Novel Nuclear Nesprin-2 Variants Tether Active Extracellular Signal-regulated MAPK1 and MAPK2 at Promyelocytic Leukemia Protein Nuclear Bodies and Act to Regulate Smooth Muscle Cell Proliferation

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Nuclear and cytoplasmic scaffold proteins have been shown to be essential for temporal and spatial organization, as well as the fidelity, of MAPK signaling pathways. In this study we show that nesprin-2 is a novel extracellular signal-regulated MAPK1 and 2 (ERK1/2) scaffold protein that serves to regulate nuclear signaling by tethering these kinases at promyelocytic leukemia protein nuclear bodies (PML NBs). Using immunofluorescence microscopy, GST pull-down and immunoprecipitation, we show that nesprin-2, ERK1/2, and PML colocalize and bind to form a nuclear complex. Interference of nesprin-2 function, by either siRNA-mediated knockdown or overexpression of a dominant negative nesprin-2 fragment, augmented ERK1/2 nuclear signaling shown by increased SP1 activity and ELK1 phosphorylation. The functional outcome of nesprin-2 disruption and the resultant sustained ERK1/2 signal was increased proliferation. Importantly, these activities were not induced by previously identified nuclear envelope (NE)-targeted nesprin-2 isoforms but rather were mediated by novel nuclear isoforms that lacked the KASH domain. Taken together, this study suggests that nesprin-2 is a novel intranuclear scaffold, essential for nuclear ERK1/2 signaling fidelity and cell cycle progression.

Mitogen-activated protein kinase (MAPK)2 signaling has essential roles in proliferation, differentiation, and apoptosis (1–3). The extracellular signal-regulated MAPK1 and MAPK2 (ERK1/2) are the best characterized MAPK members. In response to extracellular stimuli, ERK1/2 becomes phosphorylated via the RAS signaling pathway and active ERK1/2 translocates from the cytoplasm to the nucleus (3–5) to regulate transcription and cell cycle progression (1, 7, 8). Recently, studies have highlighted the importance of ERK1/2 scaffold proteins in determining the duration of signaling within the nucleus. ERK1/2 diffuses across the nuclear membrane, with movement in and out of the nucleus determined by both cytoplasmic and nuclear ERK1/2-binding partners (9, 10). Its diffusion is further impeded by phosphorylation, suggesting that active ERK1/2 forms large complexes of low mobility within nuclei (10). However, the precise localization and components of these complexes remain unknown.

Nesprins are a novel family of spectrin repeat-containing proteins that were originally identified from a differential cDNA screen as highly expressed in differentiated vascular smooth muscle cells (VSMCs) (11, 12). To date, four nesprin genes have been identified that encode a plethora of variants that vary greatly in size. The largest, or giant variants of nesprin-1 and nesprin-2 consist of an N-terminal-paired calponin homology domain, a central rod domain comprised of multiple spectrin repeats, and a C-terminal Klarlsicht, ANC-1, Syne Homology (KASH) domain required for NE localization (11–13). Initially, nesprins were described as components of the inner nuclear membrane (INM) where they interact with lamins A/C and emerin (14–17). In conjunction with larger, actin-binding outer nuclear membrane (ONM) nesprin isoforms, INM nesprins function to link the nuclear lamina to cytoplasmic cytoskeletal structures via association with SUN domain-containing proteins (18–21). However, studies using antibodies generated to peptides along the length of the giant nesprin proteins, have shown that nesprins are also present in other cytoplasmic and nuclear structures (14, 23, 24). Our understanding of the role of nesprins within these other subcellular compartments remains to be elucidated and to date, very few nesprin interaction partners have been described. Therefore, we set out to identify novel nesprin-2-binding partners as a means to interrogate further the functions of nesprin-2. We identified ERK1/2 and PML as nesprin-2 interaction partners and demonstrate that nesprin-2 acts to scaffold ERK2 within the nucleus at promyelocytic leukemia protein nuclear bodies (PML NBs), where this complex serves to inhibit ERK1/2 activity. Thus nesprin-2 plays fundamental roles in regulat-

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4 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; UTR, untranslated region; IP, immunoprecipitation; VSMC, vascular smooth muscle cells; EST, expressed sequence tag; NE, nuclear envelope; PML, promyelocytic leukemia protein; NB, nuclear bodies; INM, inner nuclear membrane; ONM, outer nuclear membrane; GST, glutathione S-transferase; WB, Western blot; ELISA, enzyme-linked immunosorbent assay; KASH, Klarsicht, ANC-1, Syne homology domain.
Nesprin-2 Tethers ERK1/2 at PML Nuclear Bodies

ing nuclear ERK1/2 signaling events and is essential for normal cell cycle progression.

