# Nesprin-1α self-associates and binds directly to emerin and lamin A in vitro

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**Abstract Nesprin-1α is a spectrin repeat (SR)-containing, transmembrane protein of the inner nuclear membrane, and is highly expressed in muscle cells. A yeast two-hybrid screen for nesprin-1α-interacting proteins showed that nesprin-1α interacted with itself. Blot overlay experiments revealed that nesprin-1α's third SR binds the fifth SR. The carboxy-terminal half of nesprin-1α directly bound lamin A, a nuclear intermediate filament protein. Biochemical analysis demonstrated that nesprin-1α dimers bind directly to the nucleoplasmic domain of emerin, an inner nuclear membrane protein, with an affinity of 4 nM. Binding was optimal for full nucleoplasmic dimers of nesprin-1α, since nesprin fragments SR1-5 and SR5-7 bound emerin as monomers with affinities of 53 nM and 250 mM, respectively. We propose that membrane-anchored nesprin-1α antiparallel dimers interact with both emerin and lamin A to provide scaffolding at the inner nuclear membrane. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Spectrin repeat; Crosslinker; Inner nuclear membrane; Lamin A/C; Emerin; Nuclear envelope; Emery–Dreifuss muscular dystrophy

## 1. Introduction

Spectrin repeats (SRs) consist of ~100 amino acid residues that fold into a characteristic 'triple-helical bundle' structure, with three α-helices separated by two loop regions [1–3]. The SR-containing proteins spectrin and dystrophin stabilize the plasma membrane against stress. SR-containing proteins also provide scaffolding for molecules such as myotilin and titin [4,5], and SR-motifs may directly mediate the dimerization of α- and β-spectrin, and α-actinin [6,7]. Thus, SR proteins appear to mediate a broad range of multivalent interactions at the plasma membrane and in the cytoskeleton.

The two membranes of the nuclear envelope confine the nucleoplasm and are continuous with the endoplasmic reticulum. Proteins of the inner nuclear membrane (INM) face the nucleoplasm where they contribute to nuclear envelope architecture, chromatin organization and postmitotic reassembly of the nucleus [8–11]. Nuclear intermediate filament proteins, named lamins, reside on the nucleoplasmic side of the INM and contribute significantly to nuclear structure. Lamins anchor to the INM through several mechanisms that include binding to membrane-anchored lamina-associated polypeptides (LAPs) and emerin [12,13]. LAP2 and emerin are abundant INM proteins that share a common 'LEM' domain [14]. LEM domains bind barrier-to-autointegration factor (BAF) [15–17], a 10 kDa chromatin protein [15,18,19], potentially establishing an organizational network that links the INM to lamins and chromatin.

Mutations in lamin A/C and emerin cause human disease. Point mutations distributed throughout lamin A cause cardiomyopathy and Emery–Dreifuss muscular dystrophy [20–22]. Mice with a homozygous null allele of lamin A/C die young from severe muscular dystrophy [23], suggesting specialized roles for nuclear membrane proteins in striated muscle. Mutations in emerin also cause muscular dystrophy and cardiomyopathy [24]. When lamin A/C is reduced, the majority of emerin mislocalizes to the endoplasmic reticulum, suggesting that a lamin-dependent network mediates emerin localization and function [23].

We recently identified a novel SR-containing INM protein, nesprin-1α (also known as myne-1, myocyte nuclear envelope) [25,26]. Nesprin-1α is highly expressed in cardiac, skeletal, and smooth muscle. Nesprin-1α is a predicted type II transmembrane protein, with a large nucleoplasmic domain containing seven SRs, a carboxyl-terminal membrane-spanning domain and 'interrupted' LEM-like domain [25]. A yeast two-hybrid screen for nesprin-1α-interacting proteins revealed that nesprin-1α interacted with itself. To understand the nuclear function of nesprin-1α, we also tested its binding to candidate partners lamin A, emerin and BAF. Our results, reported here, support a scaffolding role for nesprin-1α at the INM.

