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Citation for published version (APA):

Autore, F., Shanahan, C. M., & Zhang, Q. (2016). Identification and Validation of Putative Nesprin Variants. In *Methods in Molecular Biology* (Vol. 1411, pp. 211-220). Springer.

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Identification and validation of putative nesprin variants

Flavia Autore^{1,2}, Catherine M. Shanahan², Qiuping Zhang²

¹Randall Division of Cell and Molecular Biophysics, King's College London, UK

²British Heart Foundation Centre of Research Excellence, Cardiovascular Division,
King's College London, UK

+Corresponding author: Dr. Qiuping Zhang
King's College London,
Cardiovascular Division,
James Black Centre,
125 Coldharbour Lane,
London SE5 9NU
UK

Tel: +44 20 78485222

Fax: +44 20 78485193

Email: qp.zhang@kcl.ac.uk

Running Head: Challenges of studying nesprins

Summary

Nesprins are a family of multi-isomeric scaffolding proteins that were originally identified at the nuclear envelope (NE), where they bind to lamin A/C, emerin and SUN-domain containing proteins, to form the Linker of Nucleoskeleton-and-Cytoskeleton (LINC) complex that connects the NE to the actin cytoskeleton. However, nesprin genes also give rise to a variety of tissue specific variants of different sizes with potential roles beyond the NE. These variants are generated through alternative initiation, termination and splicing, which makes nesprin biology very complex to study due to the difficulty in generating specific antibodies and/or short interfering RNAs (siRNA) to particular isoforms. In order to distinguish genuine nesprin variants and eliminate confusion with degradation products of larger nesprin isoforms, in this chapter we discuss methods including 5' and 3' Rapid Amplification of cDNA Ends (RACE) and RT-PCR in combination with EST database searching, for identifying and validating putative nesprin isoforms. This information is essential to allow a better understanding of nesprin functions in different cell types.

Key words: Nesprins, Alternative Splicing, EST database, RACE, Multi-Tissue RT-PCR.

1. Introduction

Nesprins comprise a family of multi-isomeric scaffolding proteins that were originally identified as components of the nuclear envelope (NE) [1]. There are four mammalian genes (*SYNE* 1-4) encoding nesprin proteins. Nesprin-1, -2 and -3 are ubiquitously expressed in human tissues, while nesprin-4 is mainly expressed in highly polarized epithelial cells [2-5]. All four nesprin proteins contain a C-terminal Klarsicht/ANC-1/Syne Homology (KASH) domain, which mediates the interaction with SUN domain containing proteins (SUN1 and SUN2) at the nuclear membrane, forming the Linker of the Nucleoskeleton and the Cytoskeleton (LINC) complex [2-4]. The nesprins reside in the outer nuclear membrane (ONM) where they interact with cytoskeletal filaments. SUN 1/2 reside at the inner nuclear membrane (INM) and bind to lamin A/C directly via their nucleoplasmic domain. Therefore the LINC complex provides a physical connection between the nucleus and the cytoskeleton [5,6]. Nesprins-1 and -2 are also able to bind to lamin A/C and emerin at the INM [1,7]. Mutations in the genes encoding all of these proteins contribute to Emery–Dreifuss muscular dystrophy (EDMD) and dilated cardiomyopathy (DCM)[8,9], indicating that nuclear-cytoskeletal connection is fundamental to muscle cell function.

There are sequence diversities at the N-terminus of nesprins, allowing them to bind to different components of the cytoskeleton: actin filaments, microtubules (MT) and intermediate filaments (IF). Nesprin-1 and nesprin-2 are defined as giant proteins due to their molecular weights of ~1 MDa and ~800 kDa respectively. Both are comprised of N-terminal paired Calponin Homology domains (CHD) that bind to F-actin, a C-terminal KASH domain, and a central Spectrin Repeat (SR)-containing rod domain,

mediating protein-protein interactions [10,11]. Nesprin-3 and nesprin-4 are characterised by a much less extended rod domain, which reflects their lower molecular weights of 110 and 42 kDa respectively [11]. Despite lacking the CHDs, they are both able to bind cytoskeleton filaments: nesprin-3 binds IFs through a plectin-binding domain, whereas nesprin-4 interacts with kinesin-1, a motor protein associated with MT networks [12,13].