MATERIALS AND METHODS

Cell Culture—Human VSMCs were cultured as described previously (11). MG63 osteosarcoma cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma) plus 10% fetal calf serum (Sigma), and penicillin/streptomycin/glutamine (Sigma). For DNA transfection, cells were seeded onto an appropriately sized plate and incubated overnight. The next day, cells were transfected with DNA using Superfect transfection reagent (Qiagen) per the manufacturer’s instructions.

Yeast 2-Hybrid Screening—The N terminus of nesprin-2β was cloned into the GBDKT-7 matchmaker bait vector (Clontech) and transformed into the yeast strain AH109 (Clontech). Bait plasmids were tested for self activation before the library screen was performed. Bait plasmids were used to screen a cardiac muscle library per the manufacturer’s instructions (Clontech). Colonies that grew after 7 days were picked, plasmids rescued, and retransformed to recheck for reporter gene activation. Inserts were identified by DNA sequencing and BLAST search analysis.

Antibodies—Antibodies used for WB, IF, and immunoprecipitations were: pELK1 (9181, Cell Signaling Technologies); ERK2 (sc-1647), PML (sc-966), pERK1/2 (sc-7383), (Santa Cruz Biotechnology); Vinculin (V9131), β-actin (A5316) (Sigma), and nesprin-2 N3. Nesprin-2 N3 and ERK2 antibody specificities were confirmed by peptide blocking analysis on both Western blot and immunofluorescence (supplemental Fig. S1). Secondary antibodies for WB were horseradish peroxidase-conjugated anti-mouse (NA9108, GE Healthcare) or anti-rabbit (NA931) antibodies from GE Healthcare. ECL chemiluminescent kit (RPN2132, GE Healthcare) was used for detection according to the manufacturer’s instructions. Invitrogen anti-mouse Alexa fluor 568 (A11031) and anti-rabbit Alexa fluor 488 (A11034) were used as IF secondary antibodies.

EST Data Base Searches and PCR—NCBI expressed sequence tag data base (EST), consisting of one-shot sequences of cloned mRNA, was blasted with consecutive, 50-bp overlapping 1 kb Nesprin-2 giant cDNA sequences. Those sequences that did not fully align were aligned to the human genome to identify novel 5′-untranslated regions (UTR). PCR was performed to verify the novel 5′-UTRs using the Promega Taq master mix per the manufacturer’s instructions and the appropriate oligos: JAM28: AATTCCACCTACTTGAGACGCTGA and TGGGACTCTTGAAGCCCTTCA, JAM19: GGATGTCGAAGCCTGTCCTG, and N3 antibody for WB were cultured on coverslips, fixed in 50% methanol/acetone, and processed as described previously (11). Visualization was performed on a Leica SP5 confocal microscope.

GST Pull-down Assays—GST fusion proteins and GST alone were incubated from 100 ml of bacterial culture for 2 h by addition of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside. Purification of the proteins was performed according to the American Biosciences protocol using glutathione-Sepharose 4B beads (Amersham Biosciences). Binding assays were performed in VSMC extracts prepared as described previously. 100-μg extracts were incubated with 50-μl beads for 2 h at 4°C with rotation. Bound proteins were eluted into sample buffer, and Western blotting was performed.

Immunoprecipitations—In vivo immunoprecipitations were performed on confluent VSMCs, and cell extracts were prepared as described previously (11). Each immunoprecipitation
contained 100 µg of precleared cell lysates and 2 µg of antibody. Protein-antibody complexes were harvested using either protein A- or protein G-Sepharose beads (Sigma). Samples were then analyzed by Western blotting.

**ELISA**—ELISA assays were performed using the TransAM Sp1/3 kit per the manufacturer’s instructions (Active Motif). Briefly, Sp1/3 binding was achieved by incubating 5 µg of nuclear extract per well of TransAM plate for 1 h at room temperature. Detection of bound Sp1/3 was achieved by incubation with a Sp1/3 antibody followed by an anti-rabbit horseradish peroxidase-conjugated antibody. Cells were washed four times before addition of the developing reagent. Reactions were terminated by addition of the stop solution. Absorbance was read on a spectrophotometer at 450 nm.