## 2. Materials and methods

### 2.1. Constructs

The yeast two-hybrid bait clone, Y2K5-6, was amplified from a full-length nesprin-1α template, pT7M1, with primers (5′-CCGAATTCAGCCTGCTTTCTCCACAGCCTTC-3′ and 5′-CGGATCCCCTGCAGCAGGCCCCGCCACTCC-3′) and ligated into *Eco*RI/*Bam*HI sites of pGBKT7 (Clontech, Palo Alto, CA, USA). All other con-

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*Abbreviations:* SR, spectrin repeat; INM, inner nuclear membrane; BAF, barrier-to-autointegration factor

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structs were generated by amplification with primers specific to nesprin-1α (GenBank accession number AAL38031) from pT7M1 and ligated into pGBKT7 or pGEX4T-3 (Amersham Pharmacia Biotech, Piscataway, NJ, USA) vectors. The amino acid residues encoded by each construct are indicated in Fig. 1. Lamin A was amplified by reverse transcription-polymerase chain reaction (RT-PCR) from a human heart cDNA library using two primers (5′-GGAGACCCCG-TCCCAGCGGCGCGCCACC-3′ and 5′-ACCGGTCATCATCACC-ATCACCATTGA-3′). The resulting PCR product was ligated into pCR 2.1 TOPO (Invitrogen, Carlsbad, CA, USA), and then *Eco*RI digested and ligated into pGEX4T.3. All constructs were verified by sequencing. Full-length expression clones of emerin and BAF in pET15b vectors were described previously [16,27].

### 2.2. Yeast two-hybrid screen

Two-hybrid screening was performed in the AH109 yeast strain [28]. The bait, nesprin-1α residues 481–916, was used to screen $1\times10^7$ clones of an adult human skeletal muscle library (Clontech, Palo Alto, CA, USA). Interacting cDNAs were isolated with the Pierce Y-DER extraction kit (Pierce, Rockford, IL, USA), and inserts were amplified with vector-specific primers (pACT2F 5′-GATCTG-TATGGCTTACCCATACGATGTTC-3′/pACT2R 5′-GTGCACGA-TGCACAGTTGAAGTGAACTTG-3′). Amplified inserts were sequenced and analyzed using Sequencher (Gene Codes, Ann Arbor, MI, USA) and BLAST.

### 2.3. Preparation of recombinant proteins

Bacterial proteins were expressed in *Escherichia coli* BL21 (DE3) by inducing log phase cultures with 1 mM isopropylthiol-β-D-galactosidase. The pellet was washed in 10 mM Tris–Cl pH = 7.5 and resuspended in lysis buffer (50 mM Tris–Cl pH = 8.0, 1 mM EDTA, 100 mM NaCl) with 200 mg/ml lysozyme and incubated on ice for 30′. NaCl and NP-40 were added to final concentrations of 0.35 M and 0.8% and incubated on ice for 30′. After sonication, the insoluble fraction was pelleted at $30\,000\times g$ for 30′, washed once with 1 M urea/0.5% Triton X-100, centrifuged at $30\,000\times g$ for 30′, and solubilized in lysis buffer with 8 M urea for 1 h. Solubilized proteins were then boiled in loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS), 0.1% bromophenol blue, 10% glycerol).

### 2.4. Gel electrophoresis and blot overlay

Approximately 100 μg of solubilized recombinant GST-lamin or GST-nesprin-1α protein was separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Blue or transferred onto PVDF membrane (Millipore, Bedford, MA, USA) overnight. Blot overlay using $^{35}$S-labeled, in vitro transcription/translation products to probe PVDF membranes was performed as described [29]. Nesprin-1α fragments and BAF were transcribed and translated in vitro using a T7 polymerase/rabbit reticulocyte lysate system (Promega, Madison, WI, USA) and $^{35}$S methionine according the manufacturer's instructions. Products were verified by running 10% of the reaction volume on SDS–PAGE. Gels were dried and exposed for 12–16 h to BioMax film.

### 2.5. Emerin solution binding assays and affinity measurements

Microtiter well-binding assays were done essentially as described [30], with the following changes. Accurate affinities for SR1-7, SR1-5, and SR5-7 were measured by titrating the amount of emerin per microtiter well 10–20-fold. Either $^{35}$S-labeled SR1-7, SR1-5, or SR5-7 was added at several concentrations ranging from 2 nM to 5 mM to microtiter wells containing either 5, 10, 20, or 30 pmol of emerin. Affinity constants were generated by double reciprocal plots and confirmed by computer modeling (data not shown).

