Bitesize bundles F-actin and influences actin remodeling in syncytial *Drosophila* embryo development

Anna R. Yeh
B.S., Brandeis University (2016)

Submitted to the Department of Biology
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

at the MASSACHUSETTS INSTITUTE OF TECHNOLOGY

September 2023

Signature of Author …….
Dept. of Biology
August 29, 2023

Certified by ……
Adam C. Martin
Professor of Biology
Thesis Supervisor

Accepted by……
Mary Gehring
Associate Professor of Biology
Member, Whitehead Institute
Director, Biology Graduate Committee

Bitesize bundles F-actin and influences actin remodeling in syncytial *Drosophila* embryo development

Anna R. Yeh

Submitted to the Department of Biology on August 30, 2023 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

Actin networks undergo rearrangements that influence cell and tissue shape. Actin assembly and organization is regulated in space and time by a host of actin binding proteins. Despite the importance of these processes, their regulation is not well understood. The *Drosophila* Synaptotagmin-like protein, Bitesize (Btsz), organizes actin at epithelial cell apical junctions in a manner that depends on its interaction with the actin-binding protein, Moesin. Here, I show that Btsz functions in actin reorganization at earlier, syncytial stages of *Drosophila* embryo development. Btsz was required for the formation of stable pseudo-cleavage furrows that prevented spindle collisions and nuclear fallout prior to cellularization. While previous studies focused on Btsz isoforms containing the Moesin Binding Domain (MBD), we found that isoforms lacking the MBD also function in actin remodeling. Consistent with this, we found that the C-terminal half of BtszB cooperatively bound and bundled F-actin with micromolar affinity, suggesting a direct mechanism for Synaptotagmin-like proteins regulating actin organization during animal development. This is the first work to show that a Slp can bind and bundle F-actin and provides additional context to past work for how Btsz is regulating actin networks.

Thesis Supervisor: Adam C. Martin
Title: Professor of Biology

Acknowledgments

I would first like to thank my advisor, Adam, for the unwavering support he's provided through the years. This project would not have been possible without your guidance. Thank you for teaching me about how to be a good scientist and for encouraging me to explore many different avenues in both my research and in career development. Moreover, thank you for all the fun we had as a lab from hiking/camping trips, to copious amounts of cake in September, to seemingly endless cheese (until you get to the platter) at lab parties. I really appreciate the time and effort you put into your mentorship of each of your trainees.

Thank you to my thesis committee members (past and present), Hazel Sive, Becky Lamason, and Pulin Li, who have given valuable guidance and feedback on both my project and professional development throughout my time here at MIT. Thank you for your support and for always cheering me on.

I also need to give a huge thank you to past and present members of the Martin Lab. Clint, Marlis, and Hannah for the warm welcome and all their help when I was just starting in the lab. Nat, for all the scientific and technical help, and especially for being a great bay-mate. Jonathan, for not only contributing very helpful expertise, but also for the fun times highlighted by tea, snacks, and following my logic in our off-the-rails conversations. Mary Ann, for being a fantastic partner in chaos the past few years- I couldn't have done anything without you. And finally, Jaci, for being the best sunset bud I could ever ask for. I'm so grateful we joined the lab together and had each other through all the trials and tribulations of grad school and beyond. I'm looking forward future shenanigans together.

Thank you to my support network of friends through many walks of life- my MIT Bio cohort, friends from Brandeis, and friends from NJ. Thank you, SC, MA, and DB, for the company and helping me stay sane through all these years with our movie nights, gaming sessions, and food forays. Thank you JZ, LZ, and AY for decades of friendship and good times.

Thank you to my family and especially to my parents, who are both amazing scientists, for everything throughout my life. I would not have gotten to this point without your love, support, and encouragement. My parents were my first role models and the ones who taught me the importance of perseverance, which became a very necessary trait in grad school. Thank you for nurturing my curiosity, and appreciation of nature and science.

Finally, for Christian, the biggest thank you for being you and for loving me. Your steadfast support and confidence in me through these years means more to me than I can express through words. You are my favorite person and the one who never fails to make me smile. I couldn't have done this without you. And of course, I can't forget about the cats- Tommy, Ori, Ami, and Emi who are always bundles of joy to be around.

Table of Contents

Chapter 1: Introduction

Overview

The introduction to this thesis provides background for how the actin cytoskeleton and membrane remodeling are regulated, with a focus on their role in promoting proper development in *Drosophila melanogaster*. Actin networks must undergo dramatic rearrangements to achieve cell shape changes necessary for essential cell processes such as cell division, migration, and morphogenesis. Therefore, actin network dynamics must be tightly regulated in space and time. Because actin acts as scaffolding for the cells' plasma membrane, regulation of actin and membrane are intertwined. The cell shape changes that drive the processes mentioned above are also dependent on membrane trafficking and remodeling. Despite the importance of these processes, the range of mechanisms that control actin and membrane remodeling during development is still not fully understood. I will highlight the work that has been done on the Synaptotagmin-like protein Bitesize (Btsz), a protein that may combine both membrane and actin-remodeling functions. Prior studies on Btsz serve as the basis for my thesis work that describes a new function and activity for Btsz in the syncytial fly embryo.

Actin cytoskeleton

Actin is highly conserved and one of the most abundant proteins in eukaryotes. It exists as a 42kDa globular monomer (G-actin) that can assemble into a filamentous polymer (F-actin) (Figure 1.1). Actin is a major component of the cytoskeleton necessary for cell shape and structure, mechanotransduction, and cell movement (Dominguez and Holmes, 2011; Pollard, 2016). In order to drive these complex processes, networks of actin polymers are tightly regulated spatiotemporally to undergo dynamic rearrangements. However, the range of mechanisms that control actin network assembly and organization during development is still not fully understood.

Regulation of actin assembly and disassembly

Actin filaments are polarized with a plus and minus end that have different properties (Figure 1.1). ATP-bound actin monomers have a higher affinity for the plus end, leading to faster assembly on that end compared to the minus end. This means that the critical concentration, or the concentration of ATP-bound G-actin where net assembly and disassembly are equal, is lower for the plus end. In cells, actin assembly occurs primarily at the plus end of filaments (Evangelista et al., 2003).

There are various levels at which actin is regulated including assembly and disassembly of individual actin filaments, and the higher-level organization of actin filaments into networks. These processes are tightly orchestrated by a myriad of actin regulators to ensure these events occur at the right time and place. First, nucleation is the rate-limiting step in actin assembly, where actin monomers must overcome kinetically unfavorable conditions to form a nucleus of three monomers (Firat-Karalar

and Welch, 2011). Spontaneous nucleation of actin is inhibited by not only the instability of actin dimers but also by proteins that can bind and sequester actin monomers from polymerizing. Once a stable nucleus is formed and the concentration of free G-actin is above the critical concentration, monomers add to the nucleus and the filament polymerizes. Therefore, actin nucleation is a critical step and must be highly regulated by actin nucleators and assembly factors to ensure that polymerization only occurs at the right time and place.

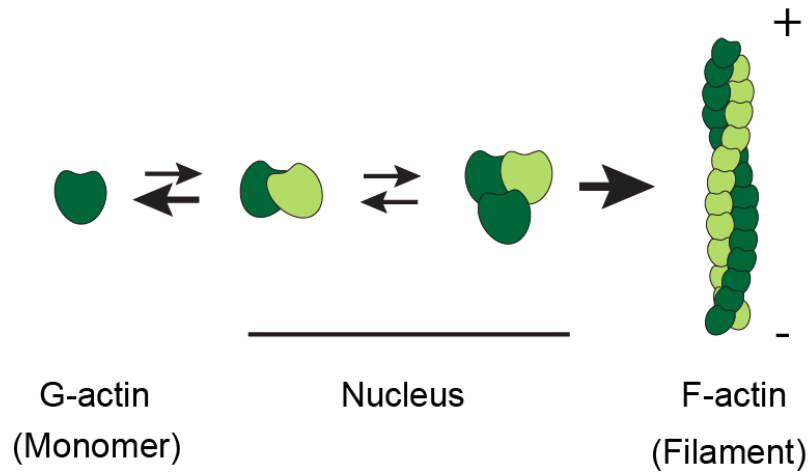


Figure 1.1 Schematic of G-actin monomers that nucleate, then undergo further assembly to form an F-actin filament. Actin assembly is only favorable once there is a stable nucleus.

Formins and the actin-related proteins-2/3 (Arp2/3) complex are some of the most well-known actin nucleators in eukaryotes that are essential in driving actin assembly in many contexts (Figure 1.2) (Breitsprecher and Goode, 2013). Formins are a family of highly conserved proteins known for promoting nucleation and elongation of unbranched actin filaments (Chesarone et al., 2010; Goode and Eck, 2007). They do this by stabilizing actin dimers and trimers, assembling F-actin while associating

processively with the growing filament, and protecting against capping at plus end of the filament (Harris et al., 2004; Pring et al., 2003). These functions are important for regulating actin in a variety of important processes such as cell polarity, migration, cytokinesis, endocytosis, and tissue morphogenesis (Breitsprecher and Goode, 2013; Lian et al., 2016; Mass et al., 1990; Sagot et al., 2002).

The Arp2/3 complex is made up of seven subunits and promotes actin nucleation off existing filaments at a 70° angle to form branched networks (Figure 1.2). These branched networks are important for forming structures necessary for, but not limited to, phagocytosis, endocytosis, and membrane-trafficking (Goley and Welch, 2006). They can also assemble beneath the plasma membrane, assembling a structure called the lamellipodium that promotes movement of the leading edge of a cell during cell migration.

Because actin filaments constantly undergo dynamic rearrangements, actin disassembly is also regulated so cells can rapidly remodel actin networks. Actin assembly is reversible such that actin monomers can dissociate from actin filament ends on their own. However, proteins that sever or destabilize F-actin enhance disassembly rates to accommodate these rapid rearrangements (Brieher, 2013). The ADF/cofilin family of proteins play a central role in regulating the disassembly of F-actin (Hawkins et al., 1993; Lappalainen and Drubin, 1997). Cofilin coats the actin filament at regions of ADP-actin, at the pointed ends of the filament, where it severs the filament and accelerates depolymerization (Figure 1.2) (Shekhar and Carlier, 2017; Wioland et al., 2017; Wioland et al., 2019). Actin turnover, the process of actin assembly and disassembly, is necessary for maintaining the coherence of actin networks. Disruption

of actin turnover by disrupting actin assembly and/or disassembly, or by depleting the G-actin pool can lead to loss of cell adhesion in *Drosophila*, and loss of network integrity and defective cell division in yeast (Jodoin, 2015; Pollard and Wu, 2010).

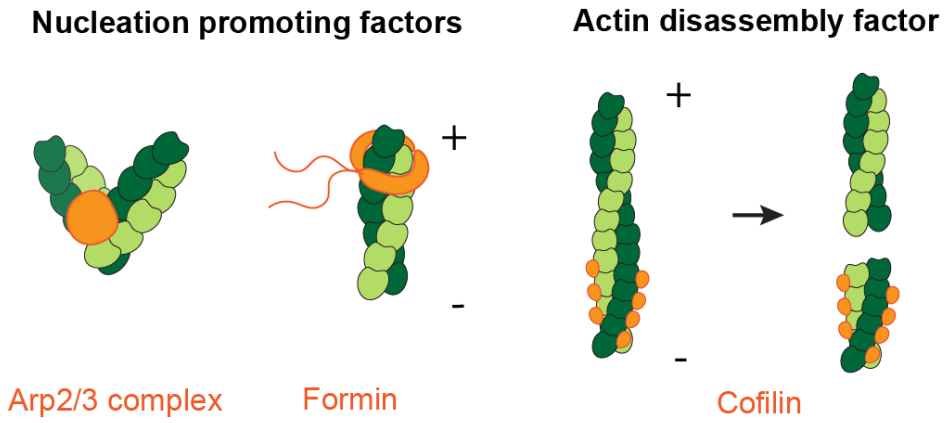


Figure 1.2 Schematic of a few different actin regulators that control actin filament assembly or disassembly. The Arp2/3 complex promotes nucleation to generate branched networks. Formins promote nucleation and generates long actin filaments by accelerating actin assembly at the plus end. Cofilin coats the actin filament at the minus end and helps sever F-actin.

Actin organization

F-actin is organized into networks which is achieved by proteins that can bundle/crosslink actin. Actin crosslinkers typically have at least two actin binding sites or oligomerize to bundle actin filaments tightly, or organize them into looser, orthogonal networks (Figure 1.3). This organization will depend on the crosslinker's structure, size, and flexibility (Otto, 1994; Uribe and Jay, 2009). Actin can be bundled tightly into parallel bundles commonly found in filopodia or actin can be organized in looser, antiparallel contractile bundles important for stress fibers and the contractile ring. Fascin and fimbrin are smaller proteins that have more than one actin-binding site, which allows them to bundle F-actin tightly (Jansen et al., 2011). On the other hand, α-actinin

is a flexible, larger protein that crosslinks F-actin around 3 times further apart than fascin or fimbrin (Courson and Rock, 2010). Filamin is a large crosslinker that organizes F-actin into orthogonal networks and can also interact with membrane-associated proteins to help cells sense the environment outside the cell (Sutherland-Smith, 2011). Filamin is also reported to be important for cell motility in some contexts (Baldassarre et al., 2009). Overall, actin crosslinking proteins change not only the actin network's architecture to perform certain functions but also can finetune the network's rigidity and contractile properties (Reichl et al., 2008).

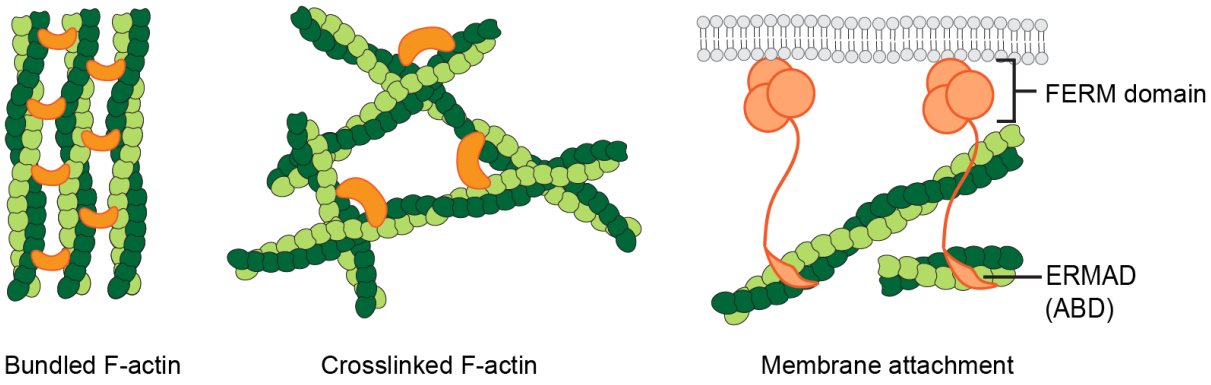


Figure 1.3 Schematics of a few types of higher-level organization of F-actin achieved through different means. Small actin cross-linkers can create tight bundles of F-actin, commonly found in structures such as filopodia. Larger actin cross-linkers can organize F-actin into orthogonal networks which is important for maintaining mechanical properties of the cell cytoskeleton. The cytoskeleton must also be attached to the membrane. One key way this occurs is through ERM proteins. The N-terminal FERM domain interacts with membrane and the C-terminal ERMAD contains the actin binding domain (ABD) which bind F-actin.

Actin cytoskeleton and morphogenesis

The actin network that makes up the cytoskeleton underlying the cell membrane is essential for migration, morphogenesis, and cell transport. The ERM (Ezrin Radixin Moesin) family of proteins is responsible for tethering F-actin to the plasma membrane.

Their N-terminal FERM domain interacts with the membrane and a C-terminal ERMAD (Ezrin Radixin Moesin Association Domain) binds F-actin (Figure 1.3) (Fehon et al., 2010). ERM proteins have an inactive, closed form that can be activated by phosphorylation through kinases. Upon phosphorylation, ERM proteins undergo a conformational change to reveal the F-actin binding and membrane protein-binding sites (Gary and Bretscher, 1995; Zaman et al., 2021). In addition to their critical role in establishing cortical stability in the cell, ERM proteins can also play a role in signaling pathways during development, establishing polarity, and regulating cell shape during mitosis (Fehon et al 2010, Carreno et al 2008). In *Drosophila*, Moesin is the only ERM protein whose depletion or inhibition leads to cell shape defects, cortical destabilization and microtubule misorganization (Kunda P 2008, Carreno et al 2008). Moreover, female flies expressing a mutant form of *moe* are sterile and cannot lay eggs, demonstrating Moesin's integral role in development (Jankovics et al., 2002).

The actin cytoskeleton must not only be tethered to the membrane, but also to cell junctions that connect F-actin networks across neighboring cells (Figure 1.4). The actin network is connected to the adherens junction (AJ) through an adhesion complex which allows for physical attachments between neighboring cells. The specific composition of the adhesion complex varies depending on the region of the cell, tissue, and organism (Clarke and Martin, 2021). In epithelia, these complexes are made up of the adhesion molecule E-cadherin along with the adaptors α-catenin and β-catenin (Figure 1.4) (Gumbiner, 2005). They help form apical junctions which are necessary for tissue integrity. Disrupting this adhesion complex by mutating β-catenin leads to tissue tearing and defective AJs can lead to defects in organ formation (Cox et al., 1996;

Greene and Copp, 2005; Harris and Peifer, 2005; Jodoin, 2015; Juriloff and Harris, 2000; Martin et al., 2010).

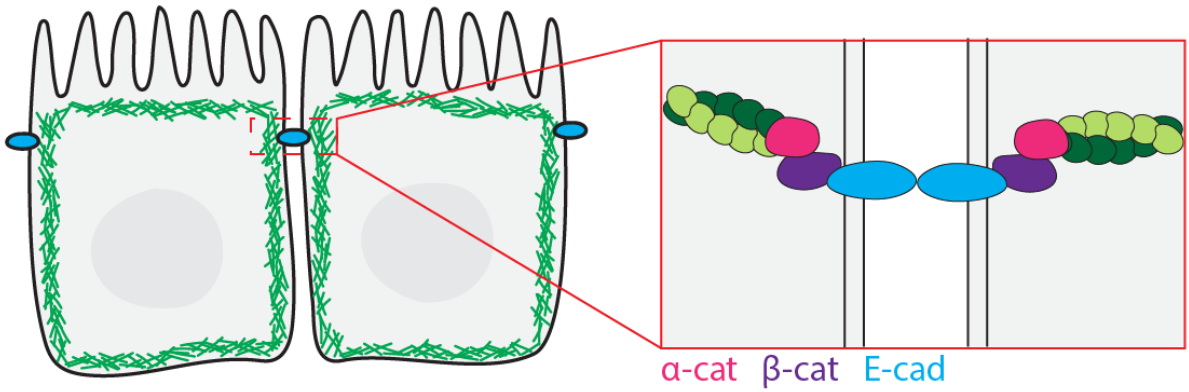


Figure 1.4 Epithelial cells are connected by the adhesion complex, made up of α-catenin, β-catenin, and E-Cadherin. α-cat helps connect the F-actin cytoskeleton to the rest of the adhesion complex, and thus also to the cytoskeleton of a neighboring cell. Disrupting these cell-cell attachments leads to loss of tissue integrity. Adapted from (Camuglia et al., 2021).

Membrane remodeling proteins and development

Cells undergo massive restructuring of their cytoskeleton and plasma membrane during morphogenesis. One family of proteins that play diverse roles in both membrane/vesicle transport and actin cortex organization are the Synaptotagmin-like proteins (Slp). Slps are related to Synaptotagmins which are a protein family are well known for their roles in vesicle trafficking (Fukuda et al., 2001). Synaptotagmins have a single N-terminal transmembrane domain and two tandem C2 domains, C2A and C2B. Synaptotagmin's C2 domains are essential for sensing and binding Ca^{2+}, which triggers membrane fusion between the target vesicle and plasma membrane (Brose et al., 2017; Striegel et al., 2012).

Slps are conserved across vertebrates and are characterized by an N-terminal Slp homology domain (SHD) and, similar to Synaptotagmins (Syt), two C-terminal C2 domains (Figure 1.5). The SHD interacts with the GTPase Rab27a and Rab27b, proteins important in the vesicle trafficking pathway (Ostrowski et al., 2010). The C2 domain consists of the C2A and C2B domains that bind membrane, typically in a calcium-dependent manner. For instance, Slp3-a and Slp5's ability to bind phosopholipids depends on Ca^{2+} (Fukuda, 2002b; Kuroda et al., 2002). The C2A domain binds with high affinity for $PI(4,5)P_2$ or $PI(3,4,5)P_3$ and the C2B domain has high affinity for PI(4,5)P2 (Galvez-Santisteban et al., 2012; Lyakhova and Knight, 2014). Overall, Slps are not as well characterized as Syts and much about their functions is still unknown.

The mammalian Slp4/granuphilin protein is present in the pancreas where it regulates the exocytosis of insulin-containing dense-core granules (Table 1.1) (Wang et al., 1999). In this secretory pathway, granules filled with insulin are tethered to membrane at release sites until a specific stimulus signals their release. Granuphilin localizes to these insulin granules and helps stably dock them beneath the plasma membrane (Gomi et al., 2005; Mizuno et al., 2016). The SNARE syntaxin-1a which is known for promoting vesicle fusion, is required for this docking activity (Gomi et al., 2005). Overall, Slp4/granuphilin's role in granule docking aligns with a general activity of Rab proteins and their effector proteins to regulate membrane trafficking by tethering vesicles to membrane. A different Slp, Slp2-a, is required for targeting vesicles to the apical surface in a PIP2-dependent manner during *de novo* epithelial lumen formation.

Slp4-a/granuphilin then couples these vesicles with apical plasma membrane to generate a lumen (Galvez-Santisteban et al., 2012).

A family of proteins related to Slps are the Synaptotagmin-like proteins lacking C2 domains (Slac2). There are 3 members of the Slac2 family that have currently been described in mammals (Slac2-a/Melanophilin, Slac2-b, and Slac2-c/MyRIP) (Table 1.1) (El-Amraoui et al., 2002; Fukuda, 2002b; Fukuda et al., 2002; Kuroda et al., 2002; Matesic et al., 2001). Slac2s contain an SHD in their N-terminal domain but lack the C-terminal C2 domains. Slac2 proteins act as adaptor proteins for cargo transport, interacting with Rab27a through its N-terminal SHD as well as Myosin Va through myosin binding sites in the middle of the protein (Kuroda and Fukuda, 2005). Slac2-a/Melanophilin helps transport melanosomes in mice and is important for their coat color (Matesic et al., 2001). Slac2-b/exophilin-5 is a Rab27b effector important for extracellular vesicle secretion in keratinocytes (Bare et al., 2021). Slac2-c/MyRIP is another Rab27a effector that also associates with melanosomes (El-Amraoui et al., 2002). Interestingly, the C-termini of Slac2-a and Slac2-c bind actin and colocalize with F-actin in cell culture (Fukuda et al., 2002; Kuroda and Fukuda, 2005).

Name	Other names	Function	Organism
Slp1	Exophilin-7/JFC1	Cytotoxic granule exocytosis in lymphocytes	Mammal
Slp2-a	Exophilin-4	Epithelial lumen formation	Mammal
Slp4	Granuphilin/exophilin-2	Epithelial lumen formation, insulin transport	Mammal
Slp4	Btsz	Gastrulation, trachea tube formation	*Drosophila*
Slac2-a	Melanophilin/exophilin-3	Melanosome transport	Mammal
Slac2-b	Exophilin-5	Vesicle secretion in keratinocytes	Mammal
Slac2-c	MyRIP/exophilin-8	Insulin transport in pancreas	Mammal

Table 1.1 List of several Slps and Slac2s with some of their known functions.

Therefore, both Slps and Slac2s are Rab27 effectors involved in vesicle trafficking. Overall, there are six Slp genes and three Slac2 genes in mammals. The reasons for such a diverse array of Slp and Slac2 genes and whether less complex metazoans have functional equivalents to this diverse gene family is unknown.

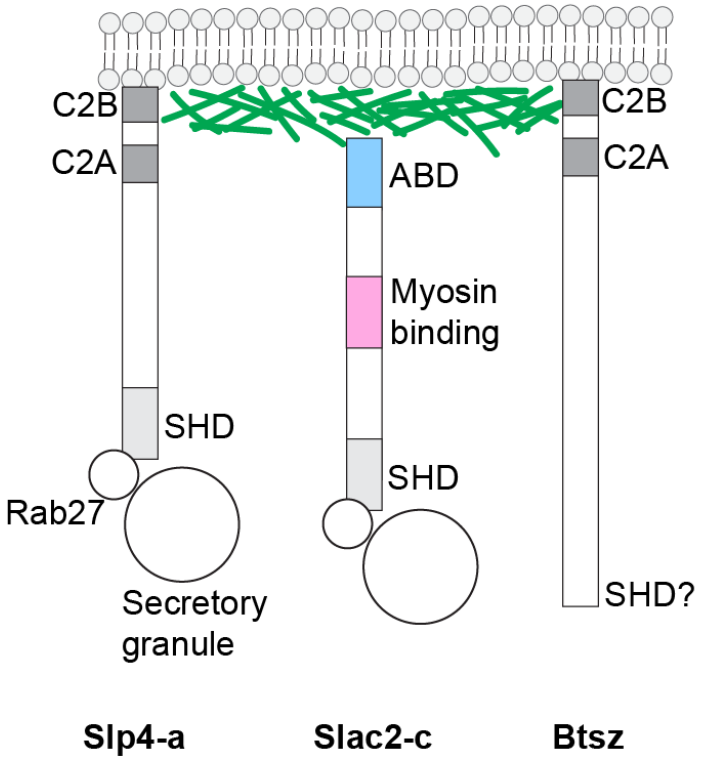


Figure 1.5 Protein schematics of Slp4-a, Slac2-c, and Btsz, the *Drosophila* homologue of Slp4. Slp4-a and Slac2-c have known functions as Rab27 effectors and are important for vesicle docking. Slp4-a and Btsz have C-terminal C2 domains. Slac2-c has a MyosinVa binding domain in the middle of the protein. Slp4-a and Slac2-c have an SHD but it is unclear whether Btsz has an SHD that mediates an interaction with a Rab. Adapted from (Imai and Tsujimura, 2017).

