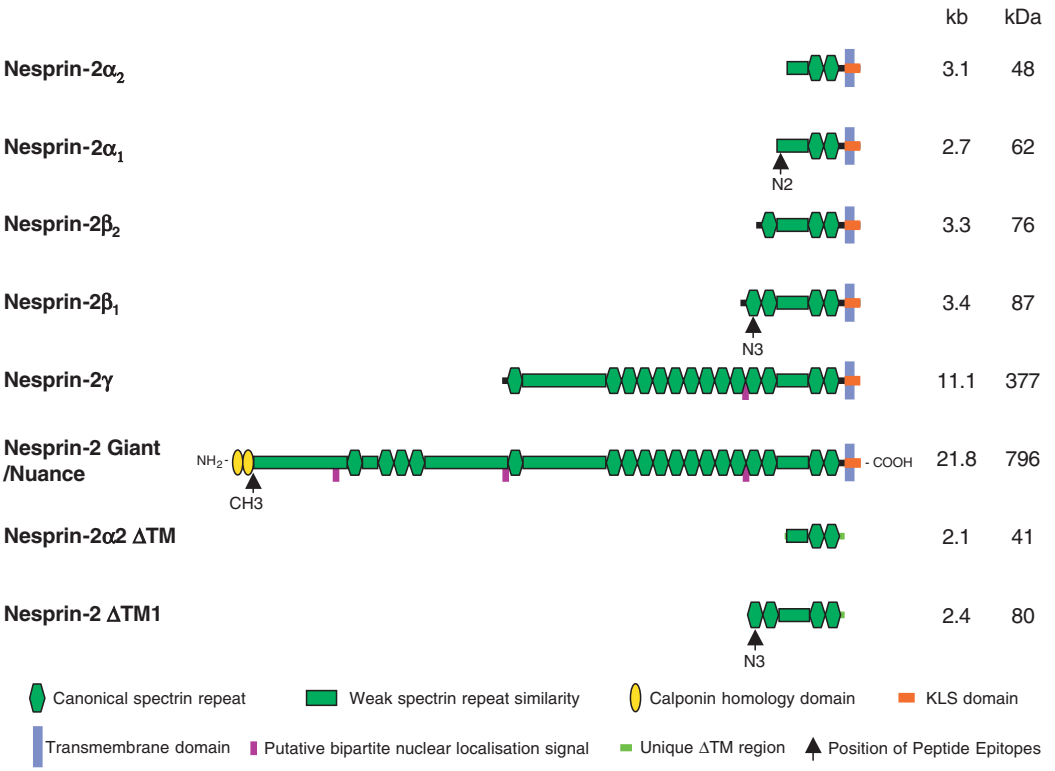


Fig. 1. Domain structure of the major nesprin-2 isoforms identified to date. Isoforms lacking the transmembrane/KLS domain are designated ΔTM. The size of the mRNA in kb is shown as well as the predicted molecular mass in kDa for each isoform. Regions shown in green are predicted to form a continuous rod domain in the presence or absence of amino acids encoded by the small alternatively spliced exons shown in green with an asterisk in Fig. 2.

binding (Mislow et al., 2002). Mutations in nuclear lamins and their NE binding partner emerin cause Emery-Dreifuss muscular dystrophy (EDMD) and cardiomyopathy with conduction defects (Bione et al., 1994; Bonne et al., 1999; Fatkin et al., 1999). Immunofluorescence using nesprin-1-specific antibodies showed that nesprin-1 co-localized with lamin A/C and emerin at the NE, and immunoprecipitation experiments showed that a small isoform of nesprin-1 (nesprin-1α), binds both lamin A and emerin (Mislow et al., 2002) (our unpublished data).

Larger isoforms of nesprin-1 and nesprin-2, including two giant isoforms of 1.01 MDa and 796 kDa respectively, have also been identified. These isoforms consist of paired N-terminal actin-binding CH domains linked by an extended rod domain to the C-terminal KLS domain (Zhang et al., 2002; Zhen et al., 2002). Nesprins have single giant orthologues in both *Drosophila* and *Caenorhabditis elegans*. The *Drosophila* protein is an extension of a known cytoskeletal protein called MSP-300, that localizes to the Z-lines in muscle; with mutations in MSP300 causing a muscle defect (Zhang et al., 2002; Rosenberg-Hasson et al., 1996; Volk, 1992). The *C. elegans* protein, ANC-1, localizes in the outer NE via its KLS/KASH domain. Its role in nuclear migration requires both the KLS/KASH domain and CH domain, which binds F-actin (Starr and Han, 2002). To date there is no evidence that the worm and fly genes are alternatively spliced and therefore capable of producing the multiplicity of isoforms observed in mammalian cells.

High expression of mammalian nesprins occurs in cardiac, smooth and skeletal muscle implying a specific role in muscle function (Apel et al., 2000; Zhang et al., 2001). The subcellular localization of nesprin-1 is altered from the NE to the nucleus and cytoplasm during in vitro differentiation of C2C12 myoblasts into myotubes while the larger isoforms of nesprin-1 are abundant in the sarcomere of both human skeletal and cardiac muscle (Zhang et al., 2002). Moreover, one isoform, nesprin-1α_2, originally identified as associated with the NE, is only detectable in skeletal, cardiac and smooth muscle, and is specifically enriched in the synaptic nuclei of syncytial muscle fibres (Apel et al., 2000; Zhang et al., 2001). However, while something is known of nesprin-1, less is known about nesprin-2, which is more abundant in skeletal and cardiac muscle. The giant isoform of nesprin-2 (NUANCE) binds F-actin and localizes predominantly to the outer NE suggesting roles in nuclear migration, similar to the *C. elegans* orthologue ANC-1 (Zhen et al., 2002). In this study we have characterized the isoform distribution, subcellular localization and binding properties of nesprin-2. We demonstrate that it is a multi-isomeric protein, located in multiple cellular compartments and that it is a new lamin- and emerin-binding protein of the NE.

Materials and Methods

PCR of human nesprin-2 and 5′ or 3′ rapid amplification of cDNA ends (RACE)

The full-length nesprin-2 cDNA sequence (accession number AF495911) was generated using 2 μl of Marathon-ready cDNA from human spleen, heart, skeletal muscle and testis (about 0.1 ng/μl;

Clontech) and a high fidelity GC-rich PCR kit (Roche). Primers were designed from the predicted sequences at overlapping 2 kb intervals (primer sequences available upon request) (Zhang et al., 2002). 5′ and 3′ RACE were performed using an Advantage-GC2 PCR Kit (Clontech) to generate the 5′ ends of nesprin-2 isoforms and to confirm predicted sequences. PCR fragments were cloned into pGEM-Easy (Promega) or pCRII vectors (Invitrogen) for automated sequencing using a BigDye sequencing kit (ABI) (Lark technologies).

Multiple human tissue Northern blots (MTN) (Clontech) were hybridised according to the manufacturer's instructions using ^{32}P-labelled probes generated by PCR for specific 5′ untranslated regions (UTR), 3′ UTR and the CH-domain of nesprin-2. The tissue distributions for nesprin-2 isoforms were generated by RT-PCR, using primers to the specific 5′ and 3′ UTR regions of the different isoforms (for details, see Fig. S1 in supplementary material). Isoform sequences were deposited into the GenBank database. Intron/exon boundaries were generated by blasting the cDNA sequences of nesprin-2 isoforms, against sequences in the human genome database.

Cell culture and cell fractionation

Human VSMCs were cultured as described previously (Zhang et al., 2001). Mouse C2C12 myoblasts (a generous gift from S. Hughes, King's College London, London, UK), human adrenal cortex carcinoma (SW13) cells and SW13/20 cells (a generous gift from C. Hutchison, University of Durham, UK) and human skin fibroblasts were cultured at 37°C/5% CO_2, in Dulbecco's modified Eagle's medium (DMEM)/15% or 10% FCS (Sigma). To generate myotubes, 80% confluent C2C12 myoblasts were moved to 2% horse serum and 10% CO_2 for 3-7 days. Cell fractions were generated as described previously (Barton and Worman, 1999) with minor modifications: cells were harvested by scraping and washed with PBS. Pelleted cells were resuspended in four pellet volumes of hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM phenylmethylsufonyl fluoride), left on ice for 5 minutes, and then sheared by seven passages through a 25-gauge needle. Lysed cells were underlaid with hypotonic lysis buffer containing 30% sucrose and centrifuged at 10,000 ***g*** for 10 minutes at 4°C. The supernatant was removed and clarified into nuclear or whole cell debris by centrifugation for 10 minutes at 14,000 ***g*** in a microcentrifuge. The cytoplasmic fraction was removed from the pellet and set aside. The sucrose pellet containing nuclei was resuspended in

