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Authors
Quinlan, Margot E
Hilgert, Susanne
Bedrossian, Anaid
et al.

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Regulatory interactions between two actin nucleators, Spire and Cappuccino

Margot E. Quinlan,1 Susanne Hilgert,1 Anaid Bedrossian,1 R. Dyche Mullins,2 and Eugen Kerkhoff1

1Bayerisches Genomforschungsnetzwerk (BayGene), Institut für funktionelle Genomik, Universität Regensburg, 93053 Regensburg, Germany
2Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94107

S
pire and Cappuccino are actin nucleation factors that are required to establish the polarity of Drosophila melanogaster oocytes. Their mutant phenotypes are nearly identical, and the proteins interact biochemically. We find that the interaction between Spire and Cappuccino family proteins is conserved across metazoan phyla and is mediated by binding of the formin homology 2 (FH2) domain from Cappuccino (or its mammalian homologue formin-2) to the kinase noncatalytic C-lobe domain (KIND) from Spire. In vitro, the KIND domain is a monomeric folded domain. Two KIND monomers bind each FH2 dimer with nanomolar affinity and strongly inhibit actin nucleation by the FH2 domain. In contrast, formation of the Spire–Cappuccino complex enhances actin nucleation by Spire. In Drosophila oocytes, Spire localizes to the cortex early in oogenesis and disappears around stage 10b, coincident with the onset of cytoplasmic streaming.

Introduction

Developing Drosophila melanogaster oocytes use both actin and microtubule cytoskeletal systems to construct and maintain internal landmarks that define the dorsal-ventral and anterior-posterior axes (Theurkauf et al., 1992; Clark et al., 1994; Pokrywka and Stephenson, 1995; Polesello et al., 2002). The cappuccino and spire genes encode actin filament nucleation factors (Quinlan et al., 2005), and mutation of either gene disrupts localization of the earliest known polarity determinants (Manseau and Schupbach, 1989; Manseau et al., 1996). spire and cappuccino were originally identified in the same genetic screen (Manseau and Schupbach, 1989), and loss of either results in the premature onset of microtubule-dependent fast cytoplasmic streaming during oogenesis, loss of oocyte polarity, and female sterility (Theurkauf, 1994; Emmons et al., 1995). Rosales-Nieves et al. (2006) demonstrated a genetic interaction between spire and cappuccino by showing that premature cytoplasmic streaming occurs in flies heterozygous for mutations in both genes. Mutation of Drosophila profilin (Chickadee) or addition of the actin-depolymerizing toxin cytochalasin D (Emmons et al., 1995; Manseau et al., 1996) also cause premature fast cytoplasmic streaming. Together, these data suggest that actin polymerization driven by Spire (Spir), Cappuccino (Capu), and Chickadee suppresses fast cytoplasmic streaming until the appropriate point in oogenesis (Serbus et al., 2005).

Consistent with genetic data, Rosales-Nieves et al. (2006) recently showed that Spir and Capu proteins interact directly. These authors found that the N-terminal region of Spir, which contains the kinase noncatalytic C-lobe domain (KIND) and a cluster of actin-binding WH2 domains (Wiskott-Aldrich syndrome protein homology domain 2), binds to the formin homology 2 (FH2) domain of Capu. Their data suggest that interaction is mediated by direct binding of the WH2 cluster to the FH2 domain. These authors report that the Spir–Capu interaction has no effect on actin nucleation by either protein but that interaction with Spir inhibits FH2-dependent cross-linking of actin filaments and microtubules. Based on these data, Rosales-Nieves et al. (2006) propose a model in which Spir and Capu inhibit premature cytoplasmic streaming by cross-linking microtubules to actin filaments in the oocyte cortex.

Interactions between Spir and Capu have been studied only in Drosophila, but there is evidence linking the two proteins in other organisms. In sequenced metazoan genomes, Capu family formins appear only in organisms that also contain Spire family genes (Higgs and Peterson, 2005). Mammals have two copies of each gene. Arthropods, including Drosophila, contain at least one spire and one cappuccino gene, whereas nematodes, such as Caenorhabditis elegans, contain neither. Because nematodes diverged from arthropods long after Deuterostomes diverged from Protostomes, it appears that nematodes lost both genes at some point in their evolution. Schumacher et al. (2004) found...
that the patterns of spir-1 and formin-2 (fnn2) expression are nearly identical in developing and adult mice.

