

# A subset of dynamic actin rearrangements in *Drosophila* requires the Arp2/3 complex

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The Arp2/3 complex has been shown to dramatically increase the slow spontaneous rate of actin filament nucleation in vitro, and it is known to be important for remodeling the actin cytoskeleton in vivo. We isolated and characterized loss of function mutations in genes encoding two subunits of the *Drosophila* Arp2/3 complex: *Arpc1*, which encodes the homologue of the p40 subunit, and *Arp3*, encoding one of the two actin-related proteins. We used these mutations to study how the Arp2/3 complex

contributes to well-characterized actin structures in the ovary and the pupal epithelium. We found that the Arp2/3 complex is required for ring canal expansion during oogenesis but not for the formation of parallel actin bundles in nurse cell cytoplasm and bristle shaft cells. The requirement for Arp2/3 in ring canals indicates that the polymerization of actin filaments at the ring canal plasma membrane is important for driving ring canal growth.

## Introduction

Biochemical studies have been instrumental in understanding how actin binding proteins shape the actin cytoskeleton. In vitro analyses of the interactions between actin and actin binding proteins have led to models that describe how the F-actin cytoskeleton can be rapidly reorganized in response to extrinsic or intrinsic cues (Pollard et al., 2000). Under physiological conditions, nucleation, the process by which actin monomers initially assemble into filaments, is too slow to account for the rapid rates of actin polymerization required in vivo. Thus, an essential feature of these models is the requirement for factors that can stimulate new actin polymerization within a cell.

Only one factor, the Arp2/3 complex, has been shown to nucleate de novo actin polymerization. The Arp2/3 complex was initially isolated from amoeba extracts on the basis of its affinity for the actin monomer binding protein profilin (Machesky et al., 1994). Since then, the complex has been shown to be a conserved nucleation factor, being present in eukaryotes ranging from yeast to humans (Machesky and Gould, 1999). The complex consists of seven proteins: the actin-related proteins Arp2 and Arp3, from which the complex takes its name, a WD repeat protein (*Arpc1*, also known as p40 or *Sop2*), and four novel proteins. Computer modeling and recently published cryo-EM structural studies have

placed the two Arp subunits at the pointed end of actin filaments, suggesting a nucleation mechanism where these subunits form a template to which actin monomers can add (Kelleher et al., 1995; Volkmann et al., 2001). Detailed biochemical and ultrastructural studies have shown that filaments nucleated by the Arp2/3 complex form a branching network in vitro (Mullins et al., 1998), and a near perfect correlation of this branching network has been observed in vivo in cultured keratocytes (Svitkina and Borisy, 1999).

Genetic analyses of Arp2/3 complex function in both budding yeast and fission yeast have provided evidence that Arp2/3 complex functions are required for the organization of the actin cytoskeleton. In budding yeast, disruption of genes encoding Arp2/3 subunits results in a slow growth phenotype characterized by defects in cortical actin patches. One subunit, Arc40p, the budding yeast *Arpc1* homologue, was found to be unconditionally essential (Winter et al., 1999). In fission yeast, *arp3*, *arp2*, and *sop2* have all been shown to be essential, and conditional mutations in these genes exhibit defects in the cortical actin cytoskeleton (Balasubramanian et al., 1996; McCollum et al., 1996; Morrell et al., 1999).

*Drosophila* provides an excellent model system to study the genetic regulation of cytoskeletal changes in a multicellular organism. The germline nurse cells in *Drosophila* egg chambers are dependent on distinct populations of actin filaments in order to complete the transfer of cytoplasm to the oocyte (Robinson and Cooley, 1997). First, large intercellular bridges called ring canals allow the flow of cytoplasm from the

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nurse cells to the oocyte during oogenesis. Ring canal stability and growth are dependent on the accumulation of an inner rim consisting of loosely bundled actin filaments. Second, during the final rapid phase of cytoplasm transport the remaining nurse cell contents are pushed into the oocyte; this requires a cortical actomyosin network to drive nurse cell contraction. Finally, a dense network of hexagonally packed parallel actin bundles is required in the cytoplasm of nurse cells to restrain their large polyploid nuclei from being pushed into the ring canals, which would otherwise block further cytoplasm transport. *Drosophila* bristle formation is also dependent on the formation of hexagonally packed bundles of actin filaments. These filaments are similar to the nurse cell cytoplasmic bundles at both the ultrastructural and molecular levels (Cant et al., 1994; Tilney et al., 1996a). Furthermore, the actin bundles in bristles and nurse cells bear striking similarity with parallel actin bundles in other systems, suggesting that the machinery used to produce these bundles is likely to be conserved (DeRosier and Tilney, 2000).

In this paper, we describe the first characterization of loss of function mutations of Arp2/3 complex components in a metazoan. We focus on how the Arp2/3 complex contributes to the assembly and organization of several well-characterized actin structures in vivo and present evidence that the Arp2/3 complex is required for specific subsets of actin polymerization. In particular, the polymerization of actin for parallel actin bundles does not depend on Arp2/3 function. In contrast, the more loosely organized actin filaments in ring canals require the function of the Arp2/3 complex. Further, we show that Arp2/3 components are localized to ring canals.

## Results

### The *Drosophila* genome encodes apparent homologs of the Arp2/3 complex

Previous work (Fyrberg and Fyrberg, 1993; Fyrberg et al., 1994) and the sequencing of the *Drosophila* genome (Adams et al., 2000) revealed apparent homologs of each of the seven subunits of the Arp2/3 complex (Table I). Single isoform homologs of each subunit are encoded, except for Arpc3 (p21) for which two *Drosophila* genes display a high degree of homology (Table I). Following the nomenclature adopted by the field (Higgs and Pollard, 2001), we designate these two genes *Arpc3A* and *Arpc3B*.

We identified loss of function mutations in the genes encoding the Arpc1 and Arpc3 subunits. Identification of *Arpc1* mutations was facilitated by the location of *Arpc1* near the

*Adh* gene, which resides within an extensively characterized region on the left arm of chromosome 2 (Ashburner et al., 1999). The saturation mutagenesis and genetic mapping studies performed by Ashburner and colleagues (Woodruff and Ashburner, 1979; Ashburner et al., 1982) allowed the mapping of the *Arpc1* gene to a region containing several lethal complementation groups within a genetic interval defined by *Df(2L)b84A9* (Fig. 1 A). A genomic transgene containing the *Arpc1*<sup>+</sup> genomic region, constructed for use in an independent study (Pflumm and Botchan, 2001), was used to rescue the lethality associated with the *l(2)34Da*<sup>CH60</sup> lethal mutation. Sequencing of the *l(2)34Da*<sup>CH60</sup> allele revealed a 207-bp genomic deletion that removes the last 62 codons of *Arpc1*; therefore, we designate this mutation as *Arpc1*<sup>CH60</sup>.

Since *Arpc1*<sup>CH60</sup> was the only allele of *Arpc1* extant at the time of our study, and this allele was likely to be hypomorphic, an ethyl methane sulfonate noncomplementation screen was performed to isolate additional *Arpc1* alleles. Five new *Arpc1* alleles were identified. Genomic DNA from the mutant chromosomes was sequenced, and molecular lesions for each allele were identified (Fig. 1 B, bottom schematic). Three alleles, *Q25sd*, *Q25st*, and *W82st*, were predicted to truncate the protein before the second WD repeat; these alleles behaved as null mutations. Another allele, *W108R*, had a missense mutation changing Trp108 to Arg, and this allele exhibited phenotypes of moderate strength. The weakest allele, *R337st*, truncated the protein in the last WD repeat (Fig. 1 B).

A mutation in the *Drosophila* *Arp3* gene was identified based on the Berkeley *Drosophila* Genome Project P-element disruption project. The EP element in *EP(3)3640* was inserted 138 bp upstream of the predicted initiating ATG methionine codon (Fig. 1 C). The lethality associated with the EP-element insertion could be reverted by excising the P element. In addition, the lethality of the *EP(3)3640* insertion could be rescued by ubiquitous expression of the Gal4 transcriptional activator; since the *EP(3)3640* insertion was oriented with the Gal4 UAS directed toward *Arp3*, ubiquitous Gal4 expression presumably restored expression of *Arp3* to viable levels. Based on these observations, we designate the *EP(3)3640* insertion as *Arp3*<sup>EP(3)3640</sup>.