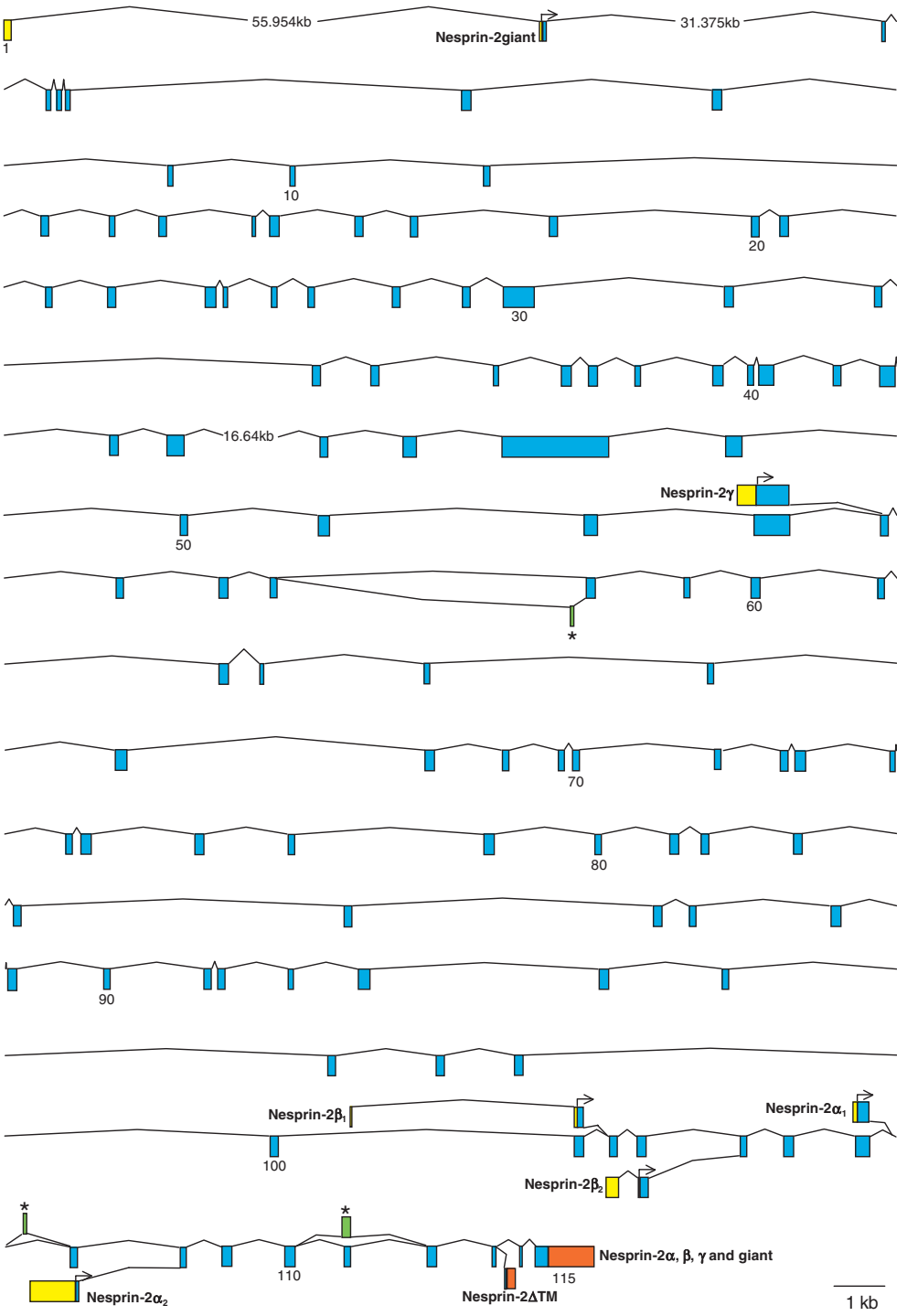
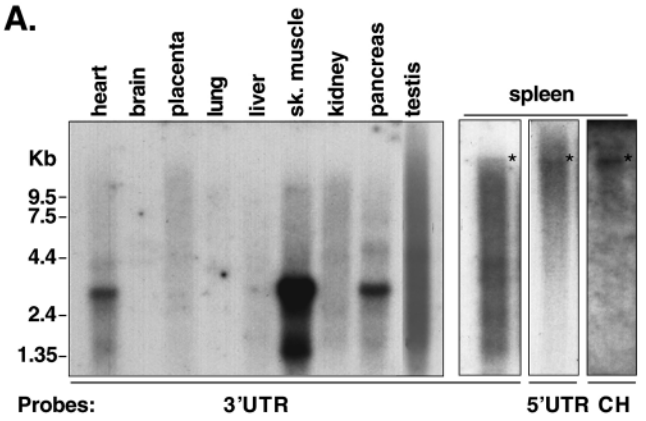


Fig. 2. Diagrammatic representation, drawn to scale, of the intron/exon splicing events that generate all nesprin-2 isoforms identified to date. Sites of alternate initiation and termination as well as alternatively spliced coding exons along the length of the giant isoform are shown. Coding exons are blue while alternatively spliced coding exons are green and starred. These are not specific to any isoform but utilized in some tissues in a number of isoforms. The 5′ and 3′ UTRs of each isoform are shown in yellow and red, respectively. Introns are represented by lines, with the majority of introns 1, 2 and 45, omitted because of size constraints.

a pellet volume of nuclear extraction buffer (20 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM phenylmethylsufonyl fluoride) and digested with 1 μg/ml DNase I and 10 μg/ml RNase A at room temperature (RT) for 15 minutes. Nuclei in all subsequent steps were pelleted by centrifugation at 14,000 ***g*** for 20 seconds. NEs were washed three times with 1 pellet volume of nuclear extraction buffer containing 0.5 M NaCl, and supernatants were saved and combined. NE pellets were then washed and resuspended in a pellet volume of nuclear extraction buffer and sonicated briefly with a Sonic Dismembrator to suspend the NEs. Protein concentrations of each fraction were estimated using the Bio-Rad protein assay system.

Antibody production and western blotting

Rabbit antibodies to different regions of human nesprin-2 were generated against three synthetic polypeptides: nesprin-2-N2, PREIQTDSWRKRGES (6369-6383aa); nesprin-2-N3, RQIHERLTQLELINKQ (6183-6198aa), and nesprin-2-CH3, KRDLDELDKDHLQLRC (353-367aa), (Immune Systems Ltd, Paignton, UK). Each polypeptide was conjugated to keyhole limpet haemocyanin and the conjugates injected into rabbits to produce polyclonal antibodies that were subsequently affinity purified and ELISA tested to confirm specificity (Immune Systems Ltd, Paignton, UK). Western blots were performed on cell fractions from human VSMCs, C2C12 myoblasts/myotubes and human tissues from heart, kidney, skeletal muscle and testis according to standard procedures. Control antibodies for cell fractions were smooth muscle (SM)-α-actin (Sigma), SF2 (Zymed Laboratories) and emerin (Novacastra). Nesprin-2 isoforms were detected using N2, N3 and CH3 antibodies (diluted 1:1000-1:2000) followed by incubation with a horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody diluted 1:2000 (NA934, Amersham Pharmacia Biotech). Antibody specificity controls were performed by pre-incubation with 5-10× peptides specific for each antibody and then western blotting. The ECL^{+Plus} chemiluminescent kit (Amersham Pharmacia Biotech) was used for signal detection.



B.

Nesprin-2 Multiple Tissue PCR Amplifications												
	Brain	Spleen	Heart	Liver	Kidney	PBL	Small Intestine	Prostate	Muscle	Lung	Ovary	Testis
Nesprin-2 CHD	+/-	++	+	+/-	+	+	+	++	+++	+/-	+/-	+
Nesprin-2 KLS	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
Nesprin-2 ΔTM	-	-	+	++	-	-	++	+	+++	-	+	+
Nesprin-2α_1	-	-	+++	-	-	-	-	-	+++	-	-	-
Nesprin-2α_2	+	+	+	+	-	-	+++	+++	+++	++	++++	-
Nesprin-2β	+	-	-	-	-	-	++++	++++	++++	++	++++	-
Nesprin-2α_2ΔTM	-	-	-	-	-	-	-	-	+	-	-	-

Fig. 3. (A) Northern blots showing nesprin-2 isoform expression in human tissues. Note that a >20 kb band (asterisks) is present in spleen using 5′ UTR (1-333 bp)-, 3′ UTR (20,673-21,364 bp)- and CH-domain (161-1787 bp)-specific probes confirming that the giant isoform predicted by RT-PCR is transcribed. Highly expressed short transcripts are present in skeletal muscle, heart and pancreas with numerous transcripts of different sizes expressed at low levels in most tissues. (B) Table showing tissue distributions and levels of expression of specific nesprin-2 domains and isoforms as determined by RT-PCR in various human tissue cDNA samples (for details, see Fig. S1 in supplementary material).

Immunohistochemistry and confocal microscopy

Frozen sections of fresh human skeletal muscle biopsies were placed onto Superfrost Plus microscope slides, fixed in acetone/methanol (1:1) for 5 minutes at –20°C and permeablized with 0.5% NP-40/TBS (Tris-buffered saline, pH 7.6) for 3.5 minutes at RT. After blocking with 5% normal goat serum/TBS at RT for 30 minutes and incubation at 4°C overnight with N2, N3 or CH3 antibodies (diluted 1:100-150 in blocking buffer), sections were incubated in the dark for 30 minutes at RT with a Alexa Fluor^{TM} 488-conjugated goat anti-rabbit IgG secondary antibody diluted 1:200 (A-11008, Vector Laboratories, Inc). Rabbit IgG (I5006, Sigma) was used as a negative control while peptide blocking with 5-10× peptides specific for N2, N3 and CH3 ablated all staining. Cultured cells or myotubes on chamber slides were stained as described above. Other markers were detected with monoclonal antibodies: lamin A/C (MM107P, COVANCE), emerin (4G5, Novocastra), myomesin (a generous gift from J.-C. Perriard, Institute of Cell Biology, Zurich, Switzerland), titin T12 and T15, Z- and M-line markers respectively (both generous gifts from M. Gautel, King's College London, London, UK), titin ab7034, A/I junction marker, (abcam), myosin (A4.1025, Alexis Corporation), SERCA2 (ab2817, abcam) and ryanodine receptor (ab2868, abcam) were incubated with Alexa Fluor^{TM} 568-conjugated goat anti-mouse IgG secondary antibody diluted 1:200 (A11004, Vector Laboratories, Inc). All samples were mounted in Vectashield^{TM} mounting medium containing DAPI (Vector Laboratories, Inc) and images were captured using a Leica TCS-NT-UV laser scanning confocal system, or digitally captured using an Olympus BX51 fluorescence microscope. Confocal images were captured using a X63, 1.2 NA, water immersion lens. Alexa dyes were excited simultaneously using 488 and 568 nm laser lines and their emitted light was captured between 510-550 nm and 610-650 nm, respectively. DAPI-stained nuclei were sequentially excited using a Tsunami (Spectra Physics) multiphoton laser tuned to 720 nm, using a capture window of 400-480 nm. A pinhole setting of 1 airy disk equivalent was used and sampling in xy and z was set to the Nyquist requirements for the objective lens and the mean wavelength of the emitted light collected in each channel. The image files were restored using the Huygens (Scientific Volume Imaging) maximum likelihood estimation (MLE) deconvolution algorithm running on a Silicon Graphics Octane 2 workstation. The deconvolved image files are presented as maximum projections of the entire z-stack.