The Slp Bitesize is an actin regulator important in fly development

Drosophila encodes a single Slp gene, Bitesize (Btsz), the fly homologue of Slp4/granuphilin (Figure 1.5). Previous work has showed that Btsz plays a role in actin cytoskeleton organization during *Drosophila* development (Pilot et al., 2006b). *btsz*

mutants have been shown to exhibit growth defects where adults are proportionally smaller than the wildtype with reduced cell size and number (Serano and Rubin, 2003). Even though Btsz is homologous to mammalian Slp4/granuphilin, it is unclear whether Btsz contains a SHD and whether it plays a direct role in exocytosis in *Drosophila*. Moreover, C2 domains are known for their ability to bind Ca^{2+} in Synaptotagmins, but Btsz lacks the conserved aspartate residues that were necessary for this activity in Synaptotagmins (Serano and Rubin, 2003). Interestingly, Btsz's C2 domains bound PIP2 in Ca^{2+}-dependent manner so there may be other residues present in these domains that can bind Ca^{2+} (Pilot et al., 2006b).

Btsz is alternatively spliced into nine Btsz isoforms and only a few have been studied more extensively. BtszC localizes apically in follicle cells, and along the membrane in S2 cells (Serano and Rubin, 2003). This membrane localization depends on Btsz's C2 domains which bind PIP2. Moreover, BtszC can bind Moesin *in vitro* and Moesin localization was affected in embryos depleted of Btsz (Pilot et al., 2006b). Yeast two-hybrid interactions have narrowed down the Moesin-Binding Domain (MBD) in Btsz to exon 8.

Pilot et al. 2006 showed that Btsz is essential for maintaining adherens junctions and epithelial integrity. They discovered that certain Btsz isoforms contain a Moesin binding domain (MBD) that allows Btsz to interact with the membrane-cortex crosslinker, Moesin. In this model, Btsz binds phosphoinositides via its C2A and B domains, localizing it to adherens junctions at the apical surface of the cell where it recruits Moesin to regulate actin organization and stabilize E-cadherin. In embryos injected with dsRNA to deplete Btsz transcripts, adherens junctions were destabilized

and the F-actin network was disorganized (Pilot et al., 2006b). These defects likely led to the observed loss of epithelial integrity. The authors propose a model where PIP2 is the upstream polarity cue that controls Btsz localization to the apical domain. There, Btsz binds Moesin to organize actin filaments. E-cadherin (E-cad) and junctional stability depend on actin regulation at these junctions so without Btsz and/or Moesin, there is a loss of epithelial integrity.

Btsz is also important for proper tracheal formation which occurs during late embryogenesis and further elongates in the third instar larva (Jayanandanan et al., 2014). In the trachea, Btsz recruits Moesin to promote proper actin and membrane organization. *btsz* mutants exhibit phenotypes indicative of defective tube morphogenesis and their trachea terminal cells decreased branch number, a common method to assess terminal cell defects. The C2 domains are also responsible for Btsz localization to membrane in tracheal cells. One difference, however, between Btsz's role in trachea development and embryogenesis is that adherens junctions remain intact in Btsz-depleted trachea. Despite this, tracheal lumen formation defects were still present in the absence of Btsz (Jayanandanan et al., 2014). Overexpression of the MBD-containing Btsz isoform C but not BtszB, which lacks the MBD, was able to suppress the mutant trachea phenotype. In *btsz* mutants, activated Moesin localization was reduced at the apical domain which was accompanied by defective membrane organization. Therefore, past work has elucidated the importance of Btsz regulation of the actin cytoskeleton through Moesin (Jayanandanan et al., 2014; Ríos-Barrera and Leptin, 2022).

The syncytial *Drosophila* embryo as a model system to study actin and membrane regulation

Overview of early Drosophila embryo development

The early *Drosophila* embryo provides a premier system to study actin and membrane regulation due to the dynamic cortical network rearrangements that occur during syncytial blastoderm development. During the first stages of development, the *Drosophila* embryo is a syncytium, or one cell with multiple nuclei (Figure 1.6). These nuclei undergo 13 rounds of synchronous modified cell divisions called nuclear divisions. The nuclear cycle lacks gap phases and cytokinesis and takes an average of 8-10 minutes to complete one round of division (Foe et al., 2000). The first nuclear cycles (NC) occur in the yolk at the center of the embryo, and nuclei migrate to the apical surface during NC 8 and 9 in a microtubule-dependent process called cortical migration (Baker et al., 1993). At the cortex, the nuclei undergo NC 10-13 and pause during interphase of the 14th round of divisions. Cellularization begins after the syncytial divisions are complete where plasma membrane furrows elongate to enclose each nucleus, generating an epithelial layer.

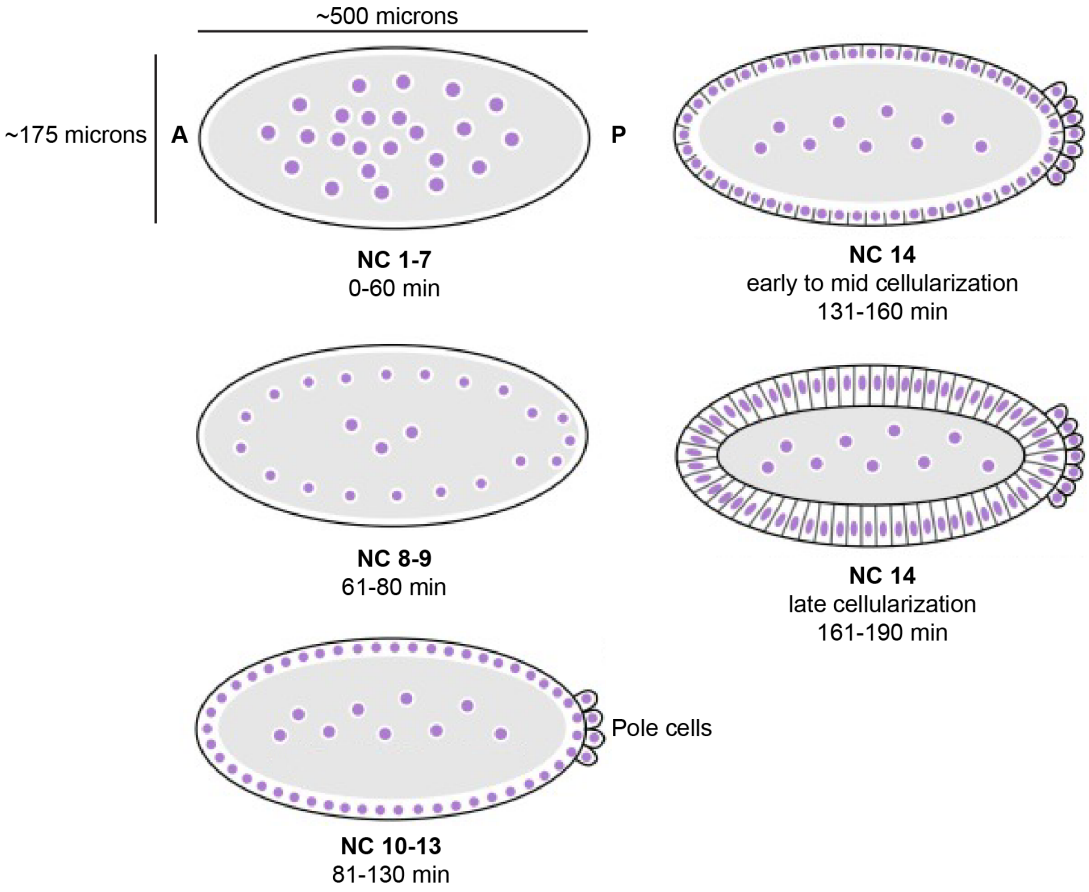


Figure 1.6 Schematic of the early stages of *Drosophila* development at 25ºC. Gastrulation occurs after cellularization at ~190 min. From (Sokac et al., 2023).

During the nuclear cycles, actin undergoes dynamic rearrangements that are orchestrated by many actin regulator proteins. Additionally, there is important crosstalk that occurs between nuclei, microtubules, and the actin at this stage. Each nucleus and centrosome pair signal to the actin network to organize into F-actin-containing structures called actin caps situated above syncytial nuclei (Raff and Glover, 1989; Stevenson et al., 2002). During each nuclear cycle, actin assembly in the actin caps drives cap expansion, leading to collisions between neighboring caps. When these caps collide with one another, the actomyosin network buckles and bends inward to form

pseudo-cleavage furrows (Zhang et al., 2018). This is a modified form of cytokinesis and many proteins involved in pseudo-cleavage furrow formation are also present at cytokinetic furrows. These pseudo-cleavage furrows are structures made up of both actin and membrane that act as a barrier between dividing spindles and are essential for proper nuclear divisions (Figure 1.7) (Foe et al., 2000; Kellogg et al., 1988; Zhang et al., 2018). At anaphase, pseudo-cleavage furrows begin regressing and actin is redistributed from the furrows back to the caps located above the nuclei. There, actin caps reform above the two daughter nuclei and the next nuclear cycle begins (Rikhy et al., 2015).

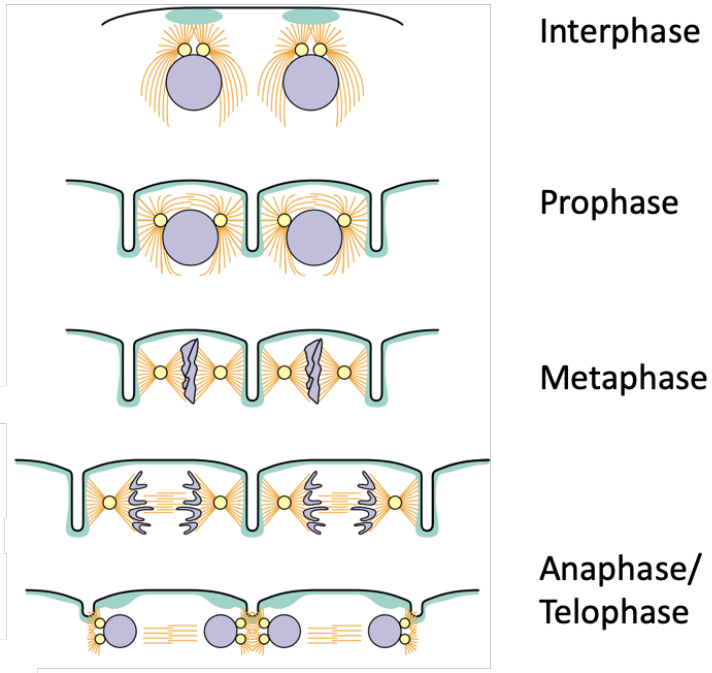


Figure 1.7 Schematic of one round of nuclear division in the syncytial *Drosophila* embryo. The nucleus is in purple, actin in green, centrosomes in yellow, and microtubules in orange. Actin assembly drives cap expansion and when neighboring caps collide, this forces the caps to buckle inward and form pseudo-cleavage furrows. During metaphase, these furrows are at their longest and they begin to retract during anaphase. Actin caps then reform above the daughter nuclei and the next nuclear cycle will begin. From Tram et al. 2002.

Both exocytosis and endocytosis of plasma membrane are important for cytokinesis and furrow growth and regression in addition to actin remodeling (Cao et al., 2008; Riggs et al., 2003; Sokac and Wieschaus, 2008). Perturbing pseudo-cleavage furrows with drugs or depleting actin-related proteins often results in spindle collision (Callaini et al., 1992; Grevengoed et al., 2003; Jiang and Harris, 2019; Zhang et al., 2018). When membrane trafficking is perturbed by knocking down components of the trafficking machinery, pseudo-cleavage furrows are shorter, or absent, and phenotypes resemble those of embryos that have defective actin assembly (Cao et al., 2008; Holly et al., 2015; Rikhy et al., 2015; Sherlekar et al., 2020).

Actin regulators during syncytial divisions

In a nuclear cycle, cap growth occurs rapidly, driven by actin nucleators such as the formin Diaphanous and the Arp2/3 complex, with the cap area expanding six fold in minutes (Xie et al., 2021). After the expansion phase, the caps stabilize and elongate as spindles duplicate and separate. Then, caps disassemble and form two caps near centrosomes during anaphase/telophase that correspond with the daughter nuclei (Figure 1.7). This will repeat with each nuclear cycle until NC13 where the cell cycle arrests.

When actin assembly is perturbed, actin cap organization and dynamics are defective. Disrupting Dia or the Arp2/3 complex results in smaller actin caps, slower expansion rate, and reduced actin intensities (Afshar et al., 2000; Stevenson et al., 2002; Xie et al., 2021). Moreover, Dia and Arp2/3 promote assembly of distinct actin structures (bundles or puncta, respectively) in these caps (Zhang et al., 2018). Both

these populations of structures are important for pseudo-cleavage furrow formation. The authors hypothesize that these bundles increase stiffness in the actin cap and allow for Arp2/3-driven assembly to promote buckling of actomyosin borders during cap growth (Jiang and Harris, 2019).

Besides Dia and Arp2/3, many other proteins have been identified for their role in promoting syncytial blastoderm development. For instance, the polarity protein and kinase Par1 helps establish or maintain Dia-based actin bundles (Jiang and Harris, 2019). The small adaptor protein Crk (CT 10 regulator of kinase) is important for Arp2/3 localization to the cortex and loss of Crk leads to spindle collisions and nuclear fallout (Spracklen et al., 2019). Actin regulators Cortactin, Dpod1, Coronin, and Scar are important for promoting actin cap growth (Xie et al., 2021). Signaling from small G proteins such as Rac and Rho1 are important for promoting actin assembly and myosin organization in actin caps (Cao et al., 2008; Crest et al., 2012; Royou et al., 2004; Stevenson et al., 2002; Zallen et al., 2002; Zhang et al., 2018).

The APC (Adenomatous polyposis coli) family is known to affect actin function, cell division, and cell adhesion. Apc2 is one of the two APC proteins expressed in *Drosophila.* In the syncytium, Apc2 localizes to actin caps and pseudo-cleavage furrows where it likely directly binds Dia and promotes pseudo-cleavage furrow growth (McCartney et al., 2001; Webb et al., 2009). This may be relevant in the context of *in vitro* results that demonstrate that mammalian mDia1 and APC can work as a complex to promote actin assembly. mDia1 and APC dimers form a nucleation complex that recruits actin monomers where APC stably associates to one end of the filament and mDia1 detaches to elongate the actin filament (Breitsprecher et al., 2012).

Membrane trafficking in the syncytium

During each syncytial division, hundreds to thousands of pseudo-cleavage furrows composed of actin and plasma membrane have only minutes to form and disassemble. Therefore, membrane trafficking and remodeling are also highly regulated by a combination of different factors. During cytokinesis, targeted vesicle trafficking delivers membrane to the ingressing furrow (Fielding et al., 2005; Lecuit and Wieschaus, 2000; Pelissier et al., 2003; Riggs et al., 2003). Because the syncytial divisions are modified cell divisions, many components are shared between cellularization and syncytial blastoderm development. The recycling endosome plays an important role in membrane trafficking and is a key component of providing membrane to growing pseudo-cleavage furrows (Riggs et al., 2003).

The mechanisms that determine when and where vesicle-mediated membrane addition occurs is still under examination. To address this, studies have turned to Nuf, Nuclear fallout, a Rab11 effector that is important for vesicle delivery to growing furrows (Brose et al., 2017; Wilcke et al., 2000). *nuf* embryos display F-actin depletion from furrows followed by membrane breakages. This creates large gaps in the network, leading to spindle collisions and nuclear fallout. Nuf is also important for RhoGEF2 localization to pseudo-cleavage furrows where it may promote actin polymerization through the Rho1 pathway (Cao et al., 2008).

Rab11, a key component of the recycling endosome, plays a role in targeted delivery of endosomes to the cytokinetic cleavage furrow (Fielding et al., 2005; Wilson et al., 2005). Rapid endocytosis is cell cycle regulated and also associated with furrow regression during cellularization (Sokac and Wieschaus, 2008). Moreover, syncytial

embryos depleted of Rab11 exhibit phenotypes similar to those of *nuf* embryos including the gaps in the actin network and membrane breakages (Riggs et al., 2003). The data also reveal that Rab11 is necessary for maintaining actin remodeling protein localization at pseudo-cleavage furrows (Riggs et al., 2003).

Another way membrane remodeling takes place is through the Ras-like protein A (RalA), a GTPase that helps regulate the exocyst complex. RalA is necessary for proper pseudo-cleavage furrow formation, as chromosome segregation is defective in RalA-depleted embryos (Holly et al., 2015). F-actin is depleted from pseudo-cleavage furrows in RalA-depleted embryos, indicating that RalA could direct F-actin assembly at furrows or that F-actin is transported to furrows through RalA-mediated membrane remodeling (Holly et al., 2015).

Myosin in the syncytium

Another component of the cytoskeleton important for its structure and mechanical properties is the motor protein myosin (Coravos and Martin, 2016; Fernandez-Gonzalez et al., 2009; Martin et al., 2010; Royou et al., 2002). In the syncytium, non-muscle myosin II accumulates at cap boundaries as the actin cap matures (Zhang et al., 2018). When the actin cap grows and meets these boundaries, the caps circularize and bend inward. The bundling of F-actin and tension generated through myosin may stiffen boundaries between caps which enables them to buckle into the cytoplasm. Perturbation of myosin regulation through Rho-kinase depletion, results in less uniform cap area and circularity. This also leads to inconsistent pseudo-cleavage furrow lengths.

When myosin regulation is disrupted in *DRhoGEF2* mutants, pseudo-cleavage furrows are affected as well which leads to nuclear fallout (Padash Barmchi et al., 2005).

Actomyosin networks, which can also bundle F-actin, are capable of generating contractility and tension in a system. Laser ablation of actin caps results in an increased actin cap area in wildtype embryos, indicating that the network was under tension from the observed recoil. This change is absent in embryos depleted of Rho-kinase, which phosphorylates and activates myosin (Zhang et al., 2018). Moreover, actomyosin surrounding actin caps contracts which exerts forces on the cytoplasm, swelling the actin caps (Zhang et al., 2018). Perturbing the mechanical properties of the actin cap may have further impacts, such as on spindle organization/dynamics. Previous work has indicated that forces exerted by both dynein and myosin synergize to promote spindle elongation during nuclear divisions (Sommi et al., 2011). The authors hypothesize that myosin can help contract “softer” actin patches and pull on astral microtubules to help spindles elongate.

Conclusion

This introduction serves as a basis for work examining the role of actin and membrane remodeling during development with a focus on the protein Btsz. Actin must be regulated at multiple levels to facilitate proper cellular processes. In addition to this, membrane remodeling often occurs hand in hand with changes in the actin network. When Nuf or Rab11 are disrupted, both membrane and F-actin organization are defective (Riggs et al., 2003). Additionally, depleting F-actin at pseudo-cleavage furrows using drug injection leads to decreased membrane stability (Cao et al., 2008). In the *Drosophila* trachea, endosomes can organize F-actin by recruiting actin nucleation

factors around endocytic vesicles (Ríos-Barrera and Leptin, 2022). There is still work that needs to be done to elucidate the intertwined mechanisms that connect membrane and actin remodeling. The syncytial *Drosophila* embryo provides a great system to study these two processes. One protein of particular interest that can potentially facilitate both actin and membrane remodeling is the Synaptotagmin-like protein Bitesize, which is the focus of my thesis work.

Findings presented in this thesis

I used both *in vivo* and *in vitro* systems to understand the role of Btsz in actin regulation. First, I observed that Btsz-depletion in the early fly embryo resulted in phenotypes earlier in development than previously described, and I determined that Btsz affects nuclear divisions in the syncytial embryo. In the absence of Btsz, actin caps are smaller and pseudo-cleavage furrows break improperly more frequently, which likely lead to spindle collisions and nuclear fallout that I observed. Second, I used a biochemical approach to show Btsz may be regulating actin by bundling and binding F-actin cooperatively. Finally, I will conclude this thesis with recommendations for future experiments.

References

Afshar, K., B. Stuart, and S.A. Wasserman. 2000. Functional analysis of the Drosophila diaphanous FH protein in early embryonic development. *Development*. 127:1887-1897.

Albertson, R., J. Cao, T.-S. Hsieh, and W. Sullivan. 2008. Vesicles and actin are targeted to the cleavage furrow via furrow microtubules and the central spindle. *Journal of Cell Biology*. 181:777-790.

Algrain, M., O. Turunen, A. Vaheri, D. Louvard, and M. Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *Journal of Cell Biology*. 120:129-139.

Arjonen, A., R. Kaukonen, and J. Ivaska. 2011. Filopodia and adhesion in cancer cell motility. *Cell Adh Migr*. 5:421-430.

Baker, J., W.E. Theurkauf, and G. Schubiger. 1993. Dynamic changes in microtubule configuration correlate with nuclear migration in the preblastoderm Drosophila embryo. *J Cell Biol*. 122:113-121.

Baldassarre, M., Z. Razinia, C.F. Burande, I. Lamsoul, P.G. Lutz, and D.A. Calderwood. 2009. Filamins Regulate Cell Spreading and Initiation of Cell Migration. *PLOS ONE*. 4:e7830.

Bare, Y., G.K. Chan, T. Hayday, J.A. McGrath, and M. Parsons. 2021. Slac2-b Coordinates Extracellular Vesicle Secretion to Regulate Keratinocyte Adhesion and Migration. *J Invest Dermatol*. 141:523-532.e522.

Breitsprecher, D., and B.L. Goode. 2013. Formins at a glance. *J Cell Sci*. 126:1-7.

Breitsprecher, D., R. Jaiswal, J.P. Bombardier, C.J. Gould, J. Gelles, and B.L. Goode. 2012. Rocket launcher mechanism of collaborative actin assembly defined by single-molecule imaging. *Science*. 336:1164-1168.

Brieher, W. 2013. Mechanisms of actin disassembly. *Molecular Biology of the Cell*. 24:2299-2302.

Brose, L., J. Crest, L. Tao, and W. Sullivan. 2017. Polo kinase mediates the phosphorylation and cellular localization of Nuf/FIP3, a Rab11 effector. *Mol Biol Cell*. 28:1435-1443.

Callaini, G., R. Dallai, and M.G. Riparbelli. 1992. Cytochalasin induces spindle fusion in the syncytial blastoderm of the early Drosophila embryo. *Biology of the Cell*. 74:249-254.

Camuglia, J.M., A.R.A. Yeh, and A.C. Martin. 2021. Extracellular | Tension and Tissue Morphogenesis. *In* Encyclopedia of Biological Chemistry III (Third Edition). J. Jez, editor. Elsevier, Oxford. 317-325.

Cant, K., B.A. Knowles, M.S. Mooseker, and L. Cooley. 1994. Drosophila singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. *Journal of Cell Biology*. 125:369-380.

Cao, J., R. Albertson, B. Riggs, C.M. Field, and W. Sullivan. 2008. Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. *Journal of Cell Biology*. 182:301-313.

Chabrillat, M.L., C. Wilhelm, C. Wasmeier, E.V. Sviderskaya, D. Louvard, and E. Coudrier. 2005. Rab8 regulates the actin-based movement of melanosomes. *Mol Biol Cell*. 16:1640-1650.

Chesarone, M.A., A.G. DuPage, and B.L. Goode. 2010. Unleashing formins to remodel the actin and microtubule cytoskeletons. *Nat Rev Mol Cell Biol*. 11:62-74.
Chou, T.B., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in Drosophila. *Genetics*. 131:643-653.
Chugh, P., and E.K. Paluch. 2018. The actin cortex at a glance. *Journal of Cell Science*. 131:jcs186254.
Clarke, D.N., and A.C. Martin. 2021. Actin-based force generation and cell adhesion in tissue morphogenesis. *Current Biology*. 31:R667-R680.
Coravos, J.S., and A.C. Martin. 2016. Apical Sarcomere-like Actomyosin Contracts Nonmuscle Drosophila Epithelial Cells. *Dev Cell*. 39:346-358.
Courson, D.S., and R.S. Rock. 2010. Actin Cross-link Assembly and Disassembly Mechanics for α-Actinin and Fascin*. *Journal of Biological Chemistry*. 285:26350-26357.
Cox, R.T., C. Kirkpatrick, and M. Peifer. 1996. Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during Drosophila embryogenesis. *J Cell Biol*. 134:133-148.
Crest, J., K. Concha-Moore, and W. Sullivan. 2012. RhoGEF and positioning of rappaport-like furrows in the early Drosophila embryo. *Curr Biol*. 22:2037-2041.
Davidson, A.J., T.H. Millard, I.R. Evans, and W. Wood. 2019. Ena orchestrates remodelling within the actin cytoskeleton to drive robust Drosophila macrophage chemotaxis. *Journal of Cell Science*. 132:jcs224618.
Dominguez, R., and K.C. Holmes. 2011. Actin structure and function. *Annu Rev Biophys*. 40:169-186.
El-Amraoui, A., J.S. Schonn, P. Küssel-Andermann, S. Blanchard, C. Desnos, J.P. Henry, U. Wolfrum, F. Darchen, and C. Petit. 2002. MyRIP, a novel Rab effector, enables myosin VIIa recruitment to retinal melanosomes. *EMBO reports*. 3:463-470.
Evangelista, M., S. Zigmond, and C. Boone. 2003. Formins: signaling effectors for assembly and polarization of actin filaments. *Journal of Cell Science*. 116:2603-2611.
Fehon, R.G., A.I. McClatchey, and A. Bretscher. 2010. Organizing the cell cortex: the role of ERM proteins. *Nature Reviews Molecular Cell Biology*. 11:276-287.
Fernandez-Gonzalez, R., M. Simoes Sde, J.C. Röper, S. Eaton, and J.A. Zallen. 2009. Myosin II dynamics are regulated by tension in intercalating cells. *Dev Cell*. 17:736-743.
Fielding, A.B., E. Schonteich, J. Matheson, G. Wilson, X. Yu, G.R. Hickson, S. Srivastava, S.A. Baldwin, R. Prekeris, and G.W. Gould. 2005. Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. *Embo j*. 24:3389-3399.
Firat-Karalar, E.N., and M.D. Welch. 2011. New mechanisms and functions of actin nucleation. *Current Opinion in Cell Biology*. 23:4-13.
Foe, V.E., C.M. Field, and G.M. Odell. 2000. Microtubules and mitotic cycle phase modulate spatiotemporal distributions of F-actin and myosin II in Drosophila syncytial blastoderm embryos. *Development*. 127:1767-1787.