**siRNA-mediated Interference**—Dharmacon smart pool oligos designed specifically to regions within nesprin-2 were: 5’-CCACAGAGCUCCAAAGUUGU-3’, 5’-GAACUAUGCCACAGGUAAUU-3’, and control oligos were purchased from Dharmacon. An additional nesprin-2β oligo (Qiagen) was also used as described previously (25). All experiments were performed with either a single nesprin-2 siRNA (siRNA 1) or a pool of siRNAs (siRNA 2), to control for off-target effects. Oligos were resuspended and transfected into VSMCs using HiPerfect transfection reagent, according to the manufacturer’s instructions (Qiagen). Forty-eight hours post-transfection, samples were prepared for Western blotting, ELISA, or immunofluorescence microscopy. Each experiment was repeated three times.

**RESULTS**

**Nesprin-2 Interacts with Active ERK1/2 in VSMCs**—To identify novel binding partners for nesprin-2, we utilized the yeast 2-hybrid system. Using the N terminus of nesprin-2 (amino acids 1–219) as bait, 2 full-length clones of ERK2 were isolated by screening 1 × 10^6 clones of a human cardiac muscle library (Fig. 1A). The interaction was confirmed by GST pull-down assays using the N terminus of nesprin-2 (GST-Nt2), which was able to precipitate ERK2 from whole-cell lysates (Fig. 1B). The N terminus of nesprin-2β is comprised of 2 spectrin repeats (SR3 and SR4). To define the minimal spectrin repeat requirement for the ERK2 interaction, deletion mapping was performed. Pull-downs using single spectrin repeats (amino acids 1–125 (SR4) and 126–219 (SR3) of nesprin-2β) fused to GST and WB analysis revealed that both spectrin repeats within the N terminus of nesprin-2β were capable of interacting with ERK2 and active ERK1/2 (Fig. 1C), suggesting that this region makes multiple connections with these proteins. However, SR4 precipitated both ERK2 and active ERK1/2 more efficiently than SR3 (Fig. 1C), indicating that this region has a higher affinity for these kinases. To determine whether nesprin-2 and ERK2 form a complex in vivo, immunoprecipitations from VSMC whole cell lysates were performed using antibodies against nesprin-2 to pellet PML (C) or PML to precipitate the 75-kDa nesprin-2 isoform, ERK2, and active ERK1/2 (D). E, GST pull-downs using different nesprin-2 fragments were performed to map the PML-interacting region of nesprin-2 to a rod domain downstream of the ERK1/2 binding region.

**FIGURE 2.** Nesprin-2 and ERK2 are components of PML NBs. A, immunofluorescence microscopy was performed and showed the colocalization of nesprin-2 (green) and PML (red) (arrows); colocalization of nesprin-2 (green) and ERK2 (red) (arrowheads) in VSMCs. B, subcellular fractionations revealed that the 75-kDa isoform of nesprin-2 is predominantly nuclear. Nuclear fractions were confirmed by detection of the nuclear protein, Splicing Factor-2 (SF2). In vivo immunoprecipitations from VSMC whole cell lysates were performed using antibodies against nesprin-2 to pellet PML (C) or PML to precipitate the 75-kDa nesprin-2 isoform, ERK2, and active ERK1/2 (D). E, GST pull-downs using different nesprin-2 fragments were performed to map the PML-interacting region of nesprin-2 to a rod domain downstream of the ERK1/2 binding region.
FIGURE 3. The 75-kDa nesprin-2 variant exists in multiple cell types and identification of novel nesprin-2 5'-UTRs. A, WB analysis of the nesprin-2 N3 profile of different cell types revealed multiple bands but MG63 cells display reduced levels of the 75-kDa variant (asterisk). B, IF revealed that MG63 cells lack nesprin-2 (green) colocalization at PML NBs (red), whereas VSMCs display strong colocalization. C, schematic representation showing the position of the novel 5'-UTRs identified by EST searching. JAM28 and JAM19 encoded for truncated nesprin-2 variants starting at exon 92 and 101, respectively. These isoforms start at amino acids 5570 and 6014, respectively of the nesprin-2 giant sequence. D, PCR analysis, using primers targeting these novel 5'-UTRs, confirmed these 5'-UTRs exist in multiple tissues. E, schematic representation of potential variants generated from the C terminus of nesprin-2 giant including variants lacking the KASH domain previously described (14, 26). Predicted molecular weights correspond closely to those observed in A.
Nesprin-2 Tethers ERK1/2 at PML Nuclear Bodies

bodies against either ERK2 or active ERK1/2 pulled down a nesprin-2 band of ~75 kDa (Fig. 1E), further confirming the interaction between nesprin-2 and active ERK1/2.