In vitro transcription/translation and immunoprecipitation

Nesprin-2α and -2β, lamin A, C and B1 and emerin cDNA was inserted into pcDNA3 or pcDNA 3.1 and transcribed from the T7 promoter and translated in a TNT^{R} T7-coupled reticulocyte lysate

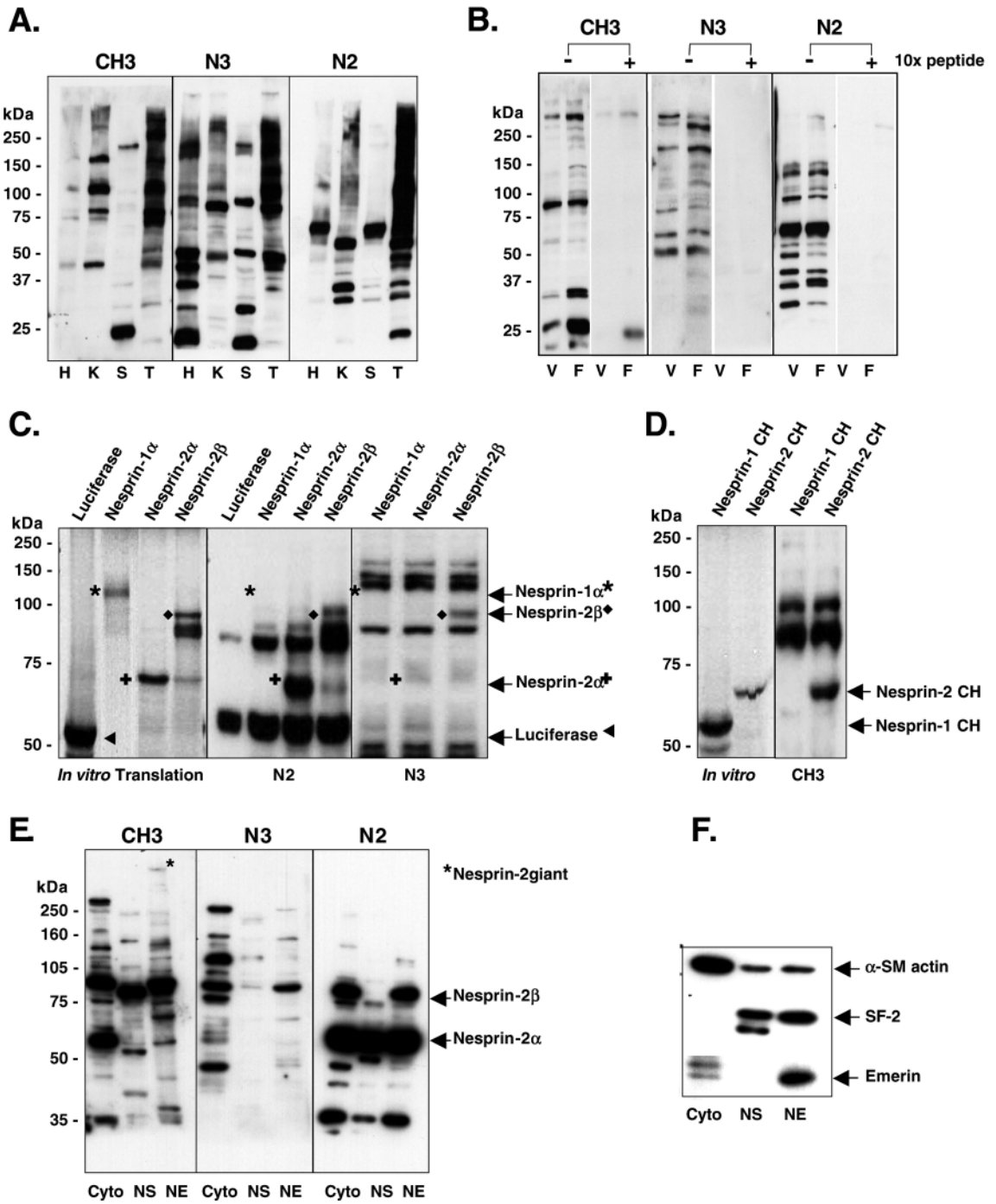


Fig. 4. (A) Western blot of different human tissues using nesprin-2 N2, N3 and CH3 antibodies demonstrating tissue-specific and multiple isoforms in heart (H), kidney (K), skeletal muscle (S) and testis (T). Note the dominant small isoforms and the similar pattern of bands using the N2 antibody in heart and skeletal muscle and the multiple bands in testis; consistent with northern blot results. (B) Multiple isoforms were identified by all antibodies in vascular smooth muscle cells (V) and fibroblasts (F) with these cell types having identical isoform patterns on western blots. All bands were competed by excess peptide confirming antibody specificity. (C) ^{35}S-labelled in vitro translation of nesprin-1α (asterisk), 2α (cross) and 2β (diamond) (first panel) and subsequent western blot analysis demonstrates specificity of N2 (middle panel) and N3 (end panel) antibodies. N2 recognised nesprin-2α and -2β while N3 recognised only nesprin-2β. Neither antibody recognised the closely related nesprin-1α. Luciferase (◀) (D) ^{35}S-labelled in vitro translation of nesprin-1 and nesprin-2 CH domains (left panel) and western blot using the CH3 antibody (right panel) shows that CH3 recognises only the nesprin-2 domain and not the closely related nesprin-1 domain confirming antibody specificity. Multiple unlabelled bands on western blots in C and D represent rabbit nesprin-2 isoforms present in the rabbit reticulocyte lysate. (E) Western blot using nesprin-2 N2, N3 and CH3 antibodies and sub-cellular fractions of human VSMCs to identify nesprin-2 isoforms in cytoplasmic and nuclear domains. Predicted sizes of isoforms identified from cDNA analysis are marked with arrowheads and it is probable that the N2 antibody identifies nesprin-2α and β isoforms. (F) Control blot shows α-SM actin, SF-2 and emerin to confirm cytoplasmic, nuclear and NE fractionation. Cyto, cytoplasmic; NS, nuclear soluble; NE, nuclear envelope.

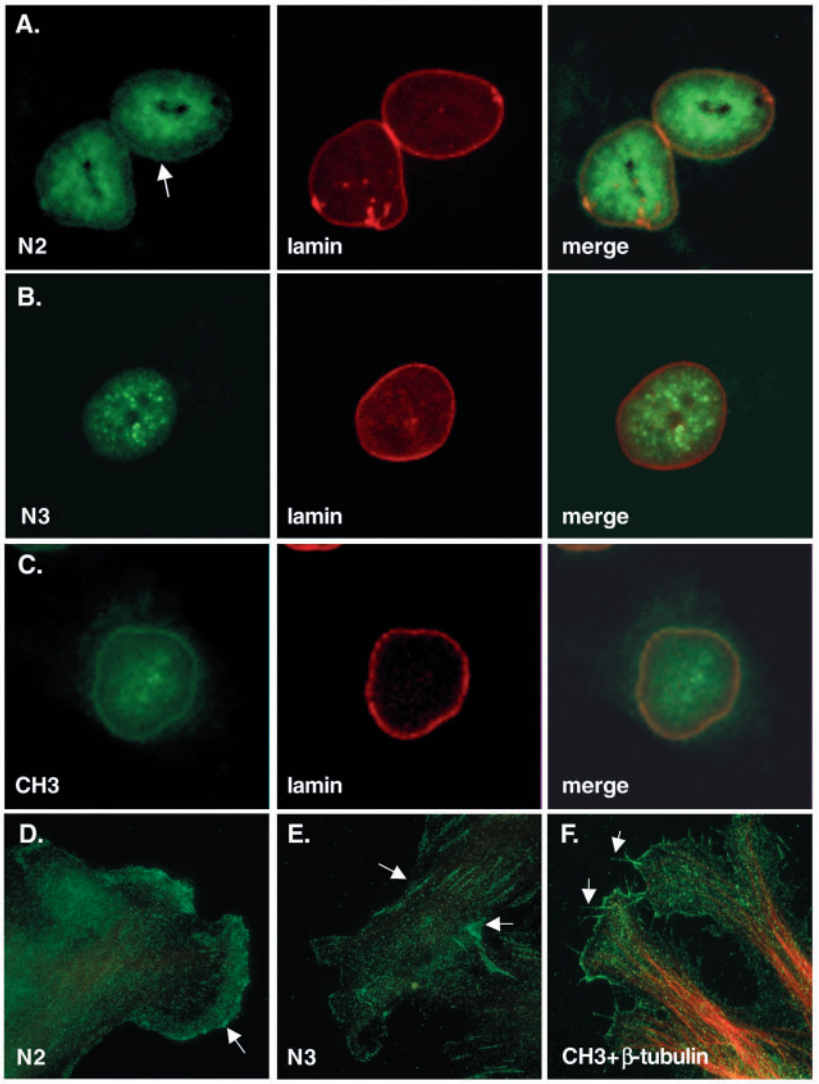


Fig. 5. (A-C) Immunofluorescent images showing the sub-cellular localization of nesprin-2 isoforms using N2, N3 and CH3 antibodies co-localized with lamin, in human VSMCs. All three antibodies localize to domains in the nucleus with N2 and CH3 showing nuclear rim staining and N2 and N3 abundant around nucleoli (identified by domain-specific antibody; not shown). Cytoplasmic staining was also observed using all three antibodies and this is shown in detail using human skin fibroblasts. (D-F) In the cytoplasm of skin fibroblasts, nesprin-2 is diffusely distributed in a reticular pattern and is localized to lamellipodia (arrow in D), to focal adhesions (arrows in E) and to filopodia (arrow in F; red stain shows β-tubulin). All stainings were competed completely by excess peptide (not shown).

system (Promega, UK), according to the manufacturer's instructions, in the presence of [^{35}S]methionine. Immunoprecipitation (IP) was performed by mixing the labelled nesprin-2α or -2β translation product with either ^{35}S-labelled lamin A, C, B1 or emerin products at 30°C for an hour, before adding lysis IP buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100) to the mixture at 4°C for 30 minutes and then incubating with nesprin-2 N2 or N3 antibodies at 4°C overnight. This was followed by addition of Protein A-agarose beads (1719408, Roche) and further incubation for 1 hour at 4°C. After washing with IP buffer four times and sedimentation of the protein complexes, the pellets were subjected to SDS-PAGE electrophoresis and autoradiography.

For in vivo IP, confluent human VSMCs were harvested, treated with IP buffer (10 mM Hepes, pH 7.4, 10 mM KCl, 5 mM EDTA, 1% Triton X-100 and protease inhibitor cocktail) and sonicated on ice, and then centrifuged at 16,000 ***g*** for 15 minutes at 4°C. Lysates was then precleared with protein A agarose beads (Roche). IPs with N2 and N3 antibodies and rabbit IgG (4 μg antibody/100 μg cell lysates) were performed at 4°C overnight, followed by incubation with protein A agarose beads at 4°C for 2 hours and centrifugation at 4,000 ***g*** for 10 minutes. After washing with IP buffer four times and sedimentation of the protein complexes, the pellets were subjected to 4-15% gradient SDS-PAGE electrophoresis and western blotting using emerin (1:250, 4G5 Novacastra) and lamin A/C (1:150, Jol2 Serotec) antibodies.

