Wash functions downstream of Rho and links linear and branched actin nucleation factors

Raymond Liu, Maria Teresa Abreu-Blanco, Kevin C. Barry, Elena V. Linardopoulou, Gregory E. Osborn and Susan M. Parkhurst*

Wiskott-Aldrich Syndrome (WAS) family proteins are Arp2/3 activators that mediate the branched-actin network formation required for cytoskeletal remodeling, intracellular transport and cell locomotion. Wasp and Scar/WAVE, the two founding members of the family, are regulated by the GTPases Cdc42 and Rac, respectively. By contrast, linear actin nucleators, such as Spire and formins, are regulated by the GTPase Rho. We recently identified a third WAS family member, called Wash, with Arp2/3-mediated actin nucleation activity. We show that Drosophila Wash interacts genetically with Arp2/3, and also functions downstream of Rho1 with Spire and the formin Cappuccino to control actin and microtubule dynamics during Drosophila oogenesis. Wash bundles and crosslinks F-actin and microtubules, is regulated by Rho1, Spire and Arp2/3, and is essential for actin cytoskeleton organization in the egg chamber. Our results establish Wash and Rho as regulators of both linear- and branched-actin networks, and suggest an Arp2/3-mediated mechanism for how cells might coordinate these structures.

KEY WORDS: Arp2/3, Rho1 GTPase, Spire, Wash, Wiskott Aldrich Syndrome, Actin nucleation, Drosophila

INTRODUCTION

The actin cytoskeleton consists of linear and branched filament networks required for processes ranging from cell division to migration (Chhabra and Higgs, 2007; Faix and Grosse, 2006; Goley and Welch, 2006). How these two networks function and are coordinated is of major interest, as their misregulation results in infertility, immunodeficiency, and tumor metastasis in humans (Bione et al., 1998; Burns et al., 2004; Yamaguchi and Condeelis, 2007). Linear actin filament networks, required for cytokinesis and filopodia formation, are regulated by nucleators and bundling proteins, which enhance filament formation rates and control filament organization, respectively (Chhabra and Higgs, 2007; Faix and Grosse, 2006; Goode and Eck, 2007; Wallar and Alberts, 2003). Examples include Spire and the formin Cappuccino (Capu), which exhibit both nucleation and bundling activity and are essential for oocyte development during Drosophila oogenesis (Chhabra and Higgs, 2007; Kerkhoff, 2006; Manseau and Schupbach, 1989; Quinlan et al., 2005; Quinlan et al., 2007; Rosales-Nieves et al., 2006; Theurkauf, 1994; Wang and Riechmann, 2008). Both Spire and Capu are regulated by the GTPase Rho1 of the Rho family of small GTPases, which is upstream of other linear nucleators, such as Diaphanous, and is considered a key regulator of linear filament formation (Goode and Eck, 2007; Wallar and Alberts, 2003).

Branch or dendritic actin filament networks, which are required for phagocytosis and lamellipodia formation, are primarily regulated by the Arp2/3 complex and by nucleation-promoting factors that associate with Arp2/3 and actin monomers to nucleate daughter filaments off of existing mother filaments (Goley and Welch, 2006; Takenawa and Suetsugu, 2007). Like Spire and Capu, Arp2/3 is essential for Drosophila oogenesis, specifically for maintaining proper nurse cell cyto-architecture and function (Hudson and Cooley, 2002). One family of Arp2/3 activators, the Wiskott-Aldrich Syndrome (WAS) protein family, has been shown to function downstream of Rho GTPases to mediate the branched-actin network formation required for cytoskeletal remodeling, intracellular transport and cell locomotion (Ben-Yaacov et al., 2001; Campellone et al., 2008; Linardopoulou et al., 2007; Stradal et al., 2004; Takenawa and Suetsugu, 2007; Zallen et al., 2002). WASP and SCAR/WAVE, the two founding subclasses of the family, are activated by the GTPases Cdc42 and Rac, respectively (Stradal et al., 2004; Takenawa and Suetsugu, 2007). Two new WAS subclasses, WASH and WHAMM, have recently been reported (Campellone et al., 2008; Linardopoulou et al., 2007) and have been shown to exhibit Arp2/3-mediated branched nucleation activity. Which GTPases might regulate them, however, is not known.

Here, we report that Drosophila Wash functions downstream of Rho1 and interacts with Spire and Capu to regulate actin and microtubule organization during Drosophila oogenesis. We show that Wash nucleates actin in an Arp2/3-dependent manner, and exhibits F-actin and microtubule bundling and crosslinking activity that is regulated by a pathway involving Rho1, Spire and Arp2/3. We find that Wash genetically interacts with Rho1, Capu, Spire and Arp2/3, and is essential for actin cytoskeleton organization during oogenesis. Our results establish Wash and Rho as regulators of both linear- and branched-actin networks, and suggest an Arp2/3-mediated mechanism of cytoskeletal control through which cells might coordinate regulate linear and branched architectures.