## 3. Results

### 3.1. Nesprin-1α-interacting proteins

To identify proteins that interact with nesprin-1α, a yeast two-hybrid screen of a human skeletal muscle library was performed using a bait that comprised SRs five and six plus the intervening sequence that contains a divergent LEM-like domain (SR5-6, Fig. 1). This construct was chosen since it did not self-activate and might attract proteins that bind SRs or the LEM-like domain. A total of 221 positive colonies were obtained from $1\times10^7$ colonies screened. Over half the positive clones encoded two proteins, but a single positive clone, 9C4, encoded nesprin-1α residues 144–417, corresponding to SR domains two and three. We then tested nesprin-1α fragment SR5-6 and controls for binding to SR2-3 in pairwise matings of yeast carrying each two-hybrid construct; α-galactosidase activity revealed that only SR5-6 interacted with SR2-3 specifically (data not shown).

### 3.2. Confirmation of nesprin-1α interaction

To confirm the self-interaction of nesprin-1α, we used a blot overlay approach. Bacterially expressed nesprin-1α was tested for interaction with $^{35}$S-labeled nesprin-1α fragments. We tested $^{35}$S-labeled fragments SR1-5 and SR5-7 for binding to a bacterially expressed GST-fusion protein containing SRs five and six and the intervening sequences (GST-SR5-6) (Fig. 1). SR1-5, but not SR5-7, bound to GST-SR5-6 (Fig. 2B,C), consistent with our two-hybrid self-interaction results. $^{35}$S-labeled SR1-5 also bound specifically to GST-SR5-6 on GST-sepharose columns (data not shown). We concluded that the

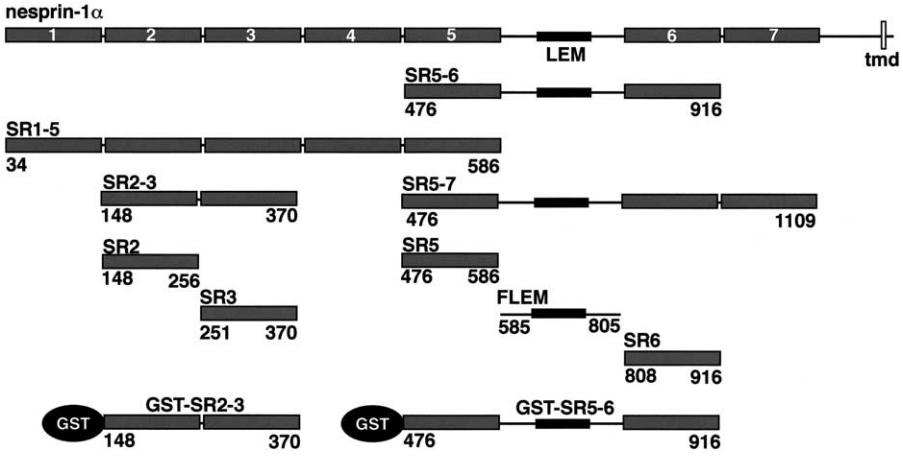


Fig. 1. Full-length nesprin-1α is shown at top of diagram for reference. The gray boxes indicate SR domains and are numbered in the top for reference. The black box indicates the LEM-like domain. The small white box indicates the transmembrane domain (tmd). Each construct is listed by name and the numbers listed below refer to the amino acid residues of nesprin-1α.