Immunogold labelling

Cells were fixed in 4% (v/v) formaldehyde in 0.1 M Pipes buffer (Sigma), cryo-protected in 25% (v/v) polypropylene glycol and frozen in melting propane before freeze-substitution in dry methanol (–90°C) containing 0.1% uranyl acetate and embedding in Lowicryl HM-20 (Taab) at –50°C. Thin sections (50 nm) were mounted on Formvar film grids and incubated in TBS at pH 7.4 containing 0.5% FCS and 10% normal goat serum for 5 minutes at RT to block non-specific binding. This was followed by incubation with primary nesprin-2 antibodies, diluted 1:100 in blocking buffer, for 16 hours at RT. After rinsing with TBS they were incubated with secondary goat anti-rabbit immunoglobulins conjugated to 10 nm gold particles (British Biocell, Cardiff, UK) diluted 1:100 in TBS at pH 8.2 for 60 minutes at RT. After extensive rinsing in TBS, followed by distilled H_2O, they were stained with uranyl acetate and lead citrate and viewed in a CM-100 transmission electron microscope (Philips, Eindhoven, Netherlands). Non-specific binding was tested by omission of the primary antibody and substitution with an irrelevant antibody.

Results

Identification and expression of nesprin-2 isoforms

RT-PCR and 5′-RACE identified at least eight major isoforms of nesprin-2 that vary in the length of their spectrin-repeat rod domains and in the presence or absence of the C-terminal KLS domain and the N-terminal paired CH domains (Fig. 1). Analysis of the intron-exon boundary structure of the nesprin-2 gene showed that these alternate transcripts/isoforms were generated by alternate initiation and termination of transcription (Fig. 2). A number of small alternatively spliced coding exons were also identified. These exons are incorporated into a number of isoforms in a tissue-specific manner resulting in the generation of a large number of minor isoforms. The protein sequences within these small insertions do not disrupt the general continuity of the coiled-coil structure of the nesprin-2 spectrin-repeat rod domain.

Northern blots using human MTNs and the 5′ and 3′ UTR and CH domain as probes demonstrated multiple transcripts for nesprin-2 as described previously (Zhang et al., 2001) (Fig. 3).

One transcript of approximately 20 kb was identified in spleen using both 5′ and 3′UTR probes, confirming the existence of the giant isoform. High expression of smaller sized transcripts was observed in skeletal muscle and heart while many transcripts of variable size were detected in testis using the 3′ UTR as a probe (Fig. 3). A number of weak transcripts of various sizes were observed in some tissues using the CH domain probe suggesting that other, as yet unidentified isoforms containing this domain, are transcribed from the nesprin-2 gene (not shown).

RT-PCR analysis using primers specific for the major isoforms demonstrated their expression in multiple tissues (Fig. 3B; see also Fig. S1 in supplementary material). One of the shorter isoforms, nesprin-2α_1, was expressed exclusively in heart and skeletal muscle corresponding to the high expression of smaller transcripts observed on northern blots.

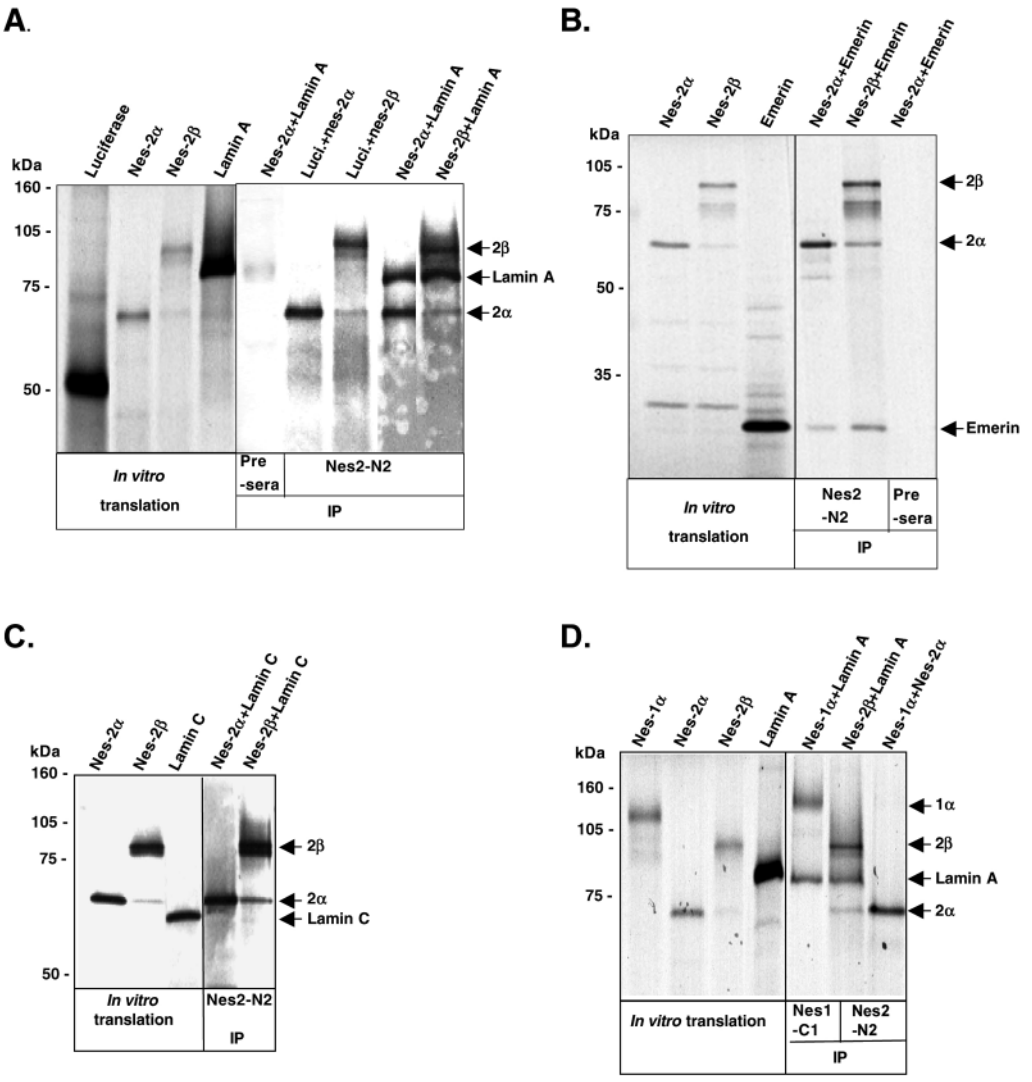


Fig. 6. In vitro co-immunoprecipitation (IP) assays demonstrating (A) nesprin-2α and -2β binding to lamin A and (B) emerin. IP of nesprin-2β with lamin A and emerin could be demonstrated with both N2 and N3 antibodies (N2 only shown). (C) Binding of nesprin-2α or -2β to lamin C could not be demonstrated. (D) Although both nesprin-1 and nesprin-2 bind lamin A, nesprin-2 does not bind to nesprin-1 (D).

Antibody characterisation, tissue-specific isoforms and cell fractionations

Affinity purified polyclonal antibodies were generated against three peptides (N2, N3 and CH3) corresponding to different regions along the length of the nesprin-2-giant protein (Fig. 1). These antibodies were not isoform specific because of the absence of unique amino acid sequences in each isoform. Western blot analysis demonstrated that numerous isoforms of nesprin-2, ranging in size from >250 kDa to 20 kDa, were present in multiple tissues (Fig. 4A). The isoform pattern for heart and skeletal muscle was similar with the N2 antibody recognising a dominant band at approximately 60 kDa corresponding to the nesprin-2α isoform that was also most abundant on northern and RT-PCR analysis. The pattern in muscle tissues differed from that in kidney and testis, as might be expected from the tissue specificity of isoforms identified by RT-PCR and northern blot, with testis showing numerous bands consistent with the multiple RNA transcripts detected on northern blots. The isoform patterns for each nesprin-2 antibody in VSMCs and fibroblasts were identical as might be expected for de-differentiated cells with very similar properties in tissue culture (Fig. 4B).

The above western analyses suggested that there were many more isoforms of nesprin-2 than had been characterized by RNA/cDNA analysis. To further confirm antibody specificity, peptide blocking was performed and demonstrated that all bands on both the tissue (not shown) and cultured cell Western blots could be competed by excess peptide (Fig. 4B). In addition nesprin-2 antibodies did not cross-react with in vitro translated proteins generated to similar spectrin-repeat and CH-domains present in either other regions of nesprin-2 itself or equivalent regions in its most closely related family member nesprin-1 (Fig. 4C,D).

Western analysis on fractionated VSMCs demonstrated that each antibody recognised multiple nuclear and cytoplasmic nesprin-2 isoforms (Fig. 4E,F). N2, the most C-terminal antibody detected two major bands migrating at 61 kDa and 87 kDa, probably corresponding to the nesprin-2α and -2β isoforms. Bands of this size were abundant in both the cytoplasmic and NE compartments of the cells, with the

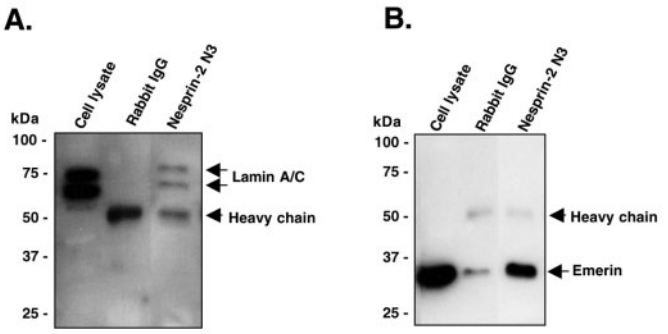


Fig. 7. In vivo co-immunoprecipitation assays demonstrating nesprin-2 binding to lamin-A/C (A) and emerin (B) (arrow) using the N3 antibody and VSMC lysates.

smaller band also abundant in the nuclear soluble fraction. N2 also detected an abundant band of 35 kDa that may correspond to the 1.35 kb transcript identified on human MTNs as expressed in muscle, or correspond to the small ΔTM isoform lacking the TM-domain; however the composition of these small isoforms remain unknown. N3 detected multiple and generally larger cytoplasmic isoforms potentially including nesprin-2β and -2γ (migrating at greater than 250 kDa) that were also present in the NE (Fig. 4E). The antibody raised to the N-terminal CH-domain region, CH3, recognised the giant nesprin-2 isoform and a large number of as yet unknown isoforms, ranging in size from 35 kDa to greater than 250 kDa abundant in the cytoplasm, NE and nucleus (Fig. 4E). The giant nesprin-2 isoform was detected only in the NE fraction (Fig. 4E). It is unknown whether all the protein bands detected by these antibodies represent splice or initiation variants or modified or cleaved proteins.

