Published in final edited form as:
J Cell Sci. 2006 April 15; 119(Pt 8): 1655–1665. doi:10.1242/jcs.02869.

Targeted Wild-Type and Jerker Espins Reveal a Novel, WH2 Domain-Dependent Way to Make Actin Bundles in Cells

Patricia A. Loomis[1], **Alexander E. Kelly**[2], **Lili Zheng**[1], **Benjarat Changyaleket**[1], **Gabriella Sekerková**[1], **Enrico Mugnaini**[1], **Adriana Ferreira**[1], **R. Dyche Mullins**[2], and **James R. Bartles**[1]

[1]Department of Cell and Molecular Biology, Feinberg School of Medicine, and Institute for Neuroscience, Northwestern University, Chicago, IL 60611, USA

[2]Graduate Group in Biophysics and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94107, USA

Summary

The espin actin-bundling proteins, which are the target of deafness mutations, are present in the parallel actin bundles of stereocilia and microvilli and appear to increase their steady-state length. Here, we report a new activity of the espins, one that depends on their enigmatic WH2 domain: the ability to assemble a large actin bundle when targeted to a specific subcellular location. This activity was observed for wild-type espins targeted to the centrosome in transfected neuronal cells and for jerker espins targeted to the nucleolus in a wide variety of transfected cells as a result of the frameshifted peptide introduced into the espin C terminus by the jerker deafness mutation. This activity, which appears specific to espins, requires two espin F-actin-binding sites and the actin monomer-binding activity of the espin WH2 domain, but can be mimicked by adding a WH2 domain to an unrelated actin-bundling protein, villin. Espins do not activate the Arp2/3 complex in vitro, and bundle assembly is not indicative of in-vitro nucleation activity. Our results suggest a novel way to build actin bundles at specific sites in cells.

Keywords

microtubule; WASP; neuron; nucleus; hearing; RS domain

Introduction

The organization and dynamics of the actin cytoskeleton are controlled by a variety of actin-binding proteins. Because cellular shape, function and behavior depend on the proper positioning of specific types of actin cytoskeletal elements, considerable attention has been focused on the mechanisms by which actin-binding proteins regulate actin polymerization and filament organization locally. One of the best-understood mechanisms involves actin filament branching via the Arp2/3 complex, which is promoted by local activation WASP family proteins: WASP, N-WASP and the Scar/WAVE proteins (Welch and Mullins, 2002; Millard et al., 2004). WASP family proteins contain homologous C-terminal peptides that include an actin monomer-binding WH2 (WASP homology 2) domain followed by the C (central) and A (acidic) peptides, which bind the Arp2/3 complex. Upon activation, the actin monomer bound to the WH2 domain is believed to contribute to a stable actin filament “nucleus” that is

Correspondence: Dr. James R. Bartles, Dept. of Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, 303 East Chicago Avenue, Chicago, IL 60611, USA, Phone: 312-503-1545; FAX: 312-503-7912, j-bartles@northwestern.edu.

elongated. Accordingly, the WH2 domain of WASP family proteins is necessary for their nucleation-promoting activity (Machesky et al., 1999). The WH2 domain appears to be a versatile motif for binding actin monomer and has been recognized in a variety of other proteins, including verprolin/WASP-interacting protein, Srv2/cyclase-associated protein, the β-thymosins, ciboulot, the Missing in Metastasis proteins and Spir (Paunola et al., 2002; Mattila et al., 2003; Woodings et al., 2003; Quinlan et al., 2005).

Recently, we discovered a WH2 domain in the espins, a class of actin-bundling protein found in microvillus-type parallel actin bundles (PABs) and Purkinje cell dendritic spines (Loomis et al., 2003; Sekerková et al., 2003; 2004). Espins are abundant in the microvillus-like projections of chemosensory and mechanosensory cells, including the stereocilia of hair cells in the inner ear (Sekerková et al., 2004), and are the target of mutations that cause deafness and vestibular dysfunction in mice and humans (Zheng et al., 2000; Naz et al., 2004; Donaudy et al., 2005). Encoded by a single gene, all espin isoforms contain the WH2 domain and a 116-aa C-terminal peptide, the actin-bundling module, which is necessary and sufficient for their potent actin-bundling activity in vitro. However, espin isoforms contain different N-terminal peptides that can include multiple ankyrin-like repeats, an additional F-actin-binding site and proline-rich peptides, which can bind profilins or the IRSp53 SH3 domain (Chen et al., 1999; Sekerková et al., 2003, 2004).

When expressed in transfected cells, espins exert profound effects on the organization of the actin cytoskeleton, ranging from the formation of coarse stress fiber-like actin bundles in fibroblastic cells (Bartles et al., 1998; Chen et al., 1999) to the dramatic barbed-end elongation of microvillar PABs in epithelial cells (Loomis et al., 2003). Here, we report that the targeting of espin constructs to centrosomes or nucleoli in transfected cells causes the de novo assembly of large actin bundles at these locations and that this activity requires two espin F-actin-binding sites and the actin monomer-binding activity of the espin WH2 domain. Moreover, we show that addition of a WH2 domain confers this activity on a different protein with two F-actin-binding sites, thereby revealing a novel way to make a large actin bundle at a specific subcellular location.

Materials and Methods

Media and sera were from Invitrogen (Carlsbad, CA). PC12 cells were cultured in DME with 7% horse serum and 5% FBS, transfected by electroporation (Yoon et al., 2001) and plated onto coverslips coated with laminin (Roche, Indianapolis, IN). LLC-PK1 cells were cultured in MEM Alpha with 5% FBS, plated onto uncoated coverslips and transfected with Lipofectamine (Loomis et al., 2003). Cell lines were transfected with the designated cDNA constructs in a pEGFP-C (BD Biosciences Clontech, Palo Alto, CA) or pcDNA3 (Invitrogen) vector and examined 18-24 h later, unless specified otherwise. In some experiments, cells were transfected with pEGFP-C vector constructs that contained deletion mutations in the cytomegalovirus promoter to give a reduced levels of expression (Loomis et al., 2003). In other experiments, cells were incubated with 1 μM nocadazole (Sigma, St. Louis, MO) or cotransfected with a myc-tagged dynamitin construct (Burkhardt et al., 1997; gift of Richard Vallee, Columbia University, New York, NY). Neuronal cultures were prepared from the hippocampi of embryonic day 18 (E18) rat embryos, transfected using a Nucleofector apparatus (Amaxa, Gaithersburg, MD) and plated on poly-L-lysine-coated coverslips in MEM with 10% horse serum and transferred to dishes containing an astroglial monolayer (Paganoni and Ferreira, 2005). The villin, fimbrin and fascin constructs and a majority of the espin constructs have been described (Loomis et al., 2003; Sekerková et al., 2004). Espin cDNA fragments containing the jerker mutation and N-WASP cDNAs were obtained by RT-PCR using RNA from jerker mouse kidney and rat cerebellum, respectively. Additional mutagenesis was carried out by PCR. Some constructs had a WH2 domain cDNA inserted between the BglII and EcoRI

sites of the pEGFP-C2 vector, upstream of an espin or villin cDNA. All constructs were checked by DNA sequence analysis.

For light microscopy (Loomis et al., 2003), cells were fixed in 2-3.5% paraformaldehyde; briefly permeabilized with 0.1% Triton X-100 or NP-40; labeled with Texas Red-phalloidin (Molecular Probes, Eugene, OR), DAPI (Sigma) or antibodies to human fibrillarin (Sigma), mouse nucleolin (gift of Lester Binder, Northwestern University, Chicago, IL), myc tag or lamin (gifts of Robert Goldman, Northwestern University, Chicago, IL) followed by Texas Red-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA); mounted in 5% n-propylgallate in 90% glycerol/10% PBS; and examined at room temperature using a Zeiss Axioplan 2 Imaging microscope system (63 ×, 1.4 N.A. oil immersion objective) equipped with an Axiocam digital camera or a Zeiss LSM 510 META confocal microscope (0.3-0.5 μm z-sections; 100 ×, 1.4 N.A. oil immersion objective). When labeling with γ-tubulin antibody (Sigma, T 3559), cells were fixed in methanol. Transient transfection inevitably results in a range of construct expression levels. Therefore, the ability of different constructs to form a large actin bundle at the centrosome or in the nucleus was scored after examination of at least 100 transfected cells on 2-4 coverslips with attention to matching expression levels visually on the basis of GFP fluorescence intensity. Actin bundles were labeled with rabbit skeletal muscle S1 (gift of Robert Goldman) as described (Svitkina and Borisy, 1998) and examined using a JEOL JEM-1200 EX electron microscope. Images were saved in TIF format, transferred to Photoshop (Adobe Systems) and assembled into composites with minor adjustments in brightness or contrast.

ATP-actin monomer binding (Uruno et al., 2001) was assayed by pull-down using GST-espin constructs (Sekerková et al., 2004). Pyrene-actin polymerization assays were performed as described (Quinlan et al., 2005) using 4 μM monomeric actin (6% pyrene-labeled) in 50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM Hepes, pH 7.0. The proteins assayed included espin constructs with an NH_2-terminal His tag (Sekerková et al., 2004), the human Scar1 VCA fragment (aa residues 489-559) with a C-terminal His tag and 10 nM Arp2/3 complex (Zalevsky et al., 2001).