Frangi, A.F., W.J. Niessen, K.L. Vincken, and M.A. Viergever. 1998. Multiscale vessel enhancement filtering. Springer Berlin Heidelberg. 130-137.
Fukuda, M. 2002a. The C2A domain of synaptotagmin-like protein 3 (Slp3) is an atypical calcium-dependent phospholipid-binding machine: comparison with the C2A domain of synaptotagmin I. *Biochemical Journal*. 366:681-687.
Fukuda, M. 2002b. The C2A domain of synaptotagmin-like protein 3 (Slp3) is an atypical calcium-dependent phospholipid-binding machine: comparison with the C2A domain of synaptotagmin I. *Biochem J*. 366:681-687.
Fukuda, M., T.S. Kuroda, and K. Mikoshiba. 2002. Slac2-a/Melanophilin, the Missing Link between Rab27 and Myosin Va. *Journal of Biological Chemistry*. 277:12432-12436.
Fukuda, M., C. Saegusa, and K. Mikoshiba. 2001. Novel splicing isoforms of synaptotagmin-like proteins 2 and 3: identification of the Slp homology domain. *Biochem Biophys Res Commun*. 283:513-519.
Galvez-Santisteban, M., A.E. Rodriguez-Fraticelli, D.M. Bryant, S. Vergarajauregui, T. Yasuda, I. Banon-Rodriguez, I. Bernascone, A. Datta, N. Spivak, K. Young, C.L. Slim, P.R. Brakeman, M. Fukuda, K.E. Mostov, and F. Martin-Belmonte. 2012. Synaptotagmin-like proteins control the formation of a single apical membrane domain in epithelial cells. *Nat Cell Biol*. 14:838-849.
Gary, R., and A. Bretscher. 1995. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Molecular Biology of the Cell*. 6:1061-1075.
Goley, E.D., and M.D. Welch. 2006. The ARP2/3 complex: an actin nucleator comes of age. *Nature Reviews Molecular Cell Biology*. 7:713-726.
Gomi, H., S. Mizutani, K. Kasai, S. Itohara, and T. Izumi. 2005. Granuphilin molecularly docks insulin granules to the fusion machinery. *Journal of Cell Biology*. 171:99-109.
Goode, B.L., and M.J. Eck. 2007. Mechanism and Function of Formins in the Control of Actin Assembly. *Annual Review of Biochemistry*. 76:593-627.
Gorfinkiel, N., and A.M. Arias. 2007. Requirements for adherens junction components in the interaction between epithelial tissues during dorsal closure in Drosophila. *Journal of Cell Science*. 120:3289-3298.
Gould, K.L., A. Bretscher, F.S. Esch, and T. Hunter. 1989. cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. *The EMBO Journal*. 8:4133-4142.
Gratz, S.J., C.D. Rubinstein, M.M. Harrison, J. Wildonger, and K.M. O'Connor-Giles. 2015. CRISPR-Cas9 Genome Editing in Drosophila. *Current Protocols in Molecular Biology*. 111.
Greene, N.D.E., and A.J. Copp. 2005. Mouse models of neural tube defects: Investigating preventive mechanisms. *American Journal of Medical Genetics Part C: Seminars in Medical Genetics*. 135C:31-41.
Grevengoed, E.E., D.T. Fox, J. Gates, and M. Peifer. 2003. Balancing different types of actin polymerization at distinct sites. *Journal of Cell Biology*. 163:1267-1279.
Gumbiner, B.M. 2005. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol*. 6:622-634.

Harris, E.S., F. Li, and H.N. Higgs. 2004. The mouse formin, FRLalpha, slows actin filament barbed end elongation, competes with capping protein, accelerates polymerization from monomers, and severs filaments. *J Biol Chem*. 279:20076-20087.
Harris, T.J., and M. Peifer. 2005. The positioning and segregation of apical cues during epithelial polarity establishment in Drosophila. *J Cell Biol*. 170:813-823.
Hawkins, M., B. Pope, S.K. Maciver, and A.G. Weeds. 1993. Human actin depolymerizing factor mediates a pH-sensitive destruction of actin filaments. *Biochemistry*. 32:9985-9993.
He, B., A. Martin, and E. Wieschaus. 2016. Flow-dependent myosin recruitment during Drosophila cellularization requires zygotic dunk activity. *Development*. 143:2417-2430.
Hickson, G.R., J. Matheson, B. Riggs, V.H. Maier, A.B. Fielding, R. Prekeris, W. Sullivan, F.A. Barr, and G.W. Gould. 2003. Arfophilins are dual Arf/Rab 11 binding proteins that regulate recycling endosome distribution and are related to Drosophila nuclear fallout. *Mol Biol Cell*. 14:2908-2920.
Holly, R.M., L.M. Mavor, Z. Zuo, and J.T. Blankenship. 2015. A rapid, membrane-dependent pathway directs furrow formation through RalA in the earlyDrosophilaembryo. *Development*. 142:2316-2328.
Homem, C.C., and M. Peifer. 2008. Diaphanous regulates myosin and adherens junctions to control cell contractility and protrusive behavior during morphogenesis. *Development*. 135:1005-1018.
Hosokawa, N., M. Kuragano, A. Yoshino, K. Shibata, T.Q.P. Uyeda, and K. Tokuraku. 2021. Unidirectional cooperative binding of fimbrin actin-binding domain 2 to actin filament. *Biochemical and Biophysical Research Communications*. 552:59-65.
Hume, A.N., L.M. Collinson, A. Rapak, A.Q. Gomes, C.R. Hopkins, and M.C. Seabra. 2001. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *J Cell Biol*. 152:795-808.
Hutson, M.S., Y. Tokutake, M.S. Chang, J.W. Bloor, S. Venakides, D.P. Kiehart, and G.S. Edwards. 2003. Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science*. 300:145-149.
Imai, A., and M. Tsujimura. 2017. The small GTPase, Rab27, and its effectors and regulators participate in granule exocytosis by parotid acinar cells. *Journal of Oral Biosciences*. 59:12-16.
Jankovics, F., R. Sinka, T. Lukácsovich, and M. Erdélyi. 2002. MOESIN Crosslinks Actin and Cell Membrane in Drosophila Oocytes and Is Required for OSKAR Anchoring. *Current Biology*. 12:2060-2065.
Jansen, S., A. Collins, C. Yang, G. Rebowski, T. Svitkina, and R. Dominguez. 2011. Mechanism of actin filament bundling by fascin. *J Biol Chem*. 286:30087-30096.
Jayanandanan, N., R. Mathew, and M. Leptin. 2014. Guidance of subcellular tubulogenesis by actin under the control of a synaptotagmin-like protein and Moesin. *Nature Communications*. 5.
Jiang, M., A.E. Paniagua, S. Volland, H. Wang, A. Balaji, D.G. Li, V.S. Lopes, B.L. Burgess, and D.S. Williams. 2020. Microtubule motor transport in the delivery of

melanosomes to the actin-rich apical domain of the retinal pigment epithelium. *J Cell Sci*. 133.
Jiang, T., and T.J.C. Harris. 2019. Par-1 controls the composition and growth of cortical actin caps during Drosophila embryo cleavage. *Journal of Cell Biology*. 218:4195-4214.
Jodoin, J.N., Coravos, J.S., Chanet, S., Vasquez, C.G., Tworoger, M., Kingston, E.R., Perkins, L.A., Perrimon, N., Martin, A.C. 2015. Stable Force Balance between Epithelial Cells Arises from F-Actin Turnover. *Developmental Cell*. 35:685-697.
Juriloff, D.M., and M.J. Harris. 2000. Mouse models for neural tube closure defects. *Hum Mol Genet*. 9:993-1000.
Kellogg, D.R., T.J. Mitchison, and B.M. Alberts. 1988. Behaviour of microtubules and actin filaments in living Drosophila embryos. *Development*. 103:675-686.
Koenderink, G.H., and E.K. Paluch. 2018. Architecture shapes contractility in actomyosin networks. *Current Opinion in Cell Biology*. 50:79-85.
Krueger, D., T. Quinkler, S.A. Mortensen, C. Sachse, and S. De Renzis. 2019. Cross-linker–mediated regulation of actin network organization controls tissue morphogenesis. *Journal of Cell Biology*. 218:2743-2761.
Kuroda, T.S., H. Ariga, and M. Fukuda. 2003. The Actin-Binding Domain of Slac2-a/Melanophilin Is Required for Melanosome Distribution in Melanocytes. *Molecular and Cellular Biology*. 23:5245-5255.
Kuroda, T.S., and M. Fukuda. 2005. Identification and Biochemical Analysis of Slac2-c/MyRIP as a Rab27A-, Myosin Va/VIIa-, and Actin-Binding Protein. *In* Methods in Enzymology. Vol. 403. Academic Press. 431-444.
Kuroda, T.S., M. Fukuda, H. Ariga, and K. Mikoshiba. 2002. Synaptotagmin-like protein 5: a novel Rab27A effector with C-terminal tandem C2 domains. *Biochemical and Biophysical Research Communications*. 293:899-906.
Lappalainen, P., and D.G. Drubin. 1997. Cofilin promotes rapid actin filament turnover in vivo. *Nature*. 388:78-82.
Lecuit, T., R. Samanta, and E. Wieschaus. 2002. slam Encodes a Developmental Regulator of Polarized Membrane Growth during Cleavage of the Drosophila Embryo. *Developmental Cell*. 2:425-436.
Lecuit, T., and E. Wieschaus. 2000. Polarized Insertion of New Membrane from a Cytoplasmic Reservoir during Cleavage of the Drosophila Embryo. *Journal of Cell Biology*. 150:849-860.
Lecuit, T., and A.S. Yap. 2015. E-cadherin junctions as active mechanical integrators in tissue dynamics. *Nature Cell Biology*. 17:533-539.
Lee, D.M., and T.J. Harris. 2013. An Arf-GEF regulates antagonism between endocytosis and the cytoskeleton for Drosophila blastoderm development. *Curr Biol*. 23:2110-2120.
Lee, M.T., A.R. Bonneau, and A.J. Giraldez. 2014. Zygotic Genome Activation During the Maternal-to-Zygotic Transition. *Annual Review of Cell and Developmental Biology*. 30:581-613.
Lian, G., M. Dettenhofer, J. Lu, M. Downing, A. Chenn, T. Wong, and V. Sheen. 2016. Filamin A- and formin 2-dependent endocytosis regulates proliferation via the canonical Wnt pathway. *Development*. 143:4509-4520.

Lyakhova, T.A., and J.D. Knight. 2014. The C2 domains of granuphilin are high-affinity sensors for plasma membrane lipids. *Chemistry and Physics of Lipids*. 182:29-37.
Maître, J.-L., H. Berthoumieux, S.F.G. Krens, G. Salbreux, F. Jülicher, E. Paluch, and C.-P. Heisenberg. 2012. Adhesion Functions in Cell Sorting by Mechanically Coupling the Cortices of Adhering Cells. *Science*. 338:253-256.
Martin, A.C., M. Gelbart, R. Fernandez-Gonzalez, M. Kaschube, and E.F. Wieschaus. 2010. Integration of contractile forces during tissue invagination. *Journal of Cell Biology*. 188:735-749.
Mass, R.L., R. Zeller, R.P. Woychik, T.F. Vogt, and P. Leder. 1990. Disruption of formin-encoding transcripts in two mutant limb deformity alleles. *Nature*. 346:853-855.
Matesic, L.E., R. Yip, A.E. Reuss, D.A. Swing, T.N. O'Sullivan, C.F. Fletcher, N.G. Copeland, and N.A. Jenkins. 2001. Mutations in Mlph , encoding a member of the Rab effector family, cause the melanosome transport defects observed in leaden mice. *Proceedings of the National Academy of Sciences*. 98:10238-10243.
Mavor, L.M., H. Miao, Z. Zuo, R.M. Holly, Y. Xie, D. Loerke, and J.T. Blankenship. 2016. Rab8 directs furrow ingression and membrane addition during epithelial formation in Drosophila melanogaster. *Development*. 143:892-903.
McCartney, B.M., D.G. McEwen, E. Grevengoed, P. Maddox, A. Bejsovec, and M. Peifer. 2001. Drosophila APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. *Nature Cell Biology*. 3:933-938.
Mizuno, K., T. Fujita, H. Gomi, and T. Izumi. 2016. Granuphilin exclusively mediates functional granule docking to the plasma membrane. *Scientific Reports*. 6:23909.
Nagashima, K., S. Torii, Z. Yi, M. Igarashi, K. Okamoto, T. Takeuchi, and T. Izumi. 2002. Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions. *FEBS Lett*. 517:233-238.
Nowotarski, S.H., N. McKeon, R.J. Moser, and M. Peifer. 2014. The actin regulators Enabled and Diaphanous direct distinct protrusive behaviors in different tissues duringDrosophiladevelopment. *Molecular Biology of the Cell*. 25:3147-3165.
Nozumi, M., F. Nakatsu, K. Katoh, and M. Igarashi. 2017. Coordinated Movement of Vesicles and Actin Bundles during Nerve Growth Revealed by Superresolution Microscopy. *Cell Rep*. 18:2203-2216.
Ostrowski, M., N.B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C.F. Moita, K. Schauer, A.N. Hume, R.P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M.C. Seabra, F. Darchen, S. Amigorena, L.F. Moita, and C. Thery. 2010. Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat Cell Biol*. 12:19-30; sup pp 11-13.
Otegui, M.S., K.J. Verbrugghe, and A.R. Skop. 2005. Midbodies and phragmoplasts: analogous structures involved in cytokinesis. *Trends in Cell Biology*. 15:404-413.
Otto, J.J. 1994. Actin-bundling proteins. *Current Opinion in Cell Biology*. 6:105-109.
Padash Barmchi, M., S. Rogers, and U. Häcker. 2005. DRhoGEF2 regulates actin organization and contractility in the Drosophila blastoderm embryo. *J Cell Biol*. 168:575-585.

Pelissier, A., J.-P. Chauvin, and T. Lecuit. 2003. Trafficking through Rab11 Endosomes Is Required for Cellularization during Drosophila Embryogenesis. *Current Biology*. 13:1848-1857.
Pilot, F., J.-M. Philippe, C.L. Lemmers, J.-P. Chauvin, and T. Lecuit. 2006a. Developmental control of nuclear morphogenesis and anchoring by charleston, identified in a functional genomic screen of Drosophila cellularisation. *Development*. 133:711-723.
Pilot, F., J.M. Philippe, C. Lemmers, and T. Lecuit. 2006b. Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. *Nature*. 442:580-584.
Plastino, J., and L. Blanchoin. 2019. Dynamic stability of the actin ecosystem. *Journal of Cell Science*. 132:jcs219832.
Pollard, T.D. 2016. Actin and actin-binding proteins. *Cold Spring Harbor perspectives in biology*. 8:a018226.
Pollard, T.D., and J.Q. Wu. 2010. Understanding cytokinesis: lessons from fission yeast. *Nat Rev Mol Cell Biol*. 11:149-155.
Postner, M.A., and E.F. Wieschaus. 1994. The nullo protein is a component of the actin-myosin network that mediates cellularization in Drosophila melanogaster embryos. *Journal of Cell Science*. 107:1863-1873.
Pring, M., M. Evangelista, C. Boone, C. Yang, and S.H. Zigmond. 2003. Mechanism of formin-induced nucleation of actin filaments. *Biochemistry*. 42:486-496.
Raff, J.W., and D.M. Glover. 1989. Centrosomes, and not nuclei, initiate pole cell formation in Drosophila embryos. *Cell*. 57:611-619.
Reichl, E.M., Y. Ren, M.K. Morphew, M. Delannoy, J.C. Effler, K.D. Girard, S. Divi, P.A. Iglesias, S.C. Kuo, and D.N. Robinson. 2008. Interactions between Myosin and Actin Crosslinkers Control Cytokinesis Contractility Dynamics and Mechanics. *Current Biology*. 18:471-480.
Ridley, A.J. 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol*. 16:522-529.
Riggs, B., W. Rothwell, S. Mische, G.R.X. Hickson, J. Matheson, T.S. Hays, G.W. Gould, and W. Sullivan. 2003. Actin cytoskeleton remodeling during early Drosophila furrow formation requires recycling endosomal components Nuclear-fallout and Rab11. *Journal of Cell Biology*. 163:143-154.
Rikhy, R., M. Mavrakis, and J. Lippincott-Schwartz. 2015. Dynamin regulates metaphase furrow formation and plasma membrane compartmentalization in the syncytial Drosophila embryo. *Biology Open*. 4:301-311.
Ríos-Barrera, L.D., and M. Leptin. 2022. An endosome-associated actin network involved in directed apical plasma membrane growth. *Journal of Cell Biology*. 221.
Roh-Johnson, M., G. Shemer, C.D. Higgins, J.H. McClellan, A.D. Werts, U.S. Tulu, L. Gao, E. Betzig, D.P. Kiehart, and B. Goldstein. 2012. Triggering a Cell Shape Change by Exploiting Preexisting Actomyosin Contractions. *Science*. 335:1232-1235.

Rothwell, W.F., P. Fogarty, C.M. Field, and W. Sullivan. 1998. Nuclear-fallout, a Drosophila protein that cycles from the cytoplasm to the centrosomes, regulates cortical microfilament organization. *Development*. 125:1295-1303.
Royou, A., C. Field, J.C. Sisson, W. Sullivan, and R. Karess. 2004. Reassessing the role and dynamics of nonmuscle myosin II during furrow formation in early Drosophila embryos. *Mol Biol Cell*. 15:838-850.
Royou, A., W. Sullivan, and R. Karess. 2002. Cortical recruitment of nonmuscle myosin II in early syncytial Drosophila embryos: its role in nuclear axial expansion and its regulation by Cdc2 activity. *J Cell Biol*. 158:127-137.
Sagot, I., S.K. Klee, and D. Pellman. 2002. Yeast formins regulate cell polarity by controlling the assembly of actin cables. *Nat Cell Biol*. 4:42-50.
Schejter, E.D., and E. Wieschaus. 1993. bottleneck acts as a regulator of the microfilament network governing cellularization of the Drosophila embryo. *Cell*. 75:373-385.
Serano, J., and G.M. Rubin. 2003. The Drosophila synaptotagmin-like protein bitesize is required for growth and has mRNA localization sequences within its open reading frame. *Proc Natl Acad Sci U S A*. 100:13368-13373.
Shekhar, S. 2017. Microfluidics-Assisted TIRF Imaging to Study Single Actin Filament Dynamics. *Current Protocols in Cell Biology*. 77.
Shekhar, S., and M.F. Carlier. 2017. Enhanced Depolymerization of Actin Filaments by ADF/Cofilin and Monomer Funneling by Capping Protein Cooperate to Accelerate Barbed-End Growth. *Curr Biol*. 27:1990-1998.e1995.
Sherlekar, A., G. Mundhe, P. Richa, B. Dey, S. Sharma, and R. Rikhy. 2020. F-BAR domain protein Syndapin regulates actomyosin dynamics during apical cap remodeling in syncytial Drosophila embryos. *J Cell Sci*. 133.
Simpson, L., and E. Wieschaus. 1990. Zygotic activity of the nullo locus is required to stabilize the actin-myosin network during cellularization in Drosophila. *Development*. 110:851-863.
Sokac, A.M., N. Biel, and S. De Renzis. 2023. Membrane-actin interactions in morphogenesis: Lessons learned from Drosophila cellularization. *Seminars in Cell & Developmental Biology*. 133:107-122.
Sokac, A.M., and E. Wieschaus. 2008. Local Actin-Dependent Endocytosis Is Zygotically Controlled to Initiate Drosophila Cellularization. *Developmental Cell*. 14:775-786.
Sommi, P., D. Cheerambathur, I. Brust-Mascher, and A. Mogilner. 2011. Actomyosin-dependent cortical dynamics contributes to the prophase force-balance in the early Drosophila embryo. *PLoS One*. 6:e18366.
Spiering, D., and L. Hodgson. 2011. Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr*. 5:170-180.
Spracklen, A.J., T.N. Fagan, K.E. Lovander, and T.L. Tootle. 2014. The pros and cons of common actin labeling tools for visualizing actin dynamics during Drosophila oogenesis. *Developmental Biology*. 393:209-226.
Spracklen, A.J., E.M. Thornton-Kolbe, A.N. Bonner, A. Florea, P.J. Compton, R. Fernandez-Gonzalez, and M. Peifer. 2019. The Crk adapter protein is essential

forDrosophilaembryogenesis, where it regulates multiple actin-dependent morphogenic events. *Molecular Biology of the Cell*. 30:2399-2421.
Spudich, J.A., and S. Watt. 1971. The Regulation of Rabbit Skeletal Muscle Contraction: I. BIOCHEMICAL STUDIES OF THE INTERACTION OF THE TROPOMYOSIN-TROPONIN COMPLEX WITH ACTIN AND THE PROTEOLYTIC FRAGMENTS OF MYOSIN. *Journal of Biological Chemistry*. 246:4866-4871.
Stevenson, V., A. Hudson, L. Cooley, and W.E. Theurkauf. 2002. Arp2/3-Dependent Psuedocleavage Furrow Assembly in Syncytial Drosophila Embryos. *Current Biology*. 12:705-711.
Stevenson, V.A., J. Kramer, J. Kuhn, and W.E. Theurkauf. 2001. Centrosomes and the Scrambled protein coordinate microtubule-independent actin reorganization. *Nature Cell Biology*. 3:68-75.
Strickland, L.I., E.J. Donnelly, and D.R. Burgess. 2005. Induction of Cytokinesis Is Independent of Precisely Regulated Microtubule Dynamics. *Molecular Biology of the Cell*. 16:4485-4494.
Striegel, A.R., L.M. Biela, C.S. Evans, Z. Wang, J.B. Delehoy, R.B. Sutton, E.R. Chapman, and N.E. Reist. 2012. Calcium binding by synaptotagmin's C2A domain is an essential element of the electrostatic switch that triggers synchronous synaptic transmission. *J Neurosci*. 32:1253-1260.
Strom, M., A.N. Hume, A.K. Tarafder, E. Barkagianni, and M.C. Seabra. 2002. A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. *J Biol Chem*. 277:25423-25430.
Sullivan, W., P. Fogarty, and W. Theurkauf. 1993. Mutations affecting the cytoskeletal organization of syncytial Drosophila embryos. *Development*. 118:1245-1254.
Sutherland-Smith, A.J. 2011. Filamin structure, function and mechanics: are altered filamin-mediated force responses associated with human disease? *Biophys Rev*. 3:15-23.
Tan, H.L., S. Bungert-Plümke, D. Kortzak, C. Fahlke, and G. Stölting. 2022. Determination of oligomeric states of proteins via dual-color colocalization with single molecule localization microscopy. *eLife*. 11:e76631.
Tilney, L.G., P.S. Connelly, K.A. Vranich, M.K. Shaw, and G.M. Guild. 2000. Regulation of Actin Filament Cross-linking and Bundle Shape in Drosophila Bristles. *Journal of Cell Biology*. 148:87-99.
Uribe, R., and D. Jay. 2009. A review of actin binding proteins: new perspectives. *Molecular Biology Reports*. 36:121-125.
Wang, J., T. Takeuchi, H. Yokota, and T. Izumi. 1999. Novel Rabphilin-3-like Protein Associates with Insulin-containing Granules in Pancreatic Beta Cells. *Journal of Biological Chemistry*. 274:28542-28548.
Webb, R.L., M.-N. Zhou, and B.M. McCartney. 2009. A novel role for an APC2-Diaphanous complex in regulating actin organization in Drosophila. *Development*. 136:1283-1293.

Wenzl, C., S. Yan, P. Laupsien, and J. Großhans. 2010. Localization of RhoGEF2 during Drosophila cellularization is developmentally controlled by slam. *Mechanisms of Development*. 127:371-384.
Wilcke, M., L. Johannes, T. Galli, V. Mayau, B. Goud, and J. Salamero. 2000. Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. *J Cell Biol*. 151:1207-1220.
Wilson, G.M., A.B. Fielding, G.C. Simon, X. Yu, P.D. Andrews, R.S. Hames, A.M. Frey, A.A. Peden, G.W. Gould, and R. Prekeris. 2005. The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis. *Mol Biol Cell*. 16:849-860.
Winder, S.J., and K.R. Ayscough. 2005. Actin-binding proteins. *Journal of Cell Science*. 118:651-654.
Wioland, H., B. Guichard, Y. Senju, S. Myram, P. Lappalainen, A. Jégou, and G. Romet-Lemonne. 2017. ADF/Cofilin Accelerates Actin Dynamics by Severing Filaments and Promoting Their Depolymerization at Both Ends. *Current Biology*. 27:1956-1967.e1957.
Wioland, H., A. Jegou, and G. Romet-Lemonne. 2019. Quantitative Variations with pH of Actin Depolymerizing Factor/Cofilin's Multiple Actions on Actin Filaments. *Biochemistry*. 58:40-47.
Wu, X., B. Bowers, K. Rao, Q. Wei, and J.A. Hammer, 3rd. 1998. Visualization of melanosome dynamics within wild-type and dilute melanocytes suggests a paradigm for myosin V function In vivo. *J Cell Biol*. 143:1899-1918.
Wu, X.S., K. Rao, H. Zhang, F. Wang, J.R. Sellers, L.E. Matesic, N.G. Copeland, N.A. Jenkins, and J.A. Hammer, 3rd. 2002. Identification of an organelle receptor for myosin-Va. *Nat Cell Biol*. 4:271-278.
Xie, Y., R. Budhathoki, and J.T. Blankenship. 2021. Combinatorial deployment of F-actin regulators to build complex 3D actin structures in vivo. *eLife*. 10.
Xue, F., D.M. Janzen, and D.A. Knecht. 2010. Contribution of Filopodia to Cell Migration: A Mechanical Link between Protrusion and Contraction. *Int J Cell Biol*. 2010:507821.
Zallen, J.A., Y. Cohen, A.M. Hudson, L. Cooley, E. Wieschaus, and E.D. Schejter. 2002. SCAR is a primary regulator of Arp2/3-dependent morphological events in Drosophila. *Journal of Cell Biology*. 156:689-701.
Zaman, R., A. Lombardo, C. Sauvanet, R. Viswanatha, V. Awad, L.E.-R. Bonomo, D. McDermitt, and A. Bretscher. 2021. Effector-mediated ERM activation locally inhibits RhoA activity to shape the apical cell domain. *Journal of Cell Biology*. 220.
Zhang, Y., J.C. Yu, T. Jiang, R. Fernandez-Gonzalez, and T.J.C. Harris. 2018. Collision of Expanding Actin Caps with Actomyosin Borders for Cortical Bending and Mitotic Rounding in a Syncytium. *Developmental Cell*. 45:551-564.e554.
Zheng, L., L.A. Sepúlveda, R.C. Lua, O. Lichtarge, I. Golding, and A.M. Sokac. 2013. The Maternal-to-Zygotic Transition Targets Actin to Promote Robustness during Morphogenesis. *PLoS Genetics*. 9:e1003901.