Nesprin-2 Colocalizes with ERK2 at PML Bodies—Confocal immunofluorescence (IF) microscopy was performed to determine whether ERK2 and nesprin-2 colocalize within cells. In primary VSMCs, using the N3-antibody, nesprin-2 was present diffusely in the cytoplasm and in a perinuclear pattern. Within the nucleus, it localized to the nucleolus; however its most prominent location was at subnuclear foci, identified as PML NBs (Fig. 2A). Nesprin-2 and ERK2 colocalized at PML NBs (Fig. 2A). Subcellular fractions confirmed that the 75-kDa nesprin-2 band was predominantly nuclear (Fig. 2B).

To determine whether nesprin-2 and PML formed physical connections, immunoprecipitation was performed using nesprin-2 antibodies. This showed that nesprin-2 could efficiently immunoprecipitate PML (Fig. 2C). Similar to ERK1/2 IPs, PML specific antibodies could also immunoprecipitate the 75-kDa nesprin-2 band (Fig. 2D) from VSMC lysates. Importantly, PML could also efficiently precipitate ERK2 and active ERK1/2 (Fig. 2D). Taken together, these data demonstrate that nesprin-2 and active ERK1/2 are components of PML NBs in VSMCs. To further define the nesprin-2/PML association we again utilized a GST pull-down approach and demonstrate that the nesprin-2β rod region specifically binds a 50-kDa PML isoform from VSMC lysates (Fig. 2E).

The ERK1/2- and PML-associated Nesprin-2 Isoform Exists in Multiple Cell Types—Next, we investigated whether the 75-kDa nesprin-2 variant was present in cell types other than VSMCs using WB and the N3 antibody. This antibody identified major bands, of ~350, 150, 100, and 75 kDa (Fig. 3A) in all cell types tested, although levels of the individual bands varied. In support of the notion that the 75-kDa band was the PML NB-associated nesprin-2 isoform, IF showed that in MG63 cells, which contain reduced 75 kDa nesprin-2 levels, PML NBs were nesprin-2 deficient (Fig. 3B), whereas VSMCs that display high levels of this isoform display prominent colocalization of nesprin-2 at PML NBs (Fig. 3B).

To elucidate the origin of the nesprin-2 variants identified by the N3 antibody, we performed comprehensive EST data base searching and identified two additional alternate initiation sites within the nesprin-2 gene (accession numbers JAM28 DA226447; JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144, JAM19 DB089560, BM805144). Previous studies have identified nesprin-2 variants lacking the KASH domain (14, 26). As shown in Fig. 3E, the molecular weights of predicted variants arising from these novel initiation sites, either containing or lacking the KASH domain, correspond to the observed molecular weights on WB using the N3 antibody, suggesting that these are bona fide nesprin-2 variants. Moreover, SMART analysis confirmed the presence of a putative NLS also within this region, supporting the notion that a 75-kDa nuclear nesprin-2 variant could be generated from this region (Fig. 3E).

Nesprin-2 Fragments Lacking the KASH Domain Accumulate within the Nucleus—Next, GFP fusion constructs containing nesprin-2 domains within, and adjacent to, the ERK1/2 binding region, were generated and transiently transfected into U20S cells (Fig. 4A). As shown in Fig. 4, A and B, IF microscopy revealed that some of these fragments could target to both the cytoplasm and the nucleus. As predicted, a large C-terminal fragment of nesprin-2 containing the KASH domain and the potential NLS localized to the NE and ER (arrowheads). However, the same fragment lacking the KASH domain but retaining the potential NLS, localized to both the nucleus and the cytoplasm with nuclear staining apparent in ~38% of cells.