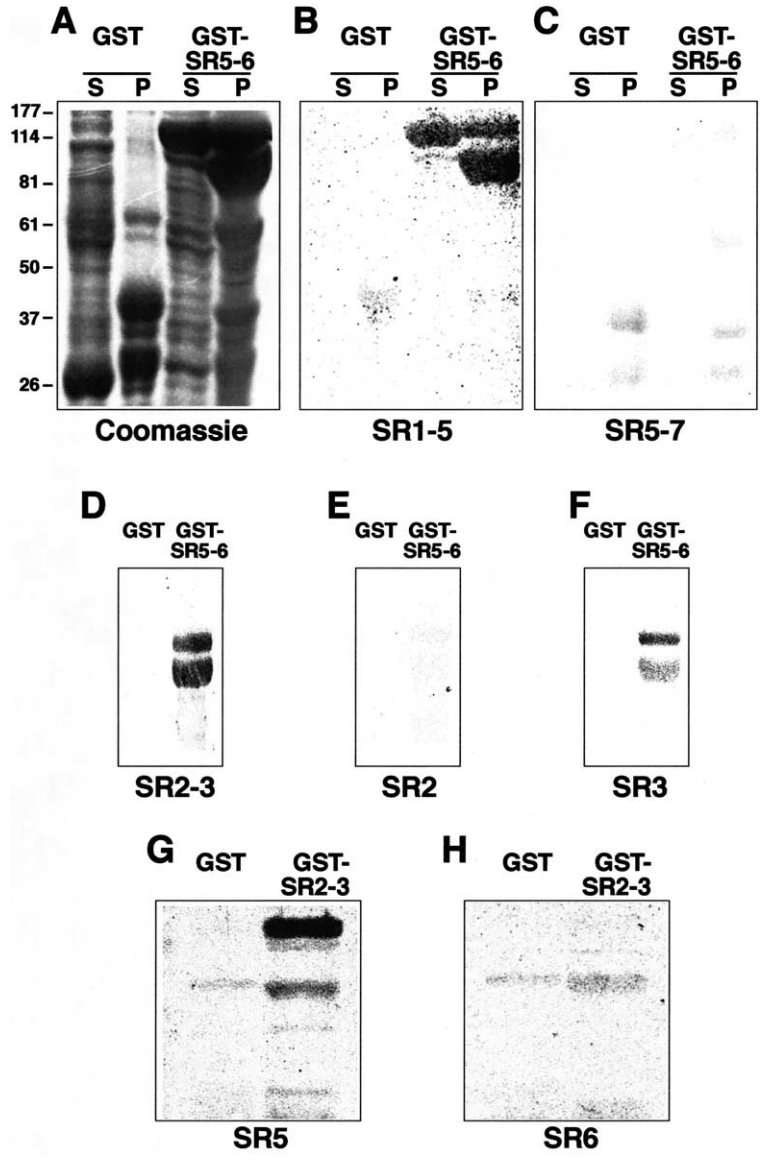


Fig. 2. A: Blot overlay of nesprin-1α self-interaction. Coomassie-stained gel of bacterially synthesized GST fusion nesprin-1α (GST-SR5-6) protein. Identical gels were blotted for overlay binding studies. P and S refer to the pellet and supernatant fractions from *E. coli* expressing empty vector (GST) or GST-fusion proteins. GST-5-6 partitions in both the supernatant and pellet fractions while GST alone (27 kDa) is largely in the supernatant. The $^{35}$S-labeled probe used is listed below each blot. B: $^{35}$S-labeled SR1-5 binding to GST-SR5-6. C: $^{35}$S-labeled SR5-7 has no detectable binding to GST-SR5-6. D, E and F represent blot overlays on to supernatant fractions from *E. coli* expressing GST or GST-5-6; $^{35}$S-labeled SR2-3 (D), SR2 (E), and SR3 (F). SR3 binds GST-SR5-6 while SR2 shows no detectable binding. G and H are duplicate blots of GST-2-3 probed with $^{35}$S-labeled SR5 or $^{35}$S-labeled SR6, respectively. SR5 binds GST-SR2-3 while SR6 has no detectable binding.

amino- and carboxy-terminal halves of nesprin-1α interact with each other. The interacting regions within nesprin-1α were further refined by blot overlay assays. $^{35}$S-labeled SR2-3 and SR3 each bound to GST-SR5-6 (Fig. 2D,F), but SR2 did not (Fig. 2E), suggesting that SR3 is sufficient to bind SR5-6.