We also note that Spir and Capu homologues are found in a variety of polarized cells, including Drosophila and Xenopus laevis oocytes (Eg6 or Xenopus spir-2; Le Goff et al., 2006), mammalian eggs (Fmn2; Leader et al., 2002), neurons (Leader and Leder, 2000; Schumacher et al., 2004), and polarized epithelial cells (formin-1; Kobielak et al., 2004). In Xenopus oocytes, the mRNA of Spir-2 (Eg6) localizes to the vegetal cytoplasm and marks the posterior end of the developing embryo (Le Goff et al., 2006). Knockout of Fmn2 in the mouse produces a maternal effect phenotype in which females are sterile as a result of mispositioning of the meiotic spindle (Leader et al., 2002).

In this study, we investigate the molecular basis of the interaction between Spir and Capu and how the interaction influences actin nucleation. We find that Spir and Capu interact in vivo as well as in vitro. Similar to Rosales-Nieves et al. (2006), we find that the Spir-WH2 cluster interacts with the Capu-FH2 domain. However, we also find that the Spir-KIND domain binds the Capu-FH2 domain with several orders of magnitude higher affinity than the WH2 cluster. This interaction has three functional consequences: (1) the KIND domain potently inhibits actin nucleation by Capu; (2) interaction between the KIND domain and Capu leads to enhanced actin nucleation by Spir; (3) the KIND domain competes with actin filaments and microtubules for binding to the FH2 domain of Capu. The KIND-FH2 interaction is evolutionarily conserved, as we observe the same results using both Drosophila and mammalian Spir and Capu family proteins. The direct interaction of Spir and Capu, the fact that the expression patterns of Spir-1 and Fmn2 exactly overlap its actin nucleation by Capu; (2) interaction between the KIND domain and Capu leads to enhanced actin nucleation by Spir; (3) the KIND domain competes with actin filaments and microtubules for binding to the FH2 domain of Capu. The KIND–FH2 interaction is evolutionarily conserved, as we observe the same results using both Drosophila and mammalian Spir and Capu family proteins. The direct interaction of Spir and Capu, the fact that the expression patterns of Spir-1 and Fmn2 exactly overlap in the developing nervous system (Schumacher et al., 2004), and the fact that their evolutionary conservation appears to be linked lead us to speculate that Spir and Capu function as part of a complex whose job is to assemble cytoskeletal landmarks for polarity in many systems.

Results

Spir in oogenesis

We find that full-length Spir is sufficient to rescue the spirale mutant phenotype. The FlyBase Genome lists four gene products, which are all derived from a single Drosophila spirale gene: Spir-PA, -PB, -PC, and -PD (GenBank/EMBL/DDBJ accession nos. NM_165323, NM_080115, NM_165325, and NM_165324, respectively). Spir-PA and -PB are ~1,000 amino acids and differ by a 29-amino acid insert. Spir-PD is equivalent to the first 584 amino acids of Spir-PA, whereas Spir-PC is approximately the N-terminal half of Spir-PA. Wellington et al. (1999) detected two distinct bands in Northern blots of RNA from fly oocytes, which they named Spire long form and short form (GenBank/EMBL/DDBJ accession nos. AF184975 and AF184976). These correspond to Spir-PA/PB and Spir-PD, respectively. There is no published evidence for the expression of Spir-PC. We made transgenic flies that express monomeric RFP (mRFP)—tagged full-length Spir (we refer to PA/PB as full length) in the germ-line. The localization of Spir fusions was enriched in the oocyte cortex and diffuse in the oocyte cytoplasm (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200706196/DC1).

Rosales-Nieves et al. (2006) expressed GFP fusions of two putative spliceoforms of Spir (GFP-SpirC and GFP-SpirD) in Drosophila egg chambers. Consistent with our observation, they found both proteins associated with the oocyte cortex. They also observed GFP-SpirC in punctae and GFP-SpirD diffuse throughout the oocyte. spir1 flies are putative nulls with the stereotypical spirale phenotypes, including female sterility. Both mRFP-Spir and Spir-mRFP rescue female sterility in spir1 flies, demonstrating that the full-length transcript is sufficient during oogenesis and that the shorter spliceoforms are not essential.