**FIGURE 4.** Fragments of nesprin-2 localize to the cytoplasm and nucleus. A, schematic representation of the constructs used for transfection into U20S cells, their predicted molecular weight and counts confirming their subcellular localization. Counts are combined data of three independent experiments. B, fluorescence microscopy was performed to observe localization of nesprin fragments. GFP fusion proteins containing the KASH domain were predominantly within the NE and ER (arrowheads), whereas fragments lacking the KASH domain were present in the nucleus (arrows) and cytoplasm. C, colocalization (arrowheads) of FLAG-nesprin-2ΔTM (green) and PML (red).

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This observation led us to investigate whether a previously identified nesprin-2 variant, nesprin-2\(\beta\)ΔTM, with a predicted molecular mass of around 75 kDa, could localize to PML NBs. IF microscopy revealed that a FLAG-nesprin-2\(\beta\)ΔTM fusion protein displayed PML NB localization in all transfected cells (Fig. 4, A and C).

To investigate further the requirements for the nuclear localization of this fragment, further deletion constructs were transfected.
into cells. Localization of the SR3–7 fragment, containing the potential NLS but lacking the lamin A/C binding region, was predominantly cytoplasmic, with only 19% of cells displaying nuclear localization. In contrast, the GFP-SR1–2 fragment, that lacks any potential NLS but retains the ability to interact with lamins A/C, was predominantly nuclear in all cells (Fig. 4, A and B). These data imply that nesprin-2 variants are capable of accumulating within the nucleus and that nuclear retention may require interactions with nuclear lamins A/C.

**Nesprin-2 Tethers ERK2 at PML NBs**—To examine the functional role of nesprin-2 within the PML NB, siRNAs designed to nesprin-2β were transiently transfected into VSMCs. 48 h post-transfection, WB analysis revealed consistent reduction of the 75-kDa nesprin-2 band using two independent siRNAs (Fig. 5A). Additional, nesprin-2 variants of around 160 and 100 kDa were also partially depleted (Fig. 5A). To confirm specificity of the siRNAs, the closely related protein, nesprin-1α was also examined by WB and found to be unchanged upon nesprin-2 depletion (Fig. 5A). IF after siRNA knockdown of nesprin-2 showed that while cytoplasmic and perinuclear localization was unaffected, nesprin-2 was absent from PML NBs in a significant proportion of VSMCs (Fig. 5, B and C). Moreover, in these cells, ERK2 was also absent from the PML NBs (Fig. 5, D and E). In contrast, VSMCs transfected with scrambled control siRNAs retained PML NB colocalization of both nesprin-2 (arrowheads) (Fig. 5, B and C) and ERK2 (arrows) (Fig. 5, D and E). Morphology and PML association with PML NBs remained unchanged in both control and nesprin-2 siRNA-treated cells (Fig. 5B), indicating that the PML NBs remain intact and structurally unaltered by nesprin-2 depletion. These data suggest that nesprin-2 acts to tether ERK2 at PML NBs but is not necessary for the formation of these subnuclear structures.

To further confirm that these siRNAs were targeting nuclear and not NE-associated variants, IF was performed for emerin, a protein dependent on nesprin-2 for its INM localization (14, 15). Importantly, emerin localization remained unaltered by nesprin-2 depletion (supplemental Fig. S2), confirming that ERK2 scaffolding by nesprin-2 at PML-NBs is independent of NE-associated nesprin-2.

**Nesprin-2 Negatively Regulates ERK Signaling within the Nucleus**—To investigate the impact of uncoupling ERK1/2 from PML NBs via nesprin-2 depletion, we analyzed the activation of downstream ERK1/2 nuclear targets by WB 48 h after transfection with nesprin-2 siRNA. Loss of the 75-kDa nesprin-2 variant did not alter total levels of phosphorylated ERK1/2 (Fig. 6A), suggesting nesprin-2 depletion does not impact on global ERK1/2 activation. However, VSMC lysates depleted of nesprin-2 displayed an increased level of phosphorylation and activation of Elk1 (Fig. 6A), indicating that ERK1/2 nuclear activity is augmented. To investigate further this increased ERK1/2 nuclear activity induced by nesprin-2 knockdown, ELISA assay was used to determine the activity of the Sp1 transcription factor in nuclear lysates purified from control and nesprin-2-depleted cells. Nesprin-2-depleted nuclei displayed a 2-fold increase in Sp1 activity compared with control nuclei (Fig. 6B), confirming an increase in nuclear ERK1/2 target activity upon nesprin-2 disruption.