MATERIALS AND METHODS

Fly strains and genetics

Flies were cultured and crossed on yeast-cornmeal-molasses-malt and maintained at 25°C. Alleles used in this study were: wasp1135, Rho116, capu1234, spire118, spire37, wimp and Sop5254. All of these alleles have been previously described (Hudson and Cooley, 2002; Linardopoulou et al., 2007; Magie and Parkhurst, 2005; Parkhurst and Ish-Horowicz, 1991; Rosales-Nieves et al., 2006), except for spire118, which is described in Fig. S2A in the supplementary material. Fly lines for our RNAi studies were obtained from the VDRC Stock Center (Wash RNAi stocks 24642 and 39769) and were driven with a maternal-Ga4 line (7063; Bloomington Stock Center).

*Author for correspondence (e-mail: susanp@fhcrc.org)

Accepted June 5 2009
Microscopy and image analysis
Immunofluorescence, confocal microscopy, and live imaging of egg chambers were performed as previously described (Rosales-Nieves et al., 2006). Movies 1-5 in the supplementary material are provided at 200X real time; Movie 6 in the supplementary material is provided at 45X real time. Swirling speeds were analyzed with ImageJ by individually tracking the distance traveled per unit time of 10 yolk granules in an active swirling region of mutant oocytes, or in a random region of a wild-type oocyte. Average speeds were calculated for at least three oocytes, and oocytes from capu^{EY12344} and spirix^{18}/spiri^{Wm} females were used for comparison. For electron microscopy, 10 μl of an F-actin/microtubule crosslinking reaction was pipetted onto a glow-discharged 200 mesh formvar/carbon-coated grid, washed with 0.1 M cacodylate buffer and water, stained with 1% uranyl acetate, and deissected overnight. Images were acquired with a JEOL 1230 transmission electron microscope (TEM).

Plasmids and constructs
Constructs used in this study have been previously described (Linardopoulou et al., 2007; Rosales-Nieves et al., 2006). Additional constructs used were: Wash FL (full-length Wash, amino acids 1-500), Wash A (amino acids 1-124), Wash B (amino acids 125-212), Wash C (amino acids 213-322), Wash D (amino acids 323-352), Wash E (amino acids 353-500), Wash VCA (amino acids 363-500), Wasp (full-length Wasp, amino acids 1-527), Wasp VCA (amino acids 401-527), Scar (full-length Scar, amino acids 1-613), Scar VCA (amino acids 496-613), Whamy VCA (amino acids 419-515) and Whamy FL (full-length Whamy, amino acids 1-515).

Protein expression and biochemistry
Protein expression, bacterial lysate preparation, protein purification, GST pulldowns, and immunoprecipitations were performed as previously described (Linardopoulou et al., 2007; Rosales-Nieves et al., 2006). For expression and purification of full-length Wash, Wasp, Scar and Whamy, constructs were cloned into a modified ‘double-tag’ pGEX (GE, Piscataway, NJ, USA) vector containing a GST protein epitope tag at the 5’ end and a His protein epitope tag at the 3’ end of the protein of interest within internal PreScission protease (PP) cleavage sites (GST>PP>multiple cloning site>PP>His6). For His-tag and or double-tag purification, supernatant from transmission electron microscope (TEM). For His-tag and or double-tag purification, supernatant from transmission electron microscope (TEM).

Antibody production and characterization
BALB/c BYJ Rb(8.12) 5BNR/J mice (Jackson Laboratories) were immunized with GST-Wash. The P3H3 monoclonal line was generated in the Fred Hutchinson Cancer Research Center (FHCRC) Hybridoma Production Facility as described (Magie and Parkhurst, 2005). Western blotting was used to test antibody specificity against in vitro translated Wash, Wasp and Scar, and for the detection of endogenous protein in whole-cell, nuclear and ovary extracts (see Fig. S3 in the supplementary material). Wild-type Drosophila whole-cell (from 0- to 2-hour embryos) and nuclear (from 0- to 12-hour embryos) extracts were gifts from Toshi Tsukiyama (FHCRC).

Actin and microtubule assays
Pyrene actin-polymerization and F-actin/MT-crosslinking assays were performed as previously described (Rosales-Nieves et al., 2006), using a SpectraMax M5 Fluorescence Spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Actin, MTs and Arp2/3 were obtained from Cytoskeleton (Denver, CO, USA).

RESULTS
Wash activates Arp2/3 to nucleate branched actin, and bundles F-actin and microtubules
The mammalian homologs of Wash (WASH), Wasp, Scar/WAVE, and the most recently identified WHAMM subclasses bind Arp2/3 and actin monomers through their C-terminal VCA domains to nucleate actin filaments (Campellone et al., 2008; Linardopoulou et al., 2007; Stradal et al., 2004; Takenawa and Suetsugu, 2007). Using pyrene-actin polymerization assays, we show here that the Drosophila Wash VCA activates Arp2/3 to nucleate actin in vitro, with similar activity to Drosophila Wasp and Scar VCA at equimolar concentrations (Fig. 1A,D). Mammalian homologs of several WAS family proteins have been shown to be regulated by auto-inhibition (Faix and Grosse, 2006; Stradal et al., 2004; Takenawa and Suetsugu, 2007). This does not appear to be the case in flies, as bacterially expressed and purified full-length Drosophila Wash, Wasp and Scar stimulated actin nucleation, requiring molar concentrations of less than threefold that of their VCA fragments to achieve similar levels of activity (Fig. 1B,D). Interestingly, this difference between mammalian and fly actin nucleators has also been observed with the Drosophila formin protein Capu, as it lacks the auto-inhibitory regulation found in its mammalian counterparts (Rosales-Nieves et al., 2006). Drosophila Wasp, Wasp and Scar are thus either constitutively active, or inhibited in trans, as has been suggested for Scar and Capu (Quinlan et al., 2007). Wash stimulation of actin nucleation was both concentration and Arp2/3 dependent, and was not affected by the addition of Rhodin or Rho1^{GTP} (Fig. 1C; data not shown). Whamy (CG12946; the closest Drosophila homolog of both WHAMM and JMY) VCA did not exhibit actin nucleation activity, which is consistent with the Drosophila homolog lacking the conserved tryptophan residue that is required for mammalian WHAMM nucleation (Campellone et al., 2008) (data not shown).