We next determined the localization of endogenous Spir in wild-type Drosophila egg chambers by immunofluorescence microscopy (Fig. 1). To distinguish specific from nonspecific staining, we compared wild-type egg chambers with those of homozygous spir1 mutants (Fig. S1 A and B). From early oogenesis through stage 9, Spir localizes specifically to the actin-rich cortex of the oocyte (Fig. 1, A and B). We cannot confirm the diffuse cytoplasmic localization observed in mRFP-Spir flies with immunofluorescence because we also observe it in spir1 flies (Fig. S1 B). At stage 10, near the onset of cytoplasmic streaming, Spir staining disappears from the cortex (Fig. 1 C). Because the loss of Spir produces precocious cytoplasmic streaming, this result suggests that cytoplasmic streaming is normally triggered by the destruction or displacement of Spir from the oocyte cortex.

Spir and Capu interact in vivo

Spir and Capu have been shown to interact in vitro (Rosales-Nieves et al., 2006). To determine whether these proteins interact in vivo, we immunoprecipitated Capu from wild-type Drosophila ovary lysates and probed the precipitated material with anti-Spir antibodies. Spir coimmunoprecipitates with Capu but not with beads alone or beads with nonspecific IgG, indicating that Spir and Capu are part of a protein complex in vivo (Figs. 1 D and S1 E).

To further examine the in vivo interaction between Spir and Capu, we studied their subcellular localizations when expressed individually or together in NIH 3T3 fibroblasts. We compared Drosophila and mammalian Spir and Capu family proteins and used truncation mutants to map domains required for interaction. As we reported previously, full-length Spir localizes to punctae (Fig. 2 B) that correspond to the trans-Golgi network, post-Golgi vesicles, and recycling endosomes (Kerkhoff et al., 2001). Full-length Capu (myc tagged) is distributed uniformly throughout the cytoplasm (Fig. 2 B). Coexpression of Spir together with Capu induces a striking change in Capu localization. Capu shifts from a diffuse distribution to discrete punctae that coincide with the localization of Spir (Fig. 2 C). Using truncation mutants, we found that the N-terminal portion of Spir and the C-terminal portion of Capu are necessary for colocalization (Fig. S2 B, available at http://www.jcb.org/cgi/content/full/jcb.200706196/DC1). We then coimmunoprecipitated EGF-Capu-FH2 with myc-Spir-NT from cells expressing both constructs, demonstrating that colocalization corresponds with interaction (Fig. 2 F).

The N-terminal half of the Spir proteins, which is necessary for the colocalization of Spir and Capu, contains two different structural motifs: one KIND domain and a cluster of four
WH2 domains (Fig. 2 A). Rosales-Nieves et al. (2006) mapped the interaction between Spir and Capu to the Capu-FH2 domain and the Spir-WH2 cluster. They also reported a weak interaction with the ~150-amino acid region adjacent to the WH2 cluster containing the C-terminal half of the KIND domain. However, they did not test for an interaction with the intact KIND domain. We found that the KIND domain is sufficient for colocalization with an EGFP-tagged Capu-FH2 (Fig. 2). We targeted the KIND domain to membranes using a C-terminal Ha-Ras-CAAX motif (Schaber et al., 1990). When expressed in NIH 3T3 fibroblasts, KIND-CAAX localizes to the plasma membrane and to cytoplasmic spots (Fig. 2 D, red). Coexpression of an EGFP-Capu-FH2 led to colocalization with the membrane-targeted KIND (Fig. 2 D). We could not test the WH2 domain in this context because the CAAX motif did not effectively drive WH2 localization to the plasma membrane or distinct punctae (unpublished data).

We also observed the colocalization of mammalian Spir and Capu family proteins (Spir-1 and Fmn2; Fig. S2 C). The Spir-1–KIND and Fmn2-FH2 domains were sufficient to mediate this interaction (Figs. 2 E and S2 C). The interaction is specific because a KIND domain from the protein very-KIND (VKIND-KIND-CAAX; Mees et al., 2005) does not colocalize with or pull down Fmn2-FH2, nor does the FH2 domain of the formin mDia1 (mDia1-FH2) colocalize with or pull down Spir-1–KIND (Fig. S2, D and E). These data suggest that the interaction between Spir and Capu family proteins is specific and conserved.

**Spir and Capu interact directly**

To further examine the interaction between Spir and Capu, we determined the affinity of purified KIND for purified Capu-FH1FH2 using fluorescence polarization anisotropy. Capu-FH2 and Capu-FH1FH2 behave similarly, but the longer construct is more stable, so for the majority of experiments, we used Capu-FH1FH2. We labeled an endogenous cysteine in KIND with AlexaFluor488 and measured changes in polarization anisotropy induced by Capu-FH1FH2. We determined the affinity by fitting the data with a quadratic binding curve (K_d = 1 ± 2 nM; Fig. 3 A).
To determine whether the label affected binding, we also determined the affinity of unlabeled KIND by using it to compete with the labeled protein ($K_d = 5 \pm 3$ nM; Fig. 3 A, inset). The agreement indicates that attachment of the fluorophore has little effect on the interaction. The affinity measured using Capu-FH2 rather than FH1FH2 was nearly indistinguishable ($K_d = 9 \pm 6$ nM; Fig. S2 F).