Results

Formation of an actin bundle at the centrosome in neuronal cells

When expressed in rat PC-12 pheochromocytoma cells under the control of the cytomegalovirus promoter, the GFP-espins elicited the formation of a large cytoplasmic F-actin bundle in a perinuclear location (Fig. 1A-C). This F-actin bundle was especially noticeable, because in the absence of espin these cells contained relatively low levels of F-actin, as revealed by staining with fluorescent phalloidin (compare untransfected control cell in upper right corner of Fig. 1C). The GFP-espin was distributed uniformly throughout the large actin bundle. Lower amounts of GFP-espin and levels of F-actin higher than those in control cells were also detected at the periphery of the transfected cells, in structures resembling filopodia (Fig. 1A-C). Identical results were obtained when espins were expressed without a GFP tag and localized by espin antibody. The formation of a perinuclear actin bundle in response to the expression of GFP-espin was not observed in fibroblastic (BHK, NRK, 3T3) or epithelial (LLC-PK1) lines, even though the GFP-espin was expressed at comparable levels. The perinuclear actin bundle was, however, observed in a second neuronal cell line, the mouse Neuro-2a neuroblastoma line, and in a subset (~10%) of transfected cells in primary rat hippocampal neuron cultures (Fig. 1D,E). Although observed occasionally in fully polarized hippocampal neurons, the large bundle was more commonly found in neuronal cells that were not fully polarized (Fig. 1D,E).

To infer how the espin-induced perinuclear actin bundle formed, we examined PC-12 cells at relatively early times (4-8 h) after transfection. Because GFP-espin and F-actin were colocalized throughout bundle formation, only GFP-espin localizations are shown in Fig. 1F-H to chronicle key stages. Relatively low levels of GFP-espin and F-actin first accumulated in a starburst-like collection of smaller bundles disposed in radial fashion about the centrosome (Fig. 1F), which was revealed by labeling the microtubule-organizing center with γ-tubulin antibody (red dots in Fig. 1F-H). These smaller bundles then collapsed and became consolidated into a larger bundle (Fig. 1G,H). One end of the bundle remained in close proximity to the centrosome (Fig. 1H).

When examined by transmission EM, the centrosomal actin bundle (CAB) consisted of tightly packed microfilaments (Fig. 2A). CABs resembled espin cross-linked actin bundles formed in vitro (Bartles et al., 1998;Chen et al., 1999) in that neither definitive cross-bridges nor extensive regions showing the cross-striations indicative of paracrystalline order could be recognized (Fig. 2A). Myosin S1 decoration revealed that the actin filaments in CABs were oriented in a parallel fashion throughout a given section (Fig. 2B-D). This parallel alignment was maintained for up to ~10 μm in single sections; little or no branching was observed, although the S1-decorated CABs often appeared to be composed of interwoven sub-bundles that passed in and out of the section plane (Fig. 2B-D). To infer CAB directionality, we examined sections that included >4-μm longitudinal stretches of S1-decorated CAB running unambiguously between the perinuclear region and plasma membrane. In nine of nine cells analyzed, the barbed end of the S1 faced the plasma membrane, suggesting a barbed-end-out polarity for the espin-induced CABs and their constituent filaments (Fig. 2B-D).

The spatial relationship to the centrosome (Fig. 1F-H), and especially the star-like actin bundle array observed at early times (Fig. 1F), suggested that the location of the CAB was determined by minus end-directed transport of the espins along microtubules. In fact, in our EM analysis, we frequently encountered examples of microtubules running alongside CABs (Fig. 2A,C, arrowheads). Consistent with this idea, overexpression of the dynactin p50 subunit dynamitin, which interferes with dynein-dynactin motor complex-mediated transport along microtubules (Burkhardt et al., 1997), blocked CAB formation and resulted instead in an accumulation of GFP-espin and F-actin in filopodia-like projections at the cell periphery and dorsal surface (Fig. 1I). A similar result was obtained when cells were treated with the microtubule-depolymerizing drug nocodazole (1 μM) after transfection, suggesting a requirement for an intact microtubule network. The inhibitory effect of nocodazole was reversible; a CAB formed 1-2 h after washing out the drug. Together, these results suggested that the formation of the CAB involved espin targeting along microtubules via the dynein-dynactin complex.

To examine the effect of espin concentration on CAB formation, we transfected PC12 and Neuro-2a cells with a series of GFP-espin constructs that contained deletion mutations in the cytomegalovirus promoter to give GFP-espin expression levels that were estimated previously on western blots to be 2, 10 and 40% of those attained with the full-strength (wild-type) promoter (Loomis et al., 2003). A CAB still formed when the espin was expressed at the 40% and 10% levels, but at the 10% level its frequency and size were reduced. This was especially the case for the PC12 cells, which expressed each construct at lower levels than the Neuro-2a cells. CABs were not observed in either cell line when the GFP-espin was expressed at the 2% level. Under these conditions, the GFP fluorescence was no longer visible by fluorescence microscopy, but transfected cells could still be recognized by immunofluorescence using antibody to GFP. This espin concentration dependence for CAB formation was also evident when comparing cells expressing different levels of GFP-espin on individual coverslips.

The formation of a CAB was observed in response to all espin isoforms tested: rat espins 1, 2A, 2B, 2D, 3A, 3B, and 4 and human espin 3A. Different transcriptional start sites distinguish

four major espin isoform size classes, designated 1-4 (Fig. 3A), and splice variants are further specified alphabetically (Sekerková et al., 2004). [Espin 2D is the revised name for Purkinje cell espin 1+ (Sekerková et al., 2003).] Even though different isoforms made CABs, the larger isoforms, such as 1 and 2B, tended to make CABs that appeared somewhat larger and more consolidated than those made by the smaller isoforms 3A, 3B and 4. This difference could reflect the presence of an additional F-actin-binding site in the larger isoforms (Chen et al., 1999) or the ability of the smaller isoforms to better inhibit actin polymerization in vitro (Sekerková et al., 2004; and see below under “Further characterization of the WH2 domain requirement”). The formation of a CAB was not observed when other actin-bundling proteins (mouse or chicken villin, human T-fimbrin, human fascin or “constitutively active” S39A-human fascin) were expressed at comparable levels to GFP-espins in these cells using the same vector.

To identify the espin domains required for CAB formation, we transfected PC-12 cells with espin 2B constructs missing known structural or functional domains and scored for the presence or absence of a CAB in cells expressing levels of construct similar to those of wild-type espin 2B on the basis of GFP fluorescence intensity (Fig. 3B). Espin 2 isoforms have been detected in two types of neuron: cerebellar Purkinje cells and vomeronasal sensory neurons (Sekerková et al., 2003;2004). Espin 2B, which corresponds to espin 1 missing its N-terminal ankyrin-like repeats (Fig. 3A), affords an optimal representation of other espin domains and has been characterized extensively in vitro and in vivo (Chen et al., 1999;Loomis et al., 2003;Sekerková et al., 2003;2004). A number of domains could be eliminated without inhibiting CAB formation, including the profilin-binding proline-rich peptides, singly or doubly, or the 23-aa additional F-actin-binding site adjacent to the N-terminal proline-rich peptide (Fig. 3B). Elimination of the espin COOH-terminal actin-bundling module, which is believed to contain two F-actin-binding sites disposed roughly at each end (Bartles et al., 1998), blocked formation of the CAB (Fig. 3B) and resulted instead in the GFP-espin fluorescence being distributed diffusely throughout the cytoplasm.

Through the analysis of additional deletion constructs, we identified a requirement for the espin WH2 domain (Fig. 3B). This domain is highly conserved among espins. In fact, the first 30 aa's of the peptide encoded by the WH2 domain exon (Figure 3D), including the 17-aa core of the WH2 domain, are identical in espins from Fugu to human, making this domain even more conserved than the actin-bundling module. WH2 domains, which occur in a variety of modular proteins involved in actin cytoskeletal regulation, are thought to be versatile adapters for binding ATP-actin monomer (e.g., Paunola et al., 2002). Accordingly, deletion the 17-aa core of the espin WH2 domain caused a dramatic reduction in the ability of espin 2B to bind ATP-actin in vitro in pull-down assays carried out under conditions intended to maintain the actin in monomeric form (Fig. 3E). A similar result was obtained previously for espin 3A and espin 3B (Sekerková et al., 2004). Moreover, deletion of the 17-aa core also reduced the rapid recovery of photobleached GFP-β-actin observed throughout the length of the long microvilli of espin-expressing epithelial cells, suggesting that the espin WH2 domain can also bind actin monomer in vivo (Loomis et al, 2003). Beyond its aa sequence and ability to binding actin monomer in vitro and in vivo, this peptide in the espins shares additional attributes with well-characterized WH2 domains (see below, under “Further characterization of the WH2 domain requirement”). Deletion of the 17-aa core of the espin WH2 domain eliminated CAB formation and resulted instead in the accumulation of GFP-espin and F-actin in filopodia-like structures at the cell periphery (Figs, 1J,3B). The segment required for CAB formation was narrowly centered on the 17-aa core of the WH2 domain, because deletion of a 41-aa peptide positioned only 7-aa downstream of the WH2 core did not inhibit formation of the CAB (Fig. 3B). In summary, these mutagenesis studies indicated that CAB formation required the espin actin-bundling module and the espin WH2 domain.

