Nesprin-2 Interacts with α-Catenin and Regulates Wnt Signaling at the Nuclear Envelope

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Sascha Neumann‡, Maria Schneider†, Rebecca L. Daugherty§, Cara J. Gottardi§, Sabine A. Eming§, Asa Beijer‡, Angelika A. Noegel††, and Iakovos Karakesisoglou‡

From the †Institute of Biochemistry I, Medical Faculty, Center for Molecular Medicine, University of Cologne, Cologne Excellence Cluster on Cellular Stress Responses in Aging-associated Diseases, and the ‡Department of Dermatology, University of Cologne, 50931 Cologne, Germany, the §Department of Medicine, Northwestern University Feinberg School of Medicine, Chicago, Illinois 60611, and the ††Department of Biological Sciences, School of Biological and Biomedical Sciences, University of Durham, DH1 3LE Durham, United Kingdom

Nesprins and emerin are structural nuclear envelope proteins that tether nuclei to the cytoskeleton. In this work, we identified the cytoskeleton-associated α-N/E-catenins as novel nesprin-2-binding partners. The association involves the C termini of nesprin-2 giant and α-N/E-catenins. α-E/T/N-catenins are known primarily for their roles in cadherin-mediated cell-cell adhesion. Here, we show that, in addition, α-catenin forms complexes with nesprin-2 that include β-catenin and emerin. We demonstrate that the depletion of nesprin-2 reduces both the amount of active β-catenin inside the nucleus and T-cell factor/lymphoid-enhancing factor-dependent transcription. Taken together, these findings suggest novel nesprin-2 functions in cellular signaling. Moreover, we propose that, in contrast to emerin, nesprin-2 is a positive regulator of the Wnt signaling pathway.

Nesprin-1, -2, -3, and -4 (nuclear envelope spectrin repeat-containing proteins) are integral nuclear membrane proteins, greatly varying in size and domain composition (1–3). The largest nesprin-1 (1.01 MDa) and nesprin-2 (796 kDa) isoforms encompass an N-terminal paired calponin homology domain that mediates F-actin binding. The C-terminal KASH (Klarsicht/ANC-1/Syne homology) domain targets nesprins to the nuclear envelope (NE). These conserved domains are separated by a long stretch of spectrin repeats.

Nesprins play pivotal roles in maintenance of NE integrity (4), nuclear positioning (5), and anchorage to the cytoskeleton and the centrosome (2). Although compelling evidence underlines nesprin roles in several human diseases that range from cerebellar ataxia to dystrophy-like phenotypes; the underlying molecular mechanisms remain elusive (6).

Here, we unravel novel nesprin-2 interactions with α-catenin. The latter, together with β-catenin, is known for its role in cell-cell adhesion. Cadherins are transmembrane proteins of the plasma membrane (PM), which play key roles in cell adhesion. The cytoplasmic tail of cadherin binds to β-catenin, which in turn associates with α-catenin. This adhesion complex is dynamically connected to the cortical actin cytoskeleton (7).

β-Catenin has an additional role in the canonical Wnt signaling pathway by transferring signals from the PM into the nucleus. When the Wnt pathway is not active, cytoplasmic β-catenin levels are kept low by protein degradation. Upon Wnt pathway activation, β-catenin accumulates in the cytoplasm and enters the nucleus, where it acts as a transcription factor (8).

In this work, we demonstrate α-catenin interactions that involve β-catenin and NE-associated nesprin-2 and emerin. From our data, we propose a mechanism by which these NE associations regulate nuclear β-catenin levels and Wnt signaling-dependent transcription.