We previously demonstrated that the linear-actin filament nucleators Capu and Spire, in addition to catalyzing actin polymerization, have the ability to directly bundle and crosslink F-actin and microtubules (MTs) (Rosales-Nieves et al., 2006). We were interested in determining whether these last-mentioned biochemical properties apply to Wash, and performed in vitro F-actin and stabilized MT crosslinking/bundling assays with bacterially purified full-length Wash alongside Wasp, Scar and Whamy for comparison. In the absence of additional proteins, F-actin and taxol-stabilized microtubules were not bundled together, but appeared as relatively uniform haystacks of individual short filaments across the field of view (Fig. 1E-E’). The addition of Wash bundled these filaments together to form intertwined aggregate strands of F-actin and MTs, with the concomitant loss of the diffuse haystack appearance (Fig. 1F,G). Wash also crosslinks both F-actin and MTs, as shown by the overlap of F-actin and MT bundles in reactions containing both filaments (Fig. 1H-H’). In contrast to Wash, we found that Wasp bundles only MTs (Fig. 1I-I’), whereas Scar bundles neither (Fig. 1J-J’). Whamy bundled MTs and, to a lesser extent, F-actin (Fig. 1K-K’), consistent with a recent study showing that mammalian WHAMM binds MTs and G-actin (Campellone et al., 2008). We examined the F-actin and MT bundles mediated by Wash by electron microscopy (Fig. 1L-T). In the absence of Wash, individual F-actin and microtubule filaments were scattered in a random pattern (Fig. 1L-R). The addition of Wash induced these filaments to align parallel to each other and form bundles (Fig. 1M-Q). Cross-linking of these filaments by Wash was also observed (Fig. 1S,T). To quantify the extent of bundling in these assays, we performed low-speed co-sedimentation of the proteins.
Fig. 1. See next page for legend.
following bundling assays (Fig. 1U,V). Unbundled actin and MT filaments did not pellet efficiently (1% and 12% in pellet fractions, respectively) and remained in the supernatant following centrifugation (Fig. 1U, actin+MTs lane). F-actin bundles and MT bundles pelletted efficiently (96-99% actin, 85-99% MT) in the presence of Wash, as did Wash itself when combined with F-actin (94%), MTs (90%), or both (95%; Fig. 1U, Wash lanes). To rule out the possibility that Wash pellets F-actin and MTs by forming aggregates that trap F-actin or MTs, we performed assays on Wash alone, and found that Wash did not pellet in the absence of F-actin or MTs. Similar quantifications were performed for Wasp, Scar and Whamy bundling/crosslinking (Fig. 1V). Members of both linear- and branched-actin filament nucleator classes, therefore, can bundle F-actin and MTs.

Wash is essential for oogenesis in Drosophila

Taken together, our results suggest that Wash participates in both branched-filament (Arp2/3) and linear-filament (bundled F-actin) regulation. To examine the role of these two functions of Wash in vivo, we tested whether the removal of Wash disrupted Arp2/3 and actin/MT bundling-dependent processes during Drosophila oogenesis. It is not possible to completely eliminate maternal wash function, as germ lines homozygous for the washΔ185 mutant allele (Linardopoulou et al., 2007) cannot be generated because of cell inviability (data not shown). Therefore, in order to modulate the level of Wash activity, we used the wimp mutation, which reduces transcription at the wash locus, in trans to the loss-of-function wash allele, thereby mimicking a hypomorphic mutation (female genotype is washΔ185/+; wimp+/+; designated reduced wash) (see Fig. S1A,B in the supplementary material) (Parkhurst and Ish-Horowicz, 1991). We found that reduced wash females are sterile, laying eggs with fused dorsal appendages (49%; Fig. 2B,C) or smaller eggs with no dorsal appendages (36%; n=1048; Fig. 2D).

Interestingly, the egg phenotypes we observed are reminiscent of phenotypes associated with mutants lacking Spire, Capu or Arp2/3. Fused dorsal appendages are a manifestation of improper dorsoventral patterning, and are associated with spire and capu mutants, in which premature ooplasmic streaming in the oocyte (described below) results in the mislocalization of polarity markers (Manseau and Schupbach, 1989; Theurkauf, 1994). Small eggs are a phenotype previously associated with Arp2/3 mutants, in which defects in nurse cell cyto-architecture and ring canal formation result in incomplete cytoplasmic transfer from nurse cells to the oocyte (a process called nurse cell dumping), which leads to smaller eggs (Hudson and Cooley, 2002). Our results suggest that Wash plays a role in these two processes.