Nesprin-1 and -2 are encoded by 146 and 115 exons respectively, which give rise to a large number of different variants[14]. Most variants have overlapping domains, indicating that the expression of nesprin variants is a highly flexible and regulated process. This flexibility is likely an adaptive response that cells require to meet the requirements of different cellular cues or stimuli. In particular in muscle tissue, nesprins not only switch isoform expression of the same gene, but also change expression between the different nesprin genes. For example, during human muscle development, expression level of nesprin-1 giant increases during early myogenesis *in vitro*, while nesprin-2 partially replaces nesprin-1 at the NE. Ultimately, the shorter nesprin KASH isoforms become the dominant variants during the transition from immature to mature muscle fibres *in vivo* [15].

We documented that nesprin-1 and -2 give rise to a vast array of tissue specific isoforms of different sizes [14] including i) isoforms lacking the KASH domain (14 for nesprin-1 and 5 for nesprin-2); ii) isoforms lacking the CHDs (16 for nesprin-1 and 12 for nesprin-2) [16], and iii) SR-only isoforms for nesprin-1 [17]. The isoform repertoire reflects emerging new functions of nesprins beyond the NE. The isoforms are generated by alternate promotor usage, pre-mRNA 3' end processing, and alternative splicing

(including or excluding cassette exons) [14,16,18]. For example, nesprin-2 α 1 and -2 α 2 variants are generated via different initiation within 5' untranslated region (UTR) (Figure 1A) [1,7]. Alternative splicing is another mechanism by which exons are removed from the mature nesprin mRNA. For example, nesprin-1 Δ KASH is generated through the removal of cassette exon 145 of nesprin-1 resulting in a stop codon at exon 146 and deletion of the KASH domain (Figure 1B). Similarly, nesprin-2 Δ KASH1 is generated through the removal of exons 111–112 (Figure 1C). In contrast, nesprin-2 Δ KASH2 is generated via different termination point through utilisation of an alternative 3'UTR juxtaposed to exon 115 via a retained intron, to produce an alternative variant (Figure 1D). These alternative transcript generation mechanisms have also been observed experimentally in other studies of nesprin-1 variants [19].

All the nesprin isoforms published so far were predicted mainly using the NCBI EST (database for “Expressed Sequence Tag”) [20], but only a small subset has been verified experimentally. The variety of sizes and isoforms that can be generated make nesprin biology very complex due to the difficulty in generating (1) specific antibodies and (2) short interfering RNAs (siRNA) [21]:

(1) The use of specific antibodies is limited to a small subset of isoforms, because the antibodies available detect not only the real products of alternative splicing (of which there may be many), but often degradation products that are commonly present during the manipulation of proteins of large molecular weight [14,15,22]. (2) In addition, the most common event for alternate isoform formation is the use of alternative 5' and 3' UTRs, with common coding exons which complicates the exclusive knockdown of a particular isoform as it is likely to interfere with the expression of other variants. In the literature the siRNAs currently used for knocking down small isoforms also hamper the expression of the giant nesprins due to this lack of specifically targeted exon

regions[14]. Moreover, the efficiency of knockdown can only be detected at the RNA level by qPCR using oligonucleotides designed specifically for each of the siRNA targeted exons, because the protein expression level is usually not verifiable due to lack of antibody specificity as described above in (1)[14].

We have employed multiple techniques to identify and verify transcripts encoding novel nesprin variants. In some cases, these analyses have enabled us to identify variants that do have some sequence specificity at the protein level which enables the generation of more selective antibody tools. Therefore the identification and verification of novel transcripts is essential for a complete understanding of nesprin biology [1,14,7]. In this chapter we discuss the techniques we have used including: i) 5' and 3' Rapid Amplification of cDNA Ends (RACE) for identifying cDNA ends that represent novel 5' and/or 3' UTRs, in combination with the EST database analysis, in which there is a vast collection of partial sequences of cDNA clones available; ii) RT-PCR from multi-tissue cDNAs using alternatively combined 5' and 3' UTRs for identifying and validating putative isoforms [1,16,7,23].

2. MATERIALS:

A range of gene-specific primers for amplification of the region of interest of the cDNA
(*see Note 1 and Figure 2*)

Marathon-Ready cDNA from a tissue of interest (Clontech)

Advantage 2 PCR kit (Clontech)

GC-RICH PCR system (Roche Applied Science)

First Strand cDNA (OriGene)

DreamTag Green PCR master mix (Fisher Scientific)

pGEM-T Easy vector (Promega)

Rotor-Gene RG 3000 (Corbett Research)

3. METHODS

3.1 Bioinformatics analyses

Published nesprin-1 and nesprin-2 UTRs were identified via screening the NCBI expressed sequence tag (EST) database (<http://www.ncbi.nlm.nih.gov/dbEST/>) [20], which contains partial sequences of cDNA clones ranging from ~200 to ~700 bp. To screen for novel nesprin UTRs in the EST database:

1. Submit consecutive nesprin cDNA sequences of 1 kb with 500 bp overlapping, covering the entire gene length, to the EST database [14].
2. Select the 5'UTRs containing an identifiable and viable Kozak sequence near the first start codon for verification.
3. Filter the detected 3' UTRs with polyadq software (http://rulai.cshl.org/tools/polyadq/polyadq_form.html) [24] (*see Note 1*).