Subcellular localization of nesprin isoforms

To further define the subcellular distribution of the isoforms, immunolocalisation studies were performed using nesprin-2 antibodies (N2, N3 and CH3) in human VSMC and skin fibroblasts (Fig. 5). As inferred from western analyses, nesprin-2 isoforms displayed a variety of subcellular localizations. The most C-terminal antibody N2, was present at the NE where it colocalised with lamin (Fig. 5A) and emerin (not shown). N3 labelling was less obvious at the NE. However, both N2 and N3 strongly stained small foci scattered within the nucleus, and in a region surrounding the nucleoli (Fig. 5A,B). Although weak in VSMCs, both the N2 and N3 antibodies showed diffuse cytoplasmic staining in fibroblasts in a reticular pattern throughout the cell, in lamellipodia at the leading edge of cells and in focal adhesions (Fig. 5D,E). The most N-terminal CH3 antibody gave abundant nuclear staining and clear nuclear rim staining (Fig. 5C). It was also present in the cytoplasm and at the plasma membrane in lamellipodia and filopodia (Fig. 5F) (patterns consistent with those previously demonstrated for NUANCE using a monoclonal antibody generated

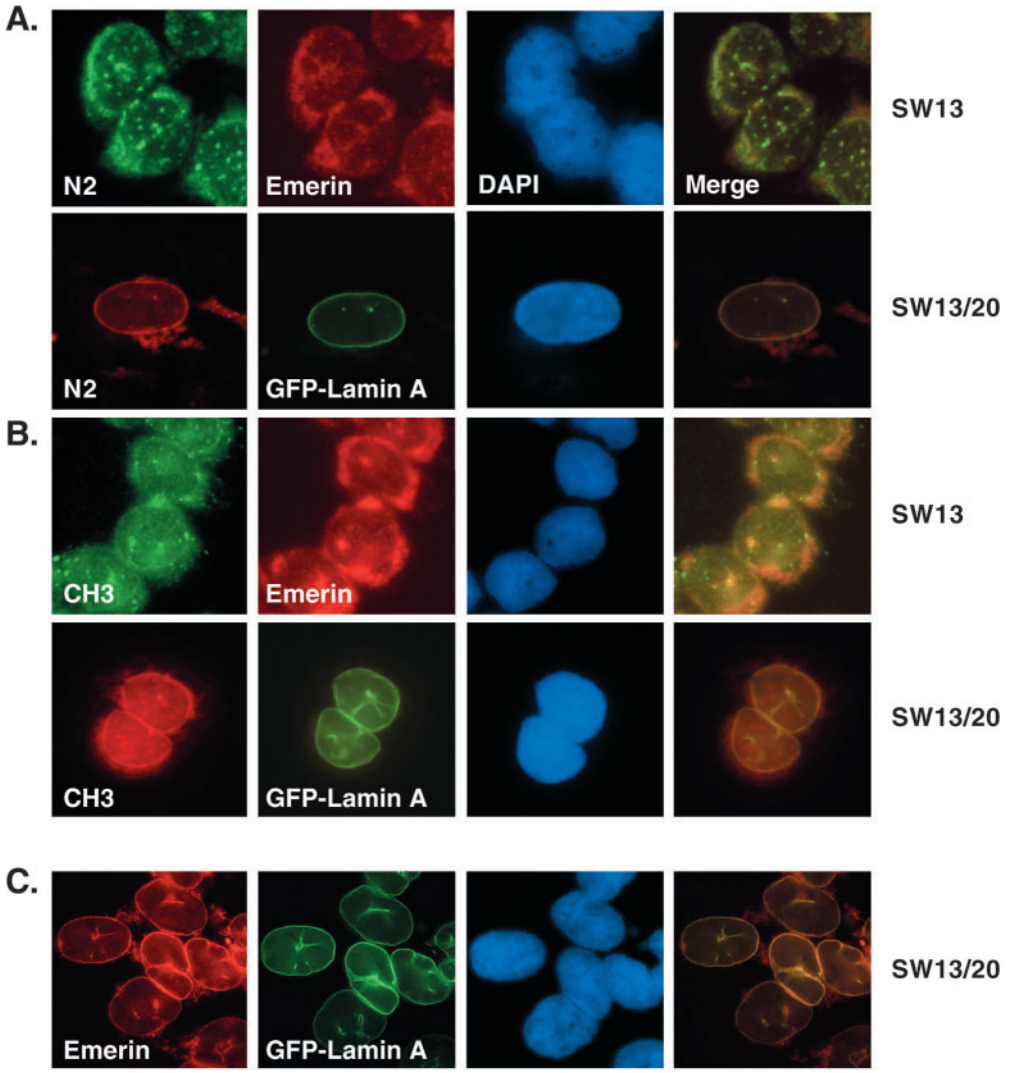


Fig. 8. Mislocalization of nesprin-2 isoforms in SW13 cells, and relocalization in SW13/20 cells. Nesprin-2 isoforms recognised by both the N2 (A, upper panel) and CH3 (B, upper panel) antibodies were mislocalized to the ER where they colocalized with emerin in lamin-A-negative SW13 cells. However, in lamin-A-GFP SW13/20 cells nesprin-2 isoforms recognised by these antibodies were correctly re-localized to the NE (A and B, lower panels) with emerin (C). With the N2 antibody there was also some mislocalization of nuclear nesprin-2 isoforms to punctate spots that were more evenly redistributed in lamin-A GFP SW13/20 cells (A).

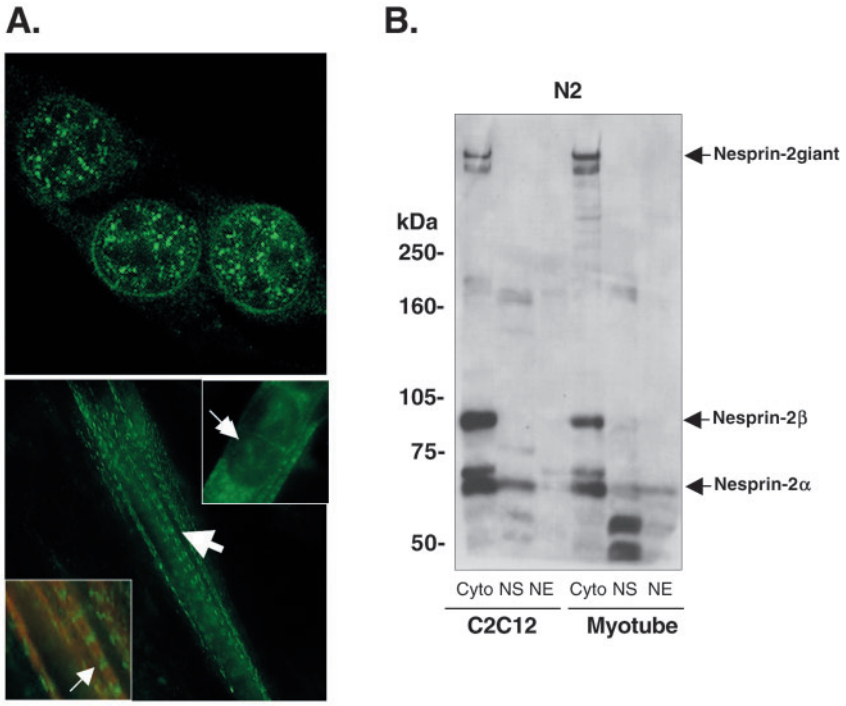


Fig. 9. The dominant sub-cellular localization of nesprin-2 isoforms changes during differentiation of C2C12 myoblasts to myotubes. (A) In myoblasts (top panel) the predominant N2 staining was nuclear, in a punctate pattern at the NE with only very weak cytoplasmic staining. In myotubes (lower panel) N2 recognised cytoplasmic structures of the muscle sarcomere organised in a striated pattern (arrows). Bottom inset: myosin staining to show differentiation and myofibre formation (small arrow) with N2 located between myosin positive bands. Top inset: the change in nuclear distribution of N2 in myotubes into a less punctate pattern (arrow). (B) Western blot analysis using the nesprin-2 N2 antibody and sub-cellular fractions of C2C12 myoblasts and myotubes demonstrated a shift in nesprin-2 isoform patterns in the cytoplasm and nucleus. The larger cytoplasmic isoforms were more highly expressed in myotubes with a reduction in smaller cytoplasmic isoforms and there was higher expression of two small nuclear isoforms in myotubes than in myoblasts.

to the CH-domain of nesprin-2, which included the region of the CH3 peptide) (Zhen et al., 2002).

C-terminal domains of nesprin-2 bind lamin and emerin in vitro and in vivo

As nesprin-2 co-localized with lamin A/C and emerin at the NE, we generated pcDNA3 constructs for full length nesprin-2α and -2β isoforms, and used in vitro transcription/translation and co-IP to further investigate the potential interactions between nesprin-2, lamin A/C, B1 and emerin. The nesprin-2, N2 antibody co-immunoprecipitated ^{35}S-labeled nesprin-2α or -2β with lamin A or emerin (Fig. 6A,B). The N3 antibody was able to co-immunoprecipitate nesprin-2β with lamin A or emerin (data not shown). Nesprin-2 failed to interact with lamin C (Fig. 6C) or lamin B1 in this system (data not shown). As these data suggested that nesprin-2, like nesprin-1, could interact with lamin A and emerin at the NE, we tested whether nesprin-1 and nesprin-2 could interact with each other (Mislow et al., 2001). We were unable to demonstrate their co-IP, which indicates that each nesprin interacts separately with both lamin A and emerin (Fig. 6D).

To investigate these interactions in vivo nesprin-2 antibodies were used to IP emerin and lamin from VSMC lysates. This demonstrated that both nesprin-2 N2 (not shown) and N3 antibodies could co-immunoprecipitate both emerin and lamin A/C (Fig. 7). The co-IP of lamin C in this system suggests that nesprin-2 may be able to interact with this protein under more physiological conditions.