EXPERIMENTAL PROCEDURES

Plasmids—The amino acid positions of nesprin-2 proteins refer to nesprin-2 giant (9). GST, GFP, and yeast two-hybrid fusion proteins were cloned into pGEX-4T (GE Healthcare), pEGFP-C (Clontech), and pGBK-T7 and pGADT-7-Rec (Clontech). Nesprin-2–SR (6146–6799), SR1 (6146–6241), SR2–3 (6348–6552), SR1+2 (6146–6347), SR2+3 (6247–6656), and SR3+4 (6553–6799) were used in this study. The amino acid positions of α-N-Cat* proteins refer to α-N-catenin-4 (NCBI accession number AK295181.1), α-N-Cat*-N-Cat* (706–860), α-N-Cat*ΔEx18 (706–765), α-N-Cat*ΔEx18/15 (706–730), α-N-Cat*ΔEx14 (731–860), Myc-α-E-catenin, and GFP-α-N-catenin-1 (according to IMAGE clone accession number 6187158). For SP-GFP-SUN-1-C, the signal peptide (SP) sequence of torsin A and enhanced GFP were isolated from pcDNA5-His-GFP-hTORA (a gift from Dr. W. T. Dauer) (10) and combined with SUN-1-C (11) in pcDNA3.1 (Invitrogen).
SP-GFP-SUN-1-C comprises the C terminus of SUN-1, including the coiled-coil regions and the SUN domain (see Fig. 4C). The N terminus is replaced with a GFP tag and a signal peptide derived from torsin A (10) that targets the protein to the perinuclear space (Fig. 4E, arrow) and the endoplasmic reticulum (Fig. 4E, arrowhead). Based on its structure, the SP-GFP-SUN-1-C protein is not anchored in the NE but is present in the perinuclear space and endoplasmic reticulum, resulting in a displacement of the nesprins from the NE (Fig. 4E/H11032, asterisk). SP-GFP is a fusion protein comprising the signal peptide of torsin and GFP.

Untransfected and transiently SP-GFP- or SP-GFP-SUN-1-C-expressing HaCaT cells were fractionated into the cytoplasm and nuclei.

**Yeast Two-hybrid Screening**—Matchmaker Two-hybrid System 3 was used following the yeast protocols handbook (PT3024-1, Clontech). SR was cloned into the yeast pGBKT-7 plasmid (9). This bait was used to screen a pretransformed human brain cDNA library in pGADT-7-Rec expression vectors used as a prey. Positive clones were isolated, sequenced, and retransformed with the bait to confirm the interaction.

**Cell Culture**—The following cell lines were employed: COS-7 (12) and HaCaT (13). Primary human keratinocytes were provided by Nils Buchstein and cultivated according to Rheinwald and Green (14).

**Cell Transfection**—COS-7 cells were transfected using GenePulser® II (Bio-Rad) at 170 V and 950 microfarads. HaCaT cells were transfected twice at intervals of 3 days using the Amaxa cell line Nucleofector® kit V (Lonza) according to the manufacturer’s instructions.

**GST Pulldown**—COS-7 cells expressing Myc- or GFP-tagged proteins were lysed in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet-P-40, and 0.5% sodium deoxycholate). After preclearing lysates for 1 h with beads, samples were incubated with GST fusion proteins, GST-coupled beads, or beads alone at room temperature for 2 h. Finally, the beads were washed with lysis buffer and PBS. For Fig. 4A (1st approach), the pulldown included two incubation steps. The first incubation was performed as described above to ensure binding of GFP-α-N-catenin-1 (Input 1) to GST-SR12. For the second incubation (Input 2), COS-7 cells were lysed in 0.5 ml of PBS with 0.075% Nonidet-P-40 (the detergent concentration was reduced by doubling the volume with PBS). Lysates were incubated with GST fractions from the first incubation step overnight at 4 °C and washed with PBS containing 0.075% Nonidet-P-40 and PBS. For Fig. 4A (2nd approach), we used only the second incubation step as described above. Samples were analyzed by Western blotting using the antibodies indicated.

**Co-immunoprecipitation**—HaCaT cells were lysed in lysis buffer or in PBS containing 0.075% Nonidet-P-40. The volume was doubled to decrease the detergent concentration. After preclearing the lysates for 1 h with protein A-Sepharose CL-4B beads (GE Healthcare), lysis buffer lysates were incubated for
2 h with 6 μg of pAbK1 to allow antigen-antibody coupling, followed by a second incubation for 2 h with protein A-Sepharose beads. Subsequently, the beads were washed twice with PBS and incubated overnight with PBS lysates. Finally, the beads were washed five times with PBS, and the fractions were analyzed by Western blotting.