GST5-6 encodes two SRs plus a LEM-like domain flanked by unique sequence. By blot overlay assays, no binding to GST-SR2-3 was detected for either the $^{35}$S-labeled flanking plus LEM-like region ('FLEM' fragment, see Fig. 1; data not shown) or SR6 (Fig. 2H). In contrast, $^{35}$S-labeled SR5 bound strongly to GST-SR2-3 (Fig. 2G). We concluded that nesprin-1α self-interaction is mediated by mutual interaction of the SR3 and SR5 domains.

### 3.3. Nesprin-1α's LEM-like domain does not bind BAF in vitro

Nesprin-1α contains an 'interrupted' LEM-like domain [25]. We tested this LEM-like domain for binding to BAF in blot overlay assays where $^{35}$S-BAF was used to probe bacterially expressed GST-SR5-6 on blots. Bacterially expressed emerin was the positive control for this assay [16]. $^{35}$S-BAF bound to emerin, but not to GST-SR5-6 (Fig. 3). Thus, the LEM-like domain of nesprin-1α does not bind BAF under these conditions.

### 3.4. Nesprin-1α binds lamin A in vitro

We previously showed that lamin A and nesprin-1α coimmunoprecipitated from cultured C2C12 muscle cells [25]. To determine if lamin A and nesprin-1α bind directly, we used

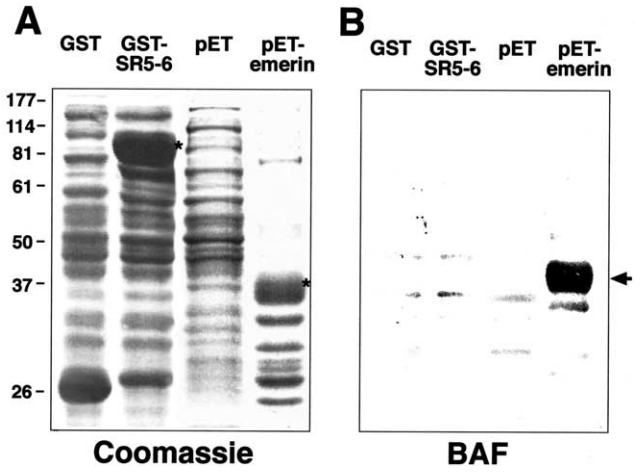


Fig. 3. A: Coomassie-stained gel showing bacterially expressed GST, nesprin-1α GST-SR5-6, pET vector and pET-emerin. The asterisks indicate nesprin-1α and emerin fusion proteins. B: An identical gel blotted and probed with $^{35}$S-labeled BAF. The arrow shows BAF binding to emerin. Nesprin-1α's LEM-like domain does not bind BAF.

$^{35}$S-labeled nesprin-1α fragments to probe blots of bacterially expressed lamin A (GST-LAMA). Neither SR1-5 (Fig. 4B) nor FLEM (data not shown) gave a signal, whereas SR5-7 bound strongly (Fig. 4C), suggesting that SR5-7 binds directly to lamin A.

### 3.5. *Nesprin-1α dimers bind to emerin with high affinity in vitro*

Because nesprin-1α and emerin colocalize at the INM of striated muscle [25], we tested their direct interaction. $^{35}$S-labeled nesprin-1α fragments SR1-5 and SR5-7 were used to probe blots of bacterially expressed emerin (residues 1–222). SR1-5 bound detectably to emerin (Fig. 5B), but no signal was detected for SR5-7 (data not shown). To quantify this interaction, we tested nesprin-1α binding to emerin in solution. Emerin was immobilized in microtiter wells in solution, washed, and incubated with soluble $^{35}$S-labeled nesprin-1α fragments SR1-7, SR1-5 and SR5-7 (Fig. 5; see Section 2). The full nucleoplasmic fragment SR1-7 bound tightly to emerin, with an equilibrium affinity of 4 nM (range, 1–11 nM; $n = 3$ triplicates; Fig. 5C). Importantly, the stoichiometry of interaction was 1.8–2 (nesprin to emerin), demonstrating that the full nucleoplasmic domain of nesprin-1α functions as a dimer. The binding affinities of SR1-5 and SR5-7 for emerin were relatively weaker. SR1-5 bound emerin with an affinity of 53 nM (range 28–120 nM; $n = 5$ triplicates; Fig. 5D), and SR5-7 bound with an affinity of 250 nM (range 90–600 nM; $n = 4$ triplicates; Fig. 5E). SR1-5 bound as a monomer (stoichiometry of 0.8–0.9; Fig. 5D), but SR5-7 binding was substoichiometric (ratio of 0.4–0.5; Fig. 5E). Based on these data, we concluded that emerin binds optimally to full-length dimers of nesprin-1α. However, the amino-terminal half of nesprin-1α (SR1-5) is sufficient to bind emerin with reasonably high affinity (53 nM).