Wash genetically interacts with Rho1 and the linear-actin nucleators Capu and Spire

We first examined whether Wash regulates ooplasmic streaming. Up to stage 10 of oogenesis, the oocyte gradually increases in size as nurse cells transport their cytoplasmic content into the oocyte. During stage 10 of development, subcortical arrays of microtubules reorganize along the oocyte periphery, and motor proteins begin to drive the coordinated movement of cytoplasmic material to redistribute the contents, in a process termed ooplasmic streaming. Premature onset of swirling results in mislocalized polarity markers and patterning defects. We previously demonstrated a complex role for Rho1, Capu and Spire in the coordinated regulation of actin and microtubule cytoskeleton dynamics to control the timing of ooplasmic streaming, and proposed that the bundling and crosslinking properties of Capu and Spire are essential for the process (Rosales-Nieves et al., 2006) (see also Discussion and Fig. S2 in the supplementary material). Given that Wash also has F-actin and microtubule bundling activity, we examined stage 7 oocytes from ovaries of reduced wash flies and found that these oocytes exhibited premature ooplasmic streaming compared with wild type (Fig. 2E,F; see also Movies 1 and 2 in the supplementary material). Yolk granules in wild-type oocytes exhibited uncoordinated, saltatory movements (20.9±2 nm/second), whereas the classical swirling mutants capu and spire exhibited higher rates of directed granule movements (165.5±29 nm/second and 41.5±3.2 nm/second, respectively). Yolk granules in reduced wash oocytes exhibited speeds that were almost twice those observed in wild type (38.6±1.7 nm/second, P<0.003, n=3) and, importantly, these movements were concerted and coordinated (Fig. 2E′-F′). In wild-type oocytes, tubulin staining showed an anterior to posterior gradient of microtubules in the oocyte and along the cortex (see arrows in Fig. 2J). Consistent with the premature swirling phenotype, reduced wash oocytes lost this organization (Fig. 2K). Interestingly, tubulin staining of reduced wash oocytes also revealed that the nucleus is mislocalized by stage 7 and continued to be mislocalized during later stages of oocyte development (arrowhead in Fig. 2K; arrow in Fig. S1E in the supplementary material). Because this is not a feature of classical swirling mutants, this nucleus mislocalization phenotype suggests that Wash is likely to have additional roles during oogenesis. To assess cortical actin organization in reduced wash oocytes, we stained oocytes with phalloidin and examined the band of actin surrounding the lateral to posterior regions of the oocyte. Wild-type oocytes exhibited a smooth and even band of actin throughout (Fig. 2O′), whereas reduced wash oocytes exhibited a disrupted and discontinuous band of actin (Fig. 2P′). We observed the same premature swirling phenotype in oocytes from a UAS-wash-RNAi line expressed using a maternal-Gal4 driver (Fig. G′; see also Fig. S1C and Movie 3 in the supplementary material). Microtubule and actin structures were also disrupted (Fig. 2L-Q′; Fig. S1D-H in the supplementary material), indicating that the reduced wash phenotypes observed were due to the knockdown of
wash. Together, this suggests that Wash, like Capu and Spire, functions to maintain proper organization of actin and microtubule bundles at the oocyte cortex.

To determine whether Wash acts in concert with Capu and Spire in the Rho1/Capu/Spire pathway (Rosales-Nieves et al., 2006), we examined oocytes from females transheterozygous for wash and Rho1, capu and spire. We found that oocytes from wash-Rho1 and wash-capu females swirl prematurely, with wash-capu oocytes exhibiting more vigorous yolk granule movements (71.8±8 nm/second, n=3, P<0.008) than wash-Rho1 oocytes (31.7±0.5 nm/second, n=3, P<0.006; Fig. 2H-I; see also Movies 4 and 5 in the supplementary material). As in reduced wash oocytes, microtubule structures were disrupted in wash-Rho1 and wash-capu mutants, and did not exhibit the cortical gradient established in wild-type oocytes (see arrows in Fig. 2J-N). Cortical actin organization was also affected in these mutants (Fig. 2O-S), although not as severely as that observed in reduced wash oocytes. Oocytes from wash-spire females were not recoverable, as these females exhibit a dominant zygotic interaction and die as early embryos with severe patterning defects (Fig. 2T-V). These results suggest that Wash acts to maintain correct microfilament and MT bundle architecture during oocyte development. Consistent with this model, Wash accumulated at the oocyte cortex throughout oogenesis (Fig. 2W-X; Figs S2, S3 in the supplementary material).