3.2 Rapid Amplification of cDNA Ends (RACE)

RACE is a suitable method to obtain full-length novel isoforms, and only requires knowledge of a small internal region of the cDNA sequence as well as both an adaptor-specific primer and a gene specific primer (GSP) [25]. The presence of a known specific adaptor sequence at the 5' or 3' end of the cDNA is essential for the libraries being used in RACE. Furthermore, a second PCR reaction (named nested PCR) is often required, by using the PCR product from the initial run to produce a more specific amplification product. GSP (including nested GSP) design is an important step to obtain efficient and specific PCR amplifications (*see Note 2*).

1. Design GSP Primers for 5' or 3' RACE against the region of interest on the gene, as illustrated in Figure 2 and in the Marathon-ready cDNA user manual (Clontech).
2. Perform an initial round of PCR reactions using Advantage 2 polymerase and the Marathon-Ready cDNA (Clontech) according to manufacturer's protocol, also briefly described in Table 1, using PCR cycling parameters as follows:

94°C for 1 min

30 cycles:

 - 94°C 30 sec (denaturation)
 - 68°C 4 min (anneal/extension)

70°C for 10 min (final extension)
3. Dilute the initial PCR product (1:50) with PCR grade water, and use 5µl from the dilution for nested PCR reactions with a nested adapter primer and a nested GSP (NGSP) (*see Note 3*). Use PCR cycling parameters as follows:

94°C for 1 min

20 cycles:

 - 94°C 30 sec
 - 68°C 4 min

70°C 10 min
4. Run the nested PCR products on an agarose gel, excise the desired fragments from the gel and clone into pGEM-T Easy vector (Promega).
5. Subject each cloned fragment to sequencing. Align the obtained sequences against the appropriate NCBI species genome to identify novel cDNA ends, which will be either upstream or downstream of the cDNA region of interest.

3.3 Multi-tissue RT-PCR

To characterise the RACE PCR products and validate expression of the putative isoform at RNA level, RT-PCR is required. To amplify short transcripts, design the 5' and 3' GSPs to either existing or newly identified regions of 5' and 3' UTRs identified from the EST database analysis and/or RACE above. For larger transcripts, design a 5' GSP or 3' GSP to the newly identified regions of 5'UTR or 3'UTR, and a 3' GSP or 5' GSP against the nearest coding exon, or primers in a range of exons (*see Note 4*) [14].

1. Perform RT-PCR on a multiple tissue cDNA panel (Origene) using GC-RICH PCR system (Roche Applied Science) according to manufacturer's protocol (Table 2 and 3), also amplifying housekeeping genes as controls. Use PCR cycling parameters as follows:

95°C for 3 min

30-40 cycles:

- 95°C 30 sec
- 48-55°C 1 min (depending on the required annealing temperature for different primers)
- 68°C 1-3 min (depending on the length of putative isoforms)

68°C for 7 min

2. Analyse the PCR products by agarose gel electrophoresis. Excise and clone the PCR product into a pGEM-T Easy vector (Promega). Subject clones to DNA sequencing for further validation.

3.4 Alternative Splicing of Cassette Exons

Putative cassette exons of nesprins can be identified using EST databases. The resulting alternative splicing events could either occur in multiple variants or be specific for a

single variant, which are also likely to be activated in a tissue specific manner [14]. These post-transcriptional events would further increase the complexity of nesprin variants [18].

1. To verify the occurrence of alternative splicing events, design specific primers that target the exons located on either side of the identified cassette exons via the EST database analysis
2. Use the designed primers to perform PCR screening of cDNA libraries from different tissues of interest (*see Note 5*) [14].
3. Perform all PCR reactions as described in section 3.3 using GC-RICH PCR system, or as in Table 4, using the following PCR cycling parameters:

95°C 1 min

30 cycles:

- 95°C 30 sec
- 55°C 1 min (depending on the required annealing temperature for different primers)
- 72°C 1 min per kb

72°C 10 min

3. Purify and clone the PCR products, then subject to DNA sequence for validation, as described in section 3.3 (*see Note 6*).