## 4. Discussion

Our results demonstrate that nesprin-1α binds directly to lamin A and emerin, but not BAF. We previously showed that nesprin-1α colocalizes with emerin in striated muscle cells [25]. However, emerin did not consistently colocalize with nesprin-1α in smooth muscle [25]. We therefore propose that nesprin-1α-mediated scaffolding of emerin and lamin A may be specific to striated muscle and may account, at least in part, for the tissue specificity of diseases caused by mutations in emerin and lamin A. We suggest that lamin A and emerin mutations contribute to striated muscle disease by disrupting a structural

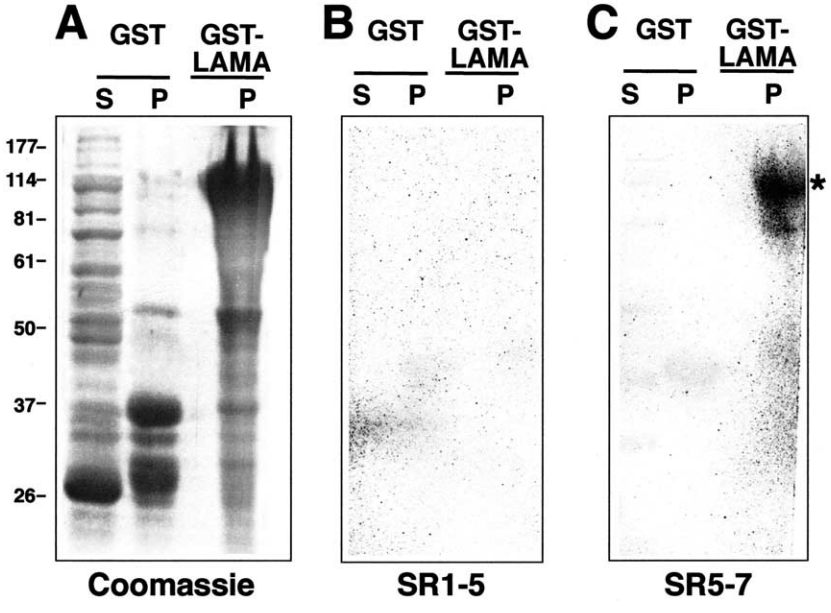


Fig. 4. A: Coomassie-stained gel of bacterially synthesized lamin (GST-LAMA). S and P refer to the pellet and supernatant from bacteria expressing GST or GST-LAMA. Lamin A is insoluble in *E. coli*, so the supernatant lane was omitted for clarity. Identical gels were blotted and probed with (B) $^{35}$S-labeled nesprin-1α SR1-5 and (C) $^{35}$S-labeled nesprin-1α SR5-7. The asterisk indicates binding of SR5-7 to lamin A. Since no binding was detected with the FLEM (data not shown), it is likely that SRs five, six, or seven mediate lamin A binding of nesprin-1α.