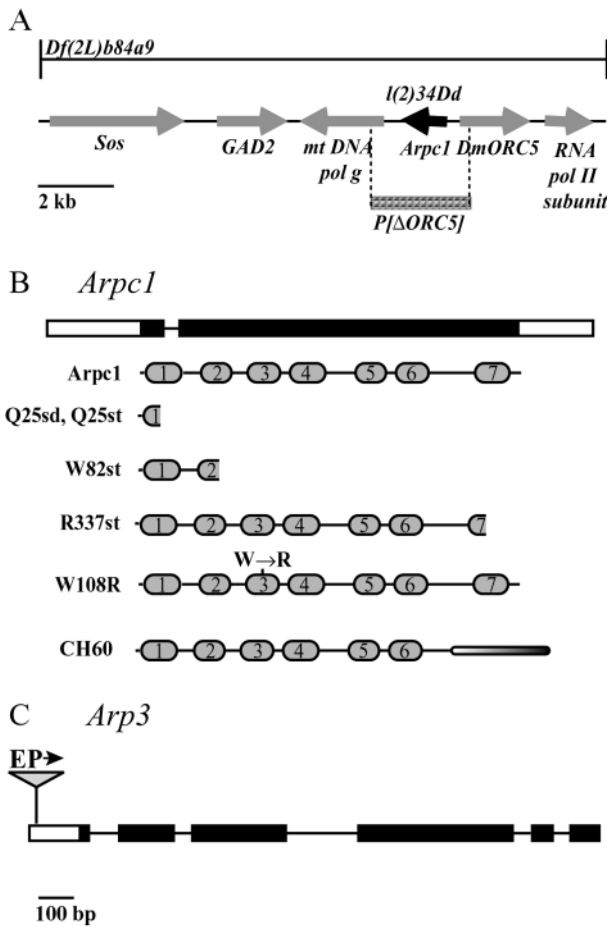
As we report below, the spectrum of phenotypes exhibited by *Arpc1* and *Arp3* mutations was identical with respect to several different actin structures in several cell types.

### Arp2/3 complex function is required for cytoplasm transport during oogenesis

All of the alleles in Arp2/3 complex genes resulted in lethality before the adult stage; therefore, the phenotypes of

Table I. The Arp2/3 complex in *Drosophila*

Arp2/3 subunit	<i>Drosophila</i> name(s)/BDGP designation	Identity/similarity ( <i>Drosophila</i> versus human) %	Cytological location
Arp2	Arp2 (Arp14D)/CG9901	82/88	14E3
Arp3	Arp3 (Arp66B)/CG7558	80/88	66B8
ARPC1 (p40)	Arpc1 (Sop2)/CG8978	53/61	34D3
ARPC2 (p34)	Arpc2/CG10954	76/83	38C9
ARPC3 (p21)	Arpc3A/CG4560	58/79	89A9
	Arpc3B/CG8936	56/75	15D4
ARPC4 (p20)	Arpc4/CG5972	83/87	26B6
ARPC5 (p16)	Arpc5/CG9881	54/63	23A1



**Figure 1. Loss of function mutations identified in the *Drosophila Arpc1* and *Arp3* genes.** (A) The *Arpc1* gene was mapped to a genetic interval defined by *Df(2L)b84a9* (bracket at top). The lethality of the *l(2)34Dd* gene was rescued by *P[ΔORC5]* (Pflumm and Botchan, 2001). This transgene contained the complete ORF of *Arpc1* and small portions of the adjacent genes; the DNA included in the transgene is indicated by the hatched box. (B) Schematic of *Arpc1* depicting genomic organization and predicted products. The white boxed regions indicate untranslated regions, black boxed regions represent coding sequence, and the thin black line represents the single intron. The seven WD repeats encoded by *Arpc1* are depicted as numbered ovals below the genomic organization. The predicted proteins produced by the original *Arpc1<sup>CH60</sup>* allele and the alleles generated in this study are diagrammed below the wild-type protein. *Arpc1<sup>Q25st</sup>* is a nonsense mutation at Gln25 (CAG→TAG), *Arpc1<sup>Q25sd</sup>* is a mutation in the conserved splice donor dinucleotide after Gln25 (C/gt→C/at), *Arpc1<sup>W82st</sup>* is a nonsense mutation at Trp82 (TGG→TGA), *Arpc1<sup>W108R</sup>* is a missense mutation changing Trp108 to an Arg (TGG→AGG), and *Arpc1<sup>R337st</sup>* is a nonsense mutation at Arg337 (CGA→TGA). The *Arpc1<sup>CH60</sup>* allele is a genomic deletion that is predicted to append 78 amino acids to the *Arpc1* reading frame. (C) Genomic organization of *Arp3* depicted as in B. The location of the *EP(3)3640* P element is shown, and the orientation of the Gal4 UAS sequences are indicated by the arrow. The *EP* element is not drawn to scale. Bar: (B and C) 100 bp.

Arp2/3 complex mutations were examined in clones of mutant cells in specific tissue types. To learn whether the Arp2/3 complex was required during oogenesis, germline clones were generated using the FLP/FRT/DFS technique, which allows the identification of egg chambers with mutant germline cells after stage 8 of oogenesis (Chou and

Perrimon, 1996). Germline mosaics using *Arp2/3* mutations revealed a requirement for the Arp2/3 complex in cytoplasm transport (Fig. 2, first column). Late stage egg chambers contained abnormally small oocytes and large residual nurse cells.

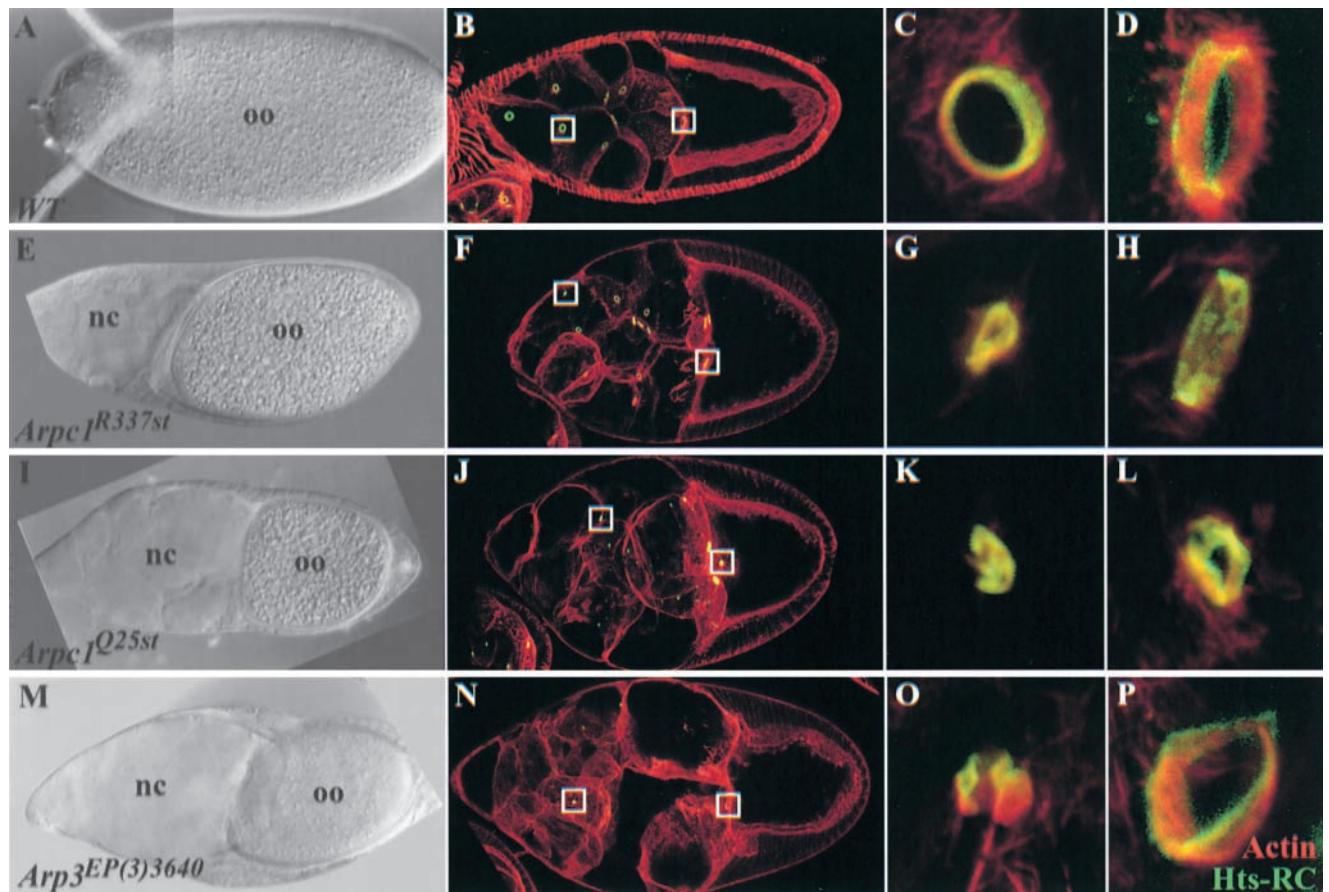
Closer examination of the *Arpc1* and *Arp3* mutant egg chambers revealed defective ring canals. Visualization of actin with fluorescent phalloidin and ring canals with Hts-RC antibodies showed that ring canal size and integrity were severely compromised (Fig. 2, second, third, and fourth columns); this phenotype was 100% penetrant. The effect on ring canals was heterogeneous; often, the four ring canals connecting the oocyte to the nurse cells appeared less severely affected than ring canals connecting nurse cells to other nurse cells (Fig. 2, third and fourth columns). Quantification of the effect on ring canal size revealed that at stage 10A, ring canals between the oocyte and adjacent nurse cells were on average 30% smaller in diameter in *Arpc1* or *Arp3* mutants than in wild type (wild-type diameter,  $10.2 \pm 0.9 \mu\text{m}$ ; *Arpc1<sup>Q25st</sup>* diameter,  $7.2 \pm 0.6 \mu\text{m}$ ; *Arp3<sup>EP(3)3640</sup>* diameter,  $7.2 \pm 0.6 \mu\text{m}$ ). Ring canals connecting pairs of nurse cells were <50% the size of wild-type ring canals in *Arpc1* and *Arp3* germline clones (wild-type diameter,  $8.0 \pm 0.3 \mu\text{m}$ ; *Arpc1<sup>Q25st</sup>* diameter,  $3.7 \pm 0.2 \mu\text{m}$ ; *Arp3<sup>EP(3)3640</sup>* diameter,  $4.0 \pm 0.3 \mu\text{m}$ ).