We found that the WH2 cluster binds weakly to Capu-FH2. The addition of Capu-FH1FH2 to AlexaFlour488-labeled WH2 produced a saturable change in fluorescence intensity,
To measure the stoichiometry of the complex, we combined AlexaFluor488-labeled Capu-FH1FH2 with KIND at three different ratios and spun the mixtures to equilibrium at multiple speeds. We determined the equilibrium distribution of Capu-FH1FH2 by measuring the absorbance of the attached fluorophore. These data were best fit by a single-species model with a molecular mass close to that predicted for two KIND domains plus one Capu-FH1FH2 dimer (predicted, 223.6 kD vs. measured, 225 kD; Fig. 4 D). The fact that the data fit a single-species model is consistent with a high affinity interaction between KIND and Capu-FH1FH2. We detected no evidence of the Capu-FH1FH2 dimer either free or bound to a single KIND domain.

Functional consequences of the Spir-Capu interaction

Spir family proteins inhibit actin nucleation by Capu family formins (Fig. 5 and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200706196/DC1). Because Spir binds the nucleation domain of Capu, we used pyrene-actin fluorescence assays to determine the effect on Capu activity. Both Capu-FH2 and Capu-FH1FH2 promote rapid actin filament assembly. The addition of KIND caused a dose-dependent decrease in nucleation activity (Figs. 5 A and S3 A). Because of local concentration effects, the addition of a weak interaction to a stronger one can have an effect on overall affinity. Thus, we also tested a mutant form of NTSpire (NTSpire[A*B*C*D*] from Quinlan et al., 2005), which includes both the KIND domain and the WH2 cluster but nucleates only very weakly (Fig. S3 B). By plotting the rate of nucleation versus the concentration of KIND (Figs. 5 B and S3 D) and fitting the data with a quadratic binding curve, we determined inhibition constants (Ki). In all cases, Spir inhibited FH2-dependent nucleation by >90% and with comparable apparent affinities (5–10 nM; Fig. 5 B). These Ki values agree well with the Kd measured by polarization anisotropy, but, from our data, we cannot determine whether the binding of one or two KIND domains is required for inhibition. We observe the same effect with the mammalian proteins (Fig. S3 C). The Kd and Ki of the Spir–1–KIND–Fmn2–FH2 interaction are higher than observed with Drosophila isoforms but, in general, agree with each other (300 ± 60 and 190 ± 40 nM; Figs. S2 G and S3 E).

Neither Drosophila nor mammalian KIND affects spontaneous actin assembly (Fig. S4, A and B; available at http://www.jcb.org/cgi/content/full/jcb.200706196/DC1), demonstrating that the effect is specific to the activity of the Capu-FH2 domain. Also, Spir–1–KIND had no effect on actin nucleation by FH2 domains from Diaphanous family formins mDia1 and mDia2 (Fig. S4 C), indicating that the inhibitory effect of Spir-KIND domains is specific to Capu family formins.

Capu does not inhibit actin nucleation by Spir (Fig. 5 C). To assess the effect of Capu on Spir-dependent nucleation, we mutated Capu-FH1FH2. Mutating Ile-706 to Ala (analogous to Ile-1431-Ala in Saccharomyces cerevisiae Bni1; Xu et al., 2004) almost completely abolishes nucleation activity. The addition of Capu-FH1FH2(1706A) to NTSpire enhanced nucleation activity (Fig. 5 C, green vs. blue traces). The effect increased with increasing concentrations of Capu-FH1FH2(1706A) until approximately equimolar concentrations of proteins were present.
Further increases in Capu-FH1FH2(I706A) concentration decreased activity. This dose response is consistent with an enhancement mechanism dependent on the dimerization of Spir via the KIND–FH2 interaction. We observed the same effect with mammalian isoforms (unpublished data). Although we detect binding between the two domains, the Capu-FH1FH2 domain has no effect on actin nucleation by the WH2 cluster alone (Fig. 5 C, inset), confirming that enhancement depends on interaction between the KIND and FH2 domains.