Formation of an actin bundle at the nucleolus by jerker espins

The analysis of jerker espins afforded a second illustration of how the targeting of espins can result in actin bundle formation at a specific subcellular site. The espin gene of the jerker mouse has a frameshift mutation (2426delG) that causes recessive deafness and vestibular dysfunction, accompanied by abnormally short and degenerated hair cell stereocilia (Zheng et al., 2000). Espins bearing the jerker mutation have the C-terminal 63 aa's of their actin-bundling module replaced with a novel 39-aa peptide – the jerker peptide – which is rich in positively charged amino acids (Fig. 3A,F). Jerker espin proteins are unstable and/or synthesized inefficiently in situ, because homozygous jerker mice are deficient in espin proteins, yet maintain normal levels of the mutated espin mRNAs (Zheng et al., 2000). Even though regulatory mechanisms must limit the accumulation of jerker espin proteins in situ in the tissues of jerker mice, we were able to express jerker espin proteins in transiently transfected mammalian cell lines. Our results were similar whether examining fibroblastic (BHK, NRK), neuronal (PC-12, Neuro-2a) or epithelial (LLC-PK1) cell lines and whether using GFP-tagged or untagged jerker espins. Therefore, only results obtained examining GFP-jerker espins in subconfluent LLC-PK1 cells are shown.

When expressed in transfected mammalian cells, espin 2B bearing the jerker mutation (jerker espin 2B) became efficiently concentrated in the nucleus and resulted in the formation of a large nuclear actin bundle (NAB; Fig. 4A-C). Although, at first glance, the outcome resembled CAB formation by wild-type espins (Fig. 1A-C), confocal microscopy confirmed that the bundle formed by the GFP-jerker espin was internal to the nuclear lamina, as revealed by lamin antibody (red in Fig. 4D). The NAB exhibited a variety of shapes, ranging from a tightly packed donut-like bundle (Fig. 4D) to a curved or wavy linear bundle, often with frayed ends (Fig. 4A-C). The GFP-espin and F-actin were colocalized throughout the NAB (Fig. 4B,C). We attempted to examine NABs using the EM methods applied to CABs (Fig. 2), but found the actin filaments difficult to resolve from other electron-dense nuclear material, even with deoxyribonuclease treatment and additional detergent extraction.

Time-course experiments revealed an unexpected pathway of assembly for the NAB – one involving the nucleolus (Fig. 4E-T). At early times after transfection, the GFP-jerker espin 2B accumulated in relatively large phase-dense subnuclear compartments (Fig. 4E-H), typically 1-3 in number. In other experiments (see below), these structures were identified as nucleoli by labeling with antibodies to nucleolar proteins, such as nucleolin or fibrillarin. Initially, the GFP-jerker espin-containing nucleoli showed no accumulation of F-actin that could be revealed by fluorescent phalloidin (Fig. 4G). Next, small actin bundles, detected as narrow strands containing GFP-jerker espin and F-actin, were observed as a thin rim at the nucleolar periphery and as small arms that radiated outward from the rim into the surrounding nucleoplasm (Fig. 4I-L). These small bundle strands grew longer and thicker, curved to form spirals (Fig. 4M-P) and eventually became consolidated into a mature NAB (Fig. 4Q-T). This assembly pathway could account for the varied shape of the NAB: donut-shaped bundles would result when there was extensive overlap of curved sub-bundles to form a closed circle, whereas linear bundles would result when there was insufficient overlap or separation of sub-bundles.

The earliest steps in the NAB assembly pathway (Fig. 4E-L) implicated GFP-jerker espin protein that was targeted to the nucleolus. This nucleolar targeting proved to be attributable to the novel 39-aa peptide (Fig. 3F) located at the distal C terminus of the proteins (Fig. 3A). The jerker peptide was sufficient to cause efficient targeting of a GFP-jerker peptide fusion construct to the nucleolus (Fig. 5A-D), suggesting that it contained a nucleolar localization sequence. However, unlike GFP-jerker espin 2B (Fig. 5E,F), the GFP-jerker peptide did not cause NABs or noticeable accumulation of F-actin in the nucleus even when expressed at comparable levels (Fig. 5G,H). Conversely, GFP-jerker espins missing the jerker peptide did not accumulate in the nucleus or assemble NABs. Thus, the 39-aa jerker peptide, which was

necessary and sufficient for the nucleolar targeting, was necessary but insufficient to form a NAB.

NAB formation showed a jerker espin 2B concentration dependence similar to that noted above for wild-type espin 2B in the formation of CABs. NABs formed when the GFP-jerker espin 2B was expressed at the 40% and 10% levels using the vectors with the mutated cytomegalovirus promoters, but at the 10% level NAB frequency and size were reduced. NABs were not observed when the GFP-jerker espin 2B was expressed at the 2% level. However, expression at the 2% level still resulted in targeting to the nucleolus, as revealed using antibody to GFP.

To identify the espin domains required for NAB formation, we transfected LLC-PK1 cells with GFP-jerker espin 2B constructs missing different domains and scored for the presence or absence of a NAB in cells expressing levels of construct comparable to those giving a strong NAB-forming response by full-length GFP-jerker espin 2B. The domain requirements for NAB formation showed many similarities to those for CAB formation, but there were some differences (Fig. 3B). For example, the proline-rich peptides could be eliminated, singly or doubly, without reducing NAB formation (Figure 3B). Moreover, NAB formation required what remained of the actin-bundling module upstream of the jerker peptide (Fig. 3B). This remaining part of the actin-bundling module is believed to contain one of two F-actin-binding sites that contribute to the actin-bundling module (Bartles et al., 1998). The espin WH2 domain also proved to be required for NAB formation (Fig. 3B). Deletion of the 17-aa core of the espin WH2 domain resulted in the accumulation of the jerker espin in nucleoli and a variable number of smaller nuclear foci, but no detectable nuclear or nucleolar F-actin (Fig. 5I,J). In contrast, deletion of the 41-aa peptide positioned only 7-aa downstream of the WH2 core did not inhibit bundle formation (Fig. 3B). Unlike the situation for CAB formation, the 23-aa additional F-actin-binding site (Fig. 3A;Chen et al., 1999) was required for NAB formation (Fig. 3B). GFP-espin 2B missing the 23-aa additional F-actin-binding site was efficiently targeted to nucleoli and smaller nuclear foci, but it did not form NABs or cause significant accumulation of F-actin (Fig. 3B; like Fig. 5I,J). This suggested that NAB formation by the jerker espin 2B required two F-actin-binding sites and that the 23-aa additional F-actin-binding site could substitute for the loss of the distal F-actin-binding site from the actin-bundling module as a result of the jerker mutation (Fig. 3A). Accordingly, jerker espin 3 and jerker espin 4, which do not contain this 23-aa additional F-actin-binding site (Fig. 3A), did not form NABs or cause an accumulation of nuclear F-actin (Fig. 3B). Although these proteins were efficiently targeted to nucleoli, they gave results similar to those obtained with the GFP-jerker peptide alone (Fig. 5G,H). Unexpectedly, jerker espin 1 – which, like the espin 2 isoforms, contains the 23-aa additional F-actin-binding site (Fig. 3A) – also failed to form NABs or cause accumulation of nuclear F-actin (Fig. 3B). This suggested that the eight ankyrin-like repeats at the N terminus of espin 1 (Fig. 3A) suppressed the activity or availability of the additional F-actin-binding site. In summary, these results suggested that, in addition to the jerker peptide, which was required for nucleolar targeting, NAB formation required two espin F-actin-binding sites and the espin WH2 domain.

Further Characterization of the WH2 Domain Requirement

We reasoned that the potent actin-bundling (Bartles et al., 1998) and barbed-end PAB elongating activities of the espins (Loomis et al., 2003), both of which stem from the actin-bundling module, could work in concert to help assemble a large actin bundle. However, since neither activity requires the espin WH2 domain, we examined further the basis for this requirement in the formation of CABs and NABs.

Although espins do not contain an obvious WASP-like C peptide, they do resemble WASP family proteins in linear domain organization: basic region (required for binding

phosphatidylinositol 4,5-bisphosphate), proline-rich peptide, WH2 domain, and C-terminal peptide containing clusters of acidic amino acids (Sekerková et al., 2004). Therefore, we tested whether espins could act as nucleation-promoting factors for Arp2/3 complex-mediated actin polymerization. Espin 3A failed to activate the Arp2/3 complex in vitro, even at relatively high concentrations (400 nM in Fig. 3G). For comparison, at this same concentration, the Scar1 VCA peptide (V, alternative abbreviation for the WH2 domain) caused pronounced activation (Fig. 3G), even though it is a relatively weak nucleation-promoting factor that is 16 and 68-fold less active than the VCA peptides of WASP and N-WASP, respectively (Zalevsky et al., 2001). To address the possibility of autoinhibition (Kim et al., 2000), we also tested an espin C-terminal construct that began just 12 aa upstream of the 17-aa core of the WH2 domain and, hence, was missing an N-terminal peptide. This espin "WA" fragment also failed to activate the Arp2/3 complex (Fig. 3G). A slight, but reproducible shortening of the polymerization lag time was noted with espin 2B at concentrations greater than 250 nM (400 nM shown in Fig. 3G), but this effect was also observed in the absence of the Arp2/3 complex (Fig. 3H). In fact, the polymerization curves obtained with the different espins constructs were similar in the presence and absence of Arp2/3 complex. Thus, espins did not appear to activate the Arp2/3 complex directly. Accordingly, the acidic aa clusters in the espin C-terminal peptide lack the conserved tryptophan that is found in most Arp2/3 activators and is required for binding the Arp2/3 complex (Marchand et al., 2001). Moreover, when examined by EM (Fig. 2), the CABs showed no evidence of the filament branching commonly associated with Arp2/3 complex involvement (Millard et al., 2004).