4. Notes

1. General rules for designing primers are applied. Efficient RACE primers must be specific for their target sequences in order to form stable duplexes without forming secondary structures such as hairpin loops, as it may interfere with polymerisation. For advice on RACE Primer design, refer to

the Marathon-ready cDNA user manual (Clontech). Adaptors required for RACE are ligated and ready for use in the Marathon-Ready cDNA kit.

2. Only the 3'UTRs containing a poly (A) tail or at least one poly (A) site downstream of the initial open reading frame (ORF) termination are selected for further experiments.
3. A nested PCR reaction is performed using the PCR product from the initial run for a more specific amplification product. For nested RACE, the nested GSP (NGSP) is designed to anneal with 1-10 nucleotides downstream of the GSP used in the first round of amplification (for 5' RACE) or upstream of the GSP (for 3' RACE).
4. For validation of 5'UTRs, the forward primer is designed within the 5'UTR and the reverse primer within the second coding exon to control for genomic contamination. For validation of 3'UTRs, the forward primer is designed within the second to last coding exon and the reverse primer within the 3'UTR to also control for genomic contamination.
5. For cassette exons (either included and excluded), forward and reverse primers should be separated by at least one exon-intron boundary to control for genomic contamination.
6. There are some examples for cassette exons described for nesprins. When PCR amplification was carried out across nesprin-1 exon 93 (one of the cassettes detected via EST database searching), two PCR products were obtained from two different cDNA libraries. One PCR product showed the presence of the exon 93 while the other showed absence of the exon, which encodes a unique 47 amino acid peptide sequence. Nesprin-2 also showed its propensity of tissue-specific alternative splicing. For example, regarding

exon 107, another cassette detected by EST, two PCR products were detected in U2OS cells, either containing or missing exon 107, which encodes a unique 23 amino acids. Only isoforms containing exon 107 were expressed in vascular smooth muscle cells [14].

Acknowledgement

This work was funded by a British Heart Foundation (BHF) project grant to QPZ (PG/11/58/29004) and programme grant to CMS (RG/11/14/29056).

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Table 1: RACE PCR Recipe (50 µl):

RACE ready cDNA	5 µl
10X Advantage 2 PCR Buffer	5 µl
dNTP mix (10 mM)	1 µl
Adaptor primer (10 µM)	1 µl
GS primer (10 µM)	1 µl
Advantage 2 Polymerase	1 µl
Nuclease-free water	to 50 µl

Table 2: GC-Rich PCR Master Mix 1 (35 µl):

First strand cDNA	50 ng
dNTP mix (10 mM)	2 µl

Forward primer (5 μ M)	2 μ l
reverse primer (5 μ M)	2 μ l
5M GC Rich Resolution	5 μ l
PCR grade water	to 35 μ l

Table 3: GC-RICH PCR Master Mix 2 (15 μ l):

PCR grade water	4 μ l
5x GC Rich PCR Buffer	10 μ l
GC Rich Enzyme mix	1 μ l

Table 4: RT-PCR recipe (50 μ l):

cDNA	50 ng
2x <i>Taq</i> master mix*	25 μ l
Forward primer (10 μ M)	1 μ l
Reverse primer (10 μ M)	1 μ l
Nuclease-free water	to 50 μ l

* DreamTag Green PCR master mix contains *Taq* polymerase, dNTP and reaction buffer at 2x concentration.

A. Nesprin-2 α 1 & 2 α 2

B. Nesprin-1 Δ KASH

C. Nesprin-2 Δ KASH1

D. Nesprin-2 Δ KASH2

Figure 1. Generation of nesprin-2 α 1 & 2 α 2, as well as nesprin-1 and -2 Δ KASH variants. Nesprin-2 α 1 and -2 α 2 variants were generated via the potential combinations of 5' and 3'UTRs (A). Nesprin-1 Δ KASH and nesprin-2 Δ KASH1 were generated through the removal of cassette exon 145 (B) and exons 111–112 (C) respectively via the indicated splicing events (splicing shown in dash lines). Nesprin-2 Δ KASH2 was generated through utilisation of an alternative 3' UTR juxtaposed to exon 115 (D).

Figure 2. Illustration of the template and primers used in Marathon RACE reactions. AP1 and AP2: adaptor primers 1 and 2; GSP and NGSP: gene specific primers, GSP1 & NGSP1 for 5'RACE, GSP2 & NGSP2 for 3'RACE.