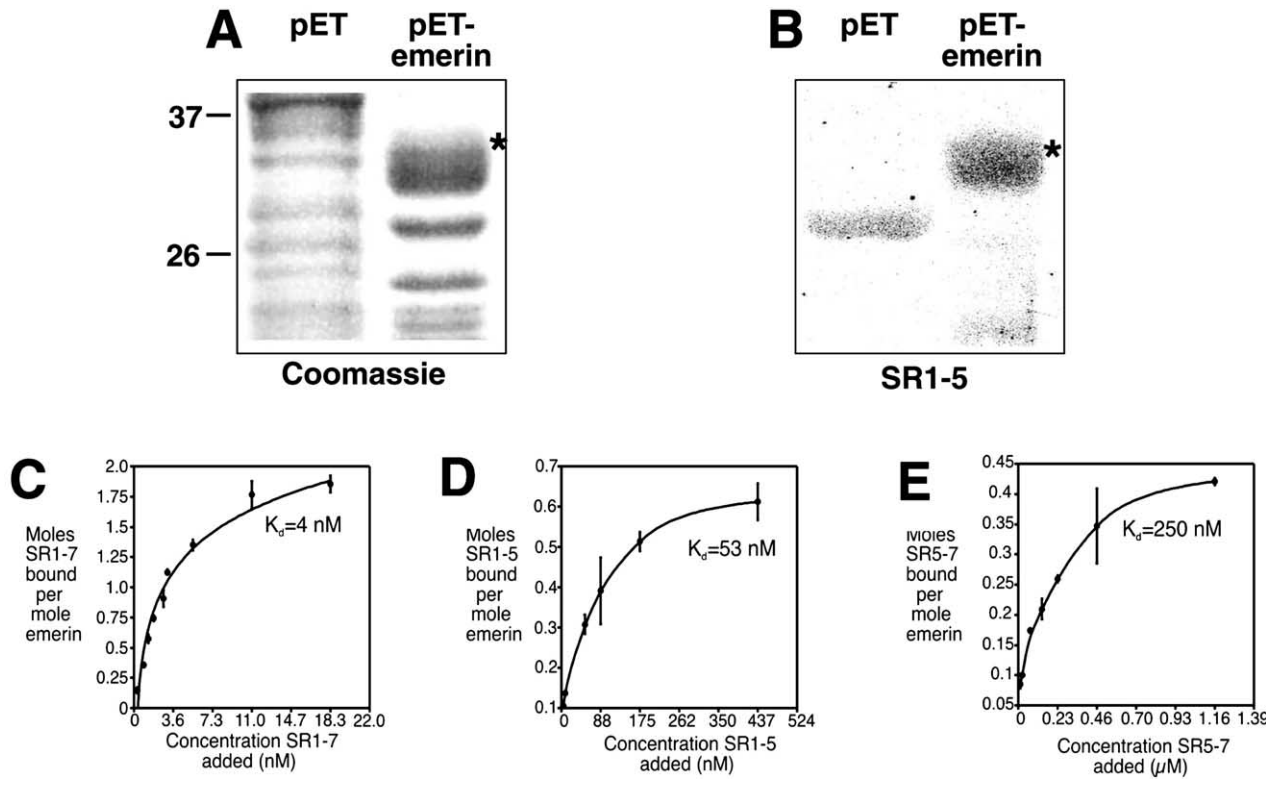


Fig. 5. A: Coomassie-stained gel of proteins isolated from bacteria expressing empty vector (pET) or emerin (pET-emerin). Duplicate gels were blotted and probed with (B) $^{35}$S-labeled SR1-5. The background band seen in the pET lane represents a non-specific BL21 *E. coli* protein that was noted with all $^{35}$S-labelled probes. C–E: Affinity of SR1-7 (C), SR1-5 (D), and SR5-7 (E) for nesprin-1α binding emerin. Affinities for each were determined by adding increasing concentrations of either $^{35}$S-labeled SR1-7, SR1-5, or SR5-7 to constant amounts of emerin immobilized in microtiter wells.

network that interlinks emerin, lamin A/C, and nesprin-1α at the INM.

SR domains provide structure and support protein–protein interactions, including homodimerization and heterodimerization with other SR proteins. Of the nesprin-1α-interacting proteins identified in our two-hybrid analysis, nesprin-1α was the only partner with SR domains. Our data show that SR domains three and five mediate dimerization of nesprin-1α. An alternative possibility is that SR3 and SR5 interact intramolecularly. However this is improbable for two reasons. First, the stoichiometry of binding is consistent with the formation of nesprin dimers. Second, SR3, SR4, and SR5 are each separated by only seven residues, and SR domains demonstrate limited flexibility [31], placing strong theoretical constraints on intramolecular SR interactions. We therefore favor models in which SR3 and SR5 mediate intermolecular dimerization of nesprin-1α.

