Drosophila Spire is an actin nucleation factor

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The actin cytoskeleton is essential for many cellular functions including shape determination, intracellular transport and locomotion. Previous work has identified two factors—the Arp2/3 complex and the formin family of proteins—that nucleate new actin filaments via different mechanisms. Here we show that the *Drosophila* protein Spire represents a third class of actin nucleation factor. *In vitro*, Spire nucleates new filaments at a rate that is similar to that of the formin family of proteins but slower than in the activated Arp2/3 complex, and it remains associated with the slow-growing pointed end of the new filament. Spire contains a cluster of four WASP homology 2 (WH2) domains, each of which binds an actin monomer. Maximal nucleation activity requires all four WH2 domains along with an additional actin-binding motif, conserved among Spire proteins. Spire itself is conserved among metazoans and, together with the formin Cappuccino, is required for axis specification in oocytes and embryos, suggesting that multiple actin nucleation factors collaborate to construct essential cytoskeletal structures.

The *Drosophila* genes *spire* and *cappuccino* are required for proper development of oocytes and embryos. Mutations in either gene result in failure of the egg, and later the developing embryo, to establish dorsal–ventral and anterior–posterior axes of polarity[1]. The molecular mechanism by which *spire* and *cappuccino* contribute to oocyte and embryo polarity is not understood but is thought to involve actin assembly. The phenotypes of *spire* and *cappuccino* mutations are mimicked by addition of cytochalasin D, a drug that inhibits actin polymerization, and by mutations in the actin-binding protein profilin[2]. The *cappuccino* gene product, Cappuccino (Capu), contains formin homology domains (FH1 and FH2) similar to previously described FH domains that nucleate actin filament formation[3,4]. The *spire* gene product, Spire (Spir), contains a cluster of four potential actin-monomer-binding sequences similar to WASP homology 2 (WH2) domains[5,6] and to the amino-terminal portion of the β-thymosin domain[7]. Other proteins that contain WH2/β-thymosin repeats have been shown to strongly inhibit spontaneous actin nucleation, probably by inhibiting interactions between monomers. In contrast, we find that Spir nucleates new filaments by a previously unknown mechanism.

Spir nucleates new actin filaments

Spir initially attracted our attention because it contains four putative WH2 domains and a stretch of acidic residues[5]—hallmarks of proteins that activate the Arp2/3 complex[8]. Spir does not, however, contain the central helical domain required for activation of the Arp2/3 complex by WASP-family proteins[9], so we tested its effect on actin assembly *in vivo* and *in vitro*. Overexpression of Spir in NIH 3T3 cells induces formation of filamentous actin clusters[5] (Fig. 1a, left panels; see also Supplementary Fig. S1) similar to those formed by overexpression of WASP-family proteins[10] (Fig. 1a, right panels). Actin clusters induced by WASP-family proteins co-localize with the Arp2/3 complex, whereas Spir-induced clusters do not (Fig. 1a). Using truncation mutants we localized the actin-cluster-forming activity of Spir to the N-terminal half of the molecule; that is, the region containing the acidic domain and the four WH2 repeats (Supplementary Fig. S1). These data suggested that Spir might promote actin filament assembly via an Arp2/3-independent pathway.

To determine the effect of Spir on actin assembly *in vitro* we expressed the N-terminal half of the protein ((NT)Spir; amino acids 1–520) fused to a 6 × -His tag in *Escherichia coli* and purified it by Ni^{+}-affinity chromatography (see Supplementary Methods). We found that (NT)Spir alone induces rapid and dose-dependent actin polymerization in the absence of the Arp2/3 complex (Fig. 1b, c). Addition of the Arp2/3 complex does not significantly alter the rate of Spir-induced polymerization, suggesting that the two nucleators do not interact (Fig. 1b; see also Supplementary Information).

Actin filament assembly may be accelerated by one of three processes: (1) an increase in the rate of filament elongation; (2) generation of new filament ends via severing; or (3) *de novo* filament nucleation. At concentrations most effective for accelerating polymerization, (NT)Spir has no effect on elongation of pre-formed actin seeds (Supplementary Fig. S2). This result rules out the first and second processes (elongation and severing) and indicates that Spir nucleates actin filaments *de novo*. *In vivo* actin filaments assemble from their fast-growing, barbed ends[11]. (NT)Spir-induced polymerization is inhibited by cytochalasin D, a factor that caps the fast-growing barbed end of actin filaments (Supplementary Fig. S3), indicating that Spir, like the Arp2/3 complex and the formins, generates filaments with elongating barbed ends. Similar to the activity of the Arp2/3 complex, Spir-dependent nucleation is slowed by the actin-monomer-binding protein profilin; however, even at a two-to-one ratio of profilin to actin, Spir's nucleation activity is significant (Supplementary Fig. S3b).