**Subcellular Fractionation**—Cells were resuspended in hypotonic buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, and 10 mM KCl) and incubated for 10 min. Cells were lysed by passage through a needle (27Gx3/4, 10 times) and centrifuged (10 min, 10,000 rpm) 5 min after the 10-min incubation step. Pellets (nuclei) were washed four to six times with PBS. Supernatants (cytoplasm) were centrifuged (20 min, 12,000 rpm) and used for protein analysis.

**Immunofluorescence and Immunoblotting Reagents**—The primary antibodies used were directed against α-catenin (epitope amino acids 890–902, Sigma), nesprin-2 (pAbK1, K20–478) (1, 9), Myc (9E10) (15), GFP (K3–184–2), emerin (4G5, Abcam), β-catenin (6F9, Sigma), active β-catenin (8E7, Millipore), actin (Ac-74, Sigma), Lap-2 (27, BD Transduction Laboratories), LamB1 (Abcam), GAPDH (71.1, Sigma), β-tubulin (WA3, U. Euteneuer), and BrdU (BD Biosciences). The secondary antibodies used were Alexa Fluor 488/568 conjugates (Sigma). Nuclei were stained with DAPI, and actin was stained with TRITC-phalloidin (Sigma). Immunofluorescence analysis and microscopy were performed as described (1).

**RNA Interference**—Human nesprin-2 knockdown was obtained by plasmid-based RNAi. Oligonucleotides were cloned into pSHAG-1 (BseRI and BamHI) (16): Nes-2ct1-s, GAG-AAGAATCAACAGGATAGCCTGACTGTGTGTTGTTCTTCTCTTTTT; Nes-2ct1-as, GAT-CAAAAAAGAGAAGAAACTCA-AACAGTCAGGCTCAGTCGTT-TAGGTCTCTTCTCGG; Nes-2ct2, CCAGGCTGCTGCACATCCG-AAGCTGGGATGTTGCAGGAGGCTGGCG; Nes-2ct2-as, GATCCCAGCTTCCTGCAACAATC-TCCAAAGCTTGAGTGTGCCTGGC-AGGAGGCTGGC; Control-s, ATCTACTCAGTCAGTACCGT-AAGCTGGGATGTTGCAGGAGGCTGGC; Control-as, GATCAAAAATCTACTCGAC-GTTAGCTCGTCAAGCTTCACGC-TCAGTGAGTATCG.

**TOP/FOP Promoter Assay**—HEK293T were transiently transfected with C-terminal nesprin-2 shRNA1 or shRNA2 or with the corresponding control using Lipofectamine 2000 (Invitrogen). The cells were transfected twice at intervals of 3 days. The first transfection was with shRNA alone. The second transfection was with shRNA-, TOPflash-, or FOPflash-luciferase reporters and a polymerase III-Renilla luciferase control reporter. 5 days after the first transfection, cells were treated overnight with 30 mM LiCl to induce the Wnt pathway. The TOP/FOP promoter assay was carried out using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer’s instructions. The experiments were done three times each in duplicate. A representative graph is shown.

**BrdU Incorporation Assay**—HaCaT cells were transiently transfected with C-terminal nesprin-2 shRNA1 or the corresponding control, plated on glass plates, and incubated with 10 μg/ml BrdU (Sigma) under growing conditions for 2 h. The cells were fixed with 4% paraformaldehyde and permeabilized...
with 0.4% Triton X-100. To allow the anti-BrdU antibody access to the antigen, cells were washed with PBS and treated with 2M HCl for 30 min at room temperature, followed by further washing with PBS. Immunofluorescence analysis was performed as described above.

RESULTS

\( \alpha \)-N-catenin, a Novel Nesprin-2-binding Partner—To gain insights into nesprin-2 biology, we performed a yeast two-hybrid screening using a nesprin-2 giant C-terminal fragment (SR), which resembles nesprin-2\( \Delta TM1 \) (17), as bait (Fig. 1A). Nesprin-2-SR represents a domain common to most nesprin-2 isoforms (4) and accumulates at the NE of COS-7 cells, although it lacks a KASH domain (Fig. 1, B and C, asterisks). We confirmed that the NE localization was not mediated by the GFP tag by transfecting the empty vector into COS-7 cells, where GFP alone could not be detected at the NE (supplemental Fig. S1, A and B, asterisks).