### The Arp2/3 complex is required for ring canal growth

Given the striking effect on ring canal integrity observed in late stage egg chambers, we examined egg chambers at earlier stages. Since the FLP/FRT/DFS method does not allow examination of mutant egg chambers before stage 8 of oogenesis, clones were generated using green fluorescent protein (GFP)\* as a marker. In this system, mutant cells were identified by loss of GFP expression. Surprisingly, ring canal development appeared to initiate normally. In the germarium, ring canals in *Arpc1* mutant clones were indistinguishable from wild type, and it was not possible to detect differences between mutant and wild type through at least stage 4 of oogenesis (unpublished data). By stages 5–6, differences between ring canals in *Arpc1* mutant egg chambers and those in comparably staged wild-type egg chambers could be discerned (Fig. 3, A and B). Actin on *Arpc1* ring canals was less robust, and ring canals appeared smaller in diameter. By stage 8, there appeared to be a reduction in ring canal actin relative to wild type, and some ring canals were misshapen (Fig. 3, D and E). By stages 9–10, ring canals appeared identical to those seen in germline clones generated using the FLP/FRT/DFS technique (Fig. 3, G and H, compared with Fig. 2, J and N). Thus, the Arp2/3 complex was not required for the initial recruitment of actin to ring canals but rather became necessary for later stages of ring canal growth.

In later stages, ring canals sometimes were detached from the nurse cell membranes, and the nurse cell subcortical actin became destabilized. This apparently resulted in the for-

\*Abbreviation used in this paper: GFP, green fluorescent protein.



**Figure 2. Germline clones of mutations in Arp2/3 complex subunits exhibit a defect in cytoplasm transport due to defective ring canals.** Wild type (A, B, C, and D); *Arpc1<sup>R337st</sup>* (E, F, G, and H); *Arpc1<sup>Q25st</sup>* (I, J, K, and L); *Arp3<sup>EP(3)3640</sup>* (M, N, O, and P). (A, E, I, and M) Stage 14 wild-type and mutant egg chambers. Oocytes (oo) from the mutant germline clones are significantly smaller than wild type and retain much of the nurse cell cluster (nc). (B, F, J, and N) Egg chambers were incubated with Alexa 488 phalloidin to visualize actin (red) and Hts-RC antibodies to mark ring canals (green). Ring canals are partially compromised in germline clones of the weak allele, *Arpc1<sup>R337st</sup>* (F), and appear as small dots in germline clones of the stronger *Arpc1<sup>Q25st</sup>* (J) and *Arp3<sup>EP(3)3640</sup>* (N) mutations. (C, G, K, and O) Higher magnification views of ring canals between nurse cells; ring canals shown are those in the left box in B, F, J, and N. Ring canal diameter and shape are severely compromised in mutants. (D, H, L, and P) Higher magnification views of oocyte ring canals; ring canals shown are those in the right box in B, F, J, and N.

mation of multinucleate cells (Fig. 3, F and I). Multinucleate cells were never observed in younger egg chambers (Fig. 3 C) and thus were unlikely to result from defective cytokinesis. Furthermore, all clonal egg chambers contained 16 germline cells, indicating that Arp2/3 was not required for germline cell division.

#### Localization of Arp2/3 complex components in egg chambers

To determine the subcellular distribution of Arp2/3 complex components, both GFP fusion constructs and antibodies were generated. Transgenes encoding Arpc1 and Arp3 fused to GFP were generated, and their distribution in the germline cells of the ovary was examined. These fusion proteins were abundant in the cytoplasm of germ cells, and both GFP fusion proteins were enriched at ring canals (Fig. 4, A and E). In addition, some enrichment on cortical membranes was present (Fig. 4, A and E). At higher magnification, both GFP fusions precisely colocalized with F-actin at the ring canal (Fig. 4 B–D and F–H). We did not observe enrichment of either GFP fusion protein on the cytoplasmic

actin bundles that form in the nurse cells at stage 10B (unpublished data).

Initial attempts at immunolocalization of the Arp2/3 complex using antibodies against Arpc1 or Arp3 were unsuccessful, since antisera generated were only useful for immunoblotting (unpublished data). More informative results were obtained with antibodies recognizing one of the two Arpc3 subunits. The two *Drosophila* Arpc3 subunits are 74% identical and 83% similar to one another (Fig. 5 A). Antisera against GST fusion proteins of Arpc3A and Arpc3B were generated. All rats immunized with GST-Arpc3A produced antibodies that strongly recognized a His-tagged Arpc3A fusion protein and also showed some affinity for a His-tagged Arpc3B fusion protein (Fig. 5 B). One rat immunized with GST-Arpc3B produced antiserum that exhibited a strong preference for His-Arpc3B (Fig. 5 A). The Arpc3B antibodies were affinity purified using recombinant His-tagged Arpc3B as an affinity matrix. Serum incubated with His-Arpc3B no longer detected specific signal in immunoblotting or in immunofluorescence.