Espin 3A inhibited actin polymerization in vitro by extending the lag time and decelerating polymerization relative to actin alone (Fig. 3G,H). As expected at this 1:10 molar ratio of espin to actin, deletion of the 17-aa core of the WH2 domain from espin 3A had little effect (Fig. 3H), suggesting that the deceleration stemmed largely from a reduced availability of filament ends because of filament bundling (Murray et al., 1996). Espins are unusually potent actin-bundling proteins (Bartles, et al., 1998; Chen et al., 1999). Some deceleration was also evident with espin 2B, but it appeared to be counteracted by that slight shortening of lag time (Fig. 3G,H). This combination of lag shortening and deceleration we observed with espin 2B was reminiscent of the effects noted previously for elongation factor 1α, which also displays actin-bundling activity (Murray et al., 1996). The weak lag-shortening effect required all three of espin 2B's actin-binding regions. Deletion of the 23-aa additional F-actin-binding site from espin 2B brought about a large increment in lag time, causing the early part of the polymerization curve to approach that for espin 3A (Fig. 3H), which does not contain the 23-aa additional F-actin-binding site (Fig. 3A). By comparison, deletion of the 17-aa core of the WH2 domain caused the early part of the curve to be more similar to that for actin alone, with deceleration being observed at later times (Fig. 3H). Deletion of the actin-bundling module caused the curve to be highly similar to that of actin alone throughout the entire time course (data not shown), reinforcing the conclusion that the deceleration resulted from filament bundling. Note that this latter construct, which contains both the 23-aa additional F-actin-binding site and the WH2 domain and is also free from the decelerating effect of the actin-bundling module, failed to shorten the lag time. In summary, although weak lag-shortening activity was observed for one wild-type espin isoform, espin 2B, the activity was observed only at relatively high protein concentrations in vitro and did not correlate with the isoform/domain requirements of CAB formation. Notably, the lag-shortening effect was not observed for espin 3A, which nonetheless made the CAB, and the 23-aa additional F-actin-binding site, which was required for the lag-shortening effect, was not required for CAB formation (Fig. 3A,B,H). Thus, CAB formation was not indicative of in-vitro nucleation activity on the part of the espins. We were unable to examine the effects of jerker espins on actin polymerization in vitro because of an inability to express and purify the mutated recombinant proteins.

We returned to cell transfection experiments to further examine the basis for the WH2 domain requirement. To investigate the importance of WH2 domain context, we reintroduced the WH2 domain into the espin 2B WH2 domain-deletion constructs, but in a different location. The 144-nt espin WH2 domain-encoding exon was positioned in-frame upstream of the cDNA coding sequence of the espin 2B WH2 domain-deletion constructs to produce wild-type and jerker espin 2B constructs that contained the espin WH2 domain ~400 aa N-terminal to its normal location (Fig. 3C). Remarkably, these constructs were just as active as intact wild-type espin 2B and jerker espin 2B at forming a CAB (Figs. 1K,3C) and NAB (Figs. 5K,L, 3C), respectively. Control constructs with the peptide encoded by the WH2 domain exon minus the 17-aa WH2 domain core reintroduced at the same upstream location remained inactive (Fig. 3C). Consistent with the requirement for espin's F-actin-binding sites, targeting of the 48-aa peptide encoded by the espin WH2-domain exon to the nucleolus as a GFP-WH2 domain-jerker peptide construct, without the other parts of espin, also failed to elicit NABs or to cause nucleolar/nuclear accumulation of F-actin (Fig. 3C). Similarly, the corresponding GFP-WH2 domain construct, without the jerker peptide attached, failed to target the centrosome (data not shown) or cause noticeable F-actin accumulation in transfected neuronal cells (Fig. 3C). These results suggested that, in the absence of the other parts of espin, the WH2 domain is not sufficient to cause F-actin accumulation or localization to the centrosome. Accordingly, when the peptide encoded by the espin WH2 domain exon was placed upstream of another actin-bundling protein, chicken villin, the resulting GFP-WH2 domain-villin construct also did not localize to the centrosome or cause CAB formation.

The aa sequence immediately C-terminal to the WH2 core domain can be of great importance in determining the functions of WH2 domain-containing proteins (Herzog et al., 2004; Irobi et al., 2004; Quinlan et al., 2005). This sequence in espins (Fig. 3D), which bears no obvious similarity to those in other WH2 domain-containing proteins, seemed unimportant for forming CABs and NABS; an upstream WH2 domain truncated only 3 aa C-terminal to the 17-aa core of the espin WH2 domain (Fig. 3D) also efficiently restored bundle-forming activity to the espin 2B WH2 domain-deletion constructs (Fig. 3C). Quinlan et al. (2005) recently reported that mutation of the three leucine residues in the WH2 domain core to alanine eliminated actin monomer binding. Restoration of bundle-forming activity to the espin 2B WH2 domain-deletion constructs was not achieved when the upstream WH2 domain had its three key leucine residues mutated to alanine (Fig. 3C), underscoring the requirement for actin monomer binding by the espin WH2 domain to build CABs and NABs. Moreover, substitution of the upstream espin WH2 domain peptide with another actin monomer-binding WH2 domain (Yamaguchi et al. 2000), the second WH2 domain (aa residues 417-466) of rat N-WASP, also restored actin bundle-forming ability to the espin 2B WH2 domain-deletion constructs (Fig. 3C). This ability of the N-WASP WH2 domain to efficiently substitute for the espin WH2 domain suggested that these two WH2 domains have a comparable affinity for binding ATP-actin monomer. Taken together, these results suggested that, in its requirement to support large actin bundle formation at the centrosome or nucleolus, the espin WH2 domain was serving as an actin monomer-binding module that could function even when placed at a markedly different location in the espin molecule.

Finally, to determine whether the espin WH2 domain was capable of conferring actin bundle-forming ability on a different targeted protein with two F-actin-binding sites, we examined the effect of introducing the WH2 domain at the N terminus of chicken villin, an actin-bundling protein that contains no obvious WH2 domain or homology to the espins. In these experiments, the cDNA encoding the 39-aa C-terminal peptide of the jerker espins was placed in frame at the 3′ end of the villin coding sequence to target the constructs to the nucleolus (Fig. 5M). The GFP-villin-jerker peptide construct was targeted to the nucleolus and, to a lesser extent, the nucleoplasm (Fig. 5M), but there was no evidence of F-actin accumulation in the nucleolus or nucleus (Fig. 5N). However, when the peptide encoded by the espin WH2 domain exon was

placed upstream of the villin-jerker peptide construct, NABs formed (Fig. 5O,P). The NABs appeared finer and less consolidated than those formed by jerker espins (Fig. 5O,P). These differences could reflect the fact that villin is less potent than espin at causing the elongation of PABs (Loomis et al., 2003) or that these targeted GFP-villin constructs appeared to be expressed at somewhat lower levels than the GFP-jerker espin constructs. Like the NABs formed by jerker espins, the GFP-WH2 domain-villin-jerker peptide construct was uniformly distributed throughout bundles (Fig. 5O,P). No NABs or nuclear F-actin accumulation were observed in response to the corresponding control construct missing the 17-aa core of the espin WH2 domain, which was nonetheless efficiently targeted to nucleoli and expressed at comparable levels. Thus, when a WH2 domain is joined to a peptide that can bundle actin filaments – a situation that occurs naturally in the case of the espins – the resultant protein can build large actin bundles when targeted to a specific cellular location.

Discussion

When targeted to a specific location in transfected cells, espins promote the local assembly of a large actin bundle. This activity, which appears to distinguish espins from other actin-bundling proteins, depends on the ability of espins to interact with actin monomer via their WH2 domain and reveals a new way to build large actin bundles at specific sites in cells.

The requirement for the WH2 domain was unexpected, because deletion of the WH2 domain has no noticeable effect on espin's actin-bundling activity in vitro (Bartles et al., 1998) or on its activities tied to actin cross-linking, such as microvillar PAB elongation in epithelial cells (Loomis et al., 2003). A remarkable aspect of the actin bundle-building activity is the flexibility we observed regarding the placement and source of the WH2 domain. Positioning the WH2 domain in a vastly different location in espin, substituting it with a WH2 domain from N-WASP or adding the WH2 domain it to a different actin-bundling protein, villin, all yielded bundle-building activity. This flexibility strongly suggests that the role of the espin WH2 domain is to increase the local concentration of polymerizable actin monomer. In this capacity, the espin WH2 domain would deliver the ATP-actin monomer needed to fuel local nucleation or elongation reactions. The multiple F-actin-binding sites of espin (or villin) would then cross-link the product and stimulate further elongation via the same mechanism by which such cross-links cause the barbed-end elongation of preformed microvillar PABs (Loomis et al., 2003). Espins do not activate the Arp2/3 complex in vitro, and bundle assembly is not indicative of in-vitro nucleation activity. Nevertheless, our mutagenesis results are entirely consistent with an actin monomer-binding function for the espin WH2 domain and substantiate the existence of multiple parallels between the espin WH2 domain and the WH2 domains of other proteins (Yamaguchi et al., 2000; Quinlan et al., 2005).