Homodimerization of nesprin-1α might involve either a parallel or antiparallel orientation. However, the predicted transmembrane domain of nesprin-1α probably anchors its carboxy-terminus at the INM. Given the known antiparallel arrangement of other SR proteins, we hypothesize that nesprin-1α forms antiparallel dimers as shown in Fig. 6B. While not all SR-containing proteins homodimerize [29,32], our predicted antiparallel orientation of nesprin-1α dimers would allow binding to lamin and emerin, and are consistent with the extremely close apposition of lamins to the nuclear membrane. Together, these data support models in which nesprin-1α crosslinks emerin and lamin A/C at the nuclear membrane (Fig. 6B). Furthermore, based on its in vitro binding to lamin A and high-afffinity binding to emerin, we suggest that nesprin-1α provides a novel mechanism for anchoring both lamin A and emerin at the INM (Fig. 6B).

Our findings suggest that nesprin-1α serves as a structural crosslinker at the INM. Although the nesprin-1α LEM-like domain does not appear to bind BAF, we predict that nesprin-1α, by virtue of its interaction with lamin A/C and emerin, is in close proximity to heterochromatin. We speculate that the scaffolding function of the INM may be important for the terminal differentiation of muscle cells, and disrupting this network may lead to disease.

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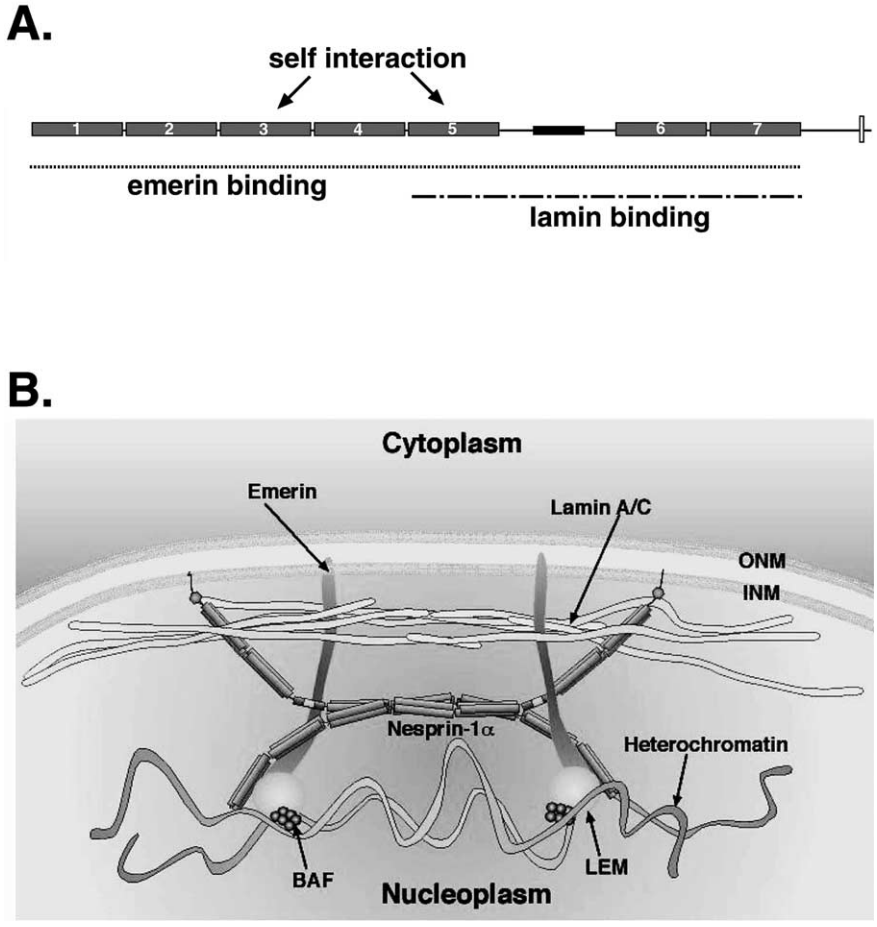


Fig. 6. A: Schematic of nesprin-1α highlighting regions implicated in self-association, emerin and lamin binding as demonstrated in this study. B: Model for the INM scaffold that includes nesprin-1α, emerin, and lamin A/C.

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