To further confirm the role of nesprin-2 in regulating nuclear ERK1/2 activity, we next performed overexpression experiments to disrupt nesprin-2 function in U2OS cells and measured the activity of a Sp1 reporter construct using luciferase assays. Overexpression of the GFP-SR1–7 fragment that retained the KASH domain and therefore showed NE localization did not alter Sp1 activity compared with control cells (Fig. 6C). However, overexpression of the same fragment lacking the KASH domain (GFP-SR1–7ΔTM) that targeted to the nucleus, induced a 4-fold increase in Sp1 activity compared with control cells (Fig. 6C). Taken together, these data confirm that nesprin-2 serves to organize ERK1/2 and regulate nuclear activity of these kinases, and this function is independent of NE targeting.

ERK1/2 signaling has been implicated in cell cycle progression and proliferation. Therefore time lapse microscopy was used to investigate whether the observed increase in nuclear ERK signaling impacted upon cell proliferation. Knockdown of nesprin-2 using siRNAs resulted in a significant increase in the number of cell divisions per 1000 cells (Fig. 6D), even when transfected into slow growing VSMCs. Increased proliferation was also indicated by an increase in Ki67-positive cells (Fig. 6E), strongly suggesting that PML NB-associated nesprin-2 serves to inhibit proliferation by scaffolding ERK1/2 away from its targets.

**DISCUSSION**

In this study, we identify nesprin-2 as a nuclear scaffold and ERK1/2 and PML as novel nesprin-2-interacting proteins. These proteins colocalize and form a complex at PML NBs that block ERK1/2-mediated signaling within the nucleus. Disruption of nesprin-2 function by siRNA-mediated knockdown or overexpression, uncoupled ERK1/2 from PML NBs, augmented nuclear ERK1/2 signaling and ultimately induced cell proliferation. This is the first study to describe an ERK1/2 scaffold that tethers these kinases to a specific subnuclear compartment and suggests that nesprin-2 functions to fine-tune ERK-initiated proliferation in multiple cell types. As such it is likely to be a novel target for modifying the function of these kinases in hyperproliferative disorders such as restenosis.

**Novel Nesprin-2 Variants Tether ERK1/2 at PML NBs**—This study demonstrates for the first time that nesprin-2 plays essential roles within the nucleus. By forming a complex with ERK1/2 and PML, nesprin-2 regulates nuclear signaling events by sequestering active ERK1/2 at PML NBs away from its nuclear targets. The 75-kDa nesprin-2 isoform precipitated by both PML and ERK2 antibodies was present in multiple cell types, implying that nesprin-2 function is highly conserved and that tethering of ERK1/2 at PML NBs is a common mechanism for regulating nuclear activity of these kinases. Disruption of nesprin-2 function, by either siRNA-mediated knockdown or overexpression of a dominant negative fragment, that failed to localize correctly to PML NBs, increased nuclear ERK1/2 activity. Importantly, nesprin-2 knockdown did not disrupt the INM, shown by emerin localization, whereas overexpression of a large nesprin-2 fragment predicted to localize to the ONM, did not affect ERK1/2 activity, confirming that this novel ERK1/2 scaffolding function of
Nesprin-2 Tethers ERK1/2 at PML Nuclear Bodies

A. Western blot analysis showing the expression of Nesprin-2, p-ERK1/2, pELK1, and Vinculin with and without Nesprin-2 siRNA.

B. Bar graph showing the relative absorbance of MCF-1, MCF-1 + blocking oligo, Control siRNA, and Nesprin-2 siRNA, with p-value 0.0213.

C. Schematic diagram of GFP constructs: GFP-Gamma, GFP-SR1-7, and GFP-SR1-7 ΔTM.

D. Bar graph showing the number of cell divisions per 1000 cells with p-value 0.0012.

E. Bar graph showing the percentage of cells displaying Ki67 staining with p-value 0.0001.
nesprin-2 is independent of the previously described NE roles of nesprin-2.

PML exists in multiple isoforms that reside in both the cytoplasm and nucleus. Our data demonstrate that nesprin-2 associates with a 50-kDa PML isoform corresponding to the approximate size of PML VIIb (27, 28). This PML isoform is truncated within the C terminus and lacks the NLS utilized by larger PML isoforms (28). Interestingly, this isoform is the predominant PML isoform in VSMC lysates and is localized within PML NBs, suggesting that this isoform can enter the nucleus without the NLS and can efficiently incorporate into nuclear bodies.