Wash binds Spire and is downstream of Rho1
Our results suggest that Wash functions within the Rho1/Spire/Capu pathway of regulation during Drosophila oogenesis. We next examined whether Wash interacts molecularly with these proteins. Rho GTPases are known to regulate other WAS family members, as
Cdc42 and Rac are upstream of Wasp and Scar, respectively (Goley and Welch, 2006) (Fig. 3A). Rho, however, has only been associated with linear-actin nucleators, such as Capu, Spire or Diaphanosus. We were therefore intrigued by the possibility that Rho might regulate the function of Wash, a branched-actin nucleator. To determine whether Rho, or other Rho family GTPases regulate Wash, we looked for a direct interaction between Wash and Rho1, Cdc42 and Rac in glutathione S-transferase (GST) pull-down assays. We found that Wash binds Rho1, and preferentially in its GDP-exchanged (active) state (Rho1GTP), but not Cdc42 or Rac (Fig. 3B). In control assays, we found that Wasp binds to Rho1GTP (Rosales-Nieves et al., 2006). As Wash exhibits similar properties to Capu and Spire, we investigated whether Wash is similarly regulated in bundling/crosslinking assays. We found that SpirD also efficiently inhibits Wash bundling and crosslinking activity (Fig. 5A-C), and, as with Capu and Spire, this inhibition is relieved by the addition of Rho1GTP (Fig. 5D-E), whereas the non-interacting SpirD3, SpirD1 and SpirD4 protein fragments do not (Fig. 5H-H'; see also Fig. S4 in the supplementary material). Moreover, inhibition by SpirD2 is not relieved by Rho1GTP (Fig. 5G-G'), suggesting that the Rho relief of inhibition requires Rho binding to Spire through the SpirD3 region (Rosales-Nieves et al., 2006). Together with our genetic data, our results suggest that Wash functions as a Rho effector together with Spire and Capu to control the timing of ooplasmic streaming (see Discussion).

Surprisingly, we found that Arp2/3 also inhibits Wash actin and microtubule bundling/crosslinking activity, as neither actin nor MT bundling are observed following the addition of Arp2/3 to Wash in our assays. Unlike SpirD inhibition, Arp2/3-mediated inhibition is not relieved by Rho1GTP. Interestingly, while Arp2/3 inhibits Wash bundling and crosslinking, we observed a wave of actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. This process is reminiscent of the dense actin lattice formation that developed over time following confocal laser (or heat) exposure, with no change to unbundled microtubules. To test whether Wash interacts with Capu and Spire in the Rho1/Spir/SpirC pathway, we expressed full-length Wash protein and Wash fragments (Fig. 4A), and assayed for binding by GST pull-down to itself, to Capu, and to the Spire protein isoforms SpirA, SpirC and SpirD (Fig. 4B-F). We found that Wash binds to itself through its N-terminal WHD1 region (Wash A), indicating possible dimerization, and binds all Spire isoforms (also through Wash A), but not Capu (Fig. 4C). To determine whether Wash associates with Rho, Spire and Capu proteins in vivo, we immunoprecipitated Wash from early embryo extracts, using an antibody specific for Wash, and probed western blots of the antibody-bound complexes for Rho, Capu and Spire. We found that Wash associates with all of these proteins, including Capu, in vivo (Fig. 4G-K). Wash therefore interacts physically with Rho1 and the three Spire isoforms, and indirectly with Capu.

**Wash bundling and crosslinking activity is regulated by Rho1, SpirD and Arp2/3**

We previously showed that Capu and SpirC bundling/crosslinking activity is regulated by SpirD inhibition, and is relieved from inhibition by Rho1GTP (Rosales-Nieves et al., 2006). As Wash exhibits similar properties to Capu and Spire, we investigated whether Wash is similarly regulated in bundling/crosslinking assays. We found that SpirD also efficiently inhibits Wash bundling and crosslinking activity (Fig. 5A-C), and, as with Capu and Spire, this inhibition is relieved by the addition of Rho1GTP (Fig. 5D-E), whereas the non-interacting SpirD3, SpirD1 and SpirD4 protein fragments do not (Fig. 5H-H'; see also Fig. S4 in the supplementary material). Moreover, inhibition by SpirD2 is not relieved by Rho1GTP (Fig. 5G-G'), suggesting that the Rho relief of inhibition requires Rho binding to Spire through the SpirD3 region (Rosales-Nieves et al., 2006). Together with our genetic data, our results suggest that Wash functions as a Rho effector together with Spire and Capu to control the timing of ooplasmic streaming (see Discussion).

