Enabled and Capping protein play important roles in shaping cell behavior during Drosophila oogenesis

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Abstract

During development, cells craft an impressive array of actin-based structures, mediating events as diverse as cytokinesis, apical constriction, and cell migration. One challenge is to determine how cells regulate actin assembly and disassembly to carry out these cell behaviors. During Drosophila oogenesis diverse cell behaviors are seen in the soma and germline. We used oogenesis to explore developmental roles of two important actin regulators: Enabled/VASP proteins and Capping protein. We found that Enabled plays an important role in cortical integrity of nurse cells, formation of robust bundled actin filaments in late nurse cells that facilitate nurse cell dumping, and migration of somatic border cells. During nurse cell dumping, Enabled localizes to barbed ends of the nurse cell actin filaments, suggesting its mechanism of action. We further pursued this mechanism using mutant Enabled proteins, each affecting one of its protein domains. These data suggest critical roles for the EVH2 domain and its tetramerization subdomain, while the EVH1 domain appears less critical. Enabled appears to be negatively regulated during oogenesis by Abelson kinase. We also explored the function of Capping protein. This revealed important roles in oocyte determination, nurse cell cortical integrity and nurse cell dumping, and support the idea that Capping protein and Enabled act antagonistically during dumping. Together these data reveal places that these actin regulators shape oogenesis.

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Introduction

Animal tissues and their constituent cells undergo dynamic changes during embryonic development and during homeostasis and remodeling of adult tissues. The actin cytoskeleton plays a key role in the dynamic behavior of individual cells during morphogenesis, mediating cell shape change and migration. One key challenge is to understand how actin dynamics are regulated during normal development.

Elegant biochemical and biophysical studies in vitro and cell biological analyses in cultured cells have begun to reveal the toolkit cells use to regulate actin dynamics. Actin filaments are asymmetric polymers that extend by monomer addition at the barbed end. An impressive array of proteins regulate actin nucleation, polymerization or capping, bundling and severing (Pollard and Borisy, 2003). One key regulated event is the decision to continue polymerization or terminate this process. Capping protein (CP) binds barbed ends to prevent further addition of actin monomers (Wear and Cooper, 2004). In contrast, Enabled (Ena)/VASP proteins act at barbed ends to promote continued polymerization, thus antagonizing CP (Barzik et al., 2005; Bear et al., 2002). Ena/VASP proteins can also accelerate polymerization by recruiting Profilin/actin complexes (Sechi and Wehland, 2004) and can bundle actin filaments in filopodia (Schirenbeck et al. 2006; Applewhite et al., 2007).

To understand how these proteins shape cell behavior, the roles of Ena/VASP proteins and CP were dissected in cultured fibroblasts and epithelial cells. In fibroblasts, Ena/VASP proteins regulate cell motility (Bear et al., 2000). Inactivating Ena/VASP proteins speeds cell migration while recruiting Ena/VASP proteins to the plasma membrane slows it, suggesting they restrain fibroblast migration. Recruiting Ena/VASP proteins to the plasma membrane leads to longer, unbranched actin filaments in lamellipodia, which may not provide sufficient mechanical strength for sustained lamellipodial extension.

Abbreviations: Ena, Enabled; Abl, Abelson kinase; AJs, adherens junctions; DE-cad, Drosophila E-cadherin; Arm, Armadillo; matGal4, matrix-Gal4-VP16; CNS, central nervous system; CP, Capping Protein; Cpa, Capping protein alpha; Cpb, Capping protein beta; Orb, Oo18 RNA-binding protein; Hts, hu-li tai shao; Fax, failed axonal connections; slbo, slow border cells.

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In keratinocytes (Vasioukhin et al., 2000) and mammary epithelial cells (Scott et al., 2006), inactivating Ena/VASP proteins impairs establishment of cadherin-based cell–cell contacts, potentially by reducing focal adhesion extensions that initiate adhesion. In contrast, depleting CP in mammalian (Mejillano et al., 2004) or Drosophila (Gates et al., 2007) cultured cells triggers explosive formation of filopodia, confirming that CP is a major player in limiting filament extension. In mammalian cells, this response requires Ena/VASP proteins (Appelwhite et al., 2007; Mejillano et al., 2004), further supporting an antagonistic relationship between Ena/VASP and CP.

Ena/VASP proteins are critical for normal development. They play important roles in axon outgrowth and guidance in mice, Drosophila and C. elegans (reviewed in Korey and Van Vactor, 2000; Krause et al., 2003). They also regulate epithelial morphogenesis. While C. elegans epithelial development is normal in the absence of its single Ena/VASP protein UNC-34, animals double mutant for UNC-34 and the Arp2/3 regulator WASP have severe defects in morphogenesis, suggesting redundant roles (Sheffield et al., 2007; Withee et al., 2004). Mice double or triple mutant for Ena/VASP proteins have defects in neural tube closure, craniofacial development, and endothelial barrier function (Furman et al., 2007; Menzies et al., 2004), while knockdown of Xenopus Ena disrupts neural tube closure (Roffers-Agarwal et al., 2008). Removal of maternal and zygotic Ena from Drosophila embryos (Gates et al., 2007) disrupts or alters many events in embryonic morphogenesis, including germ band retraction, dorsal closure and head involution, but does not disrupt cell adhesion or the overall cortical actin cytoskeleton. Less is known about the roles of CP in vivo. Functional CP is a dimer of α- and β-subunits. Null mutations in Drosophila capping protein (cpb) are zygotically larval lethal; presumably maternal contribution rescues embryogenesis. Adult mutants for weaker cpb alleles have defects in bristle development, and are female sterile (Hopmann et al., 1996). In bristles CP has antagonistic relationships with the actin monomer binding protein Profilin and the actin-nucleating Arp2/3 complex (Frank et al., 2006; Hopmann and Miller, 2003). In imaginal discs, precursors of the adult eye and epidermis, loss of CP leads to increased wing disc a subset of cells degenerate. Phenotypes of CP mutants in mice or worms have not been reported.

Together, these data provide a glimpse of some biological events that require Ena/VASP proteins and CP in vivo. To obtain additional insight into how they influence cell behavior during development, we turned to Drosophila oogenesis (Hudson and Cooley, 2002b). The ovary is a relatively simple tissue, with a small number of germline and somatic cell types (Spradling, 1993). Each ovariola is an egg assembly line, with egg chambers of different developmental stages along its length (Fig. 1A). Oogenesis involves a stereotyped set of cell behaviors driven, in part, by the actin cytoskeleton. In the germarium (Fig. 1A), germline stem cells divide asymmetrically to produce cystocytes, which undergo four rounds of cell division without fully completing cytokinesis, producing a cyst of 16 germ cells interconnected by cytoplasmic bridges called ring canals. One cell becomes the oocyte and 15 become nurse cells. Each cyst is then encapsulated by a polarized epithelial sheet of somatic follicle cells joined by adherens junctions (AJs). Nurse cells and the oocyte also have Aj proteins at the cortex, underlain by cortical actin. Cadherin-based adhesion between germ and follicle cells is critical for posterior oocyte positioning (Goddet and Tepass, 1998; González-Reyes and St Johnston, 1998; Peifer et al., 1993). Cadherins and cortical actin also maintain nurse cell integrity, likely resisting force exerted by muscles surrounding each ovariola. In the absence of AJs (Peifer et al., 1993) or when the germline is mutant for actin regulators such as Profilin (Verheyen and Cooley, 1994), the Arp2/3 complex (Hudson and Cooley, 2002a), or its regulator Scar (Zallen et al., 2002), the cortical plasma membrane is disrupted, leading to multinucleate nurse cells.

Nurse cells produce nutrients and macromolecules needed for oocytes for embryogenesis. These are transferred into the oocyte in two stages—early slow cytoplasmic transfer, and rapid late “dumping” when nurse cells transfer their entire contents into the egg, leaving only nuclei behind. Nurse cell dumping is driven by actomyosin contraction (Wheatley et al., 1995), and also requires adhesives proteins and additional cytoskeletal structures. Just prior to dumping, the nurse cell actin cytoskeleton is dramatically remodeled. Arrays of bundled actin filaments, referred to as cytoplasmic filaments, extend from the plasma membrane toward the nucleus, immobilizing it (GUILD et al., 1997; Gutzeit, 1986). These filaments initiate at the plasma membrane as filopodia/microvilli, with barbed ends oriented outward. By an unknown mechanism, many of these initial filament bundles are cross-linked together to form the full-length cytoplasmic filaments. When the germline is mutant for Profilin (Cooley et al., 1992), or the actin bundling proteins Fasclin (Cant et al., 1994, 1998), Villin (Drosophila Quail; MAHJAN-MIKLOS and Cooley, 1994), or Filamin (Li et al., 1999), cytoplasmic filaments fail to form properly. Similar failure occurs when the germline lacks AJs (Peifer et al., 1993). As a result, dumping is reduced or halted, in part because nuclei physically block the ring canals.

Follicle cells also undergo morphogenetic movements. For example, anterior polar follicle cells recruit a group of neighboring cells, undergo a partial epithelial–mesenchymal transition, leave the follicular epithelium and migrate between nurse cells to the anterior end of the oocyte (Montell, 2003; Rorth, 2002). These cells are called border cells (Fig. 1A). Cell adhesion and actin dynamics also regulate border cell migration. However, despite extensive genetic studies, essential regulators of actin dynamics in border cells have not been fully characterized. The actin-depolymerizer ADF/cofilin is required for border cell migration (Chen et al., 2001), and other actin regulators are expressed in migrating border cells (Wang et al., 2006). However, most proteins with well-characterized biochemical functions in actin dynamics have not been functionally tested in border cell migration.

Here we examine how Ena/VASP and CP influence cell behavior, using Drosophila oogenesis as a model. This revealed important roles for Ena in maintaining nurse cell cortical integrity, in forming the robust bundled cytoplasmic actin filaments in nurse cells during dumping, and in border cell migration. We examined the requirement for different domains of Ena in each process, suggesting mechanisms by which Ena acts, and explored its antagonism with CP and regulation by Abelson (Abl) kinase. We also analyzed the role of CP during germline development, revealing novel insights into events where it plays a critical role.

Materials and methods

Fly stocks and clone generation

All mutations are described at Flybase (flybase.bio.indiana.edu). ena^21, ena^24 and ena^290 germline clones were generated by heat shocking 48–72 hour old hsflp^12; FRT42Brena; FRT42BovoD1 larvae for 3 h at 37°C. ab^1 germline clones were generated in the same manner with hsflp^12; +; FRT79Dab^1b; FRT79DovoD1 larvae. Only homozygous ena or abl mutant germ cells develop in these females. ena^21 follicle cell clones were generated by heat shocking 48–72 hour old hsflp^12; FRT42B ena^21/FRT42B Ubi-GFP larvae for 3 h at 37°C. CP mutant and follicle cell clones were generated by heat shocking 48 hour old hsflp^12; FRT42D cpa^24/FRT42D Ubi-GFP larvae for 1 h at 37°C every 24 h until they eclose. Homozygous ena^21 and cpa^24 follicle and germ cells are marked by a lack of GFP. Stocks used to generate ena germline clones (hsflp^12; Sco/CyO and w; FRT42B ovoD1/CyO), ena follicle cell clones (w; FRT42B Ubi-GFP-ns), as well as mata^4-Gal4-VP16, slbo-Gal4, UAS-GFP-actin, and the hypomorphic cpa alleles were from the Bloomington Stock Center. FRT42D cpa^24 was from E. Janody, and UAS-cpa from P. Garrity.
Immunohistochemistry

Ovaries were dissected in Grace’s medium (Gibco Invitrogen) containing 10% fetal calf serum (Gibco Invitrogen, heat-inactivated) and then fixed in 4% formaldehyde (border cell experiments. Polysciences, methanol free) or 4% paraformaldehyde (other experiments, EM Sciences). Fix for cpu experiments also included 8 mM EGTA. Antibodies used: mouse anti-Ena 5G2 (1:500), mouse anti-

Fig. 1. Ena localization and mislocalization during oogenesis. Egg chambers, anterior to left. Genotypes and antigens indicated. (A) Diagram of an ovariole, showing stages of egg chamber development. Stages and cell types labeled. Modified with permission from Peifer et al., 1993. (B-H) Ena. (B) Germarium. Arrowhead = proliferating cystoblasts. Arrows = AJs in follicle cells that are encapsulating germline cells. (C-G) Stages 4, 5, 8, late 10b, and 12, respectively. (H) close-up, late stage 10b. White arrows = Ena at follicle cell AJs. Arrowheads = Ena at nurse cell cortex. Red arrow = Ena at posterior pole of oocyte. Blue arrow = Ena around ring canals. (I) Stage 9, showing border cell cluster. (J) mat-FP4mito expression relocates Ena to mitochondria. (K, L) Mutant ena23 (K) and ena210 (L) protein accumulation at cortex (arrows) is strongly reduced relative to wild-type (F) but not abolished. Scale bars = 20 μm.
ArmN27A1 (1:100), mouse anti-hrsRC (1:100), mouse anti-kel1B (1:10), rat anti-DE-cad DCAD2 (1:200, all DSHB), and rabbit anti-Fax (1:500, a gift of Dr. Eric Liebl). DNA was visualized with DAPI (1:1000, Sigma-Aldrich) and actin with Alexa Fluor®568 phalloldin (Invitrogen). Secondary antibodies (Invitrogen) were Alexa Fluor®488, Alexa Fluor®568 and Alexa Fluor®647. Tissues were mounted in Aqua Polymount (Polysciences) or Vectashield (Vector Laboratories; border cell experiments). All images were acquired using a laser scanning confocal microscope (model 510, Carl Zeiss MicroImaging), LSM510 AIM acquisition software and a 40× (Plan-Neo-Fluar: NA 1.3) or 63× (Plan-Apochromat; NA 1.4) objectives. Adobe Photoshop® 7.0 was used to adjust levels to use the full range of pixel values, and used to adjust brightness and contrast. Modifications were made on entire panel. When levels were compared, brightness and contrast alterations were performed identically on wild-type and experimental images.

Results

Ena’s dynamic localization during oogenesis suggests possible roles in actin regulation

Current models suggest that Ena/VASP proteins regulate actin capping, accelerate actin polymerization and regulate actin bundling (see Introduction). To assess when and where Ena is required during Drosophila oogenesis we examined its localization. In the gerarium, Ena is weakly cortical in all cells (Fig. 1B, arrowhead). As follicle cells envelop the germ cells, Ena becomes enriched at follicle cell AJs (Fig. 1B, arrows), where it remains as egg chambers mature (Figs. 1C–E, white arrows). During border cell migration, Ena accumulates in both central and peripheral cells in the cluster (Fig. 1I). In germline cells Ena accumulates in the cytoplasm (Figs. 1C–H) and is weakly cortically

Fig. 2. Ena localization suggests roles in regulating nurse cell actin filaments. Wild-type stage 10B egg chambers, anterior lower left. Antigens indicated. (A) As cytoplasmic actin filaments form in nurse cells, Ena localizes in a cloud near the nurse cell–nurse cell (white arrows) and nurse cell–oocyte cortex (blue arrows) and around ring canals (arrowheads). (B, D) Successively more close-up views of nurse cell cortex. Ena surrounding nurse cell–nurse cell cortex (bracket) and at barbed ends of bundled actin filaments (arrows). (C) Diagram illustrating (B, D) (E) Ena localizes to microvillar-rich membrane (arrows) surrounding ring canals (arrowhead). (E) Inset. Ena at ends (arrows) of basket of actin filaments projecting from ring canal. Scale bars = 5 μm.
enriched during early stages (Figs. 1C, E, arrowheads); cortical enrichment increases as dumping approaches (Figs. 1F, H, arrowheads) and remains high through this process (Fig. 1G, arrowhead). Ena is also enriched at the posterior end of the oocyte, the follicle cells that abut it, or both (Fig. 1E, red arrow).

Oogenesis requires multiple actin structures, and to gain insight into Ena’s role we closely examined Ena localization relative to these. During late oogenesis, the nurse cell cortical actomyosin network contracts, pushing the nurse cell contents into the oocyte through the ring canals. Just prior to dumping a set of very robust unbranched, bundled actin filaments forms between the plasma membranes and nurse cell nuclei (Fig. 2A; Guild et al., 1997). These cytoplasmic filaments are anchored in plasma membrane projections (Fig. 2C), with their barbed ends membrane proximal (Guild et al., 1997). They hold the nuclei in place, preventing them from being pushed into the ring canals and blocking transport.

The formation of cytoplasmic actin filaments requires several actin regulators, including Profilin (Cooley et al., 1992), and the actin bundling proteins Fascin (Cant et al., 1994), Villin (Drosophila Quail; Mahajan-Miklos and Cooley, 1994), and Filamin (Li et al., 1999). We thus examined whether Ena localization is consistent with a role in regulating filament formation. Interestingly, just as cytoplasmic actin filaments form, a cloud of punctate Ena appears bracketing the nurse cell cortex (Fig. 2A, white arrows, 2B, bracket) and at the nurse cell–oocyte interface (Fig. 2A, blue arrows). Strikingly, this Ena is concentrated at membrane-associated barbed ends of these bundled cytoplasmic filaments (Figs. 2B, D, arrows). During later stages, Ena also accumulates around ring canals (Figs. 1F, H, blue arrows, 2A, arrowheads). Ring canals are large assemblies of actin and other proteins (Robinson and Cooley, 1997) that are surrounded by plasma membrane rich in membrane projections (Riparbelli and Callaini, 1995; Tilney et al., 1996). During dumping, actin filaments form baskets around ring canals (inset Fig. 2E). Ena is not enriched in the ring canal proper (Fig. 2E, arrowhead), but is concentrated in the surrounding plasma membrane (Fig. 2E, arrows), and at the tips of the basket of actin filaments (Fig. 2E inset, arrows). Thus Ena is positioned to potentially help modulate the assembly of the cytoplasmic actin filaments as well as the actin filaments that surround the ring canals.

Loss of Ena results in defects in nurse cell dumping

We next examined whether Ena plays important roles in the germline during oogenesis. We inactivated Ena function using two approaches. We first adapted an approach from cultured mammalian cells, which uses the FP4mito fusion protein (Fig. 3A, top; Bear et al., 2000) to relocalize endogenous Ena/VASP proteins to mitochondria, and thus sequester them away from their normal sites of action. For use in Drosophila we put FP4mito under control of the G4a-UAS system to allow us to express it at different times and/or places in development. Expression of FP4mito rapidly relocates Ena to mitochondria, and in embryos this leads to a very strong to complete loss of Ena function (Gates et al., 2007). We expressed FP4mito in the female germline using the maternal mutants are indicated. A. Diagrams of the FP4mito construct and of Ena, showing lesions in ena alleles, ena46 has two lesions—the first is a missense change in a non-conserved amino acid and may be a polymorphism. F = F-actin binding domain, G = G-actin binding domain, T = tetramerization domain. B. Wild-type eggs. (C–E) Dumpless eggs from mat-FP4mito (C) and cpb hypomorphic (D) mutant, or ablβ (E) germline mutant mothers. (F) Frequencies of dumping defects in mat-FP4mito, ena maternal mutants, cpb hypomorphs, and ablβ maternal mutants are indicated. N = number of eggs scored. (G) Test for modification of dumping defects of FP4mito by heterozygosity for cpb or abl. Scale bar = 50 μm.

not removing the actin binding sites (Fig. 3A). ena46 truncates the protein at the very beginning of the EVH2 domain (Fig. 3A), produces only a very low level of this truncated protein, and behaves genetically like a null allele (Li et al., 2005).

When we reduced germline Ena function by either approach, female fertility was dramatically reduced; many eggs were smaller than wild-type (Fig. 3B vs. C) and most were not fertilized. These eggs exhibited the “dumpless” phenotype characteristic of failure to fully transfer the nurse cell contents into the oocyte (reviewed in Hudson and Cooley 2002b). Similar phenotypes are seen when germline cells are mutant for other cytoskeletal regulators including Ena’s binding partner Profilin (Cooley et al., 1992), the actin bundling proteins Fascin (Cant et al., 1994) or Villin (Mahajan-Miklos and Cooley, 1994), a component of the Arp2/3 complex (Hudson and Cooley, 2002a), or when they are mutant for the AJ proteins DE-cadherin (DE-cad; Godt et al., 1997) or Armadillo (Arm = fly β-catenin; Peifer et al., 1993). The dumpless phenotype of Ena sequestration using FP4mito is most severe, with nearly complete penetrance (Fig. 3F). ena23, deleting Ena’s tetramerization domain, and ena46, deleting the EVH2...
Fig. 4. Ena inactivation disrupts formation of cytoplasmic actin filaments. Egg chambers, anterior lower left, except C where anterior is at bottom. (A–C) Wild-type, antigens indicated. Arrows, filaments at tricellular junctions. Arrowheads = filaments at ring canals. (D–H) Actin. Arrows = first filaments. D1–D4. Wild-type. D3. Arrowhead = ring canal filaments. E1–E4. Ena inactivation delays formation and reduces number of cytoplasmic (arrows) and ring canal filaments (arrowheads). F1–F4. ena23 germline mutants. G1–G4. ena210 germline mutants. ena mutants have delays in filament formation (arrows) matching the severity of their dumpless phenotype. H1–H4. cpb hypomorphs. Filaments are not substantially reduced in number but are abnormal in organization. Scale bars = 40 μm.
domain (Fig. 3A), both display a similar intermediate dumpless phenotype (Fig. 3F), while ena<sup>210</sup>, a point mutation in the EVH1 domain (Fig. 3A), is relatively weak in phenotype (Fig. 3F). Thus Ena function is critical for nurse cell dumping.

**Ena stimulates formation of nurse cell cytoplasmic actin filaments**

Our localization studies suggested that Ena is positioned to potentially regulate the cortical actin network, the bundled cytoplasmic actin filaments extending from the cortex to nuclei (referred to below as cytoplasmic filaments for simplicity) or the ring canals. We hypothesized that Ena might play a role at each of these places, mediating ring canal formation or maintenance, cortical actin formation or contractility, and cytoplasmic filament formation or maintenance. Defects in any of these actin structures could potentially explain the dumping defects we saw when Ena function was reduced in the germline. To explore the mechanisms by which Ena regulates nurse cell dumping, we examined the cell biological consequences of reduced Ena function during oogenesis.

In wild-type egg chambers cytoplasmic filaments first appear in early-mid stage 10B, in nurse cells adjacent to the oocyte (Figs. 4D1,2, arrows; staging was by morphogenetic movements of follicle cells, which do not express FP4mito). Cytoplasmic filaments are often most prominent at junctions where three nurse cells meet (Figs. 4A, B arrows), or at the periphery of ring canals (Figs. 4A, C, arrowheads); interestingly, both are sites of Ena enrichment (Fig. 4A). As egg chambers mature further, cytoplasmic filaments increase in number and appear in more anterior nurse cells (Figs. 4D3, D4, arrows), and by stage 11 nurse cells near the oocyte have already expelled much of their contents (Fig. 4D4).

We first examined egg chambers expressing FP4mito using matGal4 (mat-FP4mito). MatGal4 drives expression of FP4mito in nurse cells after ring canal formation but prior to dumping, pulling Ena from the cortex to presumptive mitochondria (Fig. 1J). Based on our work with FP4mito in embryos (Gates et al., 2007), this should very strongly reduce Ena function. We did not see obvious alterations in cortical actin levels or organization prior to dumping (Fig. 4D1 vs. E1), but did observe a striking difference in the cytoplasmic filaments. Many fewer cytoplasmic filaments formed in FP4mito-expressing egg chambers, and those that formed did so later and were usually restricted to nurse cells nearest the oocyte (Figs. 4E1–4, arrows); these filaments resembled those in wild-type chambers in their banding pattern after phalloidin staining (Fig. 5J vs. K, arrows), reflecting the assembly of these filaments from short segments (Guild et al., 1997). FP4mito expression also reduced and delayed formation of ring canal-associated actin filaments (Fig. 4D2–3 vs. E3–4, arrowheads). In other dumpless mutants such as chickadee (Drosophila profillin; Cooley et al., 1992), reducing cytoplasmic filaments allows nurse cell nuclei to be forced into ring canals, blocking cytoplasmic transport. Most mat-FP4mito egg chambers display nurse cell nuclei that protrude into ring canals (Figs. 5B, D, red arrows). As a result of these defects, nurse cells in terminal chambers expressing FP4mito often retain cytoplasm (Fig. 5B, white arrow vs. 5C). Thus Ena plays an important role in the formation of the cytoplasmic actin filaments and plays a key role in nurse cell dumping.

**Differential effects of Ena's EVH1 and EVH2 domains in nurse cell cytoplasmic filament formation**

We next explored the mechanisms by which Ena acts in the formation of cytoplasmic actin filaments, by examining the requirement for...
different parts of the protein. We assessed roles of the EVH1 and EVH2 domains by examining females with germlines homozygous mutant for the three ena mutants described above, ena23, ena107, or ena46. The ena23 and ena210 alleles generate stable proteins (Ahern-Djumali et al., 1998); cortical recruitment of both mutant proteins in the germline was substantially reduced but not eliminated (Figs. 1K, L). ena23 (Figs. 4F1–4), ena46 (data not shown), and ena107 (Figs. 4G1–4) mutant germlines all had defects in cytoplasmic filaments, but these phenotypes were less severe than mat-FP4mito, with increased numbers of filaments and less delay in their appearance. ena23 had a more severe cytoplasmic filament phenotype than ena107 (Fig. 4F vs. G), consistent with its higher penetrance dumpless phenotype (24%, N = 920 vs. 4%, N = 627; Fig. 3F). ena46 is no more severe than ena23, consistent with the similar penetrance of their dumpless phenotypes (Fig. 3F). These data suggest that a functional EVH1 domain may not be essential for formation of nurse cell cytoplasmic filaments, but that the EVH2 domain and its tetramerization subdomain are more critical.

Ena is required for cortical actin integrity in nurse cells but dispensable for ring canal formation.

Ena’s localization suggested it also might mediate ring canal formation or maintenance, or cortical actin formation or contractility. We observed no defects in ring canal formation or growth in mat-FP4mito, ena23, ena46, or ena107 mutant egg chambers. They accumulated the ring canal markers actin, Hts, Kelch, and tyrosine phosphorylated proteins normally (Suppl. Figs. 1A–J). These data suggest that Ena is not essential for ring canal assembly.

Ena also localizes to the nurse cell cortex. Interestingly, cortical actin in ena23 and ena107 mutant egg chambers exhibited an additional defect not seen in mat-FP4mito: some egg chambers contained multinucleate nurse cells (Figs. 5E–G, arrows). We suspect that this resulted from rupture of the plasma membrane and cortical actin, since both actin (Figs. 5E–G) and DE-cad (data not shown) staining were lost. Consistent with this, multinucleate nurse cells often contained actin aggregates that may be ring canal remnants (Fig. 5E, arrowhead), and we occasionally saw nurse cell membranes apparently in the process of breaking down (Fig. 5F, arrow). Multinucleate nurse cells appeared at ~ stage 7, when slow transport of nurse cell contents begins (ena107% of egg chambers, N = 77; ena23% N = 30), but defect frequency increased dramatically by stage 10, when dumping is initiated (ena23% N = 105; ena107% N = 19). A similar phenotype is seen when the germline is mutant for AJ proteins (Arm, Pfeifer et al., 1993), Profilin (Cooley et al., 1992), or the Arp2/3 complex (Arp3; Hudson and Cooley, 2002a). Loss of Ena does not, however, grossly affect nurse cell Aj’s, as DE-cad levels and localization resembled wild-type (Fig. 5H vs. I). Some nurse cells successfully expelled cytoplasm even in egg chambers with multinucleate nurse cells (Fig. 5G, arrowhead), suggesting that cortical actin can retain enough integrity to allow contraction. Since cortical defects are not seen in mat-FP4mito ovaries, in which Ena inactivation does not begin until stage 6, Ena may help reorganize and strengthen cortical actin prior to contraction, so that ena mutant nurse cells are unable to cope with the mechanical stress of dumping.

Loss of Capping protein affects oocyte determination

As a further test of the hypothesis that regulated actin capping is important for oogenesis, we explored the function of CP. CP is a dimer of α- and β-subunits, both of which are essential for function (Cooper and Sept, 2008). Null alleles of either capping protein (cpa) or capping protein (cph) are lethal (Hoppman et al., 1996; Delalle et al., 2005). Analysis of mosaics in imaginal discs revealed that loss of CP function leads to a striking increase in actin levels, and in some regions of the disc this leads to cell death (Delalle et al., 2005; Janody and Treisman, 2006). We thus anticipated that eliminating CP function in the germline would elevate actin polymerization. To test this hypothesis we generated germlines mutant for a null allele of the α-subunit, cpaDNE (Janody and Treisman, 2006). We marked mutant germline cells by loss of GFP (e.g. Fig. 6B).

To our surprise, early development of egg chambers and many features of actin accumulation in cpaDNE mutant germline cells were relatively normal (Fig. 6B vs. A, C–E; wild-type oocytes are marked with an arrowhead, mutant oocytes with an arrow). Mutant germ cells formed 16-cell cysts, were encapsulated by follicle cells relatively normally, and initially grew in size at a rate relatively similar to wild-type (Fig. 6B). As they reached stage 6, mutant egg chambers became morphologically abnormal, taking on a spindle shape (Figs. 6C–E, arrows). At this point egg chambers ceased increasing in size, and ultimately degenerated. No mutant egg chambers later than ~ stage 6/7 were observed. Through stage 6/7, however, overall actin levels were relatively normal in cpaDNE mutant germ cells (Figs. 6B–E, arrows). When we looked at higher magnification, we could sometimes discern a slight increase in the number and length of fine actin filaments at the nurse cell cortex (Fig. 6I vs. J), but this was much less striking than previously observed in cpaDNE mutant imaginal disc epithelia (Delalle et al., 2005; Janody and Treisman, 2006) or than we observed in cpaDNE mutant follicle cells (see below). Ring canals also appeared to form normally, accumulating, Kelch, Hts and tyrosine phosphorylated proteins as in wild-type (Suppl. Figs. 1K–P).

There was one striking difference in cpaDNE mutant germlines: many did not contain a recognizable oocyte. Normally, one of the 16 cells in the germline cyst is specified as the oocyte soon after cyst formation. This cell accumulates the oocyte determinant Orb (Figs. 6A, B white arrowheads; Lantz et al., 1994). Most mutant egg chambers failed to accumulate Orb at higher levels in any particular cell (22/30; 73%; Figs. 6B, C, arrows; ~30% of mutant egg chambers did determine an oocyte: Figs. 6F–H, arrows). We observed a similar defect in oocyte specification in animals whose germlines were mutant for a second null allele, cpaDNE (data not shown). In wild-type, Orb enrichment in a single cell in each cyst can be detected in the gerarium, sometimes as early as the distal end of region 2b (with disc-shaped germline cysts; Fig. 6K, blue arrow). Orb enrichment is readily apparent in stage 1 (Fig. 6K, white arrows). In contrast, we did not observe Orb enrichment in any cells in many mutant germaria (Fig. 6L). As oogenesis proceeds, nurse cell genomes become highly polyploid and their nuclei enlarge, while the oocyte retains a small diploid nucleus (Fig. 6A, left inset red arrowhead). Strikingly, most cpaDNE mutant egg chambers did not contain a properly differentiated oocyte; instead they had 16 nuclei that were all larger than expected for the oocyte (73%; 19/26; we confirmed this by serial sectioning; an example is in Suppl. Fig. 2; the oocyte is one of the cells with 4 ring canals, indicated in violet). Of those without a properly differentiated oocyte, in some the 16 nuclei were equal in size (32%; 6/19; Fig. 6E, arrow), and in others one nucleus, likely the oocyte, was smaller, but not nearly as small as a normal oocyte nucleus (68%; 13/19; Suppl Fig. 2). We also saw a second defect in cpaDNE mutant egg chambers at a lower penetration. Normally, the oocyte is tightly positioned at the posterior end of the egg chamber. However, in about half the mutant egg chambers (15/26; ~61%), the oocyte was not precisely positioned at the posterior (Figs. 6G, H)—this included egg chambers that did and did not display a specified oocyte. These data suggest CP plays roles in oocyte specification and positioning.

In wild-type egg chambers, the oocyte is easily distinguished by stage 3 because it accumulates more actin at its cortex than the nurse cells (Fig. 6A, arrowheads). In cpa mutant egg chambers lacking a differentiated oocyte, no germline cell had extra actin (Figs. 6B–E, arrows). However, in the fraction of cpa mutant egg chambers that did have a properly differentiated oocyte (indicated by a small oocyte nucleus or proper Orb accumulation), actin was enriched surrounding the oocyte (Figs. 6G, H). Thus the failure to enrich actin in most cpa
mutant egg chambers is likely a secondary consequence of failed oocyte determination rather than a direct effect of loss of CP.

Reducing levels of Capping protein disrupts nurse cell dumping and disrupts integrity of the cortical membrane of nurse cells

These results revealed an important role for CP in oocyte specification, but the degeneration of mutant egg chambers after stage 7 precluded analysis of later stages, during nurse cell dumping. However, Miller and colleagues identified a transheterozygous combination of weak cph alleles (cphF19/cpb6.15) that produces viable but female sterile flies (Hopmann et al., 1996). We used these animals to examine effects of reducing CP function on oogenesis—because these are not null alleles, we cannot rule out additional roles for CP not revealed in this hypomorphic situation.

We first examined whether cphF19/cpb6.15 transheterozygotes lay eggs, and if so, whether these eggs are defective. Most cphF19/cpb6.15 eggs are shorter than wild-type (Fig. 3D), suggesting defects in nurse cell dumping. 97% were dumpless (Fig. 3F), similar to or more severe than we saw when disrupting Ena function using FP4mito. Thus reducing CP function disrupts nurse cell dumping.

To begin to determine mechanisms by which CP acts during dumping at the cellular level, we examined actin organization and Ena localization during oogenesis in cphF19/cpb6.15 mutants. Early stage egg chambers appeared largely or completely normal (Figs. 7A, B). We observed the first defects beginning at stage 8, with the appearance of
occasional egg chambers with multinucleate nurse cells (17%; N = 6). As egg chamber development progressed, multinucleate nurse cells became more frequent (57% at stage 9 were multinucleate; N = 7; Fig. 7C, pink arrow). As in ena mutants, this defect occurred without obvious overall defects in levels or localization of nurse cell cortical actin prior to the onset of nurse cell dumping (Fig. 7B), at least at the level of light microscopy. By stage 10 and later, most egg chambers had multinucleate nurse cells (66%; N = 32; Figs. 7D, G pink arrows), and many late stage egg chambers, which should have already begun dumping (Fig. 7G), exhibited little or no transfer of cytoplasm to the oocyte. There were also defects in the nurse cell–oocyte border. In wild-type this is straight (Fig. 7B, arrow), but in cbpF19/cpb6.15 mutant egg chambers nurse cell nuclei often bulged into the oocyte (Figs. 7C, E, G, yellow arrows), and at times the nurse cell–oocyte interface ruptured (Fig. 7F, arrowhead) or nurse cell nuclei were found in the oocyte (data not shown). We do not think multinucleate nurse cells result from defects in cytokinesis, as we did not observe them before stage 8. Instead, we believe they reflect breakdown of the cortical membrane. Consistent with this, we sometimes found actin aggregates in the oocyte, at least some of which appear to be ring canal remnants (Fig. 7F, white arrow). These data suggest that CP plays an important role in nurse cell cortical integrity, and in its absence these defects contribute to the highly penetrant defects in nurse cell dumping.

Capping protein is important for correct organization of cytoplasmic actin filaments in nurse cells

While cortical integrity defects are likely to explain part of the problem with nurse cell dumping in cbpF19/cpb6.15 mutants, the frequency of dumping defects (97%) is higher than that of cortical defects (~65% by stage 10). We thus examined another actin structure, the cytoplasmic actin filaments, defects in which might affect dumping.

There are striking differences in cytoplasmic actin filaments in cbpF19/cpb6.15 mutants. Prior to the onset of dumping, cortical actin appeared relatively normal in cbpF19/cpb6.15 nurse cells that did not have defects in cortical integrity (Figs. 8A, B). Unlike ena mutants, cbp hypomorphs did not have substantial delays in cytoplasmic filament production or large reductions in filament number (Figs. 4H1–4; Figs. 8H, 1 vs. F, G). However, the morphology and organization of the cytoplasmic filaments were substantially different from wild-type. As filaments formed, the oocyte (Fig. 8C, bottom arrow) and nurse cell (Fig. 8E) cortex of cbp hypomorphs appeared “furry”, with excess actin accumulating both cortically and in the form of short filaments; this contrasted with what we observed in wild-type (Fig. 8C, top arrow; D). As filament growth proceeded, the distribution of longer cytoplasmic filaments in cbp hypomorphs was less uniform around the cortex (Figs. 8L, M yellow arrows, vs. Figs. 8G, J) and filaments were more disorganized (Figs. 8L, M vs. K, J). This asymmetric filament accumulation was not effective in restraining the nurse cell nuclei, which were often positioned against the cortex, or protruding through and thus blocking ring canals (Figs. 8N, O). These defects likely contribute to the significant reduction in nurse cell dumping observed (e.g., Fig. 8I). Interestingly, despite the disorganization of the cytoplasmic actin filaments and the disruption of dumping, Ena cortical localization was not dramatically altered (Figs. 8H*, M* vs. F*, J*). Together, these data suggest that Ena and CP are essential for dumping, likely via affects on both cortical actin and cytoplasmic filaments.

Most models suggest that one function of Ena/VASP proteins is to functionally antagonize CP. These models would predict that reducing CP levels might ameliorate the reduction of Ena function. We tested this during nurse cell dumping by generating females expressing FP4mito and also heterozygous for a null mutation in CP, and comparing them to FP4mito alone. Reduction of the levels of CP reduced the severity of the dumpless phenotype from 84% to 57% (Fig. 3G), consistent with an antagonistic relationship.

Does Abl regulate Ena and actin architecture during nurse cell dumping?

During embryonic development, the tyrosine kinase Abl is a key negative regulator of Ena activity (Gertler et al., 1995). In its absence, de-regulated Ena activity leads to disruption of morphogenetic events and embryonic lethality (GrevenGoed et al., 2001; GrevenGoed et al., 2003; Fox and Peifer, 2007). We thus explored whether Abl acts as a negative regulator of Ena activity during oogenesis.

We generated females whose germlines are homozygous for the null abl allele abl–/–, and examined the actin cytoskeleton, staining them together with wild-type egg chambers marked with histone-GFP as a control. Early to mid-stages of oogenesis in abl–/– mutants were indistinguishable from wild-type in morphology and actin localization (Fig. 9A). abl–/– mutants assembled relatively normal nurse cell actin structures (Fig. 9B vs. C). There were only slight differences from wild-type. First, while most wild-type egg chambers do not assemble cytoplasmic filaments until stage 10 (Fig. 9E; we occasionally see wild-
type egg chambers with filaments in stage 9; Fig. 9F), 3/3 stage 9 abl mutants already had cytoplasmic filaments (Fig. 9D), suggesting a possible acceleration in initiating filament formation. Second, we occasionally noticed ectopic accumulations of Ena and actin in the oocyte of abl mutants (Figs. 9G, H arrows). Finally, late stage cytoplasmic filaments sometimes appeared more robust than wild-type, with additional Ena at their barbed ends (Fig. 9I vs. J). Thus, loss of Abl results in mild phenotypes consistent with modest deregulation of Ena function. These defects are associated with defects in dumping, though not as frequent as those seen upon inactivation of Ena or CP—14% of eggs laid by females whose germlines were abl mutant were dumpless (Figs. 3E, F). Given this evidence that Abl restrains Ena activity during oogenesis, we explored whether reducing Abl levels might alleviate the effects of Ena inactivation on dumping.

Fig. 8. In cpb mutants cytoplasmic actin filaments form but are disorganized. Egg chambers, anterior left, antigens and genotypes indicated. (A, B) Just prior to dumping. Actin and Ena localization are roughly normal. (C–E) When dumping begins excess actin accumulates at oocyte (C, bottom arrow) and nurse cell (E) cortex relative to wild-type (C, top arrow and D). (F, G) Mid and late stages of dumping in wild-type. (H, I) cpb mutant, mid- and late-dumping. (H) Arrow = nurse cell nucleus protruding into oocyte. (I) Arrows = disorganized actin filaments. (J–M) cpb. Filaments have a non-uniform distribution (M, yellow arrows) relative to wild-type (J), and filaments are less well organized (L vs. K). N.O. cpb. Nurse cell nuclei sometimes block ring canals (arrows). Scale Bars = 20 μm.
In fact, heterozygosity for \textit{abl} partially suppressed the effects of Ena inactivation by FP4mito, reducing the frequency of dumpless eggs from 83\% to 68\% (Fig. 3G). Together these data are consistent with a role for Abl in negatively regulating Ena during oogenesis.

Exploring roles for Ena and Capping protein in follicle cell epithelia

Work in cultured mammalian cells suggested that Ena plays an important role in cell adhesion in cultured keratinocytes and mammary epithelial cells (Scott et al., 2006; Vasioukhin et al., 2000). In \textit{Drosophila}, earlier work suggested it plays a role in epithelial integrity in follicle cells, as there were apical actin defects in follicle cells mutant for \textit{ena}^{20} or \textit{ena}^{22} (Baum and Perrimon, 2001). However, embryonic and imaginal epithelia in \textit{Drosophila} do not require Ena function for epithelial organization or cell adhesion (Gates et al., 2007). To understand this difference and test the hypothesis that Ena is required for epithelial integrity in follicle cells, we re-examined Ena function in this tissue.
Follicle cells form a polarized epithelial sheet surrounding the germ cells, with its apical surface facing inward. Follicle cells assemble an actin ring underlying the AJs, which in cross section makes a line across the apical end of the sheet (Suppl. Fig. 3B, arrowheads). We generated clones of follicle cells mutant for ena^{23}—homozygous mutant cells lost expression of a GFP-marker on the other chromosome. Loss of Ena in early egg chambers (stages 2–7) did not usually cause significant disruptions in either epithelial organization (Suppl. Fig. 3A; blue lines demarcate mutant clones), or in the assembly of cortical actin into follicle cell AJs, as illustrated in Suppl. Fig. 3B, where almost all follicle cells are mutant, or Suppl. Figs. 3C–E, illustrating boundaries between mutant and wild-type follicle cells. However, in later egg chambers, we did observe clones with reduced cortical actin (Suppl. Figs. 3F, G) and at times with disruptions in epithelial integrity (Suppl. Fig. 3H, arrowhead). These data are more consistent with a role for Ena in morphogenetic movements of follicle cells rather than in epithelial integrity per se.

In contrast, loss of CP had more dramatic consequences. Follicle cells mutant for the null allele cpa^{69E} began to accumulate excess actin on their apical and lateral surfaces in early stage egg chambers (Suppl. Fig. 3I; mutant cells lack GFP). By stages 6–8, some mutant cells began to lose their monolayer organization, especially in clones positioned near the posterior end of the egg chamber (Suppl. Figs. 3J, K, arrows). Mutant cells accumulated very high levels of actin around their entire circumference (Suppl. Figs. 3K, K′), similar to what was observed in imaginal discs mutant for CP (Delalle et al., 2005; Janody and

![slbo-Gal4,UAS-mCD8-GFP; UAS-AP4mito](image)

![slbo-Gal4,UAS-mCD8-GFP; UAS-FP4mito](image)

![slbo-Gal4,UAS-mCD8-GFP](image)

![slbo-Gal4,UAS-mCD8-GFP; UAS-FP4mito](image)

![slbo-Gal4,UAS-mCD8-GFP](image)

![slbo-Gal4,UAS-mCD8-GFP; UAS-FP4mito](image)

**Fig. 10.** Ena regulates border cell migration. Genotypes and antigens indicated. (A-D) Stage 10 egg chambers. (A) slboGal4; UAS-A4Pmito negative control. (B) slboGal4; UAS-F4Pmito. (A″, B″). High magnification views of areas indicated in A′ and B′, showing the sequestration of Ena in border cells by FP4mito but not by AP4mito. (C, D) High magnification views of border cell protrusions in (C) slboGal4,UAS-mCD8GFP and (D) slboGal4,UAS-mCD8GFP; UAS-F4Pmito. (E) Quantification of border cell migration in stage 10 egg chambers expressing indicated genes using slbo-Gal4. n = number of egg chambers scored. (F) Duration of border cell migration after detachment from the follicle cell epithelium (+/− s.d.). (G) Border cell migration speed (+/− s.d.). Scale bars = 50 μm (A, B); 10 μm (C, D).
Treisman, 2006). As wild-type cells began to accumulate basal actin in oriented filaments at stage 9 (Suppl. Fig. 3N, arrow; Gutzeit, 1990), the excess actin phenotype in cpa69E mutant cells became even more accentuated (Suppl. Fig. 3I, arrow, M′, arrowhead). Thus CP is essential for maintaining proper levels of apical actin in follicle cells, and for epithelial architecture in at least a subset of cells.

Ena regulates speed of border cell migration

The phenotypes observed in ena mutant follicle cells suggest that Ena may play a role in their morphogenetic movements. The best-characterized follicle cell movement is migration of the border cells, a cluster of 6–8 follicle cells surrounding the two anterior polar cells, which undergo a dramatic and stereotypical migration of 150–200 μm (Montell, 2003; Rorth, 2002). Early in stage 9, these cells extend long and dynamic actin-rich protrusions between the nurse cells (Murphy and Montell, 1996; Fulga and Rorth, 2002; Prasad and Montell, 2007) and then delaminate from the rest of the follicle epithelium and migrate towards the oocyte. They reach the anterior border of the oocyte by stage 10.

Ena/VASP proteins regulate the speed of migration of cultured fibroblasts; in this cell type they inhibit cell migration (Bear et al., 2000). Unlike cultured fibroblasts, border cells migrate as a group, use a cellular substrate, and must force their way in between tightly

![Diagram of Ena overexpression effects on border cell migration](image-url)

**Fig. 11.** Overexpression of Ena leads to border cell migration defects and altered protrusiveness. Genotypes and antigens indicated. (A) Wild-type (w1118), early stage 9. (B) slbo-Gal4; UAS-Ena late stage 9/early stage 10. (C-F) High magnification view of border cell cluster. Red arrows = leading cells with prominent protrusion. (C) Wild-type. (D) slboGal4; UAS-Actin-GFP. (E) slboGal4; UAS-Ena. (F) slboGal4; UAS-ActinGFP/UAS-Ena. (E, F) Arrows = numerous protrusions induced by Ena overexpression; they are even more numerous and/or more evident in the presence of Actin-GFP. G. Quantification of border cell migration in stage 10 egg chambers following overexpression of indicated genes using slbo-Gal4. n = number of egg chambers scored. (H) Wild-type stage 9 egg chamber showing incomplete border cell migration (arrow). Egg chambers staged by degree of retraction of follicle cells from nurse cells to oocyte. (I) slboGal4; UAS-Cpb stage 9 egg chamber showing border cells having reached oocyte (arrow). (J) Quantitation of completion of border cell migration during stage 9 (n = number of egg chambers scored). p value calculated using Fisher’s exact test. Scale bars = 50 μm (A, B); 10 μm (C–F).
aposed nurse cells. Thus their mechanisms of migration and their requirement for Ena function may be different. To examine Ena function in border cells we inactivated it by expressing FP4mito using slow border cells (slo)-Gal4 (Rorth et al., 1998), which drives expression in border cells, centripetal follicle cells and posterior follicle cells, but not polar cells (Fig. 10A; Geisbrecht and Montell, 2002). As a negative control, we expressed AP4mito, which localizes to mitochondria but does not bind or sequester Ena (Bear et al., 2000; Gates et al., 2007). In wild-type and AP4mito expressing border cells, Ena was largely cytoplasmic (Fig. 10A*: data not shown). When FP4mito was expressed using slo-Gal4, it altered Ena localization in follicle cells (Fig. 10B* vs. A*), consistent with its expected mitochondrial sequestration.

In contrast to what was observed in fibroblasts (Bear et al., 2000), inactivating Ena in border cells did not accelerate migration but rather slowed migration. Although the long cellular extensions required to initiate migration appeared normal (Fig. 10C vs. D), ~50% of FP4mito-expressing border cell clusters failed to reach the oocyte by early stage 10 (Fig. 10B vs. A; quantified in 10E), compared to <10% in control AP4mito or wild-type egg chambers (Fig. 10E).

This defect in arrival time could result from a change in the time required to detach from the follicle cell epithelium or an actual change in migration speed. To distinguish these, we analyzed border cells live. This confirmed that FP4mito-expressing border cells extend long protrusions seemingly normally, and detach from the follicle cell monolayer (Suppl. Fig. 4; Movie 1 vs. 2). However, migration after detachment occurred significantly more slowly than normal (Figs. 10F,G; Suppl. Fig. 4); for AP4mito-expressing controls the duration of migration was 129 min±8.1 min (Movie 1), while it was 198 min±17.9 min for FP4mito (Movie 2). Thus migration speed for border cells expressing FP4mito (1.06±0.056 μm per min) was significantly slower than that of AP4mito-expressing controls (1.56±0.26 μm per min; p = 0.038). These results indicate that Ena plays a positive role in promoting cell migration in border cells.

To explore Ena function in border cells further, we assessed effects of overexpressing Ena on border cell morphology and migration. Interestingly, Ena overexpression also delayed/disrupted migration (Fig. 11A vs. B; C; ~40% of border cell clusters did not complete migration in a timely fashion). To investigate the mechanism by which this might occur, we examined the morphology of border cell clusters, using antibodies against Failed Axonal Connections (Fax) and Singed (Sn), which are enriched in border cells (Figs. 11A, C). Wild-type border cell clusters at the beginning of migration generally exhibit one prominent protrusion in the direction of the oocyte (Figs. 11C, D). In contrast border cell clusters overexpressing Ena showed many fine protrusions (Fig. 11E). To observe actin distribution following Ena overexpression, we co-expressed Ena and GFP-actin using slbo-Gal4. Overexpression of GFP-actin by itself increases the frequency and length of the long cellular extensions from the border cell cluster (Fig. 11D vs. C), and inhibits border cell migration (Fig. 11G). When we co-expressed Ena together with GFP-actin, ~50% of border cell clusters showed numerous fine actin-rich protrusions (Fig. 11F), and border cell migration was further inhibited (Fig. 11G). Thus the proper level of Ena activity is required for border cells to generate the correct sort of protrusions and migrate effectively.

In parallel, we examined effects on border cell migration of overexpressing Cpb in border cells using slo-Gal4. Strikingly, overexpression of Cpb resulted in premature arrival of border cells at the oocyte (Fig. 11). In wild-type, we never saw border cells arrive at the oocyte during stage 9 (0/19), while in UAS-Cpb ovaries border cells had already reached the oocyte during stage 9 in 100% of those observed (28/28; p = 10−12 by Fisher's Exact test). We also assessed border cell migration in cpb hypomorphs. Border cells reached the oocyte in 28/28 stage 10 egg chambers scored. In 9/28 cases border cells arrived somewhat off-center, but this is likely due to the breakdown of some nurse cell membranes. Thus follicle cell morphogenesis is less sensitive to reduction of CP than is nurse cell dumping. However, the residual CP in the hypomorphs precludes any stronger conclusions.

Discussion

As cell biological studies provide increasing information about the toolkit cells use to modulate actin dynamics, our challenge is to determine how this toolkit is used to create diverse cell structures and behaviors during development. Drosophila oogenesis provides an outstanding model for assessing this, with diverse cell types and stereotyped cell behaviors. Our work provides new insights into the developmental mechanisms that allow Ena and CP to create a wide-range of actin structures.

Ena helps mold diverse actin structures

To address the challenge outlined above, we eliminated the function of two key actin regulators and examined the effect on the diverse actin structures cells produce during development. In the case of Ena, the results are surprising: cells use this tool to craft a diverse array of different actin assemblies that contribute to many different cell behaviors. In cultured fibroblasts (Bear et al., 2000), neurons (Lebrand et al., 2004) and epithelial cells (Meijllano et al., 2004), Ena restrains migration by modulating actin dynamics at the leading edge, and generates filopodia by anti-capping and filament bundling. Ena also regulates axon outgrowth and guidance, manipulating actin assembly in growth cones (reviewed in Krause et al., 2003), and plays roles in dendrite branching (Li et al., 2005; Lin et al., 2007). During Drosophila morphogenesis Ena plays many roles (Gates et al., 2007). Some, like promoting leading edge filopodia and thus epithelial zippering during dorsal closure, fit well with Ena’s anti-capping function. In other roles (segmental groove formation and head involution), the cell biological basis is less clear, but affected cell types exhibit striking patterns of Ena localization. Our analysis of oogenesis further broadens the diversity of cell behaviors requiring Ena, highlighting roles in nurse cell cortical integrity, formation of nurse cell cytoplasmic filaments during dumping, and border cell migration.

In the case of the cytoplasmic filaments, our analysis combined with Ena’s postulated biochemical functions provide interesting mechanistic insights into filament assembly. Ena is thought to promote filopodia by providing anti-capping activity (Barzik et al., 2005; Bear et al., 2002), promoting filament elongation (Sechi and Wehland, 2004), and helping bundle filaments (Schirenbeck et al., 2006; Applewhite et al., 2007). Work from the Tilney/Guild labs revealed that nurse cell cytoplasmic filaments form from bundled actin filaments projecting from the plasma membrane, with their barbed ends membrane proximal (Guild et al., 1997). Ena localizes to these bundled barbed ends and filament formation is depressed in its absence, supporting the idea that it plays an important role in promoting filament elongation/bundling. Consistent with anti-capping being critical, reducing CP levels partially suppressed the effects of Ena inactivation.

We used three ena mutants to begin to dissect mechanisms by which Ena acts. Ena wz protein lacks the tetramerization domain (Ahern-Djamali et al., 1998). In nurse cells, Ena tetramerization may help collect individual actin filaments into the robust bundled cytoplasmic filaments, as was suggested for filopodia in mammalian cells (Schirenbeck et al., 2006; Applewhite et al. 2007). Consistent with this, the Ena23 mutant protein, which should be able to act in anti-capping but should be reduced in the ability to bundle capped filaments, displayed a significant decrease in its ability to mediate filament formation. Ena23’s phenotype was similar to that of Ena wz, which completely lacks the EVH2 domain, suggesting tetramerization is a key part of EVH2 function. Both appeared to retain some residual
function in filament assembly, however, as their phenotypes were less severe than that of FP4mito. Alternately, FP4mito sequestration of Ena may also sequester some protein partners, thereby increasing phenotypic severity. Ena<sup>105</sup> mutant protein, with a point mutation in the EVH1 domain impairing binding to EVH1 ligands (Ahern-Djamali et al., 1998), retained significant function. This suggests either that Ena's role in this process is largely independent of an EVH1 ligand (perhaps it is recruited to the cortex by other protein interactions), or that the point mutation in ena<sup>105</sup> does not fully inactivate EVH1 function.

None of our methods of disrupting Ena function fully eliminated cytoplasmic filaments. This may suggest either that none of our approaches completely eliminate all functional Ena (our data in embryos suggest that FP4mito produces a nearly null or null phenotype; Gates et al., 2007), or that Ena is not absolutely essential for filament assembly, although it does clearly regulate the rate/success of filament initiation or polymerization. It will be interesting to further explore how Ena's structure dictates its function in future experiments.

The mechanistic role of Ena in nurse cell cortical integrity must remain more speculative, as actin substructure at this position has not been closely investigated. Cortical integrity also requires Ena's partner Profilin (Cooley et al., 1992) and Ena's antagonist CP (our data), as well as the actin-nucleating/branching Arp2/3 complex (Hudson and Cooley, 2002a). Thus correct structural integrity of cortical actin appears to require balance between anti-capping and branching. Nurse cell cortical integrity also requires the cadherin–catenin complex (Peifer et al., 1993); it may anchor cortical actin, or may have a more active role in regulating actin assembly through cadherin (Drees et al., 2005) and other actin regulators associated with AJs (Gates and Peifer, 2005).

Ena also plays a role in border cell migration. This role has an interesting twist. While decreasing Ena/VASP function increases speed of fibroblast migration in cell culture (Bear et al., 2000), it slows migration of border cells. These two cell types migrate in quite different settings. Fibroblasts move over an extracellular matrix substrate by lamellipodial protrusion, adhesion, and tail retraction. In contrast, border cells migrate by squeezing in between nurse cells, and thus their substrate is cellular rather than matrix, and the shape of the leading process is constrained by the presence of other cells surrounding it. The shapes of border cell leading protrusions (Fulga and Rorth, 2002; Prasad and Montell, 2007) are quite different from fibroblast leading edge lamella, so perhaps the differential requirements for Ena are not so surprising. In fibroblasts, too much Ena activity increases lamellipodial dynamics but prevents production of a leading edge strong enough to promote stable protrusion (Bear et al., 2000; Bear et al., 2002). In border cells Ena inactivation may inhibit migration by several mechanisms. Ena inactivation could, in principle, affect ability of cells to protrude; however protrusions appeared relatively normal following Ena sequestration. Ena inactivation could reduce formation of finer protrusions like filopodia (as it did during Drosophila dorsal closure; Gates et al., 2007), and filopodia might serve a sensory function in migration. Finally, as Ena localizes to AJs in epithelia, Ena inactivation might affect DE-cadherin-mediated adhesion, which promotes border cell migration (Niewiadomska et al., 1999). It will be important to test these alternatives, for example by exploring the migration of wild-type border cells through a germline that is env mutant.

Overexpressing Ena also causes border cell migration defects, as well as formation of excess filopodia. Neurons (Lebrand et al., 2004) and leading edge cells during Drosophila dorsal closure (Gates et al., 2007) also exhibit excess filopodia following Ena up-regulation, while fibroblasts do not. Interestingly, neurons and border cells express high levels of the actin bundler Fascin (Singed in Drosophila); whereas fibroblasts do not. Since Fascin is thought to be a key regulator of filopodia (Vignjevic et al., 2006), perhaps the explosive filopodia phenotype depends upon Fascin-mediated bundling. This remains to be tested. Singed mutants (Cant et al., 1994) do not have border cell migration defects, however, suggesting that it acts redundantly with other regulators of actin bundling under normal circumstances. Excess filopodia may slow migration several ways, from reducing the ability to produce a single leading process to altering chemosensory cues received via filopodia. More detailed analysis wild-type and mutant border cell protrusions will help address these issues.

**Balancing capping and anti-capping in generating cytoplasmic filaments?**

The simplest model of Ena/VASP protein function suggests it acts as a CP antagonist (Barzik et al., 2005; Bear et al., 2002), promoting filament elongation while CP prevents this. Studies in cultured cells support this basic hypothesis; in both mammalian B16F1 melanoma cells and Drosophila D16 cells, CP depletion triggers explosive formation of filopodia (Gates et al., 2007; Mejillano et al., 2004), and in melanoma cells this largely depends on Ena/VASP activity (Mejillano et al., 2004). In contrast Ena depletion prevents filopodial formation in both mammalian MVD7 cells and Drosophila D16 cells (Gates et al., 2007; Mejillano et al., 2004). In vivo, it is likely that cell behavior is regulated by differences in relative ratios of CP and Ena/ VASP activity in the context of other actin regulators.

Nurse cell cytoplasmic filaments provide an interesting system in which to examine this balance. Ena plays an important though possibly not essential role in filament initiation, and this role appears to be restrained by negative regulation by Abi kinase. Naively, we initially thought CP depletion might have the opposite phenotype, producing more or more robust filaments. However, reducing CP function produced a more complex phenotype. Filaments were produced, but they were not uniformly distributed around the cortex, and were not effective at anchoring nuclei during dumping. The number of filaments extending to the nuclei did not seem increased, but instead the entire nurse cell cortex became “furry” with actin.

What mechanism might explain this phenotype? Perhaps depleting CP produces so many elongating, “anti-capped” actin filaments that they exceed the available Ena and/or other tip complex proteins. This may consume much of the available G-actin in producing numerous relatively short filaments, giving the cortex its furry appearance. Recent work supports this idea of “monomer channeling” in vitro (Akin and Mullins, 2008). Another speculative possibility, which is not mutually exclusive, is that in the absence of CP, the individual “units” from which the cytoplasmic actin filaments are assembled (Guild et al., 1997) grow longer than usual, compromising their mechanical strength and leading to the disorganization observed. Similar models were offered for the reduced lamellipodial persistence in fibroblasts with too much Ena activity (Bear et al., 2002). Reducing CP levels suppressed the effects of Ena inactivation on dumping, consistent with an antagonistic relationship. Future experiments, including further exploring epistatic relationships between Ena and CP, are required to test these hypotheses and further explore Ena, CP and their joint mechanisms of action.

**An unexpected role for Capping protein in oocyte determination**

When we generated CP null germlines, we hypothesized this would dramatically increase cortical actin, as was observed in imaginal discs (Delalle et al., 2005; Janody and Treisman, 2006) and follicle cells (our data). However, this was not the case—there may be a modest increase in cortical actin, but it is not dramatic. This suggests that other factors limit actin accumulation at the cortex—these may include activity of nucleating factors like the Arp2/3 complex and Formins.

However, loss of CP function in the germline did have one dramatic and surprising consequence: oocyte determination was often
disrupted, and the oocyte determinant Orb was no longer enriched in the presumptive oocyte. Two other genes have very similar phenotypes: BicaudalD and Egalitarian. Both are thought to be co-factors for Dynin, modulating microtubule organization and mediating transport of cargos into the oocyte (Pearson and Gonzalez-Reyes, 2004). Consistent with this, microtubule depolymerization also disrupts oocyte specification. It remains unclear why loss of CP impairs this process. Eliminating CP may impair function of the dynin–dyacin complex, of which it is a part (Cooper and Sept, 2008). Alternately, cross-regulatory interactions between actin and microtubules may be important for proper cytoskeletal structure and transport in the germarium, as is true later in oogenesis (e.g., Dahlgaard et al., 2007). Finally, CP may play a more direct role in transporting or anchoring Orb, or in some other step important for oocyte determination. This can now be examined in more detail.

Together these data provide insights into the developmental mechanisms that regulate the diverse actin structures critical for oogenesis. Future explorations of the detailed mechanisms of action of Ena and CP during these dynamic events and how they cooperate with other actin regulators like Formins and the Arp2/3 complex will help further extend our understanding of this important topic.

Acknowledgments
We thank K. Miller, E. Liebl, P. Garrity, the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank for reagents, S. Beckwith, N. Kaplan and T. Tedeschi for technical assistance, and D. Applewhite, E. Rogers and G. Shemer for helpful comments.

This work was supported by NIHGM47857 to MP and NIHGM73164 to DJM. JG was a Leukemia and Lymphoma Society Special Fellow. JG was supported by NIHNSF2GM08337 and Bucknell University’s Swanlund Fellowship. JG received Cell and Molecular Biology Training Grant T32 GM008581, JPM by the Smallwood Foundation and a Swanson Fellowship, SHN by Cell and Molecular Biology Training to DJM. JG was a Leukemia and Lymphoma Society Special Fellow. JG and D. Applewhite, E. Rogers and G. Shemer for helpful comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.06.030.

References