When active Capu-FH1FH2 and NTSpir are combined, the measured nucleation rate reflects a combination of inhibition and enhancement activity (Fig. 5 D). For example, the rate of polymerization in the presence of 100 nM Capu-FH1FH2 and 250 nM NTSpir falls between the rates of either nucleator alone. Nucleation by 100 nM Capu-FH1FH2 has virtually no lag, whereas nucleation by 250 nM NTSpir has a marked lag (~30 s). When the two are combined, a long lag is observed that is consistent with the potent inhibition of Capu-FH1FH2 nucleation.
Rosales-Nieves et al. (2006) did not observe such synthetic activity when they combined Capu-FH2 and SpirD (equivalent to NTSpir). One possible explanation is that the KIND domain was not folded correctly in these experiments. When we treat NTSpir with denaturant (e.g., GdnHCl) or the KIND domain is absent (WH2 alone), Spir retains nucleation activity, but Spir and Capu do not interact in the polymerization assay (Fig. 5 C, inset; and not depicted).

Spir-KIND competes with microtubules for binding to Capu-FH2 (Fig. 6; Rosales-Nieves et al., 2006). The FH2 domain of formins is known to bind microtubules in vitro and in vivo (Wallar and Alberts, 2003). Rosales-Nieves et al. (2006) reported that Capu-FH2 cross-links actin and microtubules and that this activity is modulated by Spir. We also assessed the ability of Capu family formins to bind microtubules and tested the effect of the KIND domain on this interaction. We found that both Drosophila Capu and Fmn2 cosediment with microtubules (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200706196/DC1), whereas KIND domains do not detectably bind microtubules (Fig. S5 B). We confirmed that Capu-FH1FH2 cross-links microtubules by examining solutions of taxol-stabilized microtubules mixed with Capu-FH1FH2 by fluorescence microscopy and by performing polymerization assays under conditions that require a factor that stabilizes or cross-links tubulin nuclei.
The addition of KIND to Capu and microtubules decreased microtubule binding by Capu in a dose-dependent manner (Fig. 6 A). About 2.5 μM KIND is necessary to compete half of the Capu-FH1FH2 away from 2 μM of polymerized tubulin, indicating that microtubules and KIND bind Capu-FH1FH2 with similar affinity. By fitting the data to a competition binding curve, we measured an affinity of Capu for microtubules of \(< 1 \text{nM} \) (Fig. 6 C). We found no difference in competition with NTSpir versus KIND alone, indicating that the WH2 cluster does not contribute measurably to this inhibitory interaction (Fig. S5 C).

Spir-KIND also regulates actin bundling by Capu (Fig. 6). In addition to binding barbed ends, some formins also bind the sides of actin filaments and bundle them (Harris et al., 2004; Michelot et al., 2005). To test for actin bundling, we mixed 0.5 μM Capu-FH1FH2 with 2 μM phalloidin-stabilized actin.
We observed bundles directly by fluorescence microscopy (Fig. S5 G) and indirectly with a low speed pelleting assay, in which only cross-linked networks or bundles of actin sediment (Fig. 6 D; Harris et al., 2006). The majority of the actin was bundled in the low speed pelleting assay. As a control, we used 0.5 μM α-actinin, a known actin cross-linker. Actin is in the supernatant when alone and in the pellet when α-actinin is added. Examination of the actin showed tight bundling in the presence of Capu-FH1FH2 similar to other formins and distinct from the loose networks created by α-actinin (Fig. S5 G; Wachsstock et al., 1993; Harris et al., 2006). Capu-FH1FH2 bundles more effectively than α-actinin, most likely reflecting a difference in off rates. We measured the effect of Spir on actin bundling by mixing 0.5 μM Capu-FH1FH2 with 2 μM actin and a range of concentrations of KIND or NTspir. We then performed high speed cosedimentation assays and low speed cross-linking assays. We quantified Capu-FH1FH2 in the supernatants and pellets as a function of KIND concentration. By fitting the data to a competition binding curve, we found that the Kd of Capu for the sides of actin filaments is 7 ± 1 nM (high speed) or 6 ± 2 nM (low speed; Fig. 6, B and C; and not depicted).