The yeast two-hybrid screening revealed a C-terminal fragment of the neuron-specific \( \alpha \)-N-catenin termed \( \alpha \)-N-Cat* (Fig. 1, D and E). The corresponding gene, CTNNA2, comprises 18 exons and gives rise to three alternatively spliced isoforms (18). In contrast to \( \alpha \)-N-catenin-1, \( \alpha \)-N-catenin-2 harbors a C-terminal 48-amino acid insertion (exon 17) (Fig. 1E) (19). \( \alpha \)-N-catenin-3 possesses an alternative transcription initiation site (Fig. 1E, triangle) and does not contain the first six exons and exon 17 (18). \( \alpha \)-N-Cat* appears to belong to an additional isoform (NCBI accession number AK295181.1) lacking exons 16 and 17, which we designated as \( \alpha \)-N-catenin-4 (Fig. 1E). We verified the interaction of nesprin-2 and \( \alpha \)-N-Cat* by GST pull-down and narrowed down the respective binding sites (Fig. 2). The studies revealed that several \( \alpha \)-N-Cat* proteins, namely \( \alpha \)-N-Cat*, \( \alpha \)-N-Cat*\( \Delta Ex18 \), and \( \alpha \)-N-Cat*\( \Delta Ex18/15 \), showed the strongest binding for SR1+2. Further analysis demonstrated that SR2 is sufficient for mediating the interaction, although the binding appeared to be weaker compared with the larger proteins (Fig. 2C). Moreover, two distinct nesprin-2-binding sites were identified in \( \alpha \)-N-Cat*, including the exon 15- and 18-encoded (Fig. 2D) and exon 14-encoded (Fig. 2C) regions. The interaction is not unique to \( \alpha \)-N-Cat*, as interactions with \( \alpha \)-N-catenin-1 and \( \alpha \)-E-catenin could also be demonstrated (supplemental Fig. S2A). Because \( \alpha \)-E/T/N-catenins are conserved and tissue-specific (20), we conclude that nesprin-2 interactions can take place in a tissue-independent manner.

In an immunoprecipitation using the anti-nesprin-2 polyclonal antibody pAbK1, which detects a large number of isoforms, including the 800-kDa nesprin-2 giant, we precipitated endogenous \( \alpha \)-catenin from HaCaT cells (supplemental Fig. S2B). From
this, we additionally conclude that, *in vivo*, the interaction between both proteins is not limited to isoforms composed of the nesprin-2-SR sequence.

**α-Catenin and Nesprin-2 Localize at the NE and PM**—Bear- in mind that α-catenin is a core adherens junctions compo- nent, we analyzed its subcellular distribution in primary human keratinocytes. Cells were grown under low (50 μM CaCl₂) and high (2 mM CaCl₂) Ca²⁺ conditions to inhibit or favor adherens junction formation, respectively. Under high Ca²⁺ conditions, α-catenin localized to the PM (Fig. 3B, arrow); sig- nals surrounding the nucleus could only be observed sporadically (supplemental Fig. S3A, arrow). Nesprin-2 localized along the NE (Fig. 3B'). In contrast, under low Ca²⁺ conditions, the depletion of cell-cell contacts triggered a dra- matic redistribution of α-catenin from the PM to a perinuclear posi- tion (supplemental Fig. S3B, arrows). A continuous NE labeling was observed sporadically (Fig. 3, A and A', arrows).

Next, different GFP- and Myc- tagged α-catenin proteins were tran- siently expressed in COS-7 cells and HaCaT keratinocytes, which differ markedly in their ability to form adherens junctions (supplemental Fig. S4). In HaCaT, GFP-α-N-catenin-1 (supple- mental Fig. S4A, arrow) and Myc-α-E-catenin (Fig. 3F, arrow) were almost found exclusively at the PM. COS-7 cells exhibited a different α-catenin distribution, as both α-E-catenin-1 and α-N-catenin-1 local- ized along the NE in the majority of cells (supplemental Fig. S4, B, C, E, and F, arrows, and I). Intriguingly, N-terminally truncated α-N-cate- nin fusion proteins were distributed throughout the cytoplasm (supple- mental Fig. S4D, arrowhead) and along the NE in HaCaT cells (supple- mental Fig. S4, D, arrow, and I). In COS-7 cells, C-terminal α-cate- nin polypeptides were much more frequently found along the NE, par- alleling the full-length proteins (supplemental Fig. S4, G and H, arrows, and I). In HaCaT keratino- cytes, α-catenin seemed to be kept primarily at the PM, probably by interactions mediated by its N terminus.