Chapter 2: Bitesize bundles F-actin and influences actin remodeling in syncytial *Drosophila* embryo development

https://www.biorxiv.org/content/10.1101/2023.04.17.537198v1

AY, GJH, BLG, ACM designed and performed experiments and wrote the manuscript. AY and GH analyzed the data.

Abstract

Actin networks undergo rearrangements that influence cell and tissue shape. Actin network assembly and organization is regulated in space and time by a host of actin binding proteins. The *Drosophila* Synaptotagmin-like protein, Bitesize (Btsz), is known to organize actin at epithelial cell apical junctions in a manner that depends on its interaction with the actin-binding protein, Moesin. Here, we showed that Btsz functions in actin reorganization at earlier, syncytial stages of *Drosophila* embryo development. Btsz was required for the formation of stable metaphase pseudo-cleavage furrows that prevented spindle collisions and nuclear fallout prior to cellularization. While previous studies focused on Btsz isoforms containing the Moesin Binding Domain (MBD), we found that isoforms lacking the MBD also function in actin remodeling. Consistent with this, we found that the C-terminal half of BtszB cooperatively binds to and bundles F-actin, suggesting a direct mechanism for Synaptotagmin-like proteins regulating actin organization during animal development.

Introduction

During animal development, actin networks are tightly regulated and undergo dynamic rearrangements to promote morphogenesis (Camuglia et al., 2021; Lecuit and Yap, 2015; Sokac et al., 2023). However, the range of mechanisms that control actin network assembly and organization during development is still not fully understood. There are various levels at which actin must be regulated, which we describe here as three major levels. First, the nucleation and assembly of monomeric G-actin (globular actin) into polymeric F-actin (filamentous actin) are tightly regulated in both time and space. Spontaneous nucleation of actin is inhibited by many factors *in vivo* (Plastino and Blanchoin, 2019). Therefore, actin nucleators and assemblers such as the formin, Diaphanous (Dia), as well as the Arp2/3 complex are essential in driving actin polymerization. Dia assembles actin structures composed of linear (unbranched) filaments, whereas Arp2/3 complex assembles branched actin filament networks. Next is the organization of filamentous actin into higher-order structures, or networks, which involves a plethora of actin binding and bundling/crosslinking proteins such as Fascin/Singed, Filamin/Cheerio and Fimbrin (Cant et al., 1994; Davidson et al., 2019; Krueger et al., 2019; Tilney et al., 2000). These networks are important for structure, transport, and transducing mechanical forces across the organism. Crosslinking actin filaments not only governs the network's architecture but also its rigidity and contractile properties (Koenderink and Paluch, 2018). Finally, the actin network must be connected to the cell membrane through actin-binding proteins in order to provide structure and help induce cell shape changes (Chugh and Paluch, 2018). The ERM (Ezrin Radixin Moesin) family of proteins plays an essential role in this process (Algrain et al., 1993;

Gould et al., 1989). When activated by phosphorylation through kinases, ERM proteins undergo a conformational change to reveal an F-actin binding and membrane protein-binding site such that they can tether F-actin to membrane (Gary and Bretscher, 1995; Zaman et al., 2021). Therefore, each cell must coordinate actin nucleation, assembly and disassembly, along with higher-order levels of control such as organizing actin networks and connecting these networks to cell membrane.

The early *Drosophila* embryo provides an optimal system in which to study actin regulation due to its dynamic actin network rearrangements. At the earliest stages of development, the embryo is a syncytium consisting of one cell with multiple nuclei in a shared cytoplasm. The syncytium undergoes multiple rounds of synchronous modified cell divisions lacking cytokinesis and intervening gap phases, called nuclear divisions (Figure 1A) (Foe et al., 2000). Nuclear cycles (NC) 1-9 occur in the yolk at the center of the embryo, and nuclei migrate to the apical surface of the embryo for NC 10-13 (Foe et al., 2000). Centrosomes then help direct the formation of F-actin structures called 'caps' (Stevenson et al., 2002; Stevenson et al., 2001), which are positioned above the syncytial nuclei. During each nuclear cycle, F-actin assembly drives the growth of actin caps that expand into one another (Figure 1A). Collisions between neighboring caps lead to the formation of pseudo-cleavage furrows, structures made up of both actin and membrane (Zhang et al., 2018) (Figure 1A). Pseudo-cleavage furrows act as a barrier between dividing spindles and are essential for proper nuclear divisions. Perturbing pseudo-cleavage furrows with drugs or depleting actin-related proteins often results in spindle collisions (Afshar et al., 2000; Callaini et al., 1992; Spracklen et al., 2019; Sullivan et al., 1993; Webb et al., 2009; Zallen et al., 2002).

In addition to actin assembly, membrane trafficking is essential for syncytial blastoderm development. When membrane trafficking is perturbed by knocking down components of the trafficking machinery, such as Nuf and Rab11, pseudo-cleavage furrows are shorter and resemble those in embryos defective in actin assembly (Cao et al., 2008; Holly et al., 2015; Riggs et al., 2003; Rikhy et al., 2015; Rothwell et al., 1998; Sherlekar et al., 2020). Together, these findings indicate that actin network reorganization is coupled with membrane remodeling to create pseudo-cleavage furrows. However, there is still much to uncover about the roles that actin and/or membrane remodelers play in morphogenesis.

Synaptotagmin-like proteins (Slps) are potential candidates for coupling actin and membrane remodeling during syncytial blastoderm development. Slps are conserved across vertebrates and are characterized by an N-terminal Slp homology domain (SHD) and two C-terminal C2 domains (Figure 1B). The mammalian SHD interacts with the GTPase Rab27, and the C2 domain consists of the C2A and C2B domains that bind membrane, sometimes in a calcium-dependent manner (Fukuda, 2002a; Galvez-Santisteban et al., 2012). Specifically, the C2A domain binds with high affinity to $PI(4,5)P_2$ or $PI(3,4,5)P_3$ and the C2B domain binds with high affinity to $PI(4,5)P_2$ (Galvez-Santisteban et al., 2012; Lyakhova and Knight, 2014). A family of proteins related to Slps are Slac2s, Synaptotagmin-like proteins lacking C2 domains, which contain the SHD at their N-terminus but lack the C-terminal C2 domains (Figure 1B). Slac2 proteins function as adaptor proteins for cargo transport, interacting with Rab27A via their SHD, as well as Myosin Va via their myosin-binding sites found in the middle of Slac2 (Fukuda et al., 2002). Interestingly, the C-termini of Slac2-a and Slac2-c also bind

G- and F-actin (Fukuda et al., 2002). Both Slps and Slac2s are Rab27 effectors important for vesicle secretion. For example, Slp4 is necessary for proper exocytosis and granule docking in the pancreas (Mizuno et al., 2016). During *de novo* epithelial formation, Slp2-a is required for targeting vesicles to the apical surface in a PIP2 dependent manner, and Slp4-a couples these vesicles to apical plasma membrane to generate lumen (Galvez-Santisteban et al., 2012). Overall, there are six Slp genes and three Slac2 genes in mammals. The reasons for such a diverse array of Slp and Slac genes and whether less complex metazoans have functional equivalents to this diverse gene family are unknown.

Drosophila encodes a single Slp gene, *Bitesize* (*Btsz*) that plays a role in actin cytoskeleton organization (Pilot et al., 2006b) similar to mammalian Slp4/granuphilin. However, it is unclear whether Btsz contains an SHD, or plays a direct role in exocytosis. Pilot et al. showed that Btsz is essential for maintaining adherens junctions and epithelial integrity. They discovered that certain Btsz isoforms interact with the membrane-cortex crosslinker, Moesin (Moe), via a Moesin binding domain (MBD) (Figure 1C). Their observations supported a model in which Btsz binds phosphoinositides via its C2A and C2B domains, localizing it to adherens junctions at the apical surface of the cell where it recruits Moesin to regulate actin organization and stabilize E-cadherin (Figure 1D). During tracheal morphogenesis, Btsz recruits Moesin to the luminal membrane to promote proper actin organization (Jayanandanan et al., 2014). Therefore, previous work has established the importance of Btsz in regulating the actin cytoskeleton through Moesin. However, alternative splicing generates nine different Btsz isoforms and most of them lack the exon that encodes the MBD (Figure

1C). This raises the question of whether these other Btsz isoforms have distinct functional roles in development, and how the functions of these isoforms may be related to mammalian Slp and Slac2.

In this study, we discovered a role for Btsz in regulating actin organization during syncytial stages of *Drosophila* embryo development, which in turn impacts spindle and nuclear morphology. This function of Btsz is mediated in part by Btsz isoforms lacking the Moesin-binding domain. We demonstrated this by making a CRISPR allele that affects Btsz isoforms lacking the MBD as well as an isoform-specific UAS>Btsz::mCherry tool to assess the localization of an individual isoform. Despite lacking the MBD, we found that Btsz isoform B directly binds to F-actin and bundles F-actin *in vitro*, suggesting a more direct role for Btsz in regulating actin architecture.

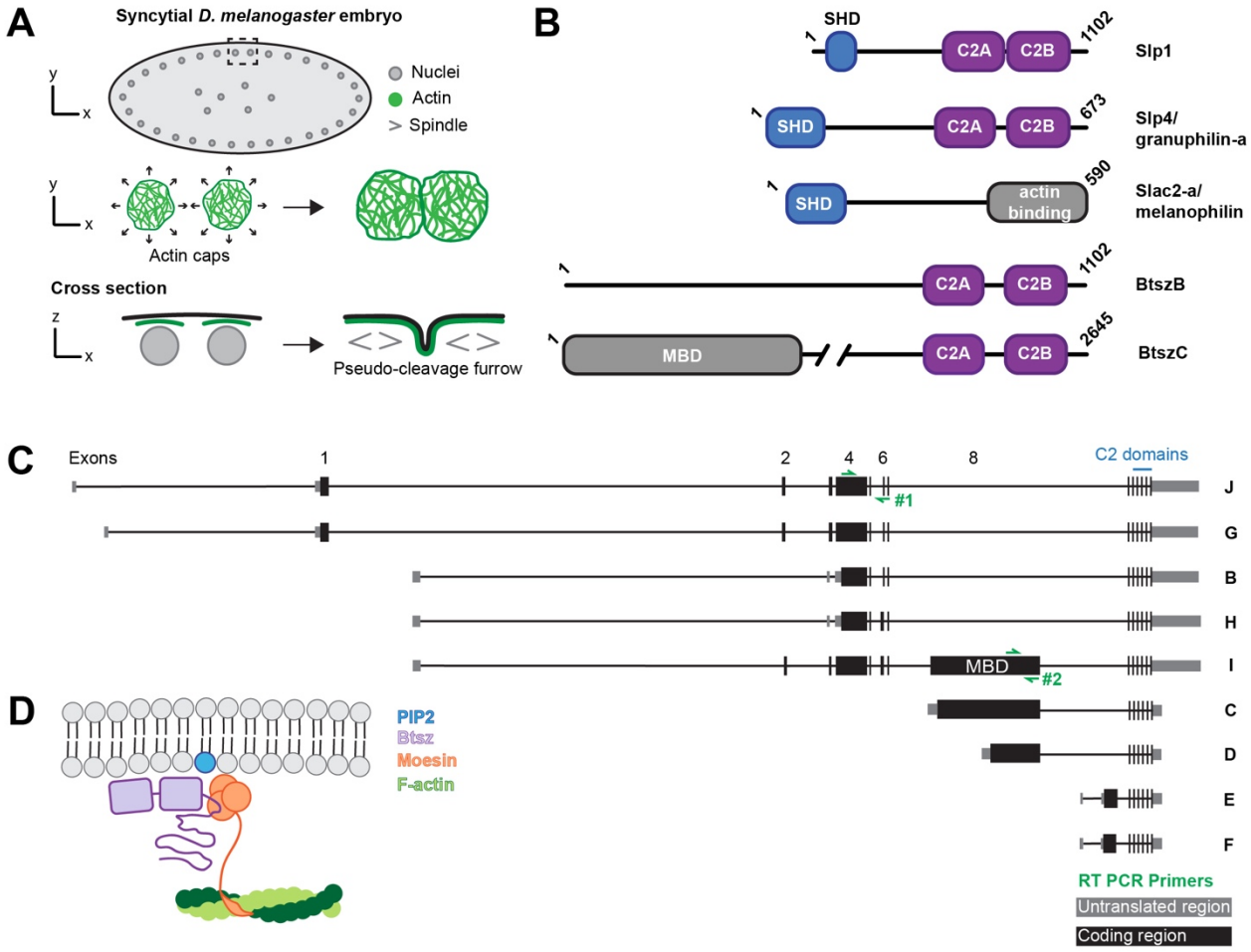
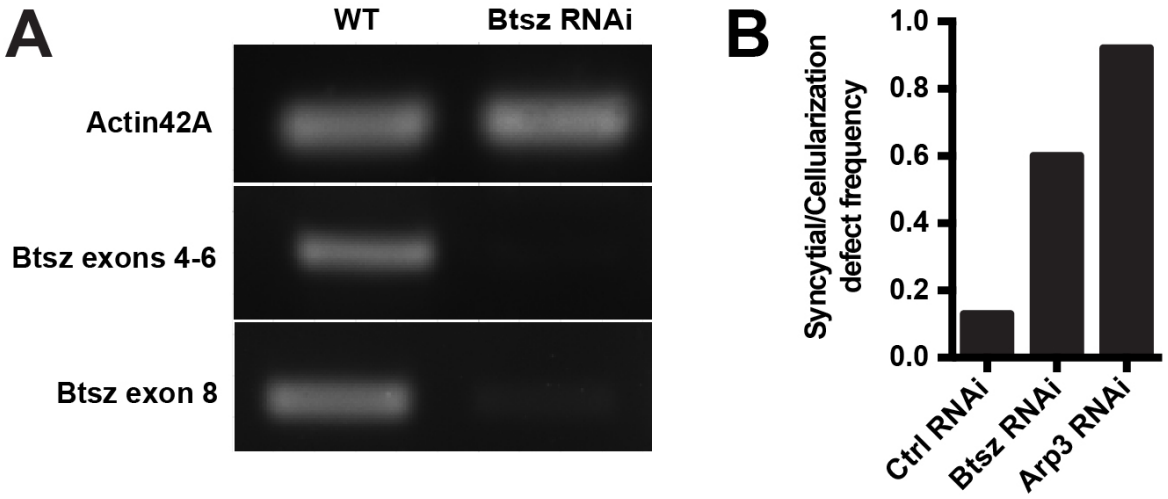


Figure 1. Btsz is a Synaptotagmin-like protein (Slp) with multiple splice isoforms. **(A)** Cartoon depiction of a syncytial embryo during a nuclear cycle. Top-down view of a syncytial embryo (top row). Zoomed in view of the actin caps in the dashed rectangular box where actin caps grow and collide with neighboring actin caps (middle row). Cross section of the zoomed in view where pseudo-cleavage furrows form from actin caps that have collided (bottom row). **(B)** Schematic of mammalian (mouse) Slp1, Slp4, Slac2-a, and fly Btsz isoform B and C proteins. **(C)** Schematic of the nine splice isoforms of the Btsz protein. Letters on the left-hand side denote the isoform name. Location of RT-PCR primer pairs #1 and #2 for exons 4-6 and exon 8, respectively, are shown with green arrows. **(D)** Model depicting Btsz's interaction with the plasma membrane and Moesin. Btsz and Moesin localized to apical membrane through interactions with PIP2. There, Btsz can bind the FERM3 domain of Moesin and Moesin binds F-actin.

Results

Btsz is required for actin remodeling during syncytial blastoderm development

To examine Btsz function in early development, we used an RNAi line to maternally deplete embryos of Btsz (Figure S1A). This shRNA targets a sequence in the 3' UTR shared by all isoforms. Past work on Btsz focused on isoforms containing the Moesin-binding domain (MBD) and their roles during gastrulation in regulating actin organization, epithelial integrity, and lumen formation (Jayanandanan et al., 2014; Pilot et al., 2006b). Upregulation of Btsz isoforms containing the MBD at the mid-blastula transition was noted in previous studies (Pilot et al., 2006a). We observed that knock-down of all Btsz isoforms revealed striking defects in syncytial embryo development as well as defects after epithelium formation, during gastrulation. We found that 60% of Btsz-RNAi embryos displayed syncytial or cellularization defects compared to 13% of control embryos (Figure S1B). Because defects prior to gastrulation in Btsz-depleted embryos have not been described, and the syncytial blastoderm is an excellent system in which to study actin remodeling, we further investigated Btsz function in the syncytial blastoderm.



Supplemental Figure 1. (A) RT-PCR Gel of Btsz cDNA showing maternal knockdown of Btsz in the early embryo. Primers used are shown in Figure 1C. Primer pair #1 was used for Btsz exons 4-6 and pair #2 was used for Btsz exon 8. **(B)** Frequency of control RNAi, Btsz RNAi, and Arp3 RNAi embryos with defects during the syncytial or cellularization stages. 4 out of 30 control, 25 out of 42 Btsz RNAi, and 12 out of 13 Arp3 RNAi embryos displayed defects.

To determine Btsz function in syncytial stages of embryonic development, we visualized actin in live embryos by driving mCherry::MoesinABD (mCh::Moe) expression with the maternal Gal4 driver mat67 to observe actin cap and pseudo-cleavage furrow structure and dynamics (Xie et al., 2021). We found that Btsz depletion affected actin cap organization dynamics (Figure 2A; Video 1). We measured cap size once the caps had finished expanding in control and Btsz-RNAi embryos. Our results show that in Btsz-depleted embryos, the average cap area is smaller compared to those of control embryos (Figure 2A-B). When comparing the distribution of cap areas, there is a bimodal distribution of cap sizes and a greater proportion of small actin caps (<80mm^2) in Btsz-shRNA embryos compared to the control (Figure 2B-B'').

Actin cap expansion and subsequent collisions between neighboring caps drive pseudo-cleavage furrow formation and growth (Zhang et al., 2018) so we hypothesized that pseudo-cleavage furrows would be affected in Btsz-RNAi embryos. Interestingly, small actin caps in Btsz-depleted embryos still formed pseudo-cleavage furrows between nuclei. Live imaging of actin in pseudo-cleavage furrows did reveal that a higher frequency of furrows prematurely retract during mitosis in Btsz-RNAi embryos (Figure 2C, D; Video 2). These unstable furrows formed normally (Figure 2C, yellow arrows) but receded during metaphase, resulting in furrows that are either very short or no longer visible (Figure 2C, red arrows). However, pseudo-cleavage furrows that formed properly and did not retract in Btsz-RNAi embryos reached their maximum length and were comparable in length to wild-type furrows (Figure S2A, B).

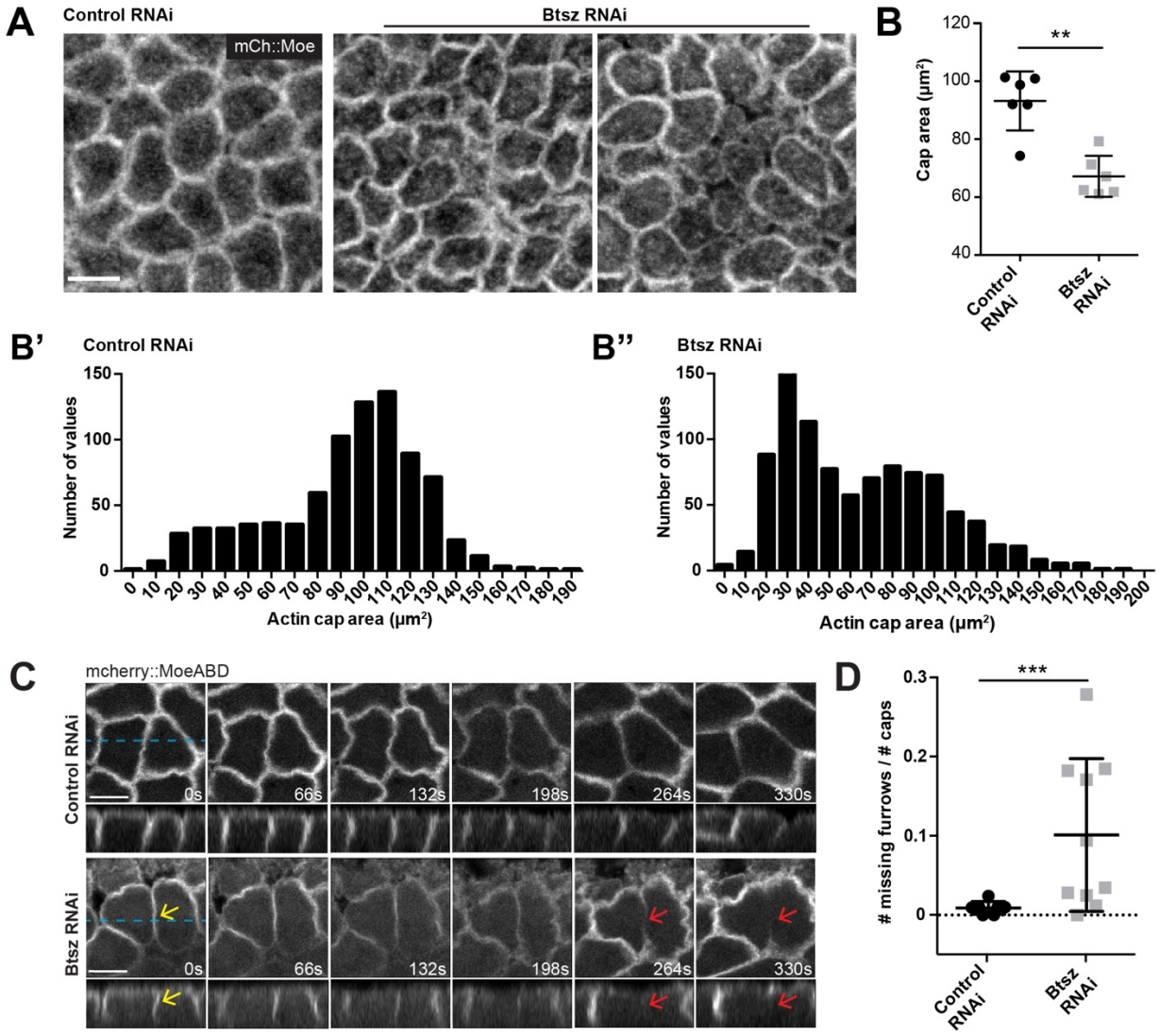
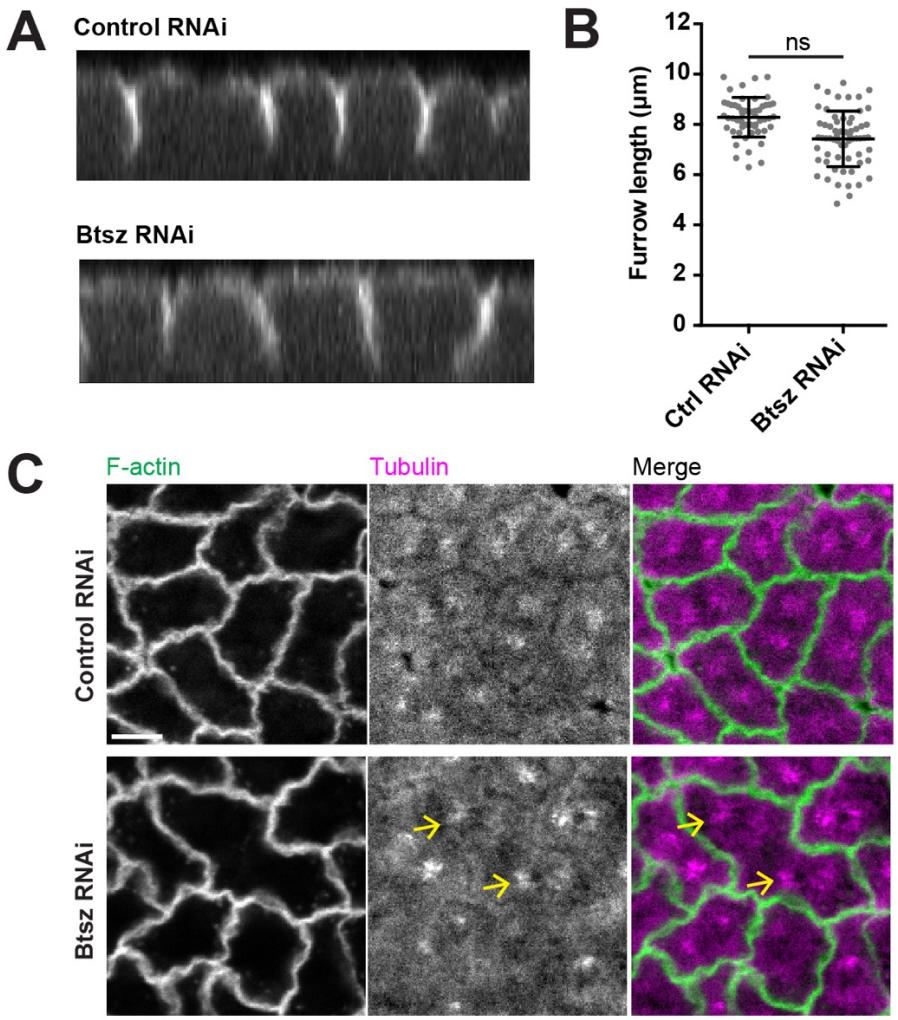


Figure 2. Btsz is required for actin remodeling during syncytial blastoderm development. (A) Maximum projection of a top-down view of control and Btsz RNAi nuclear cycle 12 actin caps. Actin was visualized live using the mCherry::MoesinABD marker. **(B)** In Btsz-depleted embryos, the average cap size is smaller compared to those in the control. Each data point is an average of all caps in the field of view per embryo. n = 6 embryos for both control RNAi and Btsz RNAi. p = 0.0043, Mann-Whitney U test. **(B', B'')** Histogram of raw data of actin cap area from Figure 2B. There is a peak of small actin caps (~30 µm^2) in Btsz RNAi embryos that is absent in the control embryos. **(C)** Time-lapse showing the formation of pseudo-cleavage furrows in control and Btsz-depleted embryos, with single slices (top panels) and orthogonal views (bottom panels). Actin was visualized live using the mCherry::MoesinABD marker. In Btsz RNAi embryo, a pseudo-cleavage furrow forms normally (yellow arrows), but breaks and recedes (red arrows) during metaphase while neighboring

furrows are at their maximum length. **(D)** Number of missing furrows normalized over total number of actin caps. In Btsz-depleted embryos, pseudo-cleavage furrows prematurely recede during mitosis more often than in wild type embryos. Each point is the ratio of missing furrows to actin caps in a field of view (212 x 106 μm) per embryo. n = 7 embryos for control RNAi, n = 10 embryos for Btsz RNAi, p = 0.0007, Mann-Whitney U test. Scale bars are 10 μm.