Relatively little is known about actin monomer availability at the centrosome and in the nucleus. Although actin has been implicated in a variety of important nuclear processes (Bettinger et al., 2004), nuclear actin levels appear to be kept low by exportin 6-mediated transport of profilin-actin complex (Stüven et al., 2003). The efficiency of this pathway may explain why the proline-rich peptides of espins, which can bind profilins (Sekerková et al., 2004) and presumably also profilin-actin complexes, cannot substitute for the WH2 domain as a source of actin monomer for building NABs. Interfering with the exportin 6 pathway (Stüven et al., 2003) or treatments such as DMSO or heat shock (Fukui and Katsu-Maru, 1979; Iida et al., 1986), can lead to the formation of nuclear actin paracrystals. Peculiar nuclear F-actin coils were observed previously in cells transfected with an N-terminal fragment of supervillin that does not include its C-terminal villin/gelsolin homology domain (Wulfkuhle et al., 1999).

Our results reveal a novel way to build large actin bundles in cells: by supplying a targeted actin-bundling protein with a WH2 domain. The combination of WH2 domain and actin-

bundling protein appears unique to espins, but may extend to the Missing in Metastasis(MIM)-B and ABBA-1 proteins, which contain a WH2 domain together with an IRSp53/MIM homology domain that can bundle actin filaments when dimerized (Woodings et al., 2003; Mattila et al., 2003; Millard et al., 2005). Interestingly, the only candidate espin orthologs currently recognized outside of the vertebrates, the forked proteins (Bartles et al., 1998), which mediate the formation of PABs in the developing neurosensory bristles of Drosophila pupae (Tilney et al. 1998), do not appear to contain a consensus WH2 domain.

The espin targeting scenarios investigated here – although exploited to reveal a novel activity of espins – may be relevant to espin biology in situ. Centrosomal targeting of expressed espins is not evident in transfected fibroblastic or epithelial cell lines (Bartles et al., 1998; Chen et al., 1999; Loomis et al., 2003), raising the possibility that espins have a propensity to become or bind cargo for the dynein-dynactin motor complex in neuronal cells. Espins are expressed endogenously in specific classes of neuron, cerebellar Purkinje cells and vomeronasal sensory neurons, and show strict compartmentalization to dendritic spines and dendritic microvilli, respectively, that could conceivably result from motor-driven transport along dendritic microtubules (Sekerková et al. 2003; 2004). Although it is also possible that the expressed espins somehow misfold or aggregate selectively in neuronal cells and are carried to aggresomes near the centrosome by the dynein-dynactin motor complex (Kawaguchi et al., 2003), the following argue against this explanation: centrosomal targeting was observed across the entire collection of structurally diverse espin isoforms, but not for other actin-bundling proteins expressed at comparably high levels, and was even apparent at early times after transfection, before the espin reached a high concentration; recombinant espins do not oligomerize or aggregate in vitro and remain monomeric, even at high concentration (Bartles et al., 1998; Chen et al., 1999); and the domain dependence noted for CAB formation suggests that many of the espin domains are functional and, therefore, folded properly. We have not yet noticed examples of CABs in the neurons of adult animals, However, CAB formation may require espin expression during a particular stage of neuronal development, and there may be ways to regulate both espin targeting and bundle-building activity in situ. However, it is possible that the type of espin-mediated, WH2 domain-dependent actin bundle assembly described here occurs on a smaller scale, locally in cells in situ, without resulting in the accumulation of such a massive actin bundle.

The targeting of jerker espins to nucleoli stems from the jerker peptide, which in our experiments behaved as a portable nucleolar localization sequence capable of specifying the nucleolar targeting of multiple heterologous proteins in a variety of cell types regardless of expression level. This 39-aa peptide, which is rich in arginine and serine residues, resembles peptides found in other nuclear proteins, including the protamines, the lamin B receptor, the Bcl2-associated transcription factor and the RS domains of SR protein splicing factors. As has been observed in other proteins targeted to nucleoli (e.g., Nagahama et al., 2004; Horke et al, 2004), the clusters of positively charged amino acids in the jerker peptide likely include signals for nuclear import and nucleolar retention. Even though the jerker espin mRNAs are maintained at wild-type levels, jerker espin proteins do not accumulate in the tissues of jerker mice (Zheng et al., 2000). In view of the jerker espin concentration dependence we noted for NAB formation, this deficiency of jerker espin protein likely explains why we do not observe NABs in situ in the cells of jerker mice. Nevertheless, the highly efficient nucleolar targeting of jerker espins, which we observed even when expressed at low levels, could be the basis for the instability of these mutated proteins in situ.

In conclusion, our results suggest that there can be more to making a PAB in cells than filament/bundle stabilization through cross-linking and that under certain circumstances issues such as the local provision of actin monomer and actin-bundling protein targeting can be critical. The robust, WH2 domain-dependent response to espins targeted to locations as diverse as the

centrosome and nucleolus suggest additional roles for these proteins in actin monomer delivery and bundle assembly as well as some possible applications in cellular engineering and nanotechnology.

Acknowledgments

We thank Richard Valle, Paul Matsudaira, Gary Borisy, Skip Bender and Robert Goldman for plasmids or proteins, and Sui Huang, Tanya Svitkina, Steve Adam, Guenter Albrecht-Buehler, Carolyn Jahn and Yoshio Fukui for advice. This work was supported by NIH grants DC004314 (J.R.B.), NS046834 (A.F.), and GM61010 (R.D.M.). J.R.B. is a Fellow of the Hugh Knowles Center for Clinical and Basic Science in Hearing and Its Disorders.

References

Bartles JR, Zheng L, Li A, Wierda A, Chen B. Small espin: a third actin-bundling protein and potential forked protein ortholog in brush border microvilli. J Cell Biol 1998;143:107–119. [PubMed: 9763424]

Bettinger BT, Gilbert DM, Amberg DC. Actin up in the nucleus. Nat Rev Mol Cell Biol 2004;5:410–415. [PubMed: 15122354]

Burkhardt JK, Echeverri CJ, Nilsson T, Vallee RB. Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. J Cell Biol 1997;139:469–484. [PubMed: 9334349]

Chen B, Li A, Wang D, Wang M, Zheng L, Bartles JR. Espin contains an additional actin-binding site in its N terminus and is a major actin-bundling protein of the Sertoli cell-spermatid ectoplasmic specialization junctional plaque. Mol Biol Cell 1999;10:4327–4339. [PubMed: 10588661]

Donaudy F, Zheng L, Ficarella R, Ballana E, Carella M, Melchonida S, Estivill X, Bartles JR, Gasparini P. Espin gene (*ESPN*) mutations associated with autosomal dominant hearing loss cause defects in microvillar elongation or organization. J Med Genet. 2005 in press. Published Online First: 1 June 2005. 10.1136/jmg.2005.032086

Fukui Y, Katsumaru H. Nuclear actin bundles in amoeba, Dictyostelium and human HeLa cells induced by dimethyl sulfoxide. Exp Cell Res 1979;120:451–455. [PubMed: 571346]

Herzog M, van Heijenoort C, Didry D, Gaudier M, Coutant J, Gigant B, Didelot G, Preat T, Knossow M, Guittet E, Carlier MF. The beta-thymosin/WH2 domain; structural basis for the switch from inhibition to promotion of actin assembly. Cell 2004;117:611–623. [PubMed: 15163409]

Horke S, Reumann K, Schweizer M, Will H, Heise T. Nuclear trafficking of La protein depends on a newly identified nucleolar localization signal and the ability to bind RNA. J Biol Chem 2004;279:26563–26570. [PubMed: 15060081]

Iida K, Iida H, Yahara I. Heat shock induction of intranuclear rods in cultured mammalian cells. Exp Cell Res 1986;165:207–215. [PubMed: 3519257]

Irobi E, Aguda AH, Larsson M, Guerin C, Yin HL, Burtnick LD, Blanchoin L, Robinson RC. Structural basis of actin sequestration by thymosin-beta4: implications for WH2 proteins. EMBO J 2004;23:3599–3608. [PubMed: 15329672]

Kawaguchi Y, Kovacs JJ, McLaurin A, Vance JM, Ito A, Yao TP. The deacetylase HDAC6 regulates aggresome formation and cell viability in response to misfolded protein stress. Cell 2003;115:727–738. [PubMed: 14675537]

Kim AS, Kakalis LT, Abdul-Manan N, Liu GA, Rosen MK. Autoinhibition and activation mechanisms of the Wiskott-Aldrich syndrome protein. Nature 2000;404:151–158. [PubMed: 10724160]

Loomis PA, Zheng L, Sekerková G, Changyaleket B, Mugnaini E, Bartles JR. Espin cross-links cause the elongation of microvillus-type parallel actin bundles. J Cell Biol 2003;163:1045–1055. [PubMed: 14657236]

Marchand JB, Kaiser DA, Pollard TD, Higgs HN. Interaction of WASP/Scar proteins with actin and vertebrate Arp2/3 complex. Nat Cell Biol 2001;3:76–82. [PubMed: 11146629]

Mattila PK, Salminen M, Yamashiro T, Lappalainen P. Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its COOH-terminal WH2 domain. J Biol Chem 2003;278:8452–8459. [PubMed: 12482861]

Millard TH, Sharp SJ, Machesky LM. Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. Biochem J 2004;380:1–17. [PubMed: 15040784]

Millard TH, Bompard G, Heung MY, Dafforn TR, Scott DJ, Machesky LM, Fütterer K. Structural basis of filopodia formation induced by the IRSp53/MIM homology domain of human IRSp53. EMBO J 2005;24:240–250. [PubMed: 15635447]

Murray JW, Edmonds BT, Liu G, Condeelis J. Bundling of actin filaments by elongation factor 1 alpha inhibits polymerization at filament ends. J Cell Biol 1996;135:1309–1321. [PubMed: 8947553]

Nagahama M, Hara Y, Seki A, Yamazoe T, Kawate Y, Shinohara T, Hatsuzawa K, Tani K, Tagaya M. NVL2 is a nucleolar AAA-ATPase that interacts with ribosomal protein L5 through its nucleolar localization sequence. Mol Biol Cell 2004;15:5712–5723. [PubMed: 15469983]

Naz S, Griffith AJ, Riazuddin S, Hampton LL, Battey JF Jr, Khan SN, Riazuddin S, Wilcox ER, Friedman TB. Mutations of ESPN cause autosomal recessive deafness and vestibular dysfunction. J Med Genet 2004;41:591–595. [PubMed: 15286153]

Pagononi S, Ferreira A. Neurite extension in central neurons: a novel role for the receptor tyrosine kinases Ror1 and Ror2. J Cell Sci 2005;118:433–446. [PubMed: 15654020]

Paunola E, Mattila PK, Lappalainen P. WH2 domain: a small, versatile adapter for actin monomers. FEBS Lett 2002;513:92–97. [PubMed: 11911886]

Quinlan ME, Heuser JE, Kerkhoff E, Mullins RD. Drosophila Spire is an actin nucleation factor. Nature 2005;433:382–388. [PubMed: 15674283]

Sekerková G, Loomis PA, Changyaleket B, Zheng L, Eytan R, Chen B, Mugnaini E, Bartles JR. Novel espin actin-bundling proteins are localized to Purkinje cell dendritic spines and bind the Src homology 3 adapter protein insulin receptor substrate p53. J Neurosci 2003;23:1310–1319. [PubMed: 12598619]

Sekerková G, Zheng L, Loomis PA, Changyaleket B, Whitlon DS, Mugnaini E, Bartles JR. Espins are multifunctional actin cytoskeletal regulatory proteins in the microvilli of chemosensory and mechanosensory cells. J Neurosci 2004;24:5445–5456. [PubMed: 15190118]

Stüven T, Hartmann E, Görlich D. Exportin 6: a novel nuclear export receptor that is specific for profilin-actin complexes. EMBO J 2003;22:5928–5940. [PubMed: 14592989]

Tilney LG, Connelly PS, Vranich KA, Shaw MK, Guild GM. Why are two different cross-linkers necessary for actin bundle formation in vivo and what does each cross-linker contribute? J Cell Biol 1998;143:121–133. [PubMed: 9763425]

Uruno T, Liu J, Zhang P, Fan Y, Egile C, Li R, Mueller SC, Zhan X. Activation of Arp2/3 complex-mediated actin polymerization by cortactin. Nat Cell Biol 2001;3:259–266. [PubMed: 11231575]

Welch MD, Mullins RD. Cellular control of actin nucleation. Annu Rev Cell Dev Biol 2002;18:247–288. [PubMed: 12142287]

Woodings JA, Sharp SJ, Machesky LM. MIM-B, a putative metastasis suppressor protein, binds to actin and to protein tyrosine phosphatase delta. Biochem J 2003;371:463–471. [PubMed: 12570871]

Wulfkuhle JD, Donina IE, Stark NH, Pope RK, Pestonjamasp KN, Niswonger ML, Luna EJ. Domain analysis of supervillin, an F-actin bundling plasma membrane protein with functional nuclear localization signals. J Cell Sci 1999;112:2125–2136. [PubMed: 10362542]

Yamaguchi H, Miki H, Suetsugu S, Ma L, Kirschner M, Takenawa T. Two tandem verprolin homology domains are necessary for a strong activation of Arp2/3 complex-induced actin polymerization and induction of microspike formation by N-WASP. Proc Natl Acad Sci USA 2000;97:12631–12636. [PubMed: 11058146]

Yoon KH, Yoon M, Moir RD, Khuon S, Flitney FW, Goldman RD. Insights into the dynamic properties of keratin intermediate filaments in living epithelial cells. J Cell Biol 2001;153:503–516. [PubMed: 11331302]

Zalevsky J, Lempert L, Kranitz H, Mullins RD. Different WASP family proteins stimulate different Arp2/3 complex-dependent actin-nucleating activities. Curr Biol 2001;11:1903–1913. [PubMed: 11747816]

Zheng L, Sekerková G, Vranich K, Tilney LG, Mugnaini E, Bartles JR. The deaf jerker mouse has a mutation in the gene encoding the espin actin-bundling proteins of hair cell stereocilia and lacks espins. Cell 2000;102:377–385. [PubMed: 10975527]

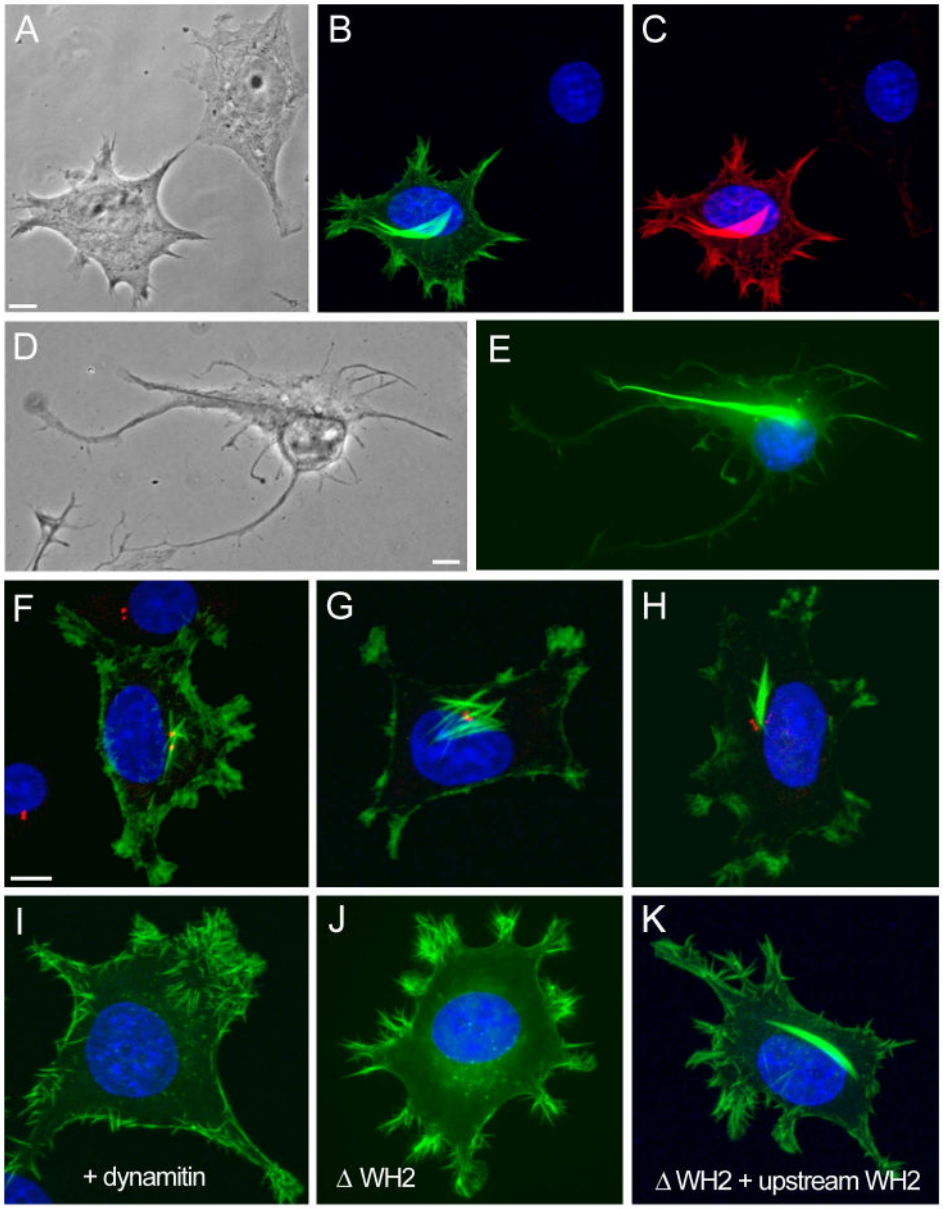

Fig. 1.

Formation of a centrosomal actin bundle (CAB) in neuronal cells transfected with GFP-espin (green). (A-C) Colcalization of GFP-espin (B) and F-actin labeled with Texas Red-phalloidin (C, red) in a large perinuclear actin bundle in a transfected PC12 cell (A, phase). (D,E) GFP-espin (E) in a large perinuclear actin bundle in a transfected primary hippocampal neuron (D, phase). (F-H) GFP-espin in bundle assembly intermediates observed in PC12 cells 4-8 h after transfection (in order of appearance) highlighting relationship to microtubule-organizing center labeled with γ-tubulin antibody (red dots). (I) Inhibition of CAB formation upon cotransfection of PC12 cell with GFP espin and myc-dynamitin constructs (dynamitin expression was confirmed with myc antibody; not shown) (J) Absence of CAB formation in PC12 cell transfected with GFP-espin WH2 domain-deletion construct. (K) Restoration of CAB formation in a PC12 cell transfected with the GFP-espin WH2 domain-deletion construct containing an upstream WH2 domain. Blue, nuclei labeled with DAPI. Bars, 5 μm.

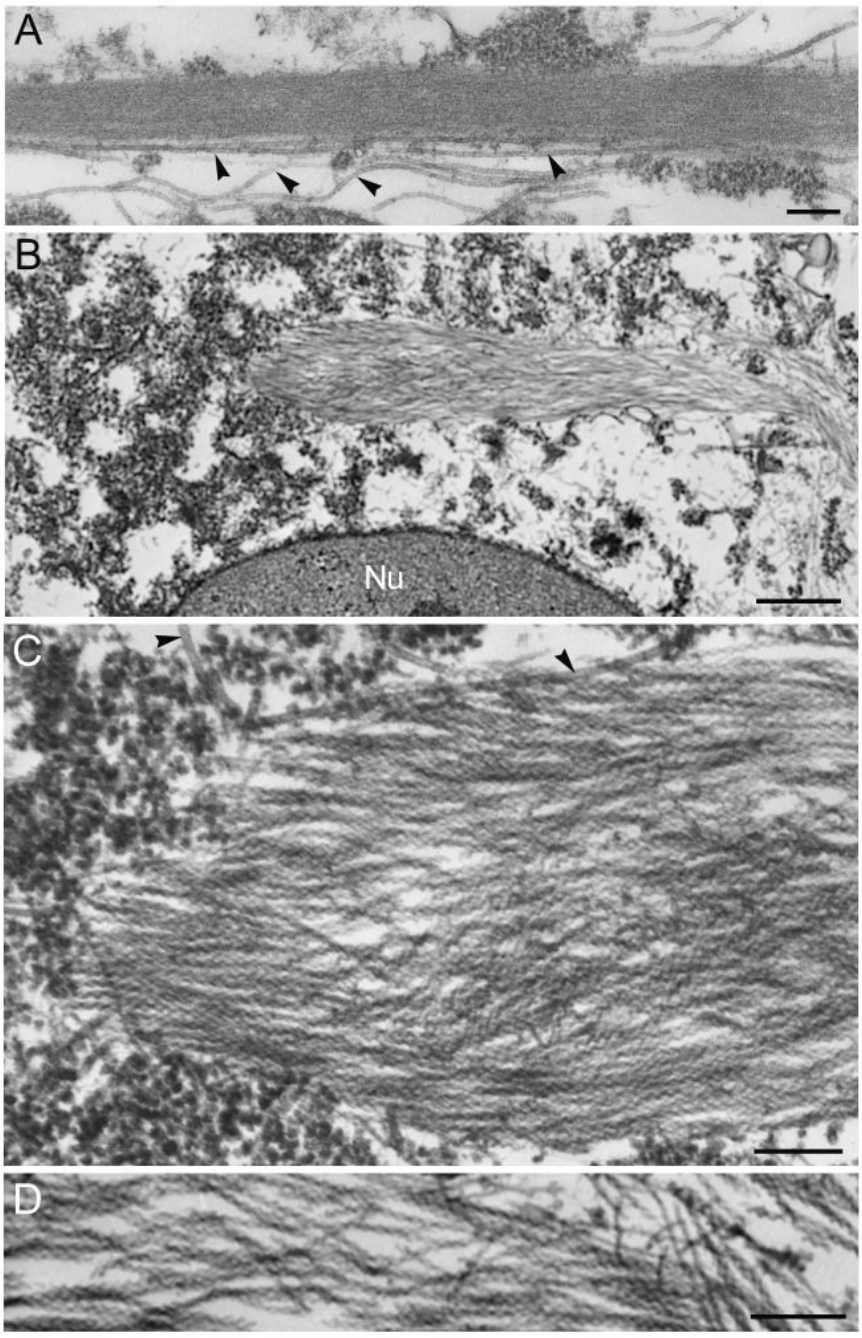

Fig. 2.

EM analysis of CABs in transfected PC12 cells without (A) and with (B-D) S1 decoration showing that CABs are PABs. Nu in B, nucleus. (C,D) Left and right ends of bundle in B, respectively. Barbed ends face to the right. Arrowheads, microtubules. Bars, 0.2 μm (A,C,D) or 1 μm (B).

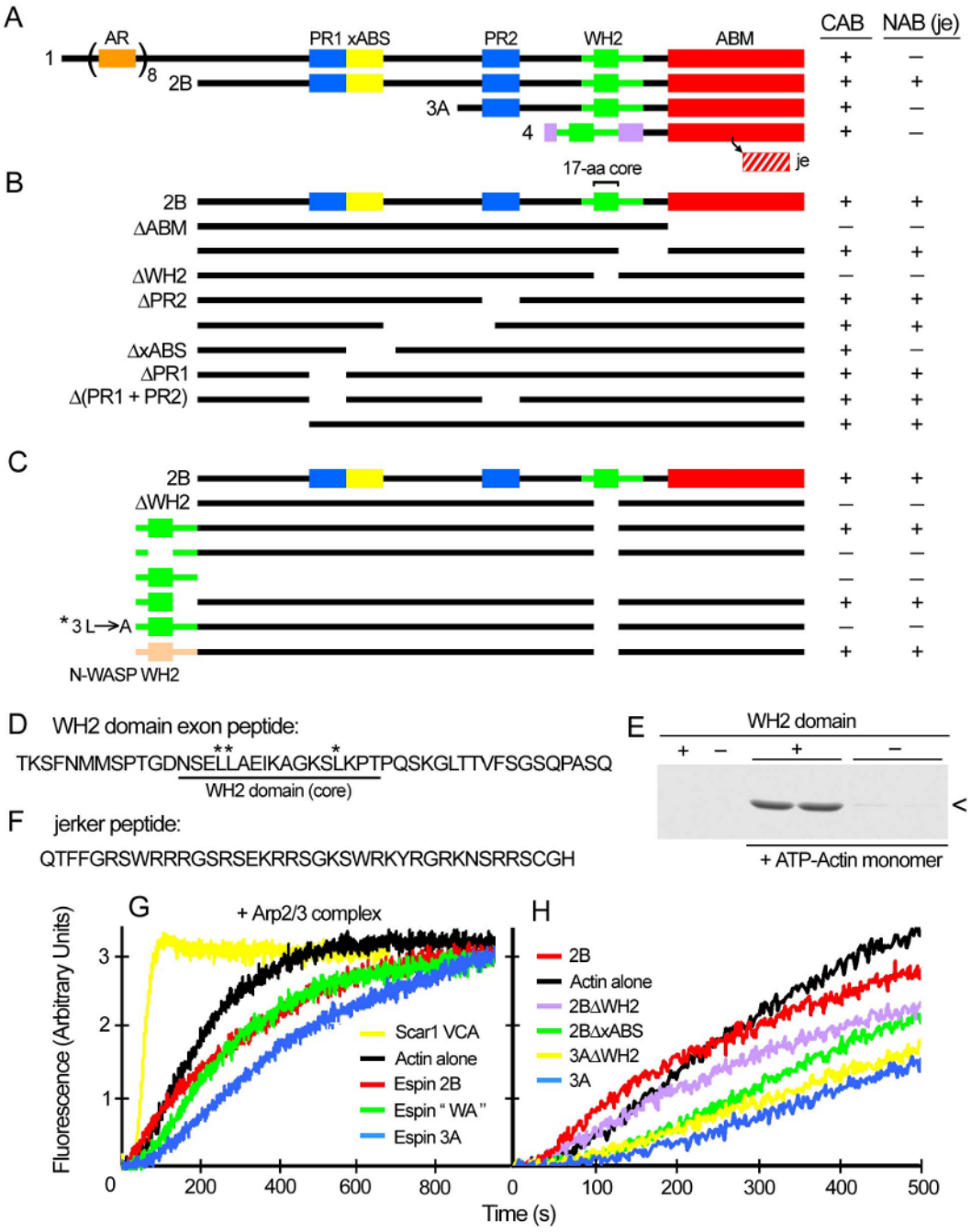

Fig. 3.

Espin isoform, domains and activities. (A-C) Isoform and domain dependence for forming the centrosomal actin bundle (CAB) by wild-type espins and the nuclear actin bundle (NAB) by jerker (je) espins. (A) Representatives of the four major espin isoform size classes. AR, ankyrin-like repeat; PR, proline-rich peptide; xABS, 23-aa additional F-actin-binding site; ABM, actin-bundling module; je, jerker peptide (red and white diagonal stripe; sequence shown in F), the frameshifted peptide that replaces the C-terminal part of the espin actin-bundling module at the site of the jerker deafness mutation (black arrow, illustrated for espin 4); purple, peptides unique to espin 4. (B) Effect of truncation or deletion mutations indicating a requirement for the actin-bundling module, WH2 domain and, in the case of jerker espin 2B, the 23-aa

additional F-actin-binding site. (C) Restoration of CAB or NAB forming activity by adding an upstream WH2 domain to the espin2B WH2 domain-deletion construct. *3L->A, WH2 domain with its 3 leucine residues (asterisks in D) mutated to alanine. (D) Peptide encoded by espin WH2 domain exon with its 17-aa core underlined. *, leucines mutated to alanine in *3L->A construct (activities shown in C). (E) Pull-down assay showing WH2 domain-dependent binding of ATP-actin monomer to espin 2B. <, actin band. (F) Sequence of jerker peptide. (G,H) Pyrene-actin polymerization assay for the designated construct (0.4 μM) in the presence (G) or absence (H) of 10 nM Arp2/3 complex showing absence of nucleation-promoting and nucleation activity of espins in vitro. Espin “WA”, espin COOH-terminal peptide that begins 12 aa upstream of the 17-aa core of the WH2 domain.

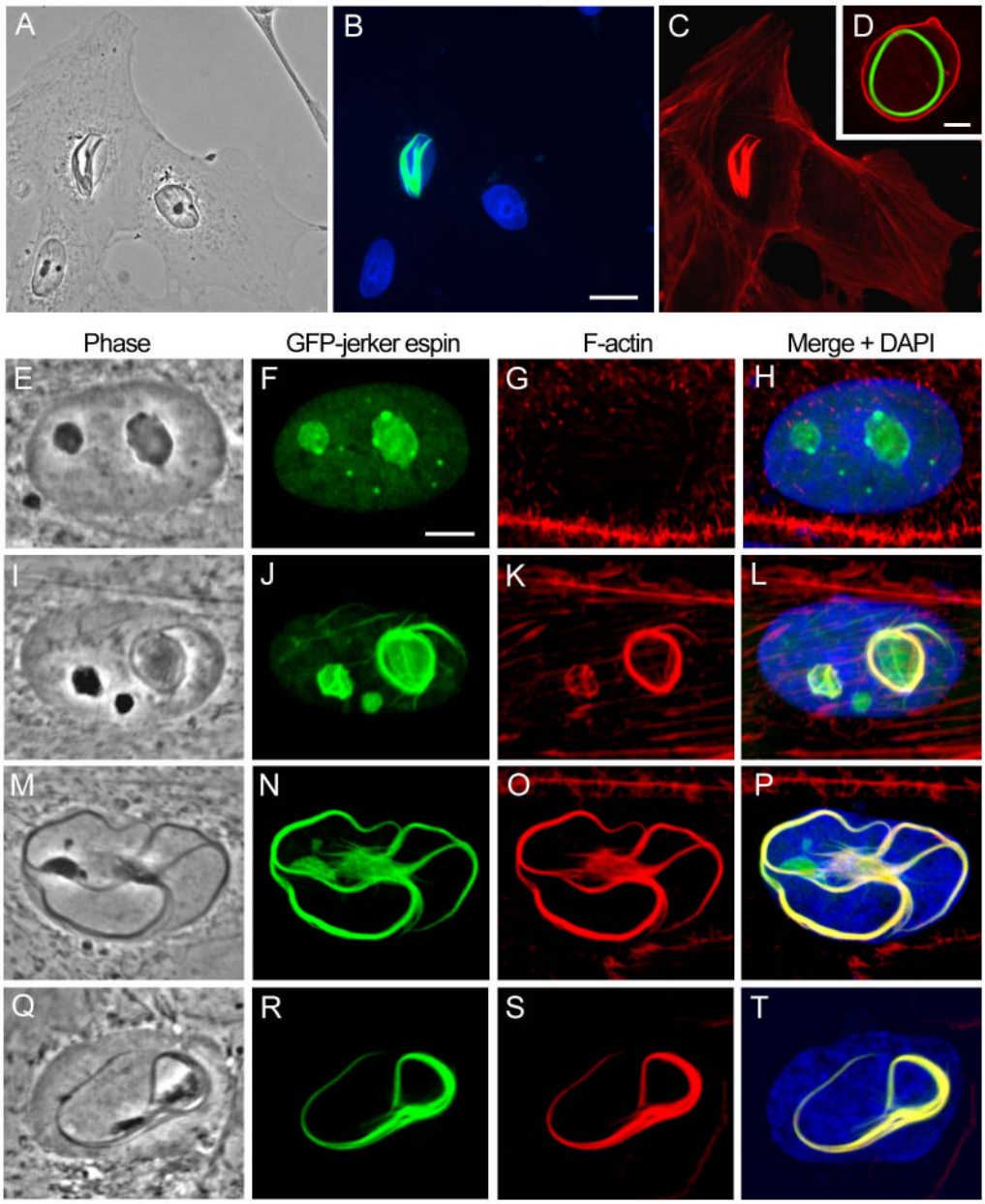

Fig. 4.

Formation of nuclear actin bundle (NAB) in LLC-PK1 cells transfected with GFP-jerker espin 2B (green). (A-C) Colocalization of GFP-jerker espin (B) and F-actin labeled with Texas Red-phalloidin (C, red) in a large actin bundle in a transfected cells (A, phase; blue in B, DAPI). (D) Confocal image showing that the GFP-jerker espin-labeled actin bundle is internal to the nuclear lamina revealed by lamin antibody (red). (E-T) Four rows of NAB assembly intermediates observed 4-8 h after transfection (in order of appearance) highlighting relationship to the nucleolus (phase dense) at early stages. Bars, 15 μm (B) or 5 μm (D,F).

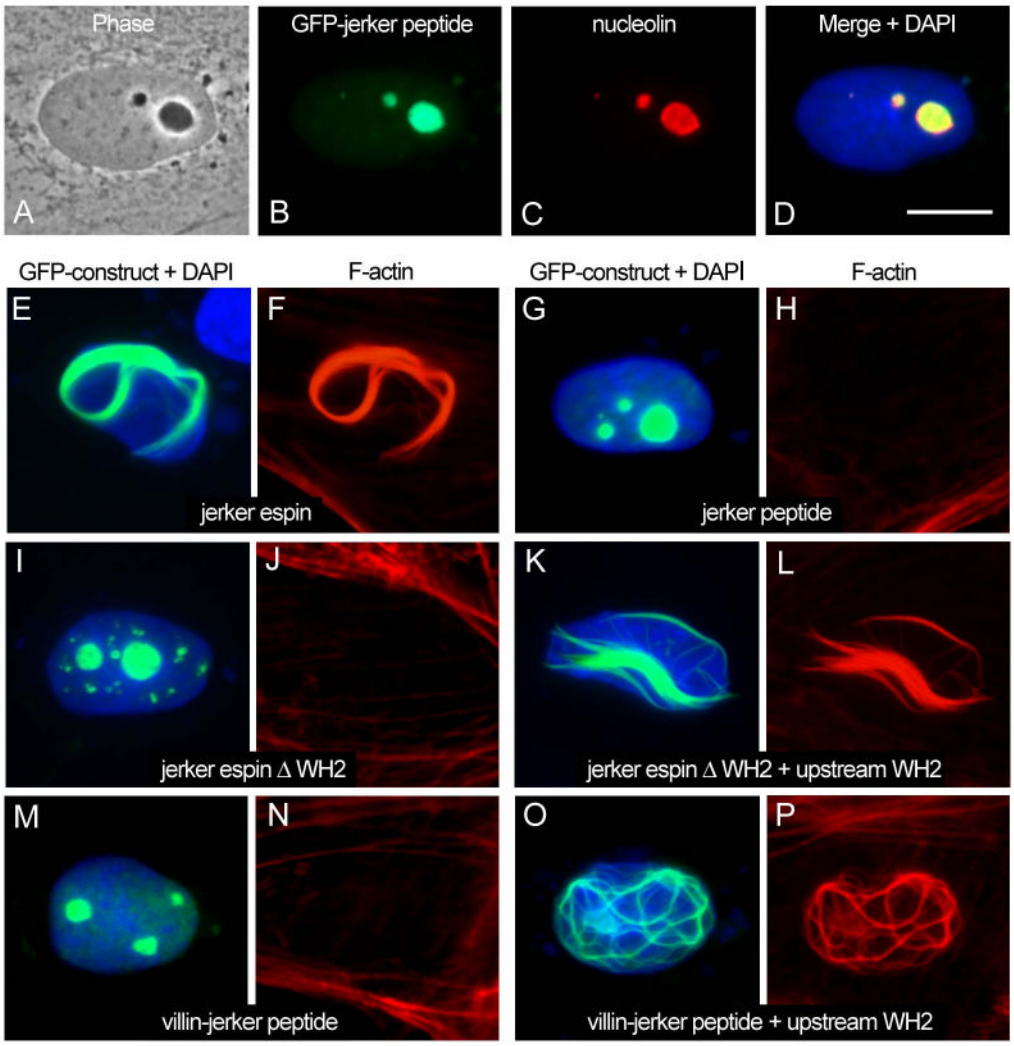

Fig. 5.

Domain dependence of NAB formation. (A-D) Targeting of the GFP-jerker peptide construct (B) to nucleoli, as revealed by labeling with nucleolin antibody (C). (E,F) Colocalization of GFP-jerker espin (E) and F-actin labeled with Texas Red-phalloidin (F) in a NAB. (G,H) Targeting of the GFP-jerker peptide construct to the nucleolus (G) does not cause F-actin accumulation (H). (I,J) The GFP-jerker espin WH2 domain-deletion construct is targeted to nucleoli and to small foci in the nucleoplasm (I), but fails to accumulate F-actin in the nucleus (J). (K,L) Reintroduction of an upstream WH2 domain into GFP-jerker espin WH2 domain-deletion construct results in restoration of NAB forming activity, causing the colocalization of GFP construct (K) and F-actin (L) in a large NAB. (M,N) The GFP-villin-jerker peptide is targeted to nucleoli (M), but does not cause accumulation of nuclear F-actin (N). (O,P) Introduction of the espin WH2 domain upstream of the villin-jerker peptide construct confers NAB forming activity and causes colocalization of the GFP-WH2 domain-villin-jerker peptide construct (O) with F-actin (P) in fine NABs. Blue, nuclei labeled with DAPI. Bar, 10 μm.