**Wash is essential for actin cytoskeleton integrity during cytoplasmic transfer**

Although Arp2/3 mutants exhibit defects in egg chamber structure (Hudson and Cooley, 2002), they do not swirl prematurely (data not shown). Our observation that reduced wash eggs are small...
that Wash might interact genetically with Arp2/3 to regulate cytoplasmic transfer during oogenesis. During the onset of ooplasmic streaming in stage 10b wild-type egg chambers, nurse cells form stress fibers and contract, expelling their contents through actin-rich ring canals into the oocyte in a process termed dumping (Fig. 6A, A/H11032, E, E/H11032). Defects in stress fiber and ring canal formation or maintenance result in incomplete dumping and thereby smaller eggs (Verdier et al., 2006) (Fig. 6I-K). Phalloidin staining of reduced wash egg chambers showed a loss of nurse cell integrity prior to dumping (Fig. 6B) and incomplete stress fiber formation during nurse cell dumping (Fig. 6F). In wild-type nurse cells, the actin network circumscribing ring canals was relatively compact during nurse cell dumping (2.3±0.3 μm width in wild type; 2.7±0.6 μm for wash/+ heterozygous siblings; n=10; Fig. 6E, E/H11032; data not shown). In reduced wash nurse cells, this actin network was abnormally expanded (4.0±0.4 μm, n=10, P<0.006; Fig. 6F, F/H11032). Inner ring canal formation, which requires Arp2/3 (Hudson and Cooley, 2002), appeared to be unaffected, suggesting that Wash is not essential for ring canal formation per se, but is important for maintaining the integrity of the actin cytoskeleton enclosing it. Indeed, we observed ring canals dissociated from the nurse cell membrane and ‘floating’ within the oocyte (see arrow in Fig. 6J). We identified dominant genetic and dose-sensitive interactions between wash and Sop2 (Arp2/3 subunit) mutants (Fig. 6C-D, G-H, H11032; data not shown) and found defects in both nurse cell cytoskeleton integrity and actin formation around ring canals (3.4±0.3 μm, n=10, P<0.01 for wash-Sop2 transheterozygotes and 6.9±0.4 μm, n=10, P<0.001 for reduced wash-Sop2 transheterozygotes compared with 2.4±0.6 μm for Sop2/+ heterozygous siblings). Consistent with these phenotypes, Wash localizes throughout nurse cells and is not specific to ring canals, which suggests a role in regulating the Arp2/3-dependent actin network in nurse cells (Fig. 2W-X). Overall, our results support a model in which Wash mediates at least two essential pathways of cytoskeletal regulation during oogenesis in two
different compartments: the bundling and crosslinking of linear actin filaments and MTs at the oocyte cortex; and the maintenance of actin cytoskeleton integrity in the nurse cells.

**DISCUSSION**

**Models of ooplasmic streaming**

We previously suggested that Rho1 regulates the timing of ooplasmic streaming by regulating the MT/microfilament crosslinking that occurs at the oocyte cortex (Rosales-Nieves et al., 2006). In this model, crosslinking antagonizes the formation of the dynamic subcortical MT arrays that are required for ooplasm streaming, but does not require the actin-nucleation activity of these proteins. Our model depends on the presence of SpirC and the cortical localization of Rho1, Capu, the Spire isoforms, and now Wash during late-stage oocytes. Support for our model comes from a recent study demonstrating that *chickadee*, encoding fly Profilin, is required for the formation of cortical actin bundles in the oocyte, and that Capu and Spire anchor the minus ends of MTs to a scaffold.

**Fig. 5. Wash F-actin/MT bundling and crosslinking activity is regulated by Rho1, Spire and Arp2/3.** (A-H) Stabilized F-actin (1 μM, A–H) and MTs (1 μM, A’–H’) were incubated with full-length Wash and/or Rho1, Arp2/3 and Spire, and observed by confocal microscopy: (A) no protein added; (B) SpirD only; (C) Wash and SpirD; (D) Wash, SpirD and Rho1(GTP); (E) Wash, SpirD and Rho1(GDP); (F) Wash and SpirD; (G) Wash, SpirD2 and Rho1(GTP); (H) Wash and SpirD3. (I) Quantification of F-actin and MT bundling/crosslinking efficiency of indicated reactions (see Fig. 3; quantification of Arp2/3 reactions were not obtained owing to co-migration of Arp2/3 with actin and MT bands). (J–M) F-actin (J–M) and microtubule bundling (J’–M’) and crosslinking assay with: (J) Arp2/3; (K) Wash and Arp2/3; (L) Wash, Arp2/3 and Rho1(GTP); (M) Wash, Arp2/3 and Rho1(GDP). All assays were performed a minimum of three times. Final protein concentrations were: Wash, 300 nM; Arp2/3, 600 nM; Rho1(GTP), 600 nM; SpirD, 300 nM; SpirD2, 300 nM; SpirD3, 300 nM; (N) Stills from a time-lapse confocal movie of branched-actin network formation by Wash and Arp2/3 in the presence of F-actin (see Movie 6 in the supplementary material). Scale bar: 10 μm.
made from these cortical actin bundles (Wang and Riechmann, 2008). These results suggest dual or multifaceted biochemical roles for these proteins in regulating developmental processes. Consistent with this concept, non-actin-nucleating roles for other formins (i.e. actin severing/dispersalization, MT stabilization, signaling, and transcriptional regulation) are beginning to be reported (Wallar and Alberts, 2003; Faix and Grosse, 2006; Bartolini et al., 2008).

St Johnston and colleagues have recently proposed an alternative model in which Capu and Spire are required to organize an isotropic mesh of actin filaments in the oocyte cytoplasm that suppresses the motility of kinesin, a plus-end directed MT motor protein that is required for ooplasmic streaming (Dahlgard et al., 2007). Their model was formulated with the assumption that the SpirC isoform does not exist, that spirRP is a null allele, and that the cortical localization of Capu and Spire is lost in late-stage oocytes. We find these assumptions not to be the case. We provide miRNA and protein evidence for the existence of the SpirC isoform (see Fig. S2B-D in the supplementary material). The existence of SpirC is also supported by ESTs from the Drosophila Genome Project. The spirRP allele only affects the SpirA and SpirD isoforms; it does not affect the SpirC isoform because this isoform has a unique 5' end (see Fig. S2A in the supplementary material). Ectopic SpirC expression would not be expected to rescue spirRP because it is already being expressed. The cortical localization of Capu and the Spire proteins during the late stages is masked by intense yolk auto-fluorescence in the green channel when using live imaging of GFP fusions, but can be observed by fixing, by antibody staining, or by the use of ChFP ('cherry' fusion protein; see Fig. S2E in the supplementary material). In addition, a subsequent study has shown that kinesin is not required for this cytoskeletal reorganization, suggesting that Capu and Spire might not act as indirect kinesin regulators, but as direct modulators of the MT cytoskeleton (Wang and Riechmann, 2008). One possibility is that Capu and Spire are bundling and crosslinking MTs to Profilin-dependent F-actin at the oocyte cortex, as has been demonstrated in vitro.