Supplemental Figure 2. (A) Cross section of pseudo-cleavage furrows during syncytial blastoderm development. Actin was visualized live with the mCherry::MoesinABD marker. **(B)** Pseudo-cleavage furrows that form normally in Btsz RNAi embryos are not significantly different in length compared to the wildtype. Each point is one furrow from n = 6 embryos for both control RNAi and Btsz RNAi. **(C)** Slice of fixed wildtype and Btsz RNAi embryos during metaphase. F-actin was visualized using Phalloidin and tubulin was visualized using an α-tubulin antibody. Pseudo-cleavage furrows that have receded lead to two sets of spindles in one compartment (yellow arrows). Scale bar is 5 μm.

Btsz depletion leads to spindle collision and nuclear defects

Because pseudo-cleavage furrows are essential for preventing spindle collisions during each nuclear cycle, we assessed the integrity of the nuclear divisions in Btsz-depleted embryos. Using the microtubule plus-end-tracking protein CLIP170 fused with GFP to visualize spindles, we found that aberrant spindle morphology accompanied *Btsz* knock-down (Figure 3A, C). In Btsz-depleted embryos, spindles were often abnormally fused between neighboring nuclei (Figure 3A). This spindle collision phenotype has been noted in various conditions where proteins that regulate actin and/or membrane remodeling are disrupted (Dia, Arp2/3, Par1, APC2, Abl, Nuf, Rab11, etc.) (Grevengoed et al., 2003; Jiang and Harris, 2019; McCartney et al., 2001; Riggs et al., 2003; Rothwell et al., 1998; Webb et al., 2009; Zhang et al., 2018).

Proper actin cytoskeleton regulation is important for centrosome migration to opposite poles during nuclear division cycles (Cao et al., 2008). Centrosome separation was abnormal in Btsz-depleted embryos (Figure 3B) with centrosomes failing to migrate to 180° opposite each other prior to nuclear envelope breakdown more often than in wild-type embryos (Figure 3B, C'). Both the distance between centrosomes (Figure 3C'') and spindle length (Figure 3C''') were shorter in Btsz-depleted embryos compared to wild-type embryos. These phenotypes were reminiscent of those observed in other actin and APC2 mutants (Cao et al., 2008; McCartney et al., 2001). Fixed imaging of both F-actin and nuclei during cellularization revealed heterogeneity in compartment sizes and nuclear morphology in Btsz RNAi embryos, indicative of improper nuclear divisions (Figure 3D).

Visualizing both F-actin and spindles confirmed that furrow breakages in the Btsz RNAi background led to two sets of spindles in the same compartment (Figure S2C). These spindle abnormalities preceded massive nuclear fallout from the cell cortex during syncytial stages and cellularization, observed through live imaging the nuclear marker Histone::GFP (Figure 3E, yellow arrows; Video 3). Quantification of relative nuclear density shows that there are fewer nuclei in Btsz-depleted embryos compared to the control (Figure 3F), likely a consequence of the pseudo-cleavage furrow defects (Figure 2C). Overall, these results demonstrate a critical role for Btsz in embryo development, well prior to its known role in epithelial integrity during gastrulation.

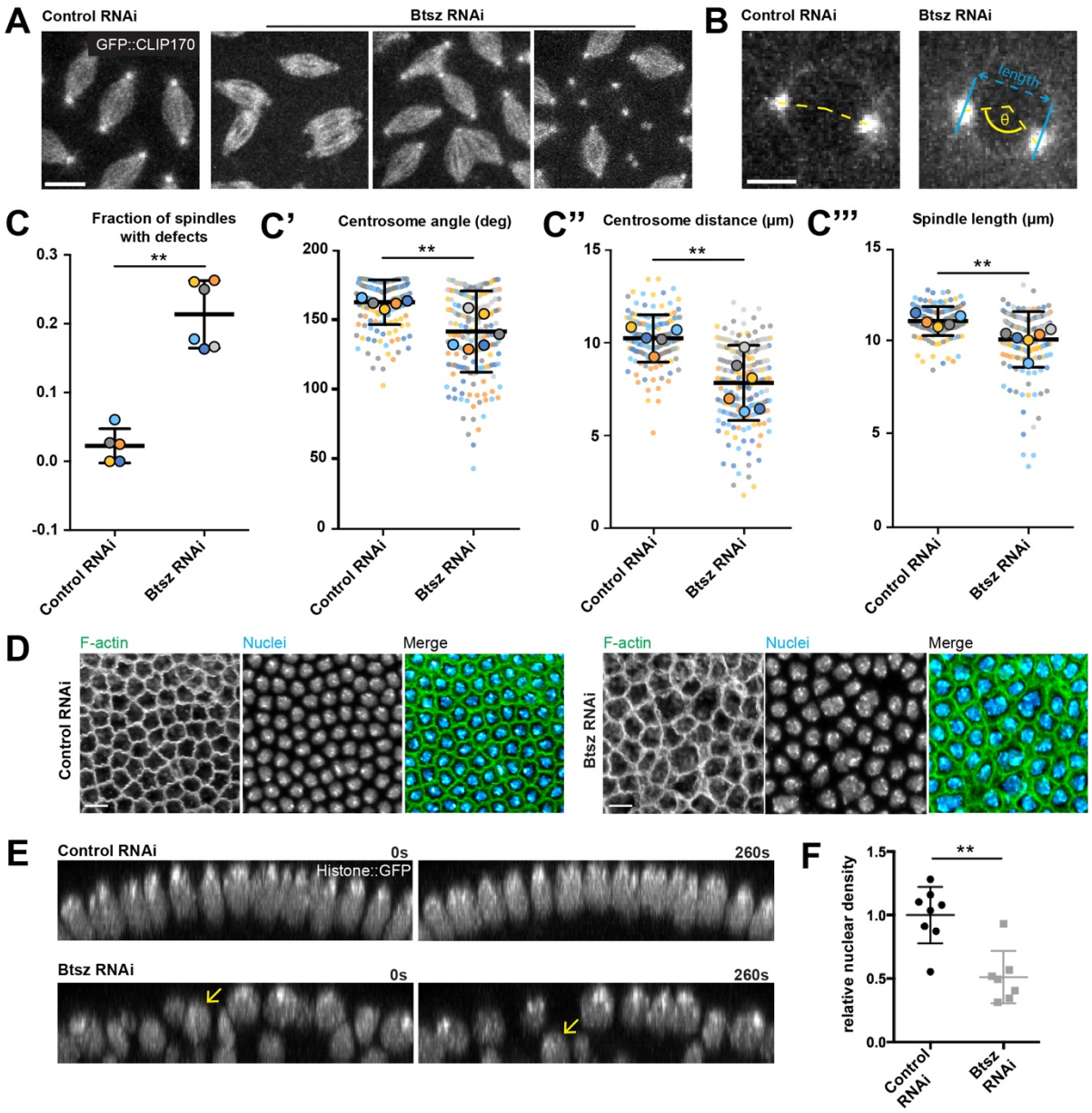


Figure 3. Btsz-depletion leads to spindle collision and nuclear defects. (A, B) Maximum projections of spindles and centrosomes visualized live using GFP::CLIP170 in control and Btsz-depleted embryos. Spindle collisions and aberrant centrosome morphology occur more frequently in Btsz-RNAi embryos. Scale bars are 10 μm for spindles, 5 μm for centrosomes. **(C)** Fraction of spindles that have collided in control and Btsz RNAi embryos. Each large, bold circle is the fraction of occurrences in an embryo **(C'-C''')** Quantification of shortest distance between a centrosome pair (cyan line), angle of a vertex drawn between a centrosome pair (yellow dashed line), and spindle length. Each large, bold circle is the average of one embryo and smaller circles of corresponding color are the centrosome pairs or individual spindles used in the analysis. n = 5 embryos for control RNAi, n = 6 embryos for Btsz RNAi. p = 0.0043

for fraction with defects, 0.0087 for centrosome distance and angle, 0.0043 for spindle length, Mann-Whitney U test. **(D)** Single slice of cellularizing control RNAi or Btsz RNAi embryos. F-actin was visualized using Phalloidin and nuclei was visualized using Hoechst in fixed samples. Both the actin cytoskeleton and nuclei are abnormal in Btsz RNAi embryos. **(E)** Orthogonal slice of nuclei visualized live using Histone::GFP at cellularization in control (top) or Btsz RNAi (bottom) embryos at 0s and 260s later. Yellow arrow indicates one nucleus at the surface of the Btsz RNAi embryo undergoing fallout. **(F)** Nuclear fallout occurs more frequently in Btsz-depleted embryos compared to the control. Each point is the number of nuclei over area (nuclear density) in one embryo. p = 0.0043, Mann-Whitney U test.

Non-MBD and MBD containing Btsz isoforms are both important for development

Previous studies have focused on how Btsz regulates actin indirectly through Moesin and demonstrated that expression of MBD isoforms was induced at the maternal to zygotic transition (MZT) which occurs around 2-3 hours into development (Pilot et al., 2006a; Pilot et al., 2006b). However, only a minority of the Btsz isoforms contain the Moesin Binding Domain (MBD). We found that non-MBD isoforms were also developmentally upregulated at the MZT (Figure 4A). Thus, both "classes" of Btsz isoforms (those with or without MBD) could be important during development.

To test whether non-MBD Btsz isoforms specifically contribute to Btsz function, we used CRISPR/Cas9 to engineer a *btsz* mutant (*btsz exon 4*) with an early truncation present in the majority of non-MBD isoforms (Figure 4B). This truncation is also present in the BtszI isoform that contains both exon 4 and exon 8 (which contains the MBD) (Figure 1C). We compared these to germline clones of the $btsz^{K13\text{-}4}$ mutant, which has a deletion in the MBD-containing exon 8 (Figure 4B) (Serano and Rubin, 2003). Because Btsz-RNAi has an obvious nuclear density

phenotype and live actin markers occasionally modified the furrow canal phenotypes (Spracklen et al., 2014), we analyzed the cytoskeleton network during cellularization using the non-muscle myosin II marker, Spaghetti squash::GFP (Sqh::GFP). We found that *btsz exon 4* embryos that are maternal and zygotic homozygous for the mutation exhibited noticeable nuclear fallout and abnormal cellularization compared to the wildtype. In $btsz^{K13\text{-}4}$ and *btsz exon 4* mutants, the myosin network constricted over the space where nuclei had fallen out, resembling Btsz-RNAi embryos though not as severe (Figure 4C, D). In addition, germline clones of the $btsz^{K13\text{-}4}$ mutant affected nuclear density, with the myosin network constricting over the space where nuclei had fallen out (Figure 4D, E). Taken together, our data suggest that Btsz functions in the early embryo in a manner that is not entirely dependent on the MBD-containing isoforms.

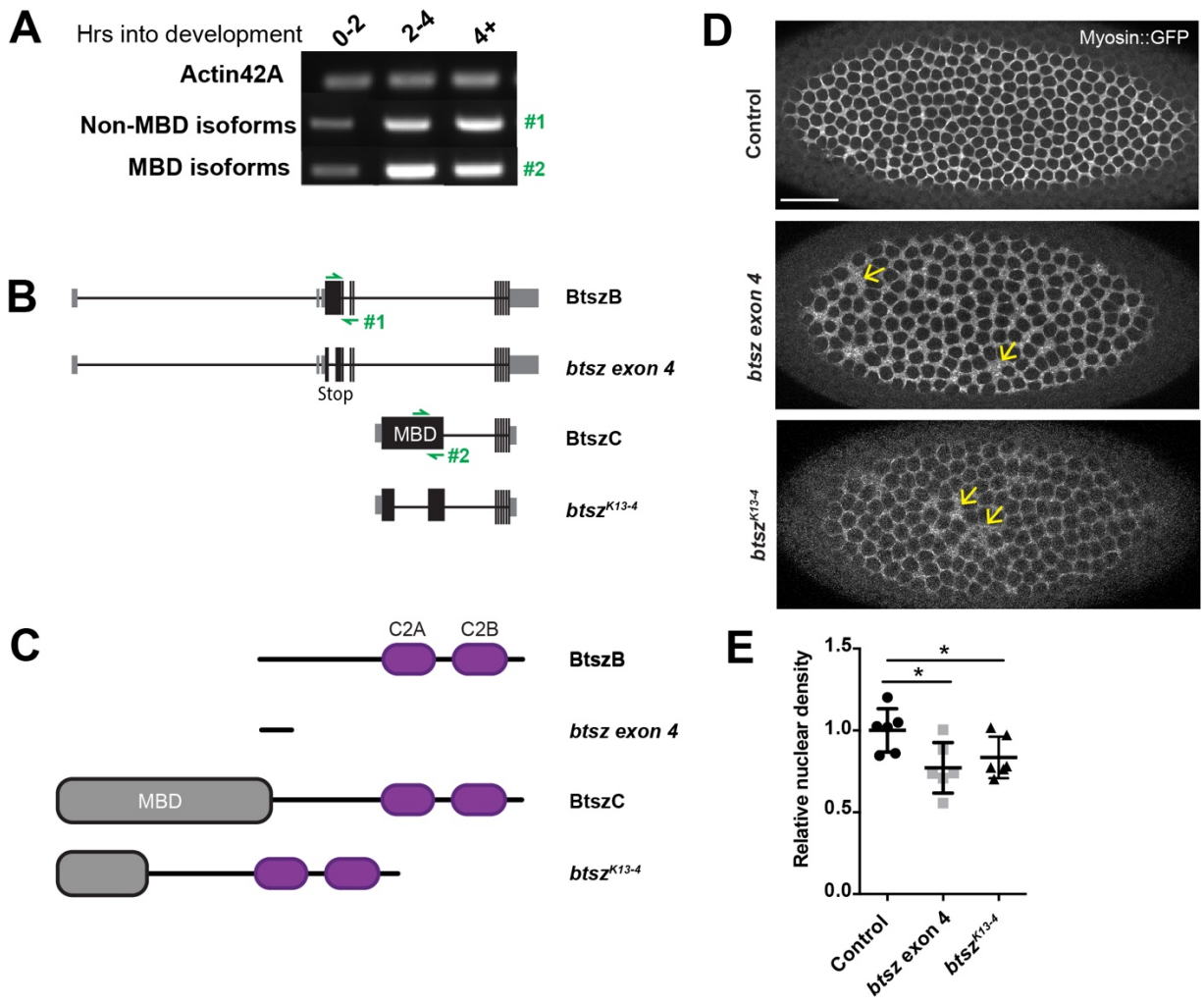


Figure 4. Non-MBD containing Btsz isoforms are important for development. **(A)** Gel of RT-PCR products measuring isoform expression over developmental time. Both non-MBD isoforms and MBD isoforms are upregulated during the maternal to zygotic transition (MZT). Primer pair #1 detected non-MBD isoforms and primer pair #2 detected MBD-containing isoforms as indicated in green. **(B)** Schematic of Btsz isoforms B and C, along with Btsz mutants *btsz exon 4 (*which was generated in this study) and *btsz*$^{K13-4}$**.** *btsz exon 4* has an early truncation in exon 4, and *btsz*$^{K13-4}$ has a deletion in exon 8. **(C)** Schematic of the protein products of the Btsz isoforms shown in (B). **(D)** Maximum projection images of cellularizing live embryos expressing Spaghetti squash::GFP (Sqh::GFP). Labeling myosin allows for visualization of the cytoskeletal network to count nuclei. Yellow arrows indicate gaps where nuclei have dropped out that have been filled in by the myosin network. Scale bar is 25 μm. **(E)** Relative nuclear density in *btsz exon 4* and *btsz*$^{K13-4}$ mutants, compared to controls. p = 0.0260 for both comparisons, Mann-Whitney U test.

BtszB binds and bundles F-actin

Given the defects in actin-related processes exhibited by Btsz-RNAi and Btsz mutants, we hypothesized that non-MBD Btsz may directly interact with actin. We examined BtszB function *in vitro* because it is the shortest isoform that contains exons 4 through 7, which are shared by the majority of Btsz isoforms, and the C2 domains which are shared by all Btsz isoforms. BtszB does not contain the MBD detailed in previous work (Pilot et al., 2006b). Full-length BtszB was prone to degradation and the N-terminal fragment of BtszB tended to pellet on its own, making F-actin cosedimentation assays with these proteins difficult to interpret. However, a purified C-terminal fragment of BtszB (BtszB-CT) demonstrated good expression and solubility. The BtszB-CT N-terminus is within exon 6 and the fragment contains the C2A and C2B domains (Figure 5A). Notably, the BtszB-CT fragment is present in the majority of Btsz isoforms.

We performed high-speed pelleting assays to test whether Btsz-CT binds to actin filaments and low-speed pelleting assays to test whether Btsz-CT bundles actin filaments. In high-speed pelleting assays, a fixed concentration of BtszB-CT was mixed with variable concentrations of F-actin, and then the amount of BtszB-CT bound to F-actin was determined by analyzing the supernatant and pellet fractions on gels. With increasing concentrations of F-actin, BtszB-CT shifted from the supernatant to the pellet (Figure 5B). The data best fit a cooperative binding model with a binding affinity (K_d) of 0.45 μM and a Hill coefficient (h) of 2.9 (Figure 5C). In low-speed pelleting assays, F-actin alone did not pellet significantly, but the addition of BtszB-CT shifted F-actin to the pellet (Figure 5D and E), suggesting that BtszB-CT organizes F-actin into higher order structures. These effects were confirmed independently through direct visualization in

TIRF microscopy assays, where BtszB-CT organized F-actin into bundles with increased fluorescence and thickness (Figure 5F and G). Overall, these results show that BtszB-CT binds directly and cooperatively to F-actin (K_d = 0.45 μM) and organizes actin filaments into bundles, suggesting that all Btsz isoforms may bind and bundle F-actin.

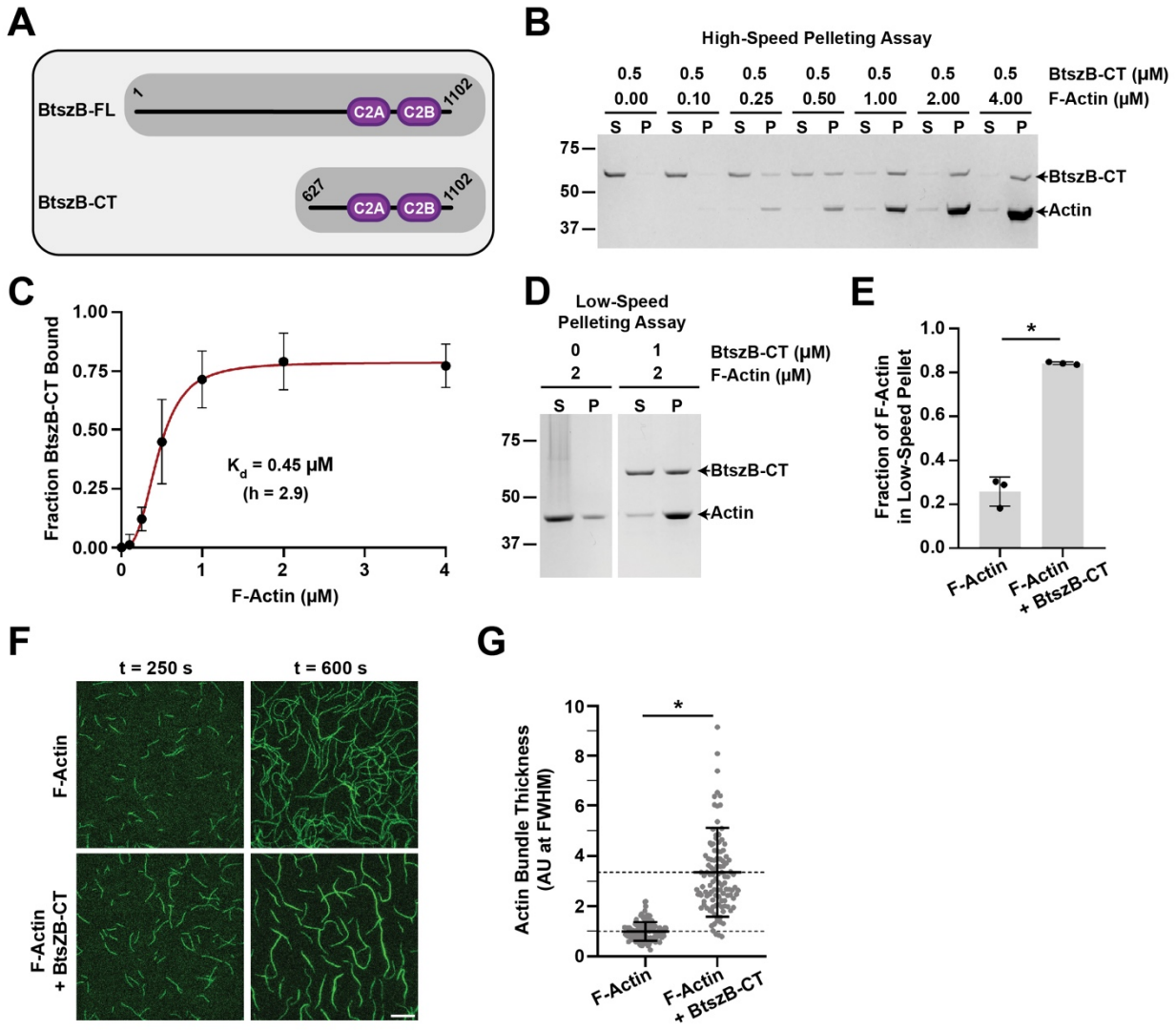


Figure 5. The C-terminus of BtszB cooperatively binds and bundles actin filaments. (A) Schematic of full length BtszB (BtszB-FL) for reference and the C-terminal fragment (BtszB-CT) that was used in the F-actin binding and bundling assays. **(B)** High-speed F-actin co-sedimentation assay varying the F-actin concentration (0.1 - 4 μM) in the presence of 0.5 μM BtszB-CT. F-actin and BtszB-CT samples were incubated together at room temperature for 30 min and then centrifuged (20 min at 25°C, 316k x g). **(C)** The fraction of BtszB-CT bound to actin filaments at increasing concentrations of F-actin was fit to a cooperative binding model with a K_d of 0.45 μM and a hill coefficient (h) of 2.9. Error bars are SD, n = 3. **(D)** Low-speed F-actin co-sedimentation assay using 2 μM F-actin with and without 1 μM BtszB-CT. F-actin and BtszB-CT were mixed together and incubated at room temperature for 30 min, then centrifuged (10 min at room temperature, 16k x g). **(E)** The fraction of F-actin pelleted with and without BtszB-CT. Error bars are SD, n = 3. p-value < 0.001, Student's t-test. **(F)** Representative time-lapse images from TIRF microscopy reactions containing 2 μM G-actin (10% Alexa 488-labeled) polymerized into filaments

10-20 μm in length, and then buffer or 1 μM BtszB-CT was flowed into the reaction chamber at 300 s and monitored for an additional 300 s. Scale bar is 20 μm. **(G)** Filament/bundle thickness was assessed by measuring the fluorescence intensity at FWHM (full width at half maximum) from line segments drawn perpendicular to the filament or bundle. Fluorescence intensity values were normalized to the mean intensity value of the control reaction (2 μM F-actin). Horizontal dashed lines are at y = 1, mean 2 μM F-actin control reaction, and y = 3.4, mean 2 μM F-actin + 1 μM BtszB-CT. ($n_{experiments}$ = 3, $n_{line\ segment}$ measurements for 2 μM F-actin = 41, 40, 42 and 1 μM BtszB-CT = 24, 35, 57. Error bars are SD, * is p-value < 0.001, Mann-Whitney U test).

Btsz localizes to apical junctions and pseudo-cleavage furrows

Next, we wanted to determine whether Btsz localizes to pseudo-cleavage furrows where actin binding and bundling are important for syncytial development. BtszF is one of the shortest Btsz isoforms (Figure 1C), containing only the C2 domains that are necessary for proper localization and one additional exon. We observed that BtszF localized to these furrows but did not completely co-localize with F-actin (Figure 6A). Given that past work indicates that the C2 domains are responsible for Btsz localization patterns (Pilot et al., 2006b), our data suggest that other Btsz isoforms localize similarly to BtszF in the syncytium.

To further explore the role of Btsz in actin binding and bundling *in vivo*, we examined actin organization in Btsz RNAi conditions. Btsz is upregulated during the MZT, similar to a subset of proteins that regulate F-actin levels in furrow canals, a distinct compartment that forms at the tip of cellularization furrows (Krueger et al., 2019; Schejter and Wieschaus, 1993; Sokac and Wieschaus, 2008; Zheng et al., 2013). Therefore, we wondered whether Btsz could be affecting F-actin at cellularization. We measured the ratio of actin intensity in the apical region of the

furrow to that in the furrow canals and saw no significant difference in Btsz-RNAi embryos compared to control (Figure 6B, C).

To determine whether non-MBD isoforms localize to a compartment that is distinct from furrow canals, we generated a fly line expressing BtszB N-terminally tagged with mCherry. Fixed imaging revealed largely apical membrane localization during cellularization and gastrulation (Figure 6D). This result aligns with previous work that described the MBD-containing Btsz isoforms' localization and role in regulating apical cell junctions (Pilot et al., 2006b). Unlike other actin cross-linkers involved in cellularization, BtszB did not localize to furrow canals, suggesting that the cellularization phenotypes we observed in Btsz mutants and Btsz RNAi (Figure 4D) were the result of prior syncytial division defects. We propose that Btsz binds the membrane through its C2 domains where Btsz organizes actin along the membrane (Figure 6E).