We further investigated these interactions using SW-13 and SW13/20 cells. SW13 cells lack lamin A and previous studies have shown that emerin is mislocalized to the ER in these cells, demonstrating that an interaction with lamin is required to retain emerin at the NE (Vaughan et al., 2001). In SW13 cells, NE nesprin-2 isoforms were mislocalized and formed aggregates in the ER that colocalized with emerin (Fig. 8A,B). Cytoplasmic staining was unaffected but nuclear staining appeared more punctate using the N2 antibody. In SW13/20 cells, which stably express GFP-lamin A, emerin was relocated to the NE, as expected (Fig. 8C) as were NE nesprin-2 isoforms recognised by both the N2 (Fig. 8A) and CH3 (Fig. 8B) antibodies. Nuclear isoforms recognised by N2 were redistributed more evenly within the nucleus suggesting a lamin A interaction may also anchor these isoforms. Relocalization of CH3 epitopes suggested that as yet unidentified isoforms with CH-domains, and therefore, actin binding potential, are present at the inner NE.

Nesprin-2 associates with myofilaments during muscle differentiation in vitro

Nesprin-2 isoforms are highly expressed in skeletal muscle. We therefore examined the subcellular localization of nesprin-2 epitopes during in vitro differentiation of C2C12 myoblasts into myotubes using the panel of antibodies, which all gave similar staining patterns (Fig. 9). In undifferentiated myoblasts, nesprin-2 isoforms was predominantly present at the NE and/or in the nucleus in a punctate pattern (Fig. 9A). Three days after induction of myotube differentiation, nesprin-2 isoforms were clearly found in both the nucleus and the cytoplasm (not shown). After myotube formation, nesprin-2 staining was predominantly cytoplasmic in a linear pattern as expected for myofibrils. Co-localization with a myosin antibody confirmed that nesprin-2 was present in bands between each A-band. Nesprin-2 isoforms were still present at the nuclear rim, however, the nuclear staining pattern was less punctate.

Western blot analysis using the N2 antibody showed subtle changes in nesprin-2 isoform patterns between myoblasts and myotubes. In myotubes there was a decreased abundance of smaller cytoplasmic isoforms and an increase in the abundance of the largest cytoplasmic isoforms together with a marked increase in some small nuclear isoforms (Fig. 9B).

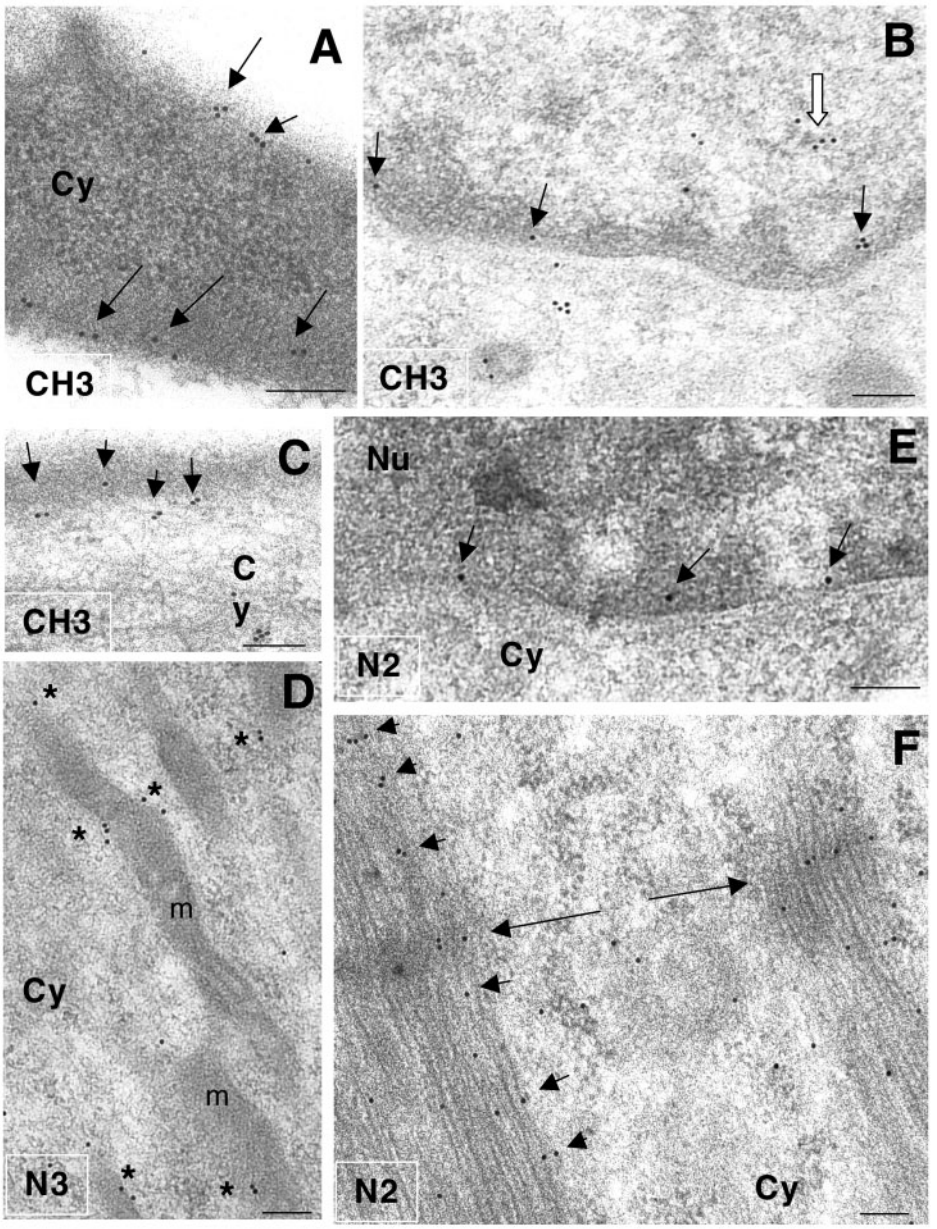


Fig. 10. EM immunogold localization of nesprin-2 epitopes in C2C12 myoblasts (A) and myotubes (B-F). In myoblasts (not shown) and myotubes, CH3 was present at the NE (black arrows in B) and in punctate nuclear (white arrow in B) localizations. In myoblasts CH3 localized to punctate zones along the plasma membrane (arrows in A) while in myotubes CH3 also decorated cytoplasmic structures, including isolated filaments associated with the plasma membrane (C). N3 was present in cytoplasmic domains in myotubes and decorated the rim of mitochondria (asterisks in D). In myoblasts (not shown) and myotubes, N2 was present at the NE (arrows in E) and in the cytoplasm at the Z-band (long arrows in F) and in a repeating pattern along the length of the myofibrils of the sarcomere (short arrows in F). Bar, 100 nm.

To further delineate the pattern of nesprin-2 localization in myoblasts and myotubes we used EM immunogold analysis. Immunogold decoration was apparent in myoblasts at both the inner nuclear membrane in association with heterochromatin (N2, CH3), the outer nuclear membrane (CH3) and within the nucleus (N3, CH3) at punctate sites in more euchromatic areas (not shown). In the cytoplasm, the CH3 antibody was distributed in clusters along the plasma membrane (Fig. 10A). In myotubes, the nuclear rim distribution of gold particles for N2 (inner NE; Fig. 10E) and nuclear rim and nuclear staining for the CH3 antibody (outer NE, punctate nuclear; Fig. 10B) was the same as that observed in myoblasts. However, both were densely distributed in the cytoplasm and localized in a repetitive, evenly spaced pattern along myofibrils and concentrated at the Z-band (N2, Fig. 10F, CH3 not shown). In addition, N3 gold labelling was clearly present in association with mitochondria (Fig. 10D) while CH3 also labelled isolated filaments positioned immediately subjacent to the plasma membrane (Fig. 10C).

Nesprin-2 associates with the Z-line and sarcoplasmic reticulum in skeletal muscle

The high expression of nesprin-2 in skeletal muscle and its association with myofilaments in C2C12 myoblasts led us to investigate its localisation in human skeletal muscle. The N2 antibody against the C-terminal region of the protein, demonstrated predominantly NE/nuclear staining, with prominent banding across the nucleus in some cells. There was also weak punctate staining in association with the sarcomere (Fig. 11L). Nuclear staining was also prominent with the N3 antibody (Fig. 11C inset) but weaker with the CH3 antibody (not shown). However, strong staining in both longitudinal and transverse lines throughout the sarcomere was observed with N3 and CH3 (Fig. 11A,E,I). Confocal de-convolution and co-staining with region-specific titin, myomesin and myosin antibodies showed that both N3 and CH3 epitopes were associated with the A/I junction and the Z-line (Fig. 11A-H). M-line staining was not observed as there was no co-localization with myomesin (Fig. 11I-K), nor was there co-localization with myosin (not shown). The association of nesprin-2 epitopes with the Z-line was observed to be punctate and suggested that nesprin-2 isoforms were associated with the sarcomere in this region. However, the pattern of staining at the A/I junction was more consistent with the localization of nesprin-2 in the sarcoplasmic reticulum (SR). Therefore, co-staining was performed with antibodies to SERCA2 ATPase to define the free SR and ryanodine receptor (RYR) to identify the junctional SR (Fig. 12). The staining pattern demonstrated that the most abundant nesprin-2 epitopes were in close apposition to SERCA2 ATPase in the free SR although the N3