When crude Arpc3A and purified Arpc3B antibodies were used to probe blots of ovarian extracts, prominent bands of

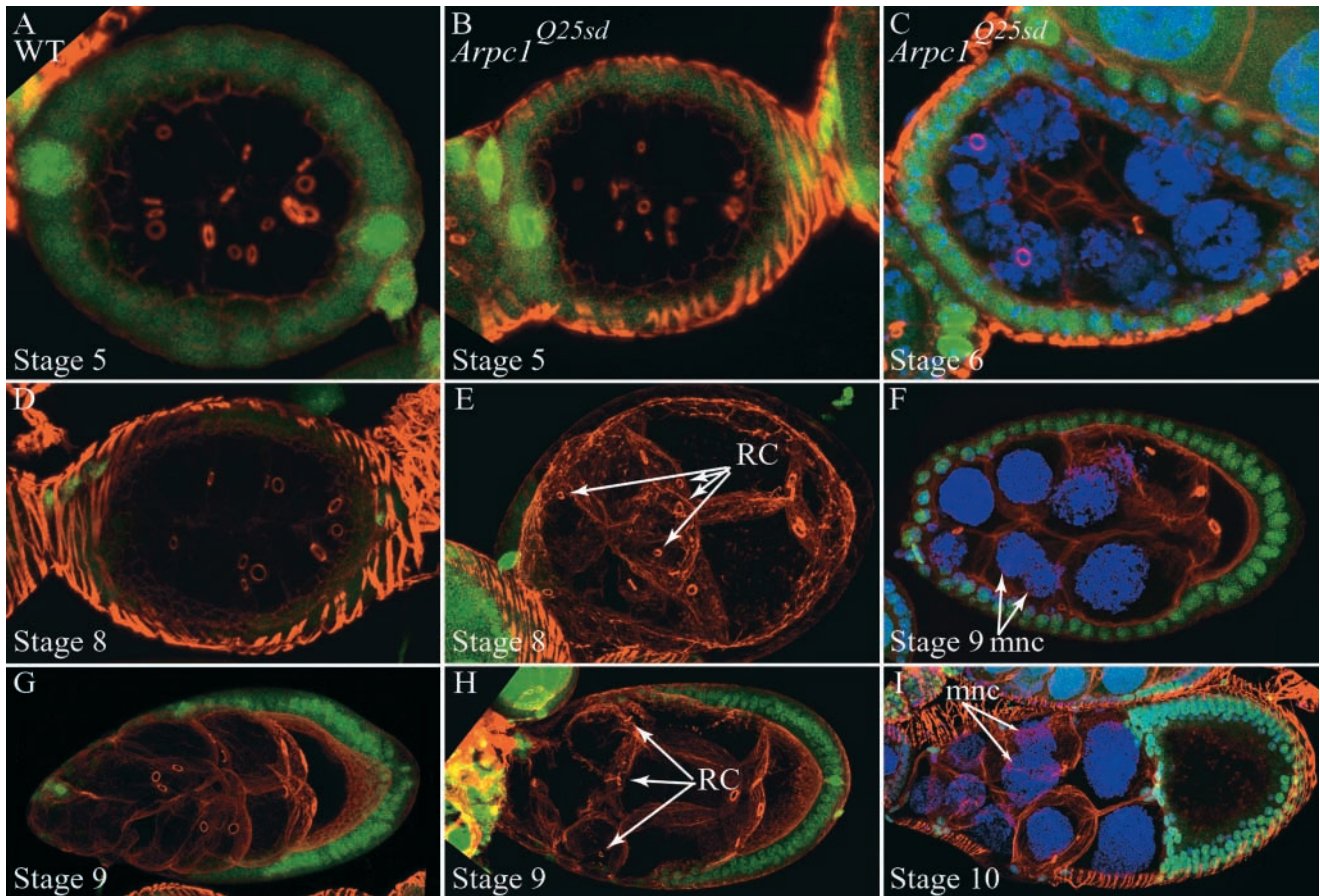


Figure 3. **Ring canal defects in *Arpc1* mutants develop during mid-oogenesis.** (A, D, and G) Progressive stages of wild-type germline clones marked by loss of GFP (green) and stained with rhodamine-phalloidin (red) to reveal F-actin distribution. (B, E, and H). Progressive stages of *Arpc1*<sup>Q25sd</sup> germline clones marked as in A, D, and F. Arrows in E and H highlight ring canals (RC) that appear small or misshapen relative to ring canals from comparably staged egg chambers. (C, F, and I). Multinucleate nurse cells in *Arpc1* germline clones result from destabilization of membranes in late stage egg chambers. *Arpc1*<sup>Q25sd</sup> germline clones marked by loss of GFP (green) were stained with rhodamine-phalloidin to reveal F-actin (red) and TO-PRO 3 to reveal DNA (blue). Multinucleate cells (mnc) become apparent in later stages of oogenesis (F and I, arrows).

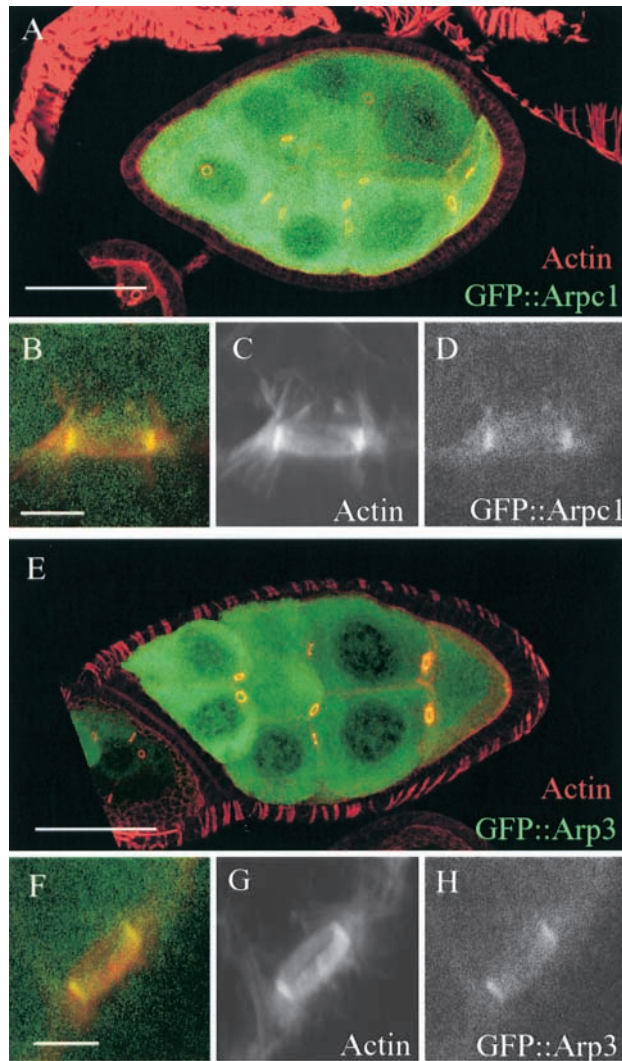
~21 and 20 kD, respectively, were observed (Fig. 5 C). These apparent molecular weights were consistent with the predicted molecular weights of the two proteins: 20.3 kD for Arpc3A and 19.9 kD for Arpc3B. The tissue distribution of these proteins was analyzed by probing blots of extracts derived from whole or dissected adult flies, revealing that the Arpc3A band was present in essentially equal amounts in all tissues tested (unpublished data). Antibodies raised against *Drosophila* Arpc1 and Arp3 showed a similar ubiquitous distribution (unpublished data). In contrast, immunoblotting with the Arpc3B antibody showed a germline-specific pattern of expression (Fig. 5 D). A prominent band was present in ovarian lysates but was absent from the female carcasses lacking ovaries and also absent from lysates of whole females that lack germ cells (Fig. 5 D, *tudor* lane). No expression was observed in extracts of whole males or isolated testes (Fig. 5 D).

Immunofluorescence using Arpc3B antibodies revealed that Arpc3B was highly enriched at ring canals throughout oogenesis (Fig. 6, A–C). Arpc3B first appeared on ring canals very early in the germarium, perhaps as early as region 2A (Fig. 6 A). This is the stage where ring canals first become distinct from their structural precursors, the arrested cytokinetic furrows. Arpc3B appeared to colocalize with

actin at ring canals (Fig. 6, D and E). At high magnification, the distribution of Arpc3B on ring canals appeared punctate compared with antibodies against phosphotyrosine, another ring canal marker (Robinson et al., 1994) (Fig. 6, F–H).