To determine whether nesprin-2 could be detected at the PM of primary human keratinocytes as well, its localization was assayed under different Ca²⁺ conditions. Under low Ca²⁺ conditions (Fig. 3, C and D), nesprin-2 was found at the NE (Fig. 3C, asterisk). Different Z-sections are shown to demonstrate the absence of cell contacts, which were visualized by phalloidin.
Nesprin-2 Interacts with α-Catenin

FIGURE 4. Nesprin-2 forms a quaternary protein complex with Wnt pathway components at the NE and influences nuclear β-catenin accumulation. 
A, GST pulldown analysis of nesprin-2 interactions with GFP-α-N-catenin-1. SR1 +2 co-precipitated with GFP-α-N-catenin-1 and endogenous β-catenin and emerin (1st approach). The absence of α-catenin did not disturb the interaction of SR1 +2, β-catenin, and emerin (2nd approach). The Coomassie Blue gel (A) and the supernatant fractions (B) show input of equal protein amounts. C, schematic of SUN-1, SP-GFP, and the dominant-negative nesprin-interfering SP-GFP-SUN-1-C expressing cells. Nesprin NE displacement resulted in decreased nuclear β-catenin levels. E, SP-GFP-SUN-1-C localized to the NE (arrow) and the endoplasmic reticulum (arrowhead) and displaced nesprin-2 (E’, asterisk) from the NE. Nesprin-2-K1, nesprin-2 visualized with pAbK1. E’’ merge, F and G, HaCaT cells were treated with control or nesprin-2 (Nes-2)-specific shRNA. F, nesprin-2 silencing efficacy was shown by Western blotting (WB) using pAbK1. G, Western blot analysis of fractionated cell lysates. Nuclear β-catenin content in nesprin-2 shRNA-treated cells was reduced. Total cell lysates (F) or cytoplasmic and nuclear fractions (G) were prepared using equal cell numbers. H, knockdown of nesprin-2 antagonized cell proliferation. BrdU incorporation was measured in HaCaT cells transiently treated with nesprin-2-specific shRNA or the corresponding control. The statistical significance was analyzed using Student’s t test (p = 0.0016). I, TOP/FOP reporter assay of WT, control shRNA-treated, and nesprin-2 shRNA-treated HEK293T cells. The TOP/FOP ratio (y axis), in which the TOPFlash-measured β-catenin-dependent transcriptional activity was normalized against unspecific FOPFlash signals, was reduced when nesprin-2 was absent. TL, total lysates.
Nesprin-2 Interacts with α-Catenin

FIGURE 5. Model of nesprin-2 and α-catenin roles in Wnt signaling. A, α- and β-catenins localize to adherens junctions linking the adhesion complex to the actin cytoskeleton. Nesprin-2 and emerin localize to the NE. Inner nuclear membrane-associated emerin supports the export of β-catenin from the nucleoplasm to the cytoplasm. Cytoplasmic β-catenin levels are kept low by degradation. B, nesprin-2 can be detected at the nuclear and plasma membranes. α-Catenin is kept primarily at the PM. Disassembling these connections by dissolving cell contacts allows α-catenin to reach the NE. C, upon Wnt pathway activation, cytoplasmic β-catenin levels increase. A quaternary complex of nesprin-2, emerin, and α- and β-catenins can be formed at the NE. When released from this complex, β-catenin can enter the nucleus. D, loss of nesprin-2 results in a reduced amount of β-catenin in the nucleus, probably due to an inefficient binding of the αβ-catenin heterodimer at the NE. LRP, lipoprotein receptor-related protein.

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