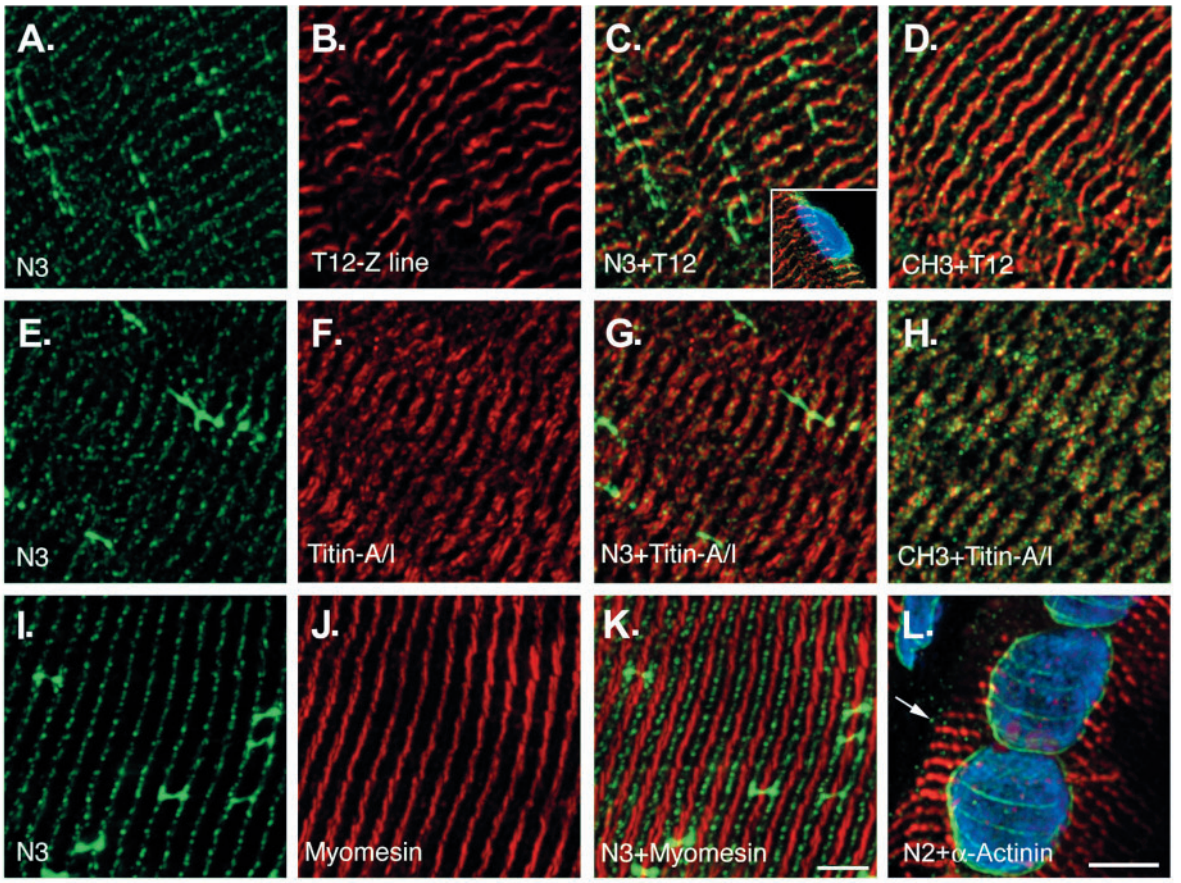


Fig. 11. Nesprin-2 epitopes are abundant in skeletal muscle. N3 and CH3 epitopes were localised to the sarcomere in a striated pattern and both colocalized in a punctate manner with titin T12 at the Z-line (A-D). N3 was also distributed around the nucleus (inset in C) and was present at the A/I junction where it colocalized with titin-A/I (E-G). The localization pattern for CH3 was essentially the same as that observed for N3 but this epitope was particularly abundant over the A/I junction where it colocalized with titin A/I (H). Nesprin-2 antibodies did not colocalize with myomesin at the M-line (N3 shown, I-K) confirming the Z-line/A/I junction distribution. The N2 antibody recognised the NE and nucleus, and was localized in a punctate pattern throughout the sarcomere in a pattern consistent with N3 and CH3, only weaker (arrow in L). Bar, 5 μm.

antibody showed some punctate colocalization with RYR. Co-localization of nesprin-2 epitopes with SERCA2, seen in cross sections, confirmed the localization of nesprin-2 isoforms to the SR (Fig. 12G-I).

Discussion

Nesprins are a conserved family of proteins present in *C. elegans, D. melanogaster* and all vertebrates analysed, suggesting an important role in basic cellular functions. We have shown that the shorter isoforms of nesprin-2 are present in the nucleus and at the inner NE, where they bind to emerin and lamin A, while larger isoforms are most probably present at the outer NE. In addition, nesprin-2 isoforms are abundant in the cytoplasm and associate with various cellular organelles, in particular the sarcomere of adult skeletal muscle.

Taken together with previous studies of nesprins in model organisms and studies of the closely related family member nesprin-1, we propose that the nesprin-2 family of proteins have multiple functions (Fig. 13). Nuclear/NE-associated nesprin-2 isoforms may perform multiple roles in maintaining the organisation and structural integrity of the nucleus and in nuclear anchorage by tethering the nucleus to actin (Starr and Han, 2002). Its distribution in multiple cytoplasmic domains, particularly in association with the SR and mitochondria, suggests it may tether such structures to the actin cytoskeleton and to myofibrils and thus be essential for the maintenance of the correct structural and spatial integrity required during sarcomeric signalling (Fig. 13). Organelle migration and sub-cellular partitioning are particularly important during muscle differentiation when dramatic changes in cytoskeletal structure and myofibril organisation occur to convert a simple cell into a highly organised differentiated cell in which accurate spatial orientation of sub-cellular components is a requisite for function (Berendse et al., 2003; Takekura et al., 2001). The abundance of muscle-specific nesprin-2 isoforms also supports a structural role. In addition, the presence of nesprin-2 isoforms at the plasma membrane and possibly in association with microfilaments and/or microtubules suggests a role in cell migration potentially by regulating the dynamics of actin and microtubule assembly and stabilization (Zhen et al., 2002; Fan and Beck, 2004).

Nesprin-2 at the NE

Nesprin-2 isoforms were present at the NE and in organized

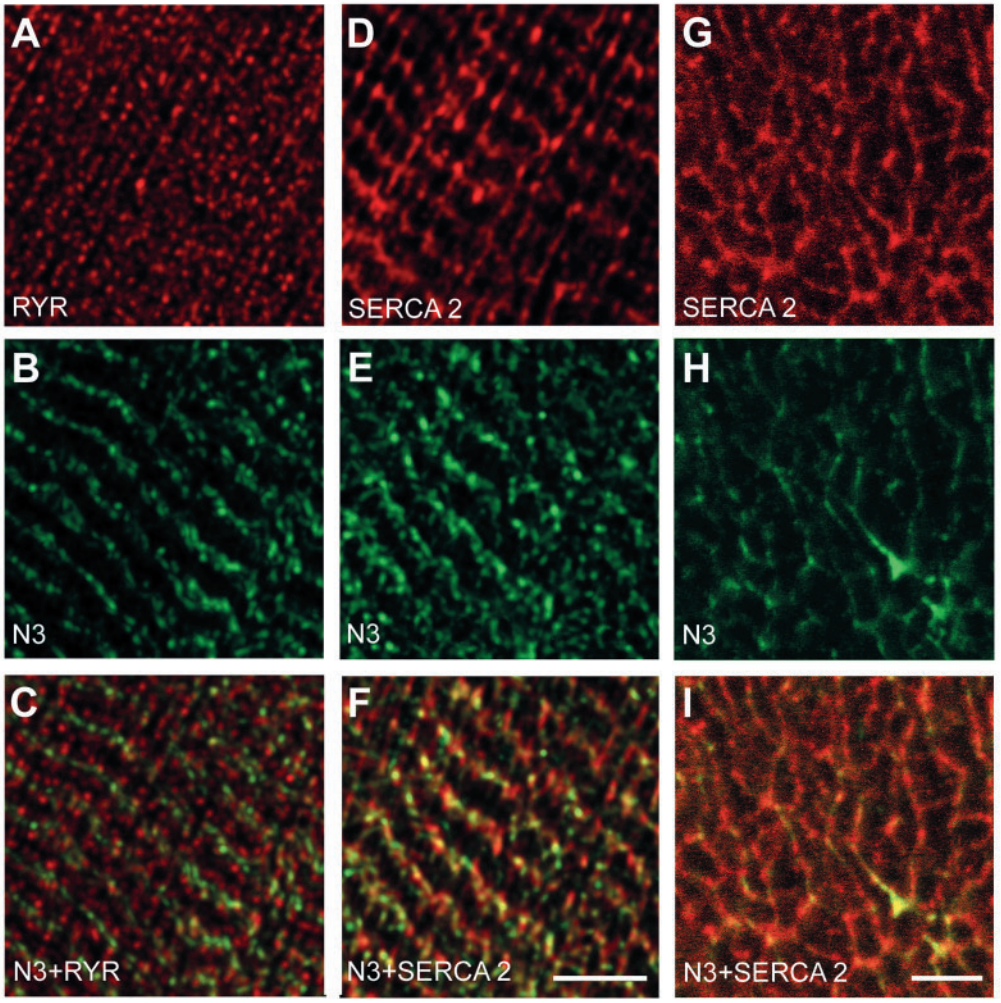


Fig. 12. Nesprin-2 epitopes are present in the sarcoplasmic reticulum in human skeletal muscle. Punctate Nesprin-2 N3 epitopes co-localize in a punctate fashion with RyR, a junctional SR marker (A-C). Larger areas of co-localization are apparent with both N3 and CH3 (not shown) and SERCA2, a free SR marker, in longitudinal sections (D-F) and confirmed in cross sections (G-I). Bar, 4 μm.

foci within the nucleoplasm and were found, using in vitro and in vivo IP, to be able to bind lamin A and emerin. Although nesprin-2 antibodies could IP lamin A/C in vivo, we could not demonstrate a nesprin-2/lamin C interaction in vitro. Potentially co-IP of lamin A/C in vivo might be due to complexing of lamin A and C suggesting that potentially the unique C-terminal region (aa 567-664) of lamin A may be responsible for nesprin-2/lamin A binding. Two conserved spectrin-repeat domains at the C terminus of nesprin-1α are sufficient to bind lamin A and homologous spectrin-repeats are also present in nesprin-2α (Mislow et al., 2002). However, further experiments are required to determine the specific domains of nesprin-2 that interact with both emerin and lamin.

We could find no evidence for co-IP of nesprin-1 with nesprin-2 in vitro. However, in SW13 cells nesprin-1, like nesprin-2 isoforms, were mislocalized with emerin in the ER (data not shown) suggesting that both nesprins separately bind emerin and lamin A to form a complex at the NE. EM immunogold localization of nesprin-2 isoforms within the interphase nucleus showed that like nesprin-1, the C-terminal region of nesprin-2 is associated with the inner NE and heterochromatin (Zhang et al., 2001). Emerin is an LEM-domain-containing protein that binds BAF, a DNA bridging-protein required for the assembly of emerin and A-type lamins during nuclear reassembly at mitosis (Haraguchi et al., 2001). Given the propensity for spectrin-repeats to participate in protein/protein interactions, it is likely that nesprins may organise a higher order complex involving lamin, heterochromatin and other inner NE proteins.