### Arp2/3 complex function is not required for nurse cell cytoplasmic actin bundles

Completion of cytoplasm transport from the nurse cells to the oocyte is dependent on the formation of dense networks of parallel hexagonally packed actin bundles that form in nurse cell cytoplasm just before the contraction that drives final cytoplasm transport (Cooley et al., 1992; Guild et al., 1997). Germline clones of null alleles of *Arpc1* or the strong hypomorph *Arp3*<sup>EP(3)3640</sup> produced abundant cytoplasmic actin bundles (Fig. 7, A–C). The relative abundance of bundles was comparable to wild type. The bundles present in mutant nurse cells were also striated in appearance as they are in wild type (Fig. 7, D–F). The distribution of the bundles was sometimes irregular; in contrast to wild type where the bundles were evenly distributed around the nuclei (Fig. 7, A and D) the bundles in *Arpc1* (Fig. 7, B and E) and *Arp3* (Fig. 7, C and F) mutant nurse cells tended to be un-



**Figure 4. Arpc1 and Arp3 GFP fusion proteins are present in the cytosol and are enriched at ring canals.** UASp-GFP::Arpc1 (A) or UASp-GFP::Arp3 (E) was expressed in ovarian germ cells by crossing to the *nos-Gal4VP16* driver (Van Doren et al., 1998). High levels of cytosolic GFP::Arpc1 and GFP::Arp3 are evident in stage 9 egg chambers, and colocalization with actin at ring canals is apparent. At higher magnification, precise colocalization with ring canal F-actin is apparent for both GFP::Arpc1 (B–D) and GFP::Arp3 (F–H). Bars: (A and E) 50  $\mu\text{m}$ ; (B and F) 5  $\mu\text{m}$ .

evenly clustered. This may be a secondary consequence of severely damaged ring canals and an unstable plasma membrane cytoskeleton.

### The Arp2/3 complex is required for the organization but not the formation of parallel actin bundles in developing bristles

Proper extension of the mechanosensory bristles is also dependent on the polymerization of parallel hexagonally packed actin bundles (Overton, 1967; Tilney et al., 1996a). To examine the consequences of loss of Arp2/3 activity in developing bristles, null alleles of *Arpc1* were used to generate mosaic flies lacking *Arpc1* in bristle precursor cells. As shown in Fig. 8 A, the mature *Arpc1* bristles were indistinguishable from wild type at low magnification. However, at

higher magnification alterations in bristle morphology were apparent (Fig. 8, B and D–G). Compared with wild type, the *Arpc1* mutant bristles had approximately twice the number of longitudinal ridges (Fig. 8, C and D). This phenotype was observed in all mutant macrochaetes. *Arp3*<sup>EP(3)3640</sup> homozygous animals die as pharate adults so the *Arp3* bristle phenotype was examined after dissecting *Arp3* adults from their pupal cases. Bristle morphology appeared normal at lower magnification (Fig. 8 B) as was the case for *Arpc1* mutant bristles. At higher magnification, alterations in the ridging pattern identical to those observed for *Arpc1* were observed (Fig. 8, E and F). The penetrance of this phenotype was  $\sim 80\%$ .

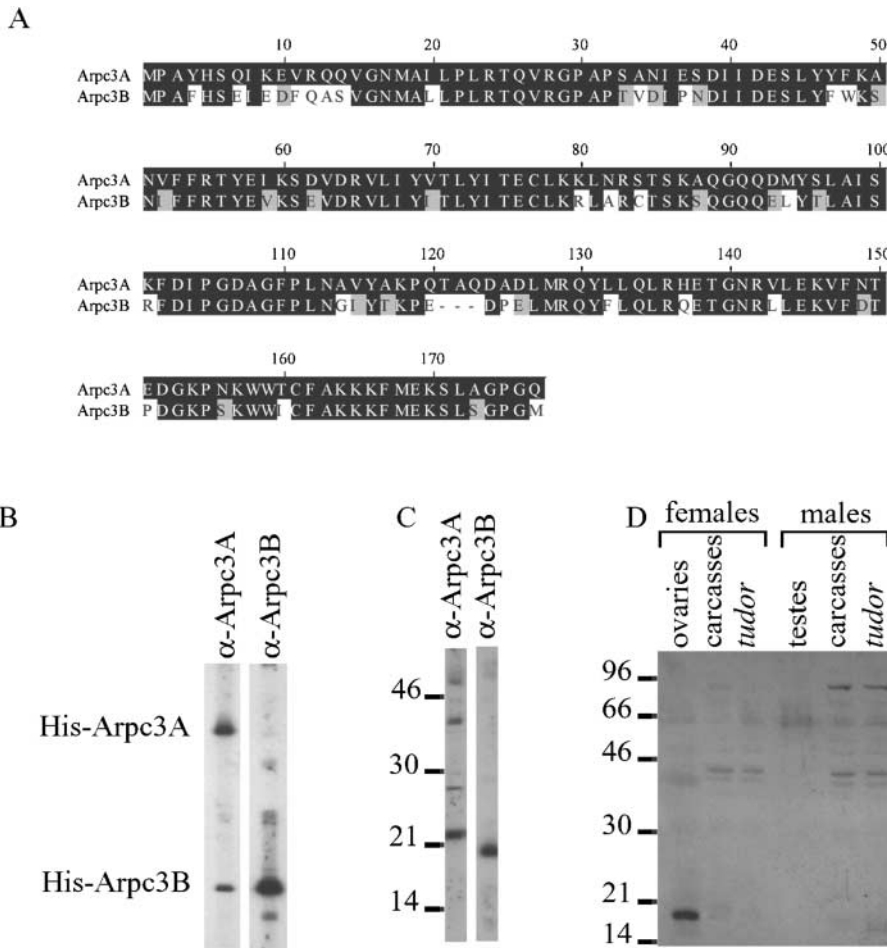
## Discussion

In this study, we present genetic and cell biological analyses of the Arp2/3 complex in *Drosophila*. We identified loss of function mutations in genes encoding two Arp2/3 complex subunits: *Arpc1* and *Arp3*. The phenotypes of the *Arp3*<sup>EP(3)3640</sup> mutation mirror those seen with the *Arpc1* mutations. The simplest interpretation of these observations is that in either case we are examining the consequences of loss of Arp2/3 complex activity and that neither subunit is required for functions apart from their shared function in the Arp2/3 complex. Reconstitution experiments have recently been reported, showing that Arp2/3 complexes lacking Arp3 or Arpc1 have little or no nucleation activity (Gournier et al., 2001), supporting the notion that mutations in these subunits abolish Arp2/3 activity.

### The Arp2/3 complex is required for ring canal growth

The onset of ring canal defects in Arp2/3 mutant egg chambers occurs relatively late in oogenesis. Interestingly, the stage at which defects are first apparent coincides with a transition in the mode of actin filament addition (Tilney et al., 1996b). The early mode of actin filament addition to ring canals involves both the expansion of the ring canal diameter and an increase in the number of filaments in cross-section. This pattern persists until stage 5 of oogenesis where the ring canals are  $\sim 5 \mu\text{m}$  in diameter and reach a maximum of  $\sim 700$  filaments in cross-section. Beginning at stage 5, the pattern of actin filament addition shifts so that the diameter of the ring canal continues to expand, whereas the number of filaments in cross-section remains constant (Tilney et al., 1996b).

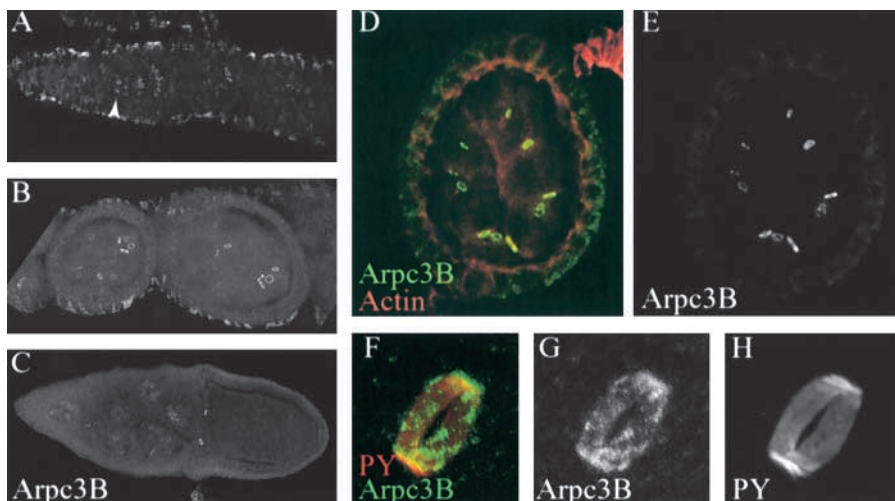
One possible explanation for the late onset of the Arp2/3 complex phenotypes is that the shift in the pattern of actin filament addition reflects an underlying mechanistic difference. An Arp2/3-independent mechanism may contribute to actin filament accumulation early in oogenesis when the number of filaments in cross-section is increasing. This Arp2/3-independent mechanism could be related to the process by which actin filaments are recruited to cleavage furrows (Cao and Wang, 1990a,b) where pre-existing filaments from the cortical actin cytoskeleton are recruited to the rings to support their expansion. From stage 5 of oogenesis onward, the Arp2/3 complex may be required to provide the additional F-actin required to support expansion of the ring canal diameter.



**Figure 5. Antibodies against Arpc3B are specific for Arpc3B and recognize a band that is enriched in ovaries.** (A) Lineup displaying peptide homology between Arpc3A and Arpc3B. (B) Purified His-tagged Arpc3A and Arpc3B proteins were blotted and probed with antibodies raised against GST-tagged Arpc3A and Arpc3B. Antibodies raised against GST-Arpc3A preferentially recognize His-Arpc3A, whereas antibodies raised against GST-Arpc3B appear specific for His-Arpc3B. (C) Crude Arpc3A and purified Arpc3B antibodies recognize distinct bands of ~21 and 20 kD, respectively, when used to probe blots of ovarian lysates. (D) Arpc3B is enriched in ovaries. In lanes 1–3, extracts were prepared from dissected ovaries, ovariectomized females, and whole *tudor* females that lack a germline. In lanes 3–6, extracts were prepared from dissected testes, the male carcasses after dissection, and whole *tudor* males.

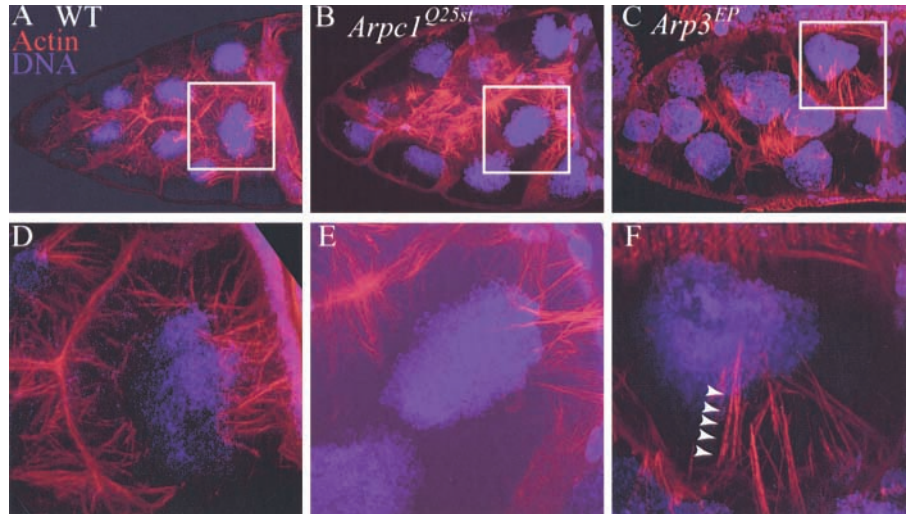
Localization of Arp2/3 components to the ring canals is consistent with a requirement for the Arp2/3 complex during ring canal expansion. However, it is curious that we find Arp2/3 complex components localized to or enriched at ring canals much earlier than the loss of function phenotypes become evident. One possible explanation for this is that the

Arp2/3 complex does contribute to actin accumulation during earlier stages of oogenesis, but the Arp2/3-independent mechanism is functionally redundant with the Arp2/3 complex during this time. Alternatively, the Arp2/3 complex may associate with ring canals in an inactive state and become activated when required later in oogenesis.



**Figure 6. Arpc3B antibodies recognize ring canals.** (A) Arpc3B localizes to ring canals at approximately region 2A in the germarium (A, arrowhead). (B and C) Arpc3B ring canal localization persists throughout oogenesis. Arpc3B is also enriched in the somatic muscle sheath in A and B. The majority of staining in the cytoplasm is background. (D and E) Arpc3B and actin colocalize at ring canals; actin monoclonal antibodies (red) were used to reveal actin distribution on methanol-fixed egg chambers. The Arpc1 channel (green) is shown in E. (F–H) Arpc3B displays a punctate distribution on ring canals. (F) Ring canal from a stage 1 egg chamber doubly labeled for Arpc3B (green) and phosphotyrosine (red), a robust ring canal marker in methanol fixed tissue. Arpc3B localization is discontinuous compared with the tight band of phosphotyrosine. G and H show the separated channels from F.

**Figure 7. Nurse cell cytoplasmic actin bundle formation in Arp2/3 complex mutations.** (A, B, and C) Stage 10B nurse cell clusters from wild-type (A), *Arpc1*<sup>Q25st</sup> (B), and *Arp3*<sup>EP(3)/3640</sup> (C) germline clones. Abundant cytoplasmic bundles are observed in all three genotypes. (D, E, and F) Higher magnification views of boxed regions in A, B, and C. Arrowheads in F highlight the striated appearance of the actin bundles in the *Arp3*<sup>EP(3)/3640</sup> mutant nurse cells.



The only known product of Arp2/3 nucleation is an orthogonal network of branching filaments reminiscent of the filaments at the leading edge of a lamellipodium. However, the actin filaments in ring canals have been described at the ultrastructural level as loosely packed parallel actin bundles (Tilney et al., 1996b). A branching F-actin network could be transformed into parallel bundles through the action of F-actin bundling proteins present at the ring canal; these include Filamin, the product of the *cheerio* gene (Li et al., 1999; Sokol and Cooley, 1999) and Kelch, which has been shown recently to bind and bundle F-actin (Kelso et al., 2002).

The requirement for Arp2/3 during ring canal growth raises the interesting possibility that new actin polymerization at the ring canal plasma membrane drives ring canal growth. There are numerous examples of actin filament-driven membrane movement including the motility of *Listeria* and viruses within cells and the advancement of the leading edge membrane in motile cells (Mitchison and Cramer, 1996). By analogy, the ring canal with its dynamic actin cytoskeleton may be equivalent to a circular leading edge membrane. Instead of being confined to one end of a cell, the Arp2/3-dependent actin is positioned between two cells in a continuous expanding ring. To drive expansion of the ring canal diameter, new actin polymerization must be directed circumferentially around the ring.

#### **Arpc3B appears to be a ring canal Arp2/3 component**

We used GFP fusions with Arpc1 and Arp3 and antibodies specific for Arpc3B to study the subcellular localization of the Arp2/3 complex. The GFP fusion proteins reveal a subcellular distribution not unlike what has been observed for the Arp2/3 complex in other systems (Kelleher et al., 1995; Welch et al., 1997): a diffuse cytoplasmic distribution and an enrichment at certain regions of active actin assembly, in this case ring canals.

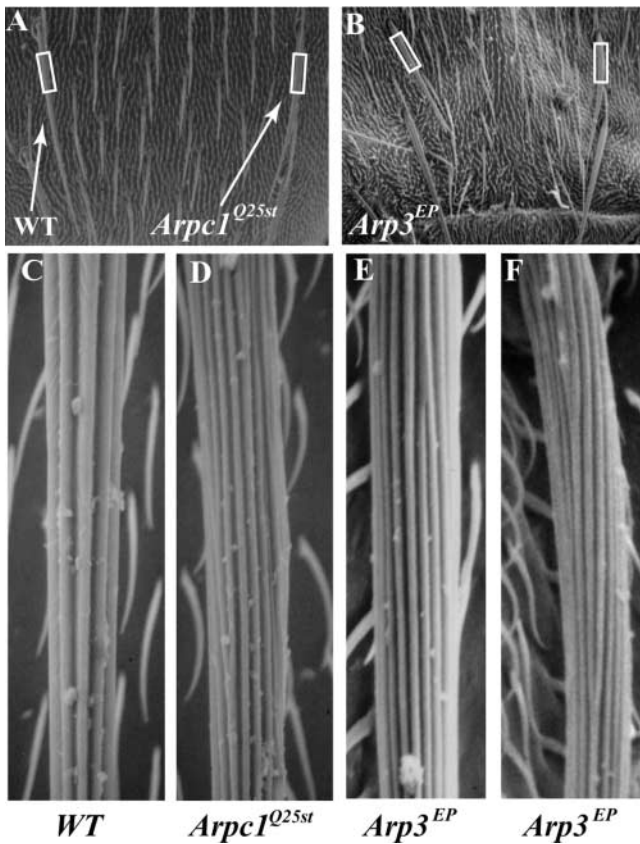
Based on immunoblotting, expression of Arpc3B in adults is restricted to the ovaries of female flies, whereas Arpc3A is ubiquitously expressed. Immunofluorescence using Arpc3B antibodies revealed that it is abundant on ring canals in the nurse cells and is present at low levels in the cytoplasm of

these cells. We speculate that Arpc3B may be a component of a specialized Arp2/3 complex that is required for actin assembly at ring canals and that Arpc3A may be a component of a more broadly distributed Arp2/3 complex. Thus, the subcellular distribution we observed with the Arp3 and Arpc1 fusion proteins may represent two distinct populations of Arp2/3 complex. The ring canal Arp2/3 complex would be expected to contain Arpc3B, whereas the cytosolic Arp2/3 complex would contain Arpc3A.

#### **The Arp2/3 complex is not required for assembly of parallel actin bundles**

Assembly of maximally crosslinked parallel bundles of actin filaments is a conserved process that occurs in tissues requiring the structural support of the actin cytoskeleton. These actin bundles are highly ordered and are assembled into a paracrystalline state through the action of multiple actin crosslinking proteins. Intestinal microvilli, stereocilia, developing *Drosophila* bristles, and *Drosophila* ovarian nurse cells all require parallel actin bundles (DeRosier and Tilney, 2000). In the case of *Drosophila* bristles and nurse cells, the long actin bundles are made up of short, 2–3  $\mu\text{m}$  modules of actin filament bundles; the striated appearance of the nurse cell bundles in wild-type and mutant nurse cells in Fig. 6 reflects the regions where there is little overlap in these short bundles (Riparbelli and Callaini, 1995; Guild et al., 1997). The growing ends of the filaments in both nurse cell and bristle bundles are located at the plasma membrane, and it has been proposed that these filaments are nucleated by factors present at the plasma membrane (Guild et al., 1997) analogous to the dense material at the tip of microvilli (Heintzelman and Mooseker, 1992). It is striking that the nurse cell cytoplasmic actin bundles are able to form in Arp2/3 complex mutants, and the striated appearance of the phalloidin-labeled bundles indicates that the mutant bundles are likely wild-type in ultrastructural organization. In addition, our finding that bristles are able to extend in Arp2/3 mutants provides further evidence that parallel actin bundles do not require Arp2/3 complex function. We conclude that the Arp2/3 complex is not involved in the nucleation of actin filaments for microvillus-





**Figure 8. Mutations in Arp2/3 complex subunits affect the ridging pattern in mechanosensory bristles.** (A) Scanning electron micrograph of a thorax with a wild-type anterior dorso-central bristle on the left and an *Arpc1* mutant anterior dorso-central bristle on the right; the regions marked by rectangles are shown at higher magnification in C and D. The *Arpc1* mutant bristle was identified by scoring the yellow ( $\gamma$ ) clonal marker under a dissecting microscope. Note that the *Arpc1* mutant bristle appears to have normal morphology overall; the bristle is slightly shorter than the wild-type counterpart due to the  $\gamma$  mutation used to mark the clone. (B) Scanning electron micrograph of a thorax from a homozygous *Arp3* pharate adult dissected from its pupal case. The regions of the anterior dorso-central bristles shown at high magnification in E and F are marked by rectangles. (C and D) High magnification comparison of wild-type (C) and *Arpc1* mutant (D) bristles; there are approximately twice as many longitudinal ridges apparent on the *Arpc1* mutant bristle. (E and F) High magnification micrographs of anterior dorso-central bristles from the thorax of *Arp3* pharate adults. As in the *Arpc1* mutant bristle, there is an increase in the number of longitudinal ridges in the *Arp3* mutant bristle compared with wild type.

type actin filament bundles; rather, an alternative nucleation factor may be involved or other mechanisms, such as filament uncapping, may play a role.

The phenotype observed in Arp2/3 mutant bristles is intriguing. Although Arp2/3 mutant cells can produce bristle extensions, the mutant bristles have twice as many ridges as in wild type. The ridges are formed between the actin bundles in the developing bristle; thus, the valleys on a mature bristle mark the location of the actin bundles during bristle development (Tilney et al., 1996a). In Arp2/3 mutants, there is an apparent increase in the number of F-actin bundles, suggesting that bundle organization requires the Arp2/3 complex. Actin filaments for the parallel actin bun-

dles are nucleated at the tips of growing bristles, and the newly polymerized filaments are initially organized into  $\sim 50$  small bundles near the bristle tip (Tilney et al., 1996a). These bundles subsequently aggregate into 7 to 10 larger bundles that mark the positions of the ridges in the mature bristle. In Arp2/3 mutants, the number of ridges is increased, suggesting a specific defect in the bundle aggregation process. Perhaps a distinct population of actin filaments nucleated by the Arp2/3 complex is required to facilitate the aggregation of the parallel actin bundles in developing bristles.

### Activation of the *Drosophila* Arp2/3 complex

It has been well established that the Arp2/3 complex requires activation by proteins of the Wasp/Scar family (for review see Higgs and Pollard, 2001). We have characterized requirements for the Arp2/3 complex in maintaining ring canal integrity and also in the production of the ridging pattern in mechanosensory bristles. In this issue, Zallen et al. (2002) report that strong alleles of *SCAR*, which encodes the single *Drosophila* Scar/Wave protein, display an oogenesis phenotype similar to Arp2/3 complex mutations. In contrast, germline clones of strong *Wasp* alleles proceed through oogenesis with no apparent defects. Thus, *Drosophila* SCAR may be an important activator of the Arp2/3 complex during oogenesis.

### Conclusion

The observations presented here begin to define the specific types of actin rearrangements that require the Arp2/3 complex in metazoan cells. Strikingly, we find no evidence for a requirement of the Arp2/3 complex in the formation of parallel actin bundles. This result suggests that other cytosolic factors are required to nucleate actin polymerization for such structures. Alternatively, these results may highlight the importance of other mechanisms for initiating new polymerization, such as severing or uncapping of existing filaments.

We do find requirements for the Arp2/3 complex in the development of ovarian ring canals and mechanosensory bristle morphology. Both of these developmental processes offer excellent model systems in which to study the function of actin regulatory proteins, and we are now poised to pursue further studies of how the Arp2/3 complex interacts with other actin binding proteins to shape the actin cytoskeleton in vivo.

## Materials and methods

### Genetics/fly strains

All crosses and culturing of *Drosophila* were performed using standard procedures (Ashburner, 1989). *l(2)34D<sup>d</sup>CH60* and *Df(2L)b84a9* were obtained from John Roote (University of Cambridge, Cambridge, UK). The *Arpc1*-rescuing transgene (designated *P[ $\Delta$ ORC5]*) has been described (Pflumm and Botchan, 2001), and was provided by M. Pflumm and M. Botchan (University of California, Berkeley, CA) before publication. The *EP(3)3640* insertion line was provided by Todd Laverty at the Berkeley *Drosophila* Genome Project. Stocks for clonal analysis were obtained from the Bloomington Stock Center.

### Mutagenesis

Isogenic *P[FRY]<sup>2L-40A</sup>* males were mutagenized with ethyl methane sulfonate and mated using standard protocols (Lewis and Bacher, 1968). 6273 F<sub>1</sub> male progeny were individually crossed to virgin females of one of the following

genotypes: *l(2)34Dd<sup>CH60</sup>/CyO* or *Df(2L)b84a9/CyO*. From 5290 viable crosses, 15 independent lines that failed to complement *l(2)34Dd<sup>CH60</sup>* or *Df(2L)b84a9* were isolated. Five of these lines contained mutations in *Arpc1* as assayed by rescue with *P[ΔORC5]*. When necessary, mutant chromosomes were backcrossed to remove extraneous lethal mutations.

### Clonal analysis

All *Arpc1* alleles used were generated on a *P{FRT}<sup>2L-40A</sup>* chromosome. *Arp3<sup>EP(3)3640</sup>* was recombined onto a chromosome containing *P{FRT}<sup>3L-2A</sup>*. For clones marked by loss of *ovo<sup>D</sup>*, flies carrying *Arpc1* or *Arp3* mutations were crossed to flies carrying *P{hsFLP}* on the X chromosome and the corresponding *P{ovo<sup>D</sup>}* FRT chromosome (Chou and Perrimon, 1996). Progeny from this cross were heat shocked as 2nd–3rd instar larvae for 1–2 h in a 38°C water bath. To mark *Arpc1* germline clones with GFP, *P{hsFLP}<sup>12</sup>*; *Arpc1*, *P{FRT}<sup>2L-40A</sup>* flies were crossed to *P{hsFLP}<sup>12</sup>*; *P{Ub-GFP}<sup>33</sup>*, *P{Ub-GFP}<sup>38</sup>*, *P{FRT}<sup>2L-40A</sup>* flies, and their progeny were heat shocked as 1st–2nd instar larvae for 1–2 h in a 38°C water bath. To mark bristle clones, *Arpc1* mutant flies were crossed to *y P{hsFLP}<sup>12</sup>*; *P{y<sup>+</sup>}*, *P{FRT}<sup>2L-40A</sup>* flies and heat shocked as 1st–2nd instar larvae for 1–2 h in a 38°C water bath.

### Sequencing of mutant alleles

Mutants were rescued either as homozygotes or hemizygotes with the *P{ΔORC5}* transgene (Pflumm and Botchan, 2001). DNA from the mutant chromosome was PCR amplified using at least one primer homologous to DNA not included in *P{ΔORC5}*. PCR products containing a single band were purified and submitted for sequencing (Keck DNA sequencing facility, Yale University). Candidate mutations were retested by sequencing both strands of an independently derived PCR product.

### Construction of GFP fusion transgene/generation of transgenic lines

A GFP tagging cassette was prepared by subcloning an EcoRI–KpnI fragment containing GFP from pUAST–GFP–actin (Verkhusha et al., 1999) into pUb–3, a derivative of pCAspeR3–Up2–RXpolyA (Fehon et al., 1997), containing the following polylinker restriction sites: EcoRI, KpnI, BamHI, HpaI, NotI, and XbaI. GFP from this construct was excised with EcoRI and XbaI and cloned into pBluescriptII KS, creating pGFP–NT. *Arpc1* and *Arp3* ORFs were PCR amplified with 5′ BamHI and 3′ NotI restriction sites, cloned into pBluescriptII KS, and sequenced to check for PCR-induced errors. *Arpc1* and *Arp3* were then excised with BamHI and NotI and cloned into pGFP–NT, creating pGFP::Arpc1 and pGFP::Arp3. NotI/KpnI partial digests were used to excise sequences encoding GFP::Arpc1 and GFP::Arp3, and these fragments were cloned into pUASp (Rorth, 1998), yielding pUASp–GFP::Arpc1 and pUASp–GFP::Arp3. These plasmids were injected into embryos according to standard techniques (Spradling, 1986), and transformant lines on each chromosome were established. To express GFP::Arpc1 or GFP::Arp3 in the ovary, the transgenes were crossed to flies carrying the nanos-Gal4:VP16 (Van Doren et al., 1998), which expresses Gal4:VP16 specifically in the germ cells.

### Antibody production/purification

To clone *Arpc3A* and *Arpc3B* cDNAs, PCR primers were designed based on the ORFs predicted by the BDGP. *Arpc3A* was amplified from a 0–4 h embryonic library (Brown and Kafatos, 1988), and *Arpc3B* cDNA was amplified from an ovarian yeast two-hybrid cDNA library (Grosshans et al., 1999). cDNAs were cloned into pBluescript II KS<sup>−</sup> using 5′ BamHI and 3′ XhoI restriction sites and sequenced to check for PCR-induced errors. *Arpc3A* and *Arpc3B* cDNAs were then cloned into pGex-6P1 (Amersham Pharmacia Biotech) and pET-28a to generate GST-tagged and His-tagged fusion proteins, respectively.

GST fusion proteins were produced in DH5α cells by inducing with 1 mM IPTG. Cells were harvested and lysed in a French press in the presence of 5 mM DTT and 1.5% *n*-lauryl sarcosine, and the soluble fusion proteins were purified as described (Frangioni and Neel, 1993).

His-tagged fusion proteins were prepared as described for the GST fusion proteins with the following changes: plasmids (pET28a–Arpc3B and pET28a–Arpc3A) were grown and induced in *E. coli* strain BL21(DE3), DTT was omitted from all steps, and proteins were purified on Ni<sup>2+</sup>/NTA agarose (QIAGEN) following the directions of the manufacturer.

GST–Arpc3A and GST–Arpc3B protein preparations were used to immunize rats, and antisera were screened for reactivity against His-tagged fusion proteins or *Drosophila* proteins. To purify Arpc3B antibodies, ~0.5 mg His–Arpc3B was loaded onto a 10 cm well of a 1-mm thick 12% polyacrylamide gel, electrophoresed, and transferred to either nitrocellulose (Amersham Pharmacia Biotech) or PVDF seqi-Blot membrane (Bio-Rad Laboratories). Following staining with Ponceau S, a thin strip containing the His–Arpc3B was excised from the blot and used as an affinity matrix as described (Pollard, 1984).

### Immunoblots

For immunoblots of His–Arpc3A and His–Arpc3B, purified proteins were quantified using the Bio-Rad Laboratories protein assay, and 5 ng of either purified protein was separated on a 12% polyacrylamide gel and transferred to nitrocellulose, blocked, and probed with primary antibodies as indicated. For immunoblots of *Drosophila* tissue, ovaries or whole flies were homogenized in RIPA buffer (Harlow and Lane, 1988), and the extract was quantitated using the Bio-Rad Laboratories protein assay to ensure equal loading. After separation on 12.5% polyacrylamide gels, proteins were transferred to Amersham Pharmacia Biotech ECL-nitrocellulose, blocked, and probed with primary antibodies as indicated.

### Immunofluorescence, image collection, and quantitation

Ovaries were dissected in ionically matched *Drosophila* saline (Singleton and Woodruff, 1994) and fixed in 6% formaldehyde as described previously (Verheyen and Cooley, 1994). To visualize F-actin, egg chambers were incubated with 1 U of either rhodamine- or Alexa 488–conjugated phalloidin in PBT. For immunofluorescence using Hts-RC antibody, egg chambers were fixed as described above and incubated with a 1:5 dilution of Hts-RC monoclonal supernatant (clone 7C) (Robinson et al., 1994) in PBT. For immunofluorescence using Arpc3B, actin, and phosphotyrosine antibodies, egg chambers were fixed in MeOH at –20°C for 10 min, washed in PBS, washed again in PBT, and incubated with anti–Arpc3B at 1:300, antiactin (Developmental studies hybridoma bank) at 1:25, or anti-phosphotyrosine (Cell Signaling) at 1:500 in PBT. All samples were viewed on a ZEISS LSM-510 confocal microscope (Center for Cell Imaging, Yale University School of Medicine), and images were processed using Adobe Photoshop™ 6.0. For quantitation of ring canal size, egg chambers were labeled with Hts-RC antibody and rhodamine-phalloidin, and three-dimensional projection images were collected. Ring canal sizes were measured using the NIH Image 1.62 software package, measuring the widest possible diameter of all ring canals in each projection image. For each genotype, the measurements for nurse cell–oocyte and nurse cell–nurse cell ring canals were combined and the mean and standard error were determined.

### Scanning EM

Flies were scored for the presence of *y* clones and photographed under a dissecting microscope. Flies were then processed as follows: selected specimens were put through an EtOH dehydration series followed by a hexamethyldisilazane/EtOH series and dried in a speed-vac for 15 min or in a fume hood overnight. Flies were mounted on stubs, rotary shadowed, and viewed on an ISI SS-40 scanning electron microscope.

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