**Discussion**

The spire and cappuccino genes have been linked since their discovery in a genetic screen 17 yr ago. We find that the KIND domain of Spir binds with high affinity to the Capu-FH2 domain at a stoichiometry of 2:2 (two KIND monomers to one FH2 dimer). We also find that the WH2 cluster of Spir interacts with Capu-FH2 but that this interaction is three orders of magnitude weaker than that between the Capu-FH2 and the KIND domain. Although we detect binding between the two domains, the Capu-FH2 domain has no direct effect on actin nucleation by the Spir-WH2 cluster. However, if the KIND domain is present and correctly folded, binding of the FH2 dimer increased nucleation activity of the WH2 cluster. On the other hand, the KIND domain potently inhibits actin nucleation by the Capu-FH2 domain. Constructs containing both the KIND and WH2 cluster do not enhance the inhibition of Capu-FH2-mediated actin nucleation or microtubule bundling over that observed for the KIND domain alone. For these reasons, we propose that the KIND–FH2 interaction is more physiologically relevant than the WH2–FH2 interaction. Additional structural and functional studies of the KIND domain are required to determine how many KIND domains are required to inhibit actin nucleation and to compete for actin and microtubule binding.

We originally identified the KIND module as a conserved region in the N-terminal half of Spir proteins (Ciccarelli et al., 2003) and named the region based on its sequence similarity to the C-lobe of the protein kinase fold (Ciccarelli et al., 2003). The KIND domain is found only in metazoa, and its consensus sequence lacks catalytic residues required for kinase activity. Because the substrates of protein kinases interact with α-helical regions in the C-lobe (Knighton et al., 1991; Tanoue and Nishida, 2002) we hypothesized that the KIND domain evolved from a functional kinase into a protein–protein interaction domain. The discovery that the Spir KIND domains bind specifically to Capu family FH2 domains supports this hypothesis.

What role do Spir and Capu play in oogenesis? Spir disappears from the oocyte cortex at stage 10, when rapid streaming normally begins and its absence in spire mutant flies leads to premature streaming. This strongly suggests that Spir plays an inhibitory role in rapid streaming. We do not yet know whether endogenous Capu has the same restricted temporal pattern observed for Spir. This information will be essential to understanding the nature of the Spir–Capu complex and its role during oogenesis. We find that Spir and Capu interact in the oocyte, and Rosales-Nieves et al. (2006) found that GFP fusions of these protein both exist at the oocyte cortex, placing them in an ideal location to coordinate actin and possibly anchor microtubules. Rapid streaming is, in part, characterized by bundling and movement of microtubules. Capu bundles microtubules, which is an activity regulated by Spir. If Spir is removed at stage 10 but Capu remains, Capu could play a role in reorganizing the microtubule cytoskeleton and possibly coordinating it with the actin cytoskeleton. A complete understanding of how Spir and Capu achieve this coordination depends on knowing when and how the Spir–Capu complex is regulated.

Capu and other members of the formin family nucleate de novo actin filament assembly and remain associated with elongating barbed ends of newly formed filaments (Pring et al., 2003; Quinlan et al., 2005). The activity of most formin family proteins is regulated by an autoinhibitory interaction between an N-terminal sequence (the Diaphanous inhibitory domain [DID]) and a C-terminal sequence (the Diaphanous autoinhibitory domain [DAD]). Small G proteins of the Rho family stimulate nucleation activity by binding to the DID domain and disrupting its interaction with DAD. However, Capu family formins lack both DID and DAD domains (Higgs and Peterson, 2005). In fact, Rosales-Nieves et al. (2006) did not observe autoinhibition when combining the N terminus of Capu with the FH2 domain, as has been observed for mDia1 (Li and Higgs, 2003). Our results argue strongly that Capu activity is regulated in trans by interaction with Spir.

The mechanism of actin nucleation by Spir is very different from that of formins like Capu. Spir binds four actin monomers using four closely apposed binding sites and then assembles them into a filament nucleus. After nucleation, Spir proteins remain associated with the slow-growing pointed end of the new filament. If Spir and Capu always function together as a single filament-forming complex, we suggest that their activities might synergize. One intriguing possibility is that Spir nucleates filaments whose free barbed ends are then handed off to Capu. Such a mechanism would enable the independent control of filament nucleation and barbed end binding. The tight binding that we measure suggests that Spir and Capu may not dissociate upon nucleation but that actin and microtubules do bind competitively. This idea begs two important questions: (1) Does the activation of Capu require the complete dissociation of Spir, or can the two proteins function together as a single filament–forming unit? (2) How is the Spir–Capu interaction modulated by upstream signaling systems? Recent data implicate the GTPase Rho as a regulator of Spir–Capu interaction in Drosophila (Rosales-Nieves et al., 2006). The Spir–Capu interaction is evolutionally conserved, but whether or not this mode of regulation is conserved remains to be tested.
Materials and Methods