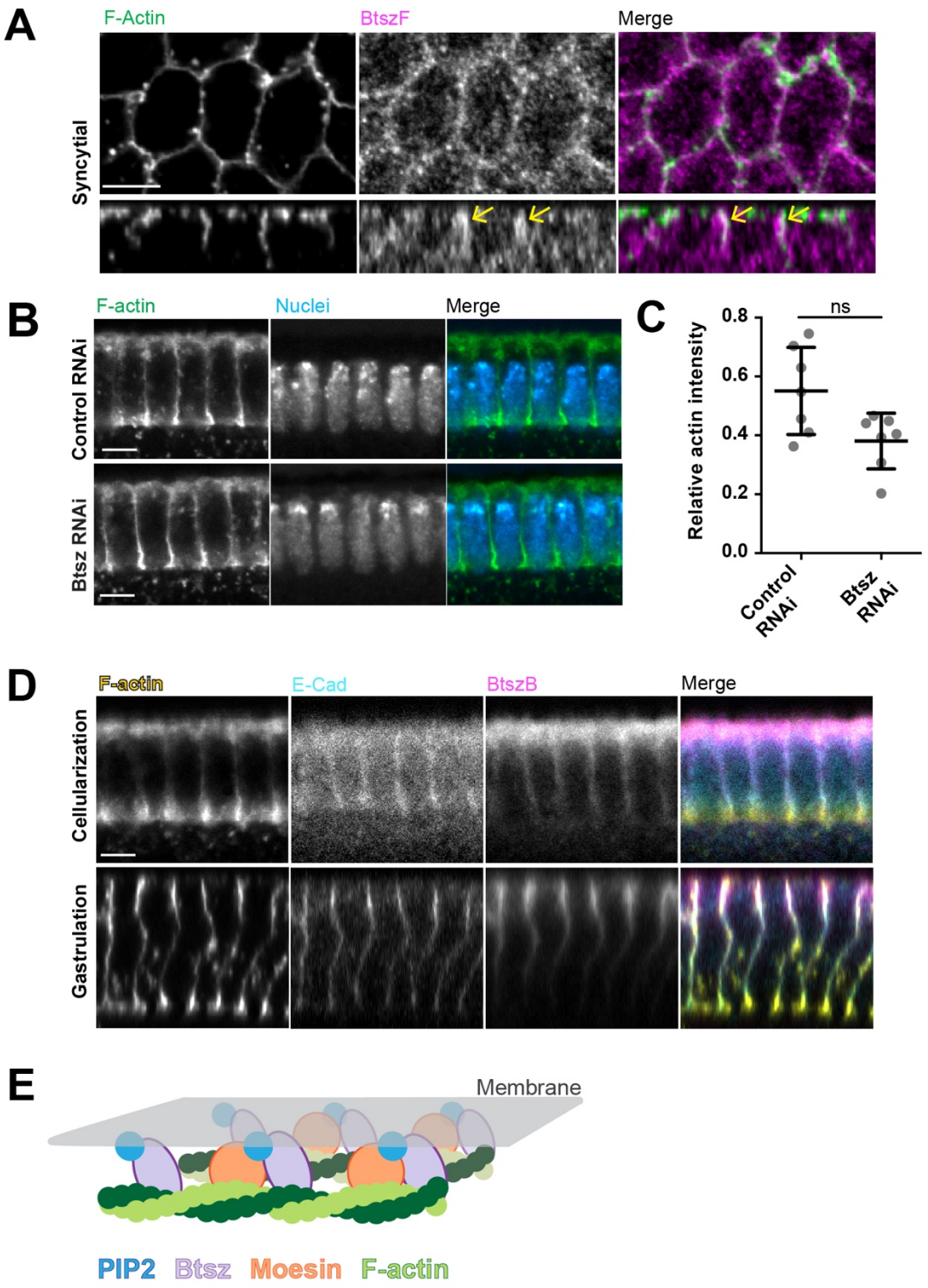


Figure 6. Btsz localizes to pseudo-cleavage furrows and BtszB localizes to apical junctions (A) Maximum projection of a top-down view of the F-actin network and Btsz isoform F localization in the syncytium. BtszF localizes to pseudo-cleavage

furrows (yellow arrows). Scale bars are 5 μm. **(B)** Cross section of fixed control RNAi or Btsz RNAi embryos during cellularization. F-actin was visualized using Phalloidin and nuclei was visualized using Hoechst. **(C)** There is no significant difference between the relative F-actin intensity (ratio of actin intensity in the apical region to that of the furrow canals) in the wildtype and Btsz-RNAi embryos. Mann-Whitney U test. **(D)** Cross sections of fixed control embryos, and embryos overexpressing mCherry::BtszB under the UASt promoter during cellularization (top) or gastrulation (bottom). F-actin (Phalloidin) in yellow, E-cadherin or junctions (α-Ecad) in cyan, and BtszB (α-RFP) in magenta. BtszB localizes apically during cellularization and gastrulation. **(E)** Schematic of how Btsz non-MBD and MBD isoforms may interact with Moesin and/or F-actin at the membrane to bundle and organize the cytoskeleton.

Discussion

Our data show that the Synaptotagmin-like protein Btsz can directly regulate actin networks in addition to functioning through Moesin. We found that depletion of either MBD or non-MBD isoforms resulted in syncytial embryo defects similar to those caused by other mutants and perturbations that affect actin organization. In addition, we showed that a Btsz C-terminal fragment can cooperatively bind to F-actin and promote F-actin bundling. This supports a model in which Btsz binds the membrane through its C2 domains and promotes actin bundling along the membrane.

Actin network organization must be tightly regulated for proper syncytial blastoderm development. This precise regulation is orchestrated by a large group of actin and membrane remodeling proteins to ensure proper nuclear divisions. Our results reveal a novel role for Btsz in organizing actin in the syncytial *Drosophila* embryo. In Btsz-RNAi embryos, actin caps were smaller and pseudo-cleavage furrows were often defective, leading to spindle collisions and nuclear fallout, which resembled phenotypes from knocking down other known actin regulators (Afshar et

al., 2000; Stevenson et al., 2002; Zallen et al., 2002). In *btsz exon 4* and *btsz*$^{K13\text{-}4}$ mutants, we saw evidence of nuclear fallout, similar to, but less severe than the Btsz-RNAi defects, suggesting that both sets of isoforms contribute to Btsz function. Taken together, the data suggest that Btsz can promote higher-order F-actin network structure by bundling F-actin, and by connecting these networks to membrane.

Btsz regulates actin organization at multiple stages of early Drosophila embryo development

Our *in vitro* data indicate that the BtszB isoform binds and bundles F-actin, shedding light on how Btsz may regulate actin *in vivo* in a complementary manner to previously published work (Jayanandanan et al., 2014; Pilot et al., 2006b; Zhang et al., 2018). While some Btsz isoforms bind Moesin, our data predict that most or all isoforms bind and bundle F-actin. In the related Slac2 protein, Melanophilin, the actin binding domain (ABD) is also at the C-terminus (Kuroda et al., 2003). Btsz is therefore another Synaptotagmin-related protein that can directly interact with actin.

In the syncytium, Btsz could promote actin filament bundles generated by the formin Diaphanous (Jiang and Harris, 2019). BtszF contains the C2 domains and localizes to syncytial pseudo-cleavage furrows. Because all Btsz isoforms contain the C2 domains responsible for membrane localization, it is possible that all isoforms localize the same way. Btsz bundling may stabilize actin at the pseudo-cleavage furrows and prevent their recession, as furrows in Btsz-depleted embryos often break and retract to the surface. Because each round of nuclear division occurs

rapidly (within ~8-10 minutes), pseudo-cleavage furrow growth requires dynamic membrane and actin remodeling. During oogenesis, the *Drosophila* Fascin homolog, Singed, is required for actin bundling when rapid actin polymerization takes place to provide structural integrity for these bundles (Cant et al., 1994). Btsz may function in a similar way, stabilizing F-actin bundles against disassembly during a time of rapid actin remodeling.

Actin bundles are also a key component of adherens junctions (AJs), which are critical for cell-cell adhesion. The dynamic coupling of the F-actin network to adherens junctions is necessary for propagating forces across cells (Gorfinkiel and Arias, 2007; Jodoin, 2015; Maître et al., 2012; Martin et al., 2010; Roh-Johnson et al., 2012). E-cadherin (E-cad) is an important component of AJs, and its localization is stabilized by F-actin. When Btsz is disrupted, so is actin organization, which in turn destabilizes E-cad, leading to defects in tissue integrity (Pilot et al., 2006b). As our data show that BtszB is present at apical junctions (Figure 6D), we hypothesize that Btsz binds and bundles F-actin to organize actin at these junctions (Figure 6E).

Multiple Btsz isoforms exhibit upregulated gene expression at the maternal-zygotic transition

Precise gene expression of actin regulators is essential for proper morphogenesis to ensure that actin networks are assembled and organized at the correct time. The MZT is a conserved process where the developing embryo transitions from being transcriptionally quiet to active (Lee et al., 2014). The expression of many actin remodeling proteins necessary for cellularization, such as Bottleneck, Nullo, Slam,

Dunk, and Serendipity-α (Sry-α), are developmentally upregulated at the MZT (He et al., 2016; Krueger et al., 2019; Lecuit et al., 2002; Postner and Wieschaus, 1994; Schejter and Wieschaus, 1993; Simpson and Wieschaus, 1990; Sokac and Wieschaus, 2008; Wenzl et al., 2010; Zheng et al., 2013). If the MZT is disrupted in *Drosophila*, cellularization does not proceed. Our work reveals that Btsz is one of this group of developmentally expressed F-actin binding/bundling proteins. However, Btsz is present uniquely in an apical-lateral compartment distinct from the other actin-remodeling proteins mentioned above, which each localize to furrow canals. In a *nullo* or *sry-α* mutant background, F-actin levels are reduced in these furrow canals. While Btsz does not appear to affect F-actin levels at the apical domain or in furrow canals (Figure 6B, C), it is possible that Btsz localizes apically during cellularization when adherens junctions form - in preparation for their role in maintaining junctions during gastrulation. Alternatively, BtszB may play an undescribed role in the apical or lateral domains of cellularization furrows.

Possible functions for Btsz in membrane trafficking

An important question that remains unanswered is whether Btsz plays a role in *Drosophila* regulating membrane trafficking like mammalian Slps. Our work here focused primarily on furthering our understanding of Btsz's role in actin organization. For instance, Nuclear fallout (Nuf) is required for actin and membrane remodeling during the syncytial divisions through its role in regulating trafficking (Riggs et al., 2003). In *nuf* mutants, pseudo-cleavage furrows are defective leading to spindle collisions and nuclear fallout (Rothwell et al., 1998). Considering the close coupling

between actin and membrane remodeling, as well as the spindle collisions and nuclear fallout observed upon Btsz depletion, we cannot rule out that the phenotypes we observe in Btsz-RNAi or *btsz* mutant embryos are in part due to defects in vesicle trafficking.

Materials and Methods

Fly stocks and genetics

Fly stocks and crosses used in this study are listed in Supplementary File 1. For crosses involving maternal gene depletion and overexpression, flies expressing maternal Gal4 drivers were crossed to UAS-driven constructs and maintained at 25°C. Nonbalancer F1 females were crossed to nonbalancer F1 males, and F2 embryos were used for imaging.

For the *btsz exon 4* mutant, a deletion was generated at the endogenous Btsz locus using CRISPR-Cas9 as previously described (Gratz et al., 2015). Two 15 base pair (bp) gRNAs targeting sites in Btsz exon 4, roughly 500 bps apart, were identified using flyrnai.org/crispr and cloned into the pCFD5 plasmid. This vector was constructed and packaged by VectorBuilder and the vector ID is VB200707-1018qzm, which can be used to retrieve further information about the vector on vectorbuilder.com. The plasmid was injected into nanos-Cas9-expressing embryos by BestGene Inc (Chino Hills, California). Surviving adults were crossed to Dr/TM3 flies and the deletion was screened for using PCR. Flies with successfully a generated deletion were sequenced for further analysis. For crosses involving *btsz exon 4*, crosses were set up using two *btsz exon 4* lines of independent CRISPR alleles.

For crosses involving *btsz*$^{K13-4}$, the FLP-DFS technique was used to generate mutant germline clones (Chou and Perrimon, 1992). We used *btszK13-4* alone and sqh::GFP; *btsz*$^{K13-4}$ mutant stocks. Briefly, hsFLP;; Dr/TM3, Sb females were crossed to FRT82B ovoD1/TM3, Sb males. hsFLP/Y;; FRT 82B ovoD1/Dr males were crossed to *btsz*$^{K13-4}$ FRT/TM3, Sb or sqh::GFP; *btsz*$^{K13-4}$ FRT/TM3, Sb females. Larvae from this cross were heat shocked for 2 hrs at 37°C for 3-4 days. hsFLP; + or sqh::GFP*; btsz*$^{K13-4}$ FRT/*ovoD* FRT females were crossed to OregonR males to collect embryos resulting from the *btsz*$^{K13-4}$ germline clones.

The pUASt-mCherry::BtszB fly line was constructed using the coding sequence of BtszB (generous gift from Thomas Lecuit, Collège de France). The vector used to overexpress mCherry::Btsz in our study was constructed and packaged by VectorBuilder (Chicago, Illinois). The vector ID is VB211103-1199vfw. This vector was injected into attP40 flies and surviving adults were crossed to Sp/CyO flies. Presence of the vector was screened for by presence of red eye color and confirmed with subsequent PCR and sequencing.

Live and fixed imaging

Crosses were maintained at 25°C unless otherwise stated. Embryos were collected from apple-juice agar plates and embryos were staged using Halocarbon 27 oil. Embryos were then dechorionated in 50% bleach (Chlorox) for 2 minutes and rinsed twice with water.

For live imaging, embryos were mounted on a slide with embryo glue (Scotch tape dissolved in heptane). No. 1.5 coverslips were placed on the slide on either side of

the mounted embryos and a No.1 coverslip was used to cover the embryos and create a chamber. This chamber was filled using Halocarbon 27 oil.

For fixed imaging involving Phalloidin staining, embryos were fixed using 4% paraformaldehyde (Electron Microscopy Sciences) in 0.1M phosphate buffer at pH 7.4 with 50% heptane (Alfa Aesar) and manually devitellinated. Embryos were washed in 0.01% Triton X-100 in PBS (PBS-T), incubated in primary antibodies followed by secondary antibodies (Supplementary File 1), and mounted onto a glass slide using AquaPolymount (Polysciences).

Live and fixed images were taken using a Zeiss LSM 710 point scanning confocal microscope with a 40x/1.2 C-Apochromat water objective lens or 63x/1.4 Plan-Apochromat oil objective lens, an argon-ion, 405 nm diode, 594 nm HeNe, 633 HeNe laser, and Zen software. Pinhole settings ranged from 1 to 2.5 Airy units. For two-color live imaging, band-pass filters were set at ~410-513 nm for Hoechst, ~490–565 nm for GFP, and ~590–690 nm for mCherry (mCh). For three-color imaging, band-pass filters were set at ~480–560 nm for Alexa Fluor 488, ~580–635 nm for Alexa Fluor 568, and ~660–750 nm for Alexa Fluor 647.

Image processing and analysis

Figure images were processed in FIJI or Matlab R2019b. For actin cap area and nuclear fallout quantifications, images were run through a segmentation analysis (detailed below) in Matlab. For all figures, brightness and contrast were adjusted linearly. For Figures 2, 3, and 5, images were processed in FIJI with a Gaussian blur of 0.5. For Figure 6, images were processed in FIJI with a Gaussian blur of 0.5 and using Subtract Background with a rolling ball radius of 50.0 pixels.

Actin cap and cellularization furrow quantifications

Actin cap area and nuclear fallout

Embryos expressing mCherry::Moe were used to visualize actin caps. To stage-match embryos, we scored the size and number of actin caps. We measured actin cap area during NC12 past the expansion phase of cap growth to minimize gaps in the actin network. This was roughly 4 minutes before pseudo-cleavage furrows reach their maximum depth during NC12. A maximum intensity projection (MIP) at this time point was then used in a segmentation pipeline in MATLAB detailed below and manual corrections were made in FIJI. To count nuclei, MIPs of embryos expressing Sqh::GFP were segmented using the same Matlab procedure and FIJI was used to measure embryo area in the given field of view and to count nuclei.

1. The intensity of each pixel was scaled up by a factor of 1.25.
2. Ridge-like structures in the image were enhanced with FrangiFilter2D using the default parameters (Dirk-Jan Kroon (2022). Hessian based Frangi Vesselness filter (https://www.mathworks.com/matlabcentral/fileexchange/24409-hessian-based-frangi-vesselness-filter), MATLAB Central File Exchange); based on (Frangi et al., 1998).
3. The resulting image was blurred via a Gaussian blur of sigma 4-10 depending on the image using Matlab's imgaussfilt() function.
4. Seeds for a watershed segmentation were automatically determined using the imregionalmin() function on the blurred, background-subtracted image.
5. The ridge-enhanced image from step 2 was blurred with a median filter of size 11 pixels, then the seeds were imposed as local minima on this image.

6. The watershed() function was used on this image to generate lines corresponding to the boundaries between actin caps.

Furrow actin intensity

Cellularizing embryos were fixed and imaged to calculate the ratio of actin intensity in the apical to basal regions in the furrows. FIJI was used to measure intensity. A ratio of intensity was taken of the most apical and basal 2 μm of the furrows. Intensities were normalized by rescaling values between 0 and 1 using $(I-I_{min})/(I_{max}-I_{min})$.

Molecular biology

For RNA extraction, thirty embryos were collected for each stage analyzed and ground up in TRIzol reagent (Life Technologies, Inc.) using a motorized pestle mixer. mRNA was extracted using TRIzol and chloroform. The aqueous phase was transferred into a fresh tube and extraction continued using the Monarch RNA Cleanup Kit (NEB). The extracted mRNA was treated with TURBO DNA-*free*™ (Invitrogen) and cDNA was produced using Invitrogen SuperScript™ III First-Strand Synthesis System. Subsequent PCR reactions were run with primers listed in (Supplementary File 1).

Purification of BtszB

Vectors of full-length BtszB and BtszB fragments were generated for protein expression and purification (ABclonal Science Inc.). Full-length Btsz isoform B was insoluble in bacteria and so we used the baculovirus system to express Btsz-B in Sf9 cells and purified protein from those (ABclonal Science Inc).

Purification and Labeling of Actin

Rabbit skeletal muscle actin was purified from acetone powder (Spudich and Watt, 1971) generated from frozen ground hind leg muscle tissue of young rabbits (PelFreez). Lyophilized acetone powder stored at -80°C was mechanically sheared in a coffee grinder, resuspended in G-buffer (5 mM Tris-HCl [pH 7.5], 0.5 mM Dithiothreitol [DTT], 0.2 mM ATP, 0.1 mM $CaCl_2$), and then cleared by centrifugation for 20 min at 50,000 × g. The supernatant was filtered through Grade 1 Whatman paper, then the actin was polymerized by the addition of 2 mM $MgCl_2$ and 50 mM NaCl and incubated overnight at 4°C. The final NaCl concentration was adjusted to 0.6 M to strip residual actin-binding proteins, incubated at 4°C for 1 h, and then the F-actin was pelleted by centrifugation for 150 min at 361,000 × g. The pellet was solubilized by dounce homogenization and dialyzed against 1 L of G-buffer at 4°C (three consecutive times at 12–18 h intervals). Monomeric actin was then precleared at 435,000 × g and loaded onto a S200 (16/60) gel-filtration column (GE Healthcare) equilibrated in G-Buffer. Peak fractions containing actin were stored at 4°C.

To fluorescently label actin, G-actin was polymerized by dialyzing overnight against modified F-buffer (20 mM PIPES [pH 6.9], 0.2 mM $CaCl_2$, 0.2mM ATP, 100 mM KCl) (Shekhar, 2017). F-actin was incubated for 2 h at room temperature with Alexa 488 NHS ester dye (Life Technologies) at a final molar concentration 5 times in excess of actin concentration. F-actin was then pelleted by centrifugation at 450,000 × g for 40 min at 4°C. The pellet was homogenized with a dounce and dialyzed overnight at 4°C against 1 L of G-buffer (three consecutive times at 12–18 h intervals). Next, the solution was centrifuged at 450,000 × g for 40 min at 4°C. The supernatant was collected and

loaded onto a S200 (16/60) gel-filtration column (GE Healthcare) equilibrated in G-Buffer. Peak fractions containing labeled G-actin were pooled and the concentration and labeling efficiency was determined by measuring the absorbance at 290 nm and 494 nm. Molar extinction coefficients used were as follows: ε_{290} actin = 26,600 M^{-1} cm^{-1} and ε_{494} Alexa 488 = 71,000 M^{-1} cm^{-1}. The labeled G-actin was stored in G-Buffer + 50% glycerol at -20°C and then dialyzed back into G-Buffer (no glycerol) for experiments.

High-Speed and Low-Speed Pelleting (Co-Sedimentation) Assays

F-actin was prepared by polymerizing 20 μM of G-actin with initiation mix (final concentration: 2 mM $MgCl_2$, 0.5 mM ATP, 50 mM KCl) overnight at room temperature. For high-speed pelleting assays, different concentrations of F-actin (0, 0.1, 0.25, 0.5, 1, 2, 4 μM) were incubated with 0.5 μM BtszB-CT for 30 min at room temperature. Reactions were then centrifuged at 316,000 × g for 20 min at 25°C using a tabletop Optima TLX Ultracentrifuge (Beckman Coulter). Supernatant and pellet fractions were analyzed on Coomassie stained gels, and quantified by scanning densitometry using a ChemiDoc Imaging System (BioRad). In calculating the fraction of BtszB-CT bound to F-actin in each reaction, the BtszB-CT that pelleted nonspecifically (without F-actin) was subtracted. Fraction of BtszB-CT bound at different concentrations of F-actin was plotted, and fit to a Hill Equation using Prism (GraphPad). For low-speed pelleting assays, 2 μM of F-actin was incubated with or without 1 μM BtszB-CT for 30 min at room temperature. Reactions were then centrifuged at 16,000 × g for 10 min at room temperature using a tabletop centrifuge (Eppendorf). Supernatant and pellet fractions were analyzed on Coomassie stained gels and quantified as above. Fraction of F-actin

pelleted +/- BtszB-CT was plotted and compared statistically using a Mann-Whitney U test from Prism (GraphPad).

F-Actin Bundling TIRF Microscopy Assays and Image Analysis

Glass coverslips (60 × 24 mm; Thermo Fisher Scientific) were first cleaned by sonication in 1% Hellmanex™ III (Sigma-Aldrich) glass cleaner for 60 min at room temperature, followed by successive sonications in 100% ethanol for 60 min and 1 M KOH for 20 min. Coverslips were then washed extensively with H_2O and dried in an N_2 stream. The cleaned coverslips were coated with 2 mg/ml methoxy-polyethylene glycol (PEG)-silane MW 2000 and 2 µg/ml biotin-PEG-silane MW 3400 (Laysan Bio, Arab, AL) in 80% ethanol, pH 2.0, and incubated overnight at 70°C. Flow cells were assembled by rinsing PEG-coated coverslips with water, drying with N_2, and adhering to µ-Slide VI0.1 (0.1 × 17 × 1 mm) flow chambers (Ibidi, Fitchburg, WI) with double-sided tape (2.5 cm × 2 mm × 120 µm) and 5-min epoxy resin (Devcon, Danvers, MA). Before each reaction, the flow cell was incubated for 1 min with 1% bovine serum albumin (BSA) in HEKG5 buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 50 mM KCl, and 5% glycerol), and then equilibrated with TIRF buffer (10 mM imidazole, pH 7.4, 50 mM KCl, 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.2 mM ATP, 10 mM DTT, 15 mM glucose, 20 µg/ml catalase, 100 µg/ml glucose oxidase) plus 0.5% methylcellulose (4000 cP). Finally, 2 µM G-actin (10% Alexa 488–labeled on Cys 374) polymerized to 10 - 20 µm, washed with TIRF buffer and then 1 µM BtszB-CT was flowed into the reaction chamber 300 s after initiation of actin assembly. Upon flowing wash TIRF buffer and BtszB-CT into the TIRF chamber, filament often would move

around but did not get flushed out because of the crowding agent (methylcellulose) in the buffer.

Single-wavelength time-lapse TIRF imaging was performed on a Nikon-Ti2000 inverted microscope equipped with a 150-mW argon laser (Melles Griot), a 60 × TIRF objective with a numerical aperture of 1.49 (Nikon Instruments), and an electron-multiplying charge coupled device (EMCCD) camera (Andor Ixon, Belfast, Ireland). One pixel was equivalent to 143 × 143 nm. Focus was maintained by the Perfect Focus system (Nikon Instruments). TIRF microscopy images were acquired every 5 s and exposed for 100 ms for at least 10 min using imaging software Elements (Nikon Instruments, New York, NY).

Images were analyzed in FIJI version 2.0.0-rc-68/1.52e (National Institutes of Health, Bethesda, MD). Background subtraction was conducted using the rolling ball background subtraction algorithm (ball radius, 5 pixels). To measure actin filament bundling, the line segment tool was used to draw a line perpendicular to actin filaments/bundles in the FOV. The intensity profile of the line segment was fit to a 2-dimensional Gaussian curve in FIJI. The intensity at full-width half-max (FWHM) for each line trace was recorded at 300 s into the reactions (where BtszB-CT was flowed in at time 0). All individual intensity measurements were normalized to the average actin filament/bundle intensity from the control reaction (2 μM F-actin). A Mann-Whitney U statistical test was performed to determine bundling activity between the control reaction (2 μM F-actin) and the 1 μM BtszB-CT reaction.

Statistical analysis

All statistical analyses were carried out using GraphPad Prism or MATLAB.

Acknowledgments

We thank members of the Martin lab for helpful discussions and comments on the manuscript. We thank Jonathan Jackson for writing the segmentation pipeline code. We thank J. Solitro, Z. Li, and J. Eskin for their assistance with experiments. We are grateful to Goode lab members for assistance with actin purification and labeling. Finally, we thank the Bloomington Stock Center and the TRiP at Harvard Medical School (National Institutes of Health/National Institute of General Medical Sciences R01-GM084947) for providing fly stocks used in this study. This research was supported by a grant from the National Institutes of Health to B.L. Goode (R35 GM134895). This work is also supported by National Institutes of Health grants R01 GM105984 and R35 GM144115 to A.C. Martin.