The binding site for ERK1/2 and PML in nesprin-2 occurs within a highly spliced region of the gene. Previous studies have highlighted the multi-isomeric nature of nesprin proteins (11, 14, 29), and in this study, using EST data base searching and PCR verification, we identified multiple novel nesprin-2 variants containing the ERK/PML binding domain. The predicted sizes of these variants were between 79 and 377 kDa, which corresponded to the size range of the nesprin-2 variants detected by WB, using the N3 antibody that recognizes an epitope very near the ERK/PML binding domain. Some of these isoforms, including those lacking the KASH domain, were predicted to be in the order of 75 kDa, the size of the nesprin-2 variant that interacts with ERK1/2 and PML. Indeed, the previously described nesprin-2βΔTM fused to a FLAG tag localized at PML NBs, suggesting that this variant scaffolds ERK1/2 at PML NBs. Although our siRNAs also depleted variants of 160 and 100 kDa, this depletion was only partial, and these did not interact with ERK1/2, further supporting nesprin-2βΔTM as the variant of interest.

Nesprin-2 Tethers ERK1/2 at PML Nuclear Bodies

Importantly, cells also need to delay the cell cycle upon differentiation and we observed that this complex appeared to be more stable in slow growing, more differentiated VSMCs that displayed prominent contractile actin filaments (data not shown). In the vessel wall VSMCs exist in a contractile, differentiated state but are not terminally differentiated and retain the ability to dedifferentiate into a non-contractile, proliferative cell in response to various stimuli, to participate in tissue repair. This unusual feature of VSMCs may explain why they display prominent nesprin-2 and ERK1/2 colocalization at PML NBs. ERK1/2 activity has been shown to determine VSMC phenotype in both health and disease with inhibition of these kinases promoting differentiation and inhibiting proliferation (30–33). Thus, VSMCs potentially utilize this complex to sequester and store active ERK1/2 in the nucleus during differentiation allowing contractile cells to be primed to proliferate upon exposure to the appropriate cues. However, the mechanisms that regulate VMSC dedifferentiation and potential roles for the nesprin-2/ERK/PML complex in this process require further investigation. In addition, the modifications and other factors that influence the assembly/disassembly of this complex remain to be elucidated.

Potential Roles for Nesprin-2 in Linking PML NBs to the Nuclear Matrix Independent of NE Function—Little is known about how nuclear substructures like PML NBs are spatially and temporally organized. Importantly, the ERK1/2 and PML binding regions are just upstream of a lamin A/C-interacting region identified in nesprin-2α, suggesting that these binding elements are present in close proximity. Evidence suggests that the 75-kDa variant identified in this study contains both regions. Indeed, only nesprin-2 fragments containing the lamin A/C binding region efficiently accumulated within the nucleus suggesting that nuclear lamina associations may be essential for the nuclear retention of nesprin-2. In addition, we previously showed that the nesprin-2 N3 antibody, used in this study, can efficiently immunoprecipitate lamins A/C both in vitro and in vivo (11, 14, 16). The lamins immunoprecipitated in vivo are likely to be the intranuclear lamins as the NE-associated lamins A/C cannot be solubilized effectively further suggesting that nesprin-2 forms intra-lamina connections.

Previous studies have demonstrated essential roles for the nuclear lamina in regulating/organizing signaling proteins. The phosphorylation and nuclear localization of retinoblastoma protein (Rb), in nuclear speckles is dependent on the integrity of the nuclear lamina in regulating/organizing signaling proteins. The phosphorylation and nuclear localization of retinoblastoma protein (Rb), in nuclear speckles is dependent on the integrity of the nuclear lamina and its disruption in cells lacking lamins A/C induces proliferation defects (6, 22, 34–36). Similarly, nesprin-2 may mediate connections between lamins A/C and PML NBs with this spatial organization important for the integrity of ERK signaling. Thus, nuclear lamina disruption may impact upon the nesprin-2/ERK/PML complex also implicat-
ing these proteins in the highly complex pathologies associated with laminopathy syndromes that arise because of mutations in lamins A/C and its NE-binding partners, including nesprin-2 (25).

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