**Wash function in Drosophila oogenesis**

Since the discovery of Arp2/3 activators and other actin-nucleation promoting factors, much of the work examining the functions of these proteins has been focused on the properties of their nucleation activities. Recent studies, however, have begun reporting novel biochemical activities for actin nucleators, including MT stabilization activity by mammalian Diaphanous (Bartolini et al., 2008; Palazzo et al., 2004), filopodia inhibition by WAVE/Arp2/3 (Stradal et al., 2004), and F-actin and MT bundling and crosslinking by Spire and Capu (Rosales-Nieves et al., 2006). Consistent with this, not all disease-associated WASP mutations are predicted to affect its actin-nucleation activity (Notarangelo et al., 2008). Our results contribute to this growing list of actin nucleators with significant non-nucleation activities, as we show that Wash is both an Arp2/3 activator and a crosslinker/bundler of F-actin and microtubules. What is unique about Wash, however, is that its combination of biochemical activities suggests that it is an important intermediary molecule functioning at the intersection of linear and branched actin architectures, with Spire, Rho and Arp2/3 acting as the factors that direct these dual functions of Wash. Based on our findings, we propose the following model for Wash function in the context of Drosophila oogenesis (Fig. 7). In the nucleation pathway, upstream signals and factors, possibly Rho, induce Wash activation, which acts with Arp2/3 to promote branched filament formation and cytoskeletal integrity in nurse cells (Fig. 7A). In the crosslinking/bundling pathway, Wash bundles and crosslinks filaments of actin and MTs, under the control of Rho and Spire, to maintain cortical bundle stability in the oocyte and to prevent premature ooplasmic streaming (Fig. 7B). Together with Capu and
family members in development. Scar has been shown to be required for axon development, egg chamber structure, adult eye morphology (Zallen et al., 2002) and myoblast fusion (Berger et al., 2008); Wasp has been demonstrated to be required for Notch-mediated cell-fate decisions (Ben-Yaacov et al., 2001), rhododermic microvilli formation (Zelhof and Hardy, 2004), bristle development (Bogdan et al., 2004; Bogdan et al., 2005; Tal et al., 2002) and myoblast fusion (Berger et al., 2008; Schafer et al., 2007); and Wash is required for pupal development (Linardopoulou et al., 2007) and oogenesis, as described in this study. Mutants in various subunits of Arp2/3 have also been described, offering additional insight into how Wash, Wasp and Scar shape the cytoskeleton during development (Hudson and Cooley, 2002; Stevenson et al., 2002). Interestingly, the spectrum of Arp2/3 mutant phenotypes reported does not completely overlap with all of the phenotypes associated with these WAS family mutants. This might be because Arp2/3 has not been examined in all of the processes in which WAS members play a role, or it might be an indication that WAS members have additional, Arp2/3-independent functions, which we find is the case for Wash. Our observations support the idea that these and other actin nucleators, such as Capu and Spire, are required at different times or locations during development, and are thus tightly regulated spatiotemporally by Rho GTPases and other factors.

Rho regulation of Wash activity
Our data indicate that Wash acts as a downstream effector of Rho. Indeed, we show that Rho regulates the bundling/crosslinking activity of Wash through the relief of SpirD inhibition. However, Rho does not enhance the ability of Wash to induce Arp2/3-mediated actin nucleation (data not shown), raising the question of how or whether Rho might regulate the Arp2/3-associated functions of Wash. Interestingly, our results are consistent with studies examining the Cdc42 regulation of Wasp in Drosophila, which conclude that Cdc42 activation of Wasp is not required for Wasp function in myoblast fusion or bristle development (Bogdan et al., 2005; Schafer et al., 2007; Tal et al., 2002). Although Wasp exhibits a strong and specific interaction with active Cdc42GTP in vitro, these studies provide strong evidence that, at least for the subset of developmental processes examined, Wasp is not regulated upstream by Cdc42GTP. As previously noted, Drosophila Wasp differs from mammalian homologs in that it is not auto-inhibited; Cdc42, therefore, might not be required for the activation of its actin nucleation-promoting functions. This might also be the case for Wash, as it too appears to be constitutively active, and might act as a downstream effector of Rho only where its bundling/crosslinking activities are concerned. Our data, however, do not rule out the possibility that the nucleation activity of Wash is regulated by a complex in vivo. In fact, recent reports have shown that two proteins originally associated with Scar regulation, Abi and Kette, control Wasp function in Drosophila as well (Bogdan and Klambt, 2003; Bogdan et al., 2005; Schafer et al., 2007). It remains to be determined whether Abi and Kette also regulate Wash function, and whether Rho might play a role in mediating these interactions.