Fly strains and immunofluorescence
Both Canton-S and w^{1118} flies were used as wild type (provided by R. Bainton, University of California, San Francisco, San Francisco, CA). sipr, cn, bnr/ CyO, pr, spire, cn/CyO flies were obtained from T. Schupbach (Bloomington Drosophila Stock Center, Indiana University, Bloomington, IN). Transgenes were cloned into a pUASP vector and expressed in the germline with Vp16nos:Gal4 or pCp:Gal4; NGT40:Gal4; Vp16nos; Gold triple maternal driver lines (provided by L. Cooley, Yale, New Haven, CT). For immunofluorescence, they were fixed and stained according to methods described by Robinson and Cooley (1997). For actin visualization, ovaries were incubated in 1–2 μM rhodamine-conjugated phalloidin (Invitrogen). For Spire immunolocalization, ovaries were incubated with ~1 μg/ml antibody, and Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Invitrogen) was used at a 1:1,000 dilution. Samples were mounted in fluorescence mounting medium (DakoCytomation). For live imaging, ovaries were dissected and teased apart under Halocarbon 700 oil (Sigma-Aldrich) at room temperature. In both cases, images were collected with a plan-Neofluor 25× 0.8 NA objective lens on a confocal microscope (LSM 510 META; Carl Zeiss MicroImaging, Inc.) with its proprietary software. Final rotations, cropping, and conversion to TIFF format were performed in ImageJ (National Institutes of Health).

DNA constructs
Standard PCR and cloning methods were used to make DNA constructs.

Cell culture and transfections
NIH 3T3 mouse fibroblasts were cultured in DME supplemented with 10% FCS, glutamate, penicillin, and streptomycin at 37°C in a CO2 (10%) incubator. The cells were transiently transfected with eukaryotic expression vectors at 4°C was incubated with glutathione–Sepharose 4B beads (GE Healthcare) for 2 h in 10 mM Tris-HCl, pH 7.4, and 0.1% Tween) and sonicated. The soluble extract was harvested after only 1.5 h. pET-20b + bacteria was transformed with E. coli Rosetta (NTSpir) were expressed in bacteria (Novagen). Cells were grown at 37°C to an optical density (A 600) of 0.6–0.8, cooled to 21°C, and diluted to 1/20 of the original volume. After centrifugation, the bacteria were suspended in TBS-Tween buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 0.1% Tween) and sonicated. The soluble extract was incubated with glutathione–Sepharose 4B beads (GE Healthcare) for 2 h at 4°C. The beads were washed twice with TBS-Tween buffer and resuspended in the same buffer for pull-down assays.

Immunoprecipitation
Immunoprecipitation from fly ovaries was performed according to the methods of Rosales-Nieves et al. (2006; ~100 flies were used for each condition). Immunoprecipitation from tissue culture cells was performed as follows: 36 h after transfection, NIH 3T3 cells were lysed with immunoprecipitation lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 0.1% NP-40, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 10 mM NaF, and 0.2 mM Na3VO4). Anti-myc 9E10 monoclonal antibodies (Santa Cruz Biotechnology, Inc.) were added to the cleared cell lysate to a final concentration of 5 μg/ml and incubated for 1 h on ice. Protein G–agarose (Roche) was added, and the sample was rotated for 150 min at 4°C. The beads were washed twice with immunoprecipitation lysis buffer, and bound proteins were analyzed by Western blotting.

Protein labeling
Acanthamoeba actin was labeled with pyrene iodoacetamide as described previously (Cooper et al., 1983). Purified KIND (human and Drosophila) was incubated with 1–2 μg GST/GST-KIND-KIND/GST-His-Spir-1–KIND/GST-His-Spir-1–KIND/VH2 fusion protein was coupled to 50 μl glutathione–Sepharose beads and washed twice with pull-down buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40, and 10% glycerol). NIH 3T3 fibroblasts transiently expressing EGFP-mDia1-F2 and EGFP-mFmn2-FH2 were lysed with pull-down buffer for 40 min at 4°C. Cleared lysate was incubated with the glutathione–Sepharose beads for 2 h. The beads were gently washed five times in pull-down buffer and boiled in SDS sample buffer. The samples were analyzed by Western blotting.