Nesprin-2 was also present at the outer NE. Previous studies have shown that the nesprin KLS domain acts as the NE targeting signal (Zhang et al., 2001). Thus, like other NE membrane-anchored proteins, this KLS domain probably inserts into the ER membrane and diffuses laterally via the nuclear pore membrane to reach the inner NE where it then anchors by binding to lamin A. However, proteins moving to the inner NE via this mechanism appear to be restricted in size (Pante and Kann, 2002; Soullam and Worman, 1995). We propose that larger nesprin-2 isoforms are excluded from the inner NE because of their size and thus remain in the outer NE where they may become tethered by binding to cytoplasmic intermediate filament proteins, and/or the actin cytoskeleton via their CH domains. In support of this, ANC-1 the worm orthologue of nesprins, has been shown to anchor nuclei in an UNC-84-dependent manner, by directly tethering the NE to the actin cytoskeleton (Star and Han, 2002; Lee et al., 2002). Similar UNC-84/SUN domain-containing proteins have been identified at the inner NE in mammals, however, their binding and colocalizations with nesprins have yet to be investigated (Malone et al., 1999; Hodzic et al., 2004). Like ANC-1, nesprin-2 may have a role in nuclear migration and anchorage. In addition, its presence at both the inner and outer NE suggests that it may form a bridging complex with SUN and other NE proteins, which spans the perinuclear space and connects the nuclear matrix to the actin cytoskeleton. This may be important for the appropriate transmission of mechanically induced signalling from the cell surface to the nucleus, for correct positioning of the nucleus within the cell, as well as having a potential role in NE breakdown (Karki and Holzbaur, 1999; Raff, 1999; Salina et al., 2002). In support of a multifunctional role of different nesprin isoforms, *Drosophila* Klarsicht, is a NE/vesicle membrane protein that shares the

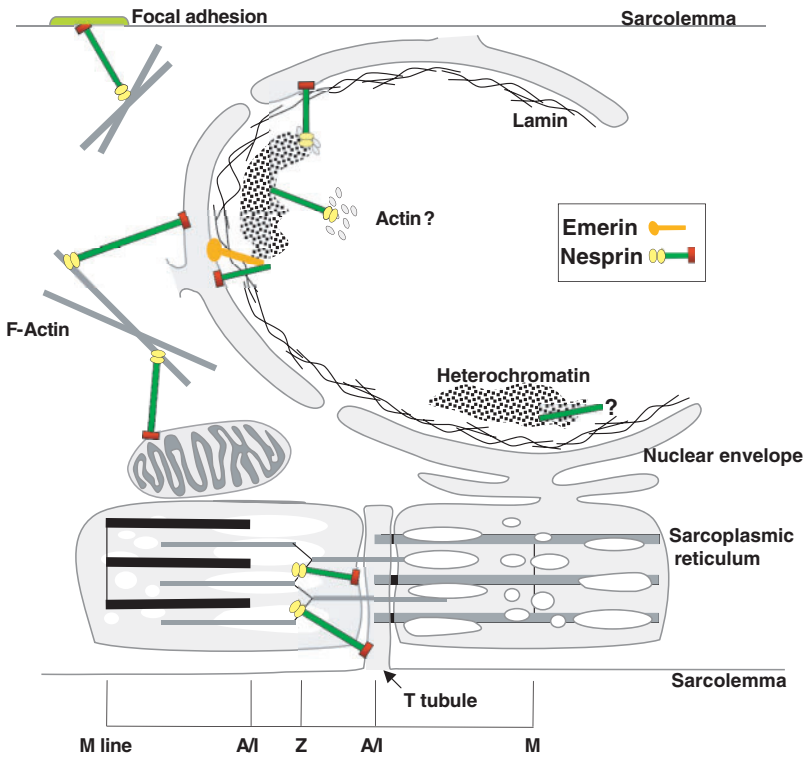


Fig. 13. Schematic of a muscle cell showing potential localizations/interactions of nesprin-2 isoforms. Isoforms potentially form a network linking the nucleus and organelles to the sarcomere and plasma membrane via the actin cytoskeleton. Continuity of interactions across the NE may occur if SUN domain proteins form a bridge across the nuclear membranes and bind to nesprin-2 isoforms on both the inner and outer faces (not shown).

conserved C-terminal KLS domain with nesprins but has no additional features in common with them. Klarsicht interacts with the dynein and dynactin complex to control lipid vesicle and nuclear migrations along microtubules during development (Mosley-Bishop et al., 1999; Welte et al., 1998) and more recently it was shown to form a complex with lamin at the NE and to have a role in connecting the microtubule organising centre to the nucleus (Patterson et al., 2004). Indeed it is possible that Klarsicht performs some of the NE and cytoplasmic functions of nesprins in *Drosophila* while MSP300, the giant orthologue of nesprins, has a more muscle-specific role in this organism.

Nesprin-2 was also abundant in the nuclear interior. It is probable that the isoforms present in this domain are those lacking the C-terminal KLS domain that is required for NE targeting. Relocalization of nesprin-2 epitopes in the nucleus in SW13/SW20 cells suggests some of these isoforms may also bind nuclear lamin. It is also clear that isoforms within the nucleus possess the CH domains and therefore may bind actin within the nucleus (Fig. 13). Roles for nuclear actin in chromatin remodelling and in RNA transcription, processing and export have been well documented (Echevarria et al., 2003; Pederson and Aebi, 2002; Rando et al., 2000; Shumaker et al., 2003). Thus nesprin-2 isoforms may contribute to actin-dependent functions in the nucleus.

Function of nesprin-2 in the sarcomere of skeletal muscle

Nesprin-2 epitopes were abundant at the free SR, particularly over the A/I junction and exhibited punctate staining at the Z-line of the adult sarcomere. The prominent nuclear banding pattern of the N2 epitope may represent continuity of the SR and the NE and the localization of nesprin-2 isoforms within these domains (Vangheluwe et al., 2003). These localization patterns are highly suggestive of a role for nesprin-2 isoforms in linking organelles such as the SR and mitochondria to nuclear or sarcomeric structures. In turn, isoforms may also link these structures to the actin cytoskeleton and to sites on the plasma membrane/T-tubule system. Nesprin-2 isoforms may provide a network of scaffolds that spatially orientate the myofibrils, SR, cytoskeleton, mitochondria and plasma membrane, thereby allowing the precise transmission of signals that couple excitation to muscle contraction. In support of this structural model, mutations in MSP300, the *Drosophila* nesprin orthologue, disrupt integrin localization and signalling at the plasma membrane of muscle cells while in ANC-1 mutant *C. elegans*, the positioning of mitochondria in mono-nucleated cells was disrupted (Starr and Han, 2002). Full-length nesprin-2-giant is predicted to be almost 0.4 μm in length, therefore it is conceivable that a single isoform may simultaneously link a number of structures, however, it is probable that a number of isoforms, of different length and specificity, perform related functions within and around the sarcomere. The subtle changes in the abundance of cytoplasmic and nuclear isoforms during differentiation of C2C12 myoblasts-myotubes in vitro support the notion that nesprin-2 isoforms act as structural scaffolds with domain-specific functions within muscle cells.

A number of spectrin-repeat proteins have now been shown to specifically interact with organelles and link these structures via intermediate filaments to other cytoskeletal structures, and nesprin-2 isoforms may have similar roles (De Matteis and Morrow, 2000; Winder et al., 1995). Indeed, recent yeast two-hybrid analysis has shown that a region of nesprin-2 specifically interacts with RyR (D.T.W. and C.M.S., unpublished), a Ca^{2+} release channel present at both the SR and the NE (Kapiloff et al., 2001). Potentially, nesprin-2 isoforms may mediate protein-binding interactions with the same molecules at both the NE and the SR.

Nesprin-2 as a candidate in muscular dystrophy syndromes

Nesprin-1 and nesprin-2 are binding partners for lamin A and emerin, the proteins mutated in EDMD and a number of overlapping diseases now referred to as laminopathies (Mounkes et al., 2003; Worman and Courvalin, 2002). It was initially proposed that the muscle-specific effects of mutations in emerin and lamin seen in EDMD were due to nuclear weakness in mechanically stressed cells, however it is now clear that pleiotropic processes contribute to the broad

spectrum of disease phenotypes (Burke and Stewart, 2002; Worman and Courvalin, 2004). Given that nesprins are likely to be involved in a cellular network that could potentially link organelles to each other and to the actin cytoskeleton, the plasma membrane and the nucleus, we propose that mechanical signals may be disrupted in patients with mutations in either emerin or lamin and recent data supports this notion (Maniotis et al., 1997; Lammerding et al., 2004). The high expression of nesprins in muscle may account for the muscle phenotypes of lamin and emerin mutations.

Other evidence that nesprins may be involved in muscular dystrophy syndromes include the striking similarities between the properties of nesprin and dystrophin, the protein affected in Duchenne muscular dystrophy (Ostlund and Worman, 2003; Zhang et al., 2002). Both these giant proteins bind actin through N-terminal CH domains and both possess extended spectrin-repeats that link the plasma membrane to intracellular structures (Djinovic-Carugo et al., 2002; Gimona et al., 2002; Ostlund and Worman, 2003). Integrin signalling at the plasma membrane can affect gene transcription in the nucleus, and mutations in *Drosophila* MSP300 caused a displacement of integrins on the plasma membrane in muscle (Maniotis et al., 1997; Mayer et al., 1997; Rosenberg-Hasson et al., 1996). In addition, incorrect localization of both nuclei and mitochondria within the sarcomere, are common defects observed in many patients with muscular dystrophy syndromes (Burke et al., 2001; Mounkes et al., 2003). Finally, several sarcomere-associated proteins reside in the nucleus, where they signal changes in the structure or function of the contractile machinery. For example the muscle-specific ring finger protein (MURF-1) binds to titin at the M-line, where titin contains a kinase domain and plays critical roles in maintaining the structural integrity of the sarcomere and in signalling (Centner et al., 2001). MURF-1 is also a nuclear protein that interacts with a transcriptional modulator, glucocorticoid modulatory element binding protein-1 (GMEB-1) (McElhinny et al., 2002), suggesting that dual interactions may link myofibril signalling pathways with muscle gene expression. Further studies are now required to determine how nesprins might participate in signalling pathways from the sarcomere to the nucleus. The identification of nesprin-binding partners in different sub-cellular domains will be the next step in identifying more specific functional roles for these proteins.

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