Arp2/3 as a possible switch between linear- and branched-actin structures
We show that Wash requires Arp2/3 for actin nucleation, but, interestingly, this association appears to disrupt the ability of Wash to bundle and crosslink F-actin and microtubules, as we observe a loss of F-actin/MT bundling in favor of branching actin filaments. This suggests that Arp2/3 might act as a molecular switch that shifts Wash function from bundling to nucleation and, in terms of
cytoskeletal remodeling, supports the hypothesis that Arp2/3 regulates the balance between linear and branched actin architectures in the cell. This is predicated on the assumption that the Wash bundling/crosslinking and nucleation-inducing activities are mutually exclusive, and would represent a previously uncharacterized function of Arp2/3. However, we cannot rule out scenarios in which nucleation and bundling might coexist. F-actin bundling might be preserved if the branched-actin structures created by Wash and Arp2/3 in vitro are bundled by Wash in parallel (form angled, branching bundles rather than the tortuous bundles observed under non-Arp2/3 conditions), or if filaments emanating from vertices are clamped together by Wash at the branching point to form angled bundles that branch from these vertices. An example of this latter case has been reported in a recent study examining the concerted actions of N-Wasp and Hsp90 to nucleate branched actin filaments (via N-Wasp activation of Arp2/3) and clamp the angled filaments to form a linear bundle (mediated by Hsp90) (Park et al., 2007). Wash therefore, in having both nucleation and bundling activities, might perform both functions simultaneously in the presence of Arp2/3. At the very least, Arp2/3 abolishes the ability of Wash to bundle MTs and crosslink them to actin, and so might contribute to regulating crosstalk between the actin and microtubule cytoskeletons. Further studies examining the molecular interactions of WAS family members and Arp2/3 will be invaluable for understanding the full range of cytoskeletal regulation in the cell.

Coordination of Wash nucleation and bundling activity in motile cells

In motile cells the actin cytoskeleton can be represented as a dynamic sum of two general geometries – strands or bundles of linear actin filaments, and broad dendritic networks of branched filaments (Chhabra and Higgs, 2007; Faix and Grosse, 2006; Goley and Welch, 2006). The mechanisms by which these two networks are remodeled and coordinated are areas of intense investigation and are important for understanding how processes such as lamellipodia and filopodia formation occur. It is intriguing to note that, in the latter case, the biochemical properties of Wash suggest that it might play a role in the convergent extension model of filopodia formation, whereby uncapped actin filaments nucleated from a dendritic branched-actin array are captured at the cell periphery and bundled to form long extensions (Mattila and Lappalainen, 2008). Wash, as both an Arp2/3 activator and an F-actin bundling protein, is in an ideal position in which to carry out both the nucleation and the bundling functions, and might thus be an important regulator of filopodia formation alongside previously discovered molecules (Mattila and Lappalainen, 2008). The presence of Spire and Arp2/3 at the dendritic bed and active Rho at the cell membrane could form two zones of differential activity to switch Wash function from nucleation to bundling and crosslinking. This form of spatial regulation is analogous to how Rho, Cdc42 and Rac define regions of differential activity during wound healing (Bement et al., 2006) and cell adhesion (Yamada and Nelson, 2007). Further investigation into the role of Wash in filopodia and lamellipodia formation will be important, as these protrusions play essential roles in wound healing, substrate adhesion and neurite outgrowth.

In humans, the misregulation of WAS members results in disorders such as Wiskott-Aldrich Syndrome, and cancer metastasis (Burns et al., 2004; Machesky, 2008; Vignjevic and Montagnac, 2008). As a new member of the WAS family, human WASH appears to also be clinically relevant. WASH has been reported to be overexpressed in a breast cancer cell line and might, like the overexpression of N-WASP and Scar/WAVEs, contribute to metastasis (Leirdal et al., 2004). Moreover, the subtelomeric location of human WASH places it at high risk for deletion and rearrangement, as subtelomers are hotspots of meiotic interchromosomal sequence transfers (Linardopoulou et al., 2005). Our data demonstrate that Wash is essential for development in Drosophila, and suggest that Wash might function in actin organization in other contexts. Further work will be required to understand how Wash and other WAS family members coordinate linear- and branched-actin networks during oogenesis and other cellular processes, and how the misregulation of these processes results in disease.

Acknowledgements
We thank Barb Trask, Sue Biggins, Valera Vasioskhin and Amir Oryan for discussions and comments on the manuscript. We thank Steve Jackson, Bobby Schneider, Roland Strong, Toshi Tsukiyama, Liz Wayner, Jennifer Zallen, the Developmental Studies Hybridoma Bank, the Bloomington Stock Center, and the Vienna Drosophila RNAi Center for antibodies, DNA, flies and other reagents used in this study. This work was supported by a Cancer Research Institute Postdoctoral Fellowship (to E.V.L.) and NIH grant GM072581 (to S.M.P.). Deposited in PMC for release after 12 months.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/16/2849/DC1

References