Table I. Summary of purified proteins used in biochemical assays (all His tagged)

| Construct name | Residues | Extinction coefficient |
|----------------|----------|------------------------|
| Drosophila isoforms | | |
| NTSpir | 1–520 | 25,575 c cm−1 |
| KIND | 1–327 | 17,452−1 c cm−1 |
| WH2 | 366–482 | 15,220 c cm−1 |
| Capu-FH2 | 573–1,058 | 53,760 c cm−1 |
| Capu-FH1-FH2 | 467–1,058 | 75,200 c cm−1 |
| Mammalian isoforms | | |
| Spir-1–KIND | 2–271 | 22,620 c cm−1 |
| Fmn2-FH2 | 1,124–1,567 | 25,445 c cm−1 |

*Quantitative SDS-PAGE with Sypro-Ruby staining.
*Comparison of absorbance at 280 nm of native protein with protein denaturated in 6 M GdnHCl.
*Analytical ultracentrifugation.
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Cross-linking assays

Two actin-cross-linking assays were used. An actin-cross-linking assay was performed according to Harris et al. (2006) with minor modifications. 50 μl solutions containing 2 μM phalloidin-stabilized actin plus 0.5 μM Capu-FH1FH2 or α-actinin or each component separately were mixed in KMEH, allowed to stand at RT for 10 min, and centrifuged at 16,000 g for 5 min. 40 μl of the supernatant was removed, and the pellet was washed once and resuspended in 50 μl. Equal amounts of supernatants and pellets were analyzed by SDS-PAGE. For microscopy experiments, we substituted AlexaFluor488-phalloidin for unlabeled phalloidin. After standing for at least 10 min, solutions were diluted 1:100 in KMEH and added to poly-L-lysine–coated flow chambers at room temperature. Images were collected with a plan Apo 60×, 1.2 NA objective lens and camera (C4742-98; Hamamatsu) on a microscope (TE300; Nikon) with Simple PCI software (Compuex, Inc.).

Two microtubule cross-linking assays were used. The first assay was performed according to the methods of Westermann et al. (2005). 50 μl containing 10 μM tubulin (doped with 10% rhodamine-tubulin [Cytoskeleton, Inc.]) plus 0.5 μM Capu-FH1FH2 or GST-Spastin(E542A) (a mutant in the Walker B site that does not sever [Roll-Mecak and Vale, 2005]) were mixed on ice in 80 μM Pipes, pH 6.9, 1 mM EGTA, 1 mM MgCl2, 1 mM GTP, and 25% glycerol. They were incubated at 37°C for 25 min and fixed with 1% glutaraldehyde. Samples were diluted 1:100, introduced into a flow chamber, and examined by fluorescence microscopy. For the second assay, the microtubules were prepolymerized at high concentration (18 μM), taxol stabilized, and diluted and combined with Capu-FH1FH2 or GST-Spastin(E542A) (10.0.5 μM). These solutions were allowed to stand at room temperature for at least 15 min before dilution (1:100) and visualization in a flow chamber, with the same equipment used for actin-cross-linking assays.

Online supplemental material

Fig S1 shows a stage 9 egg chamber expressing Spir-mRFP [A], immunofluorescence and Western blots showing the specificity of anti-Spir antibody [B–D], complete Western blots from Fig. 1 E [E], and a Western blot of an oocyte coimmunoprecipitated with and without latrunculin [F]. Fig. S2 shows additional images of Spir and Capu expression in NIH 3T3 cells [A–D], GST pull-down with mammalian isoforms of Spir and Capu [E], an anisotropy experiment with Capu-FH2 [F], and an anisotropy experiment with mammalian isoforms of Spir and Capu [G]. Fig. S3 shows the inhibition of Capu-FH2–mediated actin nucleation by KIND [A–C], sample analysis of polymerization assays [D], the inhibition curve for mammalian isoforms of Spir and Capu [E], and alternate inhibition analysis [F]. Fig. S4 shows that KIND does not influence spontaneous actin polymerization [A and B] and that Spir–1–KIND does not interact with mDia1 or mDia2 [C]. Fig. S5 shows gels of FH2 domains and KIND mixed with tubulin [A and B], a cosedimentation assay with Capu-FH1FH2, tubulin, and NTSpir (instead of KIND; C and D), and images of Capu-FH1FH2–mediated tubulin polymerization and bundling of microtubes and actin [E–G]. Online supplemental material is available at http://www.jcb.org/cgi/content/ full/jcb.200706196/DC1.

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