References

Afshar, K., B. Stuart, and S.A. Wasserman. 2000. Functional analysis of the *Drosophila* diaphanous FH protein in early embryonic development. *Development*. 127:1887-1897.

Algrain, M., O. Turunen, A. Vaheri, D. Louvard, and M. Arpin. 1993. Ezrin contains cytoskeleton and membrane binding domains accounting for its proposed role as a membrane-cytoskeletal linker. *Journal of Cell Biology*. 120:129-139.

Callaini, G., R. Dallai, and M.G. Riparbelli. 1992. Cytochalasin induces spindle fusion in the syncytial blastoderm of the early *Drosophila* embryo. *Biology of the Cell*. 74:249-254.

Camuglia, J.M., A.R.A. Yeh, and A.C. Martin. 2021. Extracellular | Tension and Tissue Morphogenesis. *In* Encyclopedia of Biological Chemistry III (Third Edition). J. Jez, editor. Elsevier, Oxford. 317-325.

Cant, K., B.A. Knowles, M.S. Mooseker, and L. Cooley. 1994. *Drosophila* singed, a fascin homolog, is required for actin bundle formation during oogenesis and bristle extension. *Journal of Cell Biology*. 125:369-380.

Cao, J., R. Albertson, B. Riggs, C.M. Field, and W. Sullivan. 2008. Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. *Journal of Cell Biology*. 182:301-313.

Chou, T.B., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila. Genetics*. 131:643-653.

Chugh, P., and E.K. Paluch. 2018. The actin cortex at a glance. *Journal of Cell Science*. 131:jcs186254.

Davidson, A.J., T.H. Millard, I.R. Evans, and W. Wood. 2019. Ena orchestrates remodelling within the actin cytoskeleton to drive robust *Drosophila* macrophage chemotaxis. *Journal of Cell Science*. 132:jcs224618.

Foe, V.E., C.M. Field, and G.M. Odell. 2000. Microtubules and mitotic cycle phase modulate spatiotemporal distributions of F-actin and myosin II in *Drosophila* syncytial blastoderm embryos. *Development*. 127:1767-1787.

Frangi, A.F., W.J. Niessen, K.L. Vincken, and M.A. Viergever. 1998. Multiscale vessel enhancement filtering. Springer Berlin Heidelberg. 130-137.

Fukuda, M. 2002. The C2A domain of synaptotagmin-like protein 3 (Slp3) is an atypical calcium-dependent phospholipid-binding machine: comparison with the C2A domain of synaptotagmin I. *Biochem J*. 366:681-687.

Fukuda, M., T.S. Kuroda, and K. Mikoshiba. 2002. Slac2-a/Melanophilin, the Missing Link between Rab27 and Myosin Va. *Journal of Biological Chemistry*. 277:12432-12436.

Galvez-Santisteban, M., A.E. Rodriguez-Fraticelli, D.M. Bryant, S. Vergarajauregui, T. Yasuda, I. Banon-Rodriguez, I. Bernascone, A. Datta, N. Spivak, K. Young, C.L. Slim, P.R. Brakeman, M. Fukuda, K.E. Mostov, and F. Martin-Belmonte. 2012. Synaptotagmin-like proteins control the formation of a single apical membrane domain in epithelial cells. *Nat Cell Biol*. 14:838-849.

Gary, R., and A. Bretscher. 1995. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Molecular Biology of the Cell*. 6:1061-1075.
Gorfinkiel, N., and A.M. Arias. 2007. Requirements for adherens junction components in the interaction between epithelial tissues during dorsal closure in *Drosophila*. *Journal of Cell Science*. 120:3289-3298.
Gould, K.L., A. Bretscher, F.S. Esch, and T. Hunter. 1989. cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. *The EMBO Journal*. 8:4133-4142.
Gratz, S.J., C.D. Rubinstein, M.M. Harrison, J. Wildonger, and K.M. O'Connor-Giles. 2015. CRISPR-Cas9 Genome Editing in *Drosophila*. *Current Protocols in Molecular Biology*. 111.
Grevengoed, E.E., D.T. Fox, J. Gates, and M. Peifer. 2003. Balancing different types of actin polymerization at distinct sites. *Journal of Cell Biology*. 163:1267-1279.
He, B., A. Martin, and E. Wieschaus. 2016. Flow-dependent myosin recruitment during *Drosophila* cellularization requires zygotic dunk activity. *Development*. 143:2417-2430.
Holly, R.M., L.M. Mavor, Z. Zuo, and J.T. Blankenship. 2015. A rapid, membrane-dependent pathway directs furrow formation through RalA in the early *Drosophila* embryo. *Development*. 142:2316-2328.
Jayanandanan, N., R. Mathew, and M. Leptin. 2014. Guidance of subcellular tubulogenesis by actin under the control of a synaptotagmin-like protein and Moesin. *Nature Communications*. 5.
Jiang, T., and T.J.C. Harris. 2019. Par-1 controls the composition and growth of cortical actin caps during *Drosophila* embryo cleavage. *Journal of Cell Biology*. 218:4195-4214.
Jodoin, J.N., Coravos, J.S., Chanet, S., Vasquez, C.G., Tworoger, M., Kingston, E.R., Perkins, L.A., Perrimon, N., Martin, A.C. 2015. Stable Force Balance between Epithelial Cells Arises from F-Actin Turnover. *Developmental Cell*. 35:685-697.
Koenderink, G.H., and E.K. Paluch. 2018. Architecture shapes contractility in actomyosin networks. *Current Opinion in Cell Biology*. 50:79-85.
Krueger, D., T. Quinkler, S.A. Mortensen, C. Sachse, and S. De Renzis. 2019. Cross-linker–mediated regulation of actin network organization controls tissue morphogenesis. *Journal of Cell Biology*. 218:2743-2761.
Kuroda, T.S., H. Ariga, and M. Fukuda. 2003. The Actin-Binding Domain of Slac2-a/Melanophilin Is Required for Melanosome Distribution in Melanocytes. *Molecular and Cellular Biology*. 23:5245-5255.
Lecuit, T., R. Samanta, and E. Wieschaus. 2002. slam Encodes a Developmental Regulator of Polarized Membrane Growth during Cleavage of the *Drosophila* Embryo. *Developmental Cell*. 2:425-436.
Lecuit, T., and A.S. Yap. 2015. E-cadherin junctions as active mechanical integrators in tissue dynamics. *Nature Cell Biology*. 17:533-539.
Lee, M.T., A.R. Bonneau, and A.J. Giraldez. 2014. Zygotic Genome Activation During the Maternal-to-Zygotic Transition. *Annual Review of Cell and Developmental Biology*. 30:581-613.

Lyakhova, T.A., and J.D. Knight. 2014. The C2 domains of granuphilin are high-affinity sensors for plasma membrane lipids. *Chemistry and Physics of Lipids*. 182:29-37.

Maître, J.-L., H. Berthoumieux, S.F.G. Krens, G. Salbreux, F. Jülicher, E. Paluch, and C.-P. Heisenberg. 2012. Adhesion Functions in Cell Sorting by Mechanically Coupling the Cortices of Adhering Cells. *Science*. 338:253-256.

Martin, A.C., M. Gelbart, R. Fernandez-Gonzalez, M. Kaschube, and E.F. Wieschaus. 2010. Integration of contractile forces during tissue invagination. *Journal of Cell Biology*. 188:735-749.

McCartney, B.M., D.G. McEwen, E. Grevengoed, P. Maddox, A. Bejsovec, and M. Peifer. 2001. *Drosophila* APC2 and Armadillo participate in tethering mitotic spindles to cortical actin. *Nature Cell Biology*. 3:933-938.

Mizuno, K., T. Fujita, H. Gomi, and T. Izumi. 2016. Granuphilin exclusively mediates functional granule docking to the plasma membrane. *Scientific Reports*. 6:23909.

Pilot, F., J.-M. Philippe, C.L. Lemmers, J.-P. Chauvin, and T. Lecuit. 2006a. Developmental control of nuclear morphogenesis and anchoring by *charleston*, identified in a functional genomic screen of *Drosophila* cellularisation. *Development*. 133:711-723.

Pilot, F., J.M. Philippe, C. Lemmers, and T. Lecuit. 2006b. Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. *Nature*. 442:580-584.

Plastino, J., and L. Blanchoin. 2019. Dynamic stability of the actin ecosystem. *Journal of Cell Science*. 132:jcs219832.

Postner, M.A., and E.F. Wieschaus. 1994. The nullo protein is a component of the actin-myosin network that mediates cellularization in *Drosophila* melanogaster embryos. *Journal of Cell Science*. 107:1863-1873.

Riggs, B., W. Rothwell, S. Mische, G.R.X. Hickson, J. Matheson, T.S. Hays, G.W. Gould, and W. Sullivan. 2003. Actin cytoskeleton remodeling during early *Drosophila* furrow formation requires recycling endosomal components Nuclear-fallout and Rab11. *Journal of Cell Biology*. 163:143-154.

Rikhy, R., M. Mavrakis, and J. Lippincott-Schwartz. 2015. Dynamin regulates metaphase furrow formation and plasma membrane compartmentalization in the syncytial *Drosophila* embryo. *Biology Open*. 4:301-311.

Roh-Johnson, M., G. Shemer, C.D. Higgins, J.H. McClellan, A.D. Werts, U.S. Tulu, L. Gao, E. Betzig, D.P. Kiehart, and B. Goldstein. 2012. Triggering a Cell Shape Change by Exploiting Preexisting Actomyosin Contractions. *Science*. 335:1232-1235.

Rothwell, W.F., P. Fogarty, C.M. Field, and W. Sullivan. 1998. Nuclear-fallout, a *Drosophila* protein that cycles from the cytoplasm to the centrosomes, regulates cortical microfilament organization. *Development*. 125:1295-1303.

Schejter, E.D., and E. Wieschaus. 1993. bottleneck acts as a regulator of the microfilament network governing cellularization of the *Drosophila* embryo. *Cell*. 75:373-385.

Serano, J., and G.M. Rubin. 2003. The *Drosophila* synaptotagmin-like protein bitesize is required for growth and has mRNA localization sequences within its open reading frame. *Proc Natl Acad Sci U S A*. 100:13368-13373.
Shekhar, S. 2017. Microfluidics-Assisted TIRF Imaging to Study Single Actin Filament Dynamics. *Current Protocols in Cell Biology*. 77.
Sherlekar, A., G. Mundhe, P. Richa, B. Dey, S. Sharma, and R. Rikhy. 2020. F-BAR domain protein Syndapin regulates actomyosin dynamics during apical cap remodeling in syncytial *Drosophila* embryos. *J Cell Sci*. 133.
Simpson, L., and E. Wieschaus. 1990. Zygotic activity of the nullo locus is required to stabilize the actin-myosin network during cellularization in *Drosophila*. *Development*. 110:851-863.
Sokac, A.M., N. Biel, and S. De Renzis. 2023. Membrane-actin interactions in morphogenesis: Lessons learned from *Drosophila* cellularization. *Seminars in Cell & Developmental Biology*. 133:107-122.
Sokac, A.M., and E. Wieschaus. 2008. Local Actin-Dependent Endocytosis Is Zygotically Controlled to Initiate *Drosophila* Cellularization. *Developmental Cell*. 14:775-786.
Spracklen, A.J., T.N. Fagan, K.E. Lovander, and T.L. Tootle. 2014. The pros and cons of common actin labeling tools for visualizing actin dynamics during *Drosophila* oogenesis. *Developmental Biology*. 393:209-226.
Spracklen, A.J., E.M. Thornton-Kolbe, A.N. Bonner, A. Florea, P.J. Compton, R. Fernandez-Gonzalez, and M. Peifer. 2019. The Crk adapter protein is essential for *Drosophila* embryogenesis, where it regulates multiple actin-dependent morphogenic events. *Molecular Biology of the Cell*. 30:2399-2421.
Spudich, J.A., and S. Watt. 1971. The Regulation of Rabbit Skeletal Muscle Contraction: I. BIOCHEMICAL STUDIES OF THE INTERACTION OF THE TROPOMYOSIN-TROPONIN COMPLEX WITH ACTIN AND THE PROTEOLYTIC FRAGMENTS OF MYOSIN. *Journal of Biological Chemistry*. 246:4866-4871.
Stevenson, V., A. Hudson, L. Cooley, and W.E. Theurkauf. 2002. Arp2/3-Dependent Psuedocleavage Furrow Assembly in Syncytial *Drosophila* Embryos. *Current Biology*. 12:705-711.
Stevenson, V.A., J. Kramer, J. Kuhn, and W.E. Theurkauf. 2001. Centrosomes and the Scrambled protein coordinate microtubule-independent actin reorganization. *Nature Cell Biology*. 3:68-75.
Sullivan, W., P. Fogarty, and W. Theurkauf. 1993. Mutations affecting the cytoskeletal organization of syncytial *Drosophila* embryos. *Development*. 118:1245-1254.
Tilney, L.G., P.S. Connelly, K.A. Vranich, M.K. Shaw, and G.M. Guild. 2000. Regulation of Actin Filament Cross-linking and Bundle Shape in *Drosophila* Bristles. *Journal of Cell Biology*. 148:87-99.
Webb, R.L., M.-N. Zhou, and B.M. McCartney. 2009. A novel role for an APC2-Diaphanous complex in regulating actin organization in *Drosophila*. *Development*. 136:1283-1293.

Wenzl, C., S. Yan, P. Laupsien, and J. Großhans. 2010. Localization of RhoGEF2 during *Drosophila* cellularization is developmentally controlled by slam. *Mechanisms of Development*. 127:371-384.
Xie, Y., R. Budhathoki, and J.T. Blankenship. 2021. Combinatorial deployment of F-actin regulators to build complex 3D actin structures in vivo. *eLife*. 10.
Zallen, J.A., Y. Cohen, A.M. Hudson, L. Cooley, E. Wieschaus, and E.D. Schejter. 2002. SCAR is a primary regulator of Arp2/3-dependent morphological events in *Drosophila*. *Journal of Cell Biology*. 156:689-701.
Zaman, R., A. Lombardo, C. Sauvanet, R. Viswanatha, V. Awad, L.E.-R. Bonomo, D. McDermitt, and A. Bretscher. 2021. Effector-mediated ERM activation locally inhibits RhoA activity to shape the apical cell domain. *Journal of Cell Biology*. 220.
Zhang, Y., J.C. Yu, T. Jiang, R. Fernandez-Gonzalez, and T.J.C. Harris. 2018. Collision of Expanding Actin Caps with Actomyosin Borders for Cortical Bending and Mitotic Rounding in a Syncytium. *Developmental Cell*. 45:551-564.e554.
Zheng, L., L.A. Sepúlveda, R.C. Lua, O. Lichtarge, I. Golding, and A.M. Sokac. 2013. The Maternal-to-Zygotic Transition Targets Actin to Promote Robustness during Morphogenesis. *PLoS Genetics*. 9:e1003901.

Supplementary Information

Table S1: Fly stocks used in this study

Stock	Genotype	Source
1	67;UAS-mCherry::MoesinABD	T. Millard/Xie et al., 2021
2	y, w; 67; UAS-GFP::CLIP170/TM3, Sb	This study, original from Ko et al., 2021
3	OreR	Bloomington Drosophila Stock Center
4	w; mat67, Sqh::GFP; mat15, Gap43::mCherry/TM3, Sb[1]	Vasquez et al., 2014
5	w; mat67; mat15	Vasquez et al., 2014
6	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS02146}attP40/CyO (Btsz RNAi line)	Bloomington Drosophila Stock Center
7	y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.GL01052}attP2 (RH3 RNAi control line)	Perkins et al., 2015
8	y,w; mat67/Cyo; Histone::GFP, gap43::mCherry-7/TM3	This study, original Histone::GFP, gap43::mCherry-7/TM3
9	w; P{UASt-mCherry::BtszB}attP20	This study
10	w; P{UASp-mCherry.btsz}attP2	Bloomington Drosophila Stock Center
11	y[1] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMJ21357}attP40 (Arp3 RNAi line)	Bloomington Drosophila Stock Center
12	w[1118]; Sqh::GFP; P{ry[+t7.2]=neoFRT}82B btsz[K13-4]/TM3, Sb[1]	This study, original w[1118]; P{ry[+t7.2]=neoFRT}82B btsz[K13-4]/TM3, Sb[1]
13	P{ry[+t7.2]=hsFLP}1, y[1] w[1118]; Dr[1]/TM3, Sb[1]	Bloomington Drosophila Stock Center
14	w[*]; P{ry[+t7.2]=neoFRT}82B P{w[+mC]=ovoD1-18}3R/st[1] betaTub85D[D] ss[1] e[s]/TM3, Sb[1]	Bloomington Drosophila Stock Center
15	sqh::GFP	Royou et al. 2002
16	sqh::GFP; btsz exon 4 mutant (independent CRISPR allele #1)	This study
16*	sqh::GFP; btsz exon 4 mutant (independent CRISPR allele #2)	This study
17	mat67, UtrophinABD::GFP	Jodoin and Martin, 2016

Figure	Cross (virgin x male) or stock
2A-D	6x1 or 7x1 for control
3A-C	6x2 or 7x2 for control
3D	6x5 or 7x5 for control
3E-F	6x8 or 7x8 for control
4A	3
4D-E	See methods section for germline clone crossing schema. 16x16* or 15x3 for control
6A	10x5
6B-C	6x5 or 7x5 for control
6D	9x5
S1A	6x5 or 7x5 for control
S1B	6x5, 11x17, or 7x5 for control
S2A-B	6x1 or 7x1 for control
S2C	6x5 or 7x5 for control

Primer	Sequence	Tm	Predicted length
Actin42A fwd	TTGGACTTCGAGCAGGAGAT	51oC Taq	~300 bp
Actin42A_rev	AATCTTCATGGTGGACGGAG		
Btsz exon 4 fwd	GGATTACTTTGGCGACAGTGCGAC	59oC Taq	~900 bp
Btsz exon 6 rev	GGATCGCCGCCCTCCTTGTT		
Btsz exon 8 fwd	CGGTGGAGGAAGCAGAAC	48oC Taq	~200 bp
Btsz exon 8 rev	CATCTTCCTCATCCTCCG		

Primers used in study

Antibodies and dyes used	Concentration
Mouse anti-E-cadherin, Developmental Studies Hybridoma Bank (DSHB)	1:50
Rabbit anti-RFP, Abcam Cat # ab124754	1:500
Mouse anti-α-tubulin, Millipore Sigma #T6199	1:500
Goat anti-Mouse Alexa Fluor 488, Invitrogen Cat #A32723	1:500
Goat anti-Mouse Alexa Fluor 568, Invitrogen Cat #A11004	1:500
Goat anti-Rabbit Alexa Fluor 647, Invitrogen Cat #A32733	1:500
Hoechst 33342 Solution, Thermo Scientific 62249	1:1000
Alexa Fluor™ 488 Phalloidin - Thermo Fisher Scientific Cat #A12379	1:40

Supplemental Video Legends

Accessible at https://www.biorxiv.org/content/10.1101/2023.04.17.537198v1

Video 1. Max intensity projection (with Gaussian blur) of control and Btsz RNAi embryos undergoing nuclear division. There are more smaller actin caps in the Btsz RNAi embryo. Actin was visualized live with the mCherry::MoesinABD marker. Scale bar is 10 μm.

Video 2. Max intensity projection (with Gaussian blur) of control and Btsz RNAi embryos undergoing nuclear division. Red arrows indicate where pseudo-cleavage furrows have started to recede in the Btsz RNAi embryo. Actin was visualized live with the mCherry::MoesinABD marker. Scale bar is 10 μm.

Video 3. Max intensity projection (with Gaussian blur) of control and Btsz RNAi embryos during cellularization. Nuclear fallout occurs in the Btsz RNAi embryo. Nuclei was visualized live with the Histone::GFP marker. Scale bar is 10 μm.

Chapter 3: Discussion

Major Conclusions

The Synaptotagmin-like protein Bitesize promotes actin remodeling in the syncytial embryo

Actin networks frequently undergo rearrangements that influence cell and tissue shape during development. A host of actin regulators control actin assembly and organization in space and time to achieve these dynamic changes. As mentioned in the introduction, the actin cytoskeleton in the syncytial *Drosophila* embryo undergoes highly choreographed changes that accompany each nuclear division cycle. My work has shown that the Synaptotagmin-like protein, Btsz, is an actin regulator that remodels actin during syncytial blastoderm development. We saw that in Btsz-depleted embryos, actin cap area, pseudo-cleavage furrow structure, and nuclear divisions are affected. These phenotypes strongly resemble those resulting from disrupted actin regulation (Afshar et al., 2000; Jiang and Harris, 2019; Spracklen et al., 2019; Stevenson et al., 2002; Xie et al., 2021; Zhang et al., 2018). Prior to this study, it was known that Btsz could organize actin at epithelial cell junctions in a manner that depended on its interaction with the actin-binding protein, Moesin (Pilot et al., 2006). However, a minority of Btsz isoforms contain the exon necessary for Moesin-binding. We showed that *btsz exon 4* mutants, where non-MBD isoforms and 1 MBD isoform are affected, exhibited phenotypes that are similar but less severe than Btsz RNAi embryos in which all isoforms are depleted. Furthermore, mutation of all MBD isoforms also does not recapitulate the phenotype of pan-isoform depletion. These data suggest that both MBD and non-MBD isoforms contribute to Btsz-dependent actin organization activity. Our *in vitro* data indicate that BtszB can interact with actin directly by binding and bundling

actin filaments. We determined that the C-terminal half of the protein, which contains the C2 domains, is responsible for the binding and bundling activity. It is likely that all Btsz isoforms, not only MBD-containing isoforms, contain this F-actin binding property and contribute to Btsz's actin regulatory functions.

Unanswered questions

The work presented in this thesis, including the discovery of a direct actin binding/bundling activity for Btsz raises many additional questions regarding the role of Btsz in development and how Btsz regulates actin cytoskeleton organization and dynamics. These questions and future possible directions to address them are detailed below.

What are the mechanisms through which Btsz binds/bundles actin?

How does Btsz bundle F-actin?

We showed that the C-terminal half of Btsz can bind and bundle F-actin *in vitro* and further examination of the mechanism of Btsz-CT bundling is a priority. In order to bundle actin, the protein must either oligomerize or have multiple actin binding sites (Jansen et al., 2011; Winkelman et al., 2014). Determining how Btsz-CT bundles F-actin would improve our understanding for how Btsz functions *in vivo.* An important piece of information to understand the bundling mechanism is to assess the oligomerization state of Btsz in actin bundling reactions, which could be determined using multi-angle light scattering with size-exclusion chromatography (SEC-MALS). This will allow for

accurate mass and size measurements of Btsz-CT and show whether Btsz-CT can dimerize/oligomerize.

Our data also indicate that BtszB-CT likely binds F-actin cooperatively, which could be important for its bundling activity. The ABD2 domain of the bundling protein fimbrin binds F-actin in a cooperative manner (Hosokawa et al., 2021). Using TIRF, the authors observed that Fimbrin-ABD2 would form clusters that coated the actin filament, which provides more information on how it generates parallel, ordered actin bundles (Hosokawa et al., 2021). Therefore, another informative experiment would be to use TIRF to visualize fluorescently tagged Btsz-CT protein with labeled actin to see how Btsz decorates the filaments to bind/bundle them. The fluorescently tagged Btsz-CT protein would also allow for step photobleaching as an alternate method to determine oligomerization state (Tan et al., 2022).

What residues are important for actin binding/bundling in Btsz?

Our *in vitro* data indicate that the BtszB isoform binds and bundles F-actin. While some Btsz isoforms bind Moesin, our data predict that most or all isoforms can bind and bundle F-actin with the C-terminal half of the protein. In the related Slac2 protein Melanophilin, the actin binding domain (ABD) is also at the C-terminus. In both Slac2 isoforms (Slac2-a and Slac2-c), mutating a cluster of basic residues in the actin binding domain greatly reduced actin binding activity (Kuroda et al., 2003). We identified two basic clusters in BtszB-CT that resemble the one in Slac2-a. One of these is within the C2A domain present in all Btsz isoforms, and the other is in exon 7, which resides in most but not all isoforms. Future work could generate fragments with mutations in these

basic residues, which would better define Btsz's actin-binding domain and reveal whether all Btsz isoforms can bind actin.

Because Slac2-a can bind F-actin and potentially G-actin *in vitro* with its C-terminus (Fukuda et al., 2002), it is possible that BtszB-FL and BtszB-CT may bind G-actin in addition to F-actin. G-actin-binding proteins such as WASp (Wiskott-Aldrich Syndrome protein), and formins promote nucleation by helping form an actin nucleus (Winder and Ayscough, 2005). Other monomer binders regulate actin by promoting nucleotide exchange, sequestration, and delivery to the growing filament (Winder and Ayscough, 2005). If Btsz does indeed bind G-actin, this would add another level of actin regulation that it is involved in. Another interesting avenue to explore involves determining if Btsz can promote actin assembly or disassembly *in vitro*. This could occur through a mechanism mentioned above through G-actin binding, or through Btsz's interactions with other actin regulators such as formins or the Arp2/3 complex to promote their actin nucleation activity. Actin turnover is important for maintaining the cytoskeleton, so it is possible that the phenotypes resulting from Btsz-depletion could be due to defects in actin assembly or disassembly.

How does the full-length Btsz protein incorporate the actin binding and bundling activity?

One limitation of this work is that we did not characterize the actin binding or bundling activity of the full-length protein. We do not know if Btsz has an auto-regulatory closed conformation that opens up to reveal its actin-binding site, similar to other actin-binding proteins such as Moesin and Dia, and how this changes the way we think about its actin regulation activity. It is possible that other actin binding proteins or actin

regulators, Rabs, and/or phosphoinositide lipids could interact with and activate Btsz *in vivo*.

Does Btsz bundle actin *in vivo*?

In this work, we did not determine whether Btsz's *in vitro* actin bundling activity is also important for its function *in vivo*. Reciprocal mutations that abolish actin-binding *in vitro* can be generated in flies using CRISPR to test the effect *in vivo.* Visualization of the actin network through a technique such as FIB-SEM could reveal more information about the network's ultrastructure (Qu et al. 2022). This technique could be used on Btsz-RNAi embryos and embryos mutant for the ABD in Btsz during syncytial blastoderm development to provide more information about possible bundling defects. Using higher resolution microscopy to visualize actin dynamics may also provide additional information on how Btsz is regulating actin *in vivo*.