Spir differs from known nucleators

Spir has no sequence homology to the formin family of proteins or to subunits of the Arp2/3 complex. To understand better its nucleation mechanism we compared the activity of Spir to that of the activated Arp2/3 complex and the *Drosophila* formin Capu (Fig. 2a). We expressed and purified a Capu construct containing the FH1 and FH2 domains (Capu(FH1FH2)) and found that it nucleates actin filament formation (Supplementary Fig. S4). Under identical conditions, the maximal nucleation activity of (NT)Spir is approximately equal to that of Capu(FH1FH2) but significantly lower than that of the activated Arp2/3 complex (Fig. 2a). At low concentrations ($<1\,\mu M$) Spir is more active than Capu. At high concentrations of Spir (approaching 1:1 stoichiometry with actin) both the rate of polymerization and the steady-state concentration of actin polymer decrease (Figs 1c and 2a). Neither the Arp2/3 complex nor the formins exhibit such a biphasic dose response,

suggesting that the biochemical mechanism of Spir activity is significantly different (see also Supplementary Fig. S5).

The Arp2/3 complex nucleates new filaments from the sides of preformed filaments and generates arrays of branched actin filaments. We tested whether Spir or Capu interact with the sides of actin filaments. Neither Capu(FH1FH2) nor (NT)Spir co-pellet with filamentous actin (our unpublished observations), suggesting that they do not bind to the sides of actin filaments. In addition, micrographs of fluorescently labelled actin filaments nucleated by Capu(FH1FH2) or (NT)Spir indicate that neither protein generates dendritically branched actin filaments characteristic of the Arp2/3 complex (Fig. 2b)[3,12,13]. Furthermore, addition of preformed filaments has no effect on the nucleation activity of (NT)Spir (Supplementary Fig. S2).

Similar to the Arp2/3 complex but unlike the formins, Spir binds the pointed end of actin filaments. Formins interact with the fast-growing barbed end of actin filaments and can alter the rate of filament elongation and disassembly from the barbed end[3,14,15], whereas the Arp2/3 complex caps the slow-growing pointed end with nanomolar affinity (Fig. 2c)[13]. (NT)Spir has no effect on elongation or shortening of actin filaments with free barbed ends (Supplementary Fig. S2); however, when the fast-growing barbed end is capped with gelsolin, Spir inhibits disassembly from the pointed end (Fig. 2c). Our data slightly underestimate the effect of Spir on pointed-end disassembly because, under our assay conditions, sequestration of monomeric actin by (NT)Spir (Supplementary Fig. S5a) should accelerate depolymerization. We estimate that (NT)Spir binds pointed ends of actin filaments with an affinity in the micromolar range. Interestingly, actobindin and Ciboulot, which contain multiple WH2-like motifs, also weakly associate with pointed ends of actin filaments[16,17].

Spir WH2 domains assemble actin nuclei

Next we tested whether the WH2-like sequences in Spir bind monomeric actin and whether they participate in filament nucleation. We refer to these domains as WH2-A through to WH2-D (N-terminal to carboxy-terminal), and we refer to the intervening sequences as linker 1 through to linker 3 (L-1 to L-3, Fig. 3a). The sequences differ by varying degrees compared with canonical WASP-family WH2 sequences (Fig. 3a). WH2-B and WH2-D are the most highly conserved and were originally identified by ref. 6, which showed that WH2-D binds actin. We expressed and purified each Spir WH2 domain fused to glutathione *S*-transferase (GST) and tested its actin binding activity *in vitro*. Similar to conventional WH2 domains[8], each Spir domain inhibits spontaneous polymerization (Supplementary Fig. S6a). Conventional WH2 domains contact actin via a set of highly conserved hydrophobic amino acid residues[16] (A. E. Kelly and R.D.M., unpublished results), and mutation of these residues in the Spir WH2-like domains (Fig. 3a) abolishes their ability to inhibit polymerization (Supplementary Fig. S6b). Together, our results indicate that each WH2-like sequence in Spir interacts with actin in a manner similar to a traditional WH2 domain.

To determine the connection between actin binding by the WH2

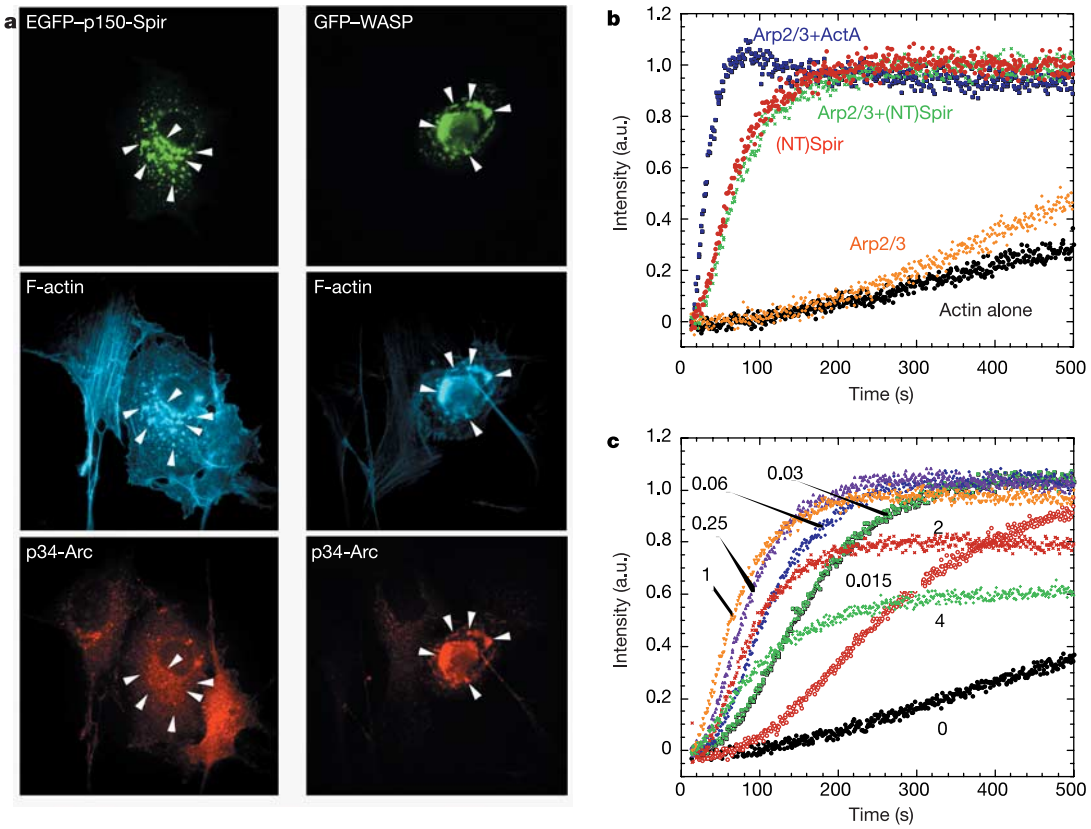

Figure 1 Spir nucleates actin filaments. **a**, Expression of either GFP-tagged Spir (EGFP–p150-Spir, green) or WASP (GFP–WASP, green) in NIH 3T3 cells leads to accumulation of filamentous actin (visualized with phalloidin, blue). The p34-Arc subunit (red) of the Arp2/3 complex co-localizes with WASP-induced actin clusters but not Spir-induced actin clusters. **b**, (NT)Spir nucleates actin independently of the Arp2/3 complex. Pyrene-actin polymerization assays with 0.5 μM of (NT)Spir alone ((NT)Spir) are as fast as those with (NT)Spir and 20 nM Arp2/3 (Arp2/3 + (NT)Spir). Polymerization induced by 20 nM of Arp2/3 alone (Arp2/3) and in the presence of saturating ActA[34] (Arp2/3 + ActA) are shown for comparison. These and all other polymerization traces are scaled (but not normalized) to an arbitrary plateau value of 1 for 0.5 μM (NT)Spir. They were not normalized so as not to mask the effect of sequestration. **c**, Spir nucleates actin filament assembly in a dose-dependent manner. The indicated amount (in μM) of (NT)Spir was added to pyrene-actin polymerization assays. See Supplementary Information for detailed Methods. a.u., arbitrary units.

domains and filament nucleation by (NT)Spir, we mutated the WH2 sequences individually and in combination (Fig. 3a, b). Mutation of the essential hydrophobic residues in individual WH2 domains produced nucleation defects of differing severity (Fig. 3b and Supplementary Table S1). Mutation of WH2-A produced the smallest effect ($t_{1/2} = 73 \pm 6$ s versus 67 ± 5 s for wild type in the presence of 4 μM actin; mean ± s.d.); mutation of WH2-B and WH2-C produced progressively greater effects ($t_{1/2} = 97 \pm 6$ s and 150 ± 10 s, respectively); and mutation of WH2-D produced the most profound effect of all on nucleation activity ($t_{1/2} = 330 \pm 20$ s). Removal of WH2 domains by truncation produced similar effects (Fig. 3c and Supplementary Table S1). A construct encompassing only the WH2 cluster (ABCD) displayed similar levels of nucleation activity as (NT)Spir (Fig. 3c). Removal of one or even two N-terminal WH2 domains had only a small effect whereas removal of WH2-D markedly reduced nucleation activity, and removal of both WH2-C and WH2-D (along with linkers 2 and 3; (NT)Spir[Δ2C3D]) nearly abolished nucleation activity (Fig. 3c and Supplementary Table S1). We detected only weak nucleation activity at high concentrations of (NT)Spir[Δ2C3D] (our unpublished observation).

A unique actin-binding site

Simultaneous mutation of all four WH2 domains ((NT)Spir [A*B*C*D*]) greatly diminishes nucleation activity but fails to abolish it ($t_{1/2} = 350 \pm 20$ s versus 420 ± 30 s for 4 μM actin alone; Fig. 3b). (NT)Spir, therefore, must contain at least one more actin-binding site with weak nucleation activity. Because the nucleation activity of ABCD is identical to that of (NT)Spir, we focused our attention on the short (~15 amino acid) sequences between the WH2 domains (Supplementary Fig. S7). To determine their role in nucleation, we replaced each linker with a set of four flexible Gly-Ser repeats that maintain the same spacing between WH2 domains (see Supplementary Information). Replacing either L-1 or L-2 ((NT)Spir[gs1] and (NT)Spir[gs2]) produced negligible effects on nucleation activity (Fig. 3d). In contrast, replacement of L-3 ((NT)Spir[gs3]) markedly diminished the nucleation activity and replacement of all three linkers was equivalent to replacing L-3 alone (Fig. 3d). Thus L-3 appeared to be a likely candidate for an actin-binding domain involved in filament nucleation.

We expressed L-3 as a 6× -His-tagged peptide (Fig. 3a and Supplementary Information) and tested its activity *in vitro*. By itself, the L-3 peptide weakly nucleates actin assembly (Fig. 3e) with activity similar to that of (NT)Spir[A*B*C*D*]. Because of its size we assume that L-3 promotes actin dimer formation by stabilizing the interaction of two actin monomers. The sequence seems to be unrelated to other known actin-binding motifs and database searches yield only Spir-like proteins (Fig. 3a; see also Supplementary S7).

Ultrastructure of the Spir–actin complex

We hypothesize that Spir forms a new actin filament by first binding several actin monomers and then assembling them stereospecifically into a filament nucleus: a dimer, trimer, or a tetramer. To test this

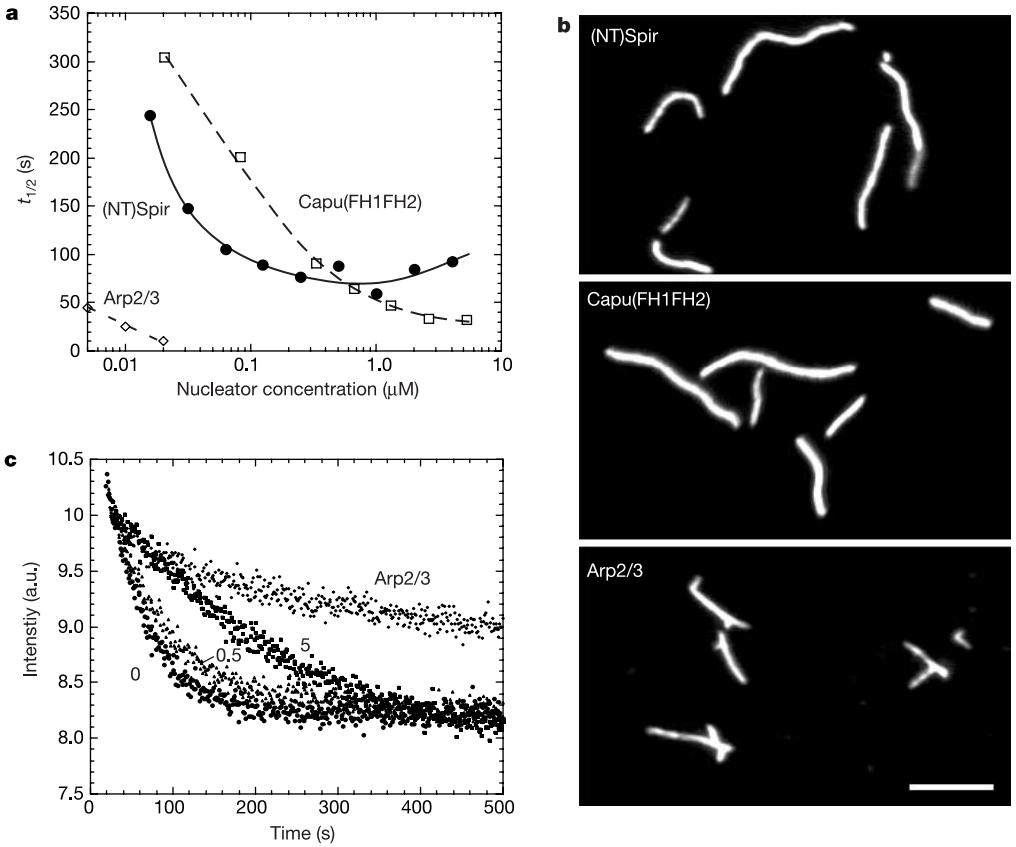

Figure 2 Spir, Capu and the Arp2/3 complex nucleate polymerization by distinct mechanisms. **a**, To compare activity levels of different nucleators, we plotted the time to half-maximal polymerization ($t_{1/2}$) as a function of (NT)Spir (filled circles), Capu(FH1FH2) (open squares) and Arp2/3 complex (open diamonds) concentrations. (NT)Spir and Capu(FH1FH2) nucleate at similar rates whereas filament formation by the Arp2/3 complex is significantly faster. **b**, Filaments produced by 0.5 μM (NT)Spir (top panel) or 0.5 μM Capu(FH1FH2) (middle panel) are not crosslinked, whereas those produced by the Arp2/3 complex (20 nM plus ActA) are branched in a characteristic dendritic pattern (bottom panel). **c**, Depolymerization of gelsolin-capped filaments in the presence of 0, 0.5 and 5 μM (NT)Spir or 100 nM Arp2/3 shows that (NT)Spir caps the pointed end of filaments.

hypothesis, we performed electron microscopy on rotary-shadowed samples of (NT)Spir bound to actin. We combined (NT)Spir and actin at a 1:8 molar ratio under non-polymerizing conditions. We then diluted the samples into polymerization buffer and rapidly fixed them. Our protocol was designed to capture the maximum number of transient nucleating structures before they became bona fide actin filaments. In many fields we did, in fact, see actin filaments (Fig. 4b, lower left panels) but in almost all fields we also observed a novel, rod-shaped structure 22 ± 4 nm in length and 6.5 ± 0.3 nm in diameter ($n = 43$; Fig. 4). We observed Spir–actin rods of the same size (6.5 ± 1 × 24 ± 3 nm, $n = 17$) in the presence of latrunculin B (Fig. 4b, lower right), which blocks actin filament formation but does not prevent binding of actin monomers to WH2 domains[16,18]; however, we never observed such structures in samples of actin or (NT)Spir alone. These Spir–actin rods were distinguishable from short actin filaments by their uniform length and smaller diameter (6.5 nm compared with 7.5 nm). The length and diameter of Spir–actin rods is consistent with four actin monomers aligned along their long axes. When we mixed the WH2 cluster (ABCD) with actin it produced rod-shaped structures nearly identical to

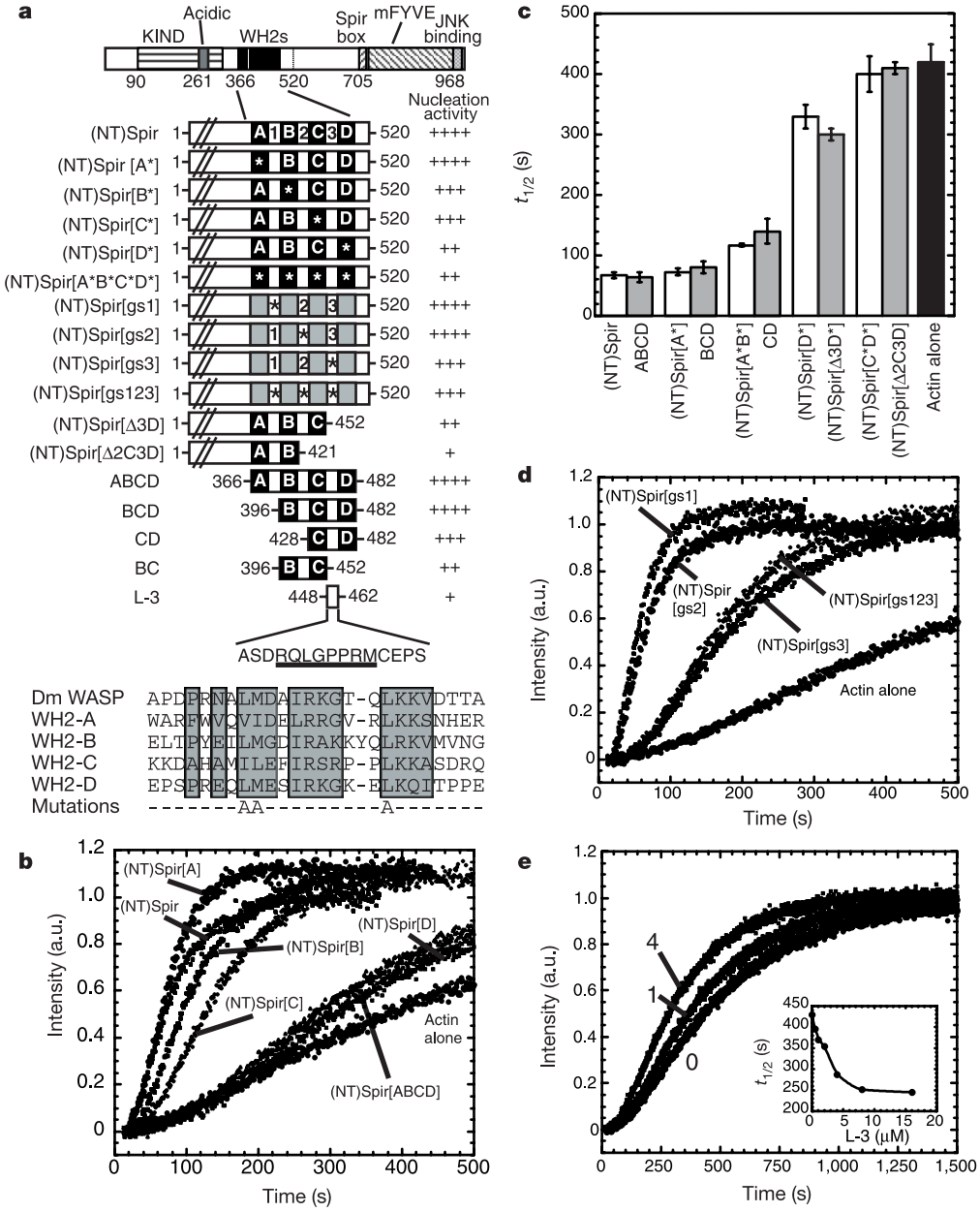

Figure 3 Molecular dissection of Spir. **a**, Diagram of full-length Spir and mutants generated in this study. The domains identified are described in Supplementary Fig. S7. The sequence of L-3 is shown with the eight residues replaced by four Gly/Ser repeats underlined. The WH2 domains are aligned with the *Drosophila* WASP WH2 domain, and the alanine mutations are shown at the bottom. Nucleation activity of each Spir mutant (0.5 μM with 4 μM actin) is indicated in the right column. Polymerization stimulated by wild-type (NT)Spir is denoted by ++++; nucleation equivalent to spontaneous actin polymerization is indicated by +. **b**, Each WH2 domain has a different role in nucleation; for example, mutation of WH2-A ((NT)Spir[A*]) had almost no effect on nucleation, whereas mutation of WH2-D ((NT)Spir[D*]) markedly decreased activity. **c**, Comparison of $t_{1/2}$ induced by truncation mutants. Activity of (NT)Spir and actin alone (black column) are shown at either end of the graph. Corresponding pairs of truncation (grey) and point mutants (white) are plotted side by side. Error bars are ±s.d. **d**, The sequences linking WH2 domains have different roles in actin nucleation. Only replacing L-3 ((NT)Spir[gs3]) markedly reduced nucleation activity. **e**, L-3 alone weakly nucleates filament formation. Concentrations of L-3 (in μM) are indicated. Inset: $t_{1/2}$ versus L-3 concentration. Note that this domain does not sequester monomeric actin at high concentrations.

those produced by (NT)Spir ($5.9 \pm 0.9 \times 26 \pm 5$ nm, $n = 111$), whereas CD and actin produced structures that were approximately half as long ($5 \pm 1 \times 9 \pm 3$ nm, $n = 85$; Supplementary Fig. S8). These data are consistent with a model in which the WH2 domains bind actin monomers and align them end-to-end, as if along one strand of the long-pitch, two-start filament helix.

Mechanism of filament nucleation by Spir

Our results indicate that the C-terminal half of the Spir WH2 cluster—composed of WH2-C, L-3 and WH2-D—is the functional core of the protein. The main kinetic barrier to nucleation is formation of an actin dimer. We propose that Spir assembles an actin dimer when WH2-C and WH2-D each bind an actin monomer and L-3 coordinates their interaction (Supplementary Fig. S9). Similar to the first dimer formed during spontaneous nucleation[19], we expect this dimer to lie along one strand of the long-pitch actin helix. On the basis of their effects on nucleation and our own electron microscopy data, we propose that WH2-B and WH2-A add a third and fourth monomer to the initial dimer (Fig. 5b).

In addition to electron microscopy (Fig. 4), spatial constraints imposed by the Spir sequence[16] (Fig. 5a) and the atomic structure of the WH2-like domain of Ciboulot[16] suggest that Spir stacks monomers along one strand of the long-pitch filament helix (Fig. 5b). The N-terminal portion of a WH2 domain would block addition of the next monomer at the barbed end. We propose that, similar to Ciboulot and the WASP-family WH2 domains, the N-terminal portion of the Spir WH2 domains dissociates rapidly upon incorporation into the nascent nucleus. Consistent with this idea, residues in the Ciboulot actin-binding site important for dissociation and promotion of actin filament assembly are conserved in Spir (Supplementary Fig. S7c). The only structure consistent with the lengths of the linker sequences is a linear arrangement of the four WH2-bound monomers, with WH2-D at the pointed end and WH2-A at the barbed end. Binding of an additional monomer to the interface between any of the WH2-bound monomers (asterisks in Fig. 5b) would result in formation of a stable nucleus and rapid filament elongation.

The fact that Spir assembles nuclei even when all three linker sequences are mutated ((NT)Spir[gs123]) suggests that tethering multiple, WH2-bound actin monomers in close proximity may be

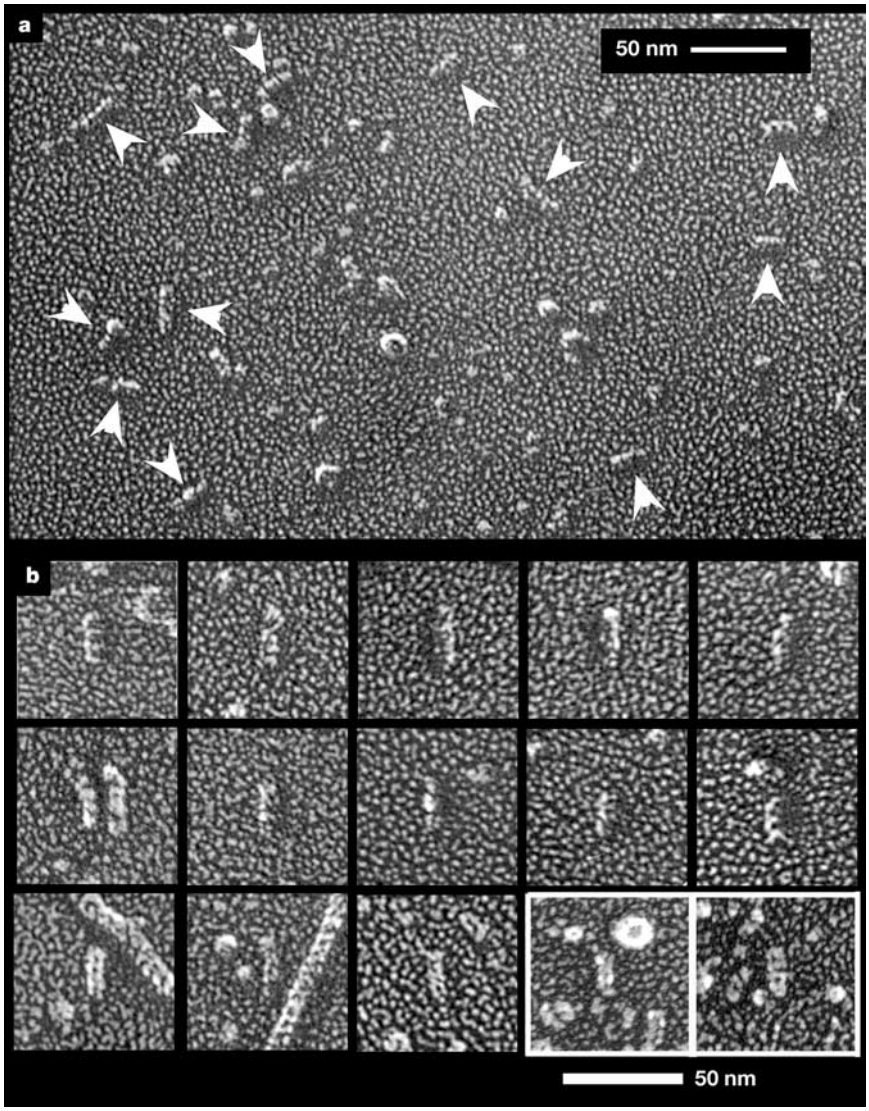

Figure 4 Spir–actin complexes are rod-like structures. **a**, Sample electron micrograph of actin and (NT)Spir (1:8 ratio) rapidly fixed after polymerizing conditions are introduced. The field contains multiple examples of an ~22 × 6.5 nm rod-shaped structure observed only in samples containing both (NT)Spir and actin. **b**, Individual Spir–actin rod complexes observed under both polymerizing and non-polymerizing conditions. The two examples in the white box at the lower right show similar structures seen in the presence of the polymerization inhibitor, latrunculin B. Typical actin filaments next to rods are seen in the two lower-left boxes.

sufficient to promote filament formation. The only other known tandem repeats of competent WH2 domains are found in N-WASP and tetra-thymosin-β. By themselves, N-WASP and tetra-thymosin-β allow elongation of the barbed ends of filaments, but strongly inhibit spontaneous nucleation. Further work is required to determine whether WH2 domains in Spir are specially adapted to promote nucleation, or whether sequences in other WH2-containing proteins are specially adapted to prevent nucleation, as in the case of thymosin-β4 (ref. 20).

Cellular function of Spir

After the Arp2/3 complex and the formins, Spir is the third actin nucleation factor to be discovered. Why do cells require more than one mechanism for constructing actin filaments? The answer probably lies in the diversity of functions performed by the actin cytoskeleton. Different functions require actin networks with different architectures, and the architecture of an actin network is determined in part by the mechanism of filament nucleation. Dendritic nucleation by the Arp2/3 complex, for example, produces space-filling actin networks capable of resisting mechanical deformation. This activity is required for amoeboid motility, phagocytosis and intracellular motility of endosomal vesicles and some pathogens[21,22]. The formins do not crosslink new filaments into branched arrays but remain attached to their growing ends and probably tether them to specific locations[15,23]. Unbranched filaments generated by formins are essential for construction of actin cables in budding yeast, and stress fibres and contractile rings in mammalian cells[24–26].

The regulation and expression pattern of Spir differ from those of the other nucleators. Unlike the Arp2/3 complex and the formins, Spir has no obvious orthologues in any sequenced protozoan genome; however, it is highly conserved across metazoan species (Supplementary Fig. S5a). Two mammalian isoforms, Spir-1 and Spir-2, are widely expressed in embryonic tissues but limited primarily to the central nervous system of adults[27]. The Arp2/3 complex and the Diaphanous-related formins are downstream of Rho-family G proteins, whereas Spir and the mammalian homologue of Capu, formin-1, are MAP kinase substrates[5,28]. Spir proteins are targeted to intracellular membranes by a C-terminal-modified FYVE zinc finger motif, and co-localize with the GTPase Rab11, which is involved in vesicle transport processes[29,30]. As with *spir* and *capu*, *Drosophila Rab11* belongs to the posterior group of genes[31,32]. In addition, *Rab11*, *spir* and *capu* mutants have similar defects in microtubule plus-end orientation during oogenesis[6,32,33]. These data together with our results suggest that Spir has evolved specifically to construct actin-based structures required for polarization of multicellular organisms. □

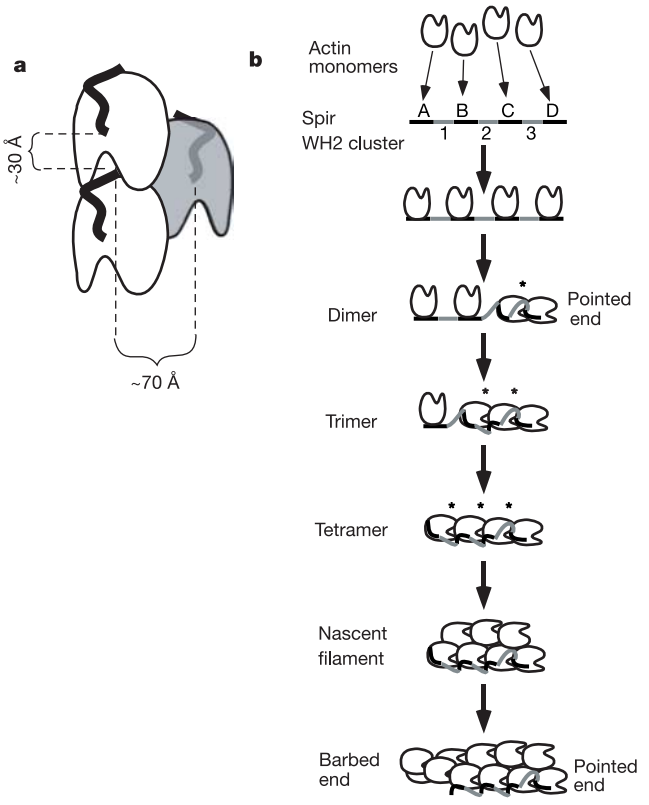

Figure 5 Mechanism of nucleation by Spir. We propose that Spir coordinates association of actin monomers along one strand of the two-start filament helix. **a**, Schematic alignment of WH2 domains on an actin trimer based on ref. 16. A 15 amino acid linker cannot connect the cross-filament dimer in this orientation. **b**, Up to four actin monomers can bind the WH2 cluster of Spir. Once monomers are bound to WH2-C and WH2-D they are coordinated and stabilized in a longitudinal dimer by L-3. Monomers from WH2-B and WH2-A are further stacked on this 'pre-nucleus' structure. Asterisks indicate positions where addition of a monomer would result in formation of a complete nucleus and subsequent rapid polymerization.

Methods

We cloned the *spire* gene and generated mutants using standard techniques (see Supplementary Methods). We expressed wild-type and mutant Spir proteins in *E. coli* and purified them using a combination of Ni^{+}-affinity and anion exchange chromatography (details in Supplementary Methods). Immuno-labelling and fluorescence microscopy were performed using standard methods (see Supplementary Methods).

Actin kinetics

All pyrene-actin polymerization assays were performed using 4 μM monomeric actin (5% pyrene labelled) in KMEI (50 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 mM imidazole pH 7.0). Ca-actin was converted to Mg-actin by incubation in ME (50 mM $MgCl_2$, 0.2 mM EGTA) for 2 min before addition of KMEI and test components. Unless otherwise indicated, 0.5 μM nucleator ((NT)Spir or mutant) was added. Fluorescence was excited at 365 nm and detected at 407 nm using an ISS PC1/K2 fluorimeter.

Depolymerization assays were performed by diluting 10 μM filamentous actin (10% pyrene labelled) to 1 μM in KMEI. Gelsolin-capped filaments were created by polymerizing actin off of gelsolin–actin seeds (ref. 13).

Electron microscopy

We first combined actin and (NT)Spir at an 8:1 molar ratio in buffer A. To capture early stages of nucleation, we rapidly diluted samples to 5 μg ml^{-1} in KMEI, adsorbed them to mica flakes at 25 °C, then quick-froze and freeze-dried them. Details regarding image acquisition and calibration are given in Supplementary Methods.

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