It would also be interesting to explore other developmental stages that involve actin bundling and probe whether Btsz-mediated actin bundling is important. For instance, filopodia, composed of crosslinked bundles of actin, are an important part of a cell's leading edge during cell migration (Arjonen et al., 2011; Xue et al., 2010). In Drosophila dorsal closure, amnioserosa cells that work together to promote epidermis migration towards the dorsal midline (Jacinto et al., 2002b). During this process, filopodia extend form the leading edge epidermis towards the dorsal midline (Jacinto et al., 2002a; Millard and Martin, 2008). In addition, there is an actomyosin cable (i.e., bundle) at the boundary between amnioserosa and epidermis that generates tension during closure (Hutson et al., 2003). Defects in actin bundles may be more easily

detected during this stage compared to in the syncytial embryo because of the distinct actin bundle structures and the high levels of tension being generated. Modulating actin assembly activity has clear, quantifiable effects on filopodia number, length, and lifetime (Nowotarski et al., 2014). Therefore, future experiments examining the effects of Btsz depletion in either leading edge cells or amnioserosa cells may elucidate how Btsz is affecting actin regulation.

Do other Slps or Slac2s bind and bundle actin?

This thesis work also raises the question of whether mammalian Slps can bundle F-actin. In various contexts, Rab proteins and their effectors directly bind motor proteins like myosin to recruit them to vesicles for transport (El-Amraoui et al., 2002; Hume et al., 2001; Wu et al., 1998; Wu et al., 2002). In a mammalian neuroblastoma × glioma hybrid cell line that models nerve growth, vesicles were enriched along actin bundles and was associated with F-actin growth (Nozumi et al., 2017). When the actin bundler Fascin was knocked down, vesicle transport was affected (Nozumi et al., 2017). It is possible that the Slac2s or Slps that dock vesicles at the membrane may have a multifunctional role to promote actin organization via actin binding and/or bundling at the membrane. Additionally, melanosomes are transported via myosin motors on actin bundles (Chabrillat et al., 2005; Jiang et al., 2020). GTP-bound Rab27a interacts with melanosomes to recruit Slac2-a/Melanophilin and myosin (MyoVa). Both Slac2-a and MyoVa can bind directly to F-actin, coupling melanosomes to the actin cytoskeleton (Fukuda et al., 2002; Nagashima et al., 2002; Strom et al., 2002). It would be interesting to test if Slac2-a can bundle F-actin *in vitro*. If Slac2-a bundles actin, it may have a

multifunctional role of docking melanosomes along with organizing the tracks on which the melanosomes are transported.

Does Btsz play a role in membrane trafficking during development?

Btsz is the only Synaptotagmin-like protein in Drosophila but there has not been definitive evidence for its role in membrane trafficking, unlike mammalian Slps. This thesis work has focused on furthering our knowledge of Btsz's role in actin remodeling but we still do not know whether Btsz can regulate membrane remodeling. We also do not know whether Btsz can interact with Rab27 or other Rabs and if so, how the Rab binding activity is coordinated with bundling. Btsz depletion in the developing *Drosophila* tracheal system leads to defects in apical actin and membrane organization, which could be affected by defective vesicle trafficking or membrane remodeling (Jayanandanan et al., 2014; Ríos-Barrera and Leptin, 2022).

Rabs are important for membrane trafficking for the rapid nuclear divisions that occur during syncytial blastoderm development to maintain pseudo-cleavage furrows (Cao et al, Riggs et al, Mavor et al 2016). When Rab11 or Rab8 mutated or knocked down, there are significant defects in the membrane and F-actin network along with failed nuclear divisions (Cao et al., 2008; Mavor et al., 2016; Riggs et al., 2003). Btsz's putative SHD, which could interact with Rabs, is located in exon 2 and is present only in three of the nine Btsz isoforms. Even so, given that many of the phenotypes that we observed in Btsz-RNAi embryos resemble those of Rab-depleted syncytial embryos, it would be an interesting avenue to explore.

Using biochemical methods, future work could test whether Btsz has a myosin-binding domain like Slac2-a does, or if Btsz can interact with Rab27a or Rab27b with its N-terminus. Given that mammalian protein Slac2-a contains F-actin binding activity located in its C-terminal domain, and that *Drosophila* has no Slac2s, it is possible that Btsz integrates functions of both Slps and Slac2s to regulate both actin and membrane remodeling. Because Btsz has 9 isoforms, perhaps there is isoform specificity in how Btsz combines its various functions. For instance, all Btsz isoforms can bind membrane, but not all of them have the putative SHD, which could be important for Rab effector activity and membrane trafficking. We have only examined BtszB's activity *in vitro* thus far. Therefore, conducting isoform-specific biochemical tests would provide information on Btsz's potential modular role in actin and membrane remodeling.

Various GTPases such as Arf (ADP-ribosylation factor), Rac, and Rho GTPases have been shown to be important for regulating both the actin cytoskeleton and plasma membrane in different contexts (Hickson et al., 2003; Ridley, 2006; Spiering and Hodgson, 2011). Arfs must be activated through an Arf-guanine nucleotide exchange factor (GEF). One Arf-GEF that has been shown to be important in the syncytial *Drosophila* embryo is Steppke, which helps maintain proper pseudo-cleavage furrow growth by restraining the membrane cytoskeleton (Lee and Harris, 2013).

The *Drosophila* protein Nuclear fallout (Nuf) shares homology with the Arf-interacting protein Arfophilin-2 and is an important component of the recycling endosome in the syncytium (Fielding et al., 2005; Riggs et al., 2003; Wilson et al., 2005). Arfophilin-2 binds Rab11 to regulate the recycling endosome (Hickson et al., 2003). In *nuf* mutant embryos, F-actin is depleted from pseudo-cleavage furrows and

membrane integrity was subsequently affected (Cao et al., 2008). The authors saw that pseudo-cleavage furrows had broken in the *nuf* mutant background. Injecting a drug to stabilize F-actin (Jasplakinolide) rescues the furrow breakage phenotype (Cao et al., 2008). Reducing Twinstar/cofilin levels also rescued this phenotype, which indicates that decreasing the amount of F-actin severing promoted pseudo-cleavage furrow integrity in the *nuf* background (Cao et al., 2008). One explanation for this is that Nuf delivers membrane and/or actin to pseudo-cleavage furrows, thus promoting actin turnover (specifically actin assembly) through its role in membrane trafficking.

We see a very similar phenotype in Btsz-depleted embryos where the pseudo-cleavage furrows break prematurely and recede. Future experiments could involve injecting Jasplakinolide into Btsz RNAi embryos to test whether this would rescue the phenotypes we have observed. As we have not yet determined the mechanism through which Btsz is promoting pseudo-cleavage furrow stability *in vivo,* these experiments could elucidate if Btsz functions through a similar mechanism to Nuf and whether it is important for the delivery of actin to furrows. If we do not see a rescue upon Jasplakinolide injection, perhaps Btsz's bundling activity is essential for maintaining the actin bundles in pseudo-cleavage furrows. Another possibility is that Nuf may directly interact with/activate Btsz to promote actin bundling. It would also be interesting to examine Btsz localization in a *nuf* mutant or Nuf localization in Btsz-depleted embryo.

Cell division is a process that requires vesicle trafficking to deliver membrane, actin, and actin-regulators to the ingressing furrow (Albertson et al., 2008; Lecuit and Wieschaus, 2000; Otegui et al., 2005; Strickland et al., 2005). During *Drosophila* cellularization, a modified form of cell division, plasma membrane furrows ingress and

constrict around nuclei to form a tissue sheet (Figure 7). Many mechanisms that drive cytokinesis are similarly essential for cellularization, such as endocytosis, which is necessary for maintaining the membrane and F-actin in growing furrows (Sokac and Wieschaus, 2008). During the cycle 14 division, which occurs after cellularization, F-actin puncta colocalize with endosomal vesicles and are recruited to cleavage furrow (Albertson et al., 2008). It is possible that Btsz recruits F-actin bundles to intracellular vesicles or actin bundles can recruit vesicles through Btsz. However, we did not see any effect on abundance of F-actin vesicles in a Btsz-depleted background. Future work can explore whether BtszB co-localizes with trafficking machinery such as Rab27, Rab8, or Rab11 or any endosomal compartments.

How is Btsz's upregulation at the MZT important for development?

The MZT is a conserved process where the developing embryo turns on zygotic gene expression and maternally supplied products are degraded (Lee et al., 2014). In *Drosophila,* the MZT occurs around cellularization. The expression of many F-actin binding/bundling proteins necessary for cellularization are developmentally upregulated at the MZT (He et al., 2016; Krueger et al., 2019; Lecuit et al., 2002; Postner and Wieschaus, 1994; Schejter and Wieschaus, 1993; Simpson and Wieschaus, 1990; Sokac and Wieschaus, 2008; Wenzl et al., 2010; Zheng et al., 2013). Other proteins important for actin regulation during cellularization include but are not limited to Dia, the Arp2/3 complex, Cofilin, Filamin, Fimbrin, and Steppke, which are maternally loaded as is Btsz (Sokac et al., 2023).

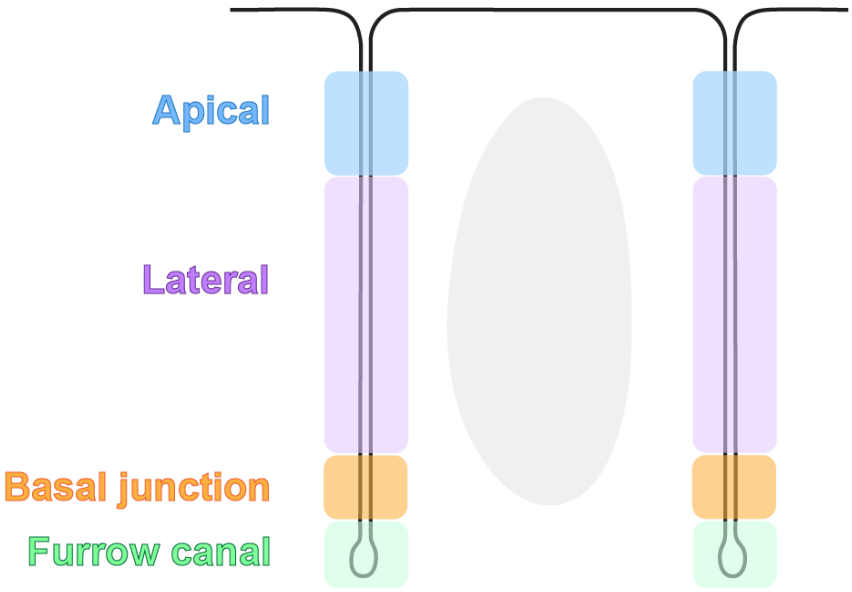


Figure 7. Schematic of plasma membrane furrows during cellularization and their different compartments. Btsz localizes primarily apical-laterally while nullo is enriched at the basal junction and furrow canals. Dia localization follows a similar pattern to that of F-actin where it is enriched at furrow canals. Adapted from (Sokac and Wieschaus, 2008).

One of the zygotically upregulated proteins essential for cellularization is Bottleneck (Bnk), an actin cross-linker essential for maintaining proper actin network organization during morphogenesis. Bnk expression is tightly regulated to turn on for only the first, slow phase (40 min) of cellularization before it is degraded during the second, fast phase (20 min) (Krueger et al., 2019; Schejter and Wieschaus, 1993). In *bnk*$^{-/-}$ embryos, actin fibers are thinner and less dense, which leads to impaired contractility and defective morphogenesis (Krueger et al., 2019). Another zygotically expressed protein is Nullo, which has a broader expression pattern than Bnk and is essential for cellularization and localizes to furrow canals (Postner and Wieschaus, 1994; Sokac and Wieschaus, 2008). There, it plays a role in actin remodeling, as F-actin furrow levels are reduced in a *nullo* mutant background (Postner and Wieschaus, 1994; Sokac and Wieschaus, 2008). Serendipity-α (Sry-α) expression is also important

for actin organization (Zheng et al., 2013). In the *sry-α* mutant background, F-actin levels are also reduced in these furrow canals. Moreover, *sry-α* mutant embryos exhibit a multinucleation phenotype common to defects in actin regulation.

Our work reveals that non-MBD Btsz isoforms are a part of this group of developmentally expressed actin-binding proteins that is upregulated at the MZT. A unique aspect of these genes discussed above is that Bnk, Nullo, and Sry-α are not known to play a role at any other point during fly development (Sokac et al., 2023). However, Btsz is important for gastrulation and tracheal tube formation (Jayanandanan et al., 2014; Pilot et al., 2006). Additionally, Btsz is present in a lateral compartment distinct from these other actin-remodeling proteins such as Nullo and Dia, which each localize to furrow canals (Figure 7) (Homem and Peifer, 2008; Sokac and Wieschaus, 2008). We did not observe a significant difference in actin levels at the membrane furrows during cellularization, so Btsz may not be playing a role during this time or is important for a process that has not yet been identified. Alternatively, Btsz may bundle F-actin in furrows at this lateral compartment and the future adherens junction belt. Laser ablation at sites of Btsz localization in control and Btsz-RNAi embryos to measure recoil could provide information of whether Btsz affects the tension and contractility of the actin cytoskeleton through its potential bundling activity.

References

Afshar, K., B. Stuart, and S.A. Wasserman. 2000. Functional analysis of the Drosophila diaphanous FH protein in early embryonic development. *Development*. 127:1887-1897.

Albertson, R., J. Cao, T.-S. Hsieh, and W. Sullivan. 2008. Vesicles and actin are targeted to the cleavage furrow via furrow microtubules and the central spindle. *Journal of Cell Biology*. 181:777-790.

Arjonen, A., R. Kaukonen, and J. Ivaska. 2011. Filopodia and adhesion in cancer cell motility. *Cell Adh Migr*. 5:421-430.

Cao, J., R. Albertson, B. Riggs, C.M. Field, and W. Sullivan. 2008. Nuf, a Rab11 effector, maintains cytokinetic furrow integrity by promoting local actin polymerization. *Journal of Cell Biology*. 182:301-313.

Chabrillat, M.L., C. Wilhelm, C. Wasmeier, E.V. Sviderskaya, D. Louvard, and E. Coudrier. 2005. Rab8 regulates the actin-based movement of melanosomes. *Mol Biol Cell*. 16:1640-1650.

El-Amraoui, A., J.S. Schonn, P. Küssel-Andermann, S. Blanchard, C. Desnos, J.P. Henry, U. Wolfrum, F. Darchen, and C. Petit. 2002. MyRIP, a novel Rab effector, enables myosin VIIa recruitment to retinal melanosomes. *EMBO reports*. 3:463-470.

Fielding, A.B., E. Schonteich, J. Matheson, G. Wilson, X. Yu, G.R. Hickson, S. Srivastava, S.A. Baldwin, R. Prekeris, and G.W. Gould. 2005. Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. *Embo j*. 24:3389-3399.

Fukuda, M., T.S. Kuroda, and K. Mikoshiba. 2002. Slac2-a/Melanophilin, the Missing Link between Rab27 and Myosin Va. *Journal of Biological Chemistry*. 277:12432-12436.

He, B., A. Martin, and E. Wieschaus. 2016. Flow-dependent myosin recruitment during Drosophila cellularization requires zygotic dunk activity. *Development*. 143:2417-2430.

Hickson, G.R., J. Matheson, B. Riggs, V.H. Maier, A.B. Fielding, R. Prekeris, W. Sullivan, F.A. Barr, and G.W. Gould. 2003. Arfophilins are dual Arf/Rab 11 binding proteins that regulate recycling endosome distribution and are related to Drosophila nuclear fallout. *Mol Biol Cell*. 14:2908-2920.

Homem, C.C., and M. Peifer. 2008. Diaphanous regulates myosin and adherens junctions to control cell contractility and protrusive behavior during morphogenesis. *Development*. 135:1005-1018.

Hosokawa, N., M. Kuragano, A. Yoshino, K. Shibata, T.Q.P. Uyeda, and K. Tokuraku. 2021. Unidirectional cooperative binding of fimbrin actin-binding domain 2 to actin filament. *Biochemical and Biophysical Research Communications*. 552:59-65.

Hume, A.N., L.M. Collinson, A. Rapak, A.Q. Gomes, C.R. Hopkins, and M.C. Seabra. 2001. Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *J Cell Biol*. 152:795-808.

Hutson, M.S., Y. Tokutake, M.S. Chang, J.W. Bloor, S. Venakides, D.P. Kiehart, and G.S. Edwards. 2003. Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science*. 300:145-149.

Jacinto, A., W. Wood, S. Woolner, C. Hiley, L. Turner, C. Wilson, A. Martinez-Arias, and P. Martin. 2002a. Dynamic Analysis of Actin Cable Function during Drosophila Dorsal Closure. *Current Biology*. 12:1245-1250.

Jacinto, A., S. Woolner, and P. Martin. 2002b. Dynamic Analysis of Dorsal Closure in Drosophila: From Genetics to Cell Biology. *Developmental Cell*. 3:9-19.

Jansen, S., A. Collins, C. Yang, G. Rebowski, T. Svitkina, and R. Dominguez. 2011. Mechanism of actin filament bundling by fascin. *J Biol Chem*. 286:30087-30096.

Jayanandanan, N., R. Mathew, and M. Leptin. 2014. Guidance of subcellular tubulogenesis by actin under the control of a synaptotagmin-like protein and Moesin. *Nature Communications*. 5.

Jiang, M., A.E. Paniagua, S. Volland, H. Wang, A. Balaji, D.G. Li, V.S. Lopes, B.L. Burgess, and D.S. Williams. 2020. Microtubule motor transport in the delivery of melanosomes to the actin-rich apical domain of the retinal pigment epithelium. *J Cell Sci*. 133.

Jiang, T., and T.J.C. Harris. 2019. Par-1 controls the composition and growth of cortical actin caps during Drosophila embryo cleavage. *Journal of Cell Biology*. 218:4195-4214.

Krueger, D., T. Quinkler, S.A. Mortensen, C. Sachse, and S. De Renzis. 2019. Cross-linker–mediated regulation of actin network organization controls tissue morphogenesis. *Journal of Cell Biology*. 218:2743-2761.

Kuroda, T.S., H. Ariga, and M. Fukuda. 2003. The Actin-Binding Domain of Slac2-a/Melanophilin Is Required for Melanosome Distribution in Melanocytes. *Molecular and Cellular Biology*. 23:5245-5255.

Lecuit, T., R. Samanta, and E. Wieschaus. 2002. slam Encodes a Developmental Regulator of Polarized Membrane Growth during Cleavage of the Drosophila Embryo. *Developmental Cell*. 2:425-436.

Lecuit, T., and E. Wieschaus. 2000. Polarized Insertion of New Membrane from a Cytoplasmic Reservoir during Cleavage of the Drosophila Embryo. *Journal of Cell Biology*. 150:849-860.

Lee, D.M., and T.J. Harris. 2013. An Arf-GEF regulates antagonism between endocytosis and the cytoskeleton for Drosophila blastoderm development. *Curr Biol*. 23:2110-2120.

Lee, M.T., A.R. Bonneau, and A.J. Giraldez. 2014. Zygotic Genome Activation During the Maternal-to-Zygotic Transition. *Annual Review of Cell and Developmental Biology*. 30:581-613.

Mavor, L.M., H. Miao, Z. Zuo, R.M. Holly, Y. Xie, D. Loerke, and J.T. Blankenship. 2016. Rab8 directs furrow ingression and membrane addition during epithelial formation in Drosophila melanogaster. *Development*. 143:892-903.

Millard, T.H., and P. Martin. 2008. Dynamic analysis of filopodial interactions during the zippering phase of Drosophila dorsal closure. *Development*. 135:621-626.

Nagashima, K., S. Torii, Z. Yi, M. Igarashi, K. Okamoto, T. Takeuchi, and T. Izumi. 2002. Melanophilin directly links Rab27a and myosin Va through its distinct coiled-coil regions. *FEBS Lett*. 517:233-238.
Nowotarski, S.H., N. McKeon, R.J. Moser, and M. Peifer. 2014. The actin regulators Enabled and Diaphanous direct distinct protrusive behaviors in different tissues duringDrosophiladevelopment. *Molecular Biology of the Cell*. 25:3147-3165.
Nozumi, M., F. Nakatsu, K. Katoh, and M. Igarashi. 2017. Coordinated Movement of Vesicles and Actin Bundles during Nerve Growth Revealed by Superresolution Microscopy. *Cell Rep*. 18:2203-2216.
Otegui, M.S., K.J. Verbrugghe, and A.R. Skop. 2005. Midbodies and phragmoplasts: analogous structures involved in cytokinesis. *Trends in Cell Biology*. 15:404-413.
Pilot, F., J.M. Philippe, C. Lemmers, and T. Lecuit. 2006. Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. *Nature*. 442:580-584.
Postner, M.A., and E.F. Wieschaus. 1994. The nullo protein is a component of the actin-myosin network that mediates cellularization in Drosophila melanogaster embryos. *Journal of Cell Science*. 107:1863-1873.
Qu, C., R. Roth, P. Puapatanakul, C. Loitman, D. Hammad, G.M. Genin, J.H. Miner, and H.Y. Suleiman. 2022. Three-Dimensional Visualization of the Podocyte Actin Network Using Integrated Membrane Extraction, Electron Microscopy, and Machine Learning. *J Am Soc Nephrol*. 33:155-173.
Ridley, A.J. 2006. Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking. *Trends Cell Biol*. 16:522-529.
Riggs, B., W. Rothwell, S. Mische, G.R.X. Hickson, J. Matheson, T.S. Hays, G.W. Gould, and W. Sullivan. 2003. Actin cytoskeleton remodeling during early Drosophila furrow formation requires recycling endosomal components Nuclear-fallout and Rab11. *Journal of Cell Biology*. 163:143-154.
Ríos-Barrera, L.D., and M. Leptin. 2022. An endosome-associated actin network involved in directed apical plasma membrane growth. *Journal of Cell Biology*. 221.
Schejter, E.D., and E. Wieschaus. 1993. bottleneck acts as a regulator of the microfilament network governing cellularization of the Drosophila embryo. *Cell*. 75:373-385.
Simpson, L., and E. Wieschaus. 1990. Zygotic activity of the nullo locus is required to stabilize the actin-myosin network during cellularization in Drosophila. *Development*. 110:851-863.
Sokac, A.M., N. Biel, and S. De Renzis. 2023. Membrane-actin interactions in morphogenesis: Lessons learned from Drosophila cellularization. *Seminars in Cell & Developmental Biology*. 133:107-122.
Sokac, A.M., and E. Wieschaus. 2008. Local Actin-Dependent Endocytosis Is Zygotically Controlled to Initiate Drosophila Cellularization. *Developmental Cell*. 14:775-786.
Spiering, D., and L. Hodgson. 2011. Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr*. 5:170-180.

Spracklen, A.J., E.M. Thornton-Kolbe, A.N. Bonner, A. Florea, P.J. Compton, R. Fernandez-Gonzalez, and M. Peifer. 2019. The Crk adapter protein is essential forDrosophilaembryogenesis, where it regulates multiple actin-dependent morphogenic events. *Molecular Biology of the Cell*. 30:2399-2421.
Stevenson, V., A. Hudson, L. Cooley, and W.E. Theurkauf. 2002. Arp2/3-Dependent Psuedocleavage Furrow Assembly in Syncytial Drosophila Embryos. *Current Biology*. 12:705-711.
Strickland, L.I., E.J. Donnelly, and D.R. Burgess. 2005. Induction of Cytokinesis Is Independent of Precisely Regulated Microtubule Dynamics. *Molecular Biology of the Cell*. 16:4485-4494.
Strom, M., A.N. Hume, A.K. Tarafder, E. Barkagianni, and M.C. Seabra. 2002. A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport. *J Biol Chem*. 277:25423-25430.
Tan, H.L., S. Bungert-Plümke, D. Kortzak, C. Fahlke, and G. Stölting. 2022. Determination of oligomeric states of proteins via dual-color colocalization with single molecule localization microscopy. *eLife*. 11:e76631.
Wenzl, C., S. Yan, P. Laupsien, and J. Großhans. 2010. Localization of RhoGEF2 during Drosophila cellularization is developmentally controlled by slam. *Mechanisms of Development*. 127:371-384.
Wilson, G.M., A.B. Fielding, G.C. Simon, X. Yu, P.D. Andrews, R.S. Hames, A.M. Frey, A.A. Peden, G.W. Gould, and R. Prekeris. 2005. The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis. *Mol Biol Cell*. 16:849-860.
Winder, S.J., and K.R. Ayscough. 2005. Actin-binding proteins. *Journal of Cell Science*. 118:651-654.
Winkelman, J.D., C.G. Bilancia, M. Peifer, and D.R. Kovar. 2014. Ena/VASP Enabled is a highly processive actin polymerase tailored to self-assemble parallel-bundled F-actin networks with Fascin. *Proceedings of the National Academy of Sciences*. 111:4121-4126.
Wu, X., B. Bowers, K. Rao, Q. Wei, and J.A. Hammer, 3rd. 1998. Visualization of melanosome dynamics within wild-type and dilute melanocytes suggests a paradigm for myosin V function In vivo. *J Cell Biol*. 143:1899-1918.
Wu, X.S., K. Rao, H. Zhang, F. Wang, J.R. Sellers, L.E. Matesic, N.G. Copeland, N.A. Jenkins, and J.A. Hammer, 3rd. 2002. Identification of an organelle receptor for myosin-Va. *Nat Cell Biol*. 4:271-278.
Xie, Y., R. Budhathoki, and J.T. Blankenship. 2021. Combinatorial deployment of F-actin regulators to build complex 3D actin structures in vivo. *eLife*. 10.
Xue, F., D.M. Janzen, and D.A. Knecht. 2010. Contribution of Filopodia to Cell Migration: A Mechanical Link between Protrusion and Contraction. *Int J Cell Biol*. 2010:507821.
Zhang, Y., J.C. Yu, T. Jiang, R. Fernandez-Gonzalez, and T.J.C. Harris. 2018. Collision of Expanding Actin Caps with Actomyosin Borders for Cortical Bending and Mitotic Rounding in a Syncytium. *Developmental Cell*. 45:551-564.e554.

Zheng, L., L.A. Sepúlveda, R.C. Lua, O. Lichtarge, I. Golding, and A.M. Sokac. 2013. The Maternal-to-Zygotic Transition Targets Actin to Promote Robustness during Morphogenesis. *PLoS Genetics*